Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 320 (2004) 145–155
BBRC
www.elsevier.com/locate/ybbrc

Dideoxynucleoside triphosphate-sensitive DNA polymerase from rice is involved in base excision repair and immunologically similar to mammalian DNA pol β☆

Sailendra Nath Sarkar, Sankar Bakshi,[1] Sanath K. Mokkapati,[2] Sujit Roy, and Dibyendu N. Sengupta*

Department of Botany, Bose Institute, 93/1, Acharya Prafulla Chandra Road, Kolkata-700 009, India

Received 21 May 2004

Abstract

A single polypeptide with ddNTP-sensitive DNA polymerase activity was purified to near homogeneity from the shoot tips of rice seedlings and analysis of the preparations by SDS–PAGE followed by silver staining showed a polypeptide of 67 kDa size. The DNA polymerase activity was found to be inhibitory by ddNTP in both in vitro DNA polymerase activity assay and activity gel analysis. Aphidicolin, an inhibitor of other types of DNA polymerases, had no effect on plant enzyme. The 67 kDa rice DNA polymerase was found to be recognized by the polyclonal antibody (purified IgG) made against rat DNA polymerase β (pol β) both in solution and also on Western blot. The recognition was found to be very specific as the activity of Klenow enzyme was unaffected by the antibody. The ability of rice nuclear extract to correct G:U mismatch of oligo-duplex was observed when oligo-duplex with ^{32}P-labeled lower strand containing U (at 22nd position) was used as substrate. Differential appearance of bands at 21-mer, 22-mer, and 51-mer position in presence of dCTP was visible only with G:U mismatch oligo-duplex, but not with G:C oligo-duplex. While ddCTP or polyclonal antibody against rat-DNA pol β inhibits base excision repair (BER), aphidicolin had no effect. These results for the first time clearly demonstrate the ability of rice nuclear extract to run BER and the involvement of ddNTP-sensitive pol β type DNA polymerase. Immunological similarity of the ddNTP-sensitive DNA polymerase β of rice and rat and its involvement in BER revealed the conservation of structure and function of ddNTP-sensitive DNA pol β in plant and animal.

Keywords: DNA polymerase; ddNTP sensitivity; Rice; Base excision repair; DNA repair; DNA polymerase β

Multiple DNA polymerases have been characterized from bacteria [1,2], yeast [3], and mammalian system [4,5] by cloning of their genes or cDNAs. Similarly the presence of multiple DNA polymerase was detected from plant system [6–8] by purification and enzymological characterization. Among all types, single polypeptide DNA polymerase β or β-polymerase (β pol) cloned from rat [9] and human [4,5] was found to be insensitive to aphidicolin but sensitive to ddNTP. Its involvement in DNA repairing specially short patch DNA repairing has been well identified from its participation in BER and presence in BER complex along with uracil DNA-glycosidase, AP_1 endonuclease, and DNA ligase [10]. Mismatch corrections through BER were demonstrated in vitro by purified β-polymerase enzyme and other enzymes from BER complex of bovine testis nuclear extract. In yeast, ddNTP-sensitive DNA polymerase IV is an ortholog of mammalian β-polymerase and has been identified from the synaptosomal complex formed during recombination at the time of meiosis in yeast [11].

☆ Financial assistance from CSIR, Govt. of India (Grant No. 38 (0944)/99/EMR-II), is gratefully acknowledged.

* Corresponding author. Fax: +91-33-2350-6790.
E-mail address: dibyendu@bosemain.boseinst.ac.in (D.N. Sengupta).
[1] Present address: Department of Botany, Vidyasagar College for Women, Kolkata-700006, India.
[2] Present address: Sealy Center for Molecular Science and Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX 77555, USA.

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.05.152

Actually the enzyme cannot discriminate ddNTPs, thereby incorporates ddNMP, as a result chain elongation terminates. Thus, inhibition depends on the ratio of ddNTPs to dNTPs. Purification and characterization of a 52-kDa polypeptide with ddNTP sensitive was reported from wheat [12]. Previously, a 67-kDa polypeptide with ddNTP-sensitive DNA-polymerase activity was purified from shoot tips of rice [13] and the enzyme showed distributive mode of DNA synthesis, aphidicolin insensitivity, etc., similar to the mammalian DNA pol β. Therefore we call this enzyme rice DNA polymerase β or rice DNA pol β. Increase in DNA damage and loss of DNA repairing ability were considered as fundamental mechanisms underlying the aging process, from the observation on declines in genomic integrity seen with age [14–18]. Aside from adult plants, seeds are subjected to DNA lesions, not only during the aging [19,20] but also from genotoxic environmental exposures [21]. For many plant species, seed viability is known to decreases with seed age (subject to storage conditions). A direct correlation between the decreased seed viability and the loss of DNA integrity in their embryo was established based on the studies of chromosomal aberrations and point mutation [22,23]. It was also reported that a progressive fragmentation of embryonic nuclear DNA occurred in dry-stored seeds of rye [19]. Thus, degree of DNA damage and efficiency of DNA repair are the molecular events that probably determine the ability of the seed to germinate. DNA polymerases are required for DNA synthesis both during DNA replication and DNA repair and ddNTP-sensitive DNA pol β is well known for its involvement in short patch DNA repairing in mammalian system. Although the ddNTP-sensitive DNA polymerase enzyme has been identified from plant system, its expression or involvement in DNA repairing is not known.

Here, we report on the detection of immunological similarity of ddNTP-sensitive DNA polymerase enzymes from rice and rat. Polyclonal antibody made against rat DNA pol β was found to recognize the rice DNA pol β on Western blot and also inhibit in vitro DNA polymerase activity of the purified rice enzyme. For the first time the existence of base excision repair (BER) to correct mismatch in plant system and involvement of ddNTP-sensitive DNA pol β in BER were detected and are reported here.

Materials and methods

Plant material, chemicals, and reagents. Seeds of *Oryza sativa* cv. IR-8 were obtained from Chinsurah Rice Research Institute, West Bengal, India, The seeds were stored at 25 °C and seeds of freshly harvested stock, 2 years 4 months old-stock, and 4 years 3 months old-stocks were used in the experiments.

Phosphocellulose (P-11) from Whatman (Germany), T4 polynucleotide kinase, activated calf thymus DNA (used as template: primer in DNA polymerase assay), dNTPs, ddNTPs from Amersham–Pharmacia (USA), magnesium chloride, sucrose, 2-mercaptoethanol, glycerol, sodium chloride, Hepes, EDTA, Triton X-100, sodium dodecyl sulfate, DEAE–Sephacel, aphidicolin, PMSF, and trichloroacetic acid from Sigma (St. Louis, USA). ssDNA agarose, urea, formamide, enzyme grade ammonium sulfate, agarose, Tris base, glycine, acrylamide, and bis-acrylamide from Gibco-BRL (now Invitrogen, USA), silver staining kit from Bio-Rad (USA) were purchased and used. All other chemicals from Qualigen (India) SRL (India) or Spectrochem (India) were used in the experiments. Polyclonal antibody (purified IgG) generated against rat DNA pol was a generous gift from Dr. S. H. Wilson, NIES, USA [10].

Radioisotopes, e.g., [α-^{32}P]CTP (Sp. Ac. > 4000 Ci/mMol) and [γ-^{32}P]ATP (Sp. Ac. > 4000 Ci/mMol), [α-^{32}P]TTP (Sp. Ac.> 3000 Ci/mMol), and [^{3}H]dTTP (Sp. Ac. > 46 Ci/mMol) were from New England Nuclear, Dupont, USA.

Radioisotopes, e.g., [α-^{32}P]CTP (Sp. Ac. > 4000 Ci/mMol) and [γ- ^{32}P]ATP (Sp. Ac. > 4000 Ci/mMol), [α-^{32}P]TTP (Sp. Ac.> 3000 Ci/mMol), and [^{3}H]dTTP (Sp. Ac. > 46 Ci/mMol) were from New England Nuclear, Dupont, USA.

