RIBOSOME BIOSYNTHESIS IN *TETRAHYMENA PYRIFORMIS*

Regulation in Response to Nutritional Changes

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ABSTRACT

Ribosome contents of growing and 12-h-starved *Tetrahymena pyriformis* (strain B) were compared. These studies indicate that (*a*) starved cells contain 74% of the ribosomes found in growing cells, (*b*) growing cells devote 20% of their protein synthetic activity to ribosomal protein production, and (*c*) less than 3% of the protein synthesized in starved cells is ribosomal protein.

Ribosome metabolism was also studied in starved cells which had been refed. For the first 1.5 h after refeeding, there is no change in ribosome number per cell. Between 1.5 and 2 h, there is an abrupt increase in rate of ribosome accumulation but little change in rate of cell division. By 3.5 h, the number of ribosomes per cell has increased to that found in growing cells. At this time, the culture begins to grow exponentially at a normal rate.

During the first 2 h after refeeding, cells devote 30–40% of their protein synthetic activity to ribosomal protein production. We estimate that the rate of ribosomal protein synthesis per cell increases at least 80-fold during the first 1–1.5 h after refeeding, reaching the level found in exponentially growing cells. This occurs before any detectable change in ribosome number per cell. The transit time for the incorporation of these newly synthesized proteins into ribosomes is from 1 to 2 h during early refeeding, whereas in exponentially growing cells it is less than 30 min. The relationship between ribosomal protein synthesis and ribosome accumulation is discussed.

Although a fairly substantial body of information exists regarding the structure, function, and genetic origin of a number of eucaryotic ribosome components (for a review, see relevant articles in reference 17), comparatively little is known about transcriptional or posttranscriptional control mechanisms, which must regulate ribosome biogenesis. An understanding of how cells coordinate the expression of the approx. 70 structural genes (or sets of genes) necessary to make a functioning ribosome will undoubtedly be fundamental to our understanding of coordinate gene control in general.

Exploiting specific characteristics of various biological systems has been an important strategy for the study of eucaryotic ribosomes (18, 20, 21, 1, 11, 23). The purpose of this paper is to demonstrate some characteristics of ribosome metabolism in the ciliated protozoan *Tetrahymena pyriformis* which indicate that it may show distinct advantages in answering questions regarding the control of ribosome biogenesis.

It has been shown that, when certain strains of *Tetrahymena* are washed from nutrients into dilute salt solutions, they remain viable but decrease their cellular RNA content by 30–50% (3), indicating a decrease in the number of ribosomes per cell. Moreover, upon refeeding, the cells reenter logarithmic growth only after a lag of several hours. These observations suggest that refed *Tetrahymena* might be an excellent system in which to study ribosome biogenesis, if at least one of the events in the lag period is a reconstitution of the normal complement of ribosomes. Thus, we wished to observe the kinetics of change in ribosome number during the early stages of refeeding and see whether attainment of the log-phase number of ribosomes per cell preceded entry into exponential growth.

In addition, Leick (14) measured the ratio of protein to RNA in exponentially growing cells and found it to be about five parts protein to one part RNA. Assuming most (~90%) of the RNA to be rRNA, and knowing that a ribosome consists of about equal parts RNA and protein, this suggests that about 20% of the protein in a log-phase *Tetrahymena* is ribosomal protein. Assuming that in growing cells the stability of ribosomal protein and general cell protein is about the same, it follows then that about 20% of the protein synthesized by log-phase cells would be ribosomal protein. As this is a strikingly high percentage for a eucaryotic cell (22), we wished to directly confirm this suggestion. Furthermore, since ribosome number fluctuates in cells under different physiological conditions, we wished to see how ribosomal protein synthesis is regulated during these periods. Specifically, determinations of the percentage of total protein synthesis devoted to ribosomal structural protein (α_r) in starved cells, log-phase cells, and starved cells which had been refed are presented.

By measuring the change in the number of functioning ribosomes in refed cells, we found it was possible to estimate the change in rate of protein synthesis in these cells. These estimates allowed us then to determine the change in rate of total ribosomal protein synthesis and compare it to the rate of accumulation of new ribosomes during this time.

MATERIALS AND METHODS
Growth Conditions

Strain BIV was used in all experiments. Cells were grown in 1% proteose peptone (Difco Laboratories,

Detroit, Mich.) at 30°C on a slowly revolving gyratory shaker (90 rpm). Under these conditions, cells divide every 2.5 h. All subsequent manipulations were also carried out at 30°C. Starved cells were obtained by pelleting log-phase cells from nutrient media, washing them once in 50 mM Tris, pH 7.4, and then resuspending them in 500–1,000 ml of 50 mM Tris at a density of 1–1.5 × 10^5 cells/ml. These cells were then maintained for 12 h on the shaker. At the end of 12 h, the cell number was nearly identical to that of the original inoculum. Refeeding was accomplished by adding one part 10% proteose peptone (already at 30°C) to nine parts starved cell suspension. As required, labeled amino acids ([^{3}H]leucine, and [^{35}S]methionine [New England Nuclear Corp., Boston, Mass.] added to give 1–5 μCi/ml) were added to the refed cultures. To obtain cells for analysis or for resuspension in nonradioactive media, 100 or 125 ml of cells were collected and washed as before. A final wash before ribosome isolation was done in 50 mM Tris and 1 mM $MgCl_2$. After washing, cells for ribosome isolation were frozen in liquid N_2 and stored at −50°C. Cells that were to be chased in nonradioactive media were washed in 50 mM Tris and then resuspended at their previous cell density in fresh 1% proteose peptone containing a 10,000-fold excess of nonradioactive amino acid. Cells were collected at the end of a chase period and stored as described above. Cell counts were made with a Coulter counter (Coulter Electronics, Inc. Hialeah, Fla.).

