DNA POLYMERASE ACTIVITY FROM *TETRAHYMENA PYRIFORMIS*

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1. Introduction

The DNA polymerase of *Tetrahymena pyriformis* was first isolated from crude extracts of the cells by Pearlman and Westergaard [1, 2]. The specific activity of this polymerase was increased if, prior to extraction, the cells were treated with either ultraviolet irradiation, electron irradiation, methotrexate plus uridine or ethidium bromide [2–4]. Using Sephadex G-200 column chromatography in 0.5 M NaCl of crude extracts of *Tetrahymena*, two DNA polymerase activities were observed after ultraviolet irradiation, electron irradiation or methotrexate plus uridine treatment [3]. The enzyme fraction showing increase in specific activity was found in the mitochondria [4].

As a first step in asking questions about the relation of "nuclear" to mitochondrial DNA polymerases and about the enzymes involved in DNA replication and DNA repair [2, 5] we have devised a fractionation scheme to partially purify the DNA polymerase from untreated exponentially growing cells. Using this fractionation scheme, we have attempted to fractionate the activity from ethidium bromide treated cells. Our fractionation method was based on an observation made with the DNA polymerases from sea urchin embryos [6] and yeast [7] and observed independently by us. Under conditions of low salt, the DNA polymerase aggregates into high molecular weight species, and in high salt dissociates into a low molecular weight species. This characteristic of the enzyme has enabled us to purify the enzyme approximately 85 fold. Part of the increase in specific activity in ethidium bromide treated cells is detected as a new activity peak when chromatographed in low salt on Sephadex G-200.

2. Materials and methods

T. pyriformis, amicronucleate strain GL, was grown at 28° in 1.5% proteose peptone (Difco) and 0.1% liver extract (Nutritional Biochemical Co.) [1, 2]. Large batches were grown in 20–30 l of medium in 40 l carboys. Vigorous aeration was obtained by bubbling filtered air into the medium. Dow Corning antifoam A spray was used to minimize foaming. Cells were harvested in middle to late log phase ($1.5–2.5 \times 10^5$ cells/ml) with a Sharples continuous flow centrifuge. Cells were washed twice by centrifugation in 0.05 M Tris-HCl pH 8.5, 0.05 M EDTA, 0.5 M NaCl and stored at −20°.

DNA polymerase was assayed as described previously [1, 2] (see table 1).

For treatment of cell cultures, ethidium bromide was added to cultures (10^5 cells/ml) to a final concentration of 15 μM. Cells were then grown in the dark with aeration for 25 hr and were harvested and washed in the same way as untreated cells. During the period of incubation with ethidium bromide, the cell number doubled.

3. Results

3.1. *Partial purification of the DNA polymerase from untreated cells*

Table 1 describes the fractionation scheme used. Frozen cells were thawed, suspended in 5 volumes of homogenizing buffer (10^{-2} M Tris-HCl pH 7.9, 10^{-2} M $MgCl_2$, 6×10^{-2} M KCl), and homogenized in a Waring blender (fraction I). After centrifugation at 30,000 *g* for 20 min and 100,000 *g* for 2 hr, (fraction II), the activity from the 100,000 *g* supernatant was precipitated with $(NH_4)_2SO_4$ at 30–70% saturation

Table 1
Purification of DNA polymerase from exponentially growing *T. pyriformis*.

Fraction	Specific activity (cpm/20 min/ 0.3 mg protein) x 10^{-3}	Recovery (%)
I Crude homogenate	2.3	100
II 100,000 *g* supernatant	6.1	71
III 30–70% $(NH_4)_2SO_4$	12	63
IV Sephadex G-200 (0 M KCl)	31	38
V Sephadex G-200 (1 M KCl)	192	33

The standard assay under optimum conditions contained in 85 μl: Tris-HCl buffer pH 7.9, 1.5 μmoles; $MgCl_2$, 0.2 μmoles; KCl, 11.25 μmoles; dATP, dCTP and dGTP, 9.8 nmoles of each; ^{3}H-TTP (New England Nuclear Corp.), 1.5 nmoles specific activity 366 μCi/μmole; heat denatured calf thymus DNA, 10–30 μg; and 35 μl of enzyme (30–100 μg of protein). Activity is cpm incorporated in 20 min [1, 2].

