CONTINUOUS NUCLEOLAR DNA SYNTHESIS IN LATE-INTERPHASE NUCLEI OF PHYSARUM POLYCEPHALUM AFTER TRANSPLANTATION INTO POST-MITOTIC PLASMODIA

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SUMMARY

In the myxomycete, Physarum polycephalum, nuclear DNA synthesis commences immediately upon completion of mitosis. While the synthesis of extranucleolar DNA is completed within a few hours, nucleolar DNA synthesis occurs during most of the S-phase and the entire G2 phase of the intermitotic period. When large (polyploid), late-interphase nuclei were allowed to bypass mitosis by transplantation into recipient plasmodia which were at early interphase and which belonged to a strain having smaller nuclei, the nucleolar DNA of the transplanted nuclei continued to be labelled (autoradiographs) after incubation of the host plasmodium with [3H]thymidine until they entered prophase along with the nuclei of the host plasmodium, approximately one intermitotic period later. This labelling was DNase-sensitive and RNase-resistant. When late-interphase nuclei were labelled with [3H]thymidine just prior to transplantation, there was no decrease of label after transplantation during the additional intermitotic period. We conclude from these experiments that there is no obligatory alternation between nucleolar DNA duplication and mitosis in Physarum polycephalum and that nucleolar DNA replication might exhibit amplification during an experimentally prolonged intermitotic period.

INTRODUCTION

In the myxomycete, Physarum polycephalum, the synthesis of extranucleolar DNA starts immediately after mitosis and is completed within a few hours (Braun, Mittermayer & Rusch, 1965; Nygaard, Guttes & Rusch, 1966) in more than 95% of the nuclei. Replication of nucleolar DNA throughout the entire G2 period preceding mitosis was first discovered by autoradiography of whole nuclei (Guttes & Guttes, 1969). It has been established since then that this DNA is not only associated with, but located within, the nucleoli (Ryser, Fakan & Braun, 1973), and that it is composed to a large extent, if not exclusively, of ribosomal cistrons (Britten & Smith, 1969; Holt & Gurney, 1969; Newlon, Sonenshein & Holt, 1973; Sonenshein, Shaw & Holt, 1970; Zellweger, Ryser & Braun, 1972). Ribosomal DNA is synthesized during S-phase and through the G2 phase (Newlon et al. 1973; Zellweger et al. 1972), and ribosomal DNA synthesis seems to be absent only during the first 60 min of the intermitotic period (Zellweger et al. 1972).
We have previously found that, when premitotic nuclei were prevented from dividing by translocation through protoplasmic streaming into post-mitotic regions of a mitotically asynchronous plasmodium, replication of the nucleolar DNA in these nuclei continued beyond the time at which the nuclei would have normally divided (Guttes & Telatnyk, 1971). Unfortunately, the period of time during which the premitotic nuclei could be distinguished by cytological criteria from the post-mitotic nuclei within an early-interphase region of the same plasmodium was limited to a few hours in these experiments. It is, therefore, possible that at least part of the continued labelling with \(^3\text{H}\)thymidine which we have previously found during this period represented delayed completion of DNA synthesis at a depressed rate following from the procedure used to produce asynchrony (Guttes & Telatnyk, 1971). We have now extended these experiments by transplantation of easily recognizable, highly polyploid nuclei at late interphase into early-interphase host plasmodia containing smaller nuclei of a much lower ploidy level. The results reported in the following indicate (1) that the nucleolar DNA of nuclei which are allowed to bypass mitosis by the above procedure continues to be synthesized for at least the duration of approximately one additional intermitotic period; and (2) that nucleolar DNA synthesized during a 'normal' intermitotic period is metabolically stable for the same period of time after transplantation.

MATERIALS AND METHODS

The organism was grown in form of microplasmodia in agitated submerged culture as previously described (Daniel & Baldwin, 1964). Mitotically synchronized surface plasmodia were obtained by coalescence of large numbers of microplasmodia (Guttes & Guttes, 1964).

The recipient plasmodia were made from a strain (McIII) which had been maintained in our laboratory for many years and which was originally obtained from the McArdle Laboratory for Cancer Research (University of Wisconsin, Madison, Wisconsin). The chromosome numbers in strains of similar origin have been determined several years ago by Ross (1966) and more recently by Mohberg, Babcock, Haugli & Rusch (1973). According to both authors, the plasmodia of McIII strains contain approximately 50 chromosomes, and Mohberg et al. (1973) found that in more recent sub-strains of McIII the number of chromosomes in about 50% of the nuclei was 75 'or more'. As this heterogeneity is also reflected by variation of nuclear sizes (Guttes & Guttes, 1969; Guttes, Hanawalt & Guttes, 1967) within a given plasmodium, it was necessary to increase drastically the overall ploidy level of the nuclei whose labelling behaviour after transplantation into another plasmodium we wanted to observe.

