Evidence for a High Proportion of Inactive Ribosomes in Slow-Growing Yeast Cells

By CLIVE WALDRON,* RICHARD JUND† and FRANÇOIS LACROUTE
Laboratoire de Genetique, I.B.M.C., 15 rue Descartes, 67084 Strasbourg, France

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From the protein and RNA content of *Saccharomyces cerevisiae* growing in different media we calculate that ribosome efficiency is changed: incorporation of amino acids into protein decreases from 8.8 amino acids/s per ribosome in fast-growing cells (0.54 doubling/h) to 5.2 amino acids/s per ribosome in slow-growing cells (0.30 doubling/h). We could not detect significant protein turnover in either fast- or slow-growing cultures, so the lower ribosome efficiency does not seem to be an artifact caused by changes in unstable protein production at different growth rates. Nor is the lower ribosome efficiency due to slower migration of ribosomes along mRNA: the times required to complete polypeptides of known molecular weights are the same in slow-growing cells as those previously determined for fast-growing cells [Waldron, Jund & Lacroute (1974) *FEBS Lett.* 46, 11–16]. We therefore deduce that ribosome efficiency changes in yeast because the fraction of ribosomes engaged in protein synthesis falls (from 84% in fast-growing cells to 50% in slow-growing cells).

Previous studies of the macromolecular composition of yeast cells growing in different media have suggested that the activity of yeast ribosomes is lower in slow-growing cells than in fast-growing cells (Boehlke & Friesen, 1975; Waldron & Lacroute, 1975). The present study was undertaken to establish the molecular basis of this growth-rate-dependent change in ribosome function.

In the reports of Boehlke & Friesen (1975) and Waldron & Lacroute (1975) ribosome efficiency, expressed as the rate of protein synthesis per ribosome, was determined from the amounts of protein and rRNA in cultures growing at different rates. The numbers of ribosomes were calculated from the rRNA contents, a reliable method because free precursor rRNA is not matured so there are no pools of free rRNA (see Warner, 1974). The rates of protein synthesis, however, were calculated from the times required to double the protein content of yeast cultures. These data can be misleading, because, if some of the protein is unstable, the rates of protein accumulation (or net rates of protein synthesis) would be underestimates of the total rates of protein synthesis. If the fraction of protein that is unstable was affected by growth rate, then changes in the total rate of protein synthesis would not be reflected in measurements of protein content. Consequently it would be incorrect to deduce that ribosome efficiency changes: the sum of stable and unstable protein synthesis could require all ribosomes to operate at

maximum efficiency irrespective of growth rate. Protein stability was not investigated in the studies of Boehlke & Friesen (1975) or Waldron & Lacroute (1975). Therefore in the present study we looked for, but were unable to detect, the high protein turnover in slow-growing yeast cells, which could invalidate the indirect evidence for large changes in ribosome efficiency.

Lower ribosome efficiency could result from (a) a decrease in the rate at which each ribosome translates messages, all ribosomes being equally affected, (b) a decrease in the proportion of ribosomes that are active, each active ribosome operating at an unchanged rate, or (c) a decrease in both the fraction of active ribosomes and their activity. Boehlke & Friesen (1975) produced evidence in favour of model (a) by measuring the polyribosome contents of spheroplasts from fast- and slow-growing yeast cells. They found a constant fraction of polyribosomal (active) ribosomes, suggesting that lower ribosome efficiency resulted from slower polypeptide-elongation rates. However, their spheroplasts from slow-growing cells were not re-equilibrated in growth medium before lysis, so this high fraction of polyribosomal ribosomes may be an artifact caused, for example, by slow amino acid-polymerization rates during spheroplast preparation. We therefore decided to measure the polypeptide elongation rate in slow-growing whole cells by the direct method that we have previously used for fast-growing yeast (Waldron *et al.*, 1974). The data show that elongation rate is not growth-rate-dependent, so we deduce that ribosome efficiency is low in slow-growing yeast cells

* Present address: Botany School, University of Oxford, South Parks Road, Oxford OX1 3RA, U.K.

† To whom reprint requests should be addressed.

because they contain a high proportion of inactive ribosomes. The significance of these extra ribosomes during times of nutritional fluctuation is discussed.

Materials and Methods

Organism and growth conditions

Saccharomyces cerevisiae strain FL530 (a wild-type diploid) was grown in liquid media at 30°C on a reciprocal shaker. Growth was monitored turbidimetrically in a Klett–Summerson colorimeter by using a blue filter (about 430 nm). The media were Yeast Nitrogen Base (Difco, Detroit, MI, U.S.A.) containing 2% (w/v) glucose and either (NH₄)₂SO₄ (0.5%, w/v) or L-proline (0.2%, w/v) as nitrogen source.

