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Biosynthesis and activity of DNA polymerase throughout the mitotic cycle of *Physarum polycephalum*

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Measurements of DNA polymerase protein levels and polymerase activity through the naturally synchronous mitotic cycle of *Physarum polycephalum* show that active DNA polymerase-α is synthesized throughout the G_2 phase, in step with the profile of general protein biosynthesis. Three main components of *P. polycephalum* DNA polymerase of 200, 112 and 70 kDa were found to be immunologically related.

DNA polymerase-α; Cell cycle; (*Physarum polycephalum*)

1. INTRODUCTION

A major requirement in reproducing the cell is to replicate the genome faithfully and completely. Although DNA replication requires the concerted action of many proteins, DNA polymerase plays a central role. The regulation of DNA polymerase during the cell cycle is conveniently studied in the plasmodial phase of *Physarum*, where all the nuclei exhibit natural mitotic synchrony [1].

The principal species of DNA polymerase in *Physarum* microplasmodia has been purified to apparent homogeneity and its molecular mass determined under denaturing conditions as 112 kDa [2]. The enzyme was designated as DNA polymerase-α [2] on the basis of its physical properties.

This page describes the pattern of synthesis of DNA polymerase-α during the cell cycle. The results provide insight into the strategies employed in order to replicate the genome of a syncitium committed to rapid growth.

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2. MATERIALS AND METHODS

2.1. *Radioimmunoassay (RIA) for DNA polymerase protein*

Polyclonal antisera to purified DNA polymerase-α isolated from *P. polycephalum* (strain M_3CVIII) were raised in rabbits [2], and a method for RIA was developed [2]. The amount of DNA polymerase-α protein present in macroplasmodial lysates was measured by RIA. Microplasmodia (strain M_3CVIII) were grown in shake culture [3,4] in the presence of [^{14}C]lysine for 24 h before use as an inoculum for the formation of a macroplasmodium grown on non-radioactive medium [3]. Samples of the macroplasmodium (approx. 200 mg wet wt) were removed at intervals during the third mitotic cycle [3]. Each sample was lysed in 0.3 ml high-salt buffer (3.4 M NaCl/2 mM EDTA/20 mM mercaptoethanol/200 mM Tris-HCl, pH 7.5). Lysates were clarified by centrifugation in an Eppendorf microfuge for 15 min. Each lysate was assayed for ^{14}C radioactivity (approx. 70 cpm/μl) by means of a Beckman LS7800 scintillation

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counter, for total protein (approx. 19 μg/μl) [3], and for DNA polymerase protein by RIA [2]. The ^{125}I-labelled DNA polymerase-α protein used in the RIA was dispensed using a micrometer syringe, and the radioactivity of the final precipitate was measured using a Packard auto-gamma (5230) counter [2]. Each RIA assay comprised duplicate measurements of 10 different amounts of a particular lysate ranging from 0.2 to 100 μg total protein. Two samples of macroplasmodia were taken after 6 h and were assayed independently.

2.2. *DNA polymerase activity measured in situ after SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

The procedure of SDS-PAGE was based on that previously described [5], except that activated (gapped) calf thymus DNA was included in the gel matrix at 150 μg/ml [6]. Proteins were denatured and DNA polymerase activity was measured as in [6]. Mini-gels (4 × 6 cm) did not have a spacer gel, and were incubated with 1 μCi/ml of [α-^{32}P]TTP (2000 Ci/mmol) at 27°C for 4–6 h. Large gels (20 × 20 cm) were incubated with 5.3 μCi/ml of [α-^{32}P]TTP (2000 Ci/mmol) at 37°C for 12 h. Radioactive label was purchased from Amersham International.

Samples of plasmodia (approx. 30 mg) were homogenized with extraction buffer (200 mM NaCl/2 mM EDTA/10 mM dithiothreitol/10%, v/v, glycerol/1 μM pepstatin/40 mM Tris-HCl, pH 6.8), and clarified by centrifugation in an Eppendorf microfuge for 5–10 min at 4°C. Total protein of each lysate was measured [7] and a volume equivalent to 50 μg total protein was used in the in situ assay for DNA polymerase activity. A sample of the lysate (50 μg total protein) was also assayed for DNA polymerase-α protein as described in section 2.3.

