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IN VIVO TRANSCRIPTION OF RIBOSOMAL RNA IN RELATION TO THE MITOTIC CYCLE IN *PHYSARUM POLYCEPHALUM**

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SUMMARY

We have investigated the transcription of ribosomal RNA in plasmodia of *Physarum polycephalum* by a combination of pulse-labelling with [^{3}H]uridine and RNA:DNA hybridization. The DNA used for the hybridization was a *Hin*dIII restriction fragment (cloned in bacteriophage λ) of *Physarum* ribosomal DNA that carries a substantial fraction of the rRNA genes, enabling the ribosomal transcripts in the newly synthesized RNA to be measured. We found that ribosomal RNA constituted about 40 % of the pulse-labelled RNA at all times during the synchronous mitotic cycle.

INTRODUCTION

The slime mould, *Physarum polycephalum*, is an interesting organism in which to study the regulation of ribosomal RNA synthesis, because the natural mitotic synchrony of its plasmodial form ensures that measurements of transcription can be related to the phases of the nuclear division cycle (for reviews, see Turnock, 1979; Seebeck & Braun, 1980). *S* phase in *Physarum* begins at the end of telophase and lasts 2–3 h and is followed by a G_2 phase of 5–6 h.

The ribosomal RNA genes of *Physarum* are located on linear, extrachromosomal DNA molecules (size: 60×10^3 bases), of which there are 150–200 copies per haploid genome (Seebeck & Braun, 1980). Each rDNA molecule is a giant palindrome and carries a set of rRNA genes towards each end. Transcription is initiated upstream from the 19 S gene and proceeds in the order, 19 S–5·8 S–26 S, the last gene being proximal to the adjacent terminus of the rDNA. The processing of the primary transcript, which includes the removal of two intervening sequences from the 26 S transcript, has been studied by hybridization of putative precursors to specific DNA probes and by R-loop mapping (Gubler, Wyler, Seebeck & Braun, 1980).

Several studies of transcription in nuclei isolated from plasmodia of *Physarum* have suggested that there may be significant changes in the relative proportions of the major classes of RNAs synthesized during the progress of the mitotic cycle (Grant, 1972; Davies & Walker, 1978*a*; Pierron & Sauer, 1980). The antibiotic, α-amanitin, was used to assess the relative contribution of nucleoplasmic RNA synthesis, which is inhibited by the drug. The results suggested that whilst the extent of rRNA synthesis,

* This paper is dedicated to the memory of Dr Ian Walker.

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directed by the α-amanitin-resistant RNA polymerase I, changes very little during the cycle, nucleoplasmic RNA synthesis shows a peak in mid-*S* phase, thereafter declining progressively during the remainder of the cycle. However, conditions, in particular the composition and ionic strength of the supporting buffer, for sustaining transcription in isolated nuclei are difficult to define unequivocally, and the most recent study (Pierron & Sauer, 1980) showed that the *in vitro* assay can be carried out in such a way that RNA polymerase II activity does not vary with the time in the mitotic cycle at which nuclei are isolated. It should be noted that the synthesis of all types of RNA ceases briefly during the actual process of nuclear division (Turnock, 1979), and nuclei isolated at metaphase exhibit very little transcriptional activity (Davies & Walker, 1978*a*).

The net rate of synthesis of mature rRNA during the balanced growth of a plasmodium increases about fivefold between early *S* and late G_2 phase (Hall & Turnock, 1976). This might seem to be in accord with those experiments (Grant, 1972; Davies & Walker, 1978*a*) that show RNA produced by RNA polymerase I as a greater proportion of total transcription in nuclei isolated in the late stages of the mitotic cycle. However, further analysis requires a procedure that can actually identify the proportion of rRNA transcripts in RNA that has been pulse-labelled *in vivo*. This paper describes such measurements made by RNA:DNA hybridization using an rDNA probe cloned in bacteriophage λ.

MATERIALS AND METHODS

Growth of plasmodia

Stock cultures of strain M_3CVIII (Daniel & Baldwin, 1964) of *Physarum polycephalum* were grown as microplasmodia as described previously (Plaut & Turnock, 1975) except that Bacto-Soytone replaced Mycological Peptone. This medium was also used to grow mitotically synchronous surface macroplasmodia in 9 cm Petri dishes (Hall & Turnock, 1976), and observations on mitosis were made by phase-contrast microscopy of ethanol-fixed smears according to Guttes, Guttes & Rusch (1961). The intermitotic time was 8–9 h.

