MORE EVIDENCE FOR REPLICATION-TRANSCRIPTION-COUPLING IN
PHYSARUM POLYCEPHALUM

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SUMMARY
Endogenous RNA polymerase activity of isolated nuclei from Physarum polycephalum was
determined at high (400 mM KCl) and low (5-100 mM KCl) ionic strength.
The activity of RNA polymerase B (α-amanitin-sensitive UMP incorporation) and of RNA
polymerase A (plus C) (α-amanitin-resistant UMP incorporation) was compared in accurately
sized nuclear samples derived from macroplasmodia at distinct points of the mitotic cycle.
Minimum total RNA polymerase activity was detected in metaphase nuclei. A constant level
of RNA polymerase B activity was detected at all other stages of the mitotic cycle, if nuclei
were assayed at high ionic strength. However, a high level in S-phase, a low level in G1-phase
and again a high level in early prophase were measured, if nuclei were assayed at low ionic
strength.
Inhibition of DNA synthesis by hydroxyurea in vivo had a selective and drastic effect on
in vitro RNA polymerase activity of isolated nuclei derived from S-phase plasmodia, yielding
up to 100% inhibition in early S-phase.

INTRODUCTION
In two previous publications it has been claimed that, in the S-phase of the mitotic
cycle of Physarum, RNA synthesis depends on concomitant DNA synthesis. In one
case (Rao & Gontcharoff, 1969) the drug fluorodeoxyuridine (FUDR) was applied
to the culture medium, which results in substantial inhibition of DNA synthesis as
measured by the incorporation of deoxycytidine.
At the same time significant inhibition of RNA synthesis has occurred. In these
experiments a more direct effect of fluorouracil (FU), a metabolite of FUDR, on
RNA synthesis has also been observed which had to be balanced by an excess of
unlabelled uridine as shown by Sachsenmaier & Rusch (1964).
In the other case (Fouquet et al. 1975) hydroxyurea (HU) or cycloheximide have
been used to block DNA synthesis, and inhibition of RNA synthesis, particularly in
the first half of S-phase has been confirmed. In addition, the extent of labelling of
poly(A)-containing RNA and its hybridization to DNA has been considerably
reduced under these conditions, suggesting that the activity of RNA polymerase B
is affected. In both previous studies, all experiments have been performed in vivo.
However, the inhibitors of DNA replication in Physarum that have to date been em-
ployed, act indirectly by disturbing precursor pools or protein synthesis.

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Therefore, it cannot be deduced with certainty that these effects do not contribute to the inhibition of RNA transcription as analysed by in vivo labelling, even though a much smaller degree of inhibition of RNA synthesis has been shown in G_2-phase as compared with S-phase. As a coupling of DNA replication with RNA transcription might have important implications for an understanding of the control of the mitotic cycle in Physarum and possibly for the regulation of cell proliferation in general (Prescott, 1976), we have studied this phenomenon in vitro to circumvent any effects the drugs may have on RNA precursor pools and processing. It has previously been shown that the amount of RNA polymerases which can be solubilized does not fluctuate during the mitotic cycle of Physarum (Hildebrandt & Sauer 1976a). However, RNA polymerase activity of isolated nuclei does vary in accordance with changes in RNA synthesis detected in the mitotic cycle (Grant, 1972; Davis & Walker, 1978) and during differentiation (Hildebrandt & Sauer, 1977). Therefore, we have analysed the effect of inhibition of DNA replication in vivo on the endogenous RNA polymerase activity assayed with isolated nuclei in vitro.

MATERIAL AND METHODS

Cultures

Physarum polycephalum strain M3C VII was grown as an agitated suspension of microplasmodia for stock cultures and macroplasmodia were prepared by fusion of microplasmodia in exponential growth phase on filter paper according to standard procedures (Daniel & Baldwin, 1964; Mittermayer, Braun & Rusch, 1965). Most experiments were done during the second and third postfusion mitosis (M_2–M_3). This cell cycle lasts 9.5 ± 0.5 h at 26 °C and metaphase of mitosis occurs with natural synchrony within 5 min throughout a whole macroplasmodium of approximately 5 cm diameter. In most experiments a single macroplasmodium was used: one half served as control, the other was incubated for 30 min in 50 mM HU (or 5 μg/ml cycloheximide), a dosage which completely inhibits DNA synthesis within 10 min (Fouquet et al. 1975). In some experiments macroplasmodia were continually exposed to 1 μCi [14C]-thymidine per ml growth medium to label DNA as an internal standard to quantitate nuclei.

