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FUNCTIONAL STATES OF RNA POLYMERASE IN THE MACRONUCLEUS OF *TETRAHYMENA PYRIFORMIS* AND THEIR DEPENDENCE ON CULTURE GROWTH

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SUMMARY

RNA polymerase I in macronuclei of late log-phase cells of the ciliate *Tetrahymena pyriformis* is found to be present in 2 functional states, the one being actively engaged in transcription of ribosomal genes, the other one in a 'free' state, i.e. free to transcribe added DNA or poly d(AT).

Stimulation of RNA synthesis induced by dilution of stationary-phase cells into fresh medium is correlated with an increase in activity of template engaged and a decrease of free RNA polymerase I.

In contrast, RNA polymerase II shows no pronounced changes in activity and is not available to transcribe poly d(AT). The data favour the assumption that factors other than the amount of RNA polymerase I molecules available control transcription of the ribosomal genes.

INTRODUCTION

The ciliate *Tetrahymena pyriformis* provides an excellent system to investigate the regulation of genes coding for ribosomal RNA because changes in the culture conditions, such as inoculation of a stationary-phase culture into fresh medium result in a rapid increase of rRNA synthesis (Cameron & Guile, 1965; Andersen & Nielsen, 1979). Incorporation studies employing whole cells are difficult to interpret because many parameters such as stability, uptake and metabolism of the RNA precursors influence the experimental results (Jauker & Hipke, 1975). On the other hand, investigations using an *in vitro* transcription system of *Tetrahymena* did not yield any evidence of a specific interaction of purified RNA polymerase with macronuclear DNA (Freiburg, 1977). Therefore I have tried a third approach by employing isolated macronuclei. The results presented here show that they represent a promising experimental system, already previously introduced for the study of transcription in *Tetrahymena* by Mita, Shiomi & Iwai (1966) and Lee & Byfield (1970). In isolated macronuclei problems concerning uptake and differences in pool sizes of precursors are negligible and the different classes of RNA polymerases within the nuclei can easily be distinguished by their different sensitivity to the mushroom poison α-amanitin. There is RNA polymerase I, the α-amanitin-insensitive enzyme which transcribes the ribosomal genes, the very sensitive RNA polymerase II which synthesizes the heterogenous nuclear RNA, and the intermediately sensitive RNA polymerase III transcribing the tRNA and 5S RNA genes (for review, see Roeder, 1976). Moreover,

isolated macronuclei are not as complex as whole cells, but still retain some of those characteristics which reflect the situation within the cell. Attempts will be reported to distinguish different functional states of RNA polymerase I which are related to gene activity.

MATERIALS AND METHODS

Tetrahymena pyriformis strain T (amicronucleate) was grown in 0·75 % proteose peptone no. 3 (Difco), 0·75 % yeast extract (Difco), 1·5 % glucose, 1 mM $MgSO_4$, 0·05 mM $CaCl_2$ and 0·003 % sequestrene at 29 °C in a New Brunswick shaker at 125 rev/min. For the isolation of macronuclei cells from 600 ml were harvested, if not otherwise indicated, during the late logarithmic growth phase (approx. $1·2 \times 10^6$ cells/ml) and resuspended in 50 ml TCM buffer (10 mM Tris-HCl, pH 7·9, 3 mM $CaCl_2$, 1 mM $MgCl_2$) containing 0·25 M sucrose, 0·2 % Nonidet P 40, 0·1 % spermidine and 35 μg phenylmethylsulphonylfluoride (PhMSF, dissolved in ethanol, added to buffers immediately before use). The cells were lysed with 2–3 strokes of a tight pestle in a Teflon-glass homogenizer. The lysate was centrifuged for 10 min at 1000 **g**, and the resulting pellet resuspended in 130 ml TCM buffer containing 1·75 M sucrose, 0·1 % spermidine and 9 μg PhMSF. The suspension was layered in 3 portions on 10-ml cushions of TCM buffer containing 1·75 M sucrose, and centrifuged for 40 min at 20000 rev/min in a SW 25·2 rotor. The purified macronuclei at the bottom of the tube were resuspended in 50 mM Tris-HCl, pH 7·9, 30 % glycerol, 5 mM $MgCl_2$, 1 mM EDTA and 0·5 mM dithiothreitol, frozen in liquid nitrogen and stored at −70 °C. The macronuclei were free of cytoplasmic contaminants as checked in the phase-contrast microscope. The isolation efficiency was approx. 90 %. All steps were carried out at 4 °C. DNA was purified from isolated macronuclei following the method given by Gross-Bellard, Oudet & Chambon (1973) and denatured by heating for 10 min in boiling water and subsequent rapidly chilling in ice. The native DNA had an average molecular weight of $3·65 \times 10^7$ (Freiburg, 1977). For measurement of RNA synthesis in a growing culture aliquots were taken at various times and incubated for 30 min at 29 °C with 5 μCi/ml [^{3}H]uridine (30 Ci/mmol). The reaction was stopped by adding an equal volume of 50 mM Tris-HCl, pH 7·9, 1 % SDS, 0·1 M NaCl and 5 mM EDTA and subsequent incubation for 1 min in boiling water. After precipitation with ice-cold 0·5 M TCA the samples were filtered through Sartorius glass fibre filters (type 13400) and washed with 0·01 M TCA. The radioactivity on the dried filters was determined by liquid scintillation spectrophotometry.

