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Saccharomyces cerevisiae Coordinates Accumulation of Yeast Ribosomal Proteins by Modulating mRNA Splicing, Translational Initiation, and Protein Turnover

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The rate of accumulation of each ribosomal protein is carefully regulated by the yeast cell to provide the equimolar ratio necessary for the assembly of the ribosome. The mechanisms responsible for this regulation have been examined by introducing into the yeast cell extra copies of seven individual ribosomal protein genes carried on autonomously replicating plasmids. In each case studied the plasmid-borne gene was transcribed to the same degree as the genomic gene. Nevertheless, the cell maintained a balanced accumulation of ribosomal proteins, using a variety of methods other than transcription. (i) Several ribosomal proteins were synthesized in substantial excess. However, the excess ribosomal protein was rapidly degraded. (ii) The excess mRNA for two of the ribosomal protein genes was translated inefficiently. We provide evidence that this was due to inefficient initiation of translation. (iii) The transcripts derived from two of the ribosomal protein genes were spliced inefficiently, leading to an accumulation of precursor RNA. We present a model which proposes the autogenous regulation of mRNA splicing as a eucaryotic parallel of the autogenous regulation of mRNA translation in procaryotes. Finally, the accumulation of each ribosomal protein was regulated independently. In no instance did the presence of excess copies of the gene for one ribosomal protein affect the synthesis of another ribosomal protein.

The ribosome is undoubtedly the most thoroughly understood organelle of the cell (2, 37, 38). It has two subunits. The smaller is composed of one RNA molecule and 20 to 30 proteins; the larger is composed of two RNA molecules and 35 to 50 proteins. With only one or two exceptions (e.g., L7/L12), there is a single molecule of each component in each *Escherichia coli* ribosome (11) and there is no reason to believe that eucaryotic ribosomes will be different.

Our interest is centered on the synthesis of the ribosome, in particular, on the mechanisms used by the cell to ensure an adequate supply of each component used for assembly without needless accumulation of excess components (7, 36). In short, we view efficient synthesis of ribosomes as a problem of molecular inventory control. That the cell can carry out this process effectively was apparent from earlier studies on both RNA (33) and proteins (9) showing that no more than 10% excess of any ribosomal component was synthesized. Since the assembly of ribosomes is presumably limited by the availability of the least abundant component, there is little margin for error.

One method of analyzing the way in which a cell regulates the synthesis of any component is to perturb the system by introducing extra copies of individual genes. We have applied this method to the yeast *Saccharomyces cerevisiae* and found that introduction of additional copies of the gene *TCM1*, coding for L3, resulted in transcription of *TCM1* in proportion to the number of copies of the gene, but with little, if any, accumulation of excess L3 (25). We concluded that the cell modulated the translation of the mRNA for L3 in response to its actual need for that ribosomal protein. Translational modulation is reminiscent of *E. coli* where the "autogenous regulation" of the translation of ribosomal protein mRNA has been well established. (19, 24).

To generalize our findings, we have repeated the gene dosage experiment with six additional ribosomal protein genes. We find that the cell uses several methods to control the accumulation of the products of these genes. Both *TCM1* and *CYH2* are subject to translational regulation, apparently at the level of initiation. In addition, both *CYH2* and *RPL32* may be regulated at the level of mRNA processing, as additional copies of each lead to elevated amounts of their unspliced transcripts. On the other hand, the presence of additional copies of *RPS7*, *RPS10A*, *RPS10B*, or *RP(29)* results in significant overproduction of their respective proteins, although not necessarily in proportion to the increase in their mRNA. The excess proteins are degraded rapidly. These results are in sharp contrast to the findings with nonribosomal protein genes, whose products accumulate roughly in proportion to gene dosage (5).

MATERIALS AND METHODS

Ribosomal protein nomenclature. The ribosomal proteins are indicated by standard nomenclature L1 . . ., S1 . . ., (36), except those such as rp29 which have not been identified in the standard system. Their genes are indicated by capitals, e.g., *RPS7*, except where they have been identified genetically: e.g., *TCM1* is the gene for L3; *CYH2* is the gene for L29. Note that L29 and rp29 are not identical (see Fig. 1 and Table 1).

Strains. The following strains were used: J40 *MATα ura3-52 his3-1 tyr1-289 lys2 ade2*; J401 *MATα ura3-52 his3-1 his7 ade1 tyr1 rna2*(Ts); J403 *MATα leu2-3 leu2-112 his4*; J409 *MATα ura3-52 his3-1 his7 ade1 tyr1*.

Vectors. YEp24′ carries portions of 2μm DNA, the *URA3* gene of yeasts, and most of pBR322 (25). YEp13 carries

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TABLE 1. Plasmids used in this study

Plasmid	Vector	Gene	Ribosomal protein[a]
YEpTCM-5	YEP24'	*TCM1*[b]	L3 (rp1)
pYERPS7	YEP24'	*RPS7*	S7 (rp5)
pYERPS10A	YEP13	*RPS10A*[c]	S10 (rp9)
pYERPS10B	YEP13	*RPS10B*[c]	S10 (rp9)
pYERPS10A'[d]	YEP24'	*RPS10A*	S10 (rp9)
pYERP(29)/L32	YEP13	*RP(29)*, *RPL32*[e]	(rp29), L32 (rp73)
YEpCYH-1	YEP24'	*CYH2*	L29 (rp44)

[a] See Warner (36) for details of the nomenclature of yeast ribosomal proteins.

[b] Both YEpTCM-5 and YEpCYH-1 carry the drug resistance alleles of their respective genes and thereby confer partial resistance to trichodermin and to cycloheximide.

[c] There are two genes which code for S10, designated *RPS10A* and *RPS10B* (W. F. Schwindinger, unpublished data). *RPS10A* is the gene sequenced by Leer et al. (18).

[d] This plasmid was used to demonstrate that the nature of the vector did not play a role in the regulation of the gene. We found no significant differences between pYERPS10A and pYERPS10A'.

[e] Both *RP(29)* and *RPL32* are contained within the same restriction fragment. (Fig. 1.)

portions of 2μm DNA, the *LEU2* gene of yeasts, and most of pBR322 (4).

Recombinant DNA techniques and preparation of yeast nucleic acids. Recombinant DNA techniques and preparation of yeast nucleic acids were carried out as previously described (25).