A stable, highly polyploid strain was produced from our original strain by heat shocks (Brewer & Rusch, 1968). For this purpose, plasmodia were placed for 20 min on preheated (37 °C) growth medium, beginning at that time of the prophase when the nucleolus had just started to disintegrate (Guttes, Guttes & Rusch, 1961). As described by Brewer & Rusch (1968), this treatment prevents further disintegration of the nucleolus and inhibits mitosis and nuclear division, although it does not interfere with chromosome division, DNA synthesis and with the formation of new nucleoli as in normal mitosis. Three heat treatments were applied and, after each heat shock, the plasmodia were allowed to grow for several cell cycles before they were heat-shocked again. The highly polyploid surface plasmodia were maintained as microplasmodial suspension cultures (Daniel & Baldwin, 1964). The nuclei of this new strain were readily distinguishable (Fig. 2), at any stage of the intermitotic period, from the much smaller nuclei of the original McIII strain. A determination of the number of chromosomes has not yet been made.

In order to have both donor and recipient plasmodia simultaneously available at the desired stages, groups of plasmodia from both strains were made from aliquots of microplasmodial
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suspensions approximately every 3 h over a period of 2 days. The density of the microplasmodial suspensions was adjusted in such a way that the prospective donor plasmodia were considerably thinner than the prospective recipient plasmodia. The growth medium for the donor plasmodia was renewed at the time of the 2nd and 3rd post-coalescence mitosis and the growth medium for the prospective host plasmodia at the time of the 2nd, 3rd, and 4th post-coalescence mitosis.

Transplantation of nuclei was achieved by allowing a small plasmodium containing the nuclei which were to be transplanted to coalesce with a much larger one serving as recipient. For this purpose, strips approximately 2.0 cm in length and 0.5 cm in width were cut from the periphery of a polyploid, late-interphase plasmodium (= donor) which was approaching the 4th post-fusion mitosis, and placed upside down along with their supporting paper, on the middle of a segment (approximately 12.0 cm²) from another plasmodium, the nuclei of which at that time were either in the process of undergoing, or had just completed, the 5th postfusion mitosis.

![Diagram of nuclear transplantation experiment](image)

Fig. 1. Schematic representation of a nuclear transplantation experiment in which the plasmodia are placed on [3H]thymidine after transplantation (the results of this experiment are shown in Figs. 4 and 5). Dashed lines represent that part of either a donor or host plasmodium which was set aside as a control prior to the transplantation. M, mitosis.

After placing the strip of donor plasmodium on the recipient plasmodium, the growth medium was replaced with non-nutrient salts medium, in order to promote locomotion and, thereby, rapid mixture of host and donor constituents. Within the next approximate 60 min, this procedure was followed by coalescence and gradual mixing of plasmodial constituents (monitored under phase-contrast in ethanol-fixed smear preparations). As the number of nuclei from the donor plasmodium was small compared to that of the recipient plasmodium, we consider this process as tantamount to transplantation. For convenience, the latter term will be applied from here on to denote the placement of a small segment from a 'donor' plasmodium on top of a much larger 'host' plasmodium.

The treatment of the plasmodia prior to and after transplantation depended on whether they were to be used to study continued nucleolar labelling or metabolic stability of the nucleolar DNA. In the former case (Fig. 1), pieces from the donor (= donor control) and from the host plasmodium (= host control) were set aside for separate observation prior to the transplantation. Donor controls, host controls, and experimental plasmodia were returned to fresh growth medium approximately 2 h after transplantation, and, subsequently, the host controls and experimental plasmodia were incubated at different times with [3H]thymidine. In the second group the donor plasmodia were incubated with [3H]thymidine (see below) at late interphase prior to the transplantation. Immediately after incubation they were floated, along with the supporting filter paper, for a few minutes on several changes of distilled water, and finally transferred to starvation medium. After 1.0 h the nuclei were transplanted into an unlabelled, early-interphase recipient plasmodium, followed by an additional 2.0 h of starvation as described above. Prior to labelling of the donor plasmodium, a small piece of this plasmodium
was set aside on growth medium in order to determine later, in retrospect, the state of the intermitotic period at which labelling had taken place.