RNA synthesis in isolated macronuclei was measured by incubation of 20 μl of the nuclear suspension in a final volume of 135 μl containing 50 mM Tris-HCl, pH 7·9, 2 mM ATP, 1 mM each of CTP and GTP (if not indicated otherwise), 0·1 mM UTP, 0·7 μCi ^{3}H-UTP (10 Ci/mmol), 0·1 M KCl and 3 mM $MnCl_2$. As an exogenous template denatured *Tetrahymena* macronuclear DNA (56 μg/ml) or poly d(AT) (80 μg/ml) were used. The choice of these templates appeared to be of advantage for a general test of RNA polymerase activity since specific initiation is no prerequisite for transcription of these templates. RNA polymerase II was measured as that fraction of total RNA polymerase activity which can be inhibited by the addition of 8 μg/ml α-amanitin/test, whereas the remaining insensitive fraction represents the activity of RNA polymerase I (III). The reactions were usually carried out for 30 min at 29 °C, stopped by the addition of 2 ml ice-cold carrier RNA (50 μg/ml in 2 M NaCl) and 0·5 ml of 3 M TCA and treated as indicated above. Each value represents the average of 4 parallel determinations.

RNA polymerase was purified from isolated macronuclei according to Roeder & Rutter (1969) except that the enzyme was eluted from DEAE-Sephadex A-25 in a 1-step procedure without separating different species.

RESULTS

RNA synthesis in isolated macronuclei

To discriminate between the activities of RNA polymerases I, II and III in isolated macronuclei, the dose-dependent inhibition of RNA synthesis by α-amanitin has been tested (Fig. 1). The initial rapid inhibition by low doses is due to the inhibi-

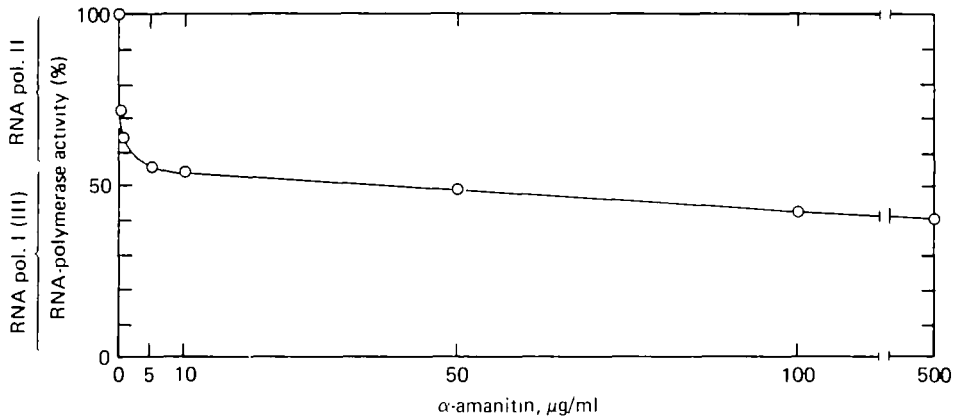

Fig. 1. Dose-dependent inhibition of RNA synthesis by α-amanitin in isolated macronuclei.

tion of RNA polymerase II (Roeder, 1976), the remaining activity represents the sum of the activity of RNA polymerases I and III. Since the inhibition observed by an increase of the α-amanitin concentration from 10 to 100 μg/ml clearly exceeds that caused by an increase from 100 to 500 μg/ml, it may be suggested that the fraction of intermediate sensitivity corresponds to RNA polymerase III. Similar characteristics have been reported for RNA polymerase III from other objects (Roeder, 1976).