2.3. *Western immunoblotting*

Lysates were prepared (see section 2.2) and the proteins were separated by electrophoresis through 1% (w/v) SDS-PAGE gels before transfer to nitrocellulose filters [8]. Immunoblotting was performed as in [9], except that dried milk (Marvel, Cadbury Ltd) was added to all solutions at 2% (w/v) [10]. Goat anti-rabbit IgG-conjugated horseradish peroxidase second antibody (Miles)

Table 1

Accumulation of DNA polymerase-α protein measured by RIA during the mitotic cycle of a macroplasmodium

Time (h) after second mitosis	Amount (μg) of total protein containing 1 U DNA polymerase-α protein[a]	Relative mass of protein per plasmodium[b]	Relative mass of DNA polymerase-α protein per plasmodium[c]
0	4.2 ± 0.4	1.0 ± 0.065	1.0 ± 0.12
1	4.2 ± 0.4	1.05 ± 0.045	1.05 ± 0.12
2	5.2 ± 0.5	1.10 ± 0.045	0.89 ± 0.08
3	4.6 ± 0.5	1.04 ± 0.035	0.95 ± 0.12
4	4.2 ± 0.4	1.12 ± 0.035	1.12 ± 0.12
5	4.4 ± 0.4	1.15 ± 0.065	1.10 ± 0.12
6	3.1 ± 0.4[d]	1.35 ± 0.065[d]	1.83 ± 0.14[d]

[a] 1 unit is defined as the amount of DNA-polymerase-α protein needed to reduce by 50% the amount of ^{125}I-labelled DNA polymerase-α precipitated by antibody in the RIA

[b] Based on triplicate measurements of total protein ($P \pm 2\%$) and on triplicate measurements of radioactivity due to [^{14}C]lysine (R) [i.e. $(P/R)_{\text{time } t}/(P/R)_{\text{time } t=0}$]

[c] Computed from columns 2 (x) and 3 (y) [i.e. $(4.2y)/x$]

[d] Average for two samples of macroplasmodium harvested at the same time

was used with 2-amino-9-ethylcarbazole (Sigma) to visualize antibody binding [11].

3. RESULTS

The experiments were designed to take into account two problems. Firstly, DNA polymerase is known to be very sensitive to proteases [12–14]. For this reason measurements were made using cell lysates instead of subcellular fractions to reduce the time during which polymerase was exposed to proteases and the protease inhibitor pepstatin was added to lysates used in the in situ gel assays and Western blotting experiments. These precautions reduce, but do not eliminate proteolysis as may be seen by inspection of the autoradiographs of the in situ assays for DNA polymerase assays. Secondly, a reference point is needed to which the amount of DNA polymerase-α and total protein may be related. This was achieved by utilizing the observation that [^{14}C]lysine is largely incorporated into metabolically stable protein [3]. Thus, the radioactivity of a macroplasmodium previously labelled with [^{14}C]lysine should remain constant during the mitotic cycle. The specific activity of total protein will diminish in the G_2 phase as new protein is synthesized reaching one-half of the initial value when the cycle is completed. In *Physarum* the S phase immediately follows mitosis (e.g. [3]).