For radioactive labelling, either plasmodial pieces (approximately 1.0 cm²) or whole plasmodia (see under Results) were placed for 20 min on growth medium containing 50 μCi/ml of [3H]thymidine (from Schwarz BioResearch, Inc., Orangeburg, New York, specific activity 6.0 Ci/mmol).

Squash preparations (Guttes & Guttes, 1969) or labelled nuclei were mounted on microscope slides and fixed with formalin (dilution: 1 vol. formalin + 3 vol. distilled water). In one set of experiments control slides were treated with DNase and RNase, respectively, as previously described (Guttes & Guttes, 1969). All slides were processed for autoradiography (Guttes & Guttes, 1969; Guttes & Telatnyk, 1971) and stored in nitrogen atmosphere for 3 months before development.

**RESULTS**

In one group of experiments we determined for how long nucleolar DNA synthesis continued to occur in nuclei which were transplanted, shortly before entering prophase, into a recipient plasmodium at the time when the nuclei of the latter were undergoing mitosis. At the time of transplantation a donor control piece was incubated with [3H]thymidine in order to determine its labelling pattern at that time. Pieces of host and host-control were incubated at different times after transplantation. The results shown in Figs. 3-5 are from one representative experiment. Fig. 3 shows the labelling pattern of the nuclei in a donor-control plasmodium at the time of transplantation. Within the limit of resolution the distribution of silver grains in the area of the autoradiographic emulsion covering the nuclei was similar to that which was previously described for late-interphase nuclei of normal size (Guttes & Guttes, 1969), i.e. labelling was limited to the nucleolar region (Fig. 3). In this experiment the number of giant nuclei found during mid-interphase in the host plasmodium was 5.6%, whereas in the host-control plasmodium we found only one nucleus of comparable size among 1000 nuclei counted during mid-interphase. We, therefore, assume that more than 90% of the giant nuclei having a large nucleolus in the host plasmodium were transplanted late-interphase nuclei.

A very few heavily labelled nuclei were found during G₂ phase in both host (1 in 1000 nuclei counted) and host-control (2 in 1000 nuclei counted) plasmodia. In these nuclei no distinction was possible between nucleolar and extranucleolar label.

In the host plasmodium, the nucleolar DNA of the implanted late-interphase nuclei became labelled at all times during the additional intermitotic period after transplantation until they were ready (Figs. 4, 5) to enter mitosis in synchrony with the nuclei of the host plasmodium approximately 11.5 h after transplantation, which occurred a little (0.5 h) earlier than mitosis in the host control. As in previous work (Guttes & Guttes, 1969), slides treated with DNase showed no nuclear label; whereas, the label was not affected by treatment with RNase.

The continued incorporation of [3H]thymidine into nucleolar DNA after transplantation might conceivably reflect metabolic instability of some of the nucleolar DNA which was present at the time of transplantation. Another group of experiments was, therefore, designed to determine if nucleolar DNA which was labelled during late G₂ phase did, or did not, stay within the nucleoli after transplantation into another
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unlabelled plasmodium. For this purpose premitotic polyploid nuclei were labelled with [³H]thymidine during late interphase (Figs. 6–9) and subsequently transplanted into recipient plasmodia which were undergoing mitosis.

Explants were removed from the donor plasmodia prior to, and from the host plasmodia at regular intervals after, transplantation and processed for autoradiography. The first set of fixations was made immediately at the end of the incubation of the donor plasmodium with [³H]thymidine. A second set was made 1·0 h later, i.e.

Table 1. Number of silver grains in autoradiographs over prelabelled nuclei at different times before and after their transplantation into an unlabelled host plasmodium

<table>
<thead>
<tr>
<th>Fixation no.</th>
<th>Nuclear label (silver grains/nucleus), $\bar{x} \pm$ s.d.</th>
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<tbody>
<tr>
<td>I</td>
<td>22·6 $\pm$ 4·9</td>
</tr>
<tr>
<td>II</td>
<td>27·0 $\pm$ 5·0</td>
</tr>
<tr>
<td>III</td>
<td>27·9 $\pm$ 3·7</td>
</tr>
<tr>
<td>IV</td>
<td>28·6 $\pm$ 5·0</td>
</tr>
</tbody>
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Approximately 90 min before prophase the donor plasmodium was placed for 20 min on medium containing [³H]thymidine. Squash preparations were fixed: at the end of incubation (I); 1·0 h later, i.e. just prior to transplantation (II); 2·0 h after transplantation (III); and just prior to the next synchronous mitosis of the transplanted nuclei along with those of the host plasmodium, approximately 12·5 h after transplantation (IV). No distinction was made between grains found in the area of the emulsion exactly above and outside the nucleolus. Each of the 4 groups of grain counts were obtained from 20 nuclei.