Ribosome Isolation

Frozen cells were thawed on ice and then suspended in a 8–20-fold excess of 0.01 M NaCl, 0.01 M Tris pH 7.5, 0.0015 M $MgCl_2$ (RS buffer). Cells were broken by homogenization in a tight-fitting Dounce homogenizer (20 strokes, Kontes Glass Co., Vineland, N. J.). Triton X-100 and deoxycholate were then added to final concentrations of 1% and 0.5%, respectively, using 20 times stock solutions. The cell homogenate was centrifuged for 15 min at 15,000 *g*; the resulting supernate was recovered and subjected to centrifugation at 100,000 *g* for 90 min. The resulting ribosome pellet was rehomogenized in 0.5 M KCl, 0.01 M Tris, pH 7.5, 0.01 M $MgCl_2$, and then spun at 15,000 *g* for 15 min. Ribosomes and ribosomal subunits were pelleted from the resulting supernate by centrifugation at 150,000 *g* for at least 5.0 h. This was necessary since most ribosomes are dissociated into subunits under these conditions. Ribosomal subunits were prepared from the 0.5 M KCl-washed ribosomes by centrifugation on a sucrose gradient in the absence of Mg^{++}. These conditions cause the complete dissociation of ribosomes. Pellets of 0.5 M KCl-washed ribosomes were homogenized in a small volume of distilled water, and enough 1.0 M KCl in 0.1 M Tris, pH 7.5, was added to give a final concentration of 0.2 M KCl. The solution was then clarified by centrifugation at 15,000 *g* for 10 min. The resulting ribosome solution was overlayed on 15–30% sucrose gradients containing 0.1 M KCl and

0.01 M Tris, pH 7.5, and spun for 15 h at 23,000 rpm and 5°C in a SW27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Fractions were collected, and their absorbances read at 259 nm to determine the locations of the large and small ribosomal subunits. Recovery of ribosomes was determined from the sum total of A_{259} in the ribosomal subunits. Ribosomal subunits prepared in this way have the following properties: (a) an absorbance maximum at 259 nm, a minimum at 236 nm and $A_{259}/A_{236} = 1.80 \pm 0.05$; $A_{259}/A_{280} = 1.95 \pm 0.05$; (b) both the large and small subunits contain 40-45% protein as determined either chemically or from their buoyant density in CsCl; and (c) the absorbance of a 1 mg/ml solution is 14.5-15 rather than the often cited 12-13 which is appropriate for less highly purified (and hence more protein-rich) ribosomes.

From the results of at least 20 separate ribosome isolations and the results presented in Table I, the above ribosome isolation procedure gave a highly reproducible yield of ribosomes. Recoveries always fell between 32% and 40% of the calculated ribosome contents, with most values falling in the 35-37% range. Consequently, all recovery corrections were made by dividing the recovered ribosomes by 0.36 to give the total ribosome amount for those cells.

Ribosomal subunits were precipitated by pooling the appropriate fractions from a sucrose gradient, adding $MgCl_2$ to 0.01 M, and then adding 0.7 vol of cold 95% ethanol. The solution was kept on ice for 20-30 min; the flocculent precipitate which formed was pelleted by centrifugation at 15,000 g for 15 min. The supernate was discarded and the pellet rinsed and frozen.

Polysome Isolations

Cells from which polysomes were to be isolated were pelleted from their growth medium, washed once in room temperature RS buffer containing 100 μg/ml heparin, and frozen immediately in liquid N_2. Cells were stored in liquid N_2. Cells were thawed in ice-cold 0.2 M KCl, 0.01 M Tris, and 0.005 M $MgCl_2$, pH 7.5, plus 100 μg/ml heparin and disrupted by three to four strokes in a Dounce homogenizer followed by the addition of Triton X-100 and deoxycholate to 1.0% and 0.5%, respectively. The cell homogenate was spun at 15,000 rpm for 15 min in a Sorvall SS-34 rotor (DuPont Instruments, Sorvall Operations, Newtown, Conn.). Aliquots of the resulting supernate containing 3-10 OD_{260} U were overlaid on 12 ml of 0.5-1.5 M sucrose gradients containing 0.1 M KCl, 0.005 M $MgCl_2$, and 0.01 M Tris, pH 7.5, and spun for 90 min at 10°C and 41,000 rpm. Gradients were pumped through a recording Pharmacia UV-monitor (Pharmacia Laboratories, Inc., Piscataway, N. J.). To determine the fraction of ribosomes in monosomes and polysomes, traces were transferred to heavy paper and the monosome and polysome regions cut out and weighed. All solutions were pretreated with diethylpyrocarbonate as described by Woodland (24).

TABLE I

Calculation of Ribosome Levels in Log-Phase and 12-Hour-Starved Cells

	Log cells			Starved cells		
Initial cell counts	37×10^6			31×10^6		
Ribosomes added to cells*	19.7 OD_{259} (133,260 cpm)			19.7 OD_{259} (133,260 cpm)		
	cpm			cpm		
Recovery at various stages of purification‡						
	Total	%	OD_{259}	Total	%	OD_{259}
Initial homogenate	132,920	99.7	–	132,560	99.5	–
15,000 supernate	96,420	72.4	–	99,200	74.4	–
Resuspended 1st high speed pellet	71,640	53.7	160	67,520	50.7	91
Resuspended second high speed pellet	50,400	37.8	112	45,920	34.5	64
Calculations						
Ribosome recovery						
from added ribosomes	7.3	OD_{259} (37.8% of 19.7)		6.8	OD_{259} (34% of 19.7)	
from cells	104.7	" (112–7.3)		57.2	" (64–6.8)	
Ribosome amounts (corrected for recovery)						
from total cells	277	" (104.7/0.378)		166	" (57.2/0.345)	
per 10^6 cells	7.49	"		5.35	"	
mass of ribosomes per 10^6 cells§	0.499	mg		0.367	mg	
mass of rRNA per 10^6 cells‖	0.275	"		0.196	"	
mass of RNA per 10^6 cells¶	0.305	"		0.218	"	
mass of RNA per 10^6 cells**	0.300	"		0.210–0.240	"	

* Ribosomes were prepared from log-phase cells labeled for 2.5 generations with [³H]leucine. They were taken through the high-salt washing step (see Materials and Methods) and redissolved in RS buffer and their concentration (OD_{259}) and radioactivity measured.

