Presence of two DNA polymerases in Tetrahymena pyriformis

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ABSTRACT

Two DNA polymerases were detected in *Tetrahymena pyriformis*, strain GL. One (enzyme I) was sensitive to N-ethylmaleimide, while the other (enzyme II) was insensitive. The molecular weight of the enzymes, as determined by glycerol gradient centrifugation analysis, were approximately 130,000 and 70,000, respectively. Optimal concentration of MgCl₂ was 10mM for enzyme I and 18mM for enzyme II. KCl inhibited enzyme I but stimulated enzyme II. Poly (dA-dT) served effectively as a template for enzyme I, while poly(dA).(dT)₁₂₋₁₈ was an effective template for enzyme II.

Enzyme I activity increased with cell growth and sharply declined after the cells reached the stationary phase. On the other hand, enzyme II activity appeared only at the end of log phase. In cells synchronized by starvation-refeeding technique enzyme I was markedly stimulated in correspondence to the rate of DNA synthesis, whereas the level of enzyme II activity changed to lesser extent. By ethidium bromide treatment, only enzyme I activity was induced.

INTRODUCTION

Evidence has been accumulated which indicates that three DNA polymerases named I, II and III are present in bacterial cells (1) and also three DNA polymerases named α, β and γ in higher eukaryotic cells (2). Combined genetic and biochemical studies have allowed characterization and functional analysis of bacterial DNA polymerases, and considerable information concerning DNA polymerases in higher eukaryotes has also been accumulated. On the other hand, the nature of DNA polymerases in lower eukaryotes is much less known, although the existence of two or more DNA polymerases was reported in such organisms (3-9).

DNA polymerase of *Tetrahymena pyriformis* was first demonstrated in the crude extracts by Pearlman and Westergaard (10), and further Westergaard (11) showed that the enzyme activity was separated into two peaks by Sephadex G-200 gel filtration. However, Crerar and Pearlman (12-13) identified and purified only one DNA polymerase species, and they have seen no evidence for a second enzyme activity.
In this paper we report evidence indicating that at least two DNA polymerases with different properties are present in the extract of *Tetrahymena pyriformis*, and their possible functional roles are also discussed.

**MATERIALS AND METHODS**

**Culture**

*Tetrahymena pyriformis*, strain GL, was grown in 2% proteose peptone-0.5% yeast extract at 28°C. The cell density was measured by counting the cells under microscope after they were fixed by NaN₃.

**Preparation of cell extracts**

The *Tetrahymena* cells were washed twice with phosphate buffered saline (7.0mM potassium phosphate, pH 7.2, containing 0.14M NaCl). To packed cells, 5 volumes of the extraction buffer (50mM Tris-HCl, pH 7.6, containing 0.25M KCl, 10mM MgCl₂, 0.5mM DTT, 0.2mM EDTA and 12.5% glycerol) was added and the cells were broken by 100 strokes in a Dounce homogenizer, then sonicated at 140W for 1 min in Kubota Insonator model 200M. The suspension was centrifuged at 90,000 x g for 40 min at 4°C. The supernatant contained all of the DNA polymerase activity in the homogenate.

**DNA polymerase assay**

Unless stated otherwise, DNA polymerase activity was measured by the incorporation of [³H]TTP into acid-insoluble material using activated DNA as template. For assay of combined activity of enzyme I and enzyme II, 0.1 ml of a reaction mixture containing 50mM Tris-HCl, pH 7.8, 10mM MgCl₂, 0.25mM each of dATP, dCTP and dGTP, 0.05mM [³H]TTP(0.1-0.5Ci/mmole), 0.25mg/ml activated calf thymus DNA and 0.5mg/ml BSA, was incubated for 30 min at 28°C. Enzyme II was assayed in the presence of 5mM NEM, which specifically inhibited enzyme I. Enzyme I activity was calculated by correcting for the contribution of enzyme II. DNA was activated by treating with pancreatic DNase I at 37°C for 20 min as previously reported (14).