Preparation of yeast polyribosomes. To a 100-ml culture, 50 μg of cycloheximide per ml was added. The culture was swirled rapidly and immediately poured over ice. The cells were collected by centrifugation and washed twice with 10 mM Tris-hydrochloride (pH 7.4)–100 mM NaCl–30 mM $MgCl_2$, containing 50 μg of cycloheximide, 200 μg of heparin, and 0.2 μl of diethylpyrocarbonate per ml. The washed cells were suspended in 1 ml of the same solution and disrupted by mixing with glass beads on a Vortex mixer. Then 1.5 ml of solution was added and the sample was spun twice at 5,000 rpm for 5 min. The supernatant was layered over a 7 to 47% (wt/wt) sucrose gradient in 50 mM Tris-acetate (pH 7.0)–50 mM NH_4Cl–12 mM $MgCl_2$–1 mM dithiothreitol and centrifuged for 150 min at 39,000 rpm and 4°C in an SW41 rotor. The gradient was pumped through a Gilford spectrophotometer, and fractions were collected into tubes containing a drop of 10% sodium dodecyl sulfate. Fractions were pooled as indicated in the legend to Fig. 5.

Hybridization techniques. The DNA was blotted by the method of Southern (29). Filters were hybridized with nick-translated probes at 37°C in 50% formamide–4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50 mM Tris (pH 7.4) and washed at 60°C in 0.1× SSC–0.2% sodium dodecyl sulfate. The RNA was blotted by the method of Thomas (31) after analysis on 1.5% agarose gels in 6% formaldehyde as described before. Filters were hybridized with nick-translated probes at 42°C in 50% formamide–5× SSC–5× Denhardt solution–50 mM $NaPO_4$ (pH 6)–200 μg of denatured calf thymus DNA per ml and washed at 50°C in 0.1× SSC–0.1% sodium dodecyl sulfate. Hybridization of pulse-labeled RNA was carried out as described previously (16).

Probes. The probe for *RPS7* was an 11-kilobase fragment isolated from a λ clone (6). This DNA fragment is complementary to a number of transcripts but the one coding for S7 was identified by hybrid selection from smaller fragments (N. J. Pearson and J. R. Warner, unpublished data). The probe for *RPL32* was a fragment from within the coding region purified by two passes through agarose gels. The probes for all other genes were from fragments cloned into derivatives of phage f1 (41). Replicative forms were prepared and labeled by nick translation.

Synthesis of ribosomal proteins. Measurement of the synthesis of individual ribosomal proteins has been described in detail (9). Briefly, a culture of control cells was labeled for more than one generation with 30 μCi of [^{3}H]methionine per ml. The test culture was labeled for 3 to 5 min with [^{35}S]methionine. (See text for details.) For the chase experiment, 100 μg of methionine per ml was added at the indicated time. Each culture was harvested by pouring over frozen ice and centrifuging at 5,000 rpm for 2 min. The cell pellet was washed, the cells were disrupted with glass beads, and total protein was extracted with 67% glacial acetic acid and dialyzed. Suitable amounts of ^{3}H-labeled protein and ^{35}S-labeled protein were mixed, lyophilized, and analyzed on a two-dimensional polyacrylamide gel. The spots for 20 to 25 ribosomal proteins and a number of nonribosomal proteins were excised, and the radioactivity was determined.

RESULTS

Transformants. We have demonstrated that a yeast cell which contains several extra copies of the ribosomal protein gene *TCM1* nevertheless synthesizes only normal amounts of its product, L3 (25). The cell appears to control the rate of translation of the mRNA derived from *TCM1*. To determine how universal such translation control is, we have subcloned several other ribosomal protein genes into autonomously replicating shuttle vectors (Table 1). In each case we know that the insert contains the complete gene (Fig. 1) along with at least 1 kilobase and in most cases more than 2 kilobases of upstream sequences.

Each plasmid described in Table 1 was used to transform a yeast strain appropriate to its selectable marker. DNA was prepared from each of the transformants, cut with appropriate restriction enzymes, displayed on an agarose gel, and hybridized to an appropriate probe (29). In some cases dot

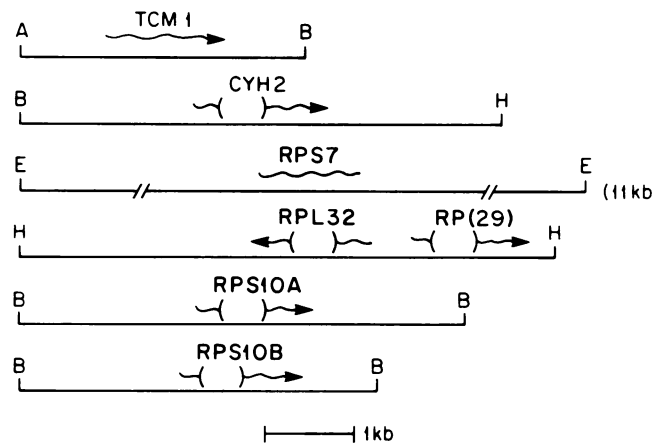

FIG. 1. Inserts of yeast DNA carrying ribosomal protein genes. The inserts carrying the yeast ribosomal protein genes, described in Table 1, are diagrammed and the locations of the genes are indicated. Arrowheads denote the direction of transcription and the parentheses denote the introns for those cases in which it has been established. The insert carrying *RPS7* is approximately 11 kilobases (kb). A, *Ava*II; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII.

TABLE 2. Summary of gene dosage experiments[a]

Plasmid	Host	Copy no.[b]	Relative transcription rate[c]	Relative mRNA concn[d]	Relative synthesis of protein[e]
YEpTCM-5	J40	6	7	3.5	1.0; 1.3
pYERPS7	J409	10	ND	3.8	1.8; 4.0
pYERPS10A	J403	10	5	4.5	2.4; 2.7
pYERPS10B	J403	10	5	4.2	2.8; 2.5
pYERPS10A'	J409	15	ND	4	3.7; 5.7
pYERP(29)/73	J403	7	(rp29) ND	3.6	2.6; 3.1
			(L32) ND	1[f]	0.8; 1.1
YEpCHY-1	J400	23	30	5[f]	1.3; 1.1

[a] Cells carrying the indicated plasmids were growing at mid-log phase in medium selecting for the outside marker on the plasmid. One sample of culture was harvested and DNA was prepared for analysis of plasmid copy number (see text). Another sample was harvested and RNA was prepared for analysis by northern blot and dot blot (Fig. 2). A third sample was pulsed with [^{35}S]methionine and protein was prepared for analysis by two-dimensional gels (Fig. 3, Table 4). On a separate occasion, parallel cultures were pulsed with [^{3}H]uracil and the RNA was prepared for hybridization to probes immobilized on filters (Table 3).

[b] These values, estimated from Southern blots (29), are subject to an error of about ±20%.

[c] From Table 3: the rate of transcription of the gene carried on the plasmid divided by the average rate of transcription in the cells carrying no plasmid and the cells carrying only vector YEP13. ND, Not determined.

[d] Northern blots were prepared from total RNA of cells with (x) and without (y) the plasmid. Each blot was probed with the ribosomal protein gene present on the plasmid (A) and with another ribosomal protein gene (B). The resulting autoradiographs were scanned. The value presented in the table is $(A/B)x \div (A/B)y$, and therefore is a measure of the excess A mRNA, after corrections for yield of RNA, irreproducibility of electrophoresis and blotting, etc. See reference 25 for details.

