Translation of Poly(riboadenylic acid)-Enriched Messenger RNAs from the Yeast, *Saccharomyces cerevisiae,* in Heterologous Cell-Free Systems†

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ABSTRACT: Poly(riboadenylic acid) [poly(A)] enriched messenger RNAs from yeast have been used to direct the synthesis of yeast polypeptides in mouse Krebs II ascites and wheat embryo extracts. Both cell-free systems synthesize polypeptides over a molecular weight range of 10,000–100,000. Autoradiograms of sodium dodecyl sulfate–polyacrylamide slab gels used to fractionate [^{35}S]methionine-labeled *in vitro* products reveal that about 25 major bands (each of them possibly representing multiple polypeptides) are produced by each cell-free system. Each of these co-electrophoreses with a major polypeptide labeled *in vivo* or in a yeast lysate. These results suggest that cell-free translational machinery from eukaryotes is not able to discriminate in an all or none fashion against messenger RNAs which are available to it. While yeast poly(A)-enriched messenger RNA directs the synthesis of similar polypeptides over approximately the same molecular weight range in both cell-free systems, the wheat germ system directs the incorporation of 45 times the amount of [^{3}H]serine into Cl_3CCOOH-precipitable polypeptides. This is in contrast to the 2.5-fold more efficient translation of hemoglobin mRNA in the wheat embryo extract. Thus, the extract from mammalian cells is able to translate mRNA from a lower plant with a much lower efficiency than it translates hemoglobin mRNA, and at a lower efficiency than is observed using a cell-free system from wheat embryos. This indicates that the wheat embryo system is the one of choice for translation of yeast messenger RNA.

The mechanism of messenger RNA synthesis in eukaryotes has not been well characterized because these organisms are highly complex and difficult to manipulate. In order to study eukaryotic messenger RNA synthesis and its control, we have chosen a simple eukaryote, *Saccharomyces cerevisiae,* whose RNA metabolism is characteristic of that of higher eukaryotes (Udem and Warner, 1972; McLaughlin *et al.,* 1973). Moreover, yeasts have other transcriptionally related characteristics similar to those of higher eukaryotes. Yeasts have a complement of histones (Wintersberger *et al.,* 1973; Gray *et al.,* 1973), multiple RNA polymerases (Adman *et al.,* 1972; Di Mauro *et al.,* 1972; Ponta *et al.,* 1971), nuclear poly(A) polymerases (Haff and Keller, 1973), and poly(adenylic acid)-containing messenger RNAs (McLaughlin *et al.,* 1973; Reed and Wintersberger, 1973). The ease of growing and manipulating yeast, its availability in large quantities, and its extensive genetics (Hartwell, 1970; Mortimer and Hawthorne, 1969) suggest that this organism might yield answers to events occurring during transcription which were heretofore poorly understood.

Before attempts can be made to study messenger RNA synthesis in yeast, it is necessary to have a reliable assay for yeast messenger RNAs. The most direct, unambiguous assay for messenger RNAs has been *in vitro* translation. A yeast cell-free system was chosen for translation of homologous RNAs as this system would most closely resemble translation *in vivo.* Because the yeast cell-free systems developed over a decade ago were never fully characterized (So and Davie, 1963; Bretthauer *et al.,* 1963), we attempted to develop a yeast translational extract based on more recently established procedures (Mathews and Korner, 1970). However, the yeast cell-free system failed to initiate the synthesis of new polypeptides (Gallis and Young, 1975), so we have turned to other cell-free systems for the assay of yeast messenger RNA.

Cell-free systems from animals and a plant have translated a variety of heterologous messenger RNAs. One of the best studied and most frequently used of these cell-free systems, that from mouse Krebs II ascites cells (Mathews and Korner, 1970), appeared to offer the capability of translating yeast messenger RNA. Messenger RNAs coding for calf lens crystallins (Mathews *et al.,* 1972a), sea urchin histones (Gross *et al.,* 1973), and mouse hemoglobin (Mathews, 1972), as well as viral RNA from QB (Aviv *et al.,* 1972) and reovirus (McDowell *et al.,* 1972), have been translated in the Krebs ascites system. Likewise, the recently developed wheat embryo cell-free system (Shih and Kaesberg, 1973; Roberts and Paterson, 1973) has been shown to translate templates of widely divergent phylogenetic origin, such as rabbit hemoglobin messenger RNA (Roberts and Paterson, 1973) and QB RNA (Davies and Kaesberg, 1973).

We show that the Krebs ascites and wheat embryo cell-free systems translate yeast poly(A)-enriched messenger RNAs with fidelity. Nearly every polypeptide labeled *in vivo* or in a yeast lysate from translation of endogenous mRNA is also made in the two heterologous cell-free systems from added yeast messenger RNAs over a molecular weight range in which these systems are capable of synthesizing polypeptides.

† From the Departments of Biochemistry and Genetics, University of Washington, Seattle, Washington 98195. *Received October 1, 1974.* This work was supported by a National Institutes of Health Training Grant to the Department of Biochemistry, a National Institutes of Health Postdoctoral Fellowship (J.E.H.), a National Institute of Dental Research Postdoctoral Fellowship (J.P.M.), and by research grants from the National Institute of Allergy and Infectious Diseases (AI-09456) and the American Cancer Society (NP-124).

Materials and Methods

Materials. L-[6-^{3}H]Serine, 1.23 Ci/mmol, L-[4,5-^{3}H]leucine, 40 Ci/mmol, and L-[^{35}S]-methionine, 71 or 148 Ci/mmol, were purchased from New England Nuclear Corporation.

Phosphocreatine and phosphocreatine kinase were purchased from Sigma, amino acids from Calbiochem, ATP and GTP from P-L, Biochemicals, cyanogen bromide activated Sepharose 4B from Pharmacia Fine Chemicals, and New Zealand white rabbits and Swiss-Webster mice from Lab Associates, Kirkland, Wash.

The following materials were gifts: pactamycin from G. B. Whitfield of the Upjohn Co., wheat germ from W. C. Mailhot of General Mills, Inc., Krebs cells from Ursula Storb, phosphorylase from Guy Pocinwong, and lysine decarboxylase from Donna Sabo.

Preparation of Krebs II Ascites S-30. Krebs ascites S-30's were prepared according to the method of Mathews and Korner (1970). Ascites cells were passed intraperitoneally by injection of 0.2 ml (2×10^8 cells/ml) of freshly drawn ascites fluid into 20–25-g Swiss-Webster mice. Cells were harvested from 10 mice 7 days after injection. Only fluid which was not darkly bloody was used for preparation of the S-30.