‡ Cells were homogenized in RS buffer containing the added radioactive ribosomes, and a standard ribosome isolation was carried out. At various steps in the isolation procedure the amount of TCA-precipitable ribosomes was measured (see Materials and Methods).

§ Calculation based on assumption that 15 OD_{259} = 1 mg ribosomes (see Materials and Methods).

‖ Calculation based on assumption that a ribosome is 55% RNA, 45% protein (see Materials and Methods).

¶ Calculation based on assumption that 90% of RNA in a cell is rRNA.

** Chemical determination of RNA per 10^6 cells by M. J. Koroly (unpublished observation).

Electrophoresis

Ribosomal subunit proteins were subjected to electrophoresis on 15% polyacrylamide gels using the procedures of Laemmli (13). Gels of either 1.0 or 1.5-mm thickness were typically run for 7-10 h at 60-100 V. Fixation and staining with Coomassie blue were done according to Fairbanks et al. (9). Stained gels were dried by vacuum filtration, and the radioactive proteins were visualized by exposing the dried gels to Kodak no-screen X-ray film (Eastman Kodak Co., Rochester, N. Y.).

Radioisotope Measurements

Measurement of incorporation of radioactive precursors into protein was done by taking 25-100-μl aliquots, diluting with 1.0 ml of cold distilled water, and then adding 2.0 ml of cold (4°C) 10% trichloroacetic acid (TCA). Precipitated protein was filtered onto Whatman GF/C filters, washed with cold 5% TCA and dried under a heat lamp. Filters were counted in a toluene-based fluor. Sometimes, aliquots of sucrose gradients fraction were diluted with water and directly counted in Aquasol (New England Nuclear). In order to correct for different counting efficiencies in calculating recoveries, several fractions of the sucrose gradient were also counted using the TCA precipitation procedure, so that cpm determined by one technique could be converted into cpm using the other.

RESULTS

Ribosomal Protein Synthesis in
Log-Phase Cells

The following pulse-chase experiments were patterned after those used by others to study ribosomal protein synthesis in *Escherichia coli* (*E. coli*) and yeast (19, 21). They were designed to roughly measure the time it takes for all newly synthesized ribosomal protein to be incorporated into mature ribosomal subunits, and to determine the accuracy of Leick's suggestion (14) that about 20% of the protein synthesized by log-phase *Tetrahymena* is ribosomal structural protein.

Exponentially growing cells (2.5-h generation time) were exposed to labeled amino acids (either [³H]leucine or [³⁵S]methionine) for times of from 15 to 45 min. Chase period intervals of 15-45 min were used totaling from 2 to 3.75 h. Ribosomes were purified from cells collected at all time-points in order to determine the fraction of newly synthesized protein that was associated with the ribosomal subunits.
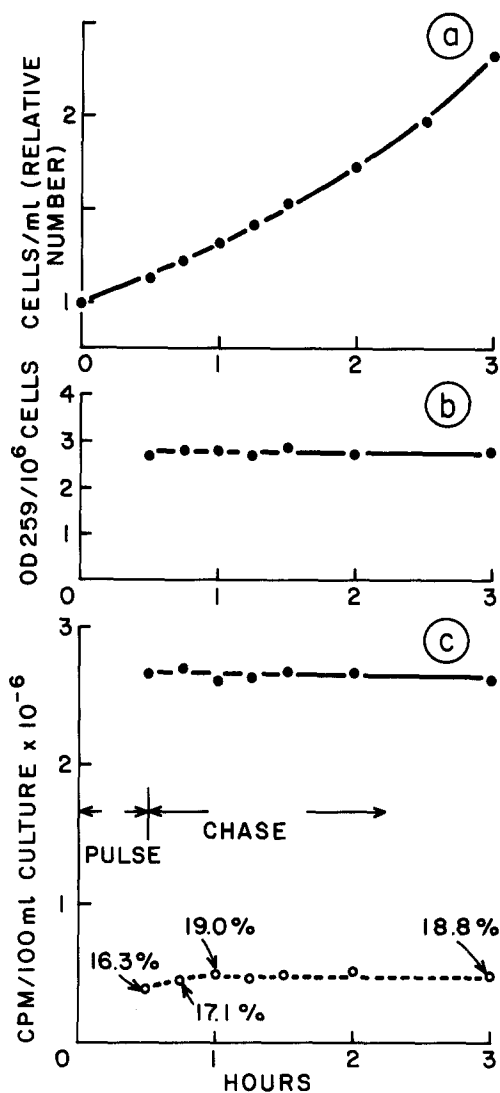

FIGURE 1 Appearance of newly synthesized protein in ribosomal subunits of exponentially growing cells. Cells (800 ml at 163,000 cells/ml) in log-phase growth (*a*) were given a pulse of [³H]leucine (2 μCi/ml; 15 Ci/mmol) for 30 min, washed and chased in fresh media. Ribosomes and their resulting subunits were prepared (see Materials and Methods) from 100 ml of cells at various times, their yield determined (*b*) and the amount of newly synthesized protein in ribosomal subunits determined (corrected for recovery) (*c*). Total TCA-precipitable cpm (●—●); TCA-precipitable cpm in ribosomal subunits (○—○). The percentages given refer to percent of counts in ribosomes relative to total counts.

