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REGULATION OF RIBOSOMAL RNA SYNTHESIS IN *TETRAHYMENA PYRIFORMIS*

J. KEIDING AND H. A. ANDERSEN
The Biological Institute of the Carlsberg Foundation,
16 *Tagensvej, DK–2200 Copenhagen N, Denmark*

SUMMARY

Ribosomal RNA is synthesized at constant rate during most of the cell cycle in heat-shock synchronized populations of *Tetrahymena pyriformis*. Early in each macronuclear *S*-period the rate of synthesis increases abruptly, concomitant with replication of the genes coding for ribosomal RNA. The increase is prevented by inhibitors of DNA replication, added prior to the *S*-period. Similarly, in cultures synchronized by starvation/refeeding, inhibition of DNA replication, at the time when the rDNA is replicated, will prevent the normal increase in rate of RNA synthesis which follows refeeding.

We conclude that inhibition of rDNA replication interferes with the synthesis of rRNA, and we suggest that with respect to rRNA synthesis a gene dosis effect is operating in fast-growing *Tetrahymena* cells.

INTRODUCTION

It seems to be a rule in eukaryote cells that DNA synthesis takes place at a discrete interval of the cell cycle, the *S*-period. Contrary to this, synthesis of RNA and protein is a continuous process occupying the entire cell cycle (Mitchison, 1971). However, in a number of different cells the rate of synthesis of the different classes of RNA has been found to vary dramatically in various phases of the cell cycle. Thus in *Schizosaccharomyces pombe* the rate of synthesis of both ribosomal RNA and messenger RNA doubles during the *S*-period (Fraser & Moreno, 1976). Fraser & Carter (1976) have reported that in fast-growing *Saccharomyces* the rate of synthesis of ribosomal RNA follows a similar pattern, but others have obtained conflicting results (Sogin, Carter & Halvorson, 1974). In HeLa cells it has been found that total cellular RNA is synthesized at constant rate during the G_1-period, and that the rate of synthesis increases during the *S*-period. After the *S*-period the new high rate of RNA synthesis is kept constant during the following G_2-period (Thilly, Arkin & Wogan, 1977). In *Physarum*, on the other hand, the rate of RNA synthesis seems to increase throughout the entire cell cycle (Hall & Turnock, 1976).

In the present paper we show that in *Tetrahymena pyriformis* synchronized with one heat shock per cell cycle the rate of RNA synthesis increases abruptly at the beginning of the macronuclear *S*-period. During exponential growth in the medium here used (PPL) it has been found that more than 90% of the total cellular RNA is ribosomal RNA (Leick, 1973). Thus changes in the overall amount of RNA mainly reflect changes in the amount of rRNA. It has previously been shown that in heat-

synchronized cells the genes coding for rRNA (rDNA) replicate in a short time interval at the beginning of the macronuclear *S*-period (Andersen & Engberg, 1975). Here we show that inhibition of the rDNA replication prevents the increase in rate of rRNA synthesis, both in heat-synchronized cells, and in cells synchronized by starvation/refeeding. The results indicate that in fast-growing, synchronized cells the number of genes is limiting for the rate of synthesis of ribosomal RNA.

MATERIALS AND METHODS

Tetrahymena pyriformis, amicronucleate strain GL, was grown in proteose peptone as previously described (Andersen, 1972*a*, *b*; Andersen & Zeuthen, 1971) and synchronized with the 'one heat-shock per cell cycle procedure' developed by Zeuthen (Zeuthen, 1971). The cells were counted using an electronic cell counter after previous fixation in 10% formalin.

DNA synthesis was inhibited by either methotrexate plus uridine (M + U) or hydroxyurea (HU). Methotrexate (gift from Cyanamid Overseas Corporation, USA, Copenhagen Division) and uridine were added under sterile conditions to final concentrations of 0·05 and 20 mM respectively. The final concentration of HU was 10 mM.

In the experiments using radioactivity cell cultures were incubated with [^{14}C]uridine, and at various times 1-ml samples were precipitated by ice-cold trichloroacetic acid (final concentration 5 %). The precipitate was filtered on Whatman GF/C filter paper disks and washed several times with 5 % TCA. Finally it was dried and counted in a liquid scintillation counter (Beckman, LS 200).

