OCCURRENCE, ISOLATION, AND CHARACTERIZATION OF POLYRIBOSOMES IN YEAST

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ABSTRACT

This report details the procedural requirements for preparing cell-free extracts of yeast rich in polyribosomes. This enabled us to demonstrate the occurrence of polyribosomes in yeast, to show their role in protein synthesis, and to devise methods for their resolution and isolation. When certain precautions are met (the use of log phase cells, rapidly halting cell growth, gentle methods of disruption, sedimentation through *exponential* density gradients, etc.), individual polyribosome size classes ranging up to the heptosome can be fractionated and separated from their nearest neighbors. Larger size classes are resolved partially among themselves, free of smaller polyribosomes. This was confirmed by extensive electron micrographic studies of material from the various fractions obtained upon density gradient centrifugation of yeast extracts. Modifications of the gradients and procedure should allow fractionation and isolation of the larger polyribosomes, including those containing polycistronic messages. Yeast polyribosomes are disaggregated to single ribosomes by longer term grinding, cell disruption by the French pressure cell, the Hughes press, or by incubation with dilute RNAse. Yeast polyribosomes are active in the incorporation of amino acids into polypeptide; the single ribosomes exhibit only slight activity. The latter activity is probably due to the presence of a small fraction of monosomes still containing mRNA. Poly-U stimulates amino acid incorporation only in the single ribosomes.

INTRODUCTION

It is well established that protein synthesis in microbial and mammalian cells occurs on polyribosomes (1–4). By electron microscope studies, polyribosome formations in rabbit reticulocytes (5, 6), HeLa cells (7), and *E. coli* (8) have been described.

Previously, we (9, 10) have presented biochemical evidence for the existence of polyribosomes in yeast, i.e. ribonucleoprotein particles sedimenting faster than the single ribosomes in a density gradient occur naturally, and only these particles incorporate amino acids in the absence of exogenous messenger. The methods employed at that time allowed but a small yield of polyribosomes partially resolved from the single ribosomes, but not among themselves.

It now seems clear from experience with various organisms and tissues that the procedures for polyribosome isolation must be carefully predetermined because the quantities of polyribosomes and the polyribosome pattern are contingent upon the conditions of preparation. This is particularly

true of yeast; preparative methods that yield ribosomes highly active in amino acid incorporation may yield negligible quantities of polyribosomes.

This paper demonstrates the occurrence of polyribosomes in yeast and describes the conditions required for the resolution and isolation of various size classes of polyribosomes. Careful manipulation of cell extracts to minimize ribonuclease action and the use of density gradient techniques allow separation and isolation of polysomes up to the heptamer class. Larger size classes are resolved partially among themselves, but are free of smaller cluster polysomes. A polyribosome fraction up to 70% of the total ribosomal population is obtained. The method described here will permit isolation of a class of any desired size. These techniques permit isolation of individual polysome classes, study of the relation between polysome size and protein product, and examination of polyribosomes large enough to contain polycistronic messages.

MATERIALS AND METHODS

The diploid yeast, *Saccharomyces dobzhanskii* X *Saccharomyces fragilis*, used in these studies was grown in a medium which contained, in grams per liter, 2.0 KH_2PO_4; 8.0 $(NH_4)_2SO_4$; 0.5 yeast extract; 0.0163 $MgCl_2$; 20 glucose and trace elements (11). The yeast was grown at 30° in vigorously shaken Erlenmeyer flasks. Growth was stopped rapidly during the late log phase by the method of pouring the culture onto ice slivers. Cells were harvested and washed twice with ice cold YTBM buffer (0.05 M Tris-HCl, pH 7.6; 0.01 M magnesium acetate; 0.01 M KCl; 0.01 M mercaptoethanol and 0.005 M spermidine), by centrifugation and disrupted by hand grinding with twice their weight of acid-washed glass beads (120 μ in diameter) or sand for 3–4 min in a mortar packed in a salt-ice bath at −5°. The mixture was extracted with a minimal quantity of cold YTBM buffer and centrifuged at 10,000 *g* for 10 min to remove debris.