The results of one such experiment are summarized in Fig. 1. In this particular experiment, cells

were given a 30-min pulse of [³H]leucine and chased for an additional 2.5 h; samples were taken at 15-min intervals immediately after the pulse period. At each time-point, ribosomal subunits were isolated to determine the amount of newly made protein associated with them. As can be seen, at the end of a half-house pulse 16.3% of the newly made protein is already associated with the ribosomal subunits, and within the next 30 min that number has increased to 19.0%, where it then remains for the duration of the chase period. The fact that total incorporation during the chase is unchanged indicates that the chase is effective, and neither total protein nor ribosome-associated protein shows any detectable turnover during this 3-h period. Although the pulse is clearly too long to give an accurate transit time value, it is clear that all newly made ribosomal proteins are incorporated into ribosomes in less than 30 min. It is impossible to tell, from these data, whether there is any difference in transit time for different ribosomal proteins. They are simply looked at as a population. Two other experiments, in which 15- and 45-min pulses, respectively, were used lead to the same conclusion. It is likely that the chase time might well be much less than 30 min, since the transit time for newly made rRNA into ribosomes is only 7-10 min (15). In any event, 30 min is certainly an upper limit and will contrast with results reported later for cells under different physiological conditions. The data also confirm that about 20% of the protein made during the pulse period can eventually be recovered in the ribosomal subunit fraction of the cells. In two other similar experiments, in both of which [³H]leucine was used, the estimated percentage of protein made as ribosomal protein was 18.1% and 22.1%. Thus, since the turnover rate of general cellular protein seems minimal under these conditions, we conclude that α_r is about 0.20.

To be certain that the radioactive protein associated with the subunits was ribosomal structural protein, a pulse-chase experiment was performed on exponentially growing cells similar to the preceding one, except that [³⁵S]methionine was used to label newly made protein. Using a 45-min pulse period followed by a 2.25 h chase, and sampling at 0.75 h intervals, we estimated that 20.7% of the newly synthesized methionine-containing protein was associated with the ribosomal subunits. The proteins from the large and small subunits of the pulsed alone, and the pulsed plus 45-min chased

cells were isolated (Fig. 2 A) and subjected to electrophoresis in SDS-containing polyacrylamide gels. After staining, the gels were dried and autoradiographed. Fig. 2 B shows that the radioactive protein from the pulsed and pulse-chased subunits are identical. It also shows that the staining and radioactive patterns, although showing some differences in relative band intensities, are qualitatively the same. Since the stain (Coomassie blue) does not obey Beer's law for all proteins and, more importantly, since all proteins would not be expected to have equimolar percentages of methionine residues, this lack of quantitive identity is not surprising. From these results, we conclude that the radioactive protein we measure is ribosomal structural protein (as defined by our operations) and not nascent polypeptides or nonspecifically absorbed protein. Furthermore, the fact that 20% of the [³⁵S]methionine-containing protein is isolated from ribosomal subunits strengthens the conclusion based on the earlier results with [³H]leucine that 20% of the total protein synthesized in log-phase cells is ribosomal structural protein.

