Yeast Protein Synthesis

PREPARATION AND ANALYSIS OF A HIGHLY ACTIVE CELL-FREE SYSTEM

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A detailed description is given of the techniques for preparing, handling and assaying a cell-free protein-synthesizing system from yeast, analogous to crude (S-30) *Escherichia coli* extracts. Its basic characteristics are described. The rate of poly(U)-directed poly-phenylalanine synthesis was at least fivefold higher than in previously reported yeast cell-free systems, approaching that of crude mammalian cell-free systems. Fractionation of the S-30 extracts lowered activity. Organelles and their fragments present in the S-30 extract neither contributed to nor inhibited cytoplasmic protein synthesis. There was a component localized in the high-speed supernatant that caused an inhibition of poly-phenylalanine synthesis. Poly(U) programmed the synthesis of long-chain polyphenylalanine, in contrast with the only other yeast system in which this has been examined (Bretthauer & Golichowski, 1968). Preincubation techniques inactivated the system and probably a small proportion only of the ribosomes was active.

The development and use of suitable cell-free systems (Nirenberg & Matthai, 1961) has been critically important in the analysis of mechanisms and controls involved in protein synthesis. Until recently detailed knowledge of these processes was mostly confined to prokaryotes, in particular *Escherichia coli*. This was mainly due to a lack of suitable cell-free systems from eukaryotes. Yeast cell-free systems active in protein synthesis are of exceptional interest: yeast is a unicellular eukaryote, has a detailed genetic and biochemical background, and a wide range of genetic techniques is available.

Previous work on yeast cell-free protein synthesis has concentrated on the functions of tRNA and the elongation factors (Perani *et al.*, 1971; Heridia & Halvorson, 1966; Richter *et al.*, 1971; Scragg, 1971), partly because synthetic polynucleotides are the only mRNA species that programme the synthesis of known protein products. To study natural mRNA function *in vitro* one has to maintain the integrity of additional complex and labile components involved in initiation and termination, and ensure fidelity of translation.

This paper describes the preparation, handling and assay techniques of a yeast cell-free protein-synthesizing system based on an S-30 extract analogous to very active *E. coli* S-30 extracts (Capechi, 1966; Bergquist *et al.*, 1968). Its activity in poly(U)-directed protein synthesis was 5–10-fold higher than that of any previously reported yeast cell-free system. The basic characteristics and limitations of the system are discussed, some possible artifacts examined

and the product of poly(U)-dependent polyphenylalanine synthesis is characterized as long-chain polyphenylalanine for the first time in yeast.

Experimental

Materials

¹⁴C-labelled amino acids were obtained from Schwartz/Mann Ltd., Orangeburg, N.Y., U.S.A., and New England Nuclear Corp., Boston, Mass., U.S.A. Ribonuclease (EC 3.1.4.22), deoxyribonuclease (EC 3.1.4.5) and pyruvate kinase (EC 2.7.1.40) were from Worthington Biochemical Co., Freehold, N.J., U.S.A. Alumina (bacteriological grade) was from Alcan Ltd., Bauxite, Arkansas, U.S.A. Poly(U) was from Miles Chemical Co., Elkhart, Ind., U.S.A.

Yeast

The yeast was a polyploid *Saccharomyces cerevisiae* strain isolated from yeast supplied by New Zealand Breweries Ltd., Auckland, New Zealand (Bergquist *et al.*, 1968). Flasks containing 2 litres of growth medium (Linnaine, 1965) were inoculated and grown at 29°C on a rotary shaker (125 rev./min) until an E_{650} of 1.2 (10^7 cells/ml) was reached. The culture was cooled on crushed ice and harvested by centrifugation in a continuous flow centrifuge (Lourdes, LCA-2). The packed cells were twice resuspended in 15 ml of ice-cold 0.01 M-Tris-HCl (pH 7.6)–0.01 M-MgCl₂/litre of culture, and re-centrifuged at 16000g_{av.} for 10 min. Finally the yeast was resuspended in 5 vol.

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of isolation buffer containing 50mM-Tris-HCl (pH 7.6), 8mM-magnesium acetate, 60mM-NH₄Cl, 0.5mM-spermidine, 0.5mM-dithiothreitol and 10% (v/v) glycerol and re-centrifuged. Small amounts of the pellet were frozen in liquid N₂ and stored at -80°C. The yield was 2g wet wt. of yeast/litre of culture.

The exact growth conditions were important. It was necessary to harvest during rapid growth to obtain maximally active cell-free systems (Lucas *et al.*, 1964). Investigation of growth rates showed that this yeast grew fastest at 28–30°C (doubling time 100min).

