The Preparation and Characterization of a Cell-free System from *Saccharomyces cerevisiae* That Translates Natural Messenger Ribonucleic Acid*

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A cell-free protein-synthesizing system has been prepared from *Saccharomyces cerevisiae* by differential centrifugation of lysed spheroplasts. The preparation, a modified 100,000 × g supernatant fraction, contains ribosomes and monosomes, ribosomal subunits, translation factors, and aminocyl-tRNA synthetases, but no polysomes. After removal of small amounts of remaining mRNA with micrococcal nuclease, protein synthesis is stringently dependent on the addition of mRNA, as well as amino acids and an energy-generating system. The 5'-cap analogue, 7-methylguanosine 5'-phosphate, inhibits translation of several natural mRNAs, but has no effect on chain elongation. Incubation of the polysome-free extract with natural mRNA leads to the formation of protein-synthesizing polysomes and eventually, to the release of protein; the molecular weight of the protein synthesized in the presence of BMV (brome mosaic virus) RNA is consistent with that of BMV coat protein.

Cell-free systems from reticulocytes, ascites cells, cultured mammalian cells, embryonic chick muscle, and wheat germ, capable of translating natural mRNAs, have been available for several years (1-7). Although yeast cell-free systems that carry out chain elongation or translation of poly(U) have been described (8-10), little or no information is available regarding the translation of natural mRNA or initiation of protein synthesis with preparations from such cells. This report describes the preparation and characterization of a cell-free system from yeast which translates exogenous natural mRNAs efficiently and faithfully.

**EXPERIMENTAL PROCEDURES**

**Preparation of Polysome-free Yeast Extract—Saccharomyces cerevisiae** (SKQ2N strain) were grown aerobically at 20°C, in YM-1 media (11), for 15 to 20 h, to a density of 1.0 to 1.2 units at 660 nm. The cells were collected by centrifugation (3000 × g, 5 min), washed once with cold sterile H2O, and resuspended in 1.0 M sorbitol, approximately 50 ml of sorbitol per liter of original cell culture. The cell suspensions received 0.4 ml of glusulase (Endo Laboratories, Inc., Garden City, N. Y.) per liter of original cell culture and then were incubated at 20°C for 1 h (12). The resulting spheroplasts were collected by centrifugation, washed once with 1.2 M sorbitol, and suspended in 120 ml of YM-5 media (11) per liter of original cell culture, plus MgSO4, to a final concentration of 0.4 mM, and incubated for 0.5 h at 20°C. The incubated spheroplasts were collected by centrifugation, resuspended in about 4 ml (per liter of original cell culture) of 20 mM Hepes/KOH buffer (pH 7.4) containing 0.1 M NH4Cl, 2 mM magnesium acetate, and 2 mM dithiothreitol, and then homogenized with 15 strokes in a Dounce homogenizer.

The yeast cell lysate was centrifuged for 15 min at 27,000 × g, and the supernatant (S-30), except for the lipid layer at the top of the solution and the flocculent material at the bottom, was centrifuged for 30 min at 100,000 × g, after reaching speed. The polysome-free supernatant obtained, representing a modified S-100 preparation, was passed through a column of Sephadex G-25 medium previously equilibrated with the Hepes/NH4H2O/Mg(OAc)2/dithiothreitol solution described above, containing 20% glycerol. A Sephadex column (25 × 80 cm) was used to chromatograph the modified S-100 extract obtained from 4 liters or original cell culture. The fractions with the highest absorbance at 260 nm (30 to 50 A260 units per ml) were pooled, and aliquots of about 0.04 ml were frozen by dropping into liquid nitrogen (S-100'). The frozen S-100' preparations were stored at -70°C, with no detectable loss of activity for at least 9 months.