The size classes of polyribosomes were separated by sedimenting 0.3–0.5 ml of the cell extract through exponential sucrose density gradients (12), containing 7–37% sucrose in YTBM, at 24,000 rpm for 3.5 hr in a Spinco L-SW 25 rotor. Stock solutions of concentrated sucrose in buffer were pretreated with colloidal macaloid (purchased from the Inerto Co., Las Vegas, Nev.) so that we could remove traces of RNAse. After centrifugation, the plastic tube was punctured at the bottom, the contents drained through a flow cell (5 mm path length; 0.2 ml volume), and the absorbancy at 260 mμ monitored in a Beckman DB spectrophotometer connected to a Sargent SRL recorder for display. Fractions were collected in tubes packed in ice.

For electron microscopy, a drop of the particular fraction was placed on a grid coated with a formvar-carbon film. The material was fixed by inverting the grid on a 10% solution of formalin adjusted to pH 7.0 with NaOH. We then floated the grid on distilled water to remove salts and sucrose, dehydrated it in absolute ethanol, and air dried it from isoamyl acetate. After shadowing with carbon-platinum, we examined the grids in a Siemens Elmiskop IIb electron microscope with a 30-μ objective aperture. Photographs were taken at a magnification of 7500 and 15,000 on Eastman Projection plates and developed in D-19.

RESULTS

The presence of polyribosomes in yeast was indicated previously by sucrose density centrifugation of extracts from cells pulse labeled with either $P^{32}O_4$ or $S^{35}O_4$ (9). In both cases, the radioactivity sedimented in a region containing ribosomes heavier than the single ribosomes. Chase experiments with nonradioactive phosphate or sulfate showed the metabolic instability of the labeled material, since in both cases radioactivity left the heavy regions. Furthermore, fractions from the region containing these heavy ribosomes incorporated amino acids into protein in the cell-free system while the single ribosomes did not (9).

Effects of Conditions of Cell Culture and Subsequent Handling on Polyribosome Content

When certain precautions were taken, it was possible to demonstrate and isolate a large number of size classes of polysomes. It was necessary to halt rapidly the growth of cells in the log phase and perform all manipulations at 0°C. Cultures allowed to cool slowly during harvest did not contain detectable quantities of polyribosomes. Similarly, yeast cultures grown into the stationary phase lacked polyribosomes. Extracts from stationary phase cells are not active in amino acid incorporation (13).

The polyribosome classes were poorly resolved when linear sucrose density gradients were used, appearing as a broad region. Polyribosomes were separable when the cell extracts were sedimented through an exponential sucrose density gradient. Fig. 1 is an absorbancy tracing of a cell extract centrifuged through an exponential gradient. Six or seven peaks in addition to the single 80S ribo-

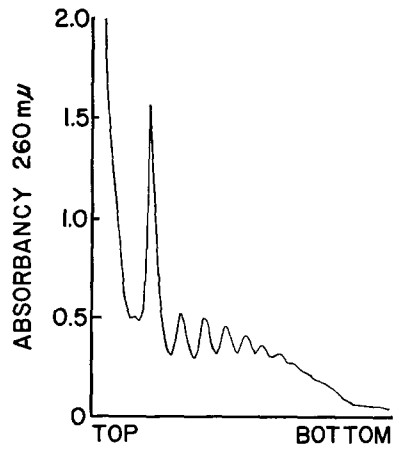


FIGURE 1 Absorbancy pattern of a yeast extract centrifuged in an SW-25 rotor for 3½ hr at 25,000 *g* through a 10–37% exponential sucrose density gradient.

somes are usually resolved. A region containing larger polyribosomes sediments ahead of the fastest resolved peak. In this extract, the polyribosome fraction contained some 67% of the total ribosome population.

It was postulated that the individual peaks contained the dimer, trimer, tetramer, etc. This was confirmed by electron micrographs of samples taken from the various peaks. Fig. 2 is an electron micrograph taken from a fraction in the single ribosome region. Fig. 3 is taken from the first peak heavier than the 80S ribosomes; the field contains doublets. Fig. 4 is taken from the third peak and contains the trimers. Figs. 5 and 6 are drawn from the next two peaks and contain the tetramers and pentamers, respectively. In this experiment, resolution decreased in the heavy side of the heptamers. The lightest portion of the unresolved area contains mostly octomers and nonomers (Fig. 7). The material from the midportion of the unresolved toe contains an heterogeneous grouping of polyribosomes ranging from the unidecimer to the tridecimer (Figs. 8 and 9). The heaviest portion of the unresolved toe contains polyribosomes up to the icosomer.