Preparation of the S-30 extract

Frozen yeast (10–20g) was ground with twice its weight of Bacto Alumina in a mortar precooled to -15°C. The alumina was added in three equal portions. Each time the mixture was ground till a paste was formed. The mortar was warmed to 0°C during the first grinding. If the alumina was added such that the yeast never became a viscous liquid during grinding the breakage decreased from about 80 to 30%.

The paste was washed into 12ml glass centrifuge tubes with an amount of isolation buffer equal to the frozen wet weight of the yeast. The extracts were centrifuged at 29000g_{av.} for 15min and the supernatant was re-centrifuged at 15000g_{av.} for 30min.

Two-thirds of the supernatant was taken, avoiding membranous fractions and floating lipid. This extract, designated the S-30 extract (Nirenberg & Matthei, 1961), was dialysed for 4h against isolation buffer and then frozen in small batches in liquid N₂. The S-30 extracts contained 300–400 E₂₆₀ units, about 30mg of biuret protein/ml (Layne, 1957) and 3–4nmol of 80S ribosomes/ml.

Analysis of subcellular fractions

The fraction to be analysed (0.1ml) was layered on 5ml linear gradients of sucrose (20–40%, w/v) in 50mM-Tris-HCl (pH 7.6)–8mM-magnesium acetate–0.5mM-spermidine–0.5mM-dithiothreitol and centrifuged for 60000g_{av.}-min. The gradients were analysed by flotation on 50% sucrose through Isco recording equipment and fractions (150μl) were collected.

Ribosome concentration was estimated by planimetry of the 80S extinction peak, identified and expressed in absolute units by calibration with washed yeast ribosomes and with solutions of known absorbance. From an extinction coefficient of 1mg of ribosomes = 13.0 E₂₆₀ units and a molecular weight of 3.4 × 10⁶ (Bruening, 1965), 1cm² = 0.365 E₂₆₀ units = 28.1 μg of ribosomes = 8.25 pmol of ribosomes.

Assay of protein synthesis

Protein synthesis was analysed by techniques based on those developed for *E. coli* (Capechi, 1966; Bergquist *et al.*, 1968) with reaction mixtures optimal for yeast protein synthesis (Sissons, 1971). Several special precautions were needed.

The standard reaction mixtures contained 25μl of diluted or preincubated S-30 extract, 30μl of 'incubation mixture', mRNA and other additives in water to make a final volume of 100μl. The 'incubation mixture' contained all components necessary to give a reaction mixture containing 50mM-Tris-HCl (pH 7.6), 8mM-magnesium acetate, 60mM-NH₄Cl, 5mM-phosphoenolpyruvate, 1.33mM-ATP, 25μg (5 EC units) of pyruvate kinase/ml, 0.167mM-GTP, 8mM-GSH, 1.0mM-spermidine, 0.04mM-amino acids except for the labelled one(s), 0.01mM-¹⁴C-labelled amino acid(s) (usually phenylalanine, sp. radioactivity 50mCi/nmol). Poly(U), if present, was 0.4mg/ml. The final concentration of dithiothreitol was 0.125mM and of glycerol, 2.5% (v/v).

The optimum S-30 extract concentration was determined for each extract. It was always 25μl at a 1:3 or 1:4 dilution (25–50pmol of ribosomes). In some experiments the S-30 extracts, instead of being diluted with isolation buffer, were preincubated after dilution with a 'preincubation mixture' which gave all the ionic components necessary for protein synthesis, but contained no radioactive amino acid.

The mixtures were incubated for 30min at 20°C (see the Results section) and stopped by addition of 0.2ml of 0.1M-KOH. They were then incubated at 30°C for 10min to hydrolyse aminoacyl-tRNA. Then 3ml of 7% (w/v) trichloroacetic acid was added, immediately mixed and the precipitated product filtered off on Whatman GF/C filters and washed with four 3ml portions of 7% trichloroacetic acid. The filters were dried at 80°C for 45min and counted for radioactivity in a scintillation counter in 5ml of toluene scintillator containing 0.5% 2,5-diphenyloxazole and 0.1% 1,4-bis-(5-phenyloxazol-2-yl)benzene. For measurement of radioactivity in polypeptide plus aminoacyl-tRNA, the KOH hydrolysis was omitted. Incorporation is given in pmol of amino acid incorporated into polypeptide, assuming for the purpose of calculation that the 4h dialysis removed endogenous amino acid. The calculated rates of protein synthesis are therefore a minimum, as endogenous amino acid would have increased the actual rate over the calculated rate.

To minimize degradation of components, the following technique for starting the reactions was rigorously followed. The reaction tubes (12ml conical centrifuge tubes) were placed in ice and the appropriate components added in the order: water, buffer, inhibitors or other compounds, 'incubation mixture', tRNA, and, immediately before starting a

set of 20 reactions, the mRNA. At 20s intervals the reactions were started by adding the S-30 extract (or ribosomes) prewarmed to the incubation temperature. Gentle mixing of the S-30 extracts every minute (care being taken to avoid foaming) was important for reliable results.