Determination of cell composition

Dry weights and cell numbers were estimated as described previously (Waldron & Lacroute, 1975).

To measure the protein content of culture samples we used the technique of Johnston *et al.* (1977): cells were precipitated with cold 5% (w/v) trichloroacetic acid, washed with 95% (v/v) ethanol, then resuspended in 1 M-NaOH. After overnight incubation at 37°C, cell debris was removed by centrifugation and the supernatant assayed for protein by the method of Lowry *et al.*, (1951), with bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) as standard. This long incubation in alkali gives values 60% higher than our previous treatment (Waldron & Lacroute, 1975), but we believe the higher estimates to be correct, because they are the same as the protein contents calculated from the incorporation of radioactive leucine (C. Waldron, unpublished work).

RNA was determined from the amount of radioactive uracil incorporated into acid-insoluble material (Waldron & Lacroute, 1975).

Labelling protocol for assays of protein turnover

All radioactive compounds were from CEA (Saclay, France).

To study release of amino acids by protein degradation, cells were grown for several generations in the media described above supplemented with [¹⁴C]leucine at 0.25 μ Ci/ml (100 μ g/ml) with isoleucine and valine (each at 100 μ g/ml) to avoid feedback inhibition affecting growth). The exponentially growing cells were harvested by filtration on sterile nitrocellulose filters, then resuspended in the same medium, but lacking radioactive leucine. At intervals during the subsequent exponential growth, samples of the culture were removed into equal volumes of cold 20% (w/v) trichloroacetic acid. Precipitated material was collected on nitrocellulose filters, washed

with 5% trichloroacetic acid, dried and placed in 10 ml of toluene containing 0.5% (w/v) 2,5-diphenyl-oxazole. These samples were counted for radioactivity in an Intertechnique SL30 spectrometer with efficiencies of about 90% for ¹⁴C and 50% for ³H.

To study changes in the molecular-weight distribution of polypeptides due to turnover, an exponentially growing culture was first labelled with [³H]leucine at 2 μ Ci/ml (1 μ g/ml). After another 4 h of exponential growth the culture received [¹⁴C]leucine at 2 μ Ci/ml (2 μ g/ml), with isoleucine and valine each at 2 μ g/ml. After 5 min incubation, non-radioactive leucine, isoleucine and valine were added (each to 100 μ g/ml) for a further 10 min before the cells were harvested into cold trichloroacetic acid as described above.

Labelling protocol for determining the polypeptide-elongation rate in slow-growing cells

An exponentially growing culture of about 3×10^8 cells in 40 ml of proline medium was labelled with [¹⁴C]leucine at 0.05 μ Ci/ml (0.03 μ g/ml). Incorporation of this label was complete within 1 h. At 4 h after addition of the [¹⁴C]leucine, [³H]leucine was added to 10 μ Ci/ml (0.6 μ g/ml), together with isoleucine and valine each to 0.6 μ g/ml. At frequent intervals during the next 10 min 2 ml samples were removed into cold trichloroacetic acid.

Electrophoretic separation of polypeptides according to their molecular weights

The methods for preparing total protein from acid-precipitated cells and its analysis by electrophoresis through slabs of 5% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulphate have been described in detail (Waldron *et al.*, 1974).

Results and Discussion

Effect of growth rate on ribosome efficiency

We determined the composition of diploid cells of *Saccharomyces cerevisiae* growing in two different media: slower-growing cells have lower contents of both protein and RNA (Table 1). From these data it appears that ribosome efficiency, expressed as the net rate of amino acid polymerization per ribosome, is also decreased in slow-growing cells (Table 1). These effects of changes in growth rate are the same as those previously observed in haploid yeast cells (Waldron & Lacroute, 1975). We did not extend our analysis of diploid cells to other media, because our object was to compare changes in ribosome efficiency with changes in polypeptide-elongation rate; the latter measurements cannot be made in other media, because uptake of radioactive amino acids is

Table 1. *Macromolecular composition of yeast growing in different media*
'Net protein synthesis' was calculated as:

$$\frac{\text{g of protein/cell} \times \text{Avogadro's number} \times \ln 2}{107 \times \text{generation time (s)}}$$

assuming the average molecular weight of amino acids in yeast protein is 107 (Weil, 1969). 'Ribosomes/cell' was calculated as:

$$\frac{\text{g of RNA/cell} \times \text{rRNA fraction} \times \text{Avogadro's number}}{2.1 \times 10^6}$$

by using the data of Waldron & Lacroute (1975) for the fraction of total RNA that is rRNA, at different growth rates, and assuming that all rRNA is present in ribosomes, each ribosome containing 2.1×10^6 daltons of rRNA (Udem & Warner, 1972).