HPLC purified oligonucleotides were synthesized from Sigma-Genosys (USA). The nucleotide sequences of the three 51-mer oligonucleotides were as follows:

Upper strand: 5′-TCGAGCTCGGTACCCGGGGATCCTCTAGA**G**TCGACCTGCAGGCATGCAAGC-3′

Lower strand (mm): 3′-AGCTCGAGCCATGGGCCCCTAGGAGA TCT**U**AGCTGGACGTCCGTACGTTCG-5′

Lower strand (nor): 3′-AGCTCGAGCCATGGGCCCCTAGGAGA TCT**C**AGCTGGACGTCCGTACGTTCG-5′

Buffers. Buffer A for protein isolation contained 50 mM Tris–HCl (pH 7.5), 1 mM 2-mercaptoethanol, 0.1 mM EDTA, 10 mM $MgCl_2$, 250 mM sucrose, and 1 mM PMSF.

Buffer B for protein storage contained 50 mM Tris–HCl, pH 6.5, 1 mM 2-mercaptoethanol, 0.1 mM EDTA, 20% glycerol, and 1 mM PMSF. Buffer A for nuclei isolation contained 10 mM Tris–Cl, pH 7.5, 5 mM $MgCl_2$, 1 M sucrose, and 10 mM 2-mercaptoethanol. Buffer D contained 20 mM Tris–Cl, pH 7.5, 5 mM $MgCl_2$, 500 mM NaCl, 20 mM PMSF, and 10 μg/ml leupeptin.

Purification of ddNTP-sensitive DNA polymerase and immunological detection. Purification of the DNA polymerase enzyme sensitive to ddNTPs was done following the protocol of Sanath Kumar et al. [13] with some modifications. Each time, 50 g of shoot tips was homogenized with 3 volumes of ice-cold buffer A in a chilled mortar and pestle at 4 °C. The homogenates were filtered through 4 layers of surgical gauze in a beaker and powdered ammonium sulfate was added slowly to reach 30% saturation on a magnetic stirrer at 4 °C. Precipitate was removed by centrifugation at 10,000*g* for 10 min at 4 °C and supernatant was brought to 70% ammonium sulfate. The precipitates were collected as pellet was resuspended in buffer A and dialyzed against 1000 vol of buffer B with several changes. The dialyzed fraction was purified through the column of P11 phosphocellulose (2.5 × 20 cm) at 4 °C. After loading the sample column was washed with three column volume buffer B. Finally, bound proteins were eluted by running a 150 ml KCl gradient (0–1 M) in buffer B and 3 ml fractions were collected by using RediFrac Fraction Collector (Pharmacia). The O.D. at $A_{280\,nm}$ of each fraction and DNA polymerase activity in absence or presence of ddCTP were measured by UV–Vis spectrophotometer (Shimadzu, Japan) and in vitro DNA polymerase assay. The fractions containing peak of ddCTP-sensitive DNA polymerase activity were pooled and dialyzed against 1000 vol. of buffer B containing 50 mM KCl. Gel filtration column was made with Sephacryl S-200 (1.6 × 80 cm) equilibrated in buffer B and dialyzed sample was run with 6 ml/h speed. The fraction collector collected aliquots of 3 ml and OD at A_{280} of each aliquot was measured. Every alternate fraction was tested for ddCTP-sensitive DNA polymerase activity. Peak fractions were pooled and loaded onto a column of ssDNA agarose (1 × 5 cm). Washing was done with 3 column volume buffer B and elution was

done by 3 volumes of buffer B. Aliquots of 0.2 ml were collected and each fraction was tested for ddCTP-sensitive DNA polymerase activity. Fractions with high activity were pooled. Protein content was measured with Bradford reagent [24] and purification was analyzed by SDS–PAGE. Gel slices from same gel (SDS–PAGE) were analyzed either by silver staining or by Western blotting so that mobility of the bands developed by two different methods can be compared. Inhibition of DNA polymerase activity was done by increasing amount of ddNTP or the antibody. To find out the specificity of inhibition of the antibody, activity of Klenow enzyme was also tested in presence of antibody.

In vitro DNA synthesis assay. DNA pol activity was monitored by measuring the incorporation of [^{3}H]dTTP or [α-^{32}P]dATP into TCA insoluble fraction, e.g., synthesized DNA, using activated calf thymus DNA as template primer. In a final volume of 50 μl, the assay mixture contained 50 mM Tris–HCl (pH 7.5), 10 mM DTT, 3 mM $MgCl_2$, 0.05 mM each of dATP, dCTP, dGTP, and 1 μCi [^{3}H]TTP (Sp. Ac. 46 Ci/mmol) or 3 μCi [α-^{32}P]CTP (Sp. Ac. 4000 Ci/mmol), 20 μg/ml activated calf thymus DNA, and 150 mM KCl. After incubation at 37 °C on a water bath shaker for 30 min, the reactions were terminated by addition of 1 ml of cold 10% TCA containing 100 mM sodium pyrophosphate. After 30 min on ice the precipitate was collected on a GFC filter (Whatman) and washed with 10 ml of 2% TCA twice using Millipore vacuum filtration device. Filters were then dried under heat lamp and the radioactivity of each filter was measured by L5 (Beckman) Liquid Scintillation Counter using scintillation fluid. Values were converted into picomoles of [^{3}H]TMP or [^{32}P]dCMP incorporated and expressed as units of enzyme activity. One unit is defined as the amount of enzyme required to incorporate 1 pmol of dTMP incorporated within 60 min at 37 °C [9].

Western blot analysis. Equal amounts [25] of protein extracts from seeds of freshly harvested stock, 2 years 4 months, and 4 years 3 months old stocks were mixed with SDS–PAGE loading buffer (125 mM Tris, [pH 6.8], 20% [v/v] glycerol, 4% [w/v] SDS, 0.01% bromophenol blue, and 100 mM DTT). Samples were incubated in boiled water for 5 min and then subjected to SDS–PAGE using 12% polyacrylamide (w/v). Proteins were electro-transferred to PVDF membrane (Pharmacia, Sweden) using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA) following the manufacturer's protocol and the transfers were found to be 100%. The membranes were then blocked with 5% BSA in TBS, overnight, on a rocker. The blots were processed by affinity purified polyclonal anti-sera (purified IgG fraction) developed against rat DNA pol β (1:20,000 dilution) followed by alkaline phosphatase (AP) conjugated anti-mouse IgG. Primary antibody recognized band on membrane was revealed by following the enzymatic assay of AP for color development as described in Sambrook et al. [25]. Similarly blots with equal amount of DNA pol β protein was probed with non-immune rabbit antiserum and monoclonal antibody against rat DNA pol β and no cross-reaction was observed.

Activity gel analysis of rice DNA polymerase. In-gel DNA polymerase activity assay was carried out following the protocols [26,27]. In these experiments special quality SDS from Boehringer Mannheim (lot No. 83794421) was used in SDS–polyacrylamide gel electrophoresis. Separating gel was made with 125 μg/ml of activated calf thymus DNA and 0.001 M EDTA. Samples were prepared by mixing 25 μl (10 μg protein) of DEAE–Sephacel purified enzyme with 20 μl sample buffer containing 4% SDS, 2% (v/v) 2-mercaptoethanol, 5 μg BSA, and 15% glycerol. The mixtures were heated for 3 min at 37 °C, loaded into wells of vertical slab gel, and electrophoresis in Tris–glycine–SDS buffer was carried out at 100 V for 2 h. After electrophoresis the gel was soaked in 250 ml buffer containing 0.01 M Tris–HCl (pH 7.5), 0.005 M β-mercaptoethanol, and 20% isopropanol with gentle shaking for 45 min to remove SDS. The gels were immersed at 25 °C in 500 ml of 50 mM Tris–HCl (pH 7.5) and after 15 min of gentle agitation the liquid was discarded and fresh 500 ml of 50 mM Tris–HCl (pH 7.5) was added and the gels were incubated for 16 h at 4 °C. The liquid was discarded and the gel was incubated for 1 h in the same solution. Gel containing lanes 1 and 2 was sliced and separately incubated with 10 ml of reaction mixture (40 mM Tris–HCl, pH 7.5; 5 mM $MgCl_2$; 50 mM KCl; 20% glycerol; 0.01 mM EDTA; 1 mM DDT; 8 μM each of dATP, dGTP, and dTTP, 10 μCi/ml [α-^{32}P]dTTP Sp. Ac. > 3000 Ci/mmol), and 80 μM ddTTP. The other part of the gel containing the lanes 3–5 was incubated in 10 ml of the same reaction mixture but without ddTTP. Gel slices were washed in 200 ml of 6% TCA and 1% sodium pyrophosphate for 1 h at room temperature with gentle shaking. This washing step was repeated 2 more times and the gel slices were immersed in 300 ml of same solution for 24 h at 4 °C. Solution was changed and the last washing was continued for 4 more hours at 4 °C. Gel slices were then transferred on Whatman 3 MM paper and dried in a gel dryer connected with a vacuum pump (Hoeffer). Autoradiograph was made by exposing a Kodak X-ray film (X-Omat type) with exposure time of 2–4 h, at room temperature.