just prior to transplantation (see under Materials and methods), a third set was made 2·0 h after transplantation, and the fourth set was fixed just before the transplanted nuclei entered mitosis along with the nuclei of the host plasmodium. The results are shown in Figs. 6–9 and in Table 1. For grain counting we chose nuclei in marginal areas of a squash preparation (Fig. 7) which were extremely flattened to the extent that the silver grains in the autoradiographic image were sufficiently spread for counting. As the nucleolus could not be identified in such thin nuclei under phase contrast, the nucleolar localization of the silver grains over the implanted nuclei was verified in less-flattened areas of the same squash preparation (Fig. 6).

As seen in Table 1, there was no appreciable decrease in nuclear label during the interval between transplantation and the next synchronous mitosis of implanted and host nuclei. This is illustrated in Figs. 8 and 9. Fig. 9 is chosen from an area in the squash preparation which contained an unlabelled giant nucleus. This nucleus is morphologically similar (comparatively small, central nucleolus) to the previously described morphologically abnormal nuclei (Guttes & Guttes, 1969). In all preparations fixed after transplantation a small number of silver grains was found over the nuclei of the recipient plasmodium. These grains showed no preferential localization over either the nucleolar or the extranucleolar chromatin. Presumably, this label is due to isotope which was carried over from the highly labelled donor plasmodium into the recipient plasmodium which was about to commence S-phase at the time of
coalescence. In this experiment the number of large nuclei present in the host plasmodium at 2.0 h after transplantation was 6.2%; whereas, only one large nucleus among 1000 nuclei counted was found in the host-control plasmodium at the same time.

**DISCUSSION**

The finding that ribosomal (= nucleolar) DNA synthesis in *Physarum polycephalum* occurs during as well as after the completion of extranucleolar DNA duplication (Guttes & Guttes, 1969; Newlon et al. 1973; Sonenshein et al. 1970; Zellweger et al. 1972), has been taken as an indication that ribosomal DNA replication is ‘continuous’ (Guttes & Telatnyk, 1971; Sonenshein et al. 1970) and that it might extend beyond mere duplication within one intermitotic period (Guttes & Telatnyk, 1971). The present experiments show that nucleolar DNA, unlike extranucleolar DNA (S. Guttes & Guttes, 1968), continues to incorporate [3H]thymidine for the duration of at least one full intermitotic period in late-interphase nuclei which were allowed to bypass mitosis by transplantation into another plasmodium which had just finished mitosis. Since nucleolar DNA replication occurs through the entire G2 phase up to the earliest stages of prophase; some residual nucleolar DNA synthesis in the transplanted, late-interphase nuclei might be expected for a short time after their transplantation.

We consider it unlikely, however, that this could account for our results, since nucleolar DNA replication was found in the transplanted nuclei as late as approximately 10–11 h after transplantation, i.e. almost 10 h after the experimental plasmodia containing the giant implant nuclei were returned to fresh growth medium. It, therefore, appears more likely that continued synthesis in addition to duplication might have taken place.

Another argument concerns the limited resolution of the localization of silver grains in light-microscopic preparations. Since a large portion of the extranucleolar chromatin is located close to the nuclear envelope (Guttes et al. 1961) it would not be possible in our preparations to distinguish silver grains caused by nucleolar DNA after incubation with [3H]thymidine from those caused by extranucleolar DNA in the close vicinity of the nucleolus. The possibility of extranucleolar DNA labelling during ‘G2’ phase has recently been pointed out by Ryser et al. (1973). However, we feel that in our experiments, extranucleolar labelling was minimized by the fact that (1) at the time of actual coalescence the donor nuclei were within approximately 1.0 h of their own impending mitosis, and (2) when incubated and fixed after transplantation, the nuclei were long past their scheduled time for mitosis; whereas, in the experiment by Ryser et al. (1973) incubation of the plasmodia with [3H]thymidine began earlier than approximately 2.5 h prior to mitosis. Furthermore, the fact that the majority of silver grains found in our autoradiographs were located within a region of the autoradiographic emulsion either directly above the nucleolar region (Figs. 6–9) or in a zone adjacent to it, argues against the possibility that stable, non-nucleolar DNA label could have significantly contributed to the labelling which we consider as ‘nucleolar.’
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The same consideration applies to the experiments which dealt with the stability after transplantation of prelabelled nucleolar DNA. Since there was no appreciable extranucleolar labelling in the prelabelled nuclei (Figs. 6, 8) to obscure metabolic instability of the nucleolar DNA, we assume that nucleolar DNA which was synthesized during late G₂ phase prior to transplantation was metabolically stable and that the incorporation of [³H]thymidine found during the experimental extension of the G₂ phase was not due to turnover of nucleolar DNA synthesized prior to the transplantation. This is in agreement with the finding by Ryser et al. (1973), that rDNA labelled during G₂ phase does not become scattered when the nucleolus is fragmented at the beginning of mitosis but, rather, condenses along with the other, non-nucleolar, DNA, as one would expect if the late-synthesized rDNA would be tightly bound to the chromosomes.

