DNA REPLICATION IN PHYSARUM POLYCEPHALUM: CHARACTERIZATION OF REPLICATION PRODUCTS MADE IN ISOLATED NUCLEI

Steinar Funderud and Finn Haugli

Institute of Medical Biology, University of Tromsø,

9000 Tromsø, Norway

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SUMMARY

Nuclei isolated from synchronous S-phase plasmodia of the myxomycete Physarum polycephalum were competent in production of low molecular weight DNA replication intermediates. Furthermore, these nuclei showed some competence in joining these fragments into DNA of intermediate molecular weight. The DNA molecules made in vitro could be correlated with products made in vivo.

INTRODUCTION

We have previously reported on the mechanism of DNA replication in vivo in Physarum (Funderud and Haugli, 1975, 1976. (1) (2)). Here we initiate studies with isolated nuclei of Physarum polycephalum, an organism well suited for such analysis because of its natural synchrony (Rusch, 1970) (3).

Schiebel and Schneck, 1974 (4) modified the nuclear isolation method of Mohberg and Rusch (1971) (5) and showed that the DNA synthesis occuring in isolated Physarum nuclei was a continuation of that started in vivo. Brewer and Ting (1975) (6) analysed DNA synthesis in crude homogenates of Physarum. Brewer (1975) (7) reported a stimulation of DNA synthesis in homogenates when dextran was included and identified two classes of products on alkaline sucrose gradients.

In the present report we wished to compare the replication in isolated nuclei with the pattern observed in vivo (Funderud and Haugli,

1975 (1)) as a first step in attempts to reconstruct physiological DNA replication in vitro.

MATERIALS AND METHODS

Strains and culture techniques have been described previously (Funderud and Haugli, 1975) (1).

Preparation of isolated nuclei. Nuclei were isolated 30 minutes past metaphase of synchronous mitosis II at the time of maximal rate of DNA synthesis. The plasmodium, containing about 10^8 nuclei and growing on a Millipore membrane (Millipore No HAW P00100) was scraped off into 12.5 ml of ice-cold homogenizing medium containing 30 mM NaCl, 30 mM KCl, 15 mM $MgCl_2$, 20 mM Tris, 5 mM mercaptoethanol and 5 mM EGTA, brought to pH 7.5 with HCl. 6 strokes with a loose fitting teflon pestle in a Potter-Elvehjem homogenizer liberated nuclei. The homogenate was diluted with 3 volumes of homogenizing medium. Debris was removed (5 minutes centrifugation at 50 x g) and nuclei pelleted (10 min centrifugation at 1000 x g). Nuclei were resuspended in the incorporation mixture (minus radioactive deoxyribonucleotide) at 0 °C at a concentration of 2.5-5 x 10^7 nuclei per ml.

Incorporation procedure. 2.5-5 x 10^6 nuclei were suspended in 100 µl of incorporation mixture containing: 50 mM MOPS, 15 mM $MgCl_2$, 2 mM EGTA, 3 mM mercaptoethanol, 0.2 M sucrose, 2.5 mM ATP, 150 µM each of dGTP, TTP, dCTP (Schwarz-Mann), and 10 µM dATP with ^{3}H-dATP at specific activity 1.4-2.0 Ci/mmole, (Amersham, Code TRK-347) pH 7.5. Incorporation of dNTP was started by removing the tube from ice to a waterbath at 28 °C, and terminated by adding 2.5 ml of ice-cold 0.6 M PCA with 0.05 M sodiumpyrophosphate. Pulse experiments were done as described above. Pulsechase experiments were performed as described above, with an additional 5-25 minute incubation period in the presence of 300 µM cold dATP. Incorporation was determined as previously reported (Funderud and Haugli, 1975) (1).

Density shift analysis. For density shift experiments the plasmodia were prelabelled with 5·bromodeoxyuridine in early prophase (20 µg/ml) in the presence of 5·fluorodoxyuridine (5 µg/ml) and Uridine (50 µg/ml). Incubation was continued until the time of nuclear isolation (30 minutes past metaphase).

After incorporation, the nuclei were washed with 3 x 5 ml of isolation medium, lysed in 1 ml of 0.25% Sarkosyl, 0.01 M Tris, 0.01 M EDTA at room temperature, and sheared by 12 passages in a 25 G canule. Neutral CsCl gradients contained 4.5 g CsCl, 0.5 ml lysate (ca 10 µg DNA) and 3.3 ml DSC (refractive index 1.400). Centrifugation was in a Beckman preparative Ultracentrifuge, with rotor SW65 at 40000 rpm, 20 °C for 60 hours. Alkaline Cs_2SO_4 gradients contained 2.75 g Cs_2SO_4, 0.5 ml lysate, 0.7 ml DSC and 2.7 ml alkaline buffer pH 12.4 (consisting of 0.06 M Na_2HPO_4 and 0.08 M NaOH). Refractive index 1.3760. Centrifugation and collection were as described above.