In some experiments enzyme II was assayed with poly(dA).(dT)₁₂-₁₈ template system containing 50mM Tris-HCl, pH 7.6, 20mM MgCl₂, 0.4 A₂₆₀nm units/ml poly (dA).(dT)₁₂-₁₈, 0.05mM [³H]TTP, 0.5mg/ml BSA and 1mM DTT. Each reaction was linear with time for at least 30 min.

The reaction products were detected as acid-insoluble radioactivity on GF/C glass fiber filters as previously described (14). One unit of DNA polymerase was defined as the incorporation of 1 nmole [³H]TTP into acid-insoluble form in 30 min. The specific activity was expressed as units per 10⁸ cells.
Synchronization by starvation-refeeding

*Tetrahymena* cells were synchronized by starvation-refeeding procedure of Bols and Zimmerman (15). The cell densities were about 3-5 x 10^4 cells/ml at the start of refeeding. The cells were grown at 28°C without shaking.

Incorporation of [^3]H]thymidine into DNA

At indicated times during the cell cycle, [^3]H]thymidine (5 Ci/mmole) was added to 2 ml of the culture to give 2.5 μCi/ml. Incubation was carried out at 28°C without shaking for 20 min, and terminated by addition of 2 ml of ice-cold 10% trichloroacetic acid. After extraction was carried out overnight at 4°C, acid-insoluble materials were collected on GF/C glass fiber filter as described by Bols and Zimmerman (15).

Glycerol gradient centrifugation analysis

The preparation of cell extract for glycerol gradient centrifugation analysis was the same as described above, except that the buffer used contained a higher concentration of KCl (0.5M). The extract (0.2 ml) was layered on 5 ml of 15-30% glycerol gradient (containing 25mM Tris-HCl, pH 7.8, 0.5M KCl, 1mM DTT and 1mM EDTA) and centrifuged at 220,000 x g for 21.5 hours at 4°C. Fractions (0.2 ml) were collected from the bottom with a peristaltic pump. BSA, yeast alcohol dehydrogenase and bovine liver catalase were used as markers. For enzyme assays 10 μl each of fraction were used.

Ethidium bromide treatment

Ethidium bromide treatment of cell cultures was performed according to the method of Westergaard and Lindberg (16). Ethidium bromide was added to culture (3.5 x 10^4 cells/ml) to a final concentration of 0.1mM. Cells were grown in the dark for 36 hours, harvested and washed in the same way as untreated cells.

Chemicals

[^3]H]TTP (30 Ci/mmole) and [^3]H]thymidine (5 Ci/mmole) were purchased from the Amersham/Searle Corporation (England). Calf thymus DNA, poly(dA)(dT)_{12-18}, dATP, dCTP, dGTP and TTP were obtained from P-L Biochemicals, Inc. (Milwaukee, Wis.). Poly(rA)(dT)_{12} and poly(dA·dT) were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Pancreatic DNase I was purchased from Sigma Chemical Company (St. Louis, Mo.). All other chemicals were of reagent grade.

RESULTS

DNA polymerase in *Tetrahymena pyriformis* cells

The extracts of *Tetrahymena* cells in late log phase were subjected to glycerol gradient centrifugation. At first, the centrifugation was performed
on 12-30% glycerol gradient for 18 hours at 165,000 x g, which were the conditions similar to those used by Pearlman and Crerar (13). One peak of DNA polymerase activity was observed with a small shoulder (not shown in figure). In order to obtain better separation, centrifugation was then performed on 15-30% glycerol gradient for 21.5 hours at 220,000 x g. As shown in Fig. 1, two peaks of DNA polymerase activity were detected, corresponding to a molecular weight of approximately 130,000 and 70,000, respectively. When enzyme activity was measured in the presence of 5mM NEM, only more slowly sedimenting one of the two peaks remained at the same level as that in the absence of 5mM NEM. The results indicated that the enzyme with a lower molecular weight is NEM-insensitive DNA polymerase and the other with a higher molecular weight is NEM-sensitive DNA polymerase.