The chemical amount of RNA was measured according to the method of Schneider (Schneider, 1945).

RESULTS

In *Tetrahymena* cells synchronized with one heat shock per generation, cell division takes place 80–90 min after the end of each heat shock. The cell division is immediately followed by a macronuclear *S*-period. The rate of RNA synthesis was measured during this cell cycle, by pulse incorporation of radioactive uridine. One-millilitre samples were withdrawn from the culture and incubated with ^{14}C-labelled uridine for 20 min. The incorporation was stopped by addition of cold trichloroacetic acid. Sampling was started at the end of heat shock no. 6 and continued for 5 h during which the culture was kept at constant temperature (28 °C).

The results are shown in Fig. 1. The amount of uridine incorporated increases stepwise during the 5-h period, with the steps located close to the 2 synchronized cell divisions, at the beginning of the macronuclear *S*-period. In this 5-h period only very small variations in the intracellular pool of ribonucleotide triphosphates have been measured (Nexø, 1975) indicating only minor influence of pool variations on the incorporation of ^{14}C-labelled uridine. Thus pulse incorporation of [^{14}C]uridine is likely to be a fairly accurate measure of the rate of RNA synthesis, and the results are also in good agreement with chemical determinations of the cellular RNA content during the cell cycle, which are shown in Fig. 2.

During a 20-min incubation with radioactive uridine essentially only stable RNA species are recorded. Less than 10% of the pulse-labelled RNA could be removed by a 3-h chase with cold uridine (data not shown), and others have obtained very similar results (Jauker, Seyfert & Sgonina, 1975). Thus most of the labelled uridine is found

in stable RNA species, i.e. in ribosomal RNA. Therefore, the results shown in Fig. 1 indicate that the rate of ribosomal RNA synthesis increases abruptly at the time of the synchronous cell divisions.

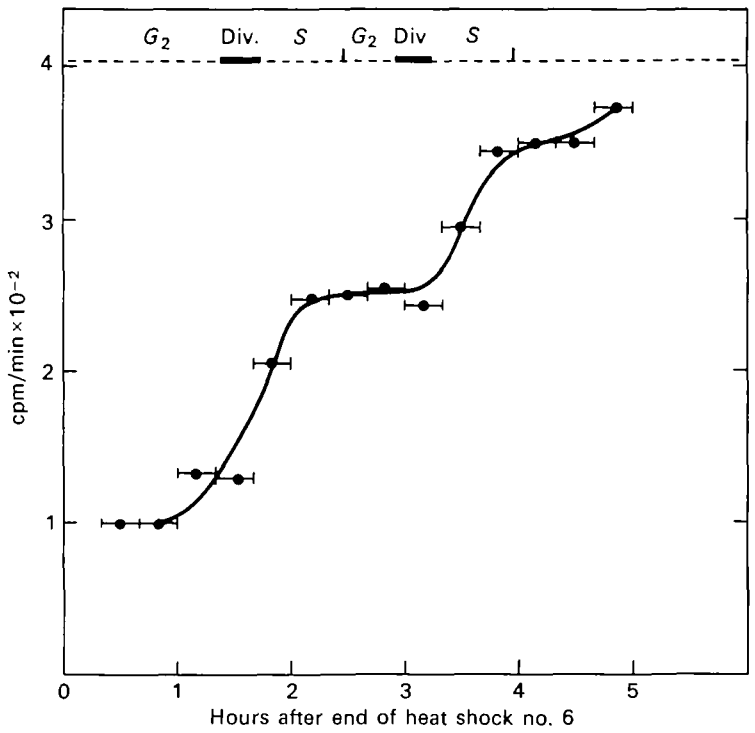

Fig. 1. Pulse labelling of RNA in a heat-synchronized population. Samples were withdrawn, labelled for 20 min with [^{14}C]uridine and subsequently precipitated with ice-cold TCA. The culture is kept at constant temperature (28 °C) after heat shock no. 6 (free-running synchrony). The cell cycle in this system is indicated at the top of the figure.