[e] See text for details. The two values represent independent experiments. The data are derived from Table 4 and from similar experiments. Although it is difficult to define a formal experimental variation, the number of control ribosomal proteins cut from each gel, as well as the fact that each cloned protein serves as a control in all other gels, gives us confidence in the conclusions drawn in the text.

[f] This represents only mature mRNA. There was also a substantial accumulation of unspliced transcripts (see Fig. 2).

blots were used instead. Scanning the resulting autoradiographs led to a measurement of the copy number of the plasmids (Table 2).

Transcription of ribosomal protein genes. The rate of transcription of several ribosomal protein genes was measured by DNA-excess hybridization (15, 16). Each of the plasmids described in Table 1 contains sequences derived from pBR322 which are transcribed to various degrees in yeast cells. To provide a probe with which to measure specifically the transcription of the ribosomal protein genes, a fragment of each was subcloned into derivatives of the single-stranded phage f1 (41).

The transformants, growing in log phase, were labeled for 5 min with 200 μCi of [^{3}H]uracil per ml. Total RNA was prepared and hybridized to the phage DNA probe as described in Materials and Methods. The results are shown in Table 3 and are summarized in Table 2.

It is evident that the transcription of each of the ribosomal protein genes is substantially higher in those cells which carry extra copies. Many yeast ribosomal proteins, including S10, are encoded by two distinct genes (3, 6, 39). The probe used to measure the transcription of RPS10 was derived from the *A* gene and contains all but three nucleotides of the coding sequence. This probe measures transcription not only from the *A* gene (Table 3, column 6) but from the *B* gene as well (Table 3, column 7), and the results show that each gene can be active when present on a multicopy plasmid. Both genes are active in the genome as well, since disruption of either still permits growth (C. Kruse and J. Warner, unpublished data). The two genes for rp51 (1) and rp28 (22) are also known to be active.

Note in Table 2 that, although the rate of transcription of both *TCM1* and *CYH2* is proportional to the copy number of the gene, the transcription of *RPS10* is proportional only to one-half the excess copies of either *RPS10A* or *RPS10B*. In normal cells, *RPS10A* and *RPS10B* together produce the same number of molecules as a single-copy gene such as *TCM1* or *CYH2* (16; unpublished data). Therefore we suggest that the inherent efficiency of transcription of an *RPS10* gene is only half that of a single-copy ribosomal protein gene. At least for this small sample it is quite clear that the yeast cell does not respond to excess copies of a ribosomal protein gene by modulating its transcription.

Transcriptional regulation of ribosomal protein genes carried on plasmids. It could be argued that the lack of transcriptional regulation in response to plasmid-borne genes might not represent the situation for these genes embedded in the chromosome. Therefore, we asked whether transcription of the plasmid-encoded genes was subject to the same regulatory factors as their chromosomal counterparts. One well-documented regulatory feature of yeast ribosomal protein genes is the precipitous, though temporary, drop in transcription which occurs upon an abrupt increase in temperature, even when the final temperature lies within the growth range (15). To determine if transcription of the ribosomal protein genes on plasmids is subject to heat-induced repression, the transformed cells were grown at 23°C and shifted to 36°C. Between 2 and 7 min after the shift, the cultures were pulsed with [^{3}H]uracil. RNA was prepared and ribosomal protein transcripts were measured by hybridization as described above. Table 3 shows that there is a 5- to 10-fold reduction in the transcription of each of the ribosomal protein genes whether or not they are on a plasmid. This result is particularly striking for *CYH2*, which is present in 20 to 30 copies per cell and contributes nearly 1% of the total transcription at 23°C. This experiment also demonstrates that long-range chromosomal effects are not involved in the alterations of transcription after a mild heat shock.

mRNAs for ribosomal proteins with elevated gene dosage. The mRNA levels for the various ribosomal proteins were measured by northern analysis of total cell RNA, probing first with the gene in question and then with another gene, to correct for experimental variations. Figure 2A shows such an analysis for the plasmids carrying either of the *RPS10* genes. After scanning the autoradiographs, we conclude that the cells with extra copies of the *RPS10A* gene have 4.5 times as much S10 mRNA, whereas those with the *RPS10B* gene have 4.3 times as much (Table 2). For most of the transformed cells in Table 2, the amount of active mRNA for the ribosomal protein of interest has been determined independently by cell-free translation (data not shown), as previously described (25). The value agreed with that obtained by northern analysis shown in Table 2.

A more intriguing result was obtained in the analysis of the *CYH2* gene (Fig. 2B). As expected, there is substantially more mature mRNA in the cells carrying the multicopy plasmid. Much of this mRNA must be transcribed from the plasmid, since the transformed cells are partly resistant to cycloheximide. More intriguing, however, is an exceptional accumulation of a larger species of *CYH2* RNA, of the correct size to be unspliced precursor RNA. There is no such accumulation of precursor RNA derived from the *RPS10* gene, either when it is present in many copies (Fig. 2A) or when it is in the presence of large amounts of unprocessed *CYH2* RNA (Fig. 2B).

TABLE 3. Transcription of ribosomal protein genes[a]

Gene hybridized	Temp (°C)	% cpm hybridized × 10^{-2}					
		None	YEP13	YEpTCM-5	pYERPS10A	pYERPS10B	YEpCYH-1
TCM1	23	2.56	4.43	[21.2]	1.53	2.64	4.04
	36	0.20	0.48	[1.39]	0.19	0.47	0.74
36/23(%)[b]		7	11	6	12	17	18
RPS10A	23	1.33	1.53	1.69	[7.35]	[7.05]	2.53
	36	0.16	0.22	0.09	[2.65]	[0.77]	0.4
36/23(%)		12	14	5	36	10	16
CYH2	23	2.13	2.48	1.96	1.02	1.77	[63.8]
	36	0.28	0.33	0.08	0.30	0.57	[9.6]
36/23(%)		13	13	4	29	32	15

[a] Cultures of cells carrying the indicated plasmids were labeled with 200 μCi of [^{3}H]uracil per ml for 5 min at 23°C or between 2 and 7 min after shifting from 23 to 36°C. The culture was chilled rapidly, the cells were harvested by centrifugation, and RNA was prepared as described in the text. RNA was hybridized as previously described to DNA from single-stranded phages, immobilized on filters (15); background hybridization, approximately 0.003 to 0.005%, has been subtracted. Note that the *RPS10A* gene cross-hybridizes to the *RPS10B* gene and its transcript. (See Fig. 2A.) The values for cloned genes are enclosed by boxes.

[b] Percent transcription remaining after the temperature shift.