Measurement of Klenow activity. Activity of Klenow enzyme was done with calf-thymus activated DNA as template-primer. The reaction was done at 37 °C for 15 min in a buffer containing 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM DTT, and 0.05 μM of all ddNTPs except dATP. Five microCurie [α-^{32}P]ATP (3000 Ci/mM) was added to the reaction.

Isolation of nuclei, preparation of nuclear extract, and detection of BER. Nuclei were prepared as reported earlier [28] with some modifications. Fifty grams of shoot tips of 72 h grown IR-8 seedlings were ground to a fine powder with liquid N_2 and homogenized with four volumes (w/v) of cold nuclei isolation buffer A in a chilled mortar and pestle at 4 °C. The mixture was filtered through three layers of cheesecloth. Triton X-100 was added dropwise to a final concentration of 0.5%. The suspension was centrifuged at 500*g* for 10 min at 4 °C to remove the debris and whole cells. Nuclear pellets were obtained by centrifugation of supernatant at 4000*g* for 20 min after carefully decanting the supernatant. The nuclear pellets were washed three times with the same buffer A after each washing of centrifugation at 4000*g* for 10 min at 4 °C. Finally, the nuclear pellets were resuspended in buffer A, purified by Percoll step gradient (50–65–80%), and centrifuged at 3500*g* for 30 min. Nuclei were collected in between 65% and 80% gradient and diluted with three volumes of buffer A, pelleted by centrifugation for 10 min at 4000*g*, and resuspended in 250 mM sucrose, 20 mM Hepes, pH 7.8, 5 mM $MgCl_2$, 1 mM DTT, and 50% glycerol. Nuclei were aliquoted and stored at −70 °C.

To prepare nuclear extract for BER assay nuclear pellet from shoot tips of 72 h grown seedlings of fresh seeds was resuspended in 5 ml of high salt buffer D (20 mM Tris-Cl, pH 7.5, 5 mM $MgCl_2$, 500 mM NaCl, 20 mM PMSF, and 10 μg/ml leupeptin) and lysed by incubating the suspension on ice for 15 min with gentle rocking. Nuclear lysates were then centrifuged for 20 min at 15,000 rpm at 4 °C. Supernatants were dialyzed against 100 volumes of dialysis buffer E (25 mM Hepes–KOH, pH 7.5, 50 mM KCl, 1 mM EDTA, pH 8.0, 10% glycerol, and 1 mM 2-mercaptoethanol) at 4 °C, overnight. Dialyzed extracts were lyophilized at −50 °C, in a lyophilizer (Secfroid, USA) and protein content was estimated using Bradford reagent [24]. Enzyme preparation was aliquoted and stored at −70 °C for further use.

The BER assay was done according to Singhal et al. [10] with some modifications. For BER assay 51 bp oligo-duplex was used as substrate and two lower strands containing either U at 22nd position, or C at 22nd position were 5′-end labeled by T4 polynucleotide kinase (NEB) and [γ-^{32}P]ATP (4000 Ci/mmol, NEN). Equimolar amount of upper strand was mixed with that of lower strand containing C, oligo-duplex without mismatch was created and used as control. Similarly equimolar concentration of upper strand was mixed with that of lower strand containing U at 22nd position to form the oligo-duplex with mismatch. To prepare 5′-^{32}P-labeled substrate for BER assay, 50 pmol of both oligonucleotides (lower strand) was 5′-end labeled by T4 polynucleotide kinase (NEB) using [γ-^{32}P]ATP (4000 Ci/mmol, NEN). Reaction was carried out at 37 °C for 45 min and stopped by heating at 68 °C for 10 min. Equal amount (50 pmol) of upper strand

oligonucleotide was added, warmed at 75 °C, and allowed to cool down slowly to room temperature so that annealing of the two oligonucleotides occurs. Both the labeled oligo-duplexes were purified by ethanol precipitation. For BER assay the standard reaction mixtures (50 μl final volume) contained 100 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 2 mM ATP, 0.5 mM NAD, four deoxynucleotide triphosphates at 20 μM each, and 20 μg of nuclear protein with either 200-fold molar excess of ddCTP over dCTP or 10 μg/ml aphidicolin. In one tube nuclear extract was first preincubated with 5 μg IgG then ^{32}P-labeled oligo-duplex was added. All reactions were incubated for 10 min at 37 °C and stopped by addition of EDTA to final concentration of 50 mM. The DNA was extracted with phenol: chloroform and precipitated with chilled ethanol keeping on ice for one hour, dried under vacuum, and resuspended in 5 μl of 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, and equal volume of "stop solution" (40 mM EDTA and 80% formamide) was added. After denaturation at 95 °C for 2 min, the DNA was separated by electrophoresis on 12% polyacrylamide 7 M urea gel normally used for DNA sequencing [25]. After 3 h run at 1200 V, the gels were dried on a piece of Whatman 3 mm paper and autoradiographs were made by exposing X-Ray (X-Omat, Kodak) films.

Results

Purification of ddNTP-sensitive, aphidicolin-insensitive DNA polymerase enzyme and its recognition by the polyclonal antibody against rat-pol β: DNA polymerase enzyme, sensitive to ddTTP, was purified from shoot tips of 72 h grown seedlings of germinated freshly harvested IR-8 rice seeds. Ammonium sulfate precipitation followed by chromatography through phosphocellulose column, gel filtrations through Sephacryl S-200 was done and finally by using affinity resin column of ssDNA agarose, purity of the enzyme near homogeneity was obtained (Fig. 1A). After each step of purification, ddNTP-sensitive DNA polymerase activity was measured by counting the incorporation of [^{3}H]dTMP into TCA insoluble fraction, using calf thymus activated DNA as template: primer. Finally, 3840-fold purification with only 3% recovery of the enzyme (Table 1) was observed. Analysis of purity as judged by SDS–PAGE followed by silver staining showed only one band of 67-kDa size (Fig. 1A) by silver staining. Polyclonal antibody (IgG fraction) made against DNA pol β from rat when used in Western blot analysis of purified enzyme cross-reaction with (Fig. 1B) the 67-kDa polypeptide was observed (Fig. 1B), suggesting the recognition of the purified enzyme by the antibody. The purified rice enzyme preparation was preincubated either with increasing concentration of the antibody or with ddTTP and DNA polymerase activity was measured. Fig. 2 shows that while increasing concentration of ddTTP sharply inhibits DNA polymerase activity, increasing amount of the rat antibody inhibits DNA polymerase activity only moderately. By double amount (ng protein basis) of antibody around 45% inhibition was obtained. When Klenow enzyme (ddNTP-sensitive DNA polymerase from *Escherichia coli*) with same amount of anti-rat-DNA pol β was preincubated no inhibitory effect on its DNA polymerase activity was detected suggesting the specificity of cross-reaction and activity neutralization. Since multiple DNA polymerases from mammalian system are called as α, β, δ, γ, ε, etc., and DNA pol β is the only enzyme sensitive to ddNTP but not to aphidicolin, from now on we will call the ddNTP-sensitive rice DNA polymerase as rice DNA pol β.

