DNA replication in Physarum polycephalum: bidirectional replication of DNA within replicons

Steinar Funderud, Rolf Andreassen and Finn Haugli

Institute of Medical Biology, University of Tromsø, 9000 Tromsø, Norway

Received 5 December 1977

ABSTRACT

The direction of replication of DNA within replicons of Physarum polycephalum was studied by pulse-labelling with 5-bromouracil-deoxyriboside (BrdUrd) and 3H-adenosine deoxyriboside (dAdo), followed by ultraviolet- (UV) - photolysis and analysis of molecular weights of single strand DNA fragments on alkaline sucrose gradients. Newly made DNA within replicons at all stages of completion is split in two equal halves upon UV irradiation when BrdUrd was given at the time of initiation of DNA synthesis. This shows that replication within replicons of Physarum polycephalum starts at an origin located in the center of each unit, proceeding bidirectionally from this origin.

INTRODUCTION

The bidirectional mode of DNA replication within replicating units of eukaryotic chromosomes was first demonstrated by Huberman and Riggs\(^1\) in Chinese hamster cells, using DNA fiber autoradiography. Bidirectional replication has since been demonstrated in a number of other eukaryotic cells using autoradiography\(^2-4\), and electron microscopy\(^5\). Biochemical evidence in support of a bidirectional mode of DNA synthesis within replicons was obtained by Weintraub (1972)\(^6\), using UV-irradiation to specifically cut newly made DNA from chick erythroblasts at points preselected by pulse labelling with BrdUrd. A similar biochemical approach was employed by McKenna and Masters (1972)\(^7\) to show that DNA replication in the E. coli replicon is also bidirectional from the origin of replication. Recently Planck and Mueller (1977)\(^8\) used UV induced photolysis of BrdUrd labelled DNA to show that repli-
cating units of HeLa cells are synthesized bidirectionally. Thus bidirectional DNA synthesis within replicons is very widespread, but not universal, since unidirectional replication occurs in some bacteriophages and in metazoan mitochondrial DNA. DNA replication in Physarum proceeds with natural, perfect synchrony at the molecular level and this organism is therefore well suited for biochemical analysis of the mechanisms of replication. The question of bidirectional vs. unidirectional replication in Physarum was never analyzed before, although Brewer (1972) and Brewer, Evans and Evans (1974) suggested that synthesis might be unidirectional. We have previously presented evidence that DNA replication in Physarum proceeds with the "discontinuous" synthesis of Okazaki fragments on both strands of progeny DNA molecules. Recently we have also obtained biochemical evidence that replicons exist in Physarum, that their single strand molecular weight is $10^7 - 3 \times 10^7$ daltons and that replicons occur in temporal clusters where little or no joining of adjacent replicons takes place during the first hour after initiation.

In the present investigation we take advantage of the precise natural synchrony, and the previous finding that the first temporal sets of replicons are not joined measurably until 40-60 minutes after initiation. Under these conditions extremely short pulses of BrdUrd allows precision photolysis of nascent DNA in order to determine the topological relationship within replicons during DNA synthesis.

**METHODS OF ANALYSIS**

**Strain and culture techniques**

Strain TU291 is used in all experiments. Culture conditions have been described.

**Labelling procedures**

Whole, synchronous surface plasmodia were pulse-labelled with 5-bromodeoxyuridine (BrdUrd) at 50 μg/ml on regular semi-defined medium without any pre-treatment. The pulse was for 30 seconds immediately after completion of anaphase, at which time DNA replication is initiated. Excess BrdUrd was removed.
by blotting on a pad of filter paper before the plasmodium was transferred to medium containing cold thymidine (dThd) at 20 μg/ml. After a 60 second chase of the BrdUrd on this medium, discs of plasmodia were pulse-labelled with ³H-deoxy-adenosine (³H-dAdo) for 2 minutes at radioactive concentration 250 μCi/ml of semidefinite medium. dAdo was chosen for radioactive labelling to have the advantage of non-competing pools for the two pulse labelling agents. The radioactive pulse was followed by chase on regular medium for the desired time periods, as given in the Results section.