Ascites fluid was diluted with an equal volume of isotonic buffer, 140 mM NaCl–35 mM Tris-HCl (pH 7.5), and centrifuged. Cells were resuspended in 20 vol of this buffer and washed three times by centrifugation at 1500 rpm in rotor #289 in an International refrigerated centrifuge. This procedure pelleted ascites cells and left most of the erythrocytes in the supernatant. The ascites cells were resuspended in two packed-cell volumes of buffer containing 1.5 mM $Mg(OAc)_2$, 10 mM Tris-HCl (pH 7.5), and 10 mM KCl. After sitting 10 min on ice, the cells were homogenized with 30–40 strokes of a tight Dounce homogenizer. One-tenth volume of 250 mM Tris-HCl (pH 7.5)–850 mM KCl–35 mM $Mg(OAc)_2$–10 mM dithiothreitol was added to the homogenate which was centrigued at 30,000*g* for 15 min. The supernatant was removed after discarding the fat at the surface. One-tenth volume of a solution of 10 mM ATP, 2 mM GTP, 50 mM phosphocreatine, and 1.5 mg/ml of creatine kinase containing 400 μM each of the 20 amino acids was added to the supernatant. The extract was then rotated in a water bath at 37° for 45 min and centrifuged again at 30,000*g* for 10 min. The supernatant was passed over a 2.5 × 30 cm, G-25 Sephadex (medium) column equilibrated with 25 mM Tris-HCl (pH 7.5), 85 mM KCl, 3.5 mM $Mg(OAc)_2$, and 1 mM dithiothreitol. The most opaque fractions in the void volume, equal to the volume applied to the column, were pooled and stored in 0.65-ml aliquots at −70°. These extracts contained 12 mg/ml of protein and 40 A_{260}units/ml.

Amino Acid Incorporation in Krebs S-30. Each 100-μl reaction mixture contained 25 mM Tris-HCl (pH 7.5), 3.5 mM $Mg(OAc)_2$, 85 mM KCl, 1 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 5 mM creatine phosphate, 0.15 mg/ml of creatine kinase, 40 μM each of the 19 nonradioactive amino acids, one labeled amino acid as indicated, 60 μl of preincubated S-30, and messenger RNA as indicated. Incubation was at 37° for 30 min or at 30° for 1 hr.

Reactions were terminated by adding 1 ml of 5% Cl_3CCOOH to 10 μl of reaction mix. The Cl_3CCOOH contained the stable isotope of the amino acid used for labeling at a concentration of 0.01 M. The precipitate was heated for 10 min at 80° to hydrolyze the aminoacyl tRNA. This mixture was cooled on ice for 5 min and the precipitate was pelleted by centrifugation for 10 min at maximum revolutions per minute in a clinical centrifuge. The pellet was dissolved by adding 0.1 ml of 0.5 N NaOH and precipitated by addition of 1.0 ml of 10% Cl_3CCOOH. The precipitate was collected on Schleicher and Schuell glass fiber filters and washed twice with 1 ml of 5% Cl_3CCOOH and once with 1 ml of 95% ethanol. The filters were dried and counted in a Beckman LS-230 liquid scintillation spectrophotometer at an efficiency of 30% for ^{3}H and 85% for ^{35}S. This efficiency was determined by spotting a known amount of each isotope on a filter.

Preparation of Wheat Embryo S-23. Wheat embryo extracts were prepared by modifications of several procedures (Stern, 1957; Shih and Kaesberg, 1973; Roberts and Paterson, 1973). Untoasted wheat germ obtained from General Mills, Inc., contained 60–70% wheat germ and 30–40% chaff. The embryos were separated from the chaff by floating 10 g of wheat germ in a solution of 150 ml of CCl_4 and 60 ml of cyclohexane in a 250-ml separatory funnel. After initial gentle swirling, the chaff settled to the bottom, and the embryos were poured off through a fine mesh nylon filter and air dried. This separation yields 6 g of wheat germ.

The wheat germ was suspended in 7 vol of lysis buffer (1 mM $Mg(OAc)_2$, 2 mM $CaCl_2$, 40 mM KCl, and 1 mM dithiothreitol) and twice its weight of acid-washed glass beads (0.45–0.55 mm diameter) were added. Cells were broken by a single 15-sec burst in the Bronwill homogenizer, or alternatively by grinding 3 min in the cold with mortar and pestle with a weight of acid washed sea sand equal to that of the embryos, in 7 vol of lysis buffer. Cell extracts prepared by either method of cell breakage yield equal levels of protein synthetic activity.

The cell lysate was centrifuged for 10 min at 23,000*g* in the Sorvall SS-34 rotor. The supernatant was removed and 0.01 vol of 1 M Hepes[1] (pH 7.5) and 0.01 vol of 0.1 M $Mg(OAc)_2$ were added. The cell sap was centrifuged again for 10 min at 23,000*g* in the SS-34 rotor. The supernatant was passed over a 2.5 × 30 cm G-25 Sephadex (medium) column equilibrated with 3.0 mM $Mg(OAc)_2$, 40 mM KCl, 20 mM Hepes (pH 7.5), and 1 mM dithiothreitol. The fractions in the void volume containing the highest A_{260} were pooled and frozen in a Dry Ice–acetone bath in 0.65-ml aliquots. The S-23's were stored at −70° at a concentration of 50 A_{260} units/ml. Extracts lost about one-third of their activity in 4–6 weeks.

Amino Acid Incorporation in Wheat Cell-Free System. The reaction conditions are identical with those used for the Krebs ascites system except that the final reaction mixture contained 3.0 mM $Mg(OAc)_2$ and 40 mM KCl. The reaction was allowed to proceed for 60 min at 30°.

Preparation of a Yeast Lysate for Amino Acid Incorporation. Yeast were grown, harvested, and lysed as before. The 30,000*g* supernatant of the yeast lysate was not preincubated but rather it was passed immediately over a 2.5 × 30 cm G-25 Sephadex (medium) column equilibrated with 90 mM KCl, 3.0 mM $Mg(OAc)_2$, 25 mM Tris-HCl (pH 7.5), and 1 mM dithiothreitol. This was also the final buffer for amino acid incorporation. All other conditions for pro-

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid; YEP medium, 2 g of glucose, 20 g of Bactopeptone, and 10 g of yeast extract per liter plus 50 μg/ml of adenine; SDS, sodium dodecyl sulfate.