A more extreme example is the gene *RPL32*. When this gene is present in excess, there is little, if any, accumulation of excess mRNA in spite of a substantial accumulation of precursor (Fig. 2C). Note that the transcription products of *RPL32* form a striking contrast to those derived from the adjacent ribosomal protein gene *RP(29)* (Fig. 1), whose mRNA accumulates in substantial excess, with little accumulation of precursor (Fig. 2C).

Therefore, it seems unlikely that the accumulation of unprocessed RNA from either *CYH2* or *RPL32* is due to a general overloading of the splicing machinery. Nor is it likely that these transcripts are spliced by a unique pathway, for there exist a number of unlinked mutations which prevent the splicing of all introns (12, 26). These observations lead us to propose that certain ribosomal proteins may regulate specifically the splicing of the transcripts of their own genes (see Discussion).

Finally, in some instances the steady-state level of mRNA in cells carrying extra genes is lower than expected, suggesting that modulation of the mRNA half-life may also play a role in controlling mRNA concentration.

Syntheses of ribosomal proteins. To determine if the plasmids carrying extra copies of ribosomal protein genes were responsible for the synthesis of extra ribosomal proteins, each of the strains listed in Table 2 was pulsed for 5 min with [^{35}S]methionine. Total protein was extracted, mixed with extract from cells carrying no plasmid, which had been labeled for more than one generation with [^{3}H]methionine, and subjected to two-dimensional electrophoresis. Twenty-five spots were cut from each gel and counted. Table 4 presents a portion of the data, normalized to the synthesis of several nonribosomal proteins (9, 25).

The results (summarized in Table 2) confirm our previous finding (25) that, in spite of the three- to fourfold excess of *TCM1* mRNA, only marginally more L3 is synthesized. A similar result is obtained for *CYH2* even with a fivefold excess of mature mRNA. These two mRNAs are translated with substantially less efficiency when they are present in excess, suggesting that there is some feedback inhibition of translation.

On the other hand, a quite different result was observed on analysis of cells carrying extra copies of the genes for S10. As seen in Table 2 and 4, severalfold more S10 was synthesized in cells carrying several copies of either *RPS10* gene. The excess synthesis of S10 is independent of the vector (see pYERPS10A'). For technical reasons we were concerned with these measurements. S10 can be phosphorylated; it is the yeast equivalent of S6 of mammalian cells (36). The phosphorylated form runs slightly more slowly in the gel, which has led to artificially high estimates of its synthesis in the past (9). Therefore, although the data in Table 4 are derived from both the phosphorylated and non-phosphorylated spots of S10, we also analyzed pulse-labeled proteins by autoradiography of a two-dimensional gel. Figure 3 compares two such gels, analyzing newly synthesized proteins from cells bearing 10 extra copies of the *RPS10A* gene (panel A) or 23 of the *CYH2* gene (panel B). It is evident from comparisons of pairs of spots that the cells represented in panel A have synthesized a substantial excess of S10, whereas those in panel B have made no detectable excess of L29.

As one examines the data presented in Table 4, and more succinctly in Table 2, it becomes clear that there is a spectrum in the response of a cell to the presence of excess copies of a ribosomal protein gene. At one extreme is the *CYH2* gene, for which a 30-fold increase in transcription yields a barely detectable increase in the synthesis of its protein. At the other extreme (thus far) is the *RPS10* gene, for which a fivefold increase in transcription yields nearly a threefold increase in the synthesis of its protein. In between are genes *RPS7* and *RP(29)*, each of which can give rise to substantial amounts of excess proteins. It should be emphasized that in no case have we observed that the synthesis of any one ribosomal protein is consistently affected by the introduction of extra copies of the gene of any other ribosomal protein.

Fate of the excess protein. To examine the fate of the excess protein, cells carrying pYERPS10A' were pulse-labeled for 3 min with [^{35}S]methionine, which was chased with excess cold methionine. In comparison with the control, there was 5.7 times the amount of S10 immediately after the pulse, 3.1 times the amount 5 min later, and a normal amount after 30 min. The relative amounts of other ribosomal proteins did not change. From these data we conclude that excess S10 decays with a half-life of 5 to 10 min. This is not surprising as excess, or unused, ribosomal proteins have been found to decay rapidly in yeast cells (10), in HeLa cells (35), and in rat liver (32). This rapid decay may account for some of the variability we have observed in trying to measure the rate of synthesis of those proteins whose genes

