DNA replication in Physarum polycephalum: UV photolysis of maturing 5-bromo-deoxyuridine substituted DNA

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ABSTRACT

Combinations of 5-bromodeoxyuridine (BrdUrd) and \(^3\)H-deoxyadenosine (\(^3\)H-dAdo) short pulses were given in the synchronous DNA-replication period of Physarum polycephalum. After a chase period, UV-photolysis products were analysed on alkaline sucrose gradients. This strategy has allowed the following conclusions:

a) at the time of master-initiation of DNA replication, points separated by 1.1-2.2x10^7 daltons of single strand DNA may initiate DNA synthesis.

b) among these, only selected groups of replicons actually proceed in DNA replication at this time, while others appear to hold (later temporal sets of replicons). The origins of the ones that proceed in replication are separated from each other by a distance corresponding to 1.1-2.2x10^7 daltons.

c) regions in actual replication are separated from each other by increasing distances (up to 1.5x10^8 daltons single strand DNA) at later times in S.

INTRODUCTION

BrdUrd is easily introduced into the DNA of most organisms, and then have several biological effects. Among the most striking is the sensitization of the DNA to damage by long-waved UV-irradiation. This effect is one of photochemically induced single strand scission which appear to be irreversible. This decomposition of DNA has been used to cut DNA photochemically at preselected places determined by a defined BrdUrd pulse during DNA replication, followed by UV irradiation. Such strategies have shown that DNA replication in replicons proceeds bidirectionally at approximately equal rates from the origin of replication.
In the present experiments we have analysed the topology of replication in *Physarum* by means of BrdUrd induced UV-photolysis. The strategy is to give short BrdUrd pulses followed by or preceeded by short $^3$H-dAdo pulses. These points of pulselabeling are varied in the S-phase and the DNA is allowed to mature before it is UV irradiated and analysed on alkaline sucrose gradients. It should then be possible, by means of the $^3$H-label, to measure the size of cut- and uncut molecules and thus measure the distance between sets of actively replicating points at the given time.

**METHODS**

**Strains**

Strains and culture techniques were as described by Funderud and Haugli (1975)\(^7\).

**Pulse labeling**

BrdUrd at 50 $\mu$g/ml were given in regular semidefined medium without any 5-fluoro deoxyuridine or other treatment. When nothing else is noted BrdUrd pulses are for 30 seconds. Control experiments with $^{14}$C-BrdUrd (data not presented) showed uptake of labeled BrdUrd into the DNA to go on for 10 minutes at approximately linear rate, and then to fall off rapidly, whereafter the label remained in the DNA. Thus the "cutting width" produced by the BrdUrd pulse and the subsequent UV-irradiation may be a maximum of 10 minutes (roughly $4 \times 10^6$ daltons), although previous results\(^5\) clearly show that the actual cut is in reality confined to a region which is less than 10-20% of this maximal width, or about $4-8 \times 10^5$ daltons, corresponding to 1-2 minutes worth of DNA synthesis.

The $^3$H-dAdo pulse, given at radioactive concentration 250 $\mu$Ci/ml of semidefined medium, was usually for 60 seconds and was separated from the BrdUrd pulse by a long chase period on regular semidefined medium, or by shorter chase periods on regular medium containing deoxythymidine at 10 $\mu$g/ml.

**Nuclear isolation and UV-photolysis**

Nuclei were isolated at $0^\circ$C with reduced light by the me-
method of Mohberg\textsuperscript{8} as described by Funderud and Haugli (1975)\textsuperscript{7}. They were resuspended in 100 μl of standard saline-citrate at concentration 10\textsuperscript{8} nuclei per ml and the nuclear suspension droplet placed in a plastic petridish in distance 20 cm from two Westinghouse FS 20 Sunlamps. The dish was covered with another plastic petrie dish containing a 1 cm thick layer of thymidine at 2 mg per ml water as a lid and irradiation shield. This shield was included in order to screen out most of the remaining 260–280 nm UV-light from the sunlamps which have maximal output at 310 nm. Control experiments showed this precaution to safeguard against a small but perceptible breakdown of non-substituted DNA which occurred in absence of thymidine shield. Irradiation was at 4°C for 30 minutes.

**Analysis of DNA molecular weight**

Conditions for alkaline lysis of irradiated or non-irradiated nuclei as well as the alkaline sucrose gradient analysis of the single stranded DNA was as described by Funderud, Andreassen and Haugli (1978)\textsuperscript{6}. The specific centrifugation conditions are given for each experiment in legends to figures.