Ribosomal Protein Synthesis in Starved Cells

Although starved cells have a lower ribosome content (a consequence of active rRNA degradation), they do continue to synthesize rRNA (4) and establish a new steady state level of ribosomes (Hallberg, R. L., unpublished observation). It is known that the overall rate of protein synthesis is depressed in starved cells, but it is not known whether the fraction of protein made as ribosomal protein is altered. To determine this, a pulse-chase experiment was carried out, in which this time, the cells that were used had been starved in 50 mM Tris for 16 h, a time sufficient to reduce the ribosome contact to a new steady state level (Hallberg, R. L., and C. A. Sutton, unpublished observations). A 30-min pulse of [³H]leucine was followed by 1.5 h of chase, with samples taken at 45 min and 90 min. Analysis of the labeled protein associated with ribosomal subunits indicated (Fig. 3) that no more than about 3% of the protein synthesized during the pulse period was ribosomal protein. This number may be somewhat in error due to the obvious turnover of protein in these starved cells as shown by the decrease in the radioactivity of total TCA-precipitable material during
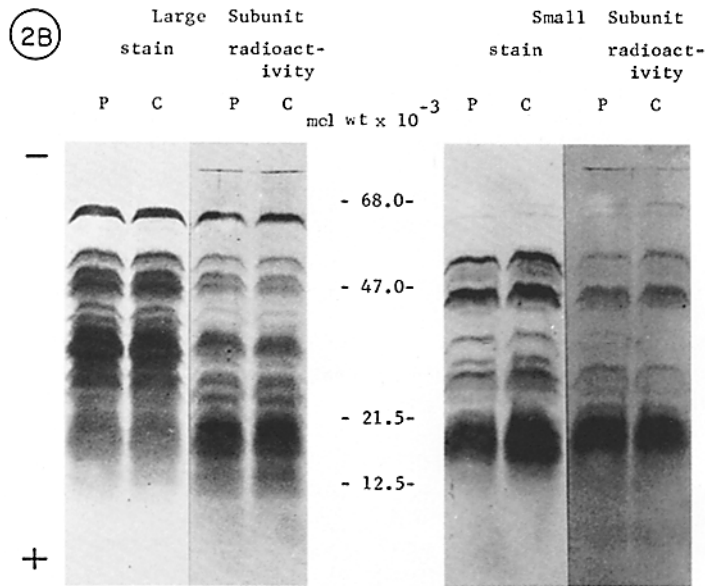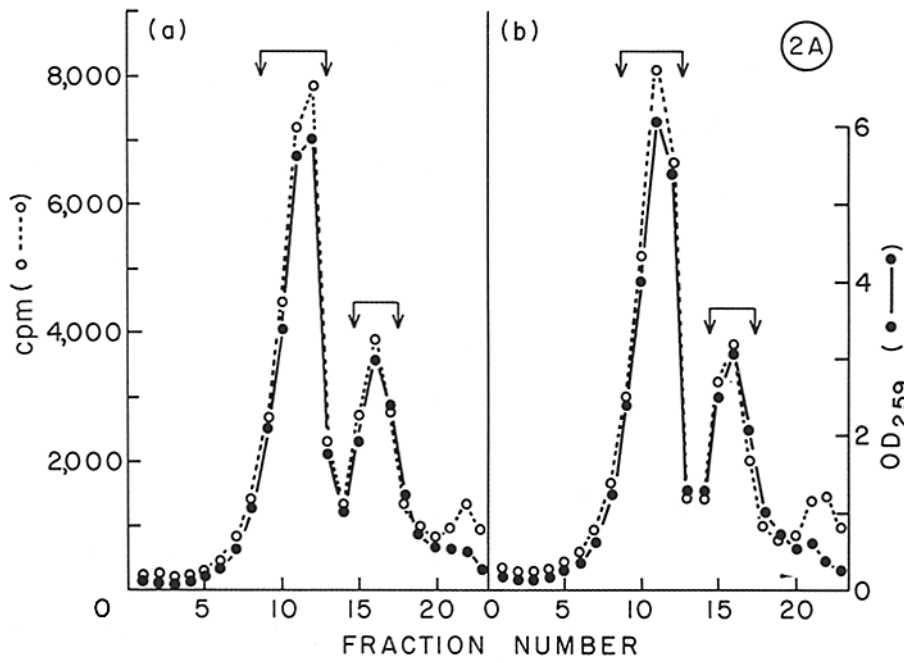
FIGURE 2   Labeling of ribosomal proteins in log-phase cells with [³⁵S]methionine. Log-phase cells (480 ml at 105,000 cells/ml) were pulsed with [³⁵S]methionine (4.2 μCi/ml; 80 Ci/mmol) for 45 min and then chased for an additional 45 min. Ribosomes were purified from the pulsed and chased cells (200-ml each) and ribosomal subunits prepared on sucrose gradients (A). (a) Pulsed cells. (b) Chased cells. Sedimentation is from right to left. Large and small ribosomal subunits were collected (bracketed fractions) from each, electrophoresed in SDS-acrylamide gels, stained, dried, and autoradiographed (B). P, pulsed; C, chased.
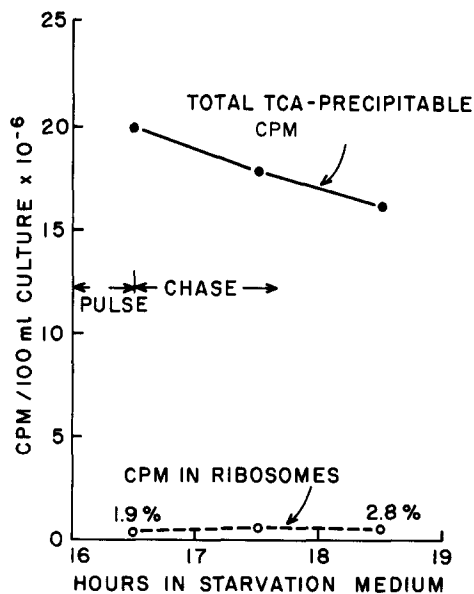
FIGURE 3 Appearance of newly made protein on ribosomal subunits of starved cells. Cells starved at a density of 122,000 cells/ml for 16 h in 50 mM Tris were pulsed with [³H]leucine (2.8 μCi/ml, 15 Ci/mmol) for 30 min. They were then washed and chased for an additional 2 h. Ribosomes were prepared and analyzed as in Fig. 1. The values for cpm in ribosomal subunits is corrected for recovery. Total TCA-precipitable cpm (●—●); TCA-precipitable cpm in ribosomal subunits (O—O). The count values in this experiment are increased due to the absence of peptone in the medium which drastically lowers the specific activity of the labeled amino acid.

the chase period. Nevertheless, it seems clear that under starvation conditions the synthesis of ribosomal protein recoverable in ribosomes relative to total protein decreases markedly. One possibility which can not be excluded is that a much larger percentage of protein is made as ribosomal protein but that because of a limited production of rRNA, only a small fraction is stabilized, with the rest being turned over. Until a direct measurement of ribosomal protein synthesis can be made which does not require ribosome isolation, this possibility cannot be excluded.

Ribosome Numbers in Log Phase, Starved, and Starved and Refed Cells

An isotope-dilution method of analysis was used to measure the absolute levels of ribosomes in cells. Ribosomes were purified from cells labeled with [³H]leucine during several generations of log-phase growth. Small quantities of purified radioactive ribosomes were added to known numbers of

starved and log-phase cells, and their ribosomes purified. The results of this experiment are summarized in Table I. Two important facts emerge: (a) the yield of ribosomes from both cell types, as judged by isotope recovery, is the same (this has been confirmed in a repeat of this experiment); and (b) the ribosome number in starved and log-phase cells is significantly different. As is shown in Table I, an estimate of the RNA amounts per cell can be made using the calculated ribosome levels; it is very close to that determined by M. J. Koroly (unpublished observations), who chemically measured RNA contents of cells both in log-phase growth and in a starved condition. Thus, our extraction procedure works equally well for both starved and log-phase cells, and is reproducible in its yield; in 15 separate isolations from starved and log-phase cells, the yield of ribosomes per cell ($OD_{259}/10^6$ cells) from each isolation fell within 3.6% of the average value for the respective physiological condition.