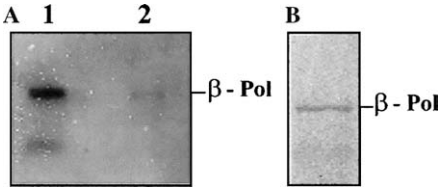

Fig. 1. Analysis of purification of ddNTP-sensitive DNA pol β enzyme by SDS–PAGE and Western blot analysis of the enzyme by polyclonal antibody made against rat DNA pol β. DNA pol β enzyme was purified from shoot tips of 72 h grown seedlings from germinated rice seed (freshly harvested stock) as described in Material and methods. The purified enzyme (50 ng) was analyzed by SDS–PAGE followed by silver staining (A) and immunological detection by Western blotting (B) by the polyclonal antibody made against rat polymerase β (1–20,000 dilution). In lane 1, 200 ng of BSA (Fr. V) and in lane 2, 50 ng purified rice DNA pol β were loaded (A).

Aging of paddy seeds and DNA pol β activity in their embryos

To measure the activities of ddNTP-sensitive DNA polymerase in embryos of fresh and aged seeds, protein extracts were prepared from the embryos either dry or 24 or 48 h water-soaked paddy seeds of three different

Table 1
Purification of ddTTP-sensitive DNA polymerase enzyme from shoot tips of 72 h grown germinated seedlings of rice (*Oryza sativa* cv. IR-8)[a]

Fraction	Total protein (mg)	Specific activity (U/mg)[b]	Purification	Percent recovery
Crude (S10)	1462	0.125	1	
70% ammonium sulfate ppt	754.34	1.54	12.3	100
Phosphocellulose	1.45	202.5	1620	25.2
Sephacryl-S-200	0.170	360	2880	5.2
ssDNA cellulose	0.076	480	3840	3.0

[a] Extractions were made from 100 g of shoot-tips from 72 h grown germinated rice seedlings, each time, and the best one recovered is shown above.

[b] One unit is defined as the amount of enzyme that incorporates 1 pmol [^{3}H]dTMP per hour into a 10% trichloroacetic acid insoluble fraction using activated DNA as template primer at 37 °C.

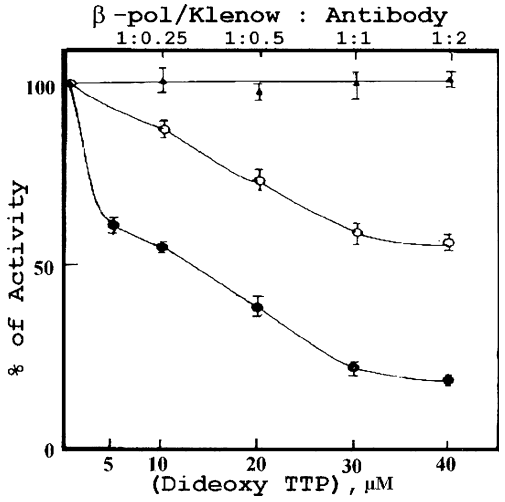

Fig. 2. Inhibition of DNA polymerase activity of the purified rice enzyme by ddTTP or by the rat DNA pol β antibody. In a 50 μl reaction mixture purified rice DNA pol β (200 ng protein) was incubated with increasing concentrations (5–40 μM) of ddTTP (●—●) or increasing concentrations (50–400 ng) of affinity purified polyclonal antibody made against rat DNA polymerase β (○—○) for 1 h at 4 °C and DNA polymerase assay was done at 37 °C for 30 min. Similarly 1 U of Klenow enzyme (▲—▲) was preincubated with the increasing amount of antibody for 1 hr at 4 °C and activity was measured as described in Materials and methods. Incorporation of dTMP in 10% TCA insoluble fraction was measured and the value obtained by enzyme alone was considered as 100% incorporation. Results clearly show that while 80% inhibition was obtained with 30 μM ddTTPs, only 45% maximum inhibition was possible by the polyclonal antibody (400 ng IgG) against rat DNA polymerase β. Antibody has no effect on the activity of Klenow suggesting the specificity of recognition of the rice enzyme by the polyclonal antibody.

age, e.g., freshly harvested, 2 years 4 months old, and 4 years 3 months old stocks. Then equal amount of protein was purified through DEAE–Sephacel column of equal size. After elution with same volume of buffer and salt gradient, DNA pol β activity of every fraction was checked and peak fractions were pooled. Aliquot with same amount of protein was used in in vitro DNA polymerase activity assay in presence or absence of 200-fold molar excess of ddTTP and contribution by DNA pol β was calculated. Results shown as histogram in Fig. 3A indicate that embryos of fresh seeds have pol β activity and the aging enhances DNA pol β activity in dry embryos. Embryos of 4 years 3 months and 2 years 4 months old seeds have 3- and 2.5-fold higher pol β activity, respectively, than the dry embryos of freshly harvested paddy seeds. When seeds were water soaked for 24 h, significant increase (2.5- to 3-fold) of ddNTP-sensitive DNA pol activity in the embryos of aged seeds over their activity in dry embryos was observed. Water treatment for 48 h showed significant decrease in DNA pol β activities in embryos of old seeds than in the freshly harvested seeds, suggesting inability of the embryos of old seeds to run synthetic machineries, e.g., synthesis of DNA polymerase enzyme, for a long time. Continuous increase of pol β activities was noticed in seeds of freshly harvested stocks when subjected to longer water treatment, showing the normal ability of growing embryos from the fresh seeds. To visualize DNA polymerase β activity of 67-kDa polypeptide, activity gel assay was done and 10 μl of the pooled fraction from the embryos of fresh and 4 years 3 months old seeds was separated by SDS–PAGE containing activated calf thymus DNA as template: primer. Renaturation of the enzyme followed by activity assay was performed. Photograph of the autoradiograph made from the activity gel is shown in Fig. 3B. From the intensity comparison by densitometric scanning (Fig. 3C), it was clear that embryos of older seeds contained 10-fold higher level of DNA polymerase activity than that of embryos of freshly harvested seeds (lanes 3 and 4 of Fig. 3B). As a positive control Klenow fragment was loaded in lane 5 of activity gel. Activity gel analysis confirms three important observations from in vitro DNA synthesis. First, it confirms the size of the DNA polymerase as 67-kDa because the enzyme migrated little slower than Klenow, which has a mol. wt. of 66-kDa. Second, it also confirms that embryos of 24 h water-treated seeds have higher level or activity of ddNTP-sensitive DNA pol β than the dry embryos of fresh seeds. Third, it contains rice DNA pol β a single subunit, ddNTP-sensitive enzyme.

Use of polyclonal antibody against rat DNA pol β to detect the expression of DNA pol β in rice plants

Since the polyclonal antibody against rat DNA pol β recognizes the rice enzyme, Western blot analysis was done to measure the level of 67-kDa DNA polymerase enzyme in the extracts of embryos of 24 h water-soaked fresh and aged seeds, partially purified through DEAE–Sephacel column. After SDS–PAGE equal amount of protein (10 μg) and transfer to PVDF membrane Western blot analysis was done with anti-rat pol β polyclonal antibody. Standard alkaline phosphatase mediated color development assay (tagged on secondary IgG) to visualize the primary antibody cross-reacted band of 67-kDa size and photograph made from one such blot is included in Fig. 3D. From the difference in the intensity of colored band, it appears that the level of the enzyme protein (67-kDa) was higher in water-soaked aged seeds (Fig. 3D) than in fresh seeds. Western blot analysis confirms the previous observation on enhancement of DNA pol β activity in solution and in activity gel after 24 h of water treatment of aged seeds and also from in vitro DNA polymerase activity.