Fig. 2 shows the results from 3 separate experiments in which the chemical amount of RNA per ml cell culture was determined at various times following the end of a heat shock. When the data are plotted in a linear graph the accumulation of RNA follows 2 straight lines, intersecting at the time of the synchronous cell division about 80 min after EH. This result also indicates that the rate of ribosomal RNA synthesis increases abruptly at the time of the cell division.

In *Tetrahymena* cells synchronized by heat shocks the G_1 period is virtually absent (Zeuthen, 1971; Andersen, 1972*b*). These cells initiate DNA replication immediately after cell division and a change in the rate of RNA synthesis can be observed at the same time. It is therefore tempting to correlate the replication of DNA early in the *S*-period with the increase in the rate of RNA synthesis. If such a correlation exists it should be possible to prevent the increase in rate of RNA synthesis by inhibition of the DNA replication in the early part of the *S*-period. The following experimental data will show that this is possible.

Fig. 3 shows how the presence of 0·05 mM methotrexate plus 20 mM uridine (M+U), a well described inhibitor of DNA replication in *Tetrahymena* (Zeuthen, 1968), affects the incorporation of labelled uridine into TCA-precipitable material. Two cultures were treated with M+U, one at the end of the fifth heat shock (EH) and the other 100 min after EH. Two parallel control cultures were treated with U alone in order to keep the specific activity of the radioactive uridine the same. It will appear from the figure that when the DNA replication is inhibited from the

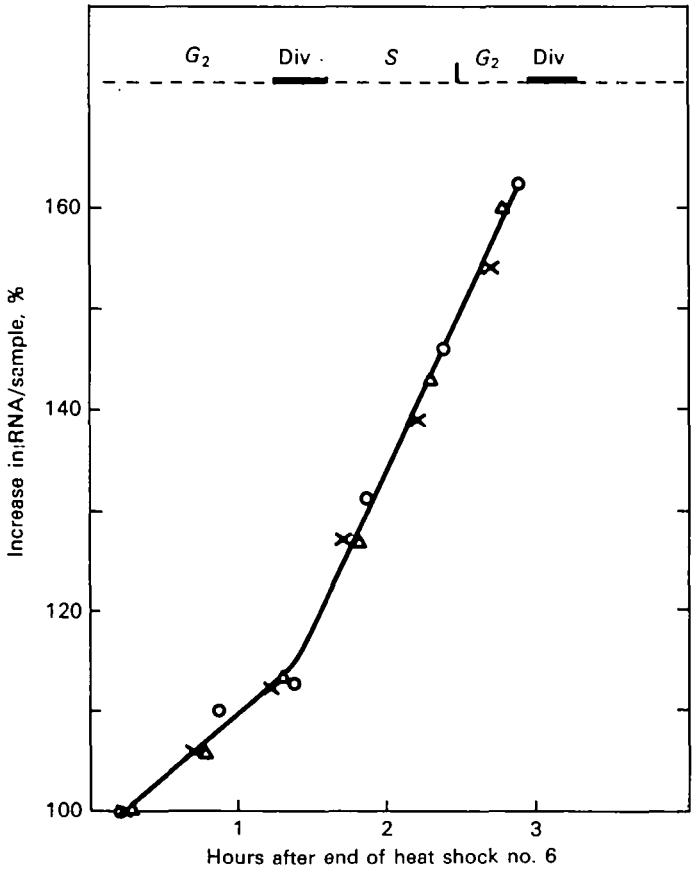

Fig. 2. Increase in amount of RNA in a heat-synchronized population. The total amount of RNA was measured at different times. Results from 3 separate experiments are shown. The culture was kept at 28 °C following heat shock no. 6.

time of EH, the RNA synthesis is influenced only at or shortly after the first synchronous cell division. If the inhibitor is added at the time of the first synchronous division RNA synthesis is affected only at the time of the second synchronous division. Uridine alone has no effect on macromolecular synthesis and the uridine-treated cultures behave in any respect like normal synchronized cultures. Fig. 4 shows results from a similar experiment in which another inhibitor of DNA synthesis, hydroxyurea (HU), has been used. The effect of HU is similar to that of M+U.

