Regulation of Translation. Analysis of Intermediary Reactions in Protein Synthesis in Exponentially Growing and Stationary Phase Chinese Hamster Ovary Cells in Culture†

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ABSTRACT: Chinese hamster ovary cells growing exponentially in serum-containing media cease to grow at relatively high cell density due to the depletion of a component or components provided in the serum. When exponentially or rapidly growing cells are incubated with radioactive amino acid, incorporation into proteins is markedly greater than in incubations containing stationary or resting phase cells. Amino acid incorporation in postmitochondrial extracts, in incubations designed to measure translation of endogenous mRNAs, is also significantly greater with preparations obtained from exponential phase cells than from stationary phase cells. This observation suggests that the level of mRNA or the ability to translate mRNA differs in stationary as compared to exponential phase cells. When extracts are depleted of endogenous mRNA and analyzed for their ability to translate exogenous natural (globin) mRNA, the activity of exponential cell extracts is much greater than that of stationary cell preparations. These results indicate that the translational system differs in stationary phase cells as compared to exponential phase cells. The formation of a complex containing 40S ribosomal subunits and [^{35}S]methionine in the absence of mRNA and the transfer of radioactive methionine to the 80S initiation complex in the presence of mRNA and cycloheximide are quantitatively similar in extracts from exponential phase and stationary phase cells. These findings suggest that transition from the exponential to the stationary phase does not significantly affect reactions involved in chain initiation such as the formation of ternary complex containing eIF-2, GTP, and Met-tRNA$_f$, the association of the ternary complex with 40S subunits, the binding of mRNA, and the joining of 60S subunits. When mRNA-depleted extracts are incubated with poly(uridylic acid), the synthesis of poly(phenylalanine) in exponential cell preparations is considerably greater than in stationary cells. Since the poly(U)-dependent synthesis of poly(phenylalanine) requires only components for polypeptide chain elongation, these results suggest that the activity of a component or components required for chain elongation, such as elongation factors or ribosomes, is altered. Analysis of individual elongation factors reveals that whereas the activity of EF-2 is similar in both preparations, the activity of EF-1 in stationary phase cells is markedly lower than in exponentially growing cells. Differences in activity between the two cell phases were not detected in analyses of chain termination and release. Thus, passage from the exponential to the stationary phase in Chinese hamster ovary cells is accompanied by a decrease in the amount of polysome-associated translatable mRNA and in the activity of EF-1; all of the other components involved in initiation, elongation, and termination of protein synthesis appear to be unaffected.

The exponential or growing phase of cultured cells in characterized by rapid cell division and growth, while at relatively high cell density the stationary or resting phase is characterized by slow cell division. In some cell types it has been shown that the inhibition of growth in the stationary phase is accompanied by a marked decline in protein synthesis, as measured by the incorporation of amino acids into the proteins of intact cells (Levine et al., 1965; Ward & Plagemann, 1969; Priest & Davies, 1969; Stanners & Becker, 1971; Lee & Engelhardt, 1977; Tanaka & Ichihara, 1977). Polysome disaggregation, concomitant with the decrease in protein synthetic activity, was also reported (Ward & Plagemann, 1969; Stanners & Becker, 1971; Rudland et al., 1975; Lee & Engelhardt, 1977). In contrast to the results obtained with some of the cell types noted above (hamster embryo fibroblasts, Vero M_3 cells, Novikoff rat hepatoma, human fibroblasts, and BHK[1] 21), there was no change in protein synthesis or in the specific activity of the aminoacyl-tRNA pool during the growth cycle in BHK 21/23 cells (Baenziger et al., 1974). Experiments with cell-free systems have been equivocal; some cells, such as Vero M_3 (Engelhardt & Sarnoski, 1975), showed lower amino acid incorporation in extracts from stationary phase cells than from exponential phase cells, while others, such as BHK 21/23 and 3T3, showed no differences (Baenziger et al., 1974; Conta & Meisler, 1977). It was demonstrated, however, that whereas there was a lower rate of protein synthesis when the parent cell BHK 21 was in the stationary phase, the BHK 21/23 line showed an increase in protein degradation (Tanaka & Ichihara, 1977). Evidence has been reported suggesting that important elements regulating protein synthesis as a function of the growth phase include the levels of mRNA (Johnson et al., 1974; Levis et al., 1976), aminoacyl-tRNA synthetases (Engelhardt & Sarnoski, 1975; Conta & Meisler, 1977), initiation of protein synthesis (Stanners & Becker, 1971; Rudland et al., 1975; Tanaka & Ichihara, 1977; Meedel & Levine, 1978), chain elongation (Engelhardt & Sarnoski, 1975), or protein degradation (Tanaka & Ichihara, 1976, 1977). However, it has been proposed that the decrease in the level of mRNA could not by itself account for the change in the rate of protein synthesis and that therefore control at the level of translation was involved (Rudland et al., 1975; Meedel & Levine, 1978).