Isolation of nuclei and alkaline sucrose gradient analysis

Isolation of nuclei was carried out according to the method of Mohberg and Rusch (1969) as previously described. 0.5 - 1.0x10⁶ nuclei suspended in 0.25 ml of 0.15 M NaCl, 10 mM EDTA, pH 9.0, were gently mixed with an equal volume of 4% sarkosyl 0.2 M NaOH, 10 mM EDTA, and left at 50°C for 20 minutes for lysis and denaturation of DNA. Centrifugation analysis was on a 4-20% alkaline sucrose gradient in a SW40 rotor in Spinco L65B centrifuge at 22°C and 30,000 rpm for 5 or 6 hours.

Ultraviolet irradiation

0.5 - 1.0x10⁶ nuclei, suspended in 125 μl 0.15 M NaCl, 10 mM EDTA, pH 9.0 were placed in a plastic Petri dish 20 cm below a Westinghouse FS20 Sunlamp and irradiated at 2°C for 10 minutes without any shield.

Marker DNA and determination of S-values and molecular weights

Phage λ ¹⁴C-DNA was prepared as previously described and included in all samples to allow calculation of sedimentation constants and molecular weights. S₂₀,w for alkaline single strand λ DNA is 40 and molecular weights are given by the relation: S₂₀,w = 0.0528·M⁰.⁴⁰⁰ (Studier, 1965). We assume that a 4-20% sucrose gradient in the 11.5 ml tubes of the SW40 rotor of Spinco L65B provides an approximation of isokinetic conditions, although the use of the λ marker to estimate the molecular weight of the Okazaki fragments in
Physarum leads to an overestimation of S-value and molecular weight. However, the deviations for DNA molecules much larger than the Okazaki fragments and closer to the X DNA in molecular weight, is much smaller and do not influence the basic conclusions of the present work.

RESULTS

Control experiments

To obtain results which reflect the ongoing processes with a maximum of clarity, we have chosen in the present investigation to analyze only the processes occurring in the very first sets of temporally isochronous replicons. From previous studies on DNA replication, we conclude that no replicons are terminated, nor joined to neighboring replicons within the first 40-60 minutes of the S-period. Thus, during this time interval only molecules at various stages of completion towards the full replicon size occurs, allowing the desired simplicity for analysis.

We attempted to maintain the precision for analysis which follows from the naturally synchronous DNA replication of Physarum by giving extremely short pulses of the photolyzes agent, BrdUrd, as well as of the radioactive marker. Control experiments showed that the labelling regimen given in the Methods section allowed scission of photo sensitized DNA, when isolated nuclei were irradiated with the Westinghouse FS20 Sunlamp from a distance of 20 cm, without shield. The temperature was 2°C and 10 minutes of irradiation proved sufficient to break essentially 100% of the labelled molecules. DNA which had not incorporated any BrdUrd remained unchanged even after 25 minutes of irradiation.

Analysis of the size of nascent DNA with and without photolysis

If replication proceeds unidirectionally from the origin, a pulse labelling regime where BrdUrd is given at initiation, immediately followed by radioactive labelling, should yield fragments of single stranded DNA which at all stages of growth within the replicon should not change its molecular weight after UV irradiation. However, if replication proceeds

---

716
bidirectionally from the origin, and joining of the discontinuously made Okazaki fragments occurs rapidly, then UV irradiation should break the nascent DNA in two fragments. These photolysis products will be of equal length if both replication forks proceed at the same rate, but will be of different size if the two rates are different. These possibilities as well as the expected patterns on alkaline sucrose gradients are displayed in Fig. 1.