(fraction III). Fraction III was dialyzed against buffer A (10^{-2} M Tris-HCl pH 7.9, 10^{-3} M EDTA, 10^{-4} M dithiothreitol (DTT), 20% glycerol) and then layered onto a Sephadex G-200 column equilibrated with buffer A. Elution was with buffer A. The DNA polymerase activity eluted close to the void volume (fig. 1a). Fractions containing the majority of the activity (fractions 28–39) were pooled and concentrated by vacuum dialysis (fraction IV). Fraction IV was made 1 M in KCl and layered onto Sephadex G-200 column equilibrated with buffer A + 1 M KCl. Elution was with buffer A + 1 M KCl. The DNA polymerase activity in high salt eluted after the bulk of the protein (A 280 nm) suggesting that the molecular weight had decreased in high salt (fig. 1b). The fractions containing activity (fractions 52–60) were pooled, concentrated by vacuum dialysis and dialyzed against buffer A (fraction V).

3.2. *Properties of fraction V*

Fraction V reveals three major protein bands under analytical disc gel electrophoresis using the method of Tobe and Loughton [8]. It is however not known which band or bands contain DNA polymerase activity.

The rate of reaction is linear for at least 1.5 hr, although there is a slight lag for the first 15 min of incubation. This lag is not eliminated when the enzyme is preincubated in the absence of triphosphates and

Table 2
Requirements of the DNA polymerase reaction with fraction V enzyme.

Omissions from complete system	Additions to complete system	Activity-(%)
None	None	100
None	PHMB (10 μM)	53
None	PHMB (100 μM)	< 1
None	DTT (45 mM after 20 min preincubation with 100 μM PHMB	< 1
Denatured calf thymus DNA	Denatured *Tetrahymena* DNA	220
Denatured calf thymus DNA	Native poly d(A-T)	98
Denatured calf thymus DNA	Denatured poly d(A-T)	67
Denatured calf thymus DNA	Native calf thymus DNA	12
Denatured calf thymus DNA	Nicked calf thymus DNA	< 1
Denatured calf thymus DNA	Native *Tetrahymena* DNA	24
KCl	None	28
KCl	NaCl	60
KCl	NH_4Cl	73
KCl	$(NH_4)_2SO_4$	88
$MgCl_2$	None	< 1
$MgCl_2$	$MnCl_2$	3
$MgCl_2$	$CaCl_2$	< 1

The complete system for assay is as described in table 1. All DNA templates were added at 20 μg/assay. Either NaCl, NH_4Cl or $(NH_4)_2SO_4$ were substituted for KCl at the same concentration as optimal for KCl (0.13 M). $MnCl_2$ or $CaCl_2$ were substituted for $MgCl_2$ at the same concentration as optimal for $MgCl_2$ (2.4 mM).

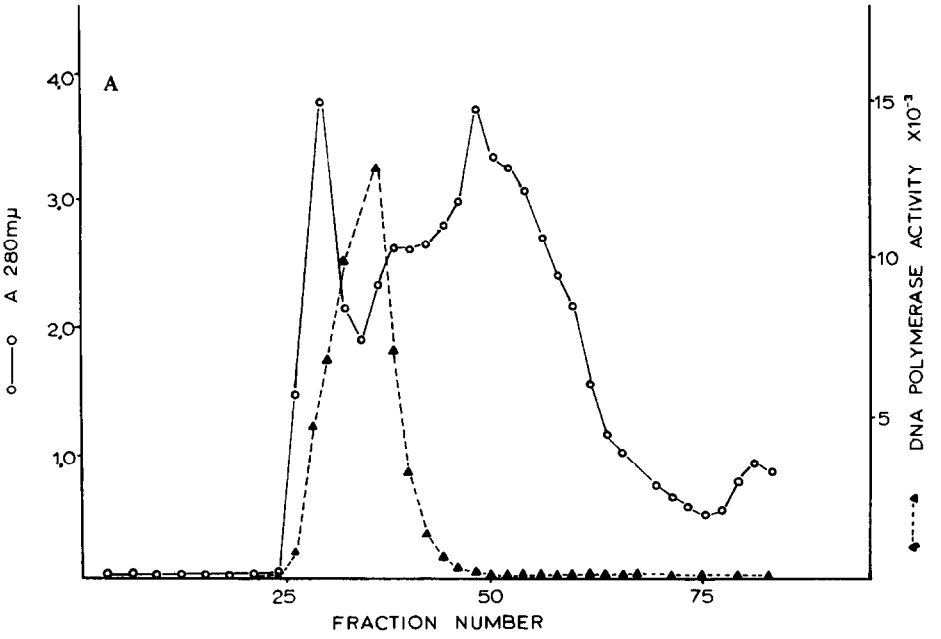

Fig. 1A.