To assure the difference between the two DNA polymerase species, the activity was assayed using various templates. The results are shown in Fig. 2. Neither polymerases could utilize native DNA as a template. Heat-denatured

Fig. 1 Glycerol density gradient centrifugation analysis of Tetrahymena DNA polymerase activities. Extract from cells in late log phase of growth (1.3 x 10^7 cells/ml) was layered on 15-30% glycerol gradient and centrifuged as described in Materials and Methods. Sedimentation was from right to left. The amount of the enzymes used in the reaction was 10 µl each of fraction. The incubation were carried out at 28°C for 60 min. O---O, total DNA polymerase activity; •••••, the enzyme activity measured in the presence of 5mM NEM (NEM-insensitive activity); O---O, the enzyme activity obtained by subtraction of NEM-insensitive activity from the total activity (NEM-sensitive activity); The sedimentation of BSA, alcohol dehydrogenase (ADH) and catalase (Cat.) in a control tube are indicated by arrows.
Fig. 2 Template specificity of *Tetrahymena* DNA polymerases. Each fraction was obtained by glycerol density gradient centrifugation for the experiment of Fig. 1. For the details, see legend to Fig. 1. Fractions were assayed for DNA polymerase activity with native calf thymus DNA (Δ--Δ), heat-denatured calf thymus DNA (Δ--Ω), poly(dA-dT) (Ο--Ο), poly(dA). (dT)_{12-18} (Δ--Ο ) and poly(rA). (dT)_{12} (Ο--Ο). MgCl$_2$ concentration was 10mM for the reaction with native and heat-denatured DNA, and 20mM for those with poly(dA-dT) and poly(dA). (dT)$_{12-18}$. When poly(rA). (dT)$_{12}$ was used as template, MnCl$_2$ was used at 0.5mM concentration.

DNA was poor template for both enzymes. The higher molecular weight enzyme catalyzed polymerization on poly(dA-dT), whereas the lower molecular weight one was relatively inactive under identical conditions. On the other hand, the latter enzyme could utilize poly(dA). (dT)$_{12-18}$, which was not used by the former one. The lower molecular weight species also used poly(rA). (dT)$_{12}$, although much less effectively.

These results show that *Tetrahymena* cells possess two DNA polymerases; a NEM-sensitive species with a molecular weight of 130,000 and a NEM-insensitive species with a molecular weight of 70,000, which are named enzyme I and enzyme II, respectively.

In an attempt to characterize further the two DNA polymerases, the optimal MgCl$_2$ and KCl concentrations for the reaction were determined. Both enzymes showed absolute requirement for a divalent cation. Enzyme I had MgCl$_2$ optimum at 10mM, while enzyme II gave highest activity at 18mM (Fig. 3). When MnCl$_2$ was added as the cation, optimal activity for enzyme I was achieved at 0.4mM, but the reaction rate was about 20% of that measured with 10mM MgCl$_2$.

Enzyme II activity was stimulated by KCl from 40 to 140mM concentration.
Fig. 3 Effect of MgCl₂ on the activity of *Tetrahymena* DNA polymerases.

Cell extracts were prepared as described in Materials and Methods, dialysed against a buffer containing 50mM Tris-HCl, pH 7.6, 0.5mM DTT, 0.2mM EDTA and 12.5% glycerol, and the enzyme activity was assayed. Standard reaction mixture was used except that varying MgCl₂ concentrations were employed. •, enzyme I activity; •—•, enzyme II activity.

and 2-fold higher than control in the presence of 80mM KCl, whereas it was inhibited by above 140mM KCl (Fig. 4). On the other hand, enzyme I activity was inhibited with increase of KCl concentration. The inhibition was 25% at 50mM KCl, and 55% at 100mM KCl.

pH optimum was 7.8 for both enzymes in 50mM Tris-HCl buffer. Similar results were obtained in 50mM potassium phosphate buffer, but the activities were lower. Based on these observations DNA polymerase reactions were routinely carried out in the presence of 50mM Tris-HCl buffer, pH 7.8 and 10mM MgCl₂.