The same isolation techniques were employed to measure the number of ribosomes as a function of time after refeeding starved cells. The results of 12 separate experiments are presented in Fig. 4. Cell number increased at a slow rate in refed cells, at first; it is only after 3 h that the cells assume logarithmic growth at the normal rate (Fig. 4a). In contrast, there is no change in ribosome content per cell (Fig. 4c) for the first 1.5 h after refeeding, but during the following 1.5 h there is an abrupt increase in ribosome number, culminating in a restoration of a log-phase level of ribosomes/cell, which coincides with the time when the cells commence normal logarithmic growth. This increase in number per cell is a direct result of an abrupt increase in rate of ribosome accumulation beginning at 1.5 h (Fig. 4b) without a concomitant increase in cell division rate. This rate of accumulation is greater than that found in log-phase cells. When the level of ribosomes per cell reaches the log-phase level (Fig. 4c), cells enter logarithmic growth, and the overall rate of ribosome accumulation decreases and parallels this growth rate. Clearly, ribosome synthesis is under strict regulation.

Ribosomal Protein Synthesis in Refed Cells

To determine what fraction of the protein synthetic activity in refed cells was directed toward ribosomal protein production, cells were pulsed with [³H]leucine from 1.0–1.5 h after refeeding
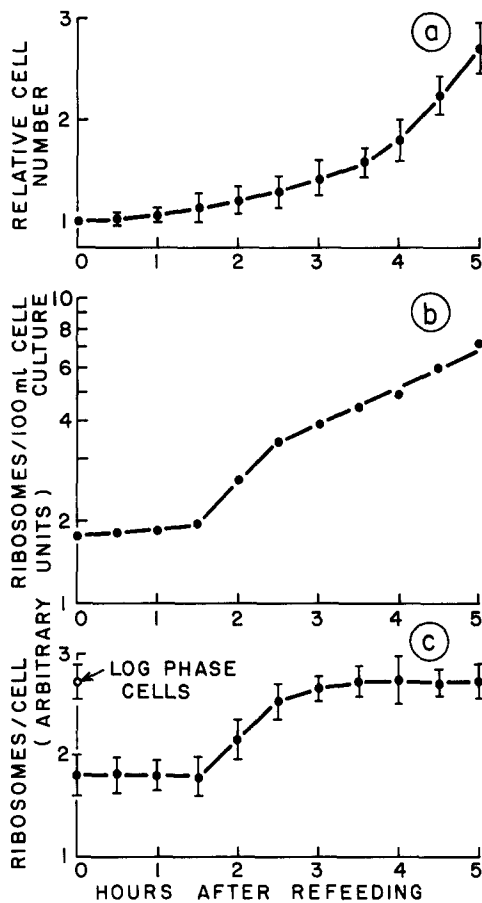
FIGURE 4 Growth characteristics and ribosome yields from starved and re-fed cells. Cells starved for 12 h (and sometimes up to 15 h) in 50 mM Tris were re-fed, and at various times afterwards cells (usually 100-150 ml at 1-2 × 10⁵ cells/ml) were collected and ribosomes isolated and measured. The data represent the average measurements from 12 separate experiments, although not all time-points are represented in each experiment. Each point is the average of four to seven measurements. The error bars represent standard errors. In (c), the average value of ribosomes recovered per cell from log-phase cells is indicated for comparison.

and then chased for an additional 3 h, with samples taken at 0.5-h intervals. The results of this analysis (Fig. 5) differ both qualitatively and quantitatively from those obtained with log-phase cells (Fig. 2). First, the amount of protein made in the one-half h pulse and ultimately chased into ribosomal subunits was almost 40% (twice that found for log-phase cells). Moreover, the length of time required to chase all newly made protein into

ribosomes was about 1.5 h, rather than the maximum of 30 min seen in log-phase cells.

To determine whether ribosomal protein synthesis resumed immediately after refeeding, a pulse of [³⁵S]methionine was administered at this time. The chase was initiated after 30 min, and was continued for 4.5 h. Ribosomes were isolated as before, and the percent of the protein in ribosomes was determined (Fig. 6). Once again, greater than 20% of the protein made during the pulse was ribosomal protein, and the entry of this protein into ribosomes took about 2 h to complete.

In order to see how α_r varied during the entire early refeeding stages, similar experiments were conducted on cells pulsed with radioactive amino acids at various other half-hour intervals after refeeding, and α_r was determined for each time interval. It can be seen in Fig. 8c that α_r reaches a maximum during the 1st h after refeeding, remains high during the 2nd h and, by 3 h, decreases to that found in log-phase cells.
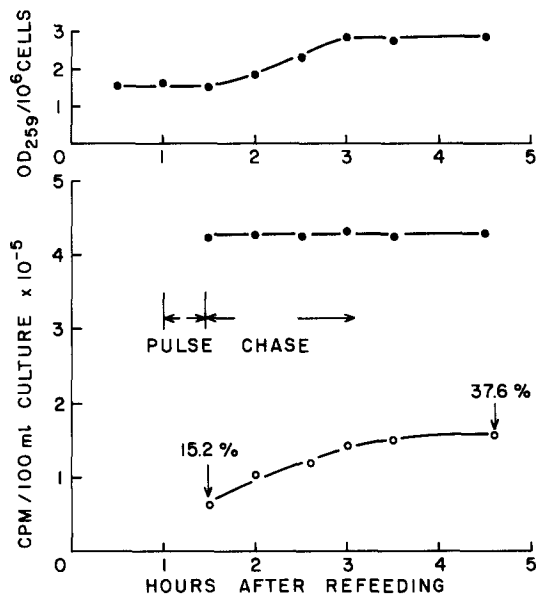