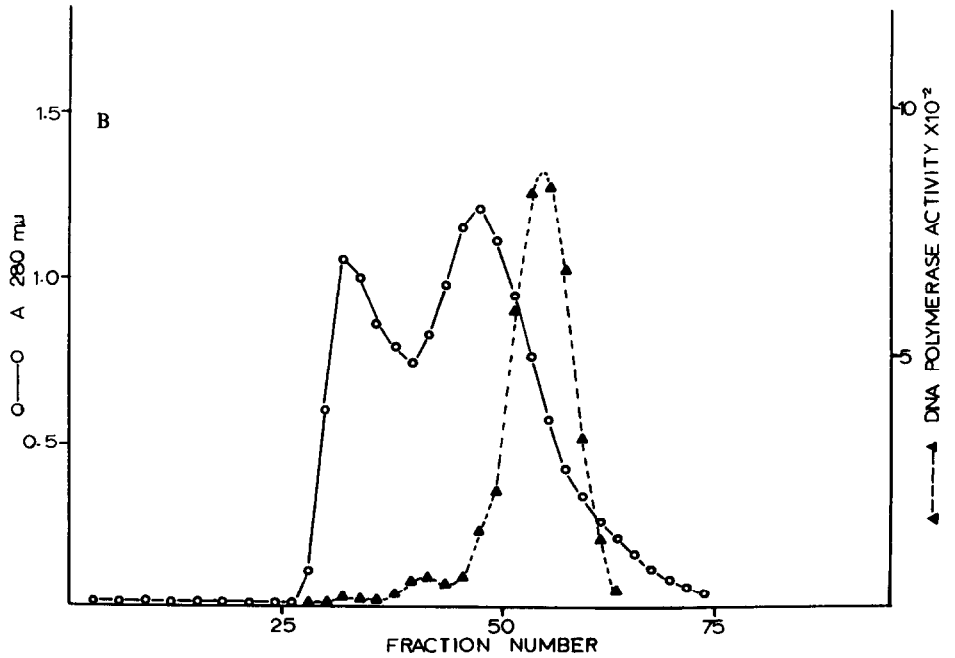

Fig. 1. Sephadex G-200 column chromatography. (A) Chromatography of fraction III. 14 ml of fraction III were layered onto the column (90 X 2.5 cm–void volume 130 ml) equilibrated with buffer A. Elution was with buffer A. 5 ml fractions were collected. DNA polymerase activity was measured as in table 1. (B) Chromatography of fraction IV. 10 ml of fraction IV made 1 M in KCl were layered onto the column (70 X 2.0 cm–void volume 90 ml) equilibrated with buffer A + 1 M KCl. Elution was with buffer A + 1 M KCl. 3 ml fractions were collected. DNA polymerase activity was measured as in table 1.