**Assay for Messenger RNA-dependent Protein Synthesis**—A two-step incubation was used; the first served to digest endogenous mRNA with Ca2+ dependent micrococcal nuclease (13); the second measured amino acid incorporation into protein in the presence of exogenous mRNA. Twenty to 40 μl (0.3 to 0.4 μg of protein) of yeast S-100' extract were incubated in a volume of about 0.683 ml with the following components, for 5 min at 20°C: 24 mM Hepes/NH4H2O buffer (pH 7.4), 108 mM NH4Cl (plus about 50 to 60 mM of monovalent cation contributed along with other components), 3.6 mM Mg(OAc)2, 3.6 mM dithiothreitol, 0.60 mM ATP, 0.12 mM GTP, 24 to 36 mM creatine phosphate, 20 μg of creatine phosphokinase, 48 μM each of 19 nonisotopic amino acids (excluding leucine), 0.8 μg of micrococcal nuclease, 0.30 mM CaCl2, and 12% glycerol. Occasionally, with less active nuclease preparations, 1.25 μg were added.

For the second incubation, the following components were added: EGTA (to a final concentration of 0.75 mM), [3H]leucine (1.46 μM final concentration, 22,000 cpm/μmol), and varying concentrations of polynucleotide templates, up to 40 μg per incubation. The final concentrations of the other components for the second incubation, in a total volume of 0.1 ml were: 20 mM Hepes buffer, 140 to 150 mM monovalent cation, 3.0 mM Mg(OAc)2, 3.0 mM dithiothreitol, 0.50 mM ATP, 0.1 mM GTP, 20 to 30 mM creatine phosphate, 40 μM amino acids (except leucine), 0.25 mM CaCl2, and 10% glycerol; the rest of the components are as stated. The second incubation was usually carried out for 60 min at 20°C. At the end of the incubation period, the hot (90°C) 5% trichloroacetic acid-insoluble fractions were prepared; the precipitates were collected on glass-fiber filters, washed, dried, and counted in a scintillation counter.

**Sucrose Gradient Centrifugation Analysis**—Some incursions and preparations were analyzed by gradient centrifugation (14). Samples...
were layered on 12 ml of linear 15 to 50% sucrose gradients containing 10 mM Tris-HCl buffer (pH 7.5), 70 mM NH₄Cl, and 4 mM Mg(OAc)₂. Centrifugation in an SW 41 (Spinco) rotor was carried out at 2°C, for 1½ h at 40,000 rpm. The gradients were then analyzed automatically at 254 nm with a scanning recording spectrophotometer; for gradients containing radioactivity, 0.4-ml fractions were collected and analyzed for hot trichloroacetic acid-insoluble radioactivity.

Preparation of Yeast Poly(A)⁺ RNA—Polysomes sedimented from the yeast cell lysate at 100,000 × g in 30 min, described above, were used as a source of mRNA. The polysomal RNA was extracted with SDS/phenol/chloroform (15, 16) and chromatographed twice on oligo(dT)-cellulose (17) to obtain the poly(A)-containing RNA, resolved from the bulk of ribosomal RNA. The RNA was precipitated with 2 volumes of ethanol (at pH 5.0, −5°C) overnight, after the addition of Escherichia coli tRNA as carrier. The RNA was then collected by sedimentation, dissolved in sterile H₂O, and stored frozen at −70°C in aliquots.

RESULTS

The postmitochondrial (S-30) fraction, obtained from spheroplasts incubated in a complete media prior to lysis, showed a small amount of 40 S and 60 S ribosomal subunits, a high proportion of 80 S ribosomes or monosomes, or both, and polysome aggregates of up to 10 or more ribosomes per polynucleotide (Fig. 1A). When similar preparations were centrifuged for 30 min at 100,000 × g, then analyzed by gradient centrifugation (Fig. 1B), only subunits and 80 S particles were evident; the polysomes appeared to have been completely removed.

As described above (see "Experimental Procedures"), translation of exogenous mRNA was carried out in a two-step incubation. If the first step designed to degrade endogenous mRNA was omitted by leaving out nuclease and CaCl₂, the S-100' preparation was capable of incorporating between 2 and 6 pmol of leucine into protein in the absence of added mRNA (Table I); such incubations measured the amount of endogenous mRNA in the preparation. The addition of mRNA to S-100' preparations containing endogenous mRNA stimulated protein synthesis 5- to 10-fold. In all subsequent experiments, incubations included the two-step procedure using micrococal nuclease.