The incorporation of ^{3}H-UMP into RNA by RNA polymerase I (III) and II in isolated macronuclei is demonstrated in Fig. 2 as a function of time. The rate of ^{3}H-UMP incorporation using endogenous DNA as template shows a gradual decline; there is only a very slight residual increase of incorporation into RNA after an incubation time of 15 min. This reflects a characteristic feature of *in vitro* transcription in isolated macronuclei: those RNA chains which have been initiated *in vivo*, are elongated and terminated *in vitro* without measurable reinitiation. This conclusion is supported by the finding that heparin which prevents initiation of free RNA polymerase molecules already at low doses, has no effect on the transcription in isolated macronuclei (Table 1).

The addition of denatured macronuclear DNA to isolated macronuclei strongly stimulates the activity of RNA polymerase I (III), while the activity of RNA polymerase II remains nearly unaffected (Fig. 2). After an incubation time of 15 min, when the transcription of the endogenous template ceases, the rate of DNA-stimulated RNA synthesis by RNA polymerase I (III) is still very high. It appears that the DNA added to macronuclei is essentially not transcribed by RNA polymerase molecules released from endogenous transcription complexes after termination, because a high proportion of RNA polymerase transcribes the exogenous DNA already at the start of the reaction, when most of the molecules engaged in transcribing the endogenous template have not yet terminated.

Template-engaged and 'free' RNA polymerase molecules in the macronucleus

The finding that denatured DNA added to isolated late log-phase macronuclei stimulates preferentially the activity of RNA polymerase I (III), gives rise to the

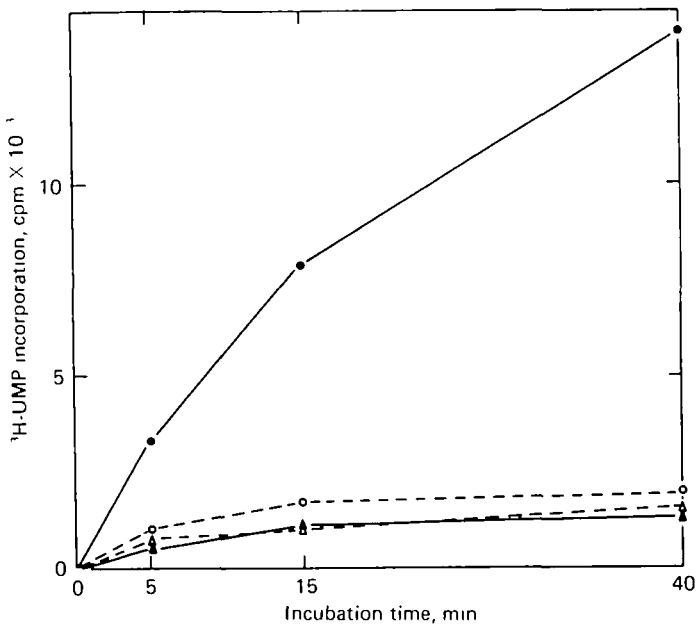

Fig. 2. Incorporation of ^{3}H-UMP into RNA in isolated macronuclei as a function of time. Activity of RNA polymerase I (III) (○ - - - - ○) and RNA polymerase II (△ - - - - △) without added DNA; activity of RNA polymerase I (III) (●——●) and RNA polymerase II (▲——▲) with added denatured *Tetrahymena* DNA. Each reaction mixture contained 2·8 × 10^6 macronuclei.

Table 1. *Effect of heparin on the total ^{3}H-UMP incorporation in isolated macronuclei and on the transcription of native Tetrahymena DNA by purified RNA polymerase*

Heparin, μg/ml	Macronuclei, cpm	RNA polymerase + DNA, cpm
0	481 ± 8	8549 ± 511
2	474 ± 57	64 ± 19
8	456 ± 59	0

Tests were carried out as described in Materials and Methods with isolated macronuclei (8·1 × 10^5 per test) or purified RNA polymerase (20 μl per test, O.D.$_{280}$ = 1·15) and native *Tetrahymena* DNA (2·6 μg per test) in the presence of different concentrations of heparin. The results are expressed as average values of 4 determinations and the corresponding standard deviation.