	Nitrogen source ... (NH ₄) ₂ SO ₄		L-Proline	
	0.54	0.50	0.31	0.30
Growth rate (doubling/h)				
Dry weight/cell (pg)	19.6	20.0	24.8	25.4
Protein/dry weight	0.277	0.278	0.200	0.196
Protein/cell (pg)	5.4	5.6	5.0	5.0
Net protein synthesis (amino acids/s per cell)	3.2×10^6	3.0×10^6	1.7×10^6	1.6×10^6
RNA/dry weight	0.072	0.074	0.051	0.048
RNA/cell (pg)	1.4	1.5	1.3	1.2
Ribosomes/cell	3.6×10^5	3.8×10^5	3.2×10^5	3.1×10^5
Ribosome efficiency (amino acids/s per ribosome)	8.8	8.0	5.2	5.2

too slow (in rich media there is an excess of non-radioactive amino acids added as supplements, and in poor media the rate of protein accumulation is too low).

Attempts to detect protein turnover

A minimum estimate of protein turnover can be obtained from the rate at which radioactive amino acids are released from protein. To detect the release of acid-soluble amino acids from acid-insoluble protein, cells were first grown in the presence of radioactive leucine, then transferred to medium containing a high concentration of unlabelled leucine. This unlabelled amino acid serves to dilute any radioactive leucine released by protein degradation and thereby decrease its re-incorporation. The amount of acid-insoluble radioactivity did not decrease during subsequent growth of cells in media with either (NH₄)₂SO₄ or proline as nitrogen source (Fig. 1). These results are consistent with only a small proportion of yeast protein being unstable. However, it must be emphasized that such a proposal is valid only if re-incorporation of degradation products is markedly decreased by high concentrations of non-radioactive amino acid in the medium. If the degradation products are re-used, in preference to exogenous amino acids, then there would be no net release of protein amino acids to indicate turnover.

As an alternative procedure, we looked for changes in the molecular-weight distribution of polypeptides during growth. In mammalian tissues large poly-

Fig. 1. *Stability of radioactively labelled yeast protein in different growth media*

Protein was labelled by growing cells overnight in the presence of [¹⁴C]leucine. At zero time cells were transferred to medium lacking radioactive leucine and sampled during subsequent exponential growth. The acid-precipitable radioactivity remaining in protein after transfer was determined as described in the Materials and Methods section. Cultures were grown with either (NH₄)₂SO₄ (●) or L-proline (○) as nitrogen source.

peptides are more susceptible to degradation, both *in vivo* and *in vitro*, than small ones (see review by Goldberg & Dice, 1974). Amino acid re-incorporation does not preclude detecting turnover by this tech-

Fig. 2. Molecular-weight distributions of stable and newly synthesized polypeptides in cells growing in different media

Cells were grown in the presence of [^3H]leucine, then transferred to medium without radioactive leucine to chase the label into stable polypeptides. Newly synthesized polypeptides were labelled by a subsequent short incubation in the presence of [^{14}C]leucine as described in the Materials and Methods section. Preparation of protein and electrophoretic separation of polypeptides according to molecular weight were as described by Waldron *et al.* (1974). The molecular-weight distributions of stable and newly synthesized polypeptides were compared by plotting the $^3\text{H}/^{14}\text{C}$ ratios for gel slices as a function of distance from the point of sample application. ----, Average $^3\text{H}/^{14}\text{C}$ ratios for each track of slab gel. The nitrogen source in each culture was: (a) $(\text{NH}_4)_2\text{SO}_4$; (b) proline.

nique, because the re-incorporated material would accumulate in more stable species. Cells were grown in the presence of [^3H]leucine, then transferred to non-radioactive medium to chase the radioactive amino acid into stable polypeptide chains. Newly synthesized protein was then labelled by incubating the cells for 5 min in the presence of [^{14}C]leucine. To ensure completion of ^{14}C -labelled polypeptide chains, the cells were incubated with an excess of non-radioactive leucine for a further 10 min before harvesting. Total protein was prepared from the cells, then separated according to molecular weight by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. A high $^3\text{H}/^{14}\text{C}$ ratio would be expected in gel fractions rich in stable polypeptides. We found that the $^3\text{H}/^{14}\text{C}$ ratio was slightly lower than average in some larger polypeptide classes of fast-growing cells, but did not vary significantly with molecular weight in protein from slow-growing cells (Fig. 2). Each gel slice contains a number of different polypeptides, so rapid turnover of minor species may not have been detected.