When the extract was depleted of mRNA with nuclease, protein synthesis was stringently dependent on added mRNA (Table II). With several preparations thus tested, amino acid incorporation was 150 to 550 times greater in the presence of mRNA (line 1) than in its absence (line 2). Although protein synthesis was only about 60% lower when ATP and GTP were omitted (line 3), suggesting that the nucleotide pool was not completely removed by passage through Sephadex, the absolute requirement for energy was demonstrated by the findings that without creatine phosphate (line 5) or the kinase (line 6), incorporation was decreased to 1% and 6%, respectively. The addition of an amino acid mixture to the complete system stimulated protein synthesis about 5-fold, as compared to incubations without it (line 7). The addition of 7-methyl guanylic acid (line 8) inhibited completely the translation of mRNA; this inhibition could be due to the effect of the modified nucleotide on the recognition of the 5'-cap in mRNA (18, 19) which may be essential for translation of some poly-

![Fig. 1. Sucrose gradient centrifugation analyses of preparations obtained from lysates of yeast cells. A, postmitochondrial (S-30) extract, 0.42 mg of protein; B, postpolysomal (S-100') extract, 0.80 mg of protein. Centrifugation and spectrophotometric procedures as described in the text.](http://www.jbc.org/)

![Table I](http://www.jbc.org/)

<table>
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<th>Incubation additions*</th>
<th>pmol of [³H]leucine incorporated into protein</th>
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<tbody>
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<td>5.6</td>
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<tr>
<td>mRNA</td>
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* Incubations contained yeast S-100' extract (prior to treatment with micrococcal nuclease and CaCl₂), magnesium acetate, NH₄Cl, ATP, GTP, creatine phosphate, creatine phosphokinase, and a 19 amino acid mixture plus [³H]leucine, as described in the text. When mRNA was present, 40 μg of unfraccionated yeast polysomal RNA were added. Incubations were carried out at 20°C for 60 min and analyzed for hot acid-insoluble radioactivity.

![Table II](http://www.jbc.org/)

<table>
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<tr>
<th>Incubation components</th>
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<td>Complete system – mRNA</td>
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<td>Complete system – ATP and GTP</td>
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<td>Complete system – creatine phosphate</td>
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<td>Complete system – creatine phosphokinase</td>
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<tr>
<td>Complete system – amino acids</td>
<td>6.6</td>
</tr>
<tr>
<td>Complete system + 7-methyl GMP</td>
<td>&lt;0.2</td>
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</tbody>
</table>

* The complete system contained micrococcal nuclease treated yeast S-100' extract and all the other components described in the text and footnote to Table I, including [³H]leucine and 40 μg of yeast polysomal RNA. When present, 7-methyl guanylate was added to a final concentration of 1.5 mM.

![Fig. 2. The effect of varying concentrations of 7-methyl guanylate on polypeptide synthesis in the presence of poly(U) or yeast poly(A)* RNA. Incubations were carried out as described in the text. One set of incubations contained 25 μg of poly(U) as template and 0.9 μM [³H]phenylalanine, 20,000 cpm/μmol (C), the other set contained 1 μg of yeast poly(A)* RNA and 1.46 μM [³H]leucine (○). Some incubations were carried out without m⁵-GMP, and some contained increasing amounts of the nucleotide, as noted. The results are expressed as percentage of activity obtained in the presence of m⁵-GMP, as compared to controls in the absence of the nucleotide. The control (100%) value for poly(U) translation was 130,000 cpm of phenylalanine incorporated, and the control value for natural mRNA translation was 180,000 cpm of leucine incorporated.](http://www.jbc.org/)
nucleotide templates. Evidence that m7-GMP inhibited only initiation of protein synthesis and that it had no effect on chain elongation is presented in Fig. 2. The mRNA-depleted yeast S-100' extract was incubated with varying concentrations of m7-GMP and poly(U) plus [3H]phenylalanine (open circles) or yeast poly(A)+ RNA and [3H]leucine, as described above. The synthesis of polyphenylalanine with poly(U), which did not require the initiation sequence of reactions but was dependent only on ribosomes and elongation factors, was not affected by concentrations of m7-GMP up to about 0.6 mM; concentrations of the nucleotide above 1 mM were required to cause a detectable (20 to 30%) inhibition of polyphenylalanine synthesis. However, 50% inhibition of natural mRNA translation was obtained with concentrations of 7-methyl guanylate as low as 0.05 mM.