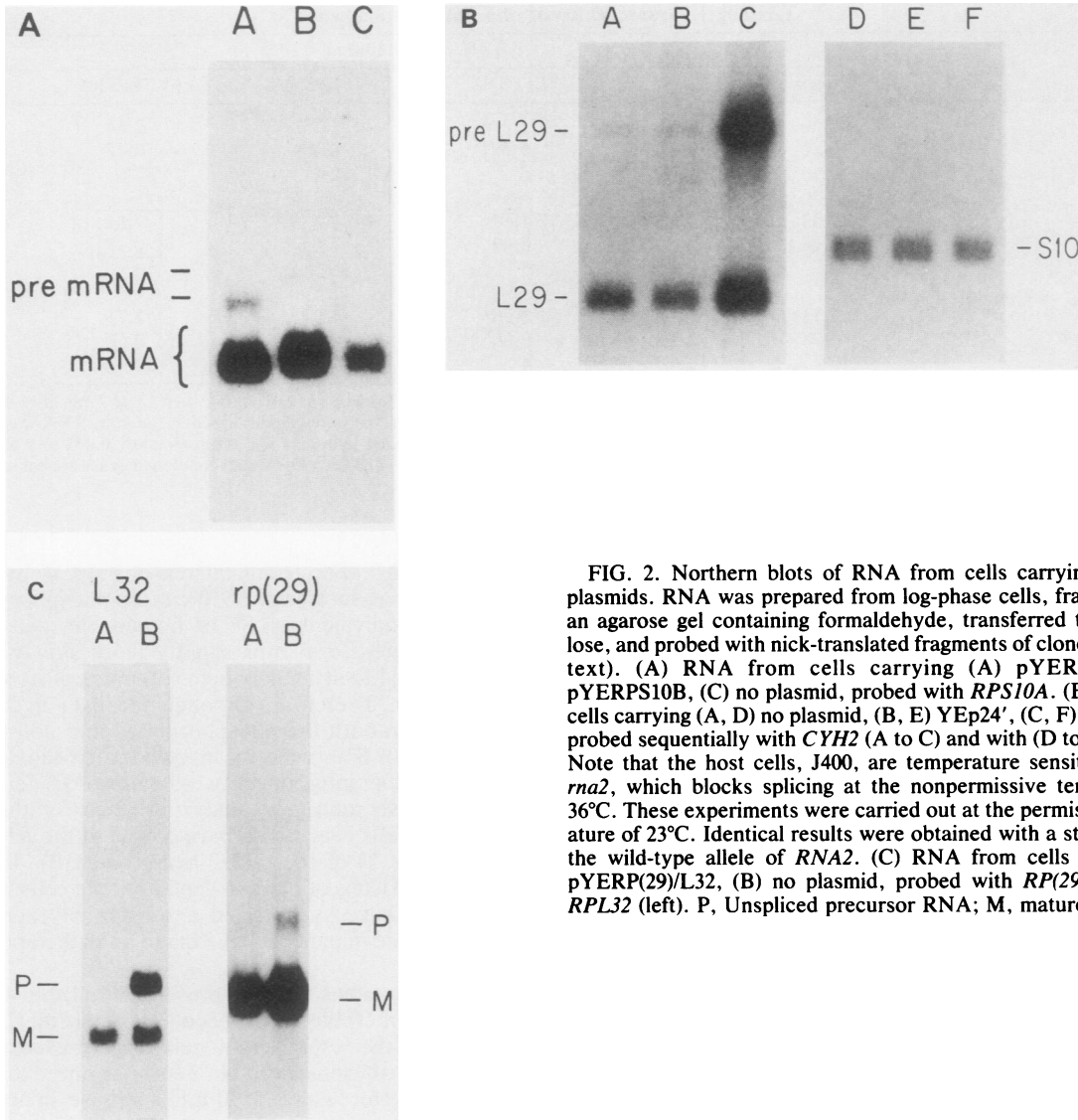

FIG. 2. Northern blots of RNA from cells carrying multicopy plasmids. RNA was prepared from log-phase cells, fractionated on an agarose gel containing formaldehyde, transferred to nitrocellulose, and probed with nick-translated fragments of cloned genes (see text). (A) RNA from cells carrying (A) pYERPS10A, (B) pYERPS10B, (C) no plasmid, probed with *RPS10A*. (B) RNA from cells carrying (A, D) no plasmid, (B, E) YEp24′, (C, F) YEpCYH-1, probed sequentially with *CYH2* (A to C) and with (D to F) *RPS10A*. Note that the host cells, J400, are temperature sensitive for gene *rna2*, which blocks splicing at the nonpermissive temperature of 36°C. These experiments were carried out at the permissive temperature of 23°C. Identical results were obtained with a strain carrying the wild-type allele of *RNA2*. (C) RNA from cells carrying (A) pYERP(29)/L32, (B) no plasmid, probed with *RP(29)* (right) and *RPL32* (left). P, Unspliced precursor RNA; M, mature mRNA.

are in excess. On the other hand, it cannot account for the dramatic difference between S10 and L29 shown in Fig. 3. To do so, the half-life of L29 would have to be measured in seconds, shorter than the translation time itself, and far shorter than the time necessary for the assembly of a ribosome (34).

How does translational regulation occur? It is clear from the foregoing results that the cell regulates the translation of the mRNAs for certain ribosomal proteins. How does this occur? Three potential mechanisms can be distinguished by determining the distribution of the mRNA in a sucrose gradient which displays the polyribosomes of the cell.

(i) By sequestering the excess mRNA to prevent its translation. The polyribosomes should have the wild-type amount of mRNA. The excess mRNA could be found either in the nucleus or in lower-molecular-weight material near the top of the gradient.

(ii) By inhibiting initiation of translation of the mRNA. If the rate of loading of an mRNA is decreased while elongation proceeds at a normal pace, the average size of the polyribosome will become smaller.

(iii) By inhibiting translation of the mRNA, either at an elongation step or at termination. If the rate of polypeptide elongation or termination is decreased while initiation continues normally, the average size of the polyribosome will remain constant or even increase, provided initiation is normally the limiting step in protein synthesis, as it appears to be in yeast cells (36).

To determine the distribution of mRNA in the polyribosomes, cells were disrupted by vortexing with glass beads and their contents were displayed on a sucrose gradient (Fig. 4). The distribution of mRNA was compared first in cells with and without plasmid YEpCYH1. RNA was prepared from total polysomes, from a region containing 80S single ribosomes and 60S subunits, and from the rest of the gradient. Northern blots were probed with two ribosomal protein genes, *CYH2* and *RPS10A* (Fig. 5). The latter was included as an internal standard. It is clear that there is substantially more *CYH2* mRNA in all fractions of cells with excess copies of the *CYH2* gene. Most of the unspliced mRNA is found at the top of the gradient. On the other hand, the bulk of the mature mRNA is found in the polyribosomes.

TABLE 4. Translation of ribosomal proteins[a]

Protein	Relative synthesis					
	YEP24'	YEpTCM-5	pYERPS7	pYERPS10A'	YEpCYH-1	YEpCYH-1
L2 (rp2)	0.74	1.01	0.87	0.99	0.81	0.85
L3 (rp1)	0.67	[0.93]	0.63	0.71	0.76	0.76
L4 (rp6)	0.73	0.98	0.85	1.22	0.81	0.89
L5 (rp8)	0.78	1.04	0.66	1.46	—[b]	0.91
L6 (rp11)	0.91	1.03	0.97	1.29	0.86	0.99
L16 (rp39)	0.87	1.13	0.86	1.03	0.99	0.85
L21 (rp22)	0.86	0.91	0.96	0.90	—	—
L22 (rp33)	0.76	0.86	0.79	0.85	0.86	—
L29 (rp44)	0.76	0.78	0.87	0.88	[1.22]	0.94
S2 (rp14)	0.78	0.68	0.84	0.99	1.03	0.64
S3 (rp13)	0.99	0.86	1.00	1.35	0.77	[0.94]
S4 (rp12)	0.90	0.86	0.86	1.35	0.95	0.87
S7 (rp5)	0.79	0.83	[1.64]	0.91	0.86	0.86
S10 (rp9)	0.78	1.07	0.79	[4.45]	—	1.01
S18 (rp41)	0.81	0.89	0.91	0.88	0.93	—
S27 (rp61)	0.83	0.97	0.89	0.96	—	0.82
(rp23)	0.80	1.00	0.83	0.96	—	—
(rp27)	0.83	0.76	0.79	1.04	—	0.89
(rp29)	0.79	0.84	0.76	0.83	—	0.56
(rp38)	0.84	0.88	0.87	1.01	1.14	—
(rp48)	0.74	0.90	1.06	0.95	0.95	—
Avg[c]	0.81	0.91	0.89	1.19	0.94	0.84
Cloned gene ÷ avg		1.02	1.84	3.73	1.30	1.12

[a] Cultures of cells carrying the indicated plasmids were labeled for 3 min with [^{35}S]methionine (30 μCi/ml). Total protein was extracted, mixed with total protein from cells labeled for one or two generations with [^{3}H]methionine, and subjected to two-dimensional polyacrylamide gel electrophoresis (9). Twenty or more ribosomal protein spots were excised, and the ^{35}S/^{3}H ratio was determined and divided by the average value for several nonribosomal proteins. (Spots containing both the unmodified and phosphorylated versions of S10 were pooled.) The values for the cloned genes are enclosed by boxes. The penultimate line shows the average ^{35}S/^{3}H for all ribosomal proteins counted, and the last line shows the relative synthesis of the protein whose gene is in excess. Two experiments on cells carrying YEpCYH-1 are shown to demonstrate the reproducibility of the measurements. Not all of the same proteins were counted, but the average was computed for more than 20 ribosomal proteins.