Pulse-labelling and extraction of RNA

Samples of [5,6-^{3}H]uridine (1·0 mCi ml^{-1}, 47·7 Ci mmol^{-1}; Amersham International) were freeze-dried and resuspended to the same concentration in growth medium. Circles (2 cm diameter) of macroplasmodia (together with the filter paper support) were cut out and floated on 0·2 ml of the radioactive medium. After 10 min the plasmodial sample was washed in 0·2 M-NaCl, 0·01 M-Tris·HCl (pH 7·4) and homogenized in 5ml of buffer that contained 0·2 M-NaCl, 0·01 M-Tris·HCl (pH 7·4), 1 % tri-isopropylnaphthalene sulphonate and 6 % 4-aminosalicylate. Total nucleic acid was extracted as described by Hall & Turnock (1976) and the final ethanol precipitate was resuspended in 0·2 ml water to an approximate concentration of 1 mg ml^{-1}. More than 90 % of the incorporated lavel was extracted by this method.

Long-term labelling

Plasmodia were grown on 15 ml medium containing [^{3}H]uridine (0·5 mCi) for 24 h. The entire plasmodium excluding the inoculum ring was scraped off into 10 ml of homogenizing buffer and total nucleic acid extracted as for pulse-labelled samples.

Purification of 19 S and 26 S rRNA

Total nucleic acid was fractionated by electrophoresis on 1 % low-melting-point agarose (Bethesda Research Laboratories) gels containing 0·015 M-iodoacetate and the bands corresponding to 19 S and 26 S rRNA were removed, after visualization with ethidium bromide. The rRNA was extracted from the gel using hexadecyltrimethylammonium bromide (Langridge, Langridge & Bergquist, 1980) or by extraction with phenol according to Maniatis, Fritsh & Sambrook (1982).

Recombinant bacteriophage probe

The λ vector λ_{NM540} (Murray & Murray, 1975) was used to construct a recombinant bacteriophage probe by insertion of a *Hin*dIII restriction fragment from *Physarum* rDNA. The resultant recombinant is designated λ_{363}. We are indebted to Mrs J. Bundick for the construction of λ_{363} and to Dr H. Matthews for supplying a purified preparation of *Physarum* rDNA. The *Hin*dIII rDNA fragment contains some 78 % of the 26 S α-fragment, the internal spacer, which includes the 5·8 S sequence, and approximately 80 % of the 19 S gene (Gubler *et al.* 1980). The inserted sequence is approximately 5 kb (1 kb = 10^3 bases) in length and the organization of DNA in λ_{363} as determined by digestion with *Bgl*III, *Hin*dIII and *Sal*I is in accord with the expected presence of the *Hin*dIII fragment of *Physarum* rDNA (results not shown). λ_{363} was grown and DNA isolated according to standard techniques (Maniatis *et al.* 1982). λ_{363} was stored at −20 °C at a concentration of 2 mg ml^{-1} and thawed out immediately before use for hybridization assays.

Hybridization of Physarum *RNA with* λ_{363} DNA

Pulse-labelled and long-term-labelled RNA in total nucleic acid preparations from *Physarum* (1–4 μg by A_{260}) were hybridized to λ_{363} DNA (up to 100 μg) in 0·12 M-NaH_2PO_4, 0·12 M-Na_2HPO_4 and 50 % (v/v) de-ionized formamide in a total volume of 1 ml. After denaturation at 85 °C for 10 min in stoppered plastic vials, the samples were incubated at 47 °C for 24 h (Hall & Braun, 1977). Under such conditions, the rate of association of RNA:DNA hybrids is favoured relative to DNA renaturation (Casey & Davidson, 1977). After incubation, samples were diluted to 4 ml with 0·12 M-Na_2HPO_4, 0·12 M-NaH_2PO_4, treated with ribonuclease A (20 μg ml^{-1}) and ribonuclease T_1 (40 U ml^{-1}) and incubated on ice for 10 min and then at room temperature for 70 min. Hybridized material was precipitated in ice-cold 10 % (w/v) trichloroacetic acid with salmon sperm DNA (500 μg) as carrier and the precipitates collected on glass fibre filters (Whatman GF/C). Nucleic acid was then hydrolysed by placing each filter in 1 ml of 0·5 M-perchloric acid and incubating at 70 °C for 30 min, after which an 0·5 ml sample was assayed for ^3H radioactivity in 4 ml Picofluor 30 scintillant (Packard) in a Packard model 3255 scintillation spectrometer.