Preparation of nuclei

Nuclei were isolated according to the established method (Mohberg & Rusch, 1971). Ca^{2+} ions were replaced by Mg^{2+} ions which were shown to be necessary for the detection of endogenous RNA polymerase activity (Grant, 1972). The number of nuclei was determined by 3 different methods: direct counting of a diluted sample in a haematocytometer, determination of radioactivity of DNA, or by the measurement of u.v. absorption at 260 nm after lysis of an aliquot of nuclei in 2 M NaCl and 5 M urea, according to Sollner-Webb & Felsenfeld (1975).

Assay of nuclear RNA polymerase activity

RNA polymerase activity was determined under the conditions specified in previous work with isolated nuclei of Physarum (Grant, 1972; Davies & Walker, 1978; Hildebrandt & Sauer, 1977). Routinely 2–4 x 10^6 nuclei were incubated for 20 min at 30 °C in a volume of 100 μl containing in final concentration: 50 mM Tris-HCl, pH 8, 5 mM MgCl_2, 1 mM dithioerythrite, 0.25 M sucrose, 0.5 mM each of GTP, ATP and CTP, and 5 μCi ^3H-UTP (10−3 Ci/μm). The incorporation of ^3H-UMP into RNA was terminated by adding 5 ml of 7.5 % trichloroacetic acid (TCA) with 2 % pyrophosphate.

TCA-precipitable material was filtered onto glass-fibre filters (Whatmann GF/A) and care-
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fully washed in 5% TCA, 1% pyrophosphate, followed by methanol, before scintillation counting. Background values at zero time of the complete incubation assay including nuclei amounted to not more than 200–300 cpm.

In most cases a high salt concentration of 400 mM KCl was used which was previously shown to yield maximum relative activity of RNA polymerase B (Hildebrandt & Sauer, 1977). In several experiments low salt concentrations, 5–100 mM KCl, were employed as was done to describe changes in RNA polymerase activity in the mitotic cycle (Davies & Walker, 1978). In each case, at the respective ionic strength, the proportion of RNA polymerase B activity was deduced from the effect of α-amanitin (added at a final concentration of 20 μg/ml) by subtracting incorporation in the presence of the drug from incorporation in its absence. In certain assay mixtures either one or several of the following substances were added: actinomycin D, heparin, poly(dA-T) according to Bünning (1978), hydroxyurea and cycloheximide. For all nuclear preparations tested, UMP incorporation was template-dependent (over 90% inhibited by actinomycin D at 200 μg/ml or by DNase (100 μg/ml) treatment prior to the RNA-polymerase assay), all 4 ribonucleotide triphosphates were required in the enzyme assay mixture, and the in vitro product was over 90% sensitive to RNase + RNase T1, as was shown previously (Hildebrandt & Sauer, 1977). In addition, the drugs HU at 10–50 mM/ml and cycloheximide at 5–10 μg/ml had no effect on the in vitro RNA-polymerase assay; values of UMP incorporation were within 5% of controls.

RESULTS

Nuclear RNA polymerase activity

One important parameter in this comparative analysis of a nuclear enzyme activity is the size of the nuclear sample. In Fig. 1A the linear relationship between thymidine label in DNA and O.D. 260 of a nuclear lysate obtained in high salt and urea can be seen. The following relationship has been established compiling the results from hematocytometer counts, thymidine label and u.v. absorption: 1 O.D. 260 = 20 × 10^6 nuclei = 20 μg DNA. As the most convenient and reliable method of quantitation, u.v. absorption of the nuclear lysate has been chosen throughout this investigation. As seen in Fig. 1B the in vitro UMP incorporation increases linearly with the size of the nuclear samples which have been quantitated by thymidine labelling of DNA in vivo. Under our assay conditions this correlation holds over a 10-fold range in nuclear concentration, from 1–10 × 10^6 nuclei per assay; we have used 2–4 × 10^6 nuclei per assay to allow for exact comparison of the data obtained from different experiments and individual plasmodia.