[b] —, No data.

[c] Average value for 20 ribosomal proteins counted.

To rule out the possibility that some of the mRNA in the polyribosomes is sequestered in a ribonucleoprotein complex sedimenting with the polyribosomes, we examined control gradients containing 10^{-5} M Mg ion, in which polyribosomes are dissociated. No mRNA was found in the "polyribosome region."

Microdensitometry of the autoradiographs in Fig. 5 showed that the polyribosomes of the cells carrying YEpCYH-1 had three times the normal amount of *CYH2* mRNA, in comparison with the level of mRNA for S10. Thus excess mRNA is not sequestered but is being translated. Yet excess protein is not being synthesized. Therefore, either the ribosomes are moving more slowly or there are fewer ribosomes per message.

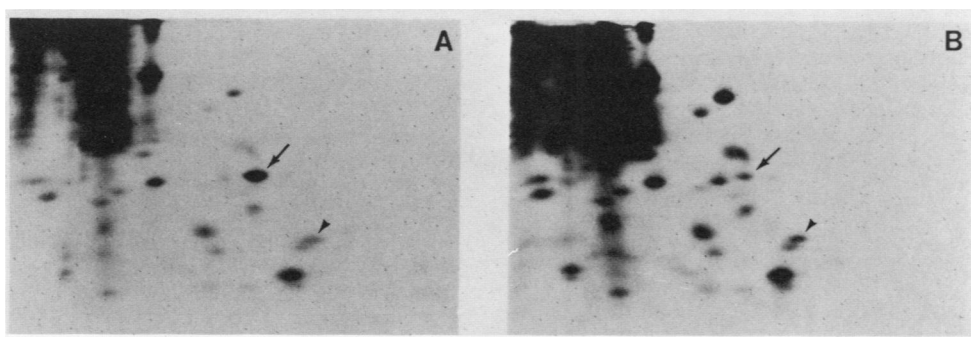

FIG. 3. Autoradiographs of two-dimensional polyacrylamide gels analyzing the basic proteins synthesized by cells carrying excess copies of the *RPS10A* gene or the CYH2 gene. Cultures of cells carrying plasmids pYERPS10A (A) and YEpCYH-1 (B) were pulsed with [^{35}S]methionine for 3 min. The cultures were quickly chilled, and the cells were collected by centrifugation. By using 67% acetic acid, total protein was extracted from cells broken with glass beads. Samples were analyzed on two-dimensional gels, run at pH 5 from left to right in the first dimension and with sodium dodecyl sulfate from top to bottom in the second dimension (9). The stained gels were subjected to fluorography. S10 is indicated by arrows; L29 is indicated by arrowheads. The left panel was underexposed to show more clearly the excess S10 synthesized in those cells.

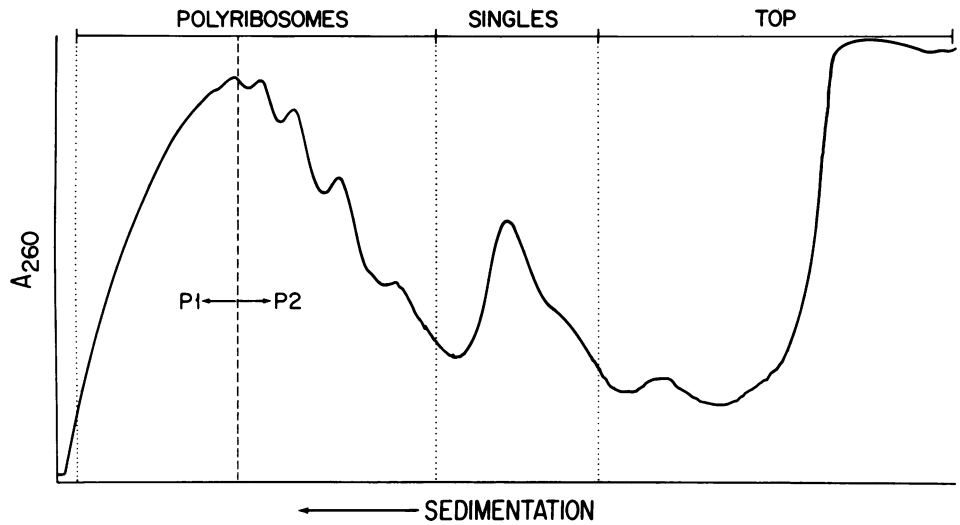

FIG. 4. Polyribosome profile of a yeast extract. Log-phase cells were chilled, harvested, and disrupted, and their contents were analyzed on a sucrose gradient as described in the text. The sections of the gradient were probed as indicated by the dotted lines for the experiment in Fig. 5 and as indicated by both the dashed and dotted lines for the experiment in Table 5.

Since the mRNA for *CYH2* has only 149 codons (13), it would be translated on a small polyribosome. However, the mRNA for *TCM1* has 388 codons (27), enough to accommodate about 10 ribosomes. It is thus large enough that one might be able to detect a decrease in the number of ribosomes on an mRNA. Extracts were prepared from host cells and from cells carrying YEpTCM-5 and were displayed on sucrose gradients as in Fig. 4. Fractions P1, P2, S, and T were taken as indicated, with the boundary between P1 and P2 being roughly in the hexasome region. RNA was prepared from each fraction and subjected to quantitative dot blot analysis, using a *TCM1* probe with the *CYH2* probe serving as a control (Table 5). In comparison to the host, cells carrying YEpTCM-5 had 3.0 times the amount of *TCM1* mRNA, but in both cells 90% was in the polyribosomes. However, it is clear that in cells carrying excess genes there has been a shift of *TCM1* mRNA from the larger polysomes to the smaller. Since the mean number of ribosomes per mRNA is substantially reduced, translational control of the synthesis of L3 most likely occurs by means of a transient inhibition of polypeptide initiation.