These crude concentrated yeast extracts sometimes completely clogged cellulose nitrate filters. Glass-fibre filters overcame this but introduced a variable background binding, up to 500c.p.m. with [^{14}C]phenylalanine. Inclusion of [^{12}C]phenylalanine in the trichloroacetic acid washes had no effect, suggesting that radioactive impurities (several could be detected by chromatography of the [^{14}C]phenylalanine; see Fig. 5) were responsible. Prewashing the filter with 7% trichloroacetic acid before filtering each reaction mixture decreased the background binding to the filter to a reproducible value (80c.p.m.). Radioactivity retained by the filter from complete reaction mixtures kept for 20s on ice was taken as a zero-reaction blank.

Preparation and partial hydrolysis of the product of poly(U)-directed polypeptide synthesis

The S-30 extract was preincubated at 0°C for 20min and then incubated with [^{14}C]phenylalanine (70c.p.m./pmol) and 0.4mg of poly(U)/ml in a total volume of 2.7ml. Parallel reactions in the presence and absence of poly(U) were followed to monitor the kinetics of reaction. After 45min, 10ml of 10% trichloroacetic acid was added and the reaction mixture was cooled on ice for 10min. The precipitate was collected by centrifugation and washed with a further 10ml of 10% trichloroacetic acid. The precipitated polyphenylalanine was extracted twice with 2M-acetic acid. The combined acetic acid extracts were freeze-dried and dissolved in a small volume of 1M-acetic acid.

Partial hydrolysis of the residue was carried out as described by Bretthauer & Golichowski (1968). The residue was dissolved in dichloroacetic acid-9M-HCl (2:1, v/v) and the mixture incubated at 60°C for 70h. The dichloroacetic acid was extracted with peroxide-free ether (twice) and the volatile residues were removed under vacuum. The residue was dissolved in 1ml of 1M-acetic acid.

Results

Kinetics and temperature dependence

Fig. 1 shows the kinetics of poly(U)-stimulated polypeptide synthesis, phenylalanyl-tRNA formation and the temperature dependence. The temperature optimum was 20°C. Polypeptide synthesis after a short lag was linear at maximal rate for 20min, then slowly decreased. At 25°C the initial rate was

Fig. 1. Kinetics of poly(U)-directed polyphenylalanine synthesis and phenylalanyl-tRNA formation

A yeast S-30 extract was preincubated with the full complement of components necessary for protein synthesis for 11min at 0°C. Three reaction mixtures were then incubated separately at 20°, 15° and 25°C with [^{14}C]phenylalanine and poly(U) added at time zero. Portions (50μl, containing 15pmol of ribosomes) were removed at the times shown and precipitated with 7% trichloroacetic acid with and without prior alkaline hydrolysis (as described under 'Assay of protein synthesis') to measure polyphenylalanine and polyphenylalanine plus phenylalanyl-tRNA. The difference represents phenylalanine present as phenylalanyl-tRNA. At 25°C, polyphenylalanine (○); at 20°C, polyphenylalanine (■), polyphenylalanine plus phenylalanyl-tRNA (□); at 15°C, polyphenylalanine (▲), polyphenylalanine plus phenylalanyl-tRNA (△).

faster but suddenly decreased after 10min. At 15°C the rate was much slower than at 20°C. Phenylalanyl-tRNA formation was greatest at 15°C, indicating increased stability of either the tRNA^{Phe} or the phenylalanyl-tRNA synthetase at lower temperatures. Temperatures higher than 25°C (not shown) gave no increase in rate of polyphenylalanine synthesis, suggesting that a specific component was inactivated at 25°C or above. Endogenous protein synthesis was linear with time for 45–60min at all temperatures.

Rate of endogenous protein synthesis

The rate of endogenous protein synthesis was high. That in Fig. 2 is 220pmol of phenylalanine/30min per mg of ribosomal protein, a rate as high as that found previously for intact yeast polyribosomes (Lamb *et al.*, 1968). Nevertheless, in my extracts the polyribosomes had degraded to monoribosomes.

Fig. 2. Preincubation of S-30 extracts: effect on endogenous and poly(U)-directed polypeptide synthesis

A S-30 extract was diluted twofold and 250 μ l was mixed with an equal volume of a 'preincubation mixture' containing all the components necessary to give optimal protein synthesis (see the Experimental section). The Mg^{2+} concentration was 5 mM. The mixture was held on ice for 10 min, then preincubated at 25°C. At the times shown, 25 μ l portions containing 20 pmol of ribosomes (37.5 μ g of ribosomal protein) were removed and incubated at 25°C for 30 min with 75 μ l of a mixture containing all the components necessary for optimal protein synthesis. Incubations were performed with (▲) and without (●) poly(U). The phenylalanine incorporation into polypeptide is given on two scales: (i) total incorporation/assay (pmol/30 min) and (ii) this incorporation adjusted to unit concentration of ribosomal protein (1 mg).