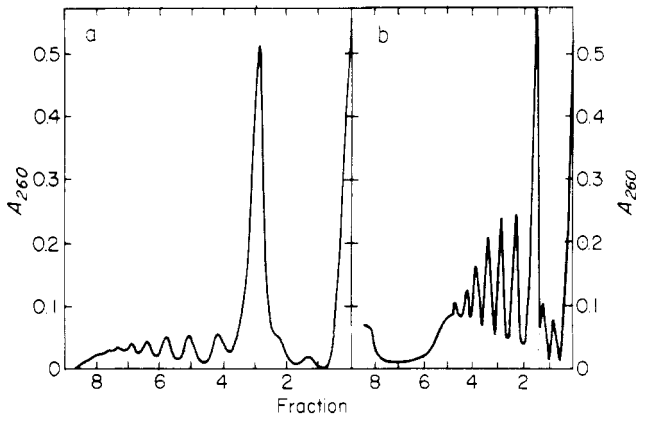

FIGURE 1: Sedimentation profile of yeast polysomes. Ten A_{260} units of yeast polysomes were sedimented in an SW 40 rotor for 90 min at 39,000 rpm, 4°, in a 15–40% sucrose gradient containing 50 mM Tris-HCl (pH 7.5)–100 mM KCl–5 mM $MgCl_2$. Gradients were monitored at A_{254} by pumping through an ISCO flow cell: (a) no cycloheximide; (b) 100 μg/ml of cycloheximide during extraction.

tein synthesis reactions were the same as those described for the Krebs ascites system, except that the reaction was carried out at 30° for 15 min.

In Vivo Labeling of Yeast Polypeptides. Yeast cells were grown logarithmically in 30 ml of YEP medium[1] at 30° to a density of 2×10^7 cells/ml. [^{35}S]Methionine (71 Ci/mmol) was added to a concentration of 3.5 μCi/ml, and growth was continued. After 30 min, the cells were centrifuged in a clinical centrifuge, resuspended in 1 ml of ice-cold 85 mM KCl–3.0 mM $Mg(OAc)_2$–30 mM Tris-HCl (pH 7.5), centrifuged again, and resuspended in 0.2 ml of the above buffer. Cells were broken in an Eaton Press at 12000 psi, and the lysate was centrifuged at 10,000 rpm for 10 min in the SS-34 rotor. The supernatant was removed, boiled for 2 min in an equal volume of twice-concentrated electrophoresis sample buffer [100 mM Tris-HCl (pH 6.8)–2% SDS[1]–20% glycerol–2% β-mercaptoethanol–0.1% Bromophenol Blue], and frozen at −20°.

Preparation of Rabbit Reticulocyte Polysomes. The method is that of Housman *et al.* (1971).

Preparation of Polysomes from Yeast. A strain (AP-1-a/α) of yeast was obtained from Dr. Anita K. Hopper. The doubling time was 75 min at 30° in YEP medium (2 g of glucose, 20 g of Bactopeptone, and 10 g of yeast extract per liter plus 50 μg/ml of adenine). Cells were grown to stationary phase, counted, and inoculated into four 1-l. cultures at 1×10^4 cells/ml. Fifteen hours later, when the cells were in log phase at $2–4 \times 10^7$ cells/ml, cycloheximide was added to a final concentration of 100 μg/ml, and shaking was continued for 5 more min.

The yeast culture was rapidly poured into centrifuge buckets on ice and pelleted by centrifugation at 5500 rpm for 5 min in the Sorvall RC-3 refrigerated centrifuge. The yield was about 10 g of cells which was resuspended in 3 vol of lysis buffer [10 mM KCl–10 mM Tris-HCl (pH 7.5)–10 mM $Mg(OAc)_2$] and pelleted at 10,000 rpm for 10 min in the Sorvall SS-34 rotor. The cells were resuspended in 2 vol of lysis buffer with twice their weight of acid-washed glass beads (0.45–0.55 mm diameter), and broken with three 15-sec bursts in a Bronwill homogenizer. The lysate was centrifuged for 19 min at 30,000*g*. The supernatant was removed and centrifuged for 3 hr at 40,000 rpm in a Type 40 rotor in an L2-65B Beckman preparative ultracentrifuge. The polysome pellet contained 1000–1500 A_{260} units when resuspended in RNA extraction buffer.

Extraction of Polysomal RNA. RNA was extracted from polysomes of rabbit reticulocytes or yeast by a modification of the procedure of Aviv and Leder (1972). The polysomes were suspended in 0.1 M Tris-HCl (pH 7.5)–0.1 M LiCl–1 mM EDTA at a concentration of 50 A_{260} units/ml and then made 0.1% in sodium dodecyl sulfate. An equal volume of phenol–chloroform–isoamyl alcohol, 50:50:1 by volume, was added, the mixture was shaken for 10 min at room temperature, and the solution was centrifuged at 10,000 rpm for 10 min in the SS-34 rotor. The aqueous phase was removed, reextracted in the same way, and made 0.2 M in NaOAc (pH 5.2). Two volumes of −20°, 95% ethanol was added and the RNA was allowed to precipitate overnight at −20°. The RNA was collected by centrifugation for 10 min at 10,000 rpm in a Sorvall SS-34 rotor, washed twice with −20°, 70% ethanol, dried, and dissolved in water. By these procedures, 240 A_{260} units of polysomal RNA can be extracted from the reticulocytes of one rabbit. In a scaled up procedure, 6000 A_{260} units of polysomal RNA have been extracted from 16 l of log phase yeast.

Poly(uridylic acid) Sepharose Chromatography. Poly(adenylic acid)-containing RNA was purified from polysomal RNA by the procedure of Firtel and Lodish (1973).

Polyacrylamide Gel Electrophoresis. The procedure for SDS[1] gels is essentially that of Studier (1973). The procedure for electrophoresing proteins at pH 3.2 or 4.5 in 8 M urea is essentially that of Panyim and Chalkley (1969).

Results

Isolation of Polysomes from Yeast. Polysomes were extracted from yeast in the exponential phase of growth in order to enhance the recovery of undegraded messenger RNA, since stationary phase yeast cultures exhibit a substantially higher rate of ribonucleic acid degradation (Halvorson, 1958). Sucrose gradient fractionation of a portion of a 30,000*g* postmitochondrial supernatant of logarithmically growing yeast reveals that about 29% of the ribosomes exist as polyribosomes (Figure 1a). The accumulation of monoribosomes is in part due to ribosome run-off during harvest of the culture and preparation of the cell extract. Hartwell and McLaughlin (1969) have shown that cycloheximide blocks conversion *in vivo* of polyribosomes to monoribosomes. Addition of cycloheximide to a final concentration of 100 μg/ml to yeast cultures 5 min prior to harvest allowed the isolation of 95% of the ribosomes as polyribosomes (Figure 1b). These polyribosomes were pelleted from the postmitochondrial supernatant for extraction of the RNA.