Fig. 3. Kinetics of incorporation of radioactive leucine into total protein of a slow-growing yeast culture

Cells were pre-labelled by growth in the presence of a small amount of [^{14}C]leucine, then at zero time [^3H]leucine was added. The amount of [^3H]leucine incorporated into protein in subsequent culture samples was determined by measuring the radioactivity in acid-precipitable material. The amount in each sample was normalized to the yield of pre-labelled ^{14}C -labelled protein for the sample and the $^3\text{H}/^{14}\text{C}$ values were plotted as a function of labelling time. The inset shows the early time points plotted on expanded scales.

However, our deduction of changing ribosome efficiency would only be invalidated by extensive turnover occurring, and then only in slow-growing cells. Our observations would be consistent with such turnover only if (a) most polypeptides of slow-growing cells were degraded at the same rate, irrespective of size, and (b) the degradation rates for most polypeptides were affected by changes in growth rate to the same extent, again irrespective of polypeptide size. Since protein degradation in mammalian cells is so markedly size-dependent, we consider that the almost constant molecular-weight distribution of yeast polypeptides is probably due to high stability of most proteins in both fast- and slow-growing cells. Our estimates of ribosome efficiency therefore reflect genuine changes in the average activity of yeast ribosomes.

Polypeptide-elongation rate in slow-growing cells

The times required to synthesize polypeptides of different molecular weights were determined from the kinetics of incorporation of a radioactive amino acid. The theoretical basis of the calculations was

Fig. 4. Kinetics of labelling of polypeptides of different molecular-weight classes in slow-growing yeast

Cells that had been pre-labelled by growth in the presence of a small amount of [^{14}C]leucine were pulsed from zero time with [^3H]leucine. Samples were taken, from which protein was prepared and separated into molecular-weight classes by electrophoresis as described by Waldron *et al.* (1974). For selected bands (a, mol.wt. 40 000; b, mol.wt. 68 000; c, mol.wt. 100 000; d, mol.wt. 153 000) the kinetics of $F(m, t)$ were followed by plotting the $^3\text{H}/^{14}\text{C}$ ratio in the band divided by the $^3\text{H}/^{14}\text{C}$ in total protein as a function of time. The times at which the kinetics change are equal to the times (T_m) required to complete a polypeptide chain of the given molecular weight.

described by Bremer & Yuan (1968) and extended by Young & Bremer (1976). Essentially we followed the variation with time of the function $F(m, t)$, which is the fraction of the total radioactivity after time t that has been incorporated into growing and completed polypeptide chains of molecular weight m . For any given molecular weight, $F(m, t)$ should increase linearly with time until a time (T_m) when the labeling period equals the time required to complete chains of that molecular weight. At T_m there is a break in the kinetics, after which $F(m, t)$ gradually approaches a constant value.

Slow-growing yeast cells were pre-labelled with [^{14}C]leucine. This label was completely incorporated into acid-insoluble protein within 1 h. At 4 h after the [^{14}C]leucine had been added, [^3H]leucine was added to the exponentially growing culture, from which samples were then removed at frequent intervals. The rate of incorporation of the [^3H]leucine into total protein was constant except for a short initial period of about 20 s (Fig. 3). During this lag the rate of incorporation was continuously increasing, presumably reflecting equilibrium of the labelled amino acid with the internal precursor pool. Protein from each sample was separated according to

molecular weight by gel electrophoresis. The molecular weights of polypeptides in selected bands were determined from the mobilities of standards of known molecular weights, which were co-electrophoresed in adjacent slots (Waldron *et al.*, 1974). To avoid problems of variation in recovery, all short-term ^3H labelling was normalized to the yield of ^{14}C pre-labelled protein. Hence the fraction $F(m, t)$ was calculated for each band as the $^3\text{H}/^{14}\text{C}$ ratio in the band divided by the $^3\text{H}/^{14}\text{C}$ ratio in total protein after the same labelling period. Fig. 4 shows the variation of $F(m, t)$ with time for four bands among which the higher-molecular-weight proteins give less precise estimates of the times at which the labelling kinetics change (Young & Bremer, 1976). The times at which the kinetics change, equal to the chain-completion times (T_m), are shown as a function of molecular weight in Fig. 5. In the molecular-weight range 40 000–200 000, the points lie on a straight line whose slope corresponds to an elongation rate of 10.5 amino acid/s. This straight line does not extrapolate back to the origin (Fig. 5). This phenomenon could be explained by polypeptides smaller than 40 000 daltons being elongated at a lower rate, or by the break points in the labelling kinetics of all