Synthesis of ribosomal DNA beyond the level of duplication is a well established phenomenon in oocytes (Brown & Dawid, 1968; Gall, 1969). However, increase of rDNA out of proportion with extranucleolar DNA synthesis, although not generally observed in somatic cells (Brown & Weber, 1968; Ritossa, Atwood & Spiegelman, 1966a; Ritossa, Atwood, Lindsley & Spiegelman, 1966b) has been reported for cells other than oocytes. Such synthesis has been found, e.g. in iris epithelium cells of Triturus during transformation into lens tissue (Collins, 1972), in liver cells in tissue culture after hormone treatment (Koch & Cruceanu, 1971), and in Cymbidium protocorms in vitro (Nagl, Hendon & Rücker, 1972). In addition the (semi-permanent) restoration of wild type in ‘bobbed’ mutants in Drosophila suggests that extra copies of rDNA can be accumulated without proportional replication of non-nuclear DNA (Boncinelli et al. 1972; Ritossa et al. 1966a, b; Ritossa et al. 1971; Tartof, 1971).

Although nucleolar DNA was not released from the prelabelled nuclei after transplantation, it seems likely that the binding of nucleolar DNA to the chromosomes and the control of its duplication is different from that of the bulk of the nuclear DNA. Evidence for this possibility comes, for example, from the finding (Braun & Evans, 1969) that nucleolar DNA, along with mitochondrial DNA, is selectively extracted from whole lysates by Hirt's (1967) method, and that nucleolar DNA synthesis is considerably less sensitive to inhibition of protein synthesis by cycloheximide (Werry & Wanka, 1972) than the synthesis of extranucleolar DNA.

The eventual fate of the presumably extra copies of nucleolar DNA in P. polycephalum made during the experimental extension of the G₂ phase after transplantation is not known. An investigation of the stability during mitosis of nucleolar DNA which is synthesized during experimental prolongation of the ‘normal’ intermitotic period is now in progress.

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REFERENCES


(Received 16 October 1973)
Fig. 2. Transplanted, polyploid mid-interphase nucleus (arrow) surrounded by smaller, mid-interphase nuclei of the host plasmodium. Ethanol-fixed smear preparation. Phase contrast. Magnification of all figures is $\times 4500$; horizontal bars represent 10 μm.

Figs. 3–9. Autoradiographs obtained from formalin-fixed squash preparations.

Fig. 3. Polyploid nuclei of a donor-control plasmodium labelled with $[^{3}H]$thymidine during a 20-min period preceding early prophase. Nucleolus (arrow) apparent in one of the nuclei. Cytoplasmic background label is probably caused by labelled mitochondria. Phase contrast.
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Figs. 4, 5. Nuclear label after incubation beginning approximately 5.0 and 10.5 h (4 and 5, respectively) after transplantation of late interphase nuclei into mitotic host plasmodium. With (Figs. 4A, 5A) and without (Figs. 4B, 5B) phase contrast. Arrows indicate transplanted nuclei.
Fig. 6. Prelabelled, early-prophase nucleus from a plasmodium which was incubated with $[^3H]$thymidine prior to transplantation into an unlabelled host plasmodium. This fixation was obtained from the donor control at the time when transplantation was occurring in the experimental plasmodium. Note close correlation between position of silver grains and nucleolus. With ($A$) and without ($B$) phase contrast.

Fig. 7. Nuclei from prelabelled polyploid plasmodium before and after transplantation into an unlabelled host; with and without phase contrast ($A, B$, respectively).
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Fig. 8. Two polyploid, preprophase nuclei of a donor control plasmodium fixed shortly after labelling, while transplantation was occurring in the experimental plasmodium. With (A) and without (B) phase contrast.

Fig. 9. Transplanted nuclei (arrows) and host nuclei fixed approximately 12:5 h after transplantation. Note one large nucleus which appears to be labelled like the smaller nuclei of the host plasmodium.