Sedimentation values of the polyribosomes calculated from a number of zonal density gradient experiments and from examination of extracts with the analytical ultracentrifuge ranged from 80S for the single ribosome to a minimum of 300S for polyribosomes larger than eleven ribosomes (Table I).

Effects of Methods of Cell Disruption on Polyribosome Content in the Resultant Extract

We have previously reported that the activity of the cell-free system in incorporating amino acids into protein was dependent upon the mode of cell disruption employed (13). For example, use of the French pressure cell, commonly employed to disrupt yeast, produces inactive extracts. Fig. 10 shows the profile of ribosomal components present in a cell extract prepared with the French pressure cell and sedimented through an exponential sucrose density gradient. Polyribosomes are absent in such preparations, whereas a control aliquot of cells disrupted by short-term grinding contains the usual classes of polyribosomes.

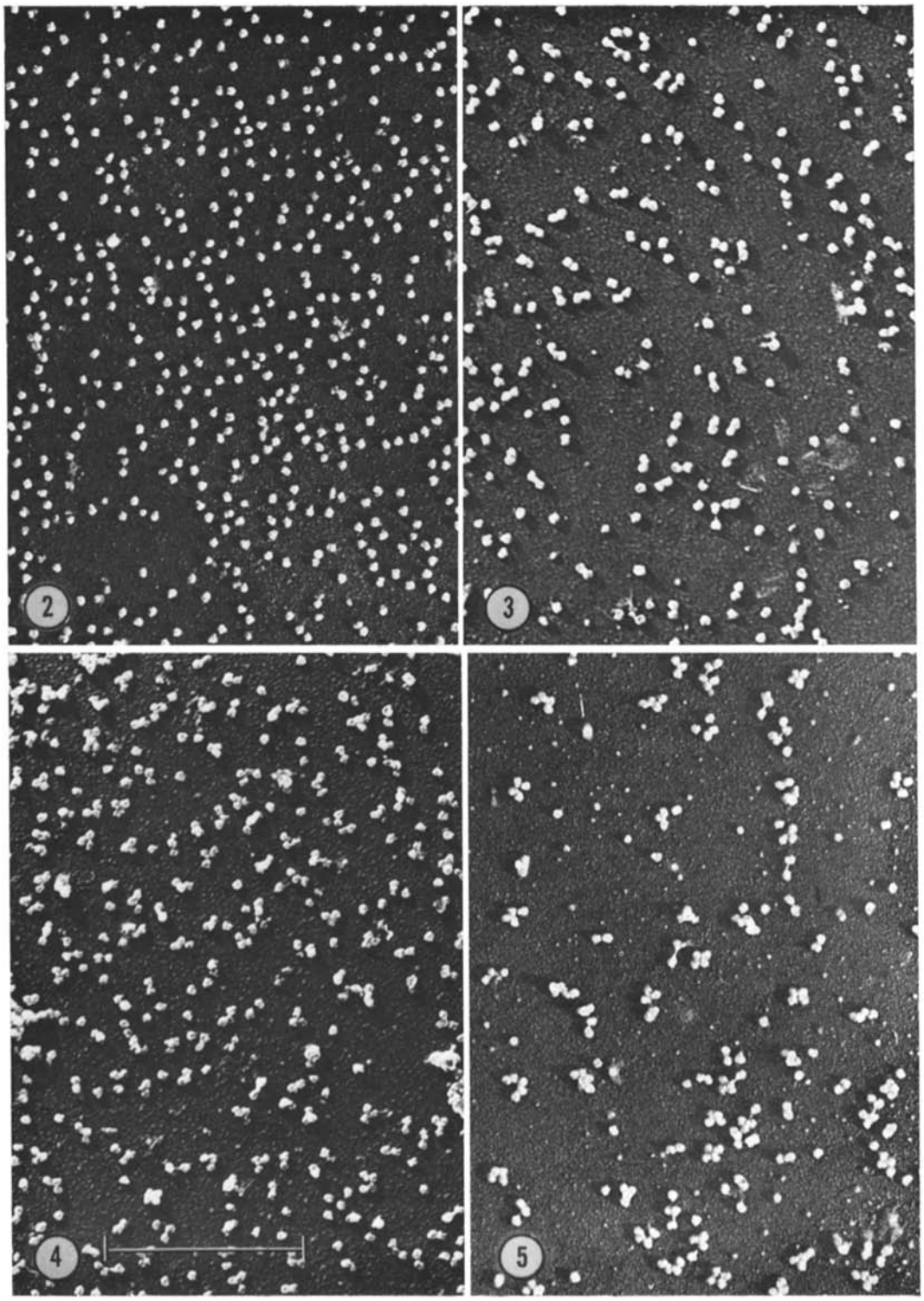
Fig. 11 shows the effects of the time of grinding on polyribosomal structure. Grinding the cells for 10 min disaggregates all of the polyribosomes larger than the dimer and markedly reduces the quantity of the dimer. Furthermore, the 60S subunit of single ribosomes is produced and appears as a shoulder on the trailing edge of the 80S ribosomes. The peak sedimenting more slowly than the 60S contains the smaller subunit of the monosome, the 40S. Some polyribosomes are disaggregated during the short-term grinding; therefore, the 70% estimate of polyribosomes normally occurring in the cell is probably low.