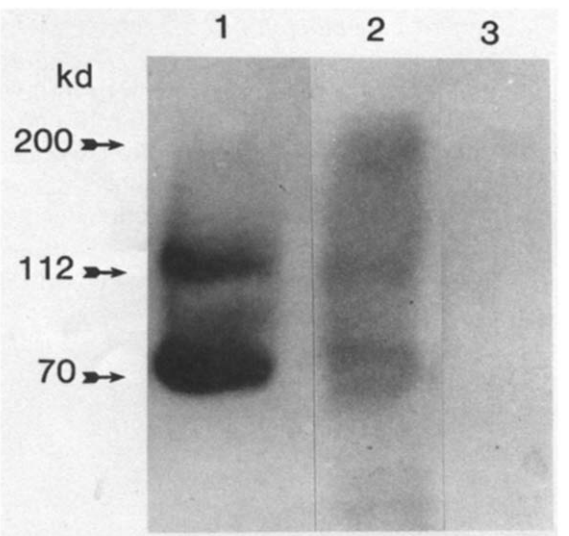

Fig.1. DNA polymerase antiserum is specific for *Physarum* DNA polymerase-α. A sample was taken in G_2 phase 7.5 h after the second mitosis (MII) from the macroplasmodium having a 10 h mitotic cycle. The sample was lysed and sufficient lysate to contain 46 μg total protein was loaded (lanes 1,2) onto a 1% (w/v) SDS-7.5% (w/v) polyacrylamide gel (4 × 6 cm) containing activated calf thymus DNA (150 μg/ml). The assay for DNA polymerase activity was carried out at 27°C for 4 h with 1 μCi/ml [α-^{32}-P]dTTP (2000 Ci/mmol). The gel was calibrated by means of *E. coli* DNA polymerase I and its Klenow fragment as detected by the in situ assay. Lanes: 1, autoradiogram of in situ assay of *Physarum* lysate; 2, autoradiogram of a Western blot of a replicate sample of *Physarum* lysate (bound DNA polymerase antibody was detected with 1 × 10^6 cpm/ml ^{125}I-labelled protein A); 3, 0.25 U of *E. coli* DNA polymerase I treated as in lane 2.

3.1. *Measurement by RIA of DNA polymerase protein*

Samples of a macroplasmodium having a 10 h mitotic cycle (3 h of S phase) were taken at the second mitosis (MII) and subsequently at hourly intervals. The samples were lysed and the amount of DNA polymerase protein present in the lysates was measured by RIA (see table 1). The amount of DNA polymerase-α protein was found to be essentially constant during the S phase (MII + 3 h) and to accumulate in G_2 phase when protein biosynthesis in general takes place [3].

3.2. *DNA polymerase activity measured in situ after SDS-PAGE*

The in situ gel assay for DNA polymerase activity [6] is valuable because the size of a polypeptide and its DNA polymerase activity may be correlated. Mobility during SDS-PAGE measures molecular size and the incorporation of ^{32}P-labelled nucleoside triphosphates into acid-insoluble DNA measures enzymatic activity. The latter is conveniently reflected in the absorbance of the X-ray film exposed to the gel.

The in situ gel assay has confirmed the earlier observation [2] that a 112 kDa polypeptide comprises the *Physarum* DNA polymerase activity. In addition, a catalytically active 70 kDa polypeptide is detected in cell extracts from G_2 cells (fig.1, lane 1) which is probably a product of partial proteolysis of the 112 kDa polypeptide. This phenomenon of proteolysis yielding polypeptides of lower molecular mass which retain catalytic activity is well documented for DNA polymerases

from many different sources [14]. The inclusion of multiple protease inhibitors during the preparation of lysates increased the proportion of 112 kDa activity relative to that of 70 kDa (Banks, G.R., unpublished).

The control non-immune serum did not react with *Physarum* extracts or *E. coli* DNA polymerase I. The specificity of the antibodies raised against the *Physarum* DNA polymerase is shown by the detection of both 112 and 70 kDa polypeptides after Western blot analysis (fig.1, lane 2). The antibodies also reacted with a catalytically less active 200 kDa polypeptide which may be, therefore, antigenically related to the 112 and 70 kDa polypeptides. In contrast, the antibodies did not cross-react with *E. coli* DNA polymerase I (fig.1, lane 3).

DNA polymerase activity was assayed throughout the cell cycle by the in situ gel assay technique (fig.2). Total enzyme activity per sample was the same irrespective of the point in the cell cycle at which the cells had been harvested (fig.2, lanes A–K). The molecular size distribution of enzyme activity also remained unchanged. A similar pattern was also found in the amoebal stage (fig.2, lane L). Because equal amounts of total cell protein were assayed from each point in the cell cycle in these experiments, DNA polymerase-α protein synthesis must take place in step with general protein synthesis throughout the G_2 phase of the cycle.