These differences in the enzyme properties gave strong evidence for the presence of two distinct DNA polymerases in *Tetrahymena pyriformis*.

**Levels of *Tetrahymena* DNA polymerases at various growth stages**

In order to evaluate the physiological role of *Tetrahymena* DNA polymerases the levels of two DNA polymerases were measured at various stages of normal growth.
growth. A typical growth curve of *Tetrahymena pyriformis* is shown in Fig. 5A. At point A, the cell growth was initiated with a small inoculum of the stationary *Tetrahymena* cells. After lag period for about 3 hours the cells entered a logarithmic growth phase and reached a stationary phase at about 36 hours. DNA polymerase activities of the extracts were measured at several growth stages as indicated. As shown in Fig. 5B, enzyme I activity increased during log phase, while enzyme II activity remained at very low levels during early log phase, followed by drastic increase at the end of log phase. During the deceleration and stationary phases the activities of both enzyme I and II decreased sharply, and returned to the values at starting point. The same result was obtained when enzyme II activity was measured with poly(dA).

![Fig. 5](image-url)  
**Fig. 5** Typical growth curve of *Tetrahymena pyriformis* and DNA polymerase activities during the growth. At point A, the culture was inoculated with cell density of $10^3$ cells/ml. The cells were grown at 28°C with shaking and harvested at point A to H for determination of enzyme activity. Cell density (A) and the activity of enzyme I (B; open bar) and enzyme II (B; solid bar) were recorded.
(dT)$_{12-18}$, which was a strictly specific template for this enzyme (data not shown).

**Levels of Tetrahymena DNA polymerases during cell cycle**

Another approach to examine the functional role of Tetrahymena DNA polymerases is to compare the rate of DNA synthesis and the levels of the DNA polymerases during the cell cycle of synchronized Tetrahymena cells. It has been reported that starvation-refeeding technique which we utilized in this work, induces synchrony in the cycles of DNA replication and cell division of Tetrahymena pyriformis, and most of the population are in $G_1$ phase in completely starved cells (17).

After refeeding of the cells with nutrient medium, no cell division occurred for about 6 hours. And then first cell division was observed and the cell population doubled by 8.5 hours. The cells began to divide again at 9 hours after refeeding (Fig. 6A). Fig. 6B shows the rate of DNA synthesis measured by incorporation of $[^3H]$thymidine into DNA. The first incorporation was observed between 2 and 3 hours after refeeding, then the major incorporation occurred at 6 and 9 hours in synchrony with the cell division. Fig. 6C shows the results of the assay of enzyme I and enzyme II in the extracts. Enzyme I activity could not be detected at time zero, then began to rise, and apparent three peaks were observed in parallel with the peak of DNA synthesis. Enzyme II activity was present at detectable level at zero time, and it increased slightly during the increase of DNA synthesis. The small change of enzyme II activity was also detected when poly(dA).[dT]$_{12-18}$ was used as a template (data not shown).

**DNA polymerases from ethidium bromide treated cells**

The effect of ethidium bromide on the enzyme I and enzyme II was investigated. Ethidium bromide (0.1mM) was added to synchronized Tetrahymena cells after refeeding for 6 hours, which provided the high activities of enzyme I and enzyme II at a cell density of $3.5 \times 10^4$ cells/ml (Fig.6). At 36 hours after addition of the dye the cells were harvested. During the period the cell number tripled. Table 1 shows that ethidium bromide treatment of the cells induced a 4-fold increase in the specific activity of enzyme I, while the specific activity of enzyme II from treated cells was almost the same as that from untreated cells. Ethidium bromide treatment of the cells in late log phase (at E or F of Fig.5) similarly induced a several-fold increase in the specific activity of enzyme I.
DISCUSSION