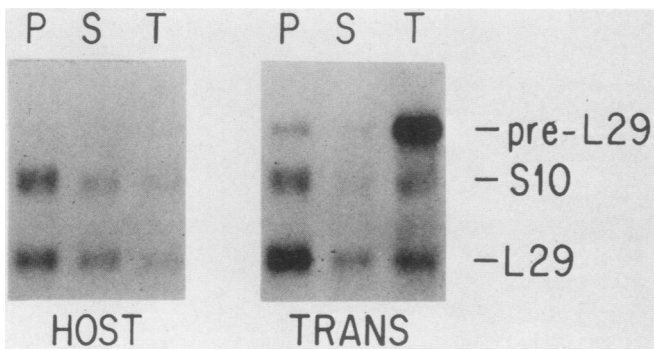

FIG. 5. Northern gel analysis of RNA from cell fractions. Cells containing YEpCYH1 (right) or no plasmid (left) were harvested and disrupted, and their polyribosomes were displayed on a sucrose gradient as in Fig. 4. RNA from pooled polyribosomes (P), from single ribosomes (S), and from the top of the gradient (T), fractionated as indicated in Fig. 4, was purified, fractionated on an agarose gel containing formaldehyde, transferred to nitrocellulose, and probed with nick-translated fragments of *CYH2* and *RPS10A* (see text).

DISCUSSION

The 70 or so ribosomal proteins constitute roughly 15% of the total protein of the yeast cell. The cell must have mechanisms to permit synthesis of only that amount of ribosomal protein necessary for the number of ribosomes demanded by given growth condition. Indeed, numerous studies have shown that each ribosomal protein is synthesized at the same rate with no significant accumulation of unassembled components (9, 10). Moreover, cells constrained by nutritional conditions to grow at a slow rate synthesize fewer ribosomes than do rapidly growing cells (14). A system as tightly regulated as that of ribosome biosynthesis is thus ideal for examining the consequences of altering the gene dosage of individual components.

We have constructed strains carrying extra copies of seven different ribosomal protein genes. It is now clear from a substantial body of previous (7) and current work that the yeast cell maintains a nearly constant amount of each of the components necessary for ribosome synthesis. It does so in a variety of ways.

TABLE 5. Distribution of mRNA in polysomes[a]

Fraction	% of total			
	Host		Host + YEpTCM-5	
	TCM1	*CYH2*	*TCM1*[b]	*CYH2*
P1	70	26	41	26
P2	20	44	49	57
S	7	21	6	14
T	2	9	3	3

[a] RNA was isolated from the two polysome fractions, the single ribosomes, and the top of the gradient. Two identical arrays of dot blots were prepared, one of which was probed with *TCM1* and the other with *CYH2*, each labeled by nick translation of the replicative form of an f1 phage derivative. The dot blots were scanned, and the integrated density of each spot was determined. By taking into account the amount of RNA originally recovered from each fraction, the distribution of each mRNA was calculated.

[b] There was 3.0-fold more *TCM1* mRNA in the gradient containing the extract of cells carrying YEpTCM-5.

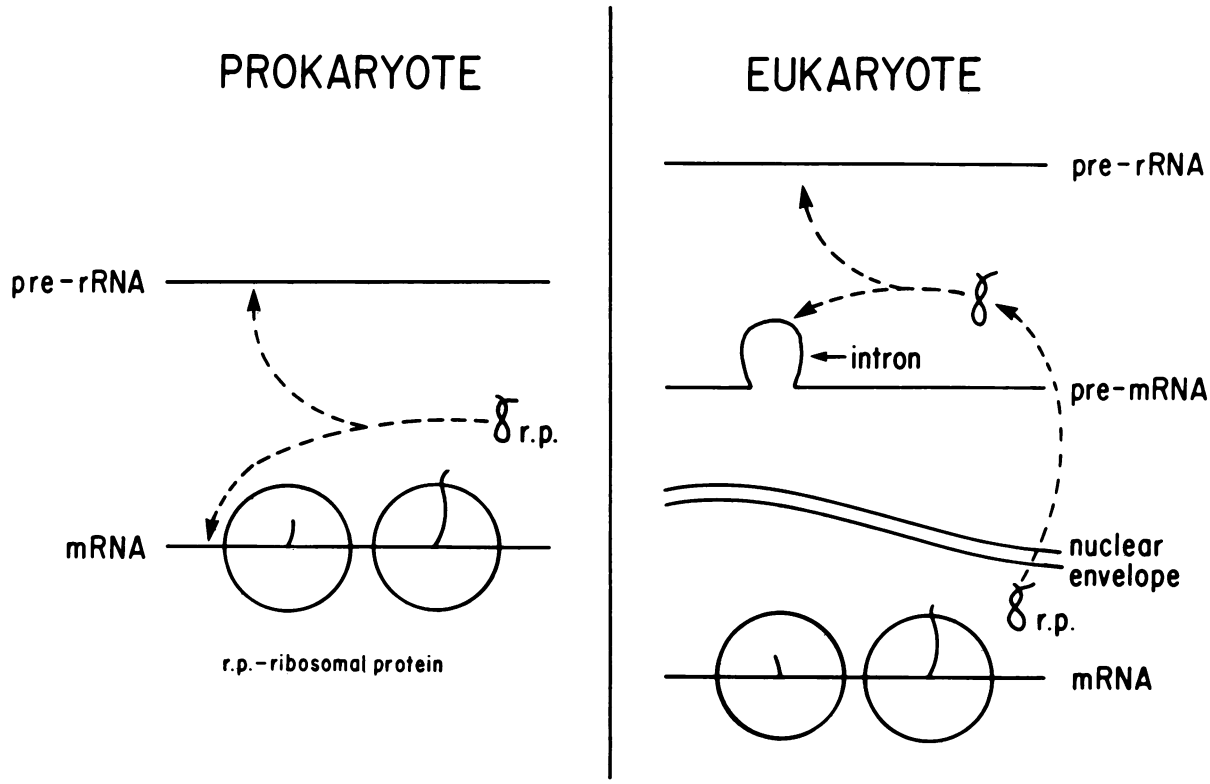

FIG. 6. Models for autogenous regulation in procaryotes and eucaryotes. See text for details.

Transcription. In none of the cells carrying multicopy plasmids have we observed a regulation of transcription. In all cases the transcription was proportional to the copy number times the rate of transcription of the host's genes. Yeasts carry single genes for some ribosomal proteins, e.g., *TCM1*, *CYH2*, and *RPL32*, and duplicate genes for others, e.g., *RPS10*, *RP(29)* (21), *RP(51)* (1), *RP(28)*, and *RPS16A* (22). When extra copies of a duplicated gene are introduced into the yeast cell, the increased synthesis of mRNA is proportional only to half the copy number (Table 3). Thus, the two *RPS10* genes, and by extrapolation the other duplicate genes, have lower inherent activity for transcription than do the single-copy ribosomal protein genes. In this way the cell maintains equimolar amounts of the mRNA for each of the ribosomal proteins (16). The rate of transcription of ribosomal protein genes is, however, subject to regulation (Table 3). The dramatic and uniform effect of a temperature change suggests that there are "signature" sequences identifying the ribosomal protein genes to RNA polymerase, either directly or through a mediating factor. Candidates for such a signature sequence have recently been identified (30). Whether such modulation of transcription also occurs during normal alterations in growth rate remains to be seen.