To find out the level of rice pol β protein expressed during germination of freshly harvested rice seeds (99% germination capability) and in other tissues of mature plants, equal amount of protein extract from lamina, anthers (just before floral emergence) embryos of fresh

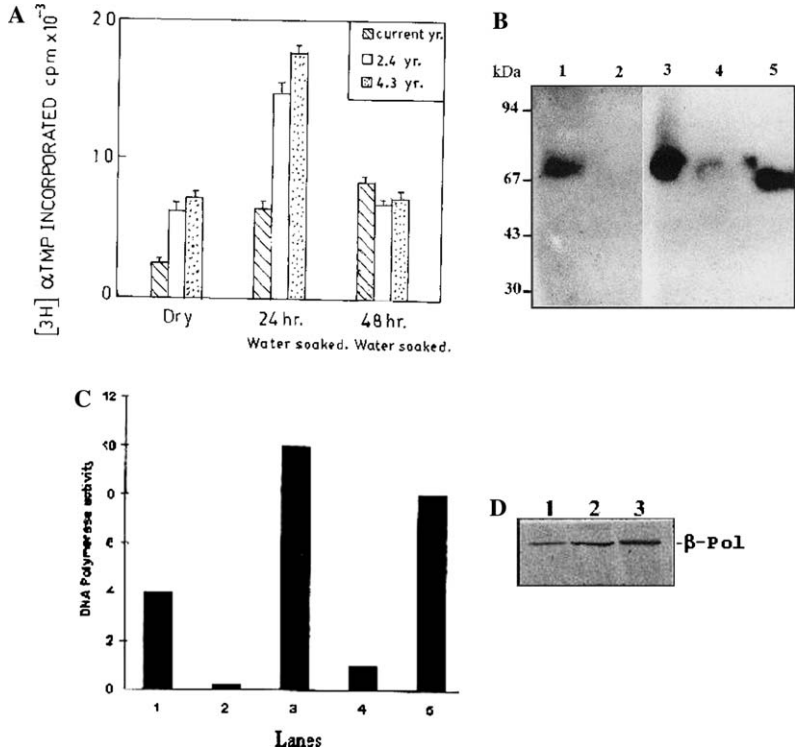

Fig. 3. (A) ddNTP-sensitive DNA polymerase activity in fresh and aged seed embryos as revealed by in vitro DNA pol β activity assay. Total and ddNTP-inhibited DNA polymerase activity was measured in vitro by the assay procedure described in Materials and methods. The activity obtained in presence of ddNTP was subtracted from the total DNA polymerase activity (without ddNTP) and calculated to draw histogram. Water soaking of rice seeds of different ages showed continuous enhancement of ddTTP-sensitivity DNA polymerase β in embryos of fresh seeds whereas transient enhancement was observed in aged seeds, as shown by histogram. (B) Demonstration of enhancement of DNA polymerase activity and sensitivity to ddNTPs directly on activity gel. In situ activity gel of partially purified rice DNA polymerase was done. DNA polymerase partially purified from embryos of 24 h water-soaked seeds of 4 years 3 months old stock (lanes 1 and 3) and from the embryos of 24 h water-soaked seeds of freshly harvested stock (lanes 2 and 4) and Klenow enzyme (one unit) (lane 5) were separated by SDS–PAGE. Twelve percentage polyacrylamide (SDS–PAGE) gel was made with activated DNA as template:primer and after renaturation gel slice with lanes 1 and 2 was incubated in [α-^{32}P]dTTP containing reaction mixture with ddTTP. The other part of the gel with lanes 3–5 was incubated with same amount of [α-^{32}P]dTTP without ddTTP. Photographs taken from the autoradiograph made from dry gel are shown above. Experiment was repeated twice and enhancement activity of the enzymes and inhibition by ddNTP were visible. (C) Densitometric Scanning of the autorad made from the activity gel. Histogram was prepared from the densitometric scanning of the autoradiogram of activity gel shown in (C) measured by LKB 2201, ultra scan. (D) Western blot analysis showing the level of expression pol β protein in embryos of fresh and aged seeds after 24 h of water treatment. Protein extracts of embryos from different year seeds after 24 h of water treatment were analyzed by Western blot using the polyclonal antibody against rat DNA pol β protein. In lane 1, 2 μg protein from embryos of 24 h water-soaked current year seed; lane 2, 2 μg of 2 years 4 months aged seed; and lane 3, 2 μg of 4 years 3 months aged seed; were loaded for electrophoresis and Western blot analysis. Photograph of the Western blot is shown above.

dry seeds, from 3 or 24 h water-soaked embryos, and shoot tips of 48 and 72 h grown rice germinated seedlings were separated by SDS–PAGE and Western blot analysis was done with the rat DNA pol polyclonal antibody as probe. Photograph of the blot included in Fig. 4 shows that dry embryo, 3 h water-soaked embryo have very low level of the protein in comparison to the embryos of 24 h water-treated seeds. This observation matches well with our previous observation on activity assay. After germination gradual enhancement of DNA pol β level during growth of germinated rice seedlings was prominent. While laminar tissue contains low level of the DNA pol β protein, anther tissue contained high level of the enzyme as in 72 h grown shoot tips in comparison to dry embryos. Immunodetection of pol β protein (not visible by non-immunized rabbit serum)

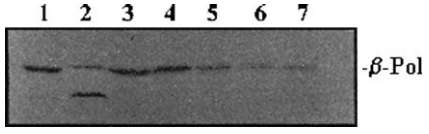

Fig. 4. Western blot analysis revealed the level of expression of pol β protein in different organs and growing shoot tips of rice plants. Western blot analysis of protein extracts prepared from anther (before anthesis) (lane 1); mature lamina (lane 2); and from shoot tips of germinated seedlings (72, 48 h, in lanes 3 and 4, respectively); from 24 h (lane 5) and 3 h (lane 6) water-soaked embryo and from embryos of freshly harvested dry seeds (lane 7) by the polyclonal antibody made against rat DNA polymerase β (pure IgG at 1:20,000 dilution). Photograph of Western blot showing high level of 67 kDa ddNTP-sensitive DNA polymerase β in anther and in shoot tips of 72 h grown germinated rice seedlings.

clearly shows regulation of expression of DNA pol β in rice plants.

Demonstration of BER activity by rice nuclear extract and its inhibition by inhibitor of DNA pol β

Uracil-initiated mismatch correction by BER has been elegantly demonstrated in bovine testis nuclear extract and different enzyme components including DNA pol β of multiprotein base excision repair complex have been purified [10] and [29]. Nothing is known about BER in plant system. Therefore, to find out the existence of BER in rice nuclear extract and involvement of ddNTP-sensitive rice DNA pol β in mismatch correction through BER, nuclear extracts were prepared from shoot tips of 72 h grown rice seedlings. Complementary oligonucleotides were custom-made and by annealing two pairs of oligonucleotide duplex 51 bp in length with either G:C normal pairing or G:U (U in lower strand at 22nd position) mismatch were made and used (Fig. 5A) as substrates for BER assay with equal amount of protein extracts (10 μg protein in each reaction) of 72 h grown shoot tip nuclear extract according to the protocol of Singhal et al. [10]. The lower strands were 5′-end labeled and annealed with equimolar amounts of upper strand. Oligo-duplex either with no mismatch or with G:U mismatch was incubated separately with rice nuclear extract and after the reaction at 37 °C for 10 min, appearance of a bands at 21-mer position and 51-mer position in the autoradiograph of DNA-sequencing gel was detected (Fig. 5B) as described in Materials and methods. In BER it is expected that only G:U mismatch oligo-duplex will be recognized by uracil DNA-glycosidase, and AP1 endonuclease, as a result 21 mer ^{32}P-labeled lower strand will be visible. Addition of dCTP will help to form 22 mer by a DNA polymerase and religation of 29 mer and 22 mer will create appearance of 51-mer band. Results presented in Fig. 5B (photograph of autoradiograph) showed that with normal duplex no band at 21-mer position was visible (lane 2), whereas with mismatch oligo-duplex as substrate, 21-mer product was visible (lane 4), which was then converted to 22 mer and then to the full-length product (51-mer DNA) in presence of dCTP (lane 5). Once the dCTP is incorporated the 51-mer band appeared as a landmark of mismatch correction. Addition of 200-fold excess of ddCTP along with dCTP shows the accumulation of 22-mer product (lane 6) as due to the incorporation of ddCMP. 51-mer products were unable to synthesize due to the absence of OH group at 3′-end of ddCMP. Whereas aphidicolin with dCTP did not show any noticeable inhibition on incorporation of dCMP as accumulation of 51-mer repaired product with very low level of 21-mer products was visible (lane 7). In addition, pre-incubation of the rice nuclear extract with polyclonal antibodies against rat β pol also showed complete inhibition of the mismatch correction or incorporation of dCMP (lane 8) as 21-mer band was visible and 51-mer band did not appear, showing inhibition of pol β activity and the specificity of the antibody. Complete inhibition of DNA pol β activity was probably due to very high level of the antibody and low level of enzyme in nuclear extract. These results clearly demonstrate the existence of mismatch correction by BER in rice nuclear extract and involvement of ddNTP-sensitive DNA polymerase β in BER.

