LIMITED DNA SYNTHESIS IN THE ABSENCE OF PROTEIN SYNTHESIS IN PHYSARUM POLYCEPHALUM

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ABSTRACT
Actidione (cycloheximide), an antibiotic inhibitor of protein synthesis, blocked the incorporation of leucine and lysine during the S phase of Physarum polycephalum. Actidione added during the early prophase period in which mitosis is blocked totally inhibited the initiation of DNA synthesis. Actidione treatment in late prophase, which permitted mitosis in the absence of protein synthesis, permitted initiation of a round of DNA replication making up between 20 and 30% of the unreplicated nuclear DNA. Actidione treatment during the S phase permitted a round of replication similar to the effect at the beginning of S. The DNA synthesized in the presence of actidione was replicated semiconservatively and was stable through at least the mitosis following antibiotic removal. Experiments in which fluorodeoxyuridine inhibition was followed by thymidine reversal in the presence of actidione suggest that the early rounds of DNA replication must be completed before later rounds are initiated.

INTRODUCTION
Previous work from this laboratory showed that nuclear DNA was replicated in Physarum polycephalum beginning immediately after mitosis, and the duration of synthesis was from 2.5 to 3.0 hr of the normal generation time of 8 to 10 hr (2, 17). Complete DNA replication appeared to be a necessary condition for normal mitosis in this organism (22), and molecules of DNA which are replicated at a particular point in one S phase are replicated during a similar temporal segment of the following S phase (2). Molecular replication of nuclear DNA thus follows an ordered temporal sequence.

The purpose of the present study was to consider the role of newly synthesized protein in the initiation and completion of nuclear DNA replication. It is suggested, as a working hypothesis, that proteins must be synthesized to initiate DNA replication and to maintain temporal order during the S phase. Much recent work suggests that protein synthesis plays a key role in DNA replication of both prokaryotes and eukaryotes. Jacob and Brenner (7) proposed an elegant hypothesis which explains bacterial DNA replication in terms of a positive control mechanism. Many eukaryotes show, however, a more complicated design of replication than do the prokaryotes (18, 25, 26). The evidence suggests that neither replication of the genome nor even replication of a chromosome is regulated by a single complex with properties similar to those of the bacterial replicon.

Physarum serves as an excellent subject for investigating the factors controlling the initiation and the completion of the S phase in a typical eukaryote, because the plasmodium of this slime mold has a highly ordered yet experimentally accessible pattern of nuclear DNA replication (2). In a previous communication, actidione (cycloheximide), an antibiotic inhibitor of protein synthesis, was observed to block amino acid incorpor-
tion into protein without inhibiting as markedly incorporation of nucleic acid precursor into RNA (3). Prior publications showed that actidione inhibited protein synthesis in a number of organisms by preventing the transfer of activated amino acids to the growing peptide chain (24, 27). This compound also inhibited DNA synthesis in vivo without markedly affecting the in vitro activity of DNA polymerase (1). In the experiments which follow, it is assumed that the primary effect of actidione is on protein synthesis, and the observed effects on DNA replication are regarded as secondary consequences of the inhibition of protein synthesis. The justification for this assumption rests on the observations cited above and on the observations that other antibiotic inhibitors of protein synthesis did not effect DNA polymerization directly (8, 26).

MATERIALS AND METHODS

Previously published methods were used in the culture of this organism and for the observation of mitosis (5, 14). These experiments were done with plasmodia at MIII (the third mitosis following fusion of microplasmodia).

The chemicals employed included the following: actidione, The Upjohn Co., Kalamazoo, Michigan; thymidine-\(^{3}H\), 6.0 c/mmole, and C\(^{14}\)-lysine, 240 \(\mu\)c/mmole, Schwarz Bio Research Inc., Orangeburg, New York; C\(^{14}\)-l-leucine, 36.4 \(\mu\)c/\(\mu\)mole, Nuclear-Chicago Corporation, Des Plains, Illinois; C\(^{14}\)-erotic acid, 5.2 mc/mmole, Commissariat à L’Énergie Atomique, Paris, France; 5-fluorodeoxyuridine, Hoffman-La Roche, Inc., Nutley, New Jersey; deoxycytidine-\(^{3}H\), 0.1 c/mmole, C\(^{14}\)-5-bromodeoxyuridine, 31.0 mc/mmole, uridine, thymidine, and 5-bromodeoxyuridine (BUDR) were from Calbiochem (Los Angeles, California). Protein determinations were made by the biuret method (4) after the plasmodia were washed twice in 5% (w/v) trichloroacetic acid in 50% aqueous acetone, washed again in 0.25 M perchloric acid, and the resultant pellet was then dissolved in 0.4 \(\times\) NaOH. Radioactivity determinations were by liquid scintillation counting of the dissolved pellet.