Purification of Yeast Poly(A)-Enriched RNA. RNAs from a variety of eukaryotic cells have been shown to contain a sequence of poly(riboadenylic acid) about 50–200 nucleotides long (Morrison *et al.*, 1973; Molloy and Darnell, 1973; McLaughlin *et al.*, 1973). Nearly all of the messenger-like RNA isolated from polyribosomes of L-cells contains poly(A) (Greenberg and Perry, 1972; Perry *et al.*, 1973). The poly(A) moiety of eukaryotic messenger RNAs has aided their purification, since they can be bound to and selectively removed from the bulk of ribosomal RNA by oligo(dT)-cellulose chromatography. These messenger RNAs have also been shown to serve as templates for specific, identifiable polypeptides by their translation in a cell-free system (Swan *et al.*, 1972; Aviv and Leder, 1972; Mach *et al.*, 1973). Thus, we have used poly(U)-Sepharose chroma-

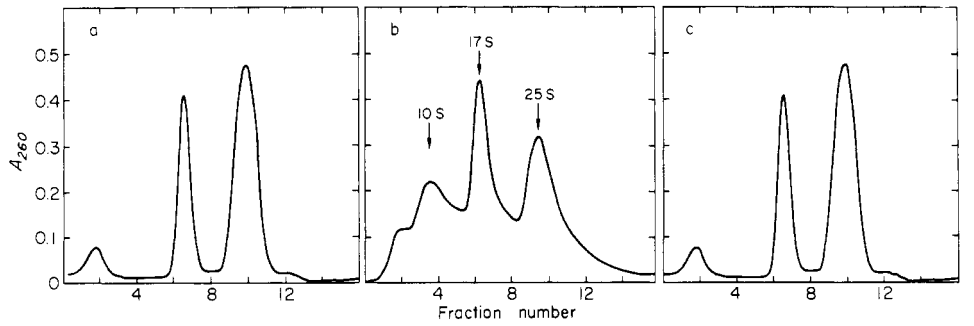

FIGURE 2: Sedimentation profiles of yeast polysomal RNAs: (a) RNA before application to poly(U)-Sepharose column (145 μg); (b) RNA which was bound to and eluted from a poly(U)-Sepharose column with 70% formamide (250 μg); (c) RNA which passed through the poly(U)-Sepharose column but did not bind (145 μg). RNA was sedimented in an SW 41 rotor for 16 hr at 25,000 rpm, 4° in 5–20% sucrose gradients containing 20 mM Tris-HCl (pH 7.5)–85 mM KCl–0.1 mM EDTA. Gradients were monitored at A_{254} by pumping through an ISCO flow cell.

tography to purify potential templates from yeast for cell-free protein synthesis.

When 10 mg of RNA extracted from yeast polysomes is applied to a poly(U)-Sepharose column, 96% of the RNA passes through the column. Four per cent of the RNA is bound and can be eluted with 70% formamide. When the bound RNA is fractionated on a sucrose gradient, it sediments heterogeneously over a range of 4–39 S, with peaks at 4, 10, 17, and 25 S (Figure 2b). The sedimentation profile (Figure 2b) suggests that about 50% of the A_{260} which is bound to and eluted from the column is either tRNA sedimenting at 4 S or rRNA which sediments at 17 and 25 S. The heterogeneity of the sedimentation profile suggests a substantial enrichment for nonribosomal RNA, presumably mRNA containing poly(A) sequences. This profile (Figure 2b), with the exception of the rRNA peaks, is essentially identical with the sedimentation profiles of radioactivity of total yeast polysomal RNA pulse labeled with [^{3}H]adenine (McLaughlin *et al.*, 1973). The sedimentation profiles of total polysomal RNA before its application to the column (Figure 2a) or of RNA which passes directly through the column (Figure 2c) indicate that neither the method of purification of the RNA nor its passage through the column produces any discernible degradation of ribosomal RNA.

Characteristics of Amino Acid Incorporation Directed by Yeast Poly(A) RNA in the Krebs Ascites Cell-Free System. To show that the poly(A)-enriched RNA has template activity, it has been added to a Krebs ascites cell-free system. In the Krebs system it stimulates amino acid incorporation linearly at 30° for 30 min (Figure 3a). The incorporation reaches a plateau by 60 min. The stimulation is dependent upon poly(A)-enriched RNA concentration. It is linear up to 70 μg/ml and reaches a maximum at 90–100 μg/ml (Figure 3b). This stimulation of amino acid incorporation is not due to protection of endogenous Krebs ascites RNA (Mathews and Korner, 1970), because 100 μg/ml of RNA from bacteriophage T7-infected *Escherichia coli* directs the incorporation of only 0.2 pmol of leucine, 1/15 the incorporation directed by the optimal concentration of yeast RNA (Figure 3a). The saturating concentration of yeast messenger RNA is closer to 40–50 μg/ml, since about 50% of the poly(U)-Sepharose purified RNA sequences are made up of tRNA or rRNA species (Figure 2b). Saturating concentrations in the range of 40–60 μg/ml have been found for ovalbumin messenger (Schutz *et al.*, 1972), mouse hemoglobin messenger (Mathews *et al.*, 1971), chick lens messenger (Mathews *et al.*, 1972b), and encephalomyocarditis RNA (Mathews and Korner, 1970; Eggen and Shatkin, 1972).

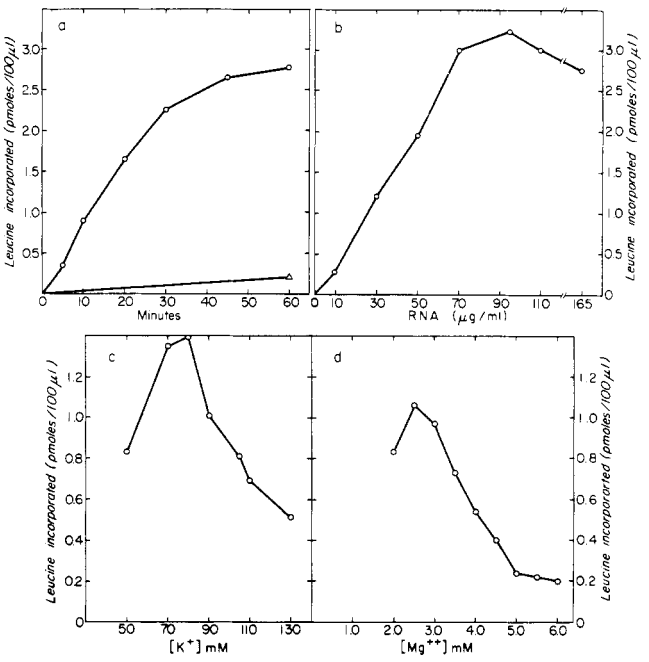

FIGURE 3: Parameters of protein synthesis directed by yeast poly(A)-enriched RNA in the Krebs ascites system: (a) kinetics of amino acid incorporation; 70 μg/ml of yeast poly(A)-enriched RNA (○); 100 μg/ml of *E. coli* RNA (△); (b) dependence of amino acid incorporation on RNA concentration; (c and d) dependence of amino acid incorporation on potassium and magnesium concentration; (c) variation of potassium at 3.0 mM magnesium; (d) variation of magnesium at 85 mM potassium. All 100-μl reactions contained 50 μCi/ml of [^{3}H]leucine (20 Ci/mmol); 1 pmol = 13,000 cpm. For the RNA and cation optima, 10-μl samples were removed for counting at 20 min, 30°, when the reaction rate was linear. For the kinetic and cation optima, the RNA concentrations were 100 and 70 μg/ml, respectively.