Inhibition of DNA replication at the time of initiation of the macronuclear *S*-period prevents the shift in the rate of RNA synthesis which is normally occurring at that time.

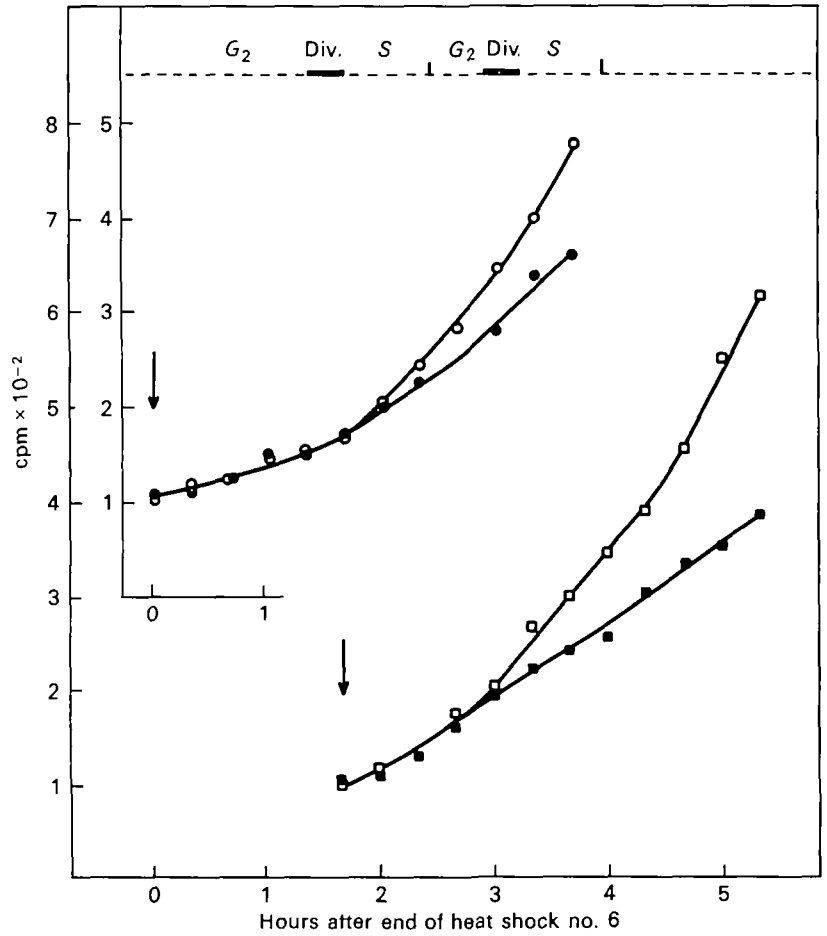

Fig. 3. The effect of M + U on incorporation of [^{14}C]uridine in heat-synchronized cells. In the upper part of the figure is shown incorporation of the radioactive uridine from the end of heat shock no. 6, at which time U (○) or M+U (●) were added together (arrow) with the radioactive uridine. In the lower part of the figure is shown results from a similar experiment beginning 100 min later, shortly after the first synchronous division. □, U; ■, M+U.

Chemical measurements of RNA in the absence and presence of M+U are presented in Fig. 5. It shows essentially the same as Fig. 3; if added before the *S*-period, M+U interferes with the rate-shift in RNA synthesis normally occurring at the very beginning of the *S*-period, but added later, after the start of the *S*-period, M+U has no effect on RNA-synthetic rate until the next expected rate shift in RNA synthesis.

Furthermore, it is apparent from the figure that during the heat shocks RNA synthesis is strongly inhibited, and it may take some time after the heat shock before

the previous rate of RNA synthesis is regained. This effect of heat on RNA synthesis has been recorded before (Bernstein & Zeuthen, 1966) and recent evidence indicates that the heat treatment selectively inhibits the synthesis of ribosomal RNA (Hermolin & Zimmerman, 1976).