RESULTS

Fig. 1 shows the effect of actidione on the incorporation of leucine and of lysine at the beginning of the period of DNA replication. The amino acid incorporation was inhibited nearly completely, soon after the antibiotic was added to the medium. A similar inhibition of methionine incorporation into both total and nuclear proteins during the same period of the cell cycle was reported previously (3).

The effect of actidione on the initiation and the completion of nuclear DNA synthesis was then determined. The effect of actidione on the rate of DNA synthesis, defined as the amount of thymidine incorporated into acid-insoluble polymer after a 20 min pulse with tritiated thymidine, is shown in Fig. 2. This experiment showed that when actidione was added to plasmodia in early prophase, during the period when mitosis is inhibited (3), no DNA synthesis was initiated during the time interval in which the S period would normally have begun. The addition of actidione in late prophase, however, permitted mitosis and nuclear reconstruction in the absence of protein synthesis (3). After this treatment, a burst of DNA synthesis was observed beginning in telophase, but the nuclear DNA complement was not completely replicated. The addition of actidione at other times during the S phase permitted similar bursts of DNA synthesis.

The pulse-labeling procedure was employed because it was a sensitive method of distinguishing whether or not DNA synthesis went on very slowly for a long time after antibiotic treatment. The results of the figure show that thymidine incorporation stopped completely after the period of actidione limited synthesis.
The significance of differences in the "rates" of DNA synthesis between treated and control plasmodia is questionable, because "rates" of synthesis as defined by pulse-labeling procedures are subject to uncertainty owing to the undefined status of the metabolic pool of thymine derivatives. It should be noted that the general features of the control curve of Fig. 2 are in agreement with a similar curve given in Braun, Mittermayer and Rusch (2). Minor points of difference can be ascribed to differences in the duration of the thymidine pulse.

The effect of cycloheximide on the time course of DNA synthesis is given in Fig. 3. Actidione was added during different segments of the S phase while thymidine was present continuously. In this experiment, thymidine was added at a relatively high level in the presence of fluorodeoxyuridine and uridine, so that it would be certain that a major part of the DNA thymine would be derived from exogenous thymidine. Thymidine incorporation reached a plateau after the limited synthesis following actidione addition. The presence of fluorodeoxyuridine and uridine tended to extend the normal S phase by about 30 min. This effect was probably due to the limited penetration of exogenous thymidine, and the effect had no obvious bearing on the interpretation of the experiment.

Various levels of actidione were tested to determine whether the amount of DNA synthesized in the presence of actidione was affected by the actidione level in the medium. In these experiments, actidione was added to the culture medium at the beginning of the S phase along with thymidine-H³ (1 μc/ml), and the amount of thymidine incorporation was measured at the end of S. No significant differences in the amount of incorporated thymidine were observed at actidione levels between 10 and 100 μg/ml.

Deoxyuridine and orotic acid were also employed as precursors of DNA synthesis. Table I shows that actidione treatment inhibited DNA synthesis to about the same extent when the different precursors were tested.