In addition, polyribosomes have not been found in extracts prepared from yeast disrupted by sonic oscillation or in the Hughes press (CoLab Inc., Chicago). However, yeast cells disrupted in the colloid mill[1] do contain variable quantities of polysomes.

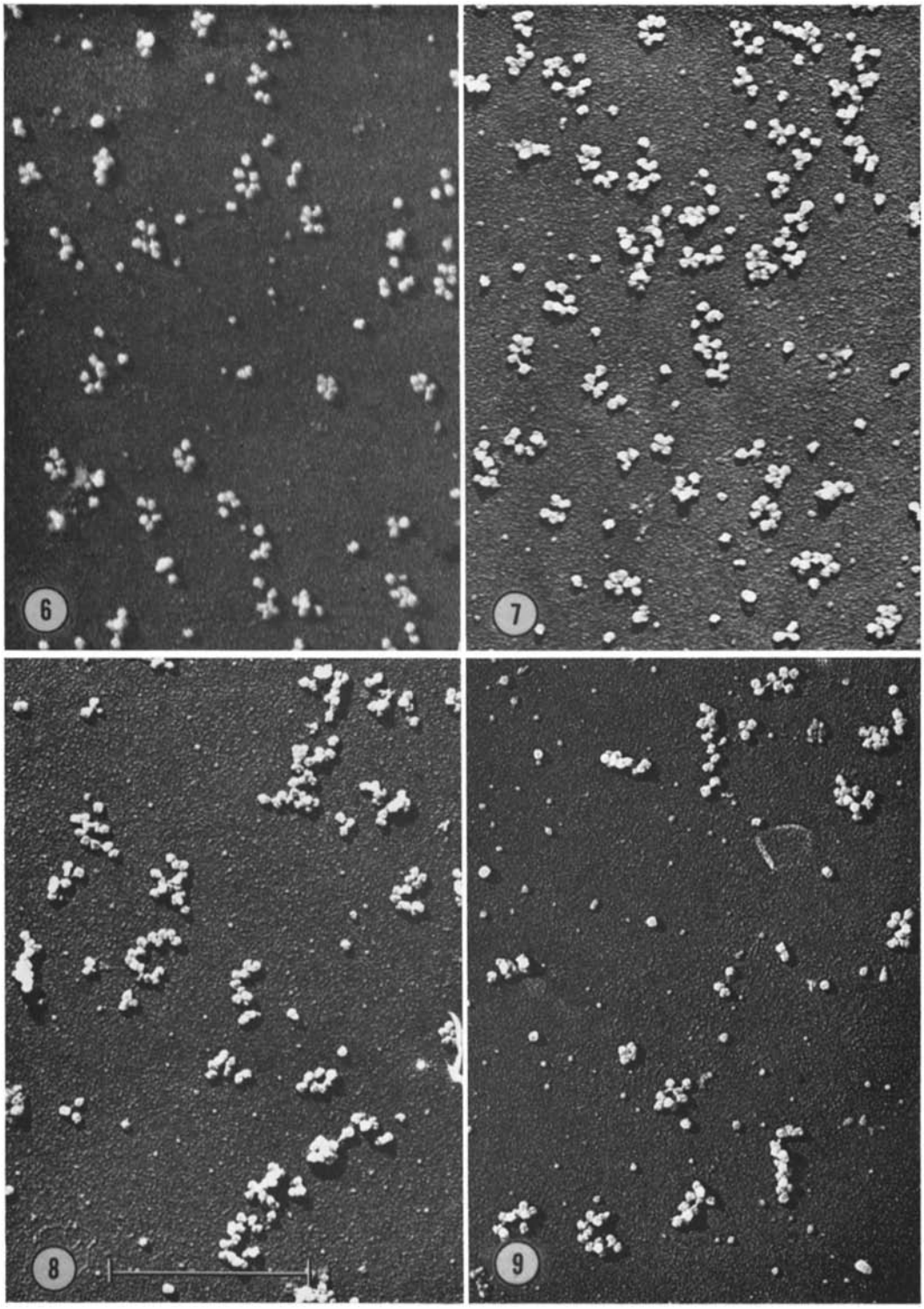
Messenger RNA in Yeast

Pulse-labeled RNA in yeast has a base composition similar to that of DNA and unlike that of the bulk RNA (14, 15). Recently, we have observed that this rapidly labeled RNA (mRNA) is associated with polyribosome structures (16). Chromatography of phenol or LiCl extracts of the ribosomes or of polyribosomes from $P^{32}O_4$ pulse labeling experiments shows the label to reside in material heavier than the 16S or 23S RNA from the ribosomes (17).

[1] The Eppenbach mini-mill Model No. 9000-1A is available from the Gifford-Wood Company, Hudson, New York.



FIGURES 2–5 Electron micrographs of ribosomes sedimenting in peak 1, peak 2, peak 3, and peak 4. The bar at the bottom of Fig. 4 represents 1 μ. × 60,000.



FIGURES 6–9 Electron micrographs of polyribosomes sedimenting in peak 5, in the light section of the heavy toe, in mid toe, in a heavier portion of toe. The bar at the bottom of Fig. 8 represents 1 μ.

TABLE I

Sedimentation Values of Yeast Ribosomes, Polyribosomes, and Ribosomal Subunits