Fig. 5. Polypeptide-chain completion times as a function of molecular weight in slow-growing yeast

The times required to complete polypeptide chains of selected molecular weights were determined from the kinetics of $F(m, t)$, as illustrated in Fig. 4. These completion times (T_m) are here plotted as a function of polypeptide size, which is expressed both as molecular weight and as the number of amino acid residues, assuming that the average molecular weight of amino acids in yeast protein is 107 (Weil, 1969).

polypeptides being delayed by the 20s period during which exogenous leucine equilibrated with the endogenous pool (Fig. 2). Whatever the explanation for slower completion times, the important observation for the present study is that these times, which were here measured in cells growing at 0.26 doubling/h, are identical with those previously determined for cells growing at 0.56 doubling/h (Waldron *et al.*, 1974). If either elongation or termination were the growth-rate-dependent rate-limiting step of protein synthesis, the slow-growing cells would take longer to make polypeptides of any given molecular weight than fast-growing cells. Since this is not observed we conclude that protein synthesis is controlled largely by the rate at which new polypeptide chains are initiated.

Calculation of the fraction of inactive ribosomes in yeast

The rate at which ribosomes elongate polypeptide chains is unaffected by growth rate, so we deduce that ribosome efficiency is determined solely by the fraction of the ribosome population that is actively engaged in protein synthesis [see model (b) in the introduction]. Since each active ribosome is polymerizing 10.5 amino acids/s, then in fast-growing cells 84% of the ribosomes must be active to

Fig. 6. Effect of nutritional shift-up on protein accumulation in yeast

A culture growing exponentially with L-proline as sole nitrogen source was divided into two at zero time. One half of the culture was immediately enriched by the addition of $(\text{NH}_4)_2\text{SO}_4$ (○). The control half of the culture was not supplemented (●). The protein contents of samples taken before and after the shift-up were determined chemically as described in the Materials and Methods section.

produce an overall efficiency of 8.8 amino acids/s per ribosome (Table 1). In slow-growing cells, however, ribosome efficiency is decreased to 5.2 amino acids/s per ribosome (Table 1), which would require only 50% of the ribosomes to be active at 10.5 amino acids/s.

Ribosomes are an expensive item in cellular economy, so they would be expected to function generally at maximum efficiency. This is indeed the case in bacteria: at most growth rates a high proportion of ribosomes are active (Forchhammer & Lindahl, 1971), albeit at a lower polymerization rate in slow-growing cells (Young & Bremer, 1976). Nevertheless at very low growth rates bacterial cells do contain inactive excess ribosomes, which Koch (1971) has suggested are advantageous to organisms living in a 'feast and famine' regime. Activation of these excess ribosomes enables cells to begin growth soon after an improvement in their environment. Yeasts are also exposed to fluctuating nutrient supply in their natural environments (Do Carmo-Sousa, 1969), so production of inactive ribosomes seems to be advantageous to slow-growing yeast as well as to bacteria. The effect of a store of inactive

ribosomes can be seen by comparing the time taken after culture enrichment to establish a new rate of protein synthesis with the time that would be required if only the active ribosomes were present in slow-growing yeast. From the data presented above we expect that a shift in growth rate from 0.30 to 0.54 doubling/h would produce a maximum increase in the fraction of active ribosomes from 0.50 to 0.84, i.e. an increase of 68% in the number of active ribosomes. We can estimate how long it would take to synthesize this number of ribosomes *de novo* from the rate of rRNA production, given that there is only a short lag between the time when a radioactive precursor appears in mature rRNA and its appearance in functional (polyribosomal) ribosomes (Udem & Warner, 1972). When a yeast culture is enriched rRNA synthesis reaches the maximum post-shift rate after about 15 min and is constant thereafter (Waldron, 1977). At a growth rate of 0.54 doubling/h it would take about 85 min to increase the amount of any cellular component such as rRNA by 68%, so after a shift-up to this growth rate a total time of about 100 min would be required to make the necessary rRNA and hence increase ribosome number by 68%. This is much longer than the observed period required to reach a new steady-state rate of protein synthesis when a culture of yeast cells growing with L-proline as sole nitrogen source is enriched by the addition of $(\text{NH}_4)_2\text{SO}_4$ (Fig. 6). Although we cannot determine precisely when protein synthesis is stimulated, it is clear that the final post-shift rate is established in much less than 100 min (probably within 15 min). Thus the existence of a pool of inactive ribosomes in a slow-growing yeast cells enables the

organism to take advantage rapidly of an improvement in its nutritional status.

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