The previous experiments showed that a limited portion of the complement of nuclear DNA was replicated in the presence of actidione. To discover whether this DNA was replicated in a "normal" manner, bromodeoxyuridine was employed as a density label to establish whether the DNA synthesized in the presence of actidione was replicated semiconservatively. The results of a cesium chloride density gradient analysis of the DNA exposed to bromodeoxyuridine in the presence of actidione are shown in Fig. 4, which shows that the DNA was separated into two bands. The light major band was unreplicated DNA, whereas

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**Figure 2** The effect of actidione on the rate of DNA synthesis (30-min thymidine-H³ pulses). The plasmodia were treated with 10 μg/ml actidione in the broth medium, then transferred to glucose + salts mixture containing 10 μg/ml actidione and 1.5 μg/ml thymidine-H³ for the 30 min period of incubation. Extraction of plasmodia, protein determinations, and radioactivity analysis were as described in the text. In this figure, \( A = \) actidione and \( M = \) metaphase. \( M + 30 \) and \( M + 60 \) refer to 30 and 60 min following \( M \), and \( M - 10 \) means 10 min prior to \( M \). The right and left ordinates have similar units. Zero time on the abscissa refers to the time that metaphase was observed or to the time of actidione treatment in the \( M - 10 \) case. Arrows indicate the point at which actidione was added.

**Figure 3** The effect of actidione on DNA replication. These incubations were in broth medium containing 50 μg/ml fluorodeoxyuridine, 200 μg/ml uridine, and 50 μg/ml actidione. The thymidine-H³ level was 100 μg/ml, and the specific activity was adjusted to 4.9 μc/μmole. Extraction of plasmodia, protein determinations, and radioactivity analysis were as described in the text. The thymidine counts are normalized with regard to protein content. \( M = \) metaphase.
Effect of Actidione on DNA Incorporation of Deoxycytidine and Orotic Acid during the S Phase

<table>
<thead>
<tr>
<th>Isotope and Actidione</th>
<th>Control</th>
<th>% of control</th>
<th>Actidione-treated</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orotic acid</td>
<td>5620</td>
<td>988</td>
<td>19.7</td>
<td></td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td>5250</td>
<td>1050</td>
<td>19.6</td>
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Isotope and actidione (50 µg/ml) were added at the time mitosis was observed. The plasmodia were harvested after 3 hr incubation. The orotic acid level was 0.32 µc/ml, and the deoxycytidine level was 1.0 µc/ml. The plasmodia were extracted as described in the text; however, the plasmodia were suspended in 2 ml of 0.4 M NaOH following the last perchloric acid wash. The extracted plasmodia were incubated for 12 hr in 0.9 N NaOH, and then the DNA and protein were precipitated by adding cold 5% trichloroacetic acid. Following centrifugation in the cold, the supernatant was discarded and the pellet resuspended in 0.4 N NaOH for determinations of protein and radioactivity as described in the text.

The dense labeled band was formed by the DNA molecules which had incorporated bromodeoxyuridine in place of thymidine. This experiment shows that "actidione" DNA was replicated semi-conservatively, since the density of this hybrid band is similar to that of the BUDR hybrid band described by Braun, Mittermayer, and Rusch (2).

The other experiment, concerned with the normality of DNA replication in the presence of actidione, was an evaluation of the stability of the DNA replicated in the presence of actidione (Table II). Plasmodia were labeled with thymidine in the presence of actidione; then the plasmodia were cut into halves. One-half of each plasmodium was assayed for radioactivity immediately, and the other half was transferred to actidione-free medium. The results show that the thymidine label was conserved through at least one subsequent division. This experiment suggests that "actidione" DNA is stable at the time of its synthesis or that it is stabilized in some manner during the recovery period.

The previous experiments suggest that the product of limited DNA replication in the presence of actidione was normal but that complete replication of nuclear DNA depended upon the synthesis of replication proteins during the S period. The next experiments were designed to find out whether or not these replication proteins accumulate during an S period in which DNA synthesis is reversibly blocked. Another way of stating the problem is to ask whether or not completion of early rounds of DNA replication is necessary in order to trigger later rounds. The plan of this experiment was to block DNA synthesis for an extended period by employing fluorodeoxyuridine in the manner described by Sachsenmaier and Rusch (22), and then to reverse this fluorodeoxyuridine blockage by adding thymidine to the inhibited cultures along with actidione. If replication proteins accumulated during the period of fluorodeoxyuridine inhibition, then, when DNA synthesis was stimulated by thymidine addition, further protein synthesis would not be necessary and nuclear DNA would be completely replicated in the presence of actidione.