Rate of poly(U)-stimulated protein synthesis

Poly(U) stimulated [^{14}C]phenylalanine incorporation into polypeptide 20-fold in most preparations (see e.g., Fig. 2). Thus individual extracts most active in endogenous protein synthesis were also those most active in poly(U)-directed polyphenylalanine synthesis, indicating that the high rate of protein synthesis probably reflected considerable integrity of the protein-synthesizing machinery (Schreier & Staehelin, 1973).

The highest rate of protein synthesis in previously reported yeast cell-free systems is rather low: only 400–500 pmol of phenylalanine polymerized/30 min per mg of ribosomal protein (Bretthauer *et al.*, 1963; Dietz *et al.*, 1965; Perani *et al.*, 1971).

The poly(U)-specific rate of protein synthesis described in the present paper is at least fivefold higher. That shown in Fig. 2 is 4300 pmol of phenylalanine polymerized/30 min per mg of ribosomal protein (7.7 phenylalanine molecules incorporated/30 min per ribosome). This is about the

same rate as found for non-preincubated rat liver ribosomes (Staehelin, 1969). The maximum rate of synthesis in most extracts was similar, e.g. in Fig. 1 the maximum rate at 20°C was 6 phenylalanine molecules polymerized/30 min per ribosome. Therefore these extracts are exceptionally active compared with previous yeast systems.

Ionic conditions

The conditions described in the Experimental section are optimal in Mg^{2+} and NH_4^+ for poly(U)-dependent synthesis with the described concentrations of chelators, i.e. nucleotides, phosphoenolpyruvate, RNA and anions (Manchester, 1970). Changing any one of these components or the polyamines changed the optima for the others (Sissons, 1971). The conditions are also close to optimal for endogenous protein synthesis.

Cation optima were mRNA-specific and should be reinvestigated for new mRNA species. The Mg^{2+} optimum for poly(U)-dependent polyphenylalanine synthesis sometimes varied in the range 6–10 mM between individual extracts, but 8 mM always gave within 5% of the highest activity. The optimum for endogenous protein synthesis varied between 4 and 8 mM- Mg^{2+} . This variation was probably caused by different concentrations of the endogenous polyamines. The polyamine interactions were especially complex, and 1 mM-spermidine, although not quite giving maximal protein synthesis on poly(U), is probably a better routine concentration than the optimal (3 mM) for investigating mRNA species other than poly(U) because of effects on mRNA secondary structure.

Inactivation of the extracts by preincubation procedures

Preincubation of cell-free systems under conditions allowing protein synthesis is a standard procedure to remove endogenous mRNA from ribosomes, freeing them for exogenous mRNA (Nirenberg & Matthaei, 1961). Preincubating this yeast system left endogenous protein synthesis comparatively unaffected, but inactivated poly(U)-dependent polyphenylalanine synthesis instead of increasing it (Fig. 2). This rapid decrease in activity ruled out preincubation of yeast extracts under these conditions as a procedure preceding experiments with exogenous mRNA. Sometimes preincubation at 0°C in low (5 mM) Mg^{2+} with all the components required for protein synthesis activated the extracts for poly(U)-dependent polyphenylalanine synthesis.

Elongation rate on poly(U)

The elongation rate is the rate of peptide-bond synthesis/active ribosome. The use of unpreincubated reaction mixtures raises a problem concerning

Table 1. *Effect of ribosome purification on activity in polypeptide synthesis*

Duplicate reactions, with and without poly(U), were incubated at 20°C for 30 min in the presence and absence of the S-100 fraction concentration (5 μ l/assay), which was optimal for stimulating unwashed ribosomes (Fig. 4a). Appropriate zero-reaction blanks were subtracted (see the legend to Fig. 4). The E_{260}/E_{280} ratio of the different fractions is shown.