Saturation values, H_s, were obtained by linear regression analysis of the single reciprocal transformation D/H against D (where D is the amount of λ_{363} DNA and H is the extent of hybridization, corrected for non-specific hybridization in controls using no DNA). H_s was expressed as a percentage of total radioactivity in each sample, which was estimated by omitting the ribonuclease step. Counts recovered at zero DNA concentration were less than 5 % of total counts and controls using λ wild-type DNA at up to 100 μg were not significantly different from these.

RESULTS

Quality of RNA

It is difficult to extract intact RNA molecules from *Physarum* due to the high activity of endogenous ribonucleases and the presence of substantial quantities of polysaccharide (Melera & Rusch, 1973). The phenol extraction procedure was chosen for its convenience and the fact that it was not strictly necessary to isolate RNAs of high structural integrity provided that any degradation products were not too small to hybridize to the λ_{363} DNA probe. Using formamide-denatured samples,

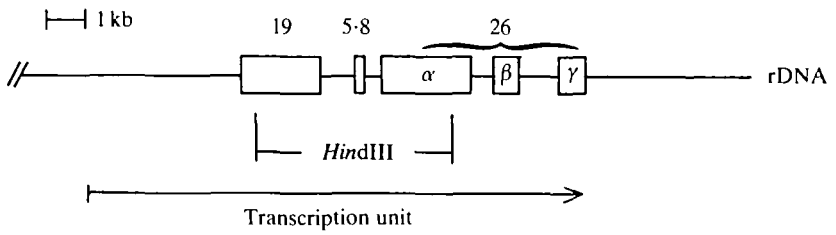

Fig. 1. The structure of the terminal region of rDNA from *Physarum* (after Campbell *et al.* 1979). The 5 kb *Hin*dIII restriction fragment incorporated into the genome of λ_{363} is shown, whilst the arrow indicates the size and direction of synthesis of the putative primary rRNA transcript. Initiation occurs approximately 4 kb upstream from the 19 S gene and the total length of the molecule is 13·3 kb.

the two major rRNAs, 19 s and 26 s, were visible on denaturing gels as two sharp bands (results not shown). Low molecular weight (< 19 S rRNA) degradation products were present in small amounts, only being detectable in denaturing gels by hybridizing [^{32}P]λ_{363} DNA to dried gels (results not shown). Such bands seem to correspond with degradation products observed in *Physarum* RNA preparations using other extraction procedures and are apparently both specific and reproducible (I. O. Walker & O. Schofield, personal communication).

Hybridization of Physarum *RNA to* λ_{363} *DNA*

The structure of the *Physarum* rDNA *Hin*dIII restriction fragment in relation to *Physarum* rDNA is shown in Fig. 1. It contains some 80 % of the mature 19 S rRNA

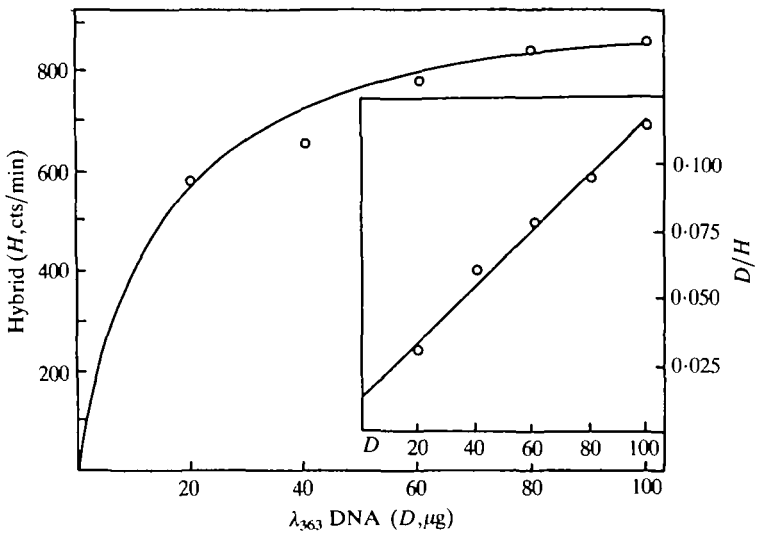

Fig. 2. Hybridization of pulse-labelled RNA to λ_{363} DNA. The inset shows a plot of D/H against D, the slope of this line being the reciprocal of H_s, the maximum quantity of [^{3}H]RNA in the sample that can hybridize with the DNA. In the example shown, H_s is 1062 cts/min relative to a value of 6574 cts/min for the total radioactivity in the sample of 2 μg of RNA.