Some of the characteristics of the S-100' yeast extract depleted of mRNA with nuclease are described in Fig. 3. The monovalent cation concentration optimum was approximately 150 mM (A), of which about one-third was contributed with other components of the incubation. The magnesium ion optimum was between 3 and 4 mM with yeast poly(A)+ RNA, and rather sharp (B); the shape of the activity versus Mg2+ concentration curve and the optimum itself varied slightly with the template used. Incorporation was dependent on the concentration of the S-100' extract protein (C) and of leucine (D) used in the incubation; maximum activity was obtained with about 0.3 mg of protein and 3 μM leucine, respectively; most incubations described here contained approximately 0.3 mg of protein and 1.46 μM [3H]leucine.

Additional characteristics of the exogenous messenger RNA-translating system are presented in Fig. 4. Kinetic analysis (A) revealed that the rate of incorporation of [3H]leucine into protein was linear for almost 1 h in the presence of yeast polysomal RNA (closed circles); the total extent of incorporation was about 20 pmol of radioactive leucine when mRNA was included, but less than 0.1 pmol in its absence (open circles), even after 1½ h. This figure (B) also shows that the yeast S-100' nuclease-treated system was capable of translating nucleic acid templates from several sources including reticulocytes, Brome mosaic virus, and yeast; the poly(A)-containing RNA from yeast (closed circles) was markedly more active at low concentrations in promoting translation than unfractionated RNA isolated from polysomes (open circles), and the nonpolyadenylated RNA (broken line) was essentially inactive.

The ribonucleoprotein particles in the S-100' preparation, obtained by centrifugation of the yeast lysate at 100,000 × g for 30 min, consisted primarily of ribosomal subunits and 80S particles, as shown in Fig. 1B; polysomes were not evident in this extract. The remaining small amount of endogenous

![Fig. 3. Characterization of the yeast cell-free mRNA-translating system. Incubations in the presence of [3H]leucine, with (●) and without (○) yeast poly(A)+ RNA, were carried out as described above with varying concentrations of monovalent cations (A), magnesium ions (B), S-100' extract protein (C), and leucine (D).](http://www.jbc.org/)

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In Vitro Translation of mRNA in Yeast

Fig. 4. The effect of time of incubation and concentration of various templates on mRNA translation. A, yeast S-100' extract was incubated with [3H]leucine in the presence (C) and absence (O) of yeast poly(A)* RNA for varying periods of time, as indicated. B, incubations for 1 h were carried out with [3H]leucine and varying concentrations of yeast poly(A)* RNA (C), unfractionated yeast polysomal RNA (O), poly(A) RNA (---), BMV RNA (A), or reticulocyte poly(A)* RNA (O).

Messenger RNA was removed with micrococcal nuclease, although the gradient centrifugation pattern (not shown here) did not differ from that shown in Fig. 1B. When the nuclease-treated S-100' extract was incubated with yeast poly(A)* RNA, as shown in Fig. 5, sucrose gradient analysis revealed that polysomes which carried out protein synthesis were reformed. After 2 (A), 10 (B), and 40 (C) min of incubation, radioactive protein was associated with 80 S monosomes and with ultraviolet-absorbing peaks representing polysomes containing up to 5 or more ribosomes. The size of the polysomes and the amount of radioactivity associated with them was dependent on the time of incubation. After 10 or 40 min of incubation, a considerable amount of radioactive protein was also obtained at the top of the gradients (fractions numbered 1 to 3), in the cytosol fraction, which could represent polypeptide chains released from polysomes after completion. However, radioactive protein was not detected in the region of the ribosomal subunits.