	E_{260}/E_{280}	Incorporation per ribosome (% of activity in S-30 extract)			
		With poly(U)		Without poly(U)	
		Without S-100	With S-100	Without S-100	With S-100
S-30 extract	1.70	100	—	100	—
Unwashed ribosomes	1.86	48	45	27	47
Washed ribosomes	1.91	0	1	0	14
Membrane fraction	1.53	0	—	0	—

the proportion (and origin) of ribosomes active in poly(U)-dependent protein synthesis. Polyribosomes comprise about 87% of the total ribosome population (Martin & Hartwell, 1970). The rate of protein synthesis on endogenous mRNA was constant for 45 min, suggesting that little termination was occurring unless there was also reinitiation. Therefore the activity on poly(U) probably took place on the 13% non-polyribosomal components. This consideration is important for estimating the elongation rate on poly(U)-containing ribosomes since, if only the 13% non-polyribosomal ribosomes are active, the true rate of peptide bond formation per ribosome is about 7–8-fold higher than the rate per ribosome in the total population. This would make the rate of polypeptide synthesis in Fig. 2, 50–60 amino acids polymerized/30 min per ribosome, a rate equal to that in an exceptionally active liver cell-free system (Falvey & Staehelin, 1970a).

An attempt was made to estimate directly the proportion of ribosomes active with poly(U). By analogy with bacterial systems, active ribosomes should be seen as a contribution to the area under the 80S extinction peak due to poly(U)-ribosomes in gradients where all ribosomes lacking peptidyl-tRNA and poly(U) had dissociated and most of those containing mRNA and peptidyl-tRNA had not (Jost *et al.*, 1968). It was established that these conditions occurred when the gradients contained isolation buffer with 0.1 mM-Mg²⁺ and 1 mM-spermidine. No poly(U)-specific increase was detected, although polyphenylalanine label indicated that the poly(U)-containing ribosomes were in the 80S peak, confirming that only a small proportion was active.

Further fractionation of S-30 extracts

Having obtained a crude S-30 system active *in vitro* it is desirable to refine it. Since ribosome integrity is probably crucial for success, especially gentle methods were used to separate different fractions and great care was taken to avoid tightly pelleting the ribosomes.

Crude fractions were isolated by centrifuging S-30 extracts at 150000g_{av.} for 4 h, giving: (i) a clear, loosely packed ribosome pellet which resuspended easily in isolation buffer (designated 'unwashed ribosomes'); (ii) a clear red layer and a fluffy white membranous layer above the ribosomes, removed together and designated the 'membrane' fraction; (iii) a clear supernatant, the S-100 fraction; (iv) about 1 cm from the meniscus, another cloudy layer designated the 'S-100 membrane' fraction. The ribosome and 'membrane' fractions were further purified by centrifugation into sucrose layers as follows and then designated 'washed' fractions. For this 5 ml of 0.25 M-sucrose in isolation buffer was layered on 5 ml of 1.7 M-sucrose in isolation buffer, and 2.2 ml of crude fraction was layered on top and the tubes were centrifuged at 250000g_{av.} for 3 h. The ribosomes could be seen as a faintly turbid band in the 1.7 M-sucrose layer, well separated from the interface. The membranous fractions banded at the interfaces. After a fourfold dilution with isolation buffer the fractions were resedimented at 250000g_{av.} for 3 h. To avoid tight pelleting the ribosomes were sedimented into a cushion of 0.5 ml of isolation buffer containing 50% (v/v) glycerol.

The effect of purification on ribosome activity, and its dependence on added S-100 fraction, were examined (Table 1). Differential centrifugation decreased activity by half, even though it gave very loosely packed ribosomes. There was limited dependence on added S-100 fraction. Washing through sucrose layers decreased poly(U)-dependent polyphenylalanine synthesis a further threefold and virtually eliminated endogenous protein synthesis. These changes were accompanied by a decrease in the ratio of protein to RNA measured by the E_{260}/E_{280} ratio. The 'membrane' fraction by itself was inactive in protein synthesis, so active ribosomes were not concentrated into it. Since even very careful purification of yeast ribosomes is accompanied by considerable inactivation, the crude S-30 extract should probably be used for initial studies of yeast

Fig. 3. Effect of S-30 extract concentration on poly(U)-directed polyphenylalanine synthesis

Different concentration of two different S-30 extracts, the less active one (owing to overgrinding) 330 E_{260} units/ml (●) and the more active one 365 E_{260} units/ml (▲), were incubated at 20°C for 30 min in standard protein-synthesis reaction mixtures containing 0.4 mg of poly(U)/ml as described in the Experimental section.

protein synthesis. All previous yeast systems programmed by exogenous polynucleotides appear to have been prepared from isolated ribosomes and a high-speed supernatant fraction. It seems reasonable to conclude that one should fractionate the system only when sure that the separation procedure is adequate and necessary.

Handling and stability

The exceptionally high activity of the yeast extracts in protein synthesis was due to minimal handling of the extracts and rigorous adherence to the procedures and precautions described. Since the system is so labile it seems useful to describe some of the conditions under which it can be handled.