Table 1. *Hybridization of [^{3}H]RNA to DNA of* λ_{363}

RNA	% Radioactivity in hybrid
A. *Purified RNAs*	
19 S rRNA	51·8 ± 7·0 (4)
26 S rRNA	48·8 ± 5·7 (6)
B. *Total RNA*	
Long-term-labelled	42·9 ± 2·5 (5)
Pulse-labelled	17·5 ± 2·3 (38)

sequence, the transcribed internal spacer with the 5·8 S rRNA sequence and 49 % of the mature 26 S rRNA sequence (Gubler *et al.* 1980; Otsuka *et al.* 1983).

A typical hybridization curve, using a pulse-labelled [^{3}H]RNA from *Physarum*, with λ_{363} DNA is presented in Fig. 2. The saturation value, H_s (calculated as the reciprocal of the slope as shown in the inset to Fig. 2), was found to be proportional to the amount of RNA used, up to at least 3 μg for purified 19 S and 26 S rRNAs, and for pulse-labelled and long-term-labelled total RNA. For 19 and 26 S rRNAs, H_s values can be compared with the estimates of radioactive RNA:DNA hybrid that should be formed based on the structure of the *Hin*dIII probe. The results of such a comparison are given in Table 1A and show excellent agreement for 26 S rRNA where the expected value of 49 % is calculated from the DNA sequence (Otsuka *et al.* 1983). For 19 S rRNA the recovery of hybrid was significantly lower than the 80 % expected. In this case, however, the sequence is not known, so that there is more uncertainty in the theoretical value, and in isolating 19 S rRNA there is always the possibility that it may be contaminated with degradation products of 26 S rRNA.

Differential rate of rRNA synthesis during the mitotic cycle

When RNA from plasmodia that had been labelled with [^{3}H]uridine for 10 min was fractionated by gel electrophoresis under denaturing conditions, the radioactivity was found (results not shown) predominantly in high molecular weight (> 26 S) material with a similar size distribution to that reported by Davies & Walker (1977, 1978*b*). The radioactive RNA molecules are a mixture of nascent and primary transcripts from all classes of genes. The fraction of [^{3}H]rRNA transcripts in RNA from plasmodia that had been pulse-labelled for 10 min was determined by RNA:DNA hybridization for several independent cultures throughout the mitotic cycle. The results are presented in Fig. 3 and clearly indicate that rRNA is synthesized as a constant proportion of total RNA synthesis at all times during the cycle.

Initiation of transcription of *Physarum* rRNA begins 17·7 kb from each end of the rDNA palindrome (Sun, Johnson & Allfrey, 1979), with termination at the 3' end of the 26 S gene corresponding to a primary transcript of 13·3 kb. However, the most abundant pre-rRNA is only 11·8 kb in length, and it has been suggested (Gubler *et al.* 1980) that there is rapid, spontaneous loss of the intervening sequences from the

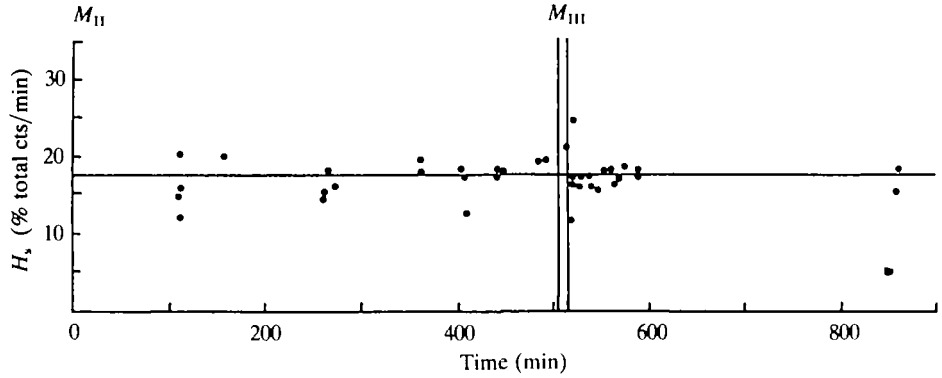

Fig. 3. The proportion of radioactivity in RNA from plasmodia pulse-labelled with [^{3}H]uridine at different times during the mitotic cycle that hybridized to λ_{363} DNA. M_{II} and M_{III} are, respectively, the second and third synchronous mitoses that follow the fusion of microplasmodia to initiate a surface plasmodium.