FIGURE 5 Appearance of newly made ribosomal proteins on ribosomes in re-fed cells. A 30-min pulse of [³H]leucine (1.3 μCi/ml, 40 Ci/mmol) was given starting at 1 h after refeeding cells (96,000 cells/ml) starved for 12 h. The measurement of newly made protein on ribosomes of pulsed and chased cells was carried out as described in Fig. 1. The values for cpm in ribosomal subunits is corrected for recovery. Total TCA-precipitable cpm (●—●); TCA-precipitable cpm in ribosomes (○—○).
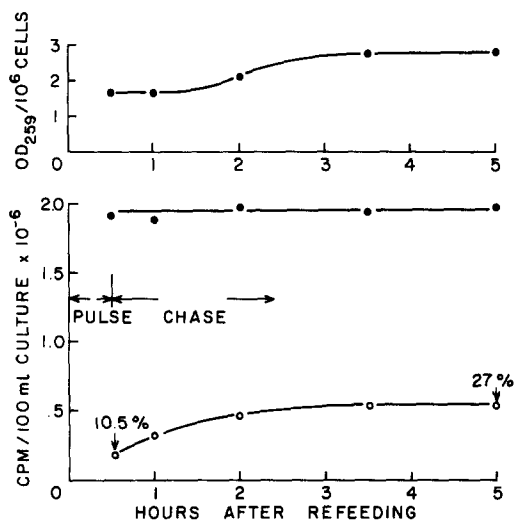
FIGURE 6 Appearance of newly made ribosomal proteins on ribosomes in re-fed cells. This experiment was conducted the same as that in Fig. 5, except that [³⁵S]methionine (5 μCi/ml, 337 Ci/mmol) was used and the pulse was given during the first 30 min after refeeding.

From these data alone, however, it was impossible to tell how the absolute rate of ribosomal protein synthesis was changing during the early refeeding stages. In order to determine that, we needed to know the change in rate of total protein synthesis in these cells. This was estimated in the following way. Polysomes were isolated from starved cells and cells re-fed for various lengths of time (Fig. 7), and the percentage of ribosomes in polysomes was measured for each time-point. It can be seen in Fig. 8a that by 1.5–2.0 h 70–75% of the ribosomes in the cell have been recruited for protein synthesis. This represents a 7–15-fold increase in percent of ribosomes in polysomes, since in starved cells between 5% and 10% of their ribosomes is in polysomes. The absolute number of ribosomes in polysomes is not indicated by Fig. 8a because the ribosome content per cell changes between 1.5 and 3.0 h (Fig. 4). Correcting for this change, the absolute amount of polysomal ribosomes per cell can be determined and is shown in Fig. 8b. It is probably a good assumption that the change in relative rate of total protein synthesis in refed cells is also described by Fig. 8b. This assumes that the ribosome translocation rate (and hence, amino acid polymerization rate) is relatively constant at a given temperature, no matter what fraction of ribosomes are messen-

ger bound. In one system in which this has been tested, this assumption appears to be correct (24). Fig. 8d then shows the change in rate of ribosomal protein synthesis during the early hours of refeeding. It is striking that by 1.25 h, a time before any change in ribosome content per cell, ribosomal protein synthesis has increased at least 80-fold (α_r = 0.03 to α_r = 0.41 and percent of ribosomes in polysomes up at least sevenfold) and has reached a rate equal to that found in exponentially growing cells. It remains to be determined how the change in rate of ribosomal protein synthesis is coupled to the change in rate of ribosome accumulation. This will require an examination of rRNA synthesis and processing in these refed cells.

DISCUSSION

Validity of the Quantitation Methodology

The accuracy of the calculations contained in this paper are based on the assumption that the recovered ribosomes (about three-eighths of the total) represent a *random* selection from the entire ribosome population, rather than some specific subpopulations. No steps were taken to select either for or against active (polysomal) vs. inactive ribosomes, nor for or against membrane vs. non-membrane bound ribosomes. Consequently, all
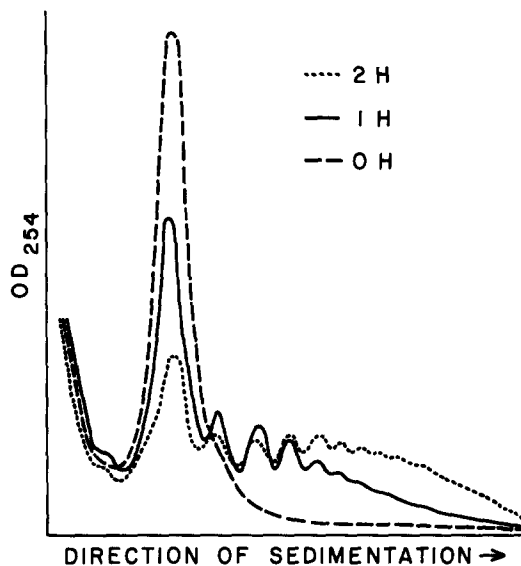

FIGURE 7 Polysome profiles from cells starved for 12 h (−) and from re-fed cells at 1 h (—) and 2 h (····) after refeeding. The extracts contained ribosomes from equal numbers of cells.
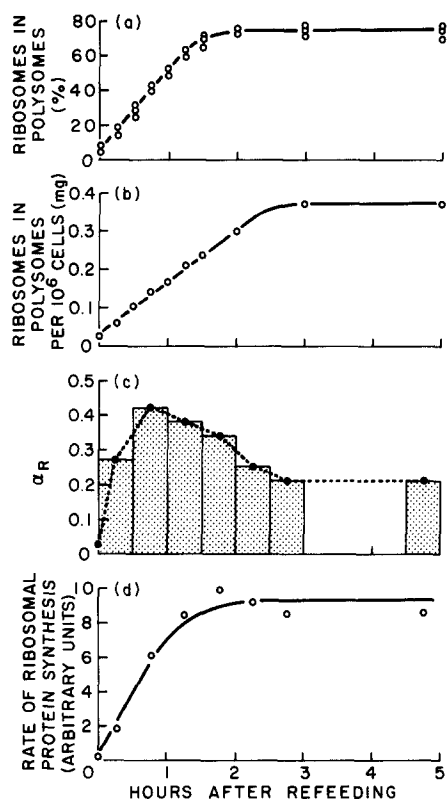
FIGURE 8 (a) Change in percentage of ribosomes in polysomes as a function of time after refeeding. Polysome profiles were obtained as in Fig. 7 for cells at various times after refeeding. The percentage expresses the fraction of ribosomes sedimenting faster than a monosome. Each point is an individual determination. (b) The absolute amount of polysomal ribosomes in cells at various stages of refeeding. The average value for each time-point in (a) was multiplied by the absolute value of ribosomes per cell (as determined from Fig. 4c and Table I). (c) The fraction of protein made as ribosomal protein during successive one-half h intervals after refeeding. α_r was determined for each interval as shown in Figs. 5 and 6. The point represents the average α_r for each time interval, and the point at time 0 represents α_r for starved cells. (d) The rate of ribosomal protein synthesis at various times in (b) were multiplied by their corresponding α_r in (c) to give an estimate of the relative rate of ribosomal protein synthesis. Since the absolute rate of protein synthesis was not measured, the values given are in arbitrary units.