If, however, early rounds of DNA synthesis had to

![Figure 4 Incorporation of bromodeoxyuridine-C14 (BUDR) into DNA in the presence of actidione. Four plasmodia were incubated, beginning immediately after MIII, in u+c medium containing 100 µg/ml bromodeoxyuridine (2 cultures incubated with 0.05 µc/ml bromodeoxyuridine-C14), 10 µg/ml fluorodeoxyuridine (FUDR), 200 µg/ml uridine, and 50 µg/ml actidione for 3 hr. Nuclei were then isolated according to Mohberg and Rusch (18), and the pellet of isolated nuclei was lysed in 0.6 ml of standard saline-citrate containing 5% sodium dodecyl sulfate for 10 min at 55°C in water bath. The lysed nuclei were mixed with 3 ml of saturated cesium chloride. Centrifugation and analysis of the gradients were as described in Braun, Mittermayer, and Rusch (2). Open circles indicate C14 cpm. Tube 1 is the fraction from the bottom of the gradient.]

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TABLE II
Conservation of Thymidine Label following Recovery after Actidione Treatment

Plasmodia incubated with thymidine-H$^3$ and actidione during MIII and for 1 hr thereafter and then cut in halves

<table>
<thead>
<tr>
<th></th>
<th>dpm/half</th>
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<tbody>
<tr>
<td>Immediate assay</td>
<td>3340</td>
</tr>
<tr>
<td>Recovery through MIV plus 3 hr</td>
<td>3570</td>
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</tbody>
</table>

These cultures were labeled with 0.21 μmoles/ml of thymidine-H$^3$. The thymidine-H$^3$ specific activity had been adjusted to 4.9 μc/μmole. The actidione level was 50 μg/ml. The recovery medium contained 0.21 μmoles/ml of thymidine. Plasmodial extraction and radioactivity determinations were as described in the text; the average values for three plasmodia are given. MIV was observed about 20 hr after MIII.

![Diagram](image)

**Figure 5** Failure of replication proteins to accumulate during fluorodeoxyuridine (FUDR) blockage. The plasmodium was incubated in a medium containing 10 μg/ml of FUDR and 500 μg/ml of uridine. Reversal of the fluorodeoxyuridine blockage was accomplished by adding thymidine-H$^3$ (TdR-H$^3$), 50 μg/ml, with the specific activity adjusted to 8.9 μc/μmole. Extraction of plasmodia, protein determinations, and radioactivity analysis are given in the text.

be completed in order to trigger the later rounds, a burst of DNA synthesis, similar to the burst at the beginning of S, would be expected. The results of this experiment are shown in Fig. 5. The complement of nuclear DNA was not completed following reversal of the fluorodeoxyuridine block, and this experiment therefore supports the hypothesis that early rounds of DNA synthesis must be completed before the replication proteins for later rounds are synthesized. Fluorodeoxyuridine blockage in *Physarum* is never absolute; very slow replication proceeds in the presence of fluorodeoxyuridine (22), but this slow replication has little bearing on the results of the present experiment.

**DISCUSSION**

The results of the present experiment show that when protein synthesis is inhibited in late prophase a burst of DNA synthesis is observed, beginning in late telophase. Inhibition of protein synthesis at other times during the S phase permits similar bursts of DNA synthesis. These bursts of DNA synthesis can be described as "rounds" of replication, because this term is consistent with the temporal order of the replication process. A round of replication is defined as the quantity of DNA synthesized after adding inhibitor during the S phase. From the degree of inhibition, it is possible to suggest that there are between three and five rounds of replication during the S period of *Physarum*. These rounds are probably not clearly delineated by discontinuous periods of protein synthesis, but they probably arise from the average replication of a large number of individual units which vary in the duration of their activity. The present experiments show that the DNA synthesized during a round is probably replicated normally, first, because the DNA is replicated semiconservatively as it is during the normal S period (2), and second, because the DNA appeared to be stable. In these respects, the DNA can be considered, at least tentatively, to be replicated in the usual fashion even though protein synthesis is inhibited and replication is not complete.