Yeast poly(A)-directed amino acid incorporation is sharply dependent upon monovalent and divalent cation concentrations in the Krebs ascites system. The optimum monovalent cation concentration is 75 mM KCl (Figure 3c). The optimum magnesium concentration is 2.5 mM Mg^{2+} (Figure 3d). These optima are the same as those found for the translation of hemoglobin messenger in the Krebs ascites system (Mathews, 1972).

Characteristics of Amino Acid Incorporation Directed by Yeast Poly(A) RNA in the Wheat Embryo Cell-Free System. Yeast poly(A)-enriched mRNA-directed amino acid incorporation is also sharply dependent upon monovalent and divalent cation concentrations in the wheat embryo cell-free system. The optimum divalent cation concentration is 40 mM KCl (Figure 4c). The optimal magnesium

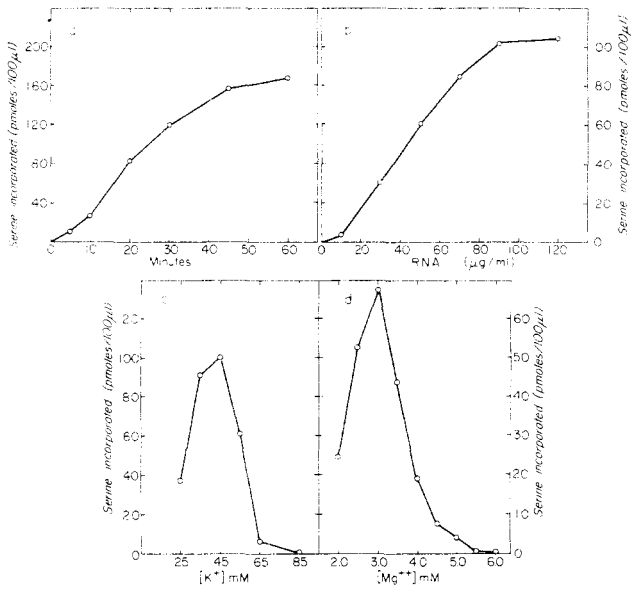

FIGURE 4: Parameters of protein synthesis directed by yeast poly(A)-enriched RNA in the wheat embryo system: (a) kinetics of amino acid incorporation; reaction mixtures contained 3.0 mM Mg^{2+} and 40 mM KCl; (b) dependence of amino acid incorporation on RNA concentration; ion concentrations same as above; (c and d) dependence of amino acid incorporation on potassium and magnesium concentration; (c) variation of potassium at 3.6 mM Mg^{2+}; (d) variation of magnesium at 37 mM KCl. All 100-μl reactions contained 50 μCi/ml of [^{3}H]serine (1.23 Ci/mmol); 1 pmol = 800 cpm. Endogenous incorporation of 4 pmol was subtracted from each point. For the RNA and cation optima, 10-μl samples were removed for counting at 30 min, 30°, when the reaction rate was linear. Kinetic and cation optima studies were done at a concentration of 100 μg/ml of poly(A)-enriched RNA.

concentration is 3.0 mM Mg (Figure 4d). These are below the optima of 55 mM KCl and 4.5 mM Mg found for the translation of hemoglobin messenger in the wheat embryo cell-free system (Roberts and Paterson, 1973). The wheat embryo system exhibits kinetics of incorporation (Figure 4a) and poly(A) RNA saturation concentrations (Figure 4b) similar to those described for the Krebs ascites system.

Relative Efficiency of Translation of Yeast Poly(A) RNA and Rabbit mRNA in Mammalian and Plant Cell-Free Systems. Yeast messengers are not translated as efficiently in the Krebs or wheat systems as is the rabbit hemoglobin messenger. Using optimal concentrations of each RNA preparation, under otherwise identical conditions, about 5 μg of yeast mRNA (assuming the poly(A) preparation is about 50% mRNA) stimulates incorporation of 3.75 pmol of [^{3}H]serine whereas 0.70 μg of Hb mRNA (assuming hemoglobin messenger is 2% of polysomal RNA) stimulates incorporation of 20 pmol of serine in the Krebs system (Table I). Thus, per microgram of mRNA, 40 times the amount of serine can be incorporated in the Krebs system from a mammalian mRNA than from yeast mRNAs. Under the same conditions in the wheat system (Table I), per microgram of mRNA, rabbit reticulocyte mRNA directs the incorporation of two times the amount of amino acid compared to yeast mRNA.

Capacities of the Mammalian and Plant Cell-Free Systems. When all conditions for cell-free translation are optimal, the wheat germ cell-free system has a greater protein synthetic capacity than does the mouse Krebs ascites system, regardless of whether or not the input RNA is from rabbit or yeast. Yeast poly(A)-enriched mRNA directs the incorporation of 45 times the amount of amino acid in the wheat compared to the Krebs ascites system (Table I). Rabbit reticulocyte RNA, the more homologous RNA for the Krebs ascites system, directs the incorporation of 2.5 times the amount of amino acid in the wheat germ as compared to the Krebs ascites system (Table I).

Polypeptides Directed by Yeast Poly(A)-Enriched RNA in the Krebs Ascites System. Yeast poly(A)-enriched RNA directs the incorporation of [^{35}S]methionine into polypeptides in Krebs ascites extracts. When the products of this translation are fractionated on 15% SDS–polyacrylamide slab gels and analyzed by autoradiography (Figure 5), about 25 polypeptides migrate at the same rate as polypeptides labeled with [^{35}S]methionine *in vivo* in yeast (Figure 5c). This similarity of polypeptides, although not absolute, occurs over a molecular weight range of 15,000–94,000. There is some nonidentity of polypeptides above mol wt 60,000. More species of polypeptides appear *in vivo* than *in vitro*. Furthermore, a greater amount of each high molecular weight polypeptide appears to be made *in vivo* than *in vitro* relative to the synthesis of low molecular weight polypeptides *in vivo* and *in vitro*. Minor polypeptides of a molecular weight greater than 60,000 probably do not appear in the Krebs ascites system due to a slow elongation rate (Mathews and Osborn, 1974). A slow elongation rate *in vitro* would also cause a decreased synthesis of the most prominent high molecular weight polypeptides due to a slower rate of completion of chains.

Below a molecular weight of 60,000, the polypeptides made *in vivo* and *in vitro* are qualitatively similar. All messenger RNAs coding for major low molecular weight polypeptides *in vivo* are also translated *in vitro*. Moreover, *in*

Table I: Amino Acid Incorporation Directed by Mammalian and Yeast Messenger RNAs in the Krebs Ascites and Wheat Embryo Cell-Free Systems.[a]

	[RNA] (μg/ml)		Serine Incorpd (pmol)			
			Krebs Ascites		Wheat Embryo	
Source of RNA	Total	mRNA[b]	Total	Per μg of mRNA	Total	Per μg of mRNA
Rabbit reticulocyte	360	7.2	20	28	52.5	73
Yeast	100	50	3.75	0.75	165	33

[a] Each 100-μl reaction mix contained 50 μCi/ml of [^{3}H]serine (1.23 Ci/mol); 1 pmole = 800 cpm. Conditions of translation are optimal for each source of RNA in each cell-free system (see Materials and Methods). Yeast RNA is poly(A)-enriched RNA and rabbit RNA is total polysomal RNA from rabbit reticulocytes. [b] See text for estimation of mRNA concentration.