Alkaline sucrose gradient analysis. 4.9 ml linear gradients of 5-20% sucrose in 0.1 N NaOH, 0.2% sarkosyl was used. Nuclear pellets containing 1 µg DNA were lysed in 0.1 ml of 0.3 M NaOH,

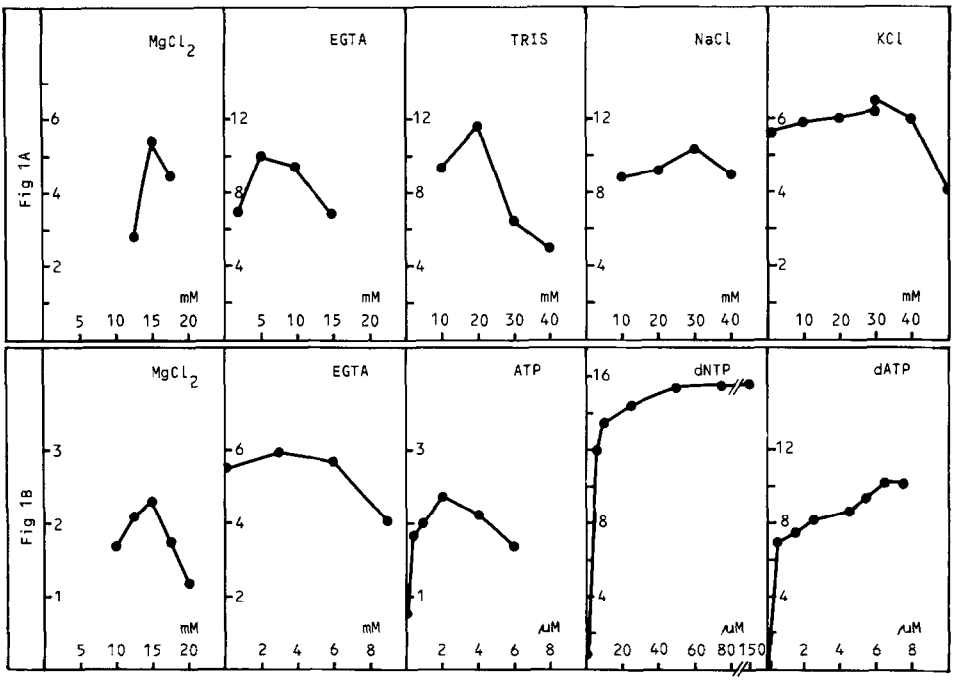

Figure 1. Optimalization of nuclear isolation and incorporation media. Ordinates 10^{-3} cpm per 10^6 nuclei.
Abscissa: concentrations in mM or µM as shown. Concentrations of other constituents than the one tested were as described in Methods.
Fig. 1A: nuclear isolation medium
Fig. 1B: incorporation mixture

2% sarkosyl, 0.01 M EDTA. 15 minutes at 0 °C was allowed for lysis and the lysate gently layered on top of 4.8 ml gradients. Centrifugation conditions described in legend to Fig. 5.

RESULTS and DISCUSSION

Optimalization of isolation and incorporation procedures. The extensively used nuclear isolation method of Mohberg and Rusch (1969) (5) yields clean nuclei. However, this method involves the use of the nonionic detergent Triton X 100 which is known to damage the nuclear membrane and to inhibit DNA synthesis. To avoid damage of nuclei we chose a gentle homogenization using 4-6 strokes with a loose fitting teflon pestle in a Potter-Elvehjem homogenizer at 0 °C, in absence of Triton X 100. Fig. 1A