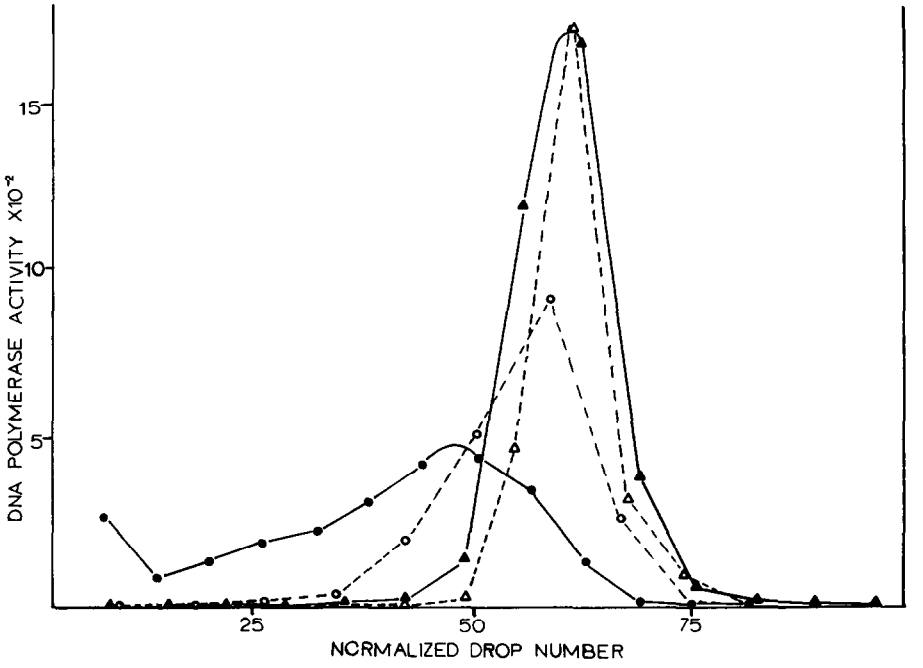

Fig. 2. 10–30% glycerol gradient centrifugation of fraction V activity. 10–30% glycerol gradients containing various concentrations of KCl in 10^{-2} M Tris-HCl pH 7.9, 10^{-3} M EDTA, 10^{-4} M DTT were centrifuged at 4° for 15 hr at 38,000 rpm in International B60 ultracentrifuge in the SB 405 rotor. Fraction V was diluted to approximately 8% glycerol before being applied to the gradient. Equal amounts of activity were applied to each gradient. Samples of fractions collected were assayed for DNA polymerase activity as in table 1. Sedimentation is from right to left. Molecular weights from glycerol gradient analyses were determined by the method of Martin and Ames [10] using γ globulin as a molecular weight standard (MW = 150,000). ●—● 0 M KCl; ○- - -○ 0.1 M KCl; ▲—▲ 0.25 M KCl; △- - -△ 0.4 M KCl.

DNA. The enzyme is stable for at least 3 months when stored at −76°. The reaction is irreversibility inhibited by p-hydroxymercuribenzoate (PHMB) (table 2).

Fraction V polymerase requires Mg^{2+}, K^+, the four deoxyribonucleoside triphosphate and DNA for optimal activity. The polymerase utilizes denatured *Tetrahymena* DNA as its best template. Denatured calf thymus DNA and native and denatured poly d(A-T) also serve as good templates whereas native *Tetrahymena* and calf thymus DNA and native calf thymus DNA partially digested with pancreatic DNase [5] are poor templates (table 2). All templates were assayed only under the optimal ionic conditions for denatured calf thymus DNA.

The polymerase without Mg^{2+} loses all of its activity, while it retains 30% of its activity if K^+ is omitted. When either NaCl, NH_4Cl or $(NH_4)_2SO_4$ are substituted at the same concentration as optimal for KCl, slightly lower activity is obtained. $MnCl_2$ or $CaCl_2$ added at the same concentration as optimal for $MgCl_2$ are poor substitutes for $MgCl_2$ (table 2).

3.3. *Nuclease activity*

Fraction V contains nuclease activity, measured by the method of Lehman [9], which requires $MgCl_2$ and a pH of 8.8 for optimal activity. Denatured *E. coli* DNA is a better substrate than native *E. coli* DNA. Nuclease activity is very low when measured under the same ionic conditions as the polymerization assay but omitting the four deoxyribonucleoside triphosphates. Preliminary experiments suggest the nuclease activity is exonucleolytic. Further purification of the polymerase activity will show whether or not nuclease activity is associated with the polymerase activity.

3.4. *Glycerol gradient centrifugation*

The association-dissociation characteristics of fraction V have been studied by employing 10–30% glycerol gradients at various concentrations of KCl. In