With the exception of the studies by Baenziger et al. (1974) with BHK 21/23, most of the cell-free systems used carried

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[1] Abbreviations used: BHK, baby hamster kidney cell line; CHO, Chinese hamster ovary cell line; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Mops, morpholinopropanesulfonic acid.

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out chain elongation only on endogenous templates or synthetic polynucleotides that did not require the initiation sequence of reactions. A cell-free preparation has been developed in this laboratory from CHO cells that actively and accurately translates endogenous mRNAs and exogenous natural mRNAs or synthetic polynucleotide templates after degradation of endogenous mRNA with nuclease. In nuclease-treated extracts, activity was stringently dependent on added mRNA, ATP, GTP, and a nucleoside triphosphate generating system. Procedures for the analysis of intermediary reactions involved in protein synthesis have also been devised. This communication describes and compares the activities of various reactions in chain initiation, elongation, and termination in extracts prepared from exponentially growing and stationary phase CHO cells.

Experimental Procedures

Preparation of Postmitochondrial Extracts. Chinese hamster ovary cells (CHO, GAT$^-$) were grown at 34 °C in spinner flasks in Minimal Essential Medium Eagle/Alpha Modified (KC Biological, Inc., Lenexa, KS) supplemented with heat-inactivated (55 °C, 30 min) serum (5% fetal calf serum and 5% calf serum), 10 μg/mL each of adenosine and deoxythymidine, and 2.2 mg/mL $NaHCO_3$ (Thompson et al., 1977). Between 1 and 3 L of culture, at a density of (2–4) × 10^5 cells/mL, was centrifuged at 2 °C, 2000*g*, for 5 min and washed 4 times by centrifugation with 50–100 mL of an isotonic solution containing 35 mM Tris-HCl buffer, pH 7.3, 146 mM NaCl, and 11 mM glucose. All subsequent steps were carried out at 2–4 °C. The cells were resuspended in and washed by centrifugation with a hypotonic solution containing 10 mM Tris-HCl buffer, pH 7.3, 15 mM KCl, 6 mM β-mercaptoethanol, and 1.5 mM $Mg(OAc)_2$ and then resuspended in 3 volumes of the same hypotonic solution.

The washed cells were homogenized with 40 strokes in a tight-fitting Dounce glass–glass homogenizer and centrifuged at 30000*g* for 20 min, and 0.1 volume of the following solution was added to the supernatant: 200 mM Hepes–KOH buffer, pH 7.3, 1.2 M KCl, 50 mM $Mg(OAc)_2$, and 60 mM β-mercaptoethanol. The supernatant was then passed through a column (20 × 1.6 cm) of Sephadex G-25 medium previously equilibrated with a solution containing 20 mM Hepes–KOH buffer, pH 7.3, 120 mM KCl, 1.5 mM $Mg(OAc)_2$, and 6 mM β-mercaptoethanol. Two-milliliter fractions were collected from the column, those with the highest absorbance at 260 nm (over 15 A_{260} units/mL) were pooled, and aliquots of ~0.04 mL were frozen by dropping into liquid nitrogen (S-30). The frozen S-30 preparations were stored at −70 °C and were stable for at least 4–6 weeks. Approximately one-third of the total protein was associated with ribosomes and subunits.

Incubation Conditions for Protein Synthesis. The translation of endogenous mRNAs was carried out by incubating S-30 extract as such with the components required for protein synthesis described below in the absence of added template. The translation of exogenous mRNA required a two-step incubation: the first, with micrococcal nuclease (Pelham & Jackson, 1976), degraded endogenous mRNA (S-30′) and the second, with radioactive amino acid and added natural or synthetic templates, measured protein synthesis.

(*a*) *Translation of Endogenous mRNAs.* Five to twenty microliters (50–200 μg of protein) of S-30 extract was incubated with 25 mM Hepes–KOH buffer, pH 7.2 (of which ~7 mM was contributed by other components of the system), 100 mM KCl (including 40 mM K^+ contributed by other components), 2.9 mM $Mg(OAc)_2$ (including 0.54 mM Mg^{2+} contributed by other components), 1 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 20 mM creatine phosphate, 24 μg of creatine phosphokinase, 0.1 mM each of 19 nonisotopic amino acids (excluding leucine), and 1.4 μM [^{3}H]leucine (22000 cpm/pmol). Incubations, in a total volume of 0.05 mL, were at 30 °C for varying periods of time. At the end of the incubation period, the hot (90 °C) 5% trichloroacetic acid insoluble protein fractions were prepared, collected on glass fiber filters, washed, dried, and counted in a scintillation counter (Sadnik et al., 1975).

(*b*) *Translation of Exogenous Natural mRNA.* The first part of the two-step incubation, in a total volume of ~0.04 mL, contained all