assumption that there are 2 different states of RNA polymerase I (III) within these macronuclei, one of which being engaged in transcription, the other existing in a 'free' form, i.e. free to transcribe added DNA. (Presumably this RNA polymerase is not part of an active ternary transcription complex before DNA is added.) The activity of 'free' RNA polymerase was measured by the transcription of added denatured *Tetrahymena* DNA or by transcription of poly d(AT) and compared with the activity of the template-engaged enzyme (Table 2). Using the endogenous DNA as template,

Table 2. *Template-engaged and free RNA polymerase molecules in isolated macronuclei*

	Substrates			
	ATP CTP GTP ^{3}H-UTP		ATP — — ^{3}H-UTP	
Enzyme activity \ Template . . .	Endog. DNA	Endog. DNA + denatured DNA	Endog. DNA	Endog. DNA + poly d(AT)
RNA polymerase I (III), cpm	1289	7796	79	5224
RNA polymerase II, cpm	1016	1543	0	0

Using endogenous DNA as template and the 4 nucleoside triphosphates all of the RNA polymerase molecules being in a specific transcription complex are active. Addition of poly d(AT) and the 2 nucleoside triphosphates ATP and UTP leads to ribo AU synthesis, which is completely dependent on the exogenous template and is catalysed by RNA polymerase molecules existing in a free state.

the activities of RNA polymerase I (III) and II are present in a ratio of 1:1. The addition of denatured DNA stimulates the α-amanitin-insensitive fraction approx. 6-fold, while the activity of RNA polymerase II increases only slightly compared to the stimulation of RNA polymerase I (III). Omission of the 2 nucleoside triphosphates CTP and GTP inhibits the transcription of the endogenous template almost completely, whereas addition of poly d(AT) leads to a ribo AU synthesis by RNA polymerase molecules which are not template-engaged and exclusively of the α-amanitin-resistant type.

Functional states of RNA polymerase during culture growth

Changes in transcriptional activities can be induced experimentally either by starvation and refeeding of *Tetrahymena* cultures (Hallberg & Bruns, 1976; Nilsson, 1976) or by dilution of stationary phase cultures into fresh medium (Cameron & Guile, 1965; Conner & Koroly, 1973; Andersen & Nielsen, 1979). The latter method was chosen to test the effect of different growth characteristics on the functional states of RNA polymerase.

A *Tetrahymena* culture was grown up in 300 ml culture medium to a cell density of 10^6 cells/ml, and harvested after further incubation for 14 h, when cells have reached the stationary phase. To start new culture growth, cells were then inoculated into 900 ml fresh prewarmed medium. Aliquots of the culture were taken at the indicated times and the incorporation of [^{3}H]uridine/cell and the cell number determined (Fig. 3A). One hour after inoculation into fresh medium, the incorporation of [^{3}H]-uridine/cell was increased approx. 4-fold, whereas cell multiplication had not yet been started. With the beginning of the exponential growth after 2 h the [^{3}H]uridine incorporation reached its maximum and declined during the following exponential