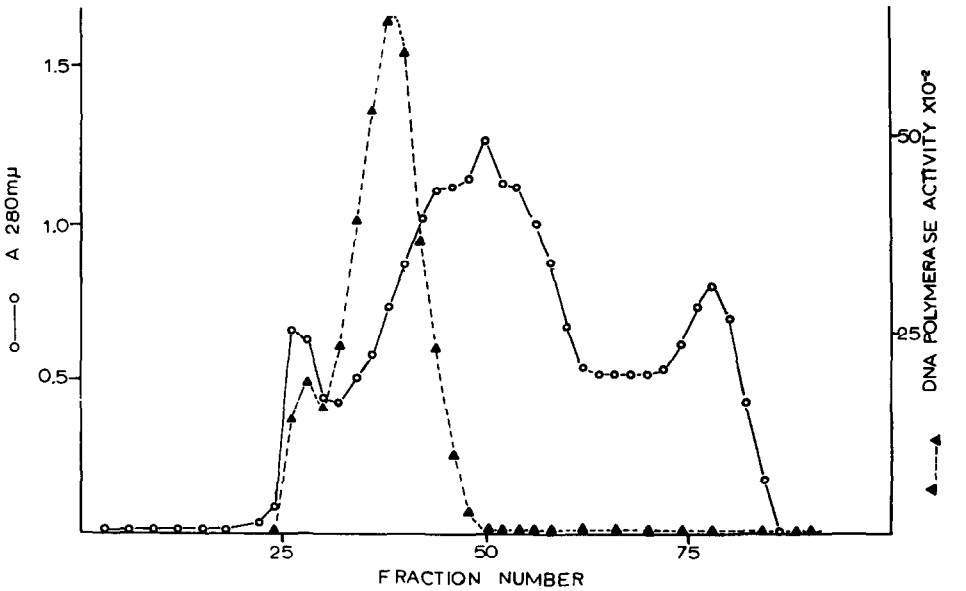

Fig. 3. Sephadex G-200 column chromatography of the 30–70% $(NH_4)_2SO_4$ fraction from ethidium bromide treated cells. 7.9 ml of the 30–70% $(NH_4)_2SO_4$ fraction was layered onto the Sephadex column (90 × 2.5 cm, void volume 130 ml) equilibrated with buffer A. Elution was with buffer A. 5 ml fractions were collected. DNA polymerase activity was measured as in table 1. Fractions 26–30 (peak A) were pooled as were fractions 32–44 (peak B).

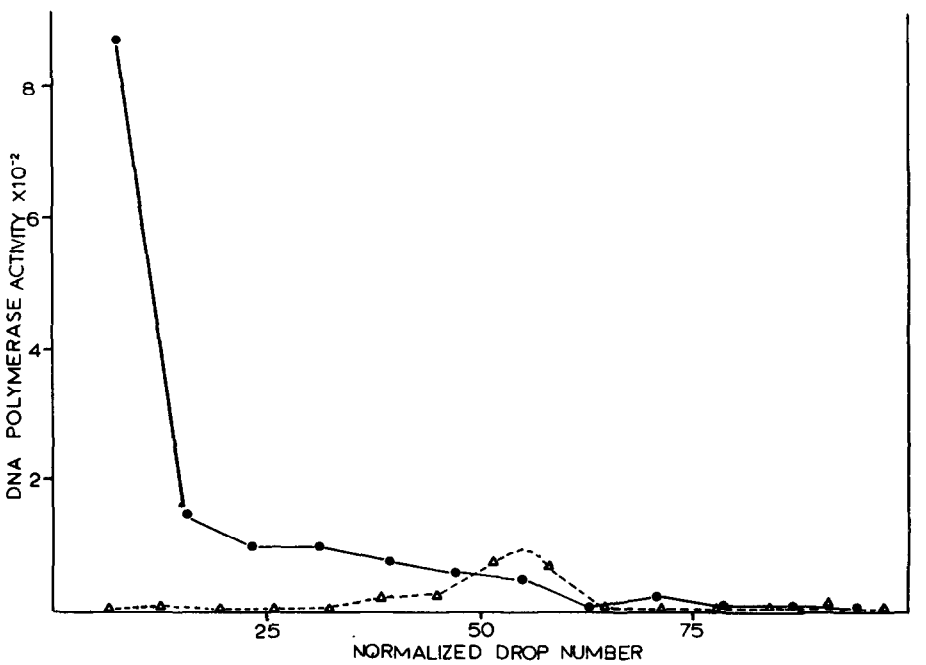

Fig. 4. 10–30% glycerol gradient centrifugation of the activity from peak A from Sephadex G-200 column chromatography of the 30–70% $(NH_4)_2SO_4$ fraction from ethidium bromide treated cells. Glycerol gradient centrifugation was as described in fig. 2. Equal amounts of activity were applied to each gradient. ●—● 0 M KCl; △- - - △ 0.25 M KCl.

the absence of KCl, the main peak of activity has a molecular weight of approximately 150,000. Higher molecular weight aggregates are also observed. In higher salt concentrations, the activity sediments more slowly and has a molecular weight between 80,000–100,000. Partial dissociation (i.e. lower molecular weight activity) is observed at 0.1 M KCl and complete dissociation at 0.25 M KCl (fig. 2). The dissociation is dependent on ionic strength since $MgCl_2$ at comparable ionic strengths to KCl gives similar results. It therefore appears that under optimal assay conditions (μ = 0.14 M) the dissociated species is more abundant although reassociation of the complex may occur due to the presence of template or triphosphate.