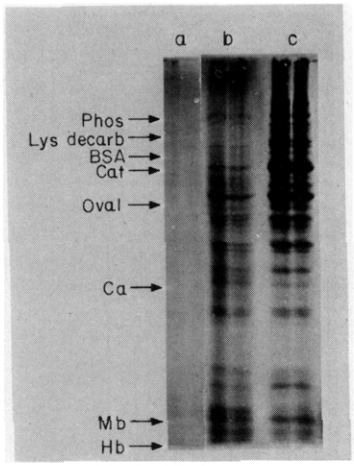

FIGURE 5: Autoradiograms of polypeptides synthesized *in vivo* in yeast and in Krebs ascite cell-free extracts and fractionated by SDS–polyacrylamide gel electrophoresis. Protein synthesis conditions were identical with those in Figure 3. Polypeptides synthesized in the Krebs ascites system after addition of: (a) no (endogenous) messenger RNA; (b) 100 μg/ml of yeast poly(A)-enriched RNA; (c) yeast polypeptides pulse labeled *in vivo* with [^{35}S]methionine. The positions of molecular weight markers are shown on the side. They are phosphorylase *b*, 100,000; lysine decarboxylase, 80,000; bovine serum albumin, 67,000; catalase, 58,000; ovalbumin, 43,000; carbonic anhydrase, 29,000; myoglobin, 17,200; and hemoglobin, 15,500.

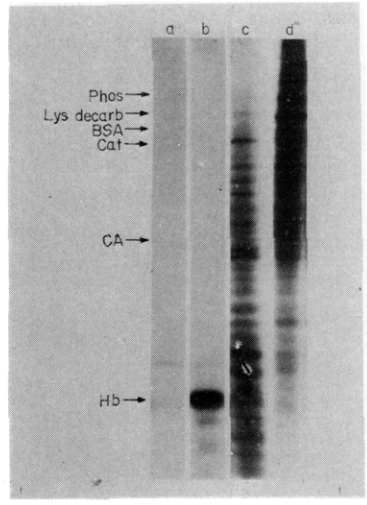

FIGURE 6: Autoradiograms of polypeptides synthesized in yeast and wheat embryo cell-free systems and fractionated by SDS–polyacrylamide gel electrophoresis. Conditions are identical with Figure 5. Polypeptides synthesized in the wheat embryo cell-free system after addition of: (a) no (endogenous) RNA; (b) 200 μg/ml of rabbit reticulocyte polysomal RNA; (c) 100 μg/ml of yeast poly(A)-enriched RNA; and (d) polypeptides directed by endogenous mRNA in a yeast cell-free system. For the molecular weights of the markers, see the legend to Figure 5.

general the polypeptides most strongly labeled *in vivo* are also the most strongly labeled *in vitro*. This observation suggests that it is the amount of a given mRNA and its structure that determine its rate of translation compared to any other mRNA, not species-specific initiation factors or ribosomes. Exceptions to this observation can also be found, however.

Polypeptides Directed by Yeast Poly(A)-Enriched RNA in the Wheat Embryo Cell-Free System. Polypeptides whose synthesis is directed by yeast poly(A)-enriched RNA in a wheat embryo system (Figure 6c) were compared with polypeptides labeled in a yeast lysate (Figure 6d). Electrophoresis of the [^{35}S]methionine-labeled products from each system in adjacent slots of a 15% polyacrylamide slab gel, followed by autoradiography, reveals at least 22 major bands which migrate at the same rate whether they are synthesized *in vivo* or *in vitro*. These polypeptides cover a molecular weight range of 10,000–94,000. Bands in the upper region of Figure 6d representing polypeptides synthesized in the yeast lysate are obscured because the lower half of the gel slot was overexposed photographically in order to display faint, lower molecular weight polypeptides. Bands below mol wt 15,000 which appear distinctly on autoradiographs are difficult to photograph; they do, however, match every band directed by poly(A)-enriched RNA in the Krebs and wheat extracts. The probable identity of such bands produced in the heterologous cell-free systems is suggested by comparison of densitometric tracings of the autoradiographs (Figure 8) of products made in the Krebs and wheat extracts (see below).

In order to further demonstrate that the wheat embryo system synthesized *bona fide* polypeptides, such as those which constitute hemoglobin, polysomal RNA from rabbit reticulocytes was added to a wheat embryo reaction mixture. The wheat embryo system directs the synthesis of a single band (Figure 6b) upon addition of polysomal RNA from rabbit reticulocytes. This band comigrates with marker hemoglobin. Rabbit hemoglobin messenger RNA has been previously translated in the wheat embryo system (Roberts and Patterson, 1973). Several polypeptides appear to be faintly labeled in the wheat embryo system under the direction of endogenous messenger RNA (Figure 6a).

Errors in polypeptide chain elongation which substitute one amino acid for another but do not terminate the polypeptide chain would not be detected by SDS–gel electrophoresis which measures only the number of amino acids per chain. Another way to assess the fidelity of translation of a complex mixture of mRNAs is to compare the electrophoretic mobility of the polypeptides synthesized *in vitro* and *in vivo* in the absence of SDS so that their net charge also influences their mobility. Since the *in vivo* polypeptides may be rapidly assembled into multimeric proteins whereas the *in vitro* proteins may not be, we compared the proteins synthesized *in vivo* and *in vitro* under denaturing conditions. The samples were denatured in 5 M urea and electrophoresed either on a 15% polyacrylamide slab gel containing 7 M urea (pH 3.2) (C, D; Panyim and Chalkley, 1969) or on a 10% polyacrylamide gel containing 8 M urea at pH 8.3 (A, B). A comparison of *in vivo* and *in vitro* polypeptides after electrophoresis and autoradiography is shown in Figure 7. Fewer polypeptides are seen than on the SDS gels, as expected since the resolution is poorer on the urea gels. Many of the proteins have not entered the pH 3.2 gel, perhaps because they were not denatured. Of the proteins that enter the gel, there is a co-migration of many of the major polypeptides synthesized *in vivo* and *in vitro* both at pH 3.2 and 8.3. This observation makes it unlikely that extensive errors in coding are occurring in the wheat embryo cell-free system.