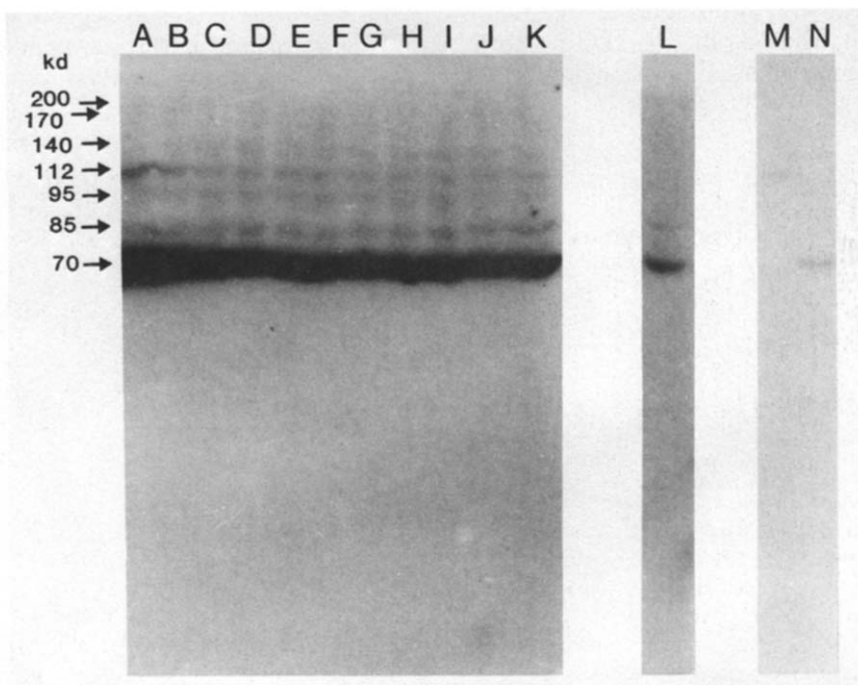

Fig.2. In situ polyacrylamide gel assay of DNA polymerase activity through the mitotic cycle. Autoradiogram of a 1% (w/v) SDS-7.5% (w/v) polyacrylamide gel (20 × 20 cm) containing 150 μg/ml of activated calf thymus DNA taken after electrophoresis and in situ assay for DNA polymerase activity. Samples were taken from a macroplasmodium after the second synchronous mitosis (MII); a portion containing 50 μg total protein was loaded onto the gel. Lanes A–K respectively, lysates prepared from samples isolated at 0, 1 h 8 min, 2 h 15 min, 3 h 23 min, 4 h 30 min, 5 h 38 min, 6 h 26 min, 7 h 53 min, 10 h (MII) and 10 h 34 min post-MII: lane L, *Physarum* myxamoebal (CLdAXE) lysate; lane M, 1 U *E. coli* DNA polymerase I (109 kDa); lane N, 1 U Klenow fragment (75 kDa). BRL prestained protein molecular mass markers were used to calibrate the gel. A series of autoradiograms were obtained. The example presented above is overexposed with respect to the 70 kDa component in order to illustrate the presence of larger species with polymerase activity. Autoradiograms obtained after shorter exposures all showed that samples A–K had essentially the same activity in the 70 kDa position.

3.3. *Western blot analysis of DNA polymerase-α throughout the cell cycle*

The polypeptide components of DNA polymerase activity were detected not only throughout the cell cycle, but also in amoebal lysates by Western blot analysis (fig.3). As described above, three polypeptides reacted with antibodies against purified DNA polymerase-α. The major component detected immunologically throughout the cell cycle was a 200 kDa polypeptide, whereas little catalytic activity was associated with this molecular size (cf. figs 2,3). Conversely, the 70 kDa polypeptide is the major contributor to catalytic activity but barely detectable by the Western blot analysis. It has been reported that proteolysis of high molecular mass catalytic subunits of eukaryotic DNA polymerases can generate proteolytic fragments more active than the intact subunit. Indeed, the 75 kDa Klenow fragment of *E. coli* DNA polymerase I is synthetically more active than the 109 kDa intact enzyme [15,16]. The 200 kDa immunoreactive polypeptide is possibly a less active precursor to the active 112 kDa polypeptide, the 70 kDa activity being derived from either of these. There is evidence that cloned *Physarum* DNA polymerase-α gene sequences are homologous to a poly(rA)$^+$ RNA transcript of about 10 kb, a size capable of endocing a 200 kDa polypeptide [17]. Both the size distribution of immunoreactive polypeptides and their relative abundance measured by antibody binding were essentially unchanged throughout the mitotic cycle (fig.3). These data support the conclusion based on RIA (table 1) and measurement of DNA polymerase activity (see fig.2) that the synthesis of DNA polymerase-α protein takes place in the G_2 phase of the mitotic cycle, in step with protein synthesis in general.