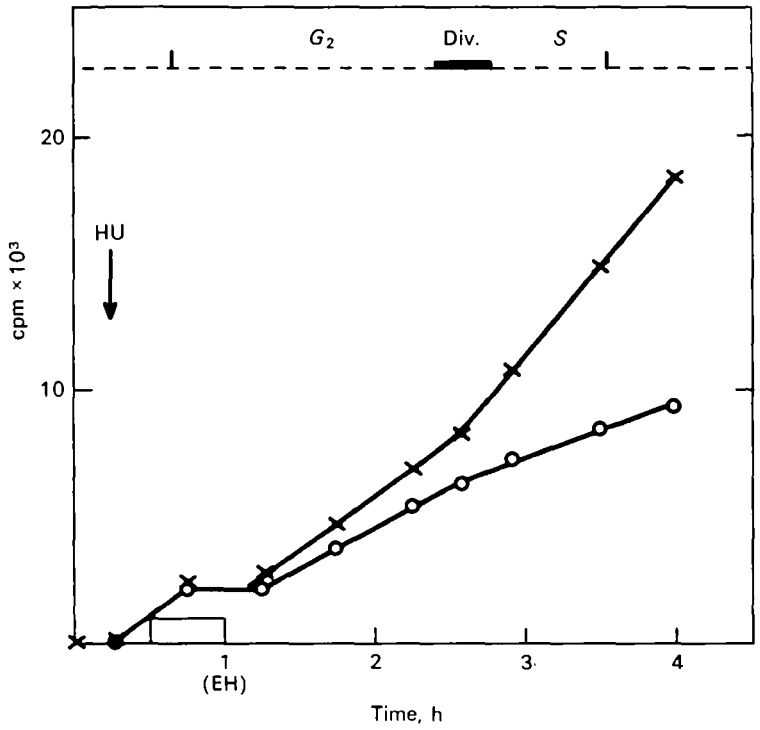

Fig. 4. The effect of HU on incorporation of [^{14}C]uridine in heat-synchronized cells. The radioactive uridine was added to the culture in a synchronized *S*-period. Then the culture was divided into halves one of which received HU (○).

The finding that the increase in rate of RNA synthesis occurs more or less simultaneously with the replication of rDNA suggests that the rate of RNA synthesis may be controlled by the number of genes coding for ribosomal RNA. This interpretation is strengthened by the observation that addition of M + U prior to rDNA replication inhibits the rate-increase in RNA synthesis, whereas the rate between the steps is not influenced by the presence of M + U.

In starved/refed *Tetrahymena* cultures rDNA replication is separated in time from bulk DNA replication. Fig. 6 shows that addition of M + U to a starved/refed *Tetrahymena* culture 30 min after refeeding inhibits a rate shift in RNA synthesis occurring several hours later, whereas addition of M + U 2 h after refeeding has no effect on this rate shift. In these cultures it has been shown, that rDNA replicates from 80 to 120 min after refeeding. Thus M + U has to be added prior to the replication of rDNA in order to interfere with the synthesis of ribosomal RNA.