**RESULTS AND DISCUSSION**

**BrdUrd and \textsuperscript{3}H-dAdo pulses at initiation of DNA replication**

The first set of experiments were designed as an extension of the analysis which showed DNA replication in Physarum to proceed bidirectionally within replicons\textsuperscript{5}. In the present experiments the chase following the two pulses at initiation, was extended to the end of S-phase. During this time the labeled DNA matures to a size of roughly 3 x 10\textsuperscript{8} daltons (single strand). Such a strategy (Figure 1A) is designed to answer the question: what is the distance between origins in those replicons that engaged in the master initiation of DNA replication? Figure 1B shows examples of the results obtained. It can be seen that up until 60 minutes the molecular weight of the photolysis products are very nearly one half of the unirradiated molecules, showing bidirectional replication as reported previously\textsuperscript{5}.
Figure 1: BrdUrd and $^3$H-dAdo pulses at start of S-phase with UV-photolysis after maturation (chase) into S-phase. 
Panel A: experimental strategy.
Panel B: experimental results. Nuclei were isolated at end of chase, irradiated, lysed and the DNA analysed on alkaline sucrose gradients as described in Methods. Centrifugation conditions: SW 40; 20°C; control, 120' and 180': 12500 rpm for 16 hours. 30': 30000 rpm for 5 hours. 60' and 90': 16600 rpm for 16 hours. Arrows show position of phage $\lambda$ DNA marker (40 s). Upper left: thymidine DNA matured to end of S-phase. (---): not irradiated. (-----): irradiated. Numbers in upper right corners of other panels show time in S-phase when nuclei were isolated. Direction of sedimentation from right to left.
Panel C: possible interpretation of results. (---) assumed location of BrdUrd, and thus of photolysis cut. (-----) assumed location of $^3$H-dAdo defining the DNA that can be observed in the gradients.
At 60 minutes and at 90, 120 and 180 minutes (near end of S-phase) two features are noteworthy. First the main class of labeled molecules stay at around 40 S, suggesting that this is the origin distance in clustered replicons (ca 1.6 x 10^7 daltons) (Figure 1C).

Second, at 60-180 minutes, but most striking at 90 and 120 minutes, a 20-25 S species of molecules (roughly 4-5 x 10^6 daltons single strand) results from photolysis, and could correspond to the terminal ends in clustered replicons, which would not become joined before flanking clusters have terminated replication.

\[ \text{H-dAdo pulse at initiation followed by BrdUrd pulses at different later times in S-phase.} \]

These experiments were designed to answer the question: What is the distance between the DNA which is initiated at anaphase (time zero in S-phase) and the DNA which is in replication at progressively later times. The experimental strategy is outlined in figure 2A, while figure 2B shows a selection of typical results and figure 2C attempts an interpretation of these data.

It can be seen that the distance which separate replicating DNA flanking the DNA that was replicated at time of master initiation is 40 S (1.6 x 10^7 daltons) at 45 minutes; 45 S (2.2 x 10^7 daltons) at 60 min; 55 S (3.4 x 10^7 daltons) at 90 min; 67 S (5.8 x 10^7 daltons) at 120 min; 77.5 S (8.4 x 10^7 daltons) at 150 min and 98.7 S (1.5 x 10^8 daltons) at 180 min. We conclude that there is an orderly increase with time in S-phase of the amount of DNA between replicating points which flanks DNA labeled at initiation, and this amount of DNA may exceed 80 S single strand equivalent.

\[ \text{BrdUrd pulse at initiation followed by H-dAdo pulse at progressively later times.} \]

These experiments were designed to measure the amount of DNA which is interspersed between the presumed clusters of replicons initiating at time zero.

The experimental design is given in figure 3A and the re-
Figure 2: $^3$H-dAdo pulse at start of S-phase; BrdUrd pulse at successively later times in S-phase. Chase into G2.

Panel A: experimental strategy.

Panel B: experimental results. Nuclei were isolated at end of chase, after an initial $^3$H-dAdo pulse at start of S-phase and a BrdUrd pulse at the time point indicated in each frame. After irradiation and lysis, DNA was analysed on alkaline sucrose gradients. Conditions were: SW 40; 20°C; 12500 rpm for 16 hours. Arrows show position of phage λ DNA marker (40 S). (---) unirradiated; (——) irradiated. Direction of sedimentation: from right to left.

Panel C: possible interpretation of results. (•••) assumed location of BrdUrd, and thus of photolysis cut. (---) assumed location of $^3$H-dAdo, defining the DNA that can be observed in the gradients.
Figure 3: BrdUrd pulse at start of S-phase; $^3$H-dAdo pulse at successively later times in S-phase. Chase into G2.

Panel A: experimental strategy.

Panel B: experimental results. Nuclei were isolated at end of chase, irradiated, lysed and DNA analysed on alkaline sucrose gradients. Conditions: SW 40; 20°C; 12500 rpm for 16 hours. Arrows show position of phage $\lambda$ DNA marker (40 S). Numbers in upper right corner give time into S-phase where $^3$H-dAdo pulse was given. (—) unirradiated; (—o—) irradiated. Direction of sedimentation: right to left.