The experiments reported in this paper demonstrate that at least two DNA polymerases (enzyme I and enzyme II) are present in Tetrahymena cells. These enzymes can be separated by use of glycerol gradient centrifugation, and the molecular weight of approximately 130,000 and 70,000 was estimated for enzyme I and enzyme II, respectively. NEM inhibited enzyme I but not enzyme II. Analysis of other properties of the reaction provided additional evidence for
Table 1 DNA polymerases from ethidium bromide treated cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific activity of DNA polymerase (U/10^8 cells)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>enzyme I</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>15</td>
</tr>
<tr>
<td>Cells treated with ethidium bromide</td>
<td>60</td>
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*Tetrahymena* cells after refeeding for 6 hours (3.5 x 10^4 cells/ml) were maintained in 0.1mM ethidium bromide for 36 hours and harvested.

distinguishing the two species. Enzyme I has MgCl₂ optimum at 10mM and is inhibited by KCl, while optimal concentration of MgCl₂ for enzyme II is 18mM and KCl stimulates enzyme II activity. Enzyme II can utilize more effectively synthetic templates than enzyme I. Analysis of the two polymerase activities through the growth and cell cycle, and the treatment by ethidium bromide also show the apparent difference between two enzymes.

In 1970 Westergaard showed that two DNA polymerase activities (enzyme A and enzyme B) were observed using Sephadex G-200 column chromatography of crude extracts of *Tetrahymena* in 0.5M NaCl, and that the two enzymes differed in their template requirements and in their ionic requirements (11). The enzyme A and enzyme B would appear to be related to the enzyme I and enzyme II in this paper, respectively, on the basis of 1) the requirement for divalent cation and 2) the effect by salt. However, there are some significant differences between enzyme B and enzyme II. Westergaard found that enzyme B was induced by various treatments to cause damage to DNA (ethidium bromide *etc.*), and was completely inhibited by sulphydryl blockers (11,16,18-20). On the other hand, not enzyme II but enzyme I in this paper was induced by ethidium bromide treatment and was sensitive to sulphydryl blockers.

In 1974 Crerar and Pearlman have identified and purified a single enzyme from exponentially growing cells (12-13). This enzyme appears to be essentially similar to enzyme I, since 1) both enzymes are the major DNA polymerase of exponentially growing cells; 2) both are sensitive to sulphydryl blockers; 3) both are induced if the cells are exposed to low concentrations of ethidium bromide; although the ability of both enzymes to copy synthetic templates is
somewhat different. They suggested (13) that this enzyme and enzyme B (11) may be the same.

The data we present here strongly suggest that enzyme II which is insensitive to NEM, is a unique DNA polymerase that differs from those previously reported. Crerar and Pearlman have seen no evidence for a second DNA polymerase activity (13). This might be explained by our finding that enzyme II activity is very low in exponentially growing cells. Purification are necessary to further demonstrate the correlation of these enzymes.

Another purpose of this work is to study the physiological role of Tetrahymena DNA polymerases. We measured the levels of enzyme I and enzyme II at various growth stages and during the synchronized cell cycle. The results showed the rise in enzyme I activity in growing cells and the fall in quiescent cells, and a clear correlation between the rate of DNA synthesis and the activity of enzyme I, indicating that enzyme I has the role as a replicative polymerase for DNA synthesis.

Enzyme II appeared at the end of log phase, and its activity was relatively constant throughout the synchronized cell cycle. These results cannot make the role of this enzyme very clear, but suggest a possibility that the enzyme may participate in the mitochondrial or nucleolar DNA replication. In Tetrahymena cells, it is well known that the replication of mitochondria and nucleolar DNA are controlled by a different mechanism from that of the bulk DNA (21-23). Besides, in Euglena gracilis, mitochondrial DNA polymerase is increased about 3-fold in stationary phase than in exponentially phase (24). Westergaard et al. described that the induced enzyme by a various chemical and physical agents that inhibit DNA replication (enzyme B) was associated with mitochondria (16,19), but it is recently suggested that the induced enzyme is likely to be the same enzyme as the purified one from exponentially growing cells, and different from the mitochondrial enzyme (25). It will be of interest to examine the intracellular location of enzyme II.

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ABBREVIATIONS

NEM : N-ethylmaleimide
DTT : dithiothreitol
BSA : bovine serum albumin
REFERENCES