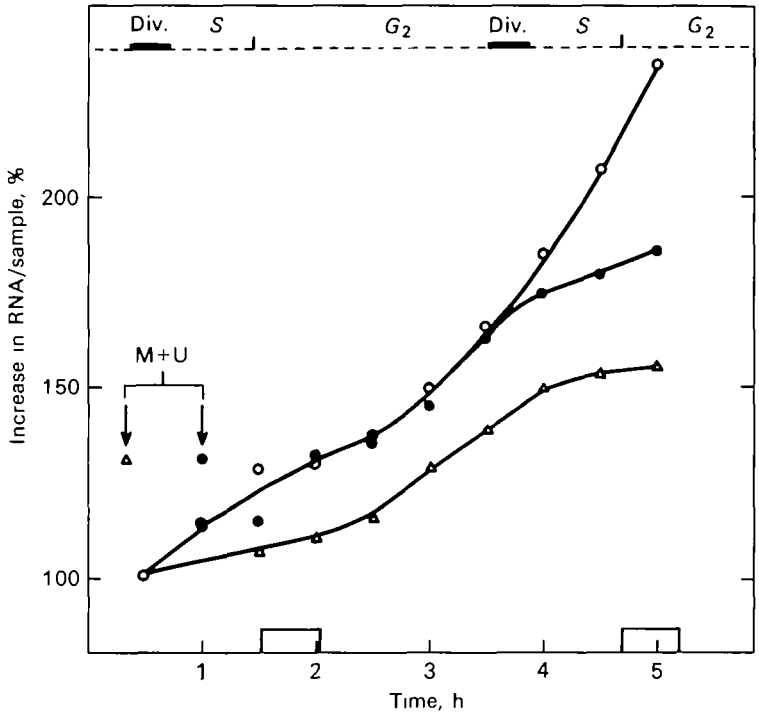

Fig. 5. The effect of M + U on the accumulation of RNA in heat-synchronized cells. Three parallel cultures were followed. One served as a control (○), and 2 received M + U: 20 min prior to (△) and 20 min after (●) a synchronous division. The heat shocks nos. 6 and 7 are indicated on the abscissa.

DISCUSSION

The DNA coding for ribosomal RNA (rDNA) exists as free linear DNA molecules in *Tetrahymena*, in contrast to most other eukaryote cells (Engberg, Andersson, Leick & Collins, 1976). This rDNA replicates very early in the macronuclear *S*-period of heat-synchronized cells (Andersen & Engberg, 1975). In cells synchronized by starvation/refeeding the rDNA replicates prior to the bulk of the DNA (Engberg, Nilsson, Pearlman & Leick, 1974).

Studies on the replication sequence in heat-synchronized *Tetrahymena* have revealed that the genes coding for other stable RNA species (5S- and tRNA) replicate throughout the *S*-period (Tønnesen & Andersen, 1977), that the replication sequence of bulk DNA changes from generation to generation (Andersen & Zeuthen, 1971) and that – except for rDNA – the buoyant density of the replicating DNA is the same in all parts of the *S*-period (Andersen, 1972*b*). Thus in *Tetrahymena* rDNA is probably the only DNA fraction which repeatedly replicates early in the *S*-period. We have now reported that replication of DNA in the early part of the *S*-period is a prerequisite of an abrupt increase in the rate of RNA synthesis, and, on the other hand, that as soon as the change in the rate of RNA synthesis has taken place, the DNA replication can be inhibited without effect on the RNA synthesis. Since we

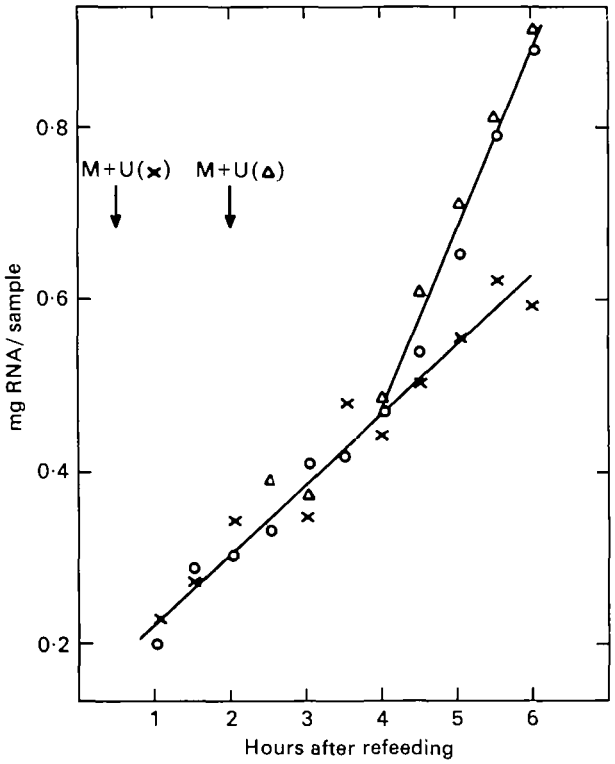

Fig. 6. The accumulation of RNA in cells after synchronization by starvation/refeeding. After 16 h of starvation in inorganic medium the cells were transferred to growth medium and divided into 3 subcultures. One served as a control (○), 2 were treated with M + U, one 30 min (x) and the other 120 min (△) after refeeding.

can assume that only rDNA is repeatedly early-*S* replicating, the results then imply that the rate of rRNA synthesis is gene-dosis dependent.