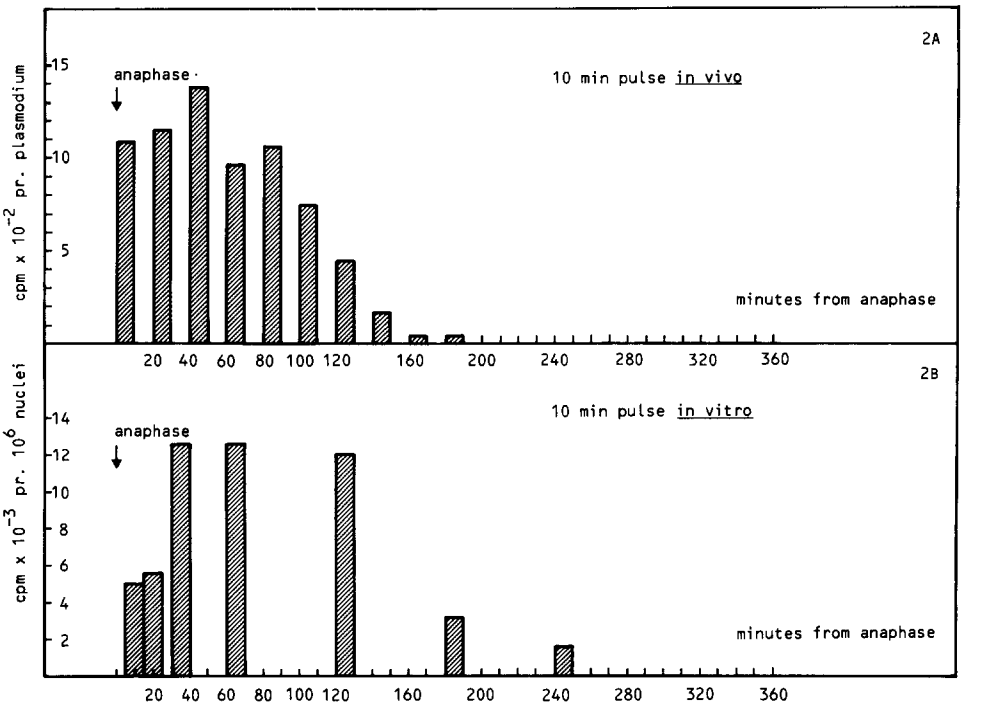

Figure 2. Rates of DNA synthesis through the nuclear cycle, in vivo and in vitro.
Fig. 2A: 1 cm^2 plasmodial discs were incubated for 10 min on 25 µl droplets of semidefined medium containing ^{3}[H]-TdR at 0.5 µCi per ml.

Fig. 2B: isolated nuclei were incubated in the optimalized incorporation mixture for 10 minutes.

shows effects on ^{3}[H]-dATP incorporation in nuclei isolated with varying concentrations of homogenizing medium constituents. Thus, concentrations of constituents described under Methods were chosen for the standard nuclear isolation method.
The incorporation mixture used is based on the one developed by Schiebel and Schneck (1974) (4). We have modified it according to our optimalization experiments shown in Fig. 1B. Thus the incorporation mixture given in Methods were chosen for the standard procedure.

DNA replication in vitro as a function of position in nuclear cycle. Fig. 2 shows 10 minute pulse incorporations of ^{3}[H]-dATP

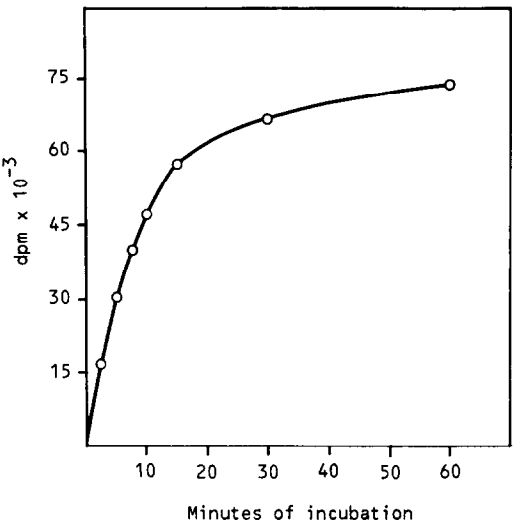

Figure 3. Incorporation of ^{3}[H]-dATP into DNA of 10^{6} isolated nuclei at 28 °C. Isolation, incubation and preparation were as described in the text.

in isolated nuclei, as well as ^{3}[H]-dT incorporation in whole plasmodia through the nuclear cycle.

We conclude that DNA synthesis in our isolated nuclei reflects the physiological DNA replication. For the rest of this study, nuclei isolated 30 minutes past metaphase have been used.

Kinetic characterization of DNA synthesis in the isolated nuclei. Fig. 3 shows the time course of incorporation into isolated nuclei. The initial rate in these experiments corresponds to approximately 10% of the *in vivo* rate. Synthesis is linear for 7-10 minutes, and continues up to 60 minutes.