Comparison of Polypeptides Directed by Yeast Poly(A)-Enriched RNA in the Krebs Ascites and Wheat Embryo Systems. We have shown that the majority of polypeptides directed by yeast poly(A)-enriched RNA in either a Krebs ascites or wheat embryo extract are similar to yeast polypeptides labeled in yeast extracts or *in vivo* in yeast. A direct comparison of the cell-free products made in both sys-

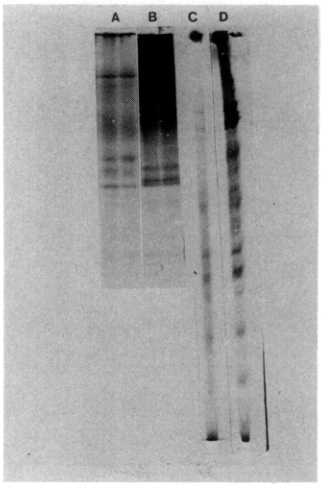

FIGURE 7: Autoradiograms of polypeptides synthesized *in vivo* or in the wheat embryo cell-free system and electrophoresed at pH 8.3 or 3.2 in urea. [^{35}S]Methionine polypeptides synthesized in yeast cells (B, D) or in the wheat embryo cell-free system containing yeast mRNA (A, C) were treated with an equal volume of deionized 8 M urea in 1 N acetic acid (pH 3.2 gels, C and D). An additional 1/8 vol of 8 M urea and 1/50 vol of dithiothreitol were added, the sample was heated to 37° for 3 min, and then 0.02 ml was electrophoresed on a 10% polyacrylamide slab gel containing 7 M urea (pH 3.2) for 5 hr at 17 mA. The gel had been pre-run for 4 hr at 15 mA. The samples to be run at pH 8.3 (A, B) were diluted into an equal volume of 8 M urea, 6% β-mercaptoethanol, and 0.002% Bromophenol Blue, and electrophoresed for 6 hr at 17 mA on a 15% polyacrylamide gel (pH 8.3) containing 8 M urea. The reservoir buffer contained Tris-glycine buffer adjusted to pH 8.3 with NaOH.

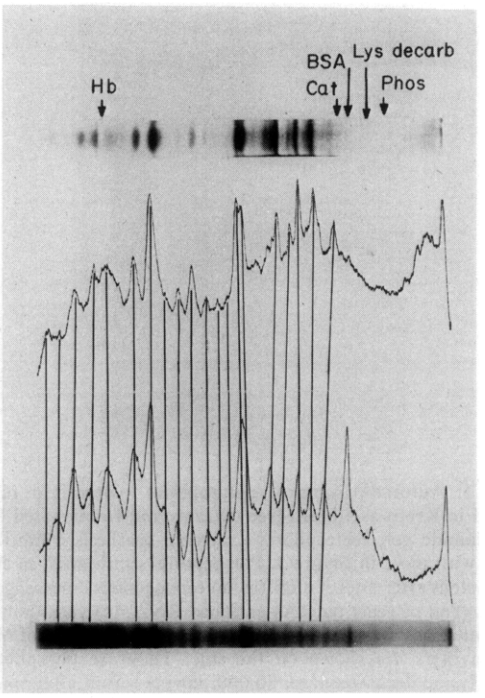

FIGURE 8: Densitometer tracings of autoradiograms of products of Krebs ascites and wheat embryo systems directed by yeast poly(A)-enriched RNA. Autoradiograms of Krebs ascites products (upper gel) and wheat embryo products (lower gel) were scanned with a Joyce-Loebl recording microdensitometer. Conditions for protein synthesis and electrophoresis were the same as in Figure 5. See the legend to Figure 5 for the molecular weights of the marker proteins.

tems is shown in Figure 8. Autoradiograms of 15% SDS–polyacrylamide gels containing products directed by yeast poly(A)-enriched RNA in both the mammalian and the plant system were scanned with a recording microdensitometer and this tracing is shown above the autoradiograph of wheat products.

Alignment of the densitometric tracings of the products of each system directed by yeast poly(A)-enriched RNA reveals that 15 bands migrate to similar position over a mol wt range of 10,000–25,000 (Figure 8). Each band in this molecular weight range appears with a characteristic intensity. That is, these bands appear at about the same intensities relative to one another in either the Krebs or wheat cell-free systems. For polypeptides greater in molecular weight than the prominent doublet at 25,000, it is not possible to align the bands with surety although each of the bands matches yeast polypeptides labeled in the yeast lysate or *in vivo* in yeast (Figures 5 and 6). The autoradiographs reveal that relatively smaller amounts of high molecular weight polypeptides are produced by either system, although the densitometric tracing clearly shows polypeptides in the wheat system as large as mol wt 94,000. Visual inspection of autoradiographs reveals that these polypeptides are similar to those labeled in a yeast lysate. Yeast polypeptides as large as those synthesized in a wheat system were synthesized in a different Krebs ascites preparation (Figure 5) than used in Figure 8. In general we find that the wheat embryo system makes more large polypeptides than the Krebs ascites system when both are translating yeast mRNAs.

Discussion

The results show that both the Krebs ascites and wheat embryo cell-free systems translate yeast messenger RNAs into polypeptides similar to those made in yeast. The fidelity with which cell-free systems from a mammal and a higher plant translate messenger RNAs from one of the simplest eukaryotes suggests that cell-free translational machinery of eukaryotes is not able to markedly discriminate qualitatively among the messengers which are available to it. This does not rule out the possibility of translational control *in vivo* (Lodish and Jacobson, 1972).

While polypeptides which are pulse labeled in yeast are also synthesized in heterologous cell-free systems, there are differences in the distribution of label among the polypeptides made *in vitro* compared to the distribution of label in the polypeptides made *in vivo*. This may be due to some extent to the selective loss or degradation of messenger RNA which could occur during extraction. It is not due to the removal of certain mRNA species which lack poly(A). We have examined the polypeptides that are translated from yeast mRNA that does not bind to poly(U)-Sepharose. The same major polypeptides are made by this mRNA and they are synthesized in the same proportion relative to one another as are the polypeptides translated from poly(U)-binding yeast mRNA (Gallis, 1974). This is not evidence that there is heterogeneity in the poly(A) content of messengers coding for the same polypeptide, however. One major reason that some yeast mRNA fails to bind to poly(U)-Sepharose is that some of the poly(U) comes off of the column and this removal is enhanced by the passage of RNA through the column (Gallis, 1974).