The experiments also provide evidence which suggests that early rounds of DNA replication are necessary to trigger the synthesis of proteins which permit the later rounds. It is possible that "all" the later replicating DNA molecules may not be triggered by the early rounds of replication. It is apparent, nevertheless, that most of the late replicating DNA molecules cannot initiate DNA synthesis until the early rounds of replication are completed. A mechanism for this triggering action is suggested by the model proposed by Lark and Lark (9) to explain the alternate replication of two chromosomes in slow-growing *Escherichia coli*. To transpose that model to the present system, it is necessary to suggest that, as the replication units of the first round reach the end of their replication cycle, information for the formation of proteins which initiate the next round is transcribed. This mechanism implies that the initiator proteins which were transcribed from the early round must be capable of specifically recognizing the following units in a replication series.

The results of the present experiments are in
good agreement with the observations of Mueller et al. (16) and Taylor (26) concerning the effect of antibiotic inhibitors of protein synthesis on DNA replication in mammalian cell culture. In both these systems, there was limited DNA replication when inhibitor was added before the beginning of S, and similar bursts of replication occurred when inhibitor was added during S. Littlefield and Jacobs (11) suggested that histone may be the class of protein required for complete replication of the nuclear DNA complement in higher organisms.

Unlike the situation in higher organisms, replication of the single bacterial chromosome is completed in the absence of protein synthesis but replication is not initiated (12). The work of Lark and his coworkers shows that replication of the bacterial genophore begins at a particular location along its length and that there are at least two proteins which regulate the replication process (8-10, 21). The present experiments show that Physarum, a relatively primitive eukaryote, has machinery for DNA replication which is similar to that of highly developed eukaryotes rather than that of prokaryotes.

In most higher organisms, replication takes place at numerous points along particular chromosomes at given times (18, 25). Plaut and Nash (19) suggested that the points of replication along the polytene chromosomes of Drosophila may be formed by independently replicating units. Plaut, Nash, and Fanning (20) suggested a model of chromosomal organization which pictures the independently replicating units as replications and suggests that the chromosome has a longitudinal array of replications. These authors also suggest that all replications are active together at some time in the over-all replicative cycle, but that some remain active over longer periods than others. The present observations suggest that, if general features of the model of Plaut, Nash, and Fanning (20) are applicable to other eukaryotes, then Physarum chromosomes must have replications which do not function simultaneously and some replications may form temporally related series. This latter observation is also consistent with the observations of Hsu (6) that chromosomal regions which begin replication early during S complete replication early and regions which begin replication late complete replication last.

It might, however, be argued that, since some DNA synthesis occurs even though protein synthesis is inhibited before the initiation of replication in Physarum and other eukaryotes (16, 26), while bacterial DNA replication can be completed but not initiated in the absence of protein synthesis (12), the two systems must be essentially dissimilar. It is, nevertheless, necessary to recognize that the time during which protein synthesis can be inhibited and still permit initiation of some replication is only about 1% of the Physarum generation cycle. This duration corresponds to about 30 sec of the generation time of a bacterial cell. A period of this short duration is difficult to resolve with present methods of analysis. If the argument is reconsidered in terms of relative mean generation times of the organisms in question, the data for bacteria presently available are not sufficient to resolve whether or not the two systems are similar or dissimilar.

The present study has not attempted to delineate the number of units making up a round of replication. The model of Plaut, Nash, and Fanning (20) provides, nevertheless, the basis for speculation concerning the number of units which might be expected on the basis of chromosomal arrays of replications. Each unreplicated Physarum nucleus contains about 100 times more DNA (15) than the nuclear bodies of E. coli (13). The present experiments suggest that each of the rounds of DNA replication is completed in 30 to 60 min; thus, the time required to complete a round of replication is of the same order as the time required to replicate the bacterial chromosome under normal growth conditions (23). If there are as many as five rounds of replication during the S phase of Physarum, each round could be made up of about 20 replications, provided these replications are about the size of the chromosome of E. coli.

The major significance of the present experiments is not the lack of positive proof concerning the number of independent units of replication nor even the lack of evidence indicating that the control of replication is basically genetic. The significant features of these observations are the segmentation of the S phase into several rounds and the suggestion that the rounds of replication form a temporal series whose order is maintained by newly synthesized protein. When it is assumed that the mechanisms of replication in prokaryotes and eukaryotes are basically similar, it is possible to suggest, in conclusion, that a system of the type described here could have evolved from the mode of replication of the bacterial genophore to insure temporal order during the replication of more complicated nuclear genetic machinery.
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