The immunoreactive polypeptides present in plasmodial lysates were also found in *Physarum*

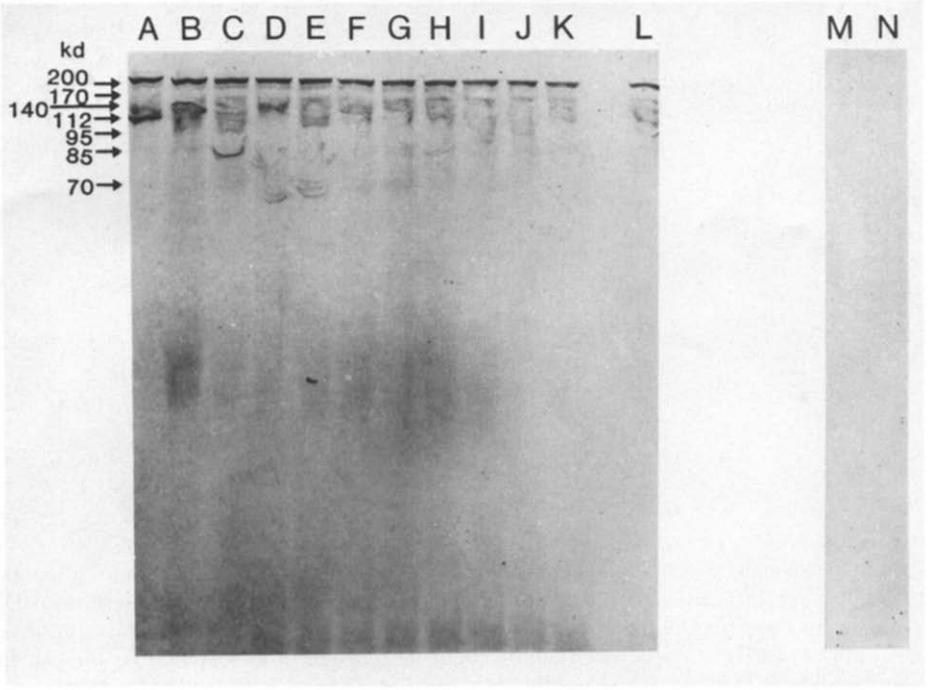

Fig.3. Assay by Western blot analysis of *Physarum* DNA polymerase protein through the cell cycle. A portion (50 μg total protein) of each of the cell lysates used in the in situ gel assay for DNA polymerase activity (see fig.2) was assayed for DNA polymerase-α protein. After electrophoresis through a 1% (w/v) SDS-10% (w/v) polyacrylamide gel (20 × 20 cm), proteins were transferred to nitrocellulose. Samples A–L were probed with DNA polymerase antiserum and samples M and N were probed with non-immune serum. Antibody binding was detected as described in section 2. Lanes A–M, see legend to fig.2; lane N, a replicate sample of K.

myxamoebal (strain CLDAxe) lysates (see fig.3), suggesting that the same polymerase proteins are present in both these phases of the life cycle.

4. DISCUSSION

Measurements of DNA polymerase protein levels (table 1 and fig.3) and of DNA polymerase activity (fig.2) of the naturally synchronous cell cycle of *Physarum* show that active DNA polymerase is synthesized throughout the G_2 phase, i.e. after DNA replication but before mitosis. At mitosis it is likely DNA polymerase is distributed equally between the two daughter nuclei, providing each of them with sufficient enzyme to replicate the genome. Thus, the cell prepares for S phase by making DNA polymerase-α in the G_2 phase of the preceding cell cycle. *Physarum* plasmodia do not have a detectable G_1 phase and it is perhaps a result of this that protein synthesis is geared to anticipate requirements in the S phase. This may be a strategy used by cells committed to rapid growth and division.

Histone genes are transcribed in the G_2 phase [18] but histone mRNA is stored until the S phase and then translated so that histone synthesis and DNA synthesis are coordinate [19]. Tubulins, which are required for spindle microtubules, are synthesized in the G_2 phase in the 2 h preceding mitosis; subsequently 75% is degraded in a brief period following mitosis. At the peak there is a 4-fold increase in the amount of tubulins compared with basal levels [20]. Unlike histones, DNA polymerase-α synthesis is not coordinate with DNA synthesis and, unlike tubulins, there is no evidence for the biosynthesis of high levels of DNA polymerase-α and its subsequent rapid degradation.

DNA polymerases-α are located in the nucleus [21,22] and yet DNA synthesis occurs only during a defined period of the cell cycle. There appears to be a mechanism that prevents DNA polymerase-α from replicating genomic DNA except during the S phase. The action of regulatory subunits on the DNA polymerase-α tightly bound to some nuclear sub-structure and/or a particular higher order structure of chromatin may affect the accessibility of enzyme and substrate and so play a crucial role in the regulation of in vivo DNA polymerase activity [23,24].

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