An alternative explanation for the different ratios among some of the polypeptides synthesized *in vivo* and *in vitro* is that the rates of chain initiation, elongation, and termination for any given mRNA *in vitro* may not be the same as they are *in vivo*. For example, in rabbit reticulocytes globin α-chain mRNA is initiated only 65% as frequently as β-

chain mRNA (Lodish, 1971), but since there appears to be 1.5 times the amount of ribosome-associated α-chain mRNA in reticulocytes, α and β chains are synthesized in equal amounts *in vivo* (Baglioni and Campana, 1967). However, reticulocyte messenger RNA added to Krebs ascites extracts directs the synthesis of more β than α chains (Housman *et al.*, 1971; Mathews, 1972; Mathews *et al.*, 1972b) while a liver cell-free system synthesized an excess of α chains (Sampson *et al.*, 1972). This difference between the ratio of α to β chains synthesized *in vivo* and *in vitro* could be due to the presence of initiation factors specific for translation of a given messenger RNA (Wigle and Smith, 1973; Nudel *et al.*, 1973; Hall and Arnstein, 1973). On the other hand, Hall and Arnstein (1973) have found that varying the amount of total messenger RNA added to the Krebs ascites system causes a variation in the ratio of α to β chains made. This phenomenon could also produce a quantitative difference between the *in vivo* and *in vitro* patterns of yeast polypeptides.

Capacity of Mammalian and Wheat Cell-Free Systems to Translate Yeast Poly(A) RNA. The wheat embryo and Krebs ascites cell-free systems differ significantly only in the amount of yeast polypeptides which each is capable of making. The wheat system synthesizes more total Cl_3CCOOH precipitable polypeptide than does the Krebs ascites system under the direction of yeast messenger RNA. While yeast messenger directs the incorporation of 3.75 pmol of serine in the Krebs system, yeast mRNA-directed incorporation in the wheat system is 165 pmol, a 45-fold difference in activity (Table I). This comparison is made between the systems when each is at its optimal temperature, ionic conditions, template concentration, and at a saturating amino acid concentration (41 μM serine). The difference in ribosome concentration in each of the reaction mixes, 24 A_{260} units/ml for the Krebs ascites system and 34 A_{260} units/ml for the wheat embryo cell-free system, is insufficient to explain the difference in incorporation, nor can these differences be explained by the possibility that the ascites lysate contains more endogenous, unlabeled serine than does the wheat lysate since both lysates were passed over a G-25 column to remove endogenous amino acids. Moreover, isotope dilution studies of the Krebs and wheat cell-free systems reveal that they contain 1 and 3.5 μM serine, respectively. These concentrations are small compared to the concentration of the added, labeled serine (41 μM), and they have not been considered in the calculation of picomoles incorporated.

The ability of the wheat system to synthesize a larger amount of polypeptides than the Krebs system could be due to several factors. First, the long preincubation of the Krebs ascites system lowers the absolute amount of messenger-directed amino acid incorporation (Mathews and Korner, 1970). This preincubation may inactivate initiation and/or elongation factors, among other components. Indeed, some ascites S-30's are initiation factor dependent (Mathews *et al.*, 1972b; Metafora *et al.*, 1972; Lebleu *et al.*, 1972). However, a 0.5 M KCl wash fraction from rabbit reticulocyte or from Krebs ascites ribosomes did not alter the rate of incorporation in our experiments. The rate of chain elongation in the Krebs ascites system is 5–10% of the *in vivo* rate (Mathews and Osborn, 1974). Limiting tRNA could lower the rate of elongation in the Krebs ascites system. Aviv *et al.* (1971) have found ascites S-30's to be tRNA limiting. However, mouse liver tRNA, when added to our ascites S-30's, failed to alter the rate or absolute amount of incorporation. While neither a high salt wash nor tRNA increased the rate of incorporation in our experiments with the Krebs system, this does not preclude the possibility that elongation or initiation factors are rate limiting, which would result in synthesis of less Cl_3CCOOH precipitable polypeptides.

Secondly, the Krebs ascites system appears to contain more ribonuclease activity. Mathews and Korner (1970) found that the Krebs S-30 solubilized [^{3}H]poly(U) at a rate of 20–50 μg/min per ml. On the other hand, polyacrylamide gel electrophoresis of ^{32}P-labeled viral messengers subsequent to their translation in the wheat system showed that 80–90% of the RNA remained undegraded (Davies and Kaesberg, 1973).

Efficiency of Translation of Yeast Poly(A) RNA and Rabbit mRNA in Mammalian and Plant Cell-Free Systems. Rabbit reticulocyte hemoglobin messenger is more efficiently translated in the wheat and Krebs ascites systems than is yeast messenger RNA (Table I). Differential affinities of wheat and mammalian translational factors or ribosomes for lower or higher eukaryotic messenger RNAs could partially explain the differences in protein synthetic abilities. For example, QB, a prokaryotic RNA, is translated with decreased efficiency in either the Krebs or wheat systems compared to translation of more closely related viral RNAs. Thus, QB is translated less efficiently (Aviv *et al.*, 1972) in the Krebs system than is EMC RNA (Aviv *et al.*, 1971). In the wheat system, QB RNA is translated less efficiently than is bromegrass mosaic virus (BMV) RNA (Davies and Kaesberg, 1973). However, the reverse is true when QB and BMV are translated in an *E. coli* cell-free system (Davies and Kaesberg, 1973). It is not known whether these differences are due to decreased rates of initiation, elongation, or termination, or some combination of these. Similarly, the relatively low activity of yeast mRNA in the Krebs ascites extract has not been further studied. Although the yeast polypeptides made in the Krebs ascites extract are smaller than those made in the wheat embryo system, the disparity is not great enough to attribute the 40-fold difference in activity in the two systems to different rates of elongation alone. Possibly yeast mRNAs lack some structural feature necessary for efficient initiation in the extract from mammalian cells.

Some Potential Applications for Cell-Free, Messenger-Directed Synthesis of Yeast Polypeptides. The ability of yeast messenger RNAs to direct the synthesis of *bona fide* yeast polypeptides in heterologous cell-free protein synthesis systems suggests experiments for which yeast is a particularly amenable organism. The presence of ochre (Gilmore *et al.*, 1971; Stewart *et al.*, 1972) and amber (Stewart and Sherman, 1972) mutations in the gene for iso-1-cytochrome *c*, and the existence of allele specific, locus nonspecific suppressors (Hawthorne and Mortimer, 1968) in yeast provide a test for the existence of suppressing tRNAs in eukaryotes. The cell-free synthesis of iso-1-cytochrome *c* directed by messenger RNA isolated from a strain bearing an iso-1-cytochrome *c* nonsense mutation in the presence of the appropriate suppressing yeast tRNA would constitute evidence that tRNA has a suppressing function in eukaryotes.

The well-defined genetics of the *his 4* region of the histidine biosynthetic pathway (Shaffer *et al.*, 1969), coupled with a potential for messenger-directed cell-free synthesis of some of the enzymes which constitute this pathway, provides one potential system for the study of gene regulation in eukaryotes.

Acknowledgments

B.M.G. wishes to thank Michael B. Mathews and Paul Kaesberg for continued interest and helpful discussions, and Anita K. Hopper for her encouragement and invaluable advice. We thank William C. Mailhot of General Mills, Inc., and G. B. Whitfield of the Upjohn Co. for their generous gifts of wheat germ and pactamycin, respectively, and Nancy Flynt for her help in preparing the manuscript.

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