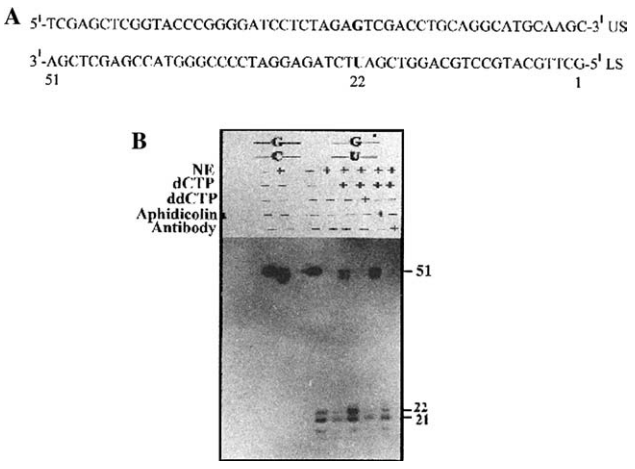

Fig. 5. Base excision repair by rice nuclear extract. (A) Sequences of duplex oligonucleotide substrate used for BER. Lower strand (LS) contained uracil (U) at position 22, relative to the 5′-end G at position 1. (B) As indicated at the top of each lane, the standard repair reaction (50 μl final volume) was carried out in the presence of rice nuclear extract (NE) alone (none) or in the presence of a 200-fold molar excess of ddCTP over dCTP or 10 μg/ml aphidicolin. Effect of antibody was tested by adding 1 μl of the antibody (5 μg IgG) to the nuclear extract on ice for 60 min before the addition of the labeled oligo-duplex as substrate. All the reactions were incubated at 37 °C for 10 min, stopped by adding EDTA and NaCl, and deproteinized by phenol:chloroform as described in Materials and methods. The number in the right-hand margin indicates the length (nts) of the radiolabeled products as judged by running DNA sequencing reactions in the four side lanes (not included in the photograph).

Discussion

Inhibitors of DNA polymerase enzyme, e.g., aphidicolin or ddNTPs are commonly used to identify the type of DNA polymerases. Multiple DNA polymerases differing in their amino acid sequence are sensitive to aphidicolin but insensitive to ddNTPs in mammalian system among them most well known are termed as α, δ, γ, and ε. The only ddNTP-sensitive and aphidicolin-insensitive, DNA polymerase in *E. coli* it is pol I, in yeast it is pol IV, and in mammalian system it is called as DNA pol β or β polymerase. The ddNTP-sensitive enzyme in mammalian system is unique from the other DNA polymerases because of non-processive type DNA

polymerase activity and active as single polypeptide enzyme [30]. Sensitive to ddNTP means that the enzyme cannot discriminate ddNTP from dNTP, thus once the ddNMP is incorporated during DNA synthesis the chain elongation stops. DNA pol I from *E. coli*, DNA pol IV from yeast are also ddNTP sensitive and aphidicolin insensitive. Although enzymological properties of DNA pol β were studied by purification from cells or tissue after cloning the cDNA from rat [9] and human [4,5], using recombinant protein structure–function relationship has been studied in great detail [5,31]. The functional significance of DNA pol β has been well correlated with DNA repairing events first by identifying the induction of the gene for pol β by MNNG [32] and then by manipulating its expression and monitoring susceptibility of the transformed cells to DNA damaging agents. When the expression of endogenous pol β gene was downregulated by antisense RNA, the transformed CHO cells were found to be more sensitive to mutagenic agents. On the other hand, the transformed CHO cells overexpressing DNA pol β showed improved resistance against mutagenic agents [32]. Presence of ddNTP-sensitive, aphidicolin-insensitive DNA polymerase was reported from wheat germ [12] and the DNA polymerase activity of the purified 52-kDa polypeptide was demonstrated [7]. Partial amino acid sequence of wheat enzyme was done and homology at the amino acid level with rat DNA pol β was revealed when the wheat partial amino acid sequence was aligned with amino acid sequence of rat DNA pol β [7].

Previously, we have reported [13] on the purification of a single polypeptide ddNTP-sensitive DNA pol β type DNA polymerase from shoot tips of 72 h grown germinated rice (*Oryza sativa* cv. IR-8) seedlings and have shown the ability of the purified enzyme to replicate homopolymer or heteropolymer DNA templates in distributive mode (non-processive). Functional significance of this enzyme in plant biology is not known and due to low abundance of the enzyme and inability to clone its cDNA. After we received the polyclonal antibody made against rat DNA polymerase β from Dr. S. H. Wilson (USA), we have repeated purification of the single polypeptide DNA polymerase enzyme sensitive to ddNTP from shoot tips of 72 h grown germinated rice seedlings following phosphocellulose column chromatography, gel filtration, and affinity chromatography. Finally, 3840-fold enrichment of specific activity with 3% recovery of the enzyme was obtained suggesting low abundance of the enzyme. Purity assessment by SDS–PAGE followed by detection of polypeptide by silver staining showed purification of 67-kDa polypeptide was near homogeneity (Fig. 1A), as no other major contaminating bands were visible. The other half of the same gel was subjected to Western blot analysis with the antibody against rat DNA polymerase β, cross-reaction of the antibody with the same polypeptide of 67-kDa was noticed even at 1:20,000 dilution of the antibody (Fig. 1B). The recognition was found to be very specific as the monoclonal antibody made against human pol β or non-immune rabbit serums failed to recognize the 67-kDa band (Data not shown) from similar enzyme preparations. The polyclonal antibody against rat DNA pol β was able to inhibit the DNA polymerase activity of the purified rice enzyme. The epitope in rice DNA pol β in native condition recognized by the antibody against rat enzyme is probably far from the active site as for activity inhibition large amount of antibody is required. The inhibition and recognition were specific as the activity of Klenow enzyme, bacterial ddNTP-sensitive DNA polymerase, was absolutely unaffected by the antibody (Fig. 2). Neither preimmune serum nor monoclonal anti rat DNA pol β antibody can recognize or inhibit. Recognition of the rice enzyme by rat DNA pol β antibody clearly suggests immunological relationship within the ddNTP-sensitive DNA polymerase β from rice and rat. Whether, the homologous domain detected by Luque et al. [7] in partial amino acid sequence of wheat and rat enzyme is also present in rice and acting as domain recognized by rat DNA pol β antibody remains a question. Conservation of amino acid sequence of many important enzymes including DNA polymerase α and DNA glycosylase during the evolution is well known [33] and [34]. Polyclonal antibody made against one protein frequently cross-reacts with the same enzyme from other heterologous source due to their similarity in amino acid sequence in the antibody recognizing epitope. In that case polyclonal antibody from one system provides opportunity to characterize the protein from other system or source [35].

Western blot analysis of rice embryo extract clearly showed enhancement of the level of pol β polypeptide in the water-soaked aged seeds in comparison to its level in the embryo of freshly harvested seeds (Fig. 3C). Activity gel assay of partially purified protein sample also showed high DNA polymerase activity from the protein sample of 24 h water-soaked embryos of 2 years 4 months rice seeds in comparison to dry embryo of fresh seeds. Through densitometric scanning (Fig. 3B) it has been found that the ddTTP-sensitive DNA pol activity in 24 h imbibed embryos of old seeds is ≈10-fold higher than in the viable embryos of 24 h water-soaked current year seeds (Fig. 3A, comparing lanes 3 and 4). Inhibition of incorporation of [α-^{32}P]dTMP by ddTTP in lanes 1 and 2 of activity gel analysis was significant and it was near around 2.5-fold as shown in histogram (lanes 1 and 2 of Fig. 3A).