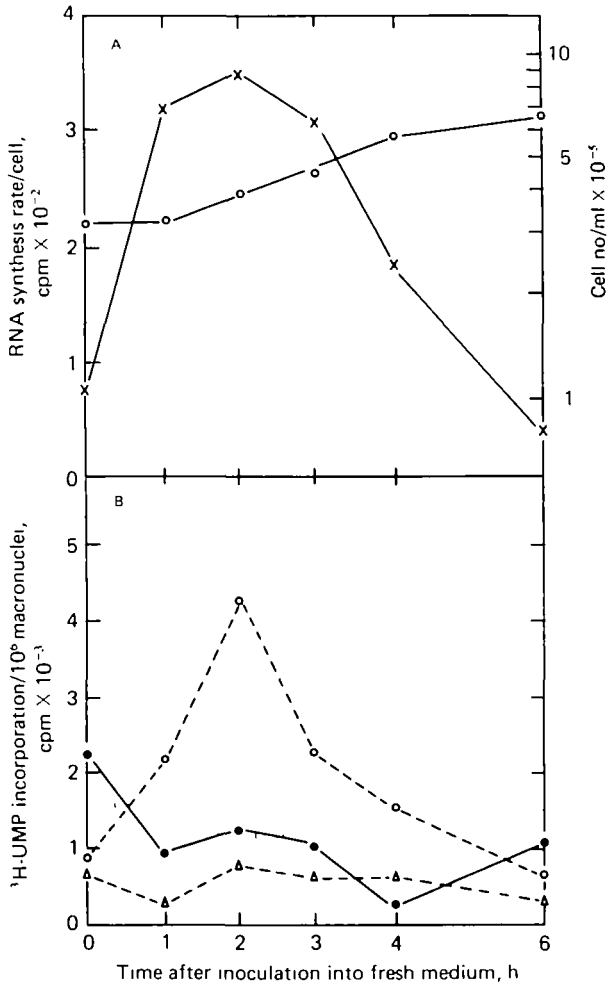

Fig. 3. Culture growth-dependent changes in RNA synthesis and the state of RNA polymerase after inoculation of a stationary culture into fresh medium. A. Cell number (○——○) and [³H]uridine incorporation/cell (×——×). B. Culture growth-dependent free and template-engaged RNA polymerases in isolated macronuclei. Parallel cultures were started by inoculation of a stationary culture into fresh medium. At different times after inoculation macronuclei were isolated and assayed for free and template-engaged RNA polymerase activities. Template-engaged RNA polymerase I (III) (○ - - - ○) and RNA polymerase II (△ - - - △) active on the endogenous DNA. Free RNA polymerase I (III) (●——●) active on added poly d(AT) and ATP and UTP as substrates only.

growth. Already 4 h after inoculation into fresh medium the cells again reached the transition phase between exponential and stationary phase.

In a similar experiment macronuclei were isolated at different times after the start of a new culture growth, and the activities of free and template-engaged RNA polymerase were determined (Fig. 3B). Two hours after inoculation the activity of bound RNA polymerase I was found to reach a maximum and declined during further

incubation. In this respect it shows a similar time course to the [^{3}H]uridine incorporation in whole cells. The activity of free RNA polymerase I decreases immediately while the activity of bound RNA polymerase I increases. In contrast, the decrease of bound RNA polymerase I activity following maximum activation is not paralleled by an increase of the free enzyme, but shows a time lag, in this case about 3 h.

DISCUSSION

The data presented in this communication show that as in other eucaryotes (for review, see Muramatsu, Matsui, Onishi & Mishima, 1979; Grummt, 1978) isolated macronuclei of the ciliate *Tetrahymena pyriformis* contain a pool of RNA polymerase I molecules which are – depending on the actual culture growth – either actively transcribing the ribosomal genes or existing in a free state, i.e. not bound to DNA. The finding that a rapid increase of RNA synthesis is correlated with the reduction of the activity of free enzyme favours the assumption that the factor limiting transcription of the ribosomal genes is not the number of RNA polymerase molecules available but their ability to initiate rRNA synthesis. There is increasing evidence that the synthesis of rRNA in eucaryotes is stringently linked to protein synthesis, whereby initiation by RNA polymerase I is positively controlled by a short-lived protein which enables the free enzyme to bind to specific sites of the DNA and to initiate new RNA chains (Gross & Pogo, 1976; Mishima, Matsui & Muramatsu, 1979). Perhaps the time lag between the decrease in activity of bound RNA polymerase I and the increase in the free enzyme indicates that there may be a second mechanism of gene regulation causing direct repression of transcription. Such a mechanism is known from *Physarum* where free RNA polymerase I is inactivated under conditions of starvation by a specific inhibitor which disappears after refeeding (Hildebrandt & Sauer, 1977*b*; Hildebrandt, Mengel & Sauer, 1979). The finding that α-amanitin-sensitive RNA polymerase II cannot be stimulated by addition of exogenous DNA or poly d(AT) to isolated macronuclei leads to the conclusion that – in contrast to RNA polymerase I – this type of enzyme remains always bound to its template. Dreyer & Hausen (1978) also reported that RNA polymerase II is always bound to DNA in lysates of *Ehrlich* ascites cells. In contrast, Hildebrandt & Sauer (1977*a*) in *Physarum*, and Yu (1974, 1975) in rat liver have found high amounts of free RNA polymerase II in isolated nuclei. These conflicting results may be due to different isolation and assay procedures or specific features of the different organisms used in the experiments. A selective loss of free RNA polymerase II in the *Tetrahymena* system can be excluded by the finding that RNA polymerase isolated from whole cells shows an elution profile on DEAE-Sephadex columns similar to that of enzyme preparations from isolated macronuclei (unpublished results).

Furthermore, RNA polymerase II shows no pronounced changes in its activity after inoculation of stationary phase cells into fresh medium. Similar results are reported from other eucaryotic systems after stimulation of RNA polymerase I (Todthunter, Weissbach & Brot, 1978; Chomczýnski, Sokól-Misiak & Kleczkowska, 1977; Cox, 1976).

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