Panel C: possible interpretation of results. (AAAA) assumed location of BrdUrd and thus of photolysis cut. (•••) assumed location of $^3$H-dAdo, defining the DNA that can be observed in the gradients.
suits are displayed in figure 3B, while figure 3C represents a possible interpretation of results.

The quite unexpected and remarkable result of this test is that the photolysis products always are about 40 S (1.6 x 10^7 daltons) in size although there is an increase from 35 S (1.1 x 10^7) at 40' to 45 S (2.2 x 10^7) at 120 and 150. This is difficult to explain. Barring any artefact of photolysis (see Final Discussion), we are forced to accept as a possibility that the DNA of Physarum initiates replication every 1.6 x 10^7 daltons at the time of master initiation, thus incorporating sufficient BrdR at these points to suffer scission when irradiated later. These points could be the origins of each replicon. Of course, the initiation of each replicon at this time do not imply that they all proceed to replicate their DNA right away. Rather, all other available evidence suggests that most "hold" and proceed only later on. Only the first temporal set of replicons proceed, while later sets await further signals 6,9,10.

FINAL DISCUSSION

Size maturation and topology of replicons in Physarum was previously studied by analysing the size increase of pulse-chase labeled DNA through the cycle 6. The present experiments were designed to yield comparable results with a different experimental approach, in order to verify or expand earlier conclusions. Here, the insertion of a defined short pulse of BrdUrd allowed size maturation to occur before UV was applied to cut the DNA at the place of the pulse. Thus we study, in essence, the event at the replication fork some time after it occurred.

From results presented in Figure 1 it appears that the first set of replicons replicate bidirectionally as previously concluded 6, and that these may occur in a clustered configuration since the size of the photolysis products remains at 40-45 S even when 180 minutes of maturation has been allowed. Because of the results presented in Figure 3, this argument for clustering of the first temporal sets of replicons can
not be made very strong (see below). At any rate, the size of the replicon estimated by the present approach is in good agreement with our previous conclusions. The next set of experiments, Figure 2, asks a quite different question. Here we have attempted to measure the distance between replicating points flanking DNA that was replicated at the start of S-phase in order to get a measure of the degree of dispersion of early and successively later replicating DNA.

The results show that DNA replicated up until 45-60 minutes is also within the first temporal set of replicons, since the size of the labeled photolysis product do not increase beyond 40-45 S. The distance between the BrdUrd scissions and the $^3$H-dAdo location increases after 90 minutes. Thus, at 120 minutes the size of labeled photolysed DNA is about 70 S, showing that the BrdUrd has been inserted outside of the proposed 70 S cluster of 3-4 replicons initiated at anaphase.

At 150 S the BrdUrd insertions are yet further removed from the $^3$H-dAdo label and may be at the ends of two such clusters. At 180 minutes, the end of S-phase, there is little difference in size between photolysed and non-photolysed DNA showing that the BrdUrd is inserted, on the average, very far from the regions which replicated at start of S-phase. Thus, it is evident that DNA replication is not occurring in a temporally disperse manner. Rather it seems that temporal clustering of replicating regions must occur.

The final set of experiments is the most interesting. Here, the $^3$H-dAdo pulse followed a BrdUrd pulse at start of S-phase. The expectation was that this would allow a measurement of distance between the points initiating replication at time zero of S-phase and successively later replicating DNA. It might be expected that the photolysis product would remain replicon-sized during the first 60 minutes until the first set terminate, and that thereafter the size would increase as the $^3$H-label was inserted into regions outside of, and further away from the first set. One might expect a limit to this size increase determined by the maximum distance between sets of temporally first replicons. The result obtained is quite different. At 40 and 90 minutes the size of the pho-
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tolysis products are 35-40 S, which can be accepted as being within the limits of expectation, if replicons occur in clusters and are, in fact, about 40 S.

However, the size increase expected do not follow. At 120 and 150 minutes the size increase of the photolysis product is very slight, and these molecules remain at around 45 S. It seems to us that there could be two explanations for this result. Either, DNA synthesis is, in fact, initiated every 40-45 S along the chromosomal DNA at start of S-phase, but not all initiated points proceed in replication immediately. Or, the 45 S fragments represent a photolysis artefact which bears no relationship to the biology of DNA replication in Physarum. Two lines of evidence argues against this possibility. First, results presented in Figure 2 show that such a BUdR induced artefact at least is not general, since a very considerable size increase is observed in the photolysis fragments. Second, experiments performed on hybrid DNA shows that the DNA strand scissions are confined to the BrdUrd containing strand (Reviewed by Hutchinson) although double strand breaks may occur at higher doses. There is, however, to our knowledge no evidence to suggest that such energy transfer can occur to distant places on the same strand. Thus we consider it unlikely that the 40-45 S photolysis product obtained as shown in Figure 3 are artefacts. We conclude, therefore, that this result must be interpreted as evidence for some kind of general master initiation occurring within each replicon at the start of S-phase. The initiated replicons may then wait for further signals before they proceed to replicate their DNA.

REFERENCES