Western blot analysis of protein extract prepared from different organs by using polyclonal antibody against rat DNA polymerase showed high level of 67-kDa polypeptide with DNA pol β activity in anther and shoot tips of growing embryo (Fig. 4). Presence of high level of pol β in developing anther clearly indicates its

involvement in the events of meiosis. Reports on detection of high level of DNA pol β in rat testis during spermatogenesis [36] and also in mouse testis [37] are already available where probable involvement of DNA pol β in recombination has been claimed. Using antibody against mouse DNA pol β, localization of DNA pol β enzyme was found in the synaptosomal complex that formed during recombination of chromosomes [38]. Thus, our result of Western blot analysis showing high level of DNA pol β could be due to the involvement of the enzyme in DNA recombination during meiosis in pollen mother cells.

In bovine testis nuclei DNA pol β was found to be an essential component of BER complex along with other proteins like uracil-DNA glycosylase, AP_1 endonuclease, and DNA ligase. DNA pol β mediated G:U mismatch correction by BER pathway has been elegantly demonstrated [10]. Therefore, DNA pol β has been considered as DNA repairing DNA polymerase, especially short patch repairing DNA polymerase [39]. Sensitivity of BER to ddNTPs but not to aphidicolin was also shown [10,29]. All organisms have developed protective mechanism to keep the integrity of their genomes against wide range of genotoxic effects induced by environmental factors (UV irradiation, bacterial, and fungal toxin) as well as by the intermediate products of normal cellular metabolism (e.g., alkylating and oxidizing agents). BER was first described in bacteria and later identified and analyzed extensively in yeast and mammals [40,41]. Unfortunately plant DNA repair machineries with few exceptions [42] and mechanism are very poorly known [43,44]. Though BER mechanism has not been demonstrated in plant system, the presence of other enzymes has been detected from different plant sources. uracil-DNA glycosylase and apurinic/apyrimidinic endodeoxyribonuclease were found in cultured cells of carrot [45]. These findings suggest that plants do have the enzyme components required to perform BER. A cDNA clone encoding adenine glycosylase was reported from *Arabidopsis* [46]. Uracil arises in DNA by two independent pathways, first deamination of cytosine to uracil, which occurs spontaneously [47] or in response to oxidizing chemical agents [48] giving rise to G:U mismatch. To find out the existence of BER to correct mismatch and involvement of ddNTP-sensitive rice DNA pol β in plant system, nuclei were made and nuclear extracts were prepared from the shoot tips of 72 h grown rice seedlings. Mismatch repair assay was done following the protocol of Singhal et al. [10] using two oligonucleotide-duplexes 51 bp in length with G:C or G:U mismatch used as substrate (Fig. 5A). In mismatch oligo-duplex two strands were complementary to each other except a G:U mismatch at 22nd position. Thus, the lower strands containing either C or U at 22nd position from 5′-end were labeled with ^{32}P and then annealed with equimolar amounts of upper strand. These oligo-duplexes when incubated with rice nuclear extract appearance of 21 mer ^{32}P-labeled lower strand due to the actions of uracil DNA glycosylase and AP_1 endonuclease, from 51 mer ^{32}P-labeled lower strand were detected only from G:U mismatch oligo-duplex, by the sequencing gel followed by autoradiography (Fig. 5B). Results of Fig. 5B indicate that while oligo-duplex with G:C was used as substrate no degradation was detectable. Whereas with G:U mismatch oligo-duplex, the 21 mer ^{32}P-labeled lower strand product was visible. The uracil base was selectively removed from mismatch oligo-duplex and dCMP was incorporated as complementary to the template G, and the full-length ^{32}P-labeled 51-mer products appeared. Addition of ddCTP (100-fold excess of dCTP) along with dCTP showed accumulation of 21- and 22-mer products, without formation of 51-mer product, suggesting incorporation of ddCTP by DNA pol β enzyme present in the rice nuclear extract and chain termination. Addition of aphidicolin to nuclear extract showed no effect on repairing ruling out the possibility of involvement of other DNA polymerases in the repair reaction. Addition of antibody against rat DNA pol β which inhibits rice DNA pol β partially, but significantly, to the nuclear extract inhibits incorporation of dCMP at 22nd position, therefore 21-mer products accumulate without appearance of 51-mer products. This also proves the specificity of the recognition of the rice DNA pol β by the antibody. The actual mechanism of mismatch repair whether by removing uracil, the base first and then by removing AP site, i.e., sugar–phosphate or by removing the UMP nucleotide, is difficult to detect.

Results presented in this paper clearly provide novel information on ddNTP-sensitive DNA polymerase from rice or plant system. Purification of the enzyme confirms the molecular weight of the enzyme as 67-kDa, as detected by SDS–PAGE followed by silver staining or Western blot. Recognition by the polyclonal antibody made against rat DNA polymerase β indicates immunological similarity with the rat pol β. Western blot analysis revealed the high level of expression of 67 kDa enzyme in developing anther and growing seedlings, probably due to its involvement in DNA repairing. Demonstration of BER by rice nuclear extract to correct mismatch in DNA and involvement of DNA pol β shows commonality of the basic mechanism to repair mismatch in DNA and the factors involved in the concerted effort, within plant and animal system, suggesting conservation of the basic mechanism and the structure–function of the DNA polymerase, whose activity can be blocked by ddNTPs.

Acknowledgments

Generous gift of polyclonal antibodies (Purified IgG preparation) made against purified DNA polymerase β from rat from Dr. S.H.

Wilson and Dr. Rajendra Prasad (NIESH, Research Triangle, NC, USA) is also gratefully acknowledged. We are thankful to Mr. Jadab Kr. Ghosh for his excellent assistance in Central Instrument Facility in the Botany Department, Bose Institute.