3.5. *DNA polymerase from ethidium bromide treated cells*

After cells have been treated with ethidium bromide, there is an approximately 6 fold increase in the specific activity of the DNA polymerase in the 30–70% $(NH_4)_2SO_4$ fraction (same fractionation scheme as for untreated cells). When this fraction is layered onto a Sephadex G-200 column in low salt (buffer A), two peaks of activity are eluted (fig. 3). The first peak of activity, peak A, elutes close to the void volume and has an A 260/280 of 1.0 whereas the second peak, peak B, elutes at the same position as fraction IV from untreated cells and has an A 260/280 of 0.65. Native calf thymus DNA is not a good template for either peak A or B activity.

Peak A was pooled and layered onto 10–30% glycerol gradients with and without KCl. The polymerase activity at 0 M KCl sediments to the bottom of the gradient. At 0.25 M KCl, the activity sediments at the same position as the polymerase from untreated cells (80,000–100,000 MW) but 90% of its activity has been lost (fig. 4). This suggests high ionic strength conditions may inactivate peak A activity.

Glycerol gradient analysis of peak B is similar to the gradients of fraction V from untreated cells and the activity from peak B elutes from a Sephadex G-200 column in buffer A plus 1 M KCl in the same position as fraction V activity from untreated cells.

4. Discussion

We have purified a DNA polymerase activity approximately 85 fold from exponentially growing cultures of *Tetrahymena pyriformis*. The enzyme is found as high molecular weight species in low salt and dissociates into a low molecular weight species in high salt. The high molecular weight species of the enzyme likely do not represent enzyme-DNA complexes but more likely represent aggregates of the low molecular weight species. This is consistent with the following data. The A 260/280 of fraction V is 0.85, the reaction catalyzed by fraction V is dependent on exogenous DNA and the DNA polymerase activity after Sephadex G-200 column chromatography in buffer A plus 1 M KCl reassociates into high molecular weight species when dialyzed against buffer A.

When working with large volumes of *Tetrahymena*, ethidium bromide is the most satisfactory reagent for inducing an increase in the specific activity of DNA polymerase. Chromatography of the 30–70% $(NH_4)_2SO_4$ fraction from treated cells on Sephadex G-200 in buffer A without KCl results in two distinct peaks of activity. Westergaard has also observed two distinct peaks of activity on Sephadex G-200 column chromatography in 0.5 M NaCl of the 12,000 *g* supernatant from cells treated with methotrexate plus uridine, electron irradiation or ultraviolet irradiation [3]. The two activities observed by Westergaard differ in their template requirements and in their ionic requirements. The two peaks observed by us are similar in their template requirements. Both activities observed by us utilize denatured DNA as their best template.

Activity in peak B from ethidium bromide treated cells is similar in chromatographic and dissociation properties to fraction V activity from untreated cells. Peak A activity from treated cells differs from peak B activity from treated cells and from fraction V activity from untreated cells in chromatographic properties on Sephadex G-200 in 0 M KCl and in dissociation properties observed on glycerol gradients. It is suggested that the activity from peak A represents a DNA polymerase distinct from the DNA polymerase isolated

from untreated cells. It is however, difficult to make any correlations between peaks A and B from ethidium bromide treated cells and the two peaks of activity observed by Westergaard. In this regard, it will be of interest to use our fractionation scheme with activity induced by agents other than ethidium bromide. Fractionation of DNA polymerase activity from mitochondria of *Tetrahymena pyriformis* and comparison of this with activity from untreated cells will also be of interest. These approaches may be valuable in examining the role of the nucleus in control of mitochondrial function [4] and in examining the enzymology of DNA replication and DNA repair in a eucaryotic organism [2, 5].

Acknowledgements

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