The product formed by the yeast S-100' cell-free system in incubations containing BMV RNA was characterized by SDS gel electrophoresis of the reaction mixture. Analysis for radioactive leucine (Fig. 6) of fractions obtained from the gel indicated that over 85% of the product migrated to a region of about 19,000 molecular weight. Besides the major product, only a small amount of lower molecular weight material could represent ribosome-bound nascent polypeptides, prior to completion and release. The distance that the major product migrated was in agreement with that of the BMV coat protein, as determined by SDS gel electrophoresis of the protein extracted from Brome mosaic virus particles.

All of the incubations for protein synthesis described above were carried out at 20°C; amino acid incorporation was reduced markedly when the incubation temperature was raised to 30°C or 37°C. Experiments to be described elsewhere, in which nuclease-treated extract was preincubated with and without mRNA, indicated that the loss of templating activity of mRNA and RNA hydrolysis were more rapid at the elevated temperatures than the loss of activity of the translational apparatus.

DISCUSSION

The postmitochondrial fraction of yeast lysates, which contains polysomes, ribosomes, ribosomal subunits, aminoacyl-tRNA synthetases, and translational factors, is capable of carrying out chain elongation, as evidenced by the ability to incorporate amino acids into proteins in the absence of added polynucleotide template. However, although S-30 preparations from reticulocytes, ascites cells, wheat germ, E. coli, etc., are active with added mRNA, numerous attempts to stimulate the yeast S-30 preparation with exogenous mRNAs were unsuccessful. In contrast, the postpolysomal (S-100') fraction derived from the S-30 of yeast is markedly stimulated by the addition of natural mRNA; stimulation is obtained with or without the removal of small amounts of remaining endogenous mRNA with nuclease. This finding suggests that the S-100' fraction contains a population of native subunits that...
readily initiate protein synthesis on addition of mRNA. The low molecular weight pool in the extract is markedly diminished by molecular sieve chromatography on Sephadex G-25; therefore, for maximal protein synthesis, the incubations must be supplemented not only with mRNA but also with nucleotides, an energy-generating system, and a pool of amino acids.

A number of natural polynucleotides such as the poly(A)-containing RNA fractions from yeast and reticulocytes and the RNA of Brome mosaic virus enhance amino acid incorporation. The fraction of yeast RNA that does not contain poly(A) is not well translated. The structural analogue of the 5'-terminal structure of some eukaryotic mRNAs, 7-methylguanosine 5'-monophosphate, which inhibits translation of capped mRNA species (21–24), completely inhibits translation of the active natural templates but has no effect on chain elongation. Rough estimates based on the amount of leucine incorporated into protein associated with ribosomal particles were synthesized per active ribosome. The rate of protein synthesis, at 20°C, is linear for about 1 h. Both the yeast S-100' extract and the polynucleotide template are relatively stable at 20°C.

The following observations have been made with respect to protein synthesis in the nuclease-treated yeast S-100' extract: (1) the level of endogenous protein synthesis is negligible; (2) the requirement for exogenous mRNA is rigorous; (3) m'GMP, a "cap" analogue, markedly inhibits translation; (4) proteins-synthesizing polysomes, containing at least 5 ribosomes per polynucleotide, are formed from a pool of ribosomal subunits and ribosomes; (5) significant amounts of labeled protein are released from polysomes and are recovered in the soluble fraction; and (6) the protein product formed with a polynucleotide template, BMV RNA, has a molecular weight consistent with that of BMV protein. These observations indicate that all of the individual phases in translation, such as initiation, elongation, and termination, are catalyzed by the yeast S-100' extract. Further, crucial intermediary steps such as the recognition of the 5' terminus and the initiation and termination coding sequences are accurately and faithfully recognized. This represents the first system from a fungi that is capable of initiating protein synthesis and translating natural mRNAs. These methods may be applicable to other biological systems that have proven to be difficult sources for in vitro protein synthesis.

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