References

[1] H. Chen, S.K. Bryan, R.E. Moses, Cloning the pol B gene of *Escherichia coli* and identification of its product, J. Biol. Chem. 264 (1989) 20591–20593.
[2] E.G. Minkley Jr., A.T. Leney, J.B. Bodner, M.M. Panicker, W.E. Brown, *Escherichia coli* DNA polymerase I. Construction of a pol A plasmid for amplification and an improved purification Scheme, J. Biol. Chem. 259 (1984) 10386–10392.
[3] Y. Kawasaki, A. Sugino, Yeast Replicative DNA polymerases and their role at the replication fork, Mol. Cells 12 (2001) 277–285.
[4] D.N. Sengupta, B.Z. Zmudzka, P. Kumar, F. Cobianchi, J. Swowrovski, S.H. Wilson, Sequence of human DNA polymerase β mRNA obtained through cDNA cloning, Biochem. Biophys. Res. Commun. 136 (1986) 341–347.
[5] J. Abbots, D.N. Sengupta, B.Z. Zmudka, S.G. Widen, V. Notario, S.H. Wilson, Expression of human DNA polymerase β in *Escherichia coli* and characterization Of the recombinant enzyme, Biochemistry 17 (1988) 901–909.
[6] M.C. Richards, S. Litvak, M. Castroviejo, DNA polymerase from wheat embryos: a plant δ like DNA polymerase, Arch. Biochem. Biophys. 287 (1991) 141–150.
[7] A.E. Luque, J.P. Benedetto, M. Castroviejo, Wheat DNA polymerase C1: homologue of rat DNA polymerase β, Plant Mol. Biol. 38 (1998) 647–654.
[8] M. Yokoy, M. Ito, M. Izumi, H. Miyazawa, H. Nakai, F. Hanaoka, Molecular cloning of the cDNA for the catalytic subunit of plant DNA polymerase α and its cell cycle dependent expression, Genes Cells. 2 (1997) 695–709.
[9] B.Z. Zmudzka, D.N. Sengupta, A. Matsukage, F. Cobianchi, P. Kumar, S.H. Wilson, Structure of rat DNA polymerase β as revealed by partial amino acid sequencing and cDNA cloning, Proc. Natl. Acad. Sci. USA 83 (1988) 5106–5110.
[10] R.K. Singhal, R. Prasad, S.H. Wilson, DNA polymerase β conducts the Gap-filling step in uracil-initiated base excision repair in a Bovine testis nuclear extract, J. Biol. Chem. 270 (1995) 949–957.
[11] S.H. Leem, P.A. Ropp, A. Sugino, The yeast *Saccharomyces cerevisiae* DNA polymerase IV: possible involvement in double strand break DNA repair, Nucleic Acids Res. 22 (1994) 3011–3017.
[12] M. Castroviejo, T.M. Gatius, S. Litvak, A low molecular weight DNA polymerase from wheat embryos, Plant Mol. Biol. 15 (1990) 383–397.
[13] M. Sanath Kumar, B. Ghosh, D.N. Sengupta, Isolation of mammalian pol β type DNA polymerase from the shoot tips of germinated seedlings of IR-8 rice (*Oryza sativa* L.), Biochem. Mol. Biol. Int. 39 (1996) 117–136.
[14] H.R. Warner, A.R. Price, Involvement of DNA repair in cancer and aging, J. Gerentol. 44 (1989) 45–54.
[15] E. Mullaart, P.H. Loahman, F. Berends, J. Vijg, DNA damage metabolism and aging, Mutat. Res. 237 (1990) 189–210.
[16] V.A. Bohr, R.M. Anson, DNA damage, mutation and fine structure DNA repair in aging, Mutat. Res. 338 (1995) 25–34.
[17] C.A. Walter, D.T. Grabowski, K.A. Street, C.C. Conrad, A. Richardson, Analysis and modulation of DNA repair in aging, Mech. Aging Dev. 98 (1997) 207–222.
[18] J. Vijg, M.E. Dolle, H.J. Martus, M.E. Boerrigter, Transgenic mouse models for studying mutations in vivo: applications in aging research, Mech. Aging Dev. 98 (1997) 189–202.
[19] K.S.E. Cheah, D.J. Osbrone, DNA lesions occur with loss of viability in embryos of ageing rye seeds, Nature (London) 272 (1978) 593–599.
[20] G. Koppen, L. Verschaeve, The alkaline single-cell electrophoresis/comet assay: a way to study DNA repair in radicle cells of germinating *Vicia faba*, Folia Biol. (Praha.) 47 (2001) 50–54.
[21] G. Ries, W. Heller, H. Puchta, H. Sandermann, H.K. Seidlitz, B. Hohn, Elevated UV-B radiation reduces genome stability in plants, Nature 406 (2000) 30–31.
[22] E.H. Roberts, Seed Aging: the genome and its expression, in: L.D. Nooden, A.C. Leopold (Eds.), Senescence and Aging in Plants, Academic Press, San Diego, 1988.
[23] M. Murta, T. Jsuchiya, E.E. Roos, Chromosome damage induced by artificial seed aging in barley: II Types of chromosomal aberration at first mitosis, Bot. Gaz. 143 (1982) 111–116.
[24] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[25] J. Sambrook, D.W. Russell, Molecular cloning, Vol. III, Cold Spring Harbor Laboratory Press, Cold Spring Harbour, New York, 2001.
[26] A. Blank, J.R. Silber, M.P. Thelen, C.A. Dekker, Detection of enzymatic activities in sodium dodecyl sulfate–polyacrylamide gels: DNA polymerase as model enzyme, Anal. Biochem. 125 (1983) 423–430.
[27] E. Karawya, J.A. Swack, S.H. Wilson, Improved conditions for activity gel analysis DNA polymerase catalytic polypeptides, Anal. Biochem. 135 (1983) 318–325.
[28] S. Gupta, M.K. Chattopadhyay, P. Chaterjee, B. Ghosh, D.N. SenGupta, Expression of abscisic acid-responsive element-binding protein in salt-tolerant indica rice (*Oryza sativa* L. cv. Pokkali), Plant Mol. Biol. 37 (1998) 629–637.
[29] R. Prasad, R.K. Singhal, D.K. Srivastava, J.T. Molina, A.E. Tomkinson, S.H. Wilson, Specific interaction of DNA polymerase β and DNA ligase I in a multiprotein base excision repair complex from bovine testis, J. Biol. Chem. 271 (1996) 16000–16007.
[30] P.M. Burgers et al., Eukaryotic DNA polymerases: proposal for a revised nomenclature, J. Biol. Chem. 276 (2001) 43487–43490.
[31] A. Kumar, S.G. Widen, K.R. Williams, S.H. Wilson, Studies of the domain structure of mammalian DNA polymerase beta. Identification of a discrete template binding domain, J. Biol. Chem. 265 (1990) 2124–2131.
[32] A.J. Fornace Jr., B. Zmudzka, M.C. Hollander, S.H. Wilson, Induction of beta-polymerase mRNA by DNA damaging agents in Chinese hamster ovary cells, Mol. Cell. Biol. 9 (1989) 851–853.
[33] W.S. Wong, A.F. Wahl, P.M. Yuan, N. Arai, B.E. Pearson, K. Arai, D. Korn, W. Hunkapiller, T.S.F. Wang, Human DNA Polymerase α gene expression is cell proliferation dependent and its primary structure is similar to both prokaryotic and eukaryotic replicative DNA polymerase, EMBO J. 7 (1988) 7–47.
[34] Y. Choi et al., Demeter, a DNA glycosylase domain protein is required for endosperm gene imprinting and seed viability in *Arabidopsis*, Cell 110 (2002) 33–42.
[35] M.L. Knetsch, M. Wang, B.E. Jagalska-Snaar, S. Dijkstra-Heimovaara, Abscisic acid induces mitogen-activation in barley aleurone protoplasts, Plant Cell 8 (1996) 1061–1067.
[36] R. Nowak, M. Woszezynski, J.A. Siedlecki, Changes in the DNA polymerase beta gene expression during development of lung, brain and testis suggest an involvement of the enzyme in DNA recombination, Exp. Cell Res. 191 (1990) 51–56.
[37] A.A. Alcivar, L.F. Hake, N.B. Hecht, DNA polymerase-beta and poly (ADP) ribose polymerase mRNAs are differentially expressed during the development of male germinal cells, Biol. Reprod. 46 (1992) 201–207.

[38] H.W. Plug, C.A. Clairmont, E. Sapi, T. Ashley, J.B. Sweasy, Evidence for a role for DNA polymerase beta in mammalian meiosis, Proc. Natl. Acad. Sci. USA 94 (1997) 1327–1331.
[39] A.J. Podlutsky, I.I. Dianova, S.H. Wilson, V.A. Bohr, G.L. Dianov, DNA synthesis and dRPase activities of polymerase beta are both essential for single nucleotide patch base excision repair in mammalian cell extract, Biochemistry 40 (2001) 809–813.
[40] A. Sancar, DNA Excision Repair, Annu. Rev. Biochem. 65 (1996) 43–81.
[41] A. Memisoglu, L. Samson, Base excision repair in yeast and mammalian, Mutat. Res. 451 (2000) 39–51.
[42] A. Li, D. Schuermann, F. Gallego, I. Kovalchuk, B. Tinland, Repair of damaged DNA by *Arabidopsis* cell extract, Plant Cell 14 (2002) 263–273.
[43] E.J. Vonarx, H.L. Mitchell, R. Karthikeyan, I. Chatterjee, B.A. Kunz, DNA repair in higher plants, Mutat. Res. 400 (1998) 187–200.
[44] A.B. Britt, Molecular genetics of DNA repair in higher plants, Trends Plant Sci. 4 (1999) 20–25.
[45] M. Talpaert-Borle, M. Liuzzi, Base-excision repair in carrot cells. Partial purification and characterization of uracil-DNA glycosylase and apurinic/apyrimidinic endodeoxyribonuclease, Eur. J. Biochem. 124 (1982) 435–444.
[46] A. Santerre, A.B. Britt, Cloning of a 3-methyladenine-DNA glycosylase from *Arabidopsis thaliana*, Proc. Natl. Acad. Sci. USA 91 (1994) 2240–2244.
[47] L.M. Montero, J. Filipski, P. Gil, J. Capel, J.M. Martinez-Zapater, J. Salinas, The distribution of 5-methylcytosine in the nuclear genome of plants, Nucleic Acids Res. 25 (1992) 3207–3210.
[48] E.C. Friedberg, DNA Repair, W.H. Freeman NY, 1985.