Fig. 2 shows the profile of RNA polymerase B activity as determined by the effect of α-amanitin at distinct stages of the mitotic cycle and assayed under conditions of low salt concentration. The lowest activity, less than 5% of total UMP incorporation, is seen at mitosis. During early S-phase a high activity of RNA polymerase B has always been detected. At the end of S-phase this enzyme activity has decreased by 50% and during G2-phase, by 80%. However, in early prophase a high level of RNA polymerase B activity is again noted. In relation to total activity, RNA polymerase B amounts to approximately 20% in S-phase and 5% in G2-phase.

Three stages of the mitotic cycle have been chosen to compare the enzyme activity assayed at high salt concentration (Fig. 2). We observe no RNA polymerase B activity in mitosis and equal activities of RNA polymerase B of 40 ± 10% of total activity in
Fig. 1. Relationships between internal thymidine label in DNA, u.v. absorption of lysed nuclei, and UMP incorporation into RNA. A, for a series of aliquots from a nuclear suspension radioactivity (cpm) and o.d. 260 after lysis of each preparation were determined as described in Material and methods. B, increasing numbers of nuclei, as measured by $^{14}$C-Tdr incorporation were added to a standard in vitro assay of RNA polymerase as detailed in Material and methods.
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5-phase and G2-phase alike; total activity being also the same at approximately 50,000 cpm UMP incorporated per 10^6 nuclei.

An attempt has been made to detect free RNA polymerase B in isolated nuclei after blocking the endogenous template with actinomycin D and supplying exogenous template in form of synthetic poly(dA-T). At not one stage of the cell cycle, including metaphase, can any UMP incorporation be detected, and variable salt concentrations between 0-400 mM KCl had no influence on the inactivity of the exogenous template. (Similar observations have been made by A. Hildebrandt in our laboratory, who suspects interferences with an endogenous nuclear inhibitor, of RNA polymerase.) Furthermore, it has been found that heparin up to 100 μg/ml has no influence on the extent of UMP incorporation by isolated nuclei from various mitotic cycle stages, which argues against in vitro initiation of transcription.

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The effect of in vivo inhibition of DNA synthesis on in vitro RNA polymerase activities

In a series of experiments one half of a macroplasmodium has been treated with hydroxyurea and nuclei have been isolated from this half and the control half. Then endogenous RNA polymerase activity has been assayed without and with α-amanitin. Enzyme assays containing 2-4 x 10^6 nuclei have been made in triplicate and the values of UMP incorporation have been within 10%. The assays have been performed at high salt concentration where RNA-polymerase B is maximally active in all mitotic cycle stages. The percent inhibition of total UMP incorporation, α-amanitin-insensitive incorporation (RNA polymerase A (+C)) and α-amanitin-sensitive incorporation (RNA polymerase B) of the nuclei obtained from the treated cultures or each respective control, are summarized in Fig. 3 with respect to distinct mitotic cycle stages. Maximum inhibition of total activity (Fig. 3A) is observed in the first 60 min of the mitotic cycle, i.e. in early S-phase. RNA polymerase A is inhibited to some
Fig. 3. Replication-transcription-coupling. Inhibition of RNA polymerase activity in isolated nuclei after a blockade of replication in vivo. Percent inhibition at distinct cell cycle stages computed from in vitro RNA synthesis of control and treated halves of one plasmodium: A, inhibition of total RNA polymerase activity; B, inhibition of RNA polymerase A (and C) activity (α-amanitin-resistant UMP incorporation); and C, inhibition of RNA polymerase B activity (α-amanitin-sensitive incorporation) after treatment with hydroxyurea (○) and after treatment with cycloheximide (□).

From Fig. 3 c it becomes evident that RNA polymerase B is completely inhibited in early S-phase. Inhibition becomes less in the second hour of the mitotic cycle, i.e. in later S-phase. No inhibition of RNA polymerase B can be detected during G2-phase of the mitotic cycle, or in prophase.