The S-30 extract is stable in isolation buffer. At room temperature (25°C) its activity decreased by only 4% in 30 min. Although the 4 h dialysis caused material to precipitate there was no difference in protein synthesis between an undialysed S-30 extract and the same extract dialysed, frozen and thawed. Analysis on sucrose gradients and analytical ultracentrifugation revealed no significant physical difference. The inactivation accompanying protein synthesis (Figs. 1 and 2) also produced no detectable change in subunits or ribosomes. Freeze-thawing five times caused some loss of activity.

Integrity was maintained for more than 8 months in liquid N_2 . Storage at -10°C for 18 h caused

inactivation with extensive ribosome degradation. Glycerol was present to stabilize labile components against freezing.

There was some variation in activity between extracts. This was probably due to differing damage to the ribosomes (Schreier & Staehelin, 1973) during preparation of the extracts. Grinding with alumina was almost certainly the most critical step. Grinding for 5 min gave a low breakage but some intact polyribosomes. Alternative procedures for breaking yeast proved unsatisfactory (Sissons, 1971).

Optimum S-30 extract concentration and an 'inhibitor' in the S-100 fraction

Poly(U)-dependent polyphenylalanine synthesis was proportional to S-30 extract concentration up to about 10 µl/100 µl of assay mixture, then decreased (Fig. 3). The concentration of S-30 extract was kept between 6 and 9 µl/100 µl of assay mixture (see the Experimental section).

The localization of the component causing this inhibition was examined (Fig. 4). To a constant, low, concentration of unwashed ribosomes were added different 'membrane' fractions, with and without additional S-100 fraction (Fig. 4a). Polyphenylalanine synthesis was stimulated at low concentrations of S-100 fraction. Higher S-100 fraction concentrations inhibited. Adding the 'membrane' fraction and the 'S-100 membrane' had exactly the same effect as adding the S-100 fraction. Since both 'membrane' fractions consisted of particular organelles suspended in the S-100 fraction and they inhibited the same as S-100 fraction, the 'membranes' themselves had no effect on protein synthesis. The unwashed ribosomes (up to 4.5 mg/ml) caused no additional inhibition in the presence of a concentration of S-100 fraction which was already inhibiting (Fig. 4b). Therefore the 'inhibitory' component was localized primarily in the S-100 fraction. It may have been liberated into the soluble phase from the organelles. It was probably not ribonuclease (assayed by the technique of Randles, 1968) since the ribosomes contained as much ribonuclease as the S-100 fraction. Inhibitors in S-100 fractions from other organisms have been reported (Englehardt, 1972).

Disruption and delocalization of many subcellular components during preparation of yeast protein-synthesizing systems gives rise to some complications in the use of crude extracts, absent with those prepared from organisms such as *E. coli*. Analysis by sucrose gradients and analytical ultracentrifugation showed that the crude 'membrane' fraction consisted of the S-100 fraction enriched in all the heavy (>20S) components of the S-30 extract except 80S ribosomes and ribosomal subunits. The failure of this fraction to effect or inhibit protein synthesis, even in combination with other subcellular

Fig. 4. Effect of fractions from the S-30 extract on poly(U)-directed polyphenylalanine synthesis

(a) Non-ribosome fractions. The amount of the fractions is given in μl for direct comparison of contents of S-100 components. Each 0.1 ml standard reaction mixture contained $5\mu\text{l}$ of unwashed ribosomes ($1.93 E_{260}$ units, 43.4 pmol), [^{14}C]phenylalanine (69.4 c.p.m./pmol) and poly(U) (0.4 mg/ml). Incubation was for 30 min at 20°C . Zero-time binding of [^{14}C]phenylalanine to the fractions has been subtracted. No added fraction ($\blacksquare$); S-100 only ($\blacktriangle$); $10\mu\text{l}$ of S-100 membrane fraction assayed with and without $10\mu\text{l}$ of S-100 fraction ($\square$); the 'membrane' fraction, $5\mu\text{l}$ ($\circ$) and $10\mu\text{l}$ ($\triangle$) assayed with and without $10\mu\text{l}$ of S-100 fraction. (b) Ribosomes. To $10\mu\text{l}$ of S-100 fraction was added the amount of unwashed ribosomes shown in a final reaction mixture with a volume of 0.1 ml. The zero-time binding blanks have been subtracted for each ribosome concentration. The ribosome concentration was $0.386 E_{260}$ unit ($8.68\text{ pmol}/\mu\text{l}$). Reaction conditions were the same as in (a). $\bullet$, With poly(U); $\blacktriangle$, without poly(U).

It also rules out the possibility that necessary components such as transfer factors, aminoacyl-tRNA synthetase or active ribosomal subunits were concentrated into the 'membrane' fraction. The presence of organelles and fragments seem to have no effect on cytoplasmic protein synthesis, another reason for using crude concentrated yeast S-30 extracts for initial experiments in cell-free protein synthesis.