The amount of ribosomal RNA per cell is not kept constant during either exponential or synchronous growth. Several reports have shown that under the growth conditions normally used, the cells produce a high amount of RNA per cell in the period following inoculation of the culture and that the amount of RNA synthesized per cell per generation is decreasing during the period of exponential growth. The cells enter stationary phase with a relatively low RNA content (Conner & Koroly, 1973; Jauker & Hipke, 1975). The RNA content per cell declines also in synchronously growing populations as indicated by the results presented in this paper.

Plesner, Hartman, Schülein & Kristiansen (1976) found that the change in rRNA synthesis which takes place shortly after cell division is followed by a change in the formation of new ribosomes as well. They have evidence which indicates that the proteins necessary for the ribosome formation were made – at least partly – in the preceeding cell cycle, thus implying that the rate of ribosome accumulation is limited primarily by the rate of rRNA formation.

In the starvation/refeeding synchronized *Tetrahymena* cells a pool of ribosomal

protein is built up prior to the start of synthesis (Hallberg & Bruns, 1976). Furthermore, a breakdown of old ribosomes has been reported to take place at the time when new rRNA synthesis starts (Elrod & Padilla, 1976). The low rate of rRNA synthesis immediately after refeeding cannot be accounted for by the reduction in the amount of rDNA resulting from starvation. Synthesis of rRNA is probably repressed under these circumstances. Later, when the ribosomal protein pool has been built up, the transcription of rDNA is derepressed. It takes 180–240 min after refeeding before the number of ribosomes per cell equals the number in logarithmically growing cells (Elrod & Padilla, 1976), and at this time the rate of ribosomal RNA becomes genedosis dependent (cf. Fig. 6).

Minassian & Bell (1976) have pointed out that in many cells – including *Tetrahymena* (Andersen & Engberg, 1975; Charret, 1969) – different authors have found different timings of the rDNA replication. They suggested that synchronization procedures might interfere with the replication of rDNA, thus leading to a different timing of replication. We would like to emphasize the importance of growth conditions. The timing of rDNA replication could be a means by which cells regulate the capacity for ribosomal RNA synthesis in response to growth conditions. If the gene number is limiting for the rate of RNA synthesis, cells which replicate rDNA early in the cell cycle have a higher capacity for rRNA synthesis than cells which replicate rDNA late in the cell cycle. Fast-growing *Tetrahymena* cells contain on an average 8×10^7 ribosomes (Leick, 1973); with a generation time of 160 min, the cell synthesizes $8 \times 10^7/9600 = 8{\cdot}3 \times 10^3$ ribosomes/s. The primary transcript from rDNA has a mol. wt. of $2{\cdot}3 \times 10^6$ D $\sim 6{\cdot}3 \times 10^3$ nucleotides (mol. wt. 340), and with $8{\cdot}3 \times 10^3$ ribosomes synthesized per s the cell has to assemble $8{\cdot}3 \times 10^3 \times 6{\cdot}5 \times 10^3 = 5{\cdot}4 \times 10^7$ nucleotides into RNA per s. Under optimal growth conditions the average cell contains about 750 nucleoli each with 18 rDNA molecules, and the rDNA molecules contain 2 genes for 17- and 25-S RNA, respectively. Therefore, the rRNA is transcribed from about $2{\cdot}7 \times 10^4$ genes. Data from both prokaryotes and eukaryotes indicate that RNA polymerase travels along the DNA molecule with a rate of about 30–40 nucleotides/s (Chambon, 1974; Richardson, 1969). Using this figure one can calculate that ~ 60 RNA polymerase molecules are constantly active on each gene, or 120 per rDNA molecule. In each actively transcribing rRNA gene from *Triturus* about 100 RNA polymerase molecules have been observed, and it is reasonable to assume that this is the maximum number of polymerase molecules which can be packed on to one gene (Lewin, 1974). On this background we propose that the early replication of rDNA in *Tetrahymena* and the concomitant increase in the rate of rRNA synthesis constitute a cellular mechanism ensuring optimal synthesis of ribosomal RNA.

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