Typical sedimentation values for polyribosomes, ribosomes, and ribosomal subunits computed from data obtained from yeast extracts sedimented in sucrose density gradients and in the Beckman Model E analytical ultracentrifuge.

Unit	S Value
Subunit a	40S
Subunit b	60
1	80
2	110
3	140
4	164
5	186
6	204
7	226
8	244
9	280
10	297
11 and larger	300+

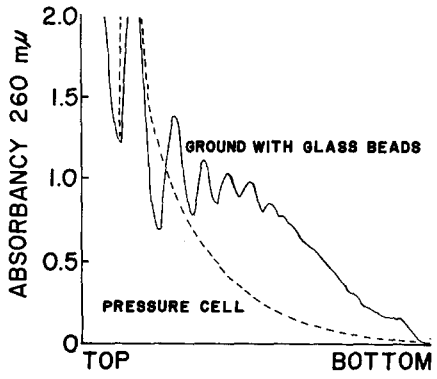


FIGURE 10 The effect of mode of disruption of yeast on the polyribosome content of the resultant extract. One aliquot of cells was passed through a French pressure cell; another (control) was ground manually with glass beads for 4 min. Both were centrifuged through the exponential sucrose density gradient under our standard conditions.

Additional evidence for the presence of mRNA in polyribosomes was gained by examination of extracts from pulse-labeled cells in a sucrose density gradient. In such experiments, the $P^{32}O_4$ incorporated follows the absorbancy trace of the polyribosomes. The quantity of $P^{32}O_4$ per polyribosome size class is a function of the number of ribosomes in a given polyribosome, suggesting a constant density of mRNA per ribosome (16).

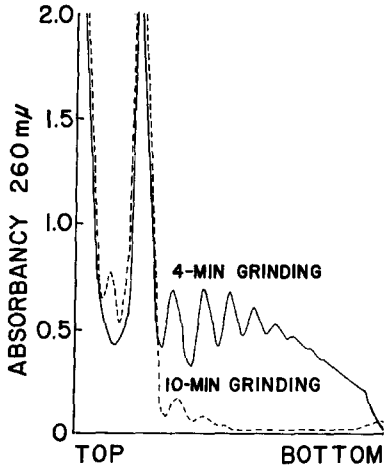


FIGURE 11 The effect of length of time of grinding on the polyribosome content of the resultant extract. One aliquot of cells was ground for 10 min; another (control) was ground with glass beads for 4 min. Both were centrifuged through the exponential sucrose density gradient under our standard conditions.