In a series of experiments with cycloheximide a significant inhibition of RNA
polymerase B activity (α-amanitin-sensitive UMP incorporation) has also been detected (Fig. 3c). As in the experiments with hydroxyurea this inhibition is restricted to S-phase and cannot be observed during G2-phase of the mitotic cycle of Physarum.

**DISCUSSION**

The main conclusion of the results presented above is that a blockade of *in vivo* DNA replication can completely abolish RNA polymerase B activity of isolated nuclei. Maximum inhibition in early S-phase gradually decreases over the S-phase and no inhibition of that enzyme is seen in G2-phase. This result completely parallels previous observations where poly (A) RNA, labelled *in vivo*, has served as the parameter to monitor RNA polymerase B activity *in vivo* (Fouquet et al. 1975). However, in that case inhibition of RNA transcription by hydroxyurea and cycloheximide could have resulted from side effects of the inhibitors on RNA precursor pools or directly on polyadenylation. These possible artifacts cannot occur under the *in vitro* assay conditions where ribonucleotide triphosphates are not limiting and RNA polymerase B is assayed by UMP incorporation and is completely sensitive to α-amanitin. Furthermore, the drugs have no effect on the *in vitro* assay per se. Consequently, these results lend further support for the hypothesis of replication-transcription-coupling in Physarum.

Such coupling would determine the appearance of DNA transcripts in an ordered temporal sequence, since DNA replication follows a strict temporal order in Physarum (Braun, Mittermayer & Rusch, 1965).

Previous hybridization experiments with poly (A) RNA (Fouquet et al. 1975) or those utilizing early replicating DNA (Wick, 1976) as well as RNA transcription with isolated RNA-polymerase B from Physarum with synthetic templates (Hildebrandt & Sauer, 1976b) or with isolated nuclei or chromatin (Schicker, Hildebrandt & Sauer, 1979) provide additional though indirect evidence for replication-transcription-coupling. However, the investigation by Schicker et al. (1979) also shows, unexpectedly, a high template activity with exogenous RNA-polymerase B of nuclei late in the mitotic cycle (early prophase). This observation correlates with one finding reported in this paper: under assay conditions which confirm minimum RNA-polymerase activity in isolated nuclei in metaphase, maximum activity in S-phase and low activity in G2-phase (Davies & Walker, 1978), we observe a high level of endogenous RNA polymerase activity in prophase. Such has also been noted with pulse-labelled poly (A) RNA *in vivo* (Wick, 1976).

The low activity of RNA polymerase B seen *in vivo* and *in vitro* in untreated plasmodia in G2-phase is consistent with previous results with isolated nuclei (Grant, 1972). In a similar situation, it has been demonstrated that nuclei with low endogenous RNA polymerase B activity during starvation can be stimulated by removal of a non-histone-protein with a detergent (Triton X-100) to the same high transcription capacity seen in growth phase (Hildebrandt & Sauer, 1977). It is conceivable that the variable template activity in G2-phase and prophase is not controlled by the amount
of RNA polymerase but by the proportion of bound but inactive enzyme molecules as discussed by Hildebrandt & Sauer (1977).

An important assumption of the replication-transcription-coupling hypothesis is that in each mitotic cycle a starting point exists for the attachment of RNA polymerase molecules to the chromatin. Since there is no G1-phase in Physarum, this point is located in metaphase, just before the onset of S-phase. At this time we observe minimum activity of endogenous RNA polymerase B, confirming a previous analysis (Davies & Walker, 1978) and no RNA synthesis has been detected in metaphase in vivo (for review see Sauer, 1978). While this is indirect evidence for a lack of RNA polymerase in mitosis, a more direct analysis employing radioactive α-amanitin has indicated a significant reduction in RNA polymerase B of at least 75% during mitosis in isolated nuclei, in dispersed chromatin, and in partly deproteinized nucleoids (Sauer, 1979).

In conclusion, we can assume that a large proportion of RNA polymerase B attaches to replicating chromatin, but is only active if replication is not disturbed. The enzyme activity is high in S-phase, depressed in G2-phase and high again in early prophase. However, the enzyme leaves the chromatin during chromosome condensation and reaches its lowest level in metaphase. Therefore, it seems to behave like a shuttle protein (Goldstein, 1974).

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REFERENCES


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