measurements were made with purified ribosomal subunits obtained from free subunits, monosomes, and polysomes. Confidence in the results is supported by the following: (a) the recoveries from both log-phase and starved cells were highly re-

producible; (b) the percent recovery of ribosomes, as determined by the isotope dilution technique, was the same for both starved and log-phase cells; and (c) our measurement of RNA per cell using ribosome recovery was strikingly similar to the measurement of RNA per cell made using chemical means. If there had been subpopulations of ribosomes whose recoverability varied, one might expect their relative concentrations to be quite different in starved and log-phase cells affecting the percent recovery data.

Ribosomal Protein Synthesis

The way in which we have determined α_r was patterned after the technique introduced by Schleif (19). This technique requires that ribosomal protein synthesis be assayed by measuring the appearance of newly made protein in fully assembled ribosomes. In steady states of growth in bacteria, this requirement appears to be no problem since synthesis of ribosome components is balanced (12). However, in starved and refed Tetrahymena, the assumption of a balance between ribosomal protein production and rRNA production may or may not be correct. If ribosomal protein production exceeds rRNA production and if ribosomal protein which does not enter ribosomes within a certain time is degraded, then α_r cannot be reliably measured by using an assay which requires the appearance of ribosomal protein in ribosomes. In this case, α_r will be underestimated. An assay for ribosomal protein production which is independent of ribosome isolation, such as that used by Dennis (6) for E. coli and Gorenstein and Warner (10) for yeast, will be necessary to directly measure α_r. Such experiments are underway. In any case, all α_r reported in this paper are either equal to or less than the true α_r.

Since our analysis of the newly made protein found in ribosomes assayed only at the level of ribosomal subunits and therefore did not look at individual proteins, we cannot tell whether all ribosomal proteins are synthesized coordinately either in log-phase cells or in starved and refed cells. It is conceivable that the population of ribosomal proteins synthesized early in refed cells differs either quantitatively and/or qualitatively from that seen in log-phase cells. We are currently examining this question by analyzing the relative synthesis of the various individual ribosomal proteins at various stages of refeeding. Nonetheless,

this does not detract from our general findings regarding changes in α_r.

It will be important to determine the mechanisms by which ribosomal protein synthesis is elevated during early refeeding. It has been reported (2) that the protein synthesis which takes place during the 1st hour after refeeding starved *Tetrahymena* may be independent of RNA synthesis. If true, this might indicate that the control of the rate of ribosomal protein synthesis early in refeeding takes place at the translational level and occurs on messages made in the starved cells. Since ribosomal protein synthesis can now be assayed in the absence of RNA synthesis (10), this possibility can easily be tested.

Regulation of Ribosome Biogenesis

In some respects the control of ribosome biogenesis in *Tetrahymena* is similar to that seen in bacteria. For example, both organisms exhibit generation time-dependent levels of ribosomes: the faster the growth rate, the higher the ribosome content per cell (19, 12, 14). Both show wide variations in percent of protein made as ribosomal protein (α_r). When shifted from nongrowth (starved) to growth conditions, *Tetrahymena* initially produces ribosomes at a rate greater than that seen in exponentially dividing cells. Later, the rate decreases to that of log-phase cells. A similar phenomenon is seen in some bacteria in shift-up experiments (12). Whether there is an analogue to stringent control of ribosome production in eucaryotes remains to be seen.

However, one distinct dissimilarity is noteworthy. When bacteria are shifted from one growth condition to another, α_r increases, rRNA synthesis increases, and ribosome accumulation immediately mirrors these changes (7, 8). In other words, the rate of ribosome accumulation reflects the rate of ribosomal protein and ribosomal RNA synthesis. Our results here indicate that ribosome accumulation in refed cells is not a direct reflection of an increase in rate of ribosomal protein synthesis. The extended lag time between synthesis of ribosomal proteins and their incorporation into ribosomes at early refeeding times and the fact that ribosomal protein synthesis reaches a maximum before any change in ribosome number indicate the possibility that ribosomal protein and rRNA synthesis may be non-coordinated at this time. Since it has been shown that ribosomal protein and rRNA synthesis can be uncoupled, albeit with drugs, in eucaryotic cells (5, 16), it is possible that

cells may show differential regulation of these components at some times. An equally likely possibility to explain these results would be that the rRNA and ribosomal protein syntheses increase in parallel but that the assembly of ribosomes is delayed in refed cells either because rRNA processing is limiting or because some step in the association of RNA and protein is delayed. In either case, we interpret the results as indicating that the ribosomal protein pool (either as free protein or in preribosomal particles) increases during early refeeding and then decreases as ribosome accumulation increases and these proteins are chased into mature ribosomal subunits. We are currently examining rRNA synthesis in refed cells to determine how ribosomal protein synthesis, rRNA synthesis and ribosome assembly are related to each other.

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