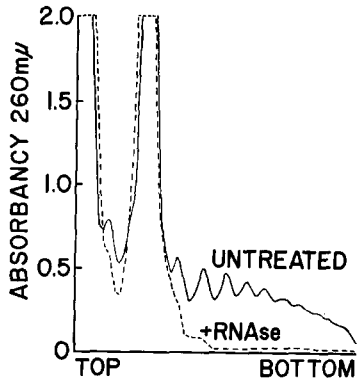


FIGURE 12 The effect of RNAse on polyribosomes. A cell extract was incubated with RNAse (10 μg/ml) for 4 min at 30°C before centrifugation through the sucrose density gradient under our standard conditions.

A further indication that mRNA binds ribosomes to form polyribosomes may be inferred from the sensitivity of the aggregate structure to RNAse. A cell-free extract of yeast was treated briefly with RNAse (10 μg/ml) and centrifuged through the sucrose density gradient (Fig. 12). The lower absorbancy trace results from the RNAse treatment. The polyribosomes are disaggregated for the most part; the bulk of the ribosomes appear as singles,

and only small quantities of dimer and trimer remain. The untreated control shows the usual number of polyribosomes.

DISCUSSION

Previous difficulties in demonstrating polyribosomes in yeast were largely methodological. The present paper demonstrates that the following minimal conditions are required for isolation of polyribosomes and resolution of polyribosomal size classes in yeast, and probably in other organisms.

Actively Growing Log Phase Cells

Polyribosomes are characteristic of actively growing yeast cells. Prime requisites for recovery of polyribosomes in yeast include the use of log phase cells. Polyribosomes rapidly disaggregate (most preexistent mRNA is translated and degraded; few new messages are transcribed) during the stationary phase. Furthermore, polyribosomes are rapidly disaggregated in cells growing at a declining rate, i.e. allowed to cool slowly during harvest. Therefore, growth of log phase cells should be terminated rapidly. Pouring the culture onto ice slivers reduces the temperature to 0°–2°C within 30 sec. All subsequent manipulations were performed at ice bath temperatures.

Gentle Cell Disruption Methods

The polyribosomal entity is extremely fragile. Most methods routinely employed to rupture yeast cells destroy polyribosomal integrity. Yeast cell disruption in the Hughes press, in the French pressure cell, or by sonic oscillation yields preparations lacking in polyribosomes. However, variable quantities of polyribosomes remain after grinding yeast in the mini-mill.[1] Thus far, only short-term grinding with glass beads or sand at low temperature has given consistently good yields of polyribosomes. Direct osmotic shock should be the gentlest method of cell disruption for polysome isolation. We are examining rapid methods for mass spheroplast formation in actively growing cultures. The yeast polyribosomes are disaggregated to single ribosomes by brief treatment with dilute RNAse; the entire structure including single ribosomes falls apart upon treatment with EDTA or citrate.

Exponential Density Gradient Centrifugation

While linear density gradients separate polyribosomes in bulk from the single ribosomes, the polyribosomes at best may fall under a broad peak. *Exponential* density gradients resolved the polyribosome size classes up to the heptamer into bands seen as individual peaks of absorbancy by the recording spectrophotometer. Larger polyribosomes were less readily isolated, but could be separated by collecting smaller fractions. Modifications in the density gradients should allow resolution of any given size class.

The presence of dimers and trimers in a cell extract may result from chain initiation or from disruption of larger polyribosomes during preparation. The finding of a constant amino acid incorporation per ribosome seems to support the latter (16). In addition, chain initiation would give prolonged incorporation. Alternatively, these size classes might reflect the final reading of the message. This may be tested by examining these classes for nascent protein; that is, for the presence of newly formed enzymes that would be found on larger polyribosomes if the message were still complete and being translated by a full complement of ribosomes.

Larger polyribosomes contain messages sufficiently large to code for proteins or protein subunits. Polyribosomes containing more than ten ribosomes would code for high molecular weight proteins. A more interesting possibility is that the mRNA binding these larger aggregates may contain the information for a number of proteins (polycistronic). Although operons have not yet been demonstrated unequivocably in yeast, genes for a given pathway usually are scattered; it is likely that they do exist in yeast.

The excellent resolution of polyribosome size classes that is afforded by exponential sucrose density gradient centrifugation should allow detection of the presence of polycistronic messages on the larger polyribosomes.

A preliminary report of this research was presented at the 1963 annual meeting of the Federation of American Societies for Experimental Biology.

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