Density shift analysis of DNA made in vitro. The DNA made *in vitro* was analysed in density-shift experiments. Fig. 4A shows result of neutral CsCl density gradient centrifugation. It is appearant that newly made DNA is heavy, indicating continuity with DNA made *in vivo*. A stronger test is in the alkaline Cs_2SO_4

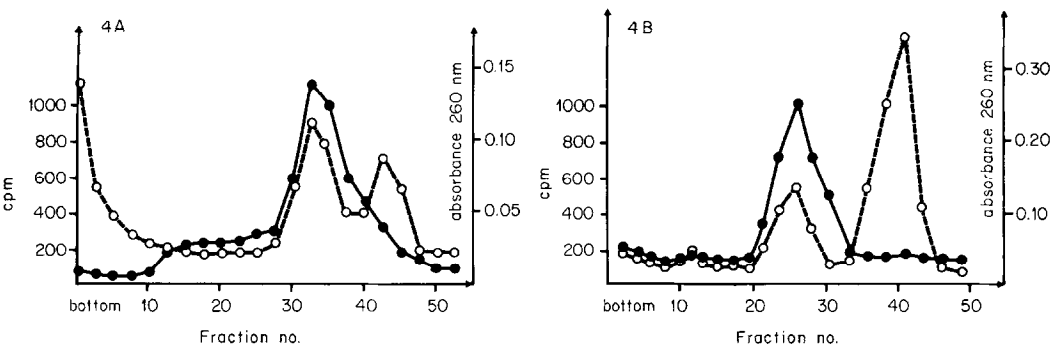

Figure 4. Density gradient centrifugation of DNA labelled with BrdU *in vivo* and ^{3}[H]-dATP *in vitro*.

Plasmodia were exposed to BrdU during the S-phase prior to isolation and nuclei isolated and incubated with the incorporation medium for 5 minutes. DNA was then analysed on neutral CsCl gradient (4A) and on alkaline $CsSO_4$ gradient (4B). —●— radioactivity. --○--absorbance at 260 nm.

density gradient analysis, results of which is shown in Fig. 4B. It is evident that DNA made *in vitro* is continous with DNA made *in vivo* just prior to nuclear isolation. This is taken as evidence against repair synthesis, and for true replication.

Alkaline sucrose gradient analysis of DNA made in vitro. Next, the species of single stranded DNA synthesized *in vitro* were examined in alkaline sucrose gradients.

Fig. 5A shows that pulse and pulse-chase experiments revealed 2 size-classes of DNA with S values and approximate molecular weights of 4.5 S (5×10^4 daltons) and 30-35 S (7×10^6-10^7 daltons). In order to evaluate the physiological meaning of the classes of DNA made in isolated nuclei (Fig. 5A), results of a pulse experiment performed *in vivo* is shown in Fig. 5B. It appears likely that the 4.5 S DNA made *in vitro* corresponds to the 4.5 S "Okazaki" pieces made *in vivo*. Also, the joining of these into 30-35 S DNA seen *in vivo*, appears to occur *in vitro*,

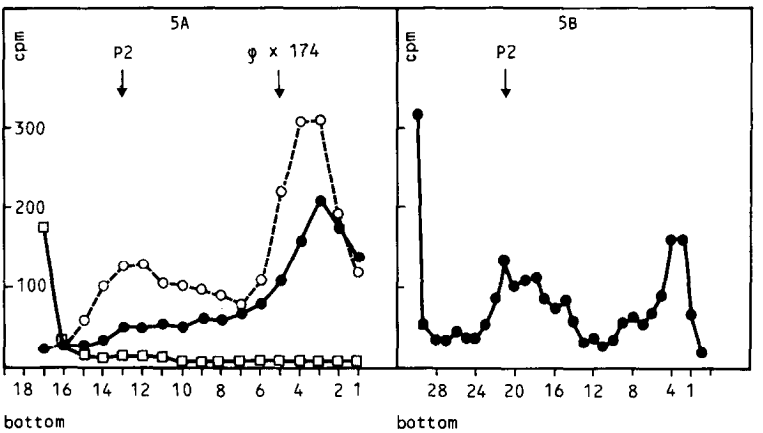

Figure 5. Alkaline sucrose gradient analysis of replication products from isolated nuclei (5A) and whole plasmodia (5B).
In 5A isolated nuclei were pulsed for 2.5 min with ^{3}[H]-dATP (—●—) and then chased for 27.5 min (-○-). (—□—) marks sedimentation of uniformly ^{14}[C]-labelled DNA. (Conditions: SW 65 rotor of Spinco L65B. 40 000 rpm, 4 hours, 20°C).
In 5B intact plasmodium was pulsed for 60 seconds with ^{3}[H]-AdR (See Funderud and Haugli, 1975) (1). (Conditions: SW 27 rotor of Spinco L65B, 27 000 rpm, 18 hours, 4 °C).
Arrow marks position of ^{14}[C]-labelled P2 DNA, S-value 30.

but at a reduced rate. Thus, the nuclei isolated here in a salts medium are defective in over-all DNA synthesis. However, analysis of the products made *in vitro* makes it possible to decide that not all aspects of the replication process are equally affected.

These nuclei, therefore have defined defects. While we continue work to isolate nuclei with better performance *in vitro*, we now plan to use the defective nuclei to assay for the presence of replication factors in protein fractions from plasmodia and nuclei.

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