Observed protein synthesis: function of cytoplasmic and mitochondrial ribosomes

Use of crude cellular extracts raises the possibility of artifacts causing apparent protein synthesis. However, the use of specific inhibitors of ribosomal protein synthesis showed that the observed reactions were protein synthesis on cytoplasmic ribosomes. Ribonuclease at $1\mu\text{g/ml}$ completely inhibited endogenous protein synthesis, indicating an absence of contaminating bacteria. Puromycin (2 mM) inhibited by 99%. Therefore aminoacyl-tRNA transferases (Soffer *et al.*, 1969) were not involved and the product was formed by ribosomal protein synthesis.

Mitochondrial ribosomes form 2% of the total yeast ribosome population (Kaempfer, 1969). The possibility that they formed a much greater proportion of the active ribosomes was examined. Cycloheximide (0.5 mM), which has no effect on protein synthesis by mitochondrial ribosomes (Lamb *et al.*, 1968; Schmitt, 1970), inhibited both endogenous and poly(U)-dependent polyphenylalanine synthesis by 97%. This is unambiguous evidence of protein synthesis on cytoplasmic ribosomes. It was confirmed by using chloramphenicol, specific to bacterial ribosomes (Vazquez & Munro, 1967) and mitochondrial systems (Lamb *et al.*, 1968; Schmitt, 1970). Chloramphenicol (0.5 mM) did not inhibit endogenous protein synthesis, although it did inhibit poly(U)-dependent polypeptide synthesis very slightly (8%). This anomalous slight inhibition was probably caused by the yeast reaction conditions.

Characterization of the product of poly(U)-directed polypeptide synthesis

After the early work on yeast cell-free systems, Bretthauer & Golichowski (1968) reported that when programmed by poly(U) the yeast cell-free system synthesized only diphenylalanine and triphenylalanine. These peptides are insoluble in trichloroacetic acid and therefore usually estimated as polyphenylalanine. Using (A) $_{18}$ Halvorson & Heridia (1967) produced polylysine (chain length greater than 20), although elongation of the mRNA by poly(A) polymerase did not occur. Both results suggest a defect in yeast translocation *in vitro*. In my extracts incorporation of [^{14}C]phenylalanine into polyphenylalanine was often greater than 3 molecules of

fractions, rules out significant inhibition by interfering enzymes such as organelle adenosine triphosphatases (Matile *et al.*, 1967; Lamb *et al.*, 1968; Cartledge & Lloyd, 1972).

Fig. 5. Sephadex G-15 chromatography of phenylalanine and polyphenylalanine hydrolysis products

The product of poly(U)-directed polypeptide synthesis was prepared, acetic acid-soluble phenylalanine peptides were extracted, and the residue was partially hydrolysed and extracted with acetic acid as described in the Experimental section. These acetic acid extracts and phenylalanine standards were chromatographed on a column (60cm $\times$ 1cm) of Sephadex G-15 which had been extensively pre-washed with acetic acid. Eluent was 1M-acetic acid at 12ml/h. The radioactive samples were monitored with an Isco u.v. analyser and fractions (1ml or 2.5ml) were collected directly in scintillation vials dried at 80°C; 100 μ l of 1M-acetic acid and 5ml of Bray's (1961) scintillant were added. To analyse [12 C]phenylalanine peptides the column was connected to a Technicon Autoanalyser, with an amino acid-ninhydrin marker identifying the solvent front. (a) [14 C]Phenylalanine. The [14 C]phenylalanine (325 mCi/nmol, from Schwartz/Mann Ltd.) contained 0.5 μ Ci of [14 C]phenylalanine and Dextran Blue 2000. $\square$, E_{254} (Dextran Blue); $\bullet$, $10^{-2} \times$ radioactivity (c.p.m.)/1 ml fraction; $\blacktriangle$, $0.5 \times 10^{-3} \times$ radioactivity (c.p.m.)/1 ml fraction. (b) The 1M-acetic acid extract of polyphenylalanine. The extract contained 2000 c.p.m. $\bullet$, Radioactivity/2.5 ml fraction. A scale copy of the Autoanalyser trace of phenylalanine di-, tri- and tetraphenylalanine (in equimolar quantities) is also shown (—). (c) Phenylalanine peptides produced by partial hydrolysis of polyphenylalanine. The extract contained 27000 c.p.m. Fractions (1ml) were collected and the radioactivity/1ml fraction was measured ($\bullet$). The profile of the [12 C]phenylalanine peptides (as in b) is also shown (—).

phenylalanine polymerized/ribosome. Nevertheless it was important to establish directly that long-chain polyphenylalanine was being formed, since it was possible that recycling poly(U)-ribosome complexes

could have formed diphenylalanyl-tRNA and triphenylalanyl-tRNA in high yield.