Splicing of mRNA. There is a convincing model for the regulation of the synthesis of ribosomal proteins in *E. coli* (24), based on a competition for newly formed ribosomal protein (see Fig. 6; reviewed in reference 24). If the protein binds to pre-rRNA, it becomes assembled permanently into a ribosome; pre-rRNA acts as a "sink" for new ribosomal proteins. On the other hand, the protein can bind to a specific site on its own mRNA, thereby inhibiting its translation. The relative abundance of pre-rRNA therefore regulates the synthesis of ribosomal proteins. This mechanism is particularly effective in *E. coli* for two reasons. (i) Both translation and ribosome assembly occur in a single compartment, leading to a ready competition for the newly formed ribosomal protein. (ii) Most of the ribosomal protein genes are in large operons, the translation of whose mRNA is controlled by a single protein, usually one which is a primary RNA-binding protein.

Neither of these conditions occur in yeasts, due to their very nature as eucaryotes. (i) The nuclear envelope separates the site of translation from the site of ribosome assembly. (ii) The mechanism of initiation of translation in eucaryotic cells precludes polycistronic mRNAs (17, 28); indeed, ribosomal protein genes are dispersed throughout the genome. However, the finding of substantial accumulation of unspliced transcripts in cells carrying excess copies of *CYH2* or *RPL32* (Fig. 2) suggests a way in which the evolution of a regulatory mechanism could proceed in parallel with the evolutionary development of a nuclear envelope (Fig. 6). The splicing of an mRNA, unlike its translation, does occur in the nucleus. Therefore the splicing of mRNA and the assembly of ribosomes occur in the same part of the cell. We can postulate that there is a site on pre-mRNA, possibly in the intron, to which the newly formed ribosomal protein can bind, and that such binding inhibits splicing. Therefore a competition for the newly formed ribosomal protein can occur between the pre-rRNA and the pre-mRNA. In such a way excess ribosomal protein would inhibit the formation of its mature mRNA. This model is attractive in that it maintains the basic concept of the regulation proposed for procaryotes, i.e., the competition between rRNA and mRNA for new ribosomal proteins, but in a eucaryotic context. It offers an explanation for the remarkable distribution of introns in yeast cells: introns are found only in the genes for ribosomal proteins, with a few exceptions, e.g., actin (8, 23) and MATa1 (20).

Two further observations are consistent with but do not directly test the hypothesis. First, some unspliced transcript of *CYH2* is usually detectable even in control cells (Fig. 2B, lanes A and B), as might be expected if pre-rRNA were limiting. Second, if 395 nucleotides of the 510-nucleotide intron are deleted from the *CYH2* gene, which is then reinserted into the yeast cell, the efficiency of processing approaches that of cells carrying a single copy of the gene (N. Kaufer and J. Warner, unpublished data). Although this result could suggest that either the size of the intron or certain sequences within it slow the splicing reaction, a more interesting possibility is that the signals which respond to the feedback on splicing have been deleted. This suggestion is readily testable.

Translation of mRNA. Cells carrying extra copies of some ribosomal protein genes, e.g., *TCM1* and *CYH2*, do accumulate excess mRNA but synthesize little if any excess protein (Table 2) (25). The results in Fig. 5 and Table 5 suggest that the initiation of translation is somehow modulated, although additional effects on elongation cannot be ruled out. No secondary effects on the synthesis of any other ribosomal proteins have been observed, in contrast to the situation in *E. coli*, where excess copies of the gene for the regulatory proteins of an operon can be lethal due to the inhibition of synthesis of the other proteins in the operon (40). It seems inescapable that excess L3 in *S. cerevisiae* leads to modulation of the translation of its mRNA, although whether L3 itself is the direct inhibitor of its own translation is not known. Nomura et al. (24) argue that neither the nuclear location of pre-rRNA nor the monocistronic nature of yeast ribosomal protein genes forms a compelling argument against the autogenous regulation of translation of some proteins. As yet, preliminary attempts to demonstrate specific translational inhibition by L3 in vitro have not been successful.

Finally, it is not clear whether mRNA derived from genes other than *TCM1* and *CYH2* is subject to such translational regulation. In most of the cases shown in Table 2, the observed synthesis of protein was less than the excess of mRNA, suggesting that some degree of translational regulation may be widespread, but not fully efficient. However, the rapid turnover of excess ribosomal proteins hinders the precise determination of their rate of synthesis.

Turnover of ribosomal proteins. In several instances, significant amounts of excess protein are synthesized (Fig. 3, Table 3). A detailed study of S10 showed that excess protein turned over very rapidly, with a half-life of approximately 5 min. Excess rp29 was also found to turn over rapidly (data not shown).

Conclusions. It is clear that the cell goes to great lengths to maintain a balanced accumulation of ribosomal proteins. To some degree this may reflect concern for an efficient utilization of resources. Yet it is likely that more pressing considerations are involved. Many ribosomal proteins bind avidly to RNA. It could be debilitating, if not dangerous, for the cell to accumulate large amounts of such RNA-binding proteins. This may explain why excess ribosomal proteins are degraded so rapidly, not only in yeast cells, but also in mammalian cells (35).

Given the need to regulate closely the accumulation of 70 ribosomal proteins, it is perhaps not surprising that yeasts should use a variety of methods to do so. Unlike *E. coli*, the yeast cell has at least one, and often two, transcription units for each ribosomal protein. Therefore the accumulation of each protein must be controlled separately. At first glance these observations tend to contradict the widespread notion that ribosomal protein genes are coordinately regulated. Yet, under conditions of balanced growth, there is no a priori requirement that ribosomal protein genes be regulated coordinately, only that ribosomal proteins accumulate coordinately. Indeed, it is difficult to imagine 70 different genes each having an identical rate of transcription and 70 different transcripts each with identical rates of processing, translation, and turnover. At present we do not understand why the accumulation of a given ribosomal protein is controlled by a particular mechanism. Perhaps it is related to the order of assembly of ribosomal proteins; i.e., the RNA-binding proteins may be more adept at binding to mRNA or its precursor. Perhaps it reflects simply the collective product of evolution.

Finally, how is the regulation associated with excess copies of a gene related to the regulation that occurs physiologically? Perhaps our experiments address only the mechanisms used to modulate the supply of ribosomal components during balanced growth. The small amount of unspliced transcript derived from the *CYH2* or *RPL32* genes in normal cells may represent such modulation. In this view, major changes in ribosome synthesis, such as accompany a nutritional shift (14), could be accomplished by other mechanisms, e.g., signals to alter transcription. Alternatively, the combination of regulatory mechanisms we have seen could be the major element of regulation under nearly all conditions.

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