26 S segment of the primary transcript, possibly by a similar mechanism to that demonstrated for the splicing of pre-rRNA in *Tetrahymena* (Cech, Zaug, Grabowski & Brehm, 1982).

It takes several minutes for a plasmodium, supported by a piece of filter paper, to achieve a significant rate of labelling when placed on medium containing [^{3}H]uridine (Birch & Turnock, 1976). Now, the *Hin*dIII fragment of rDNA in λ_{363} represents approximately 42 % of the 11·8 kb precursor, so that the 17·5 % (Table 1B) of RNA made during a pulse that hybridizes to the DNA is equivalent to a contribution of 40–45 % by the rRNA genes to the total transcriptional activity.

In contrast, when long-term-labelled RNA is hybridized to λ_{363} DNA, radioactivity in RNA:DNA hybrid must be derived almost entirely from the mature 19 S and 26 S rRNAs. To a first approximation, half of each of these molecules hybridizes to λ_{363} DNA (Table 1A), so that the 42·9 % of long-term-labelled RNA that forms hybrid must represent a value of 84–86 % for the proportion of rRNA in total RNA. This value is in agreement with direct measurements of the rRNA content of *Physarum* RNA (Plaut & Turnock, 1975).

DISCUSSION

Determination of the differential rate of rRNA synthesis, by relating ^{3}H in RNA:DNA hybrid to total [^{3}H]RNA in pulse-labelled samples, is valid only if transcription throughout a nucleus draws on a common supply of pyrimidine triphosphates that receives radioactivity uniformly from exogenous [^{3}H]uridine. We have tested this assumption by showing that the pyrimidines of RNAs derived both from the nucleoplasm and the nucleolus are labelled in an identical manner by [^{3}H]uridine (Birch & Turnock, 1982). It is therefore reasonable to conclude that the

data presented in Fig. 3 are not biased by unequal labelling of RNAs synthesized in different regions of the nucleus.

The constant contribution of the rRNA genes to the overall transcriptional activity of the *Physarum* genome throughout interphase is an interesting observation. Assuming that precursors to cytoplasmic mRNAs are major components of the radioactive RNA, labelled in a 10-min pulse, that do not hybridize to the rDNA probe, it follows that fine adjustments to the balance between the synthesis of new ribosomes and the continuing production of mRNA must be made during post-transcriptional processing. The approximately fivefold increase in the net rate of mature rRNA synthesis between early S phase and late G_2 phase (Hall & Turnock, 1976) could, for example, be achieved by control of the fraction of primary rRNA transcripts that are processed and incorporated into ribosomes.

The amount of radioactivity from [^{3}H]uridine that enters rRNA in a 10-min pulse cannot be related to the net rate of ribosome production unless the specific radioactivities attained by UTP and CTP in the nuclear fraction can be determined, a requirement that cannot be met with techniques currently available. However, the relationship between gross and net rates of rRNA synthesis has been established for the bacterium, *Escherichia coli*. The results (Gausing, 1977) demonstrated that even at fast growth rates transcription from the rRNA genes is greater than the actual demand for rRNA, whilst at low growth rates only 30 % of the newly made rRNA is incorporated into ribosomes, the remainder being degraded. It is therefore possible that control of ribosome production in a eukaryote could also be effected via the post-transcriptional processing reactions. In particular, modulation of ribosome synthesis during the mitotic cycle may operate in this way. We are developing hybridization probes specific for the pre-rRNAs of *Physarum* to allow any variations in the relative amounts of the major precursors during the cycle to be determined.

As described in the Introduction, measurements of nucleolar and nucleoplasmic RNA synthesis in isolated nuclei have been complicated by susceptibility of the assay to variation in ionic conditions. Of the several studies, our results are in accord with the experiments of Pierron & Sauer (1980), who found that, by varying the concentration of KCl, nuclei from S and G_2 phases gave the same activity for RNA polymerase II. As RNA polymerase I activity in isolated nuclei is likewise invariant during the mitotic cycle (Davies & Walker, 1978*a*; Pierron & Sauer, 1980), a constant proportion of rRNA transcripts in newly synthesized RNA would be predicted. The same result would also be anticipated from an analysis of RNA polymerases I and II in which homogenates of plasmodia were fractionated by ion-exchange chromatography to separate the two enzymes (Hildebrandt & Sauer, 1976). The ratio of the two enzymes did not change during the mitotic cycle, with RNA polymerase I representing 40 % of the total activity. RNA polymerase III was detected in the same study, although it proved to be unstable in extracts.

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