The product of the poly(U)-directed reaction, before and after partial hydrolysis with dichloroacetic acid-9M-HCl (2:1, v/v), was chromatographed on columns of Sephadex G-15 (Bretthauer & Golichowski, 1968) standardized by using phenylalanine peptides (Fig. 5b) and [14 C]phenylalanine (Fig. 5a). The product of [14 C]phenylalanine incorporation into polypeptide was prepared. Parallel reactions in the presence and absence of poly(U) showed endogenous protein synthesis to be 6% of the total. Of the alkali-resistant trichloroacetic acid-precipitable product 5% was soluble in 1M-acetic acid and chromatographed as [14 C]phenylalanine with a trace of aggregated material (Fig. 5b). This suggests that the residue consisted of polyphenylalanine chains longer than Phe₄ (Bretthauer & Golichowski, 1968) plus the residual product of endogenous protein synthesis. After partial hydrolysis of the putative polyphenylalanine, chromatography of the acetic acid-soluble products (Fig. 5c) showed that phenylalanine and short-chain phenylalanine peptides had been produced. The elution volumes relative to the void volumes were within 0.6% of the standards. This confirms the identification of the product as mostly polyphenylalanine. Since 43% of the polyphenylalanine was still longer than Phe₄, the original product was probably considerably longer than Phe₅. This reinforces the indirect evidence, from polymerization results of 7 phenylalanine molecules/ribosome and from experiments with differentially dissociated ribosomes, that only a small proportion of these ribosomes was actually synthesizing polyphenylalanine.

Coding fidelity was high. [14 C]Leucine incorporation was not stimulated by poly(U), so there was no third-position wobble in codon recognition, which is the most sensitive index of miscoding. Therefore poly(U)-directed incorporation of amino acids other than phenylalanine in polypeptide was probably very low.

Discussion

One approach to improving the relatively poor state of yeast systems for protein synthesis *in vitro* is to improve the partial system utilizing poly(U) before studying natural mRNA, initiation and termination factor function. The use of poly(U) as an mRNA probe in developing cell-free systems involves, however, at least one major limitation. Poly(U) initiates protein synthesis abnormally, in both prokaryotes (Schreier & Noll, 1970) and eukaryotes (Falvey & Staehelin, 1970b), by omitting most of the steps. Since the precise functions of eukaryotic initiation factors are not yet clear (Haselkorn &

Rothman-Denes, 1973), it is impossible to say that some initiation-factor function in controlling ribosome state (e.g. Pêtre, 1970) is not involved in poly(U) initiation. Factors analogous to bacterial factors IF-1 and IF-2 are not involved and neither, probably, is the mRNA-binding function of factor IF-3 (Haselkorn & Rothman-Denes, 1973). Therefore the high activity of my yeast cell-free system in synthesizing polyphenylalanine strongly suggests that the elongation factors and phenylalanine-tRNA synthetase (EC 6.1.1.20) were intact and, most important, shows considerable integrity of the ribosomes (Schreier & Staehelin, 1973). It leaves open the question of initiation-factor function.

If yeast initiation factors are exceptionally labile, one cannot rule out the possibility that they did not survive preparation of the S-30 extract. There is virtually no evidence about their stability. Factors distinct from elongation factors have been reported, which promote binding of *N*-acetylphenylalanyl-tRNA to ribosome-poly(U) complexes (Ayuso & Heridia, 1968; Torano *et al.*, 1972) and binding of [¹⁴C]methionyl-tRNA^{Met} (the initiation aminoacyl-tRNA, Lucas-Lenard & Lipmann, 1971; Stewart *et al.*, 1971) to ribosome-AUG complexes (Richter *et al.*, 1971). These factors may be involved in initiation, but were prepared from ribosomes isolated by procedures much more drastic than those used here to prepare the S-30 extract. High salt treatment (Richter *et al.*, 1971) is an extremely disruptive procedure for yeast ribosomes (Kaempfer, 1969; Martin & Hartwell, 1970). Therefore, these reported factors are almost certainly functional in the S-30 extract.

Probably the major limitation of the present yeast cell-free system lies in the failure, so far, to preincubate the extracts successfully and obtain 'run-off' (Falvey & Staehelin, 1970a) ribosomes (Fig. 2). In consequence, as my use of partially dissociated ribosomes suggests, only a small proportion of the total ribosome population functions on poly(U). The rest of the ribosomes contain endogenous mRNA, which is almost certainly partially degraded. The active ribosomes probably consisted of components from the 13% of non-polyribosomal ribosomes (Marcus *et al.*, 1967; Martin & Hartwell, 1970).

Now that techniques for isolating single species of mRNA molecules are available, experiments using natural mRNA species to develop further the yeast system *in vitro* are possible.

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