The results of experiments where DNA was pulse labelled at initiation and chased for various times are shown in Fig. 2. When DNA, pulsed with H-dAdo at initiation of DNA synthesis, but with BrdUrd only at the very end of a 35 minute chase, was irradiated and analyzed in the same manner, no change in

---

Fig. 1: Models displaying possible modes of replicon growth and the expected results on alkaline sucrose gradients.
A: unidirectional synthesis from origin, O
B: bidirectional synthesis with different growth rates at the two forks.
C: bidirectional synthesis with similar growth rates at the forks.
(--'): BrdUrd pulse. (...): H-deoxyadenosine.
(----): cold DNA made in progressively longer chase.
Panels: (---) unirradiated DNA, and (----) irradiated DNA on alkaline sucrose gradients.
Fig. 2: Photolysis of growing DNA molecules in replicons. A plasmodium was labelled with BrdUrd and 3H-deoxyadenosine as described in Methods. The chase was on regular semidefined medium for 15, 20, 25, 30, 35 and 40 min, as shown. All nuclear samples were divided into two equal halves, one of which was irradiated for 10 minutes as described in Methods. Irradiated and unirradiated DNA were analyzed separately with λ DNA as marker, on 4-20% alkaline sucrose gradients, SW40, 30 K, 22°C. The 15' sample was centrifuged for 6 hours, the others for 5 hours. Results for each chasestep are superimposed on each other by alignment of the λ-marker position (arrow). Direction of sedimentation is from right to left. o--o unirradiated. o---o irradiated.

molecular weight could be detected (data not shown). The molecular weights of unirradiated and irradiated DNA, calculated from the positions of peaks and marker DNA in Fig. 2, is displayed in Fig. 3.

We conclude that DNA in replicons at all stages of completion grows in a manner compatible with the model outlined in Fig. 1C. That is, initiation of replication occurs in the center of the replicon and proceeds bidirectionally at equal rates for both forks. The cumulative, average rate at these two forks is $3.2 \times 10^5$ daltons per minute as calculated from the data in
Fig. 3: Chain growth rate and reduction in molecular weight by irradiation of BrdUrd substituted nascent DNA. The molecular weights of the DNA species from Fig. 2 have been plotted in this histogram, as a function of the chase period.

DISCUSSION

We have obtained evidence elsewhere\textsuperscript{18} that replicons of size corresponding to a single strand molecular weight of \(10^7\) - \(3\times10^7\) daltons exist in Physarum. These are synthesized on both templates via the rapid joining of discontinuously made Okazaki fragments. In the present investigation we have analyzed the mechanisms of DNA synthesis in replicons in more detail by means of BrdUrd photolysis of the growing chains at the site of initiation of replicon DNA synthesis. Considering the models presented in Fig. 1, and the results given in Fig. 2 and Fig. 3 the following conclusions can be reached:

1. Replicons grow with an overall rate of about \(3.2 \times 10^5\) daltons per strand per minute, as calculated from the non-irradiated samples in Fig. 2 or Fig. 3. Calculations
based on shorter intervals give results which are higher or lower (eg. 7 \cdot 10^5 daltons per minute at 15-20 minutes and 1 \cdot 10^5 daltons per minute at 30-35 minutes). This scattering is likely to be due to timing errors, but the possibility of rate change with time can not be excluded.

2. UV-irradiation of DNA pulse-labelled at initiation with BrdUrd and \(^3\)H-dAdo and then chased for various time periods causes a reduction in molecular weight to approximately one half of the original, as can be seen from Fig. 2 or Fig. 3.

3. Rates of chain growth for the photolysis fragments and for the whole molecules respectively, are 8.0x10^5 and 1.67x10^5 daltons per minute after 15 minutes chase following the pulse label with BrdUrd and \(^3\)H-dAdo; 1.55x10^5 and 3.0x10^5 after 20 minutes of chase; 1.84x10^5 and 3.6x10^5 after 25 minutes; 1.67x10^5 and 3.5x10^5 after 30 minutes; 1.71x10^5 and 3.14x10^5 after 35 minutes; and 1.8x10^5 and 3.25x10^5 daltons per minute after 40 minutes of chase. It can be concluded that replicon chain growth occurs bidirectionally with approximately equal rates at both forks.

Since this work only includes the analysis of those replicons that initiate at the time of master initiation of DNA synthesis, immediately following anaphase, the conclusions reached here are strictly valid only for this class. We do suggest, however, that the mechanisms are likely to be similar throughout the S-phase, although it is possible that rates of chain growth and replicon size could change at later stages, as has been found in other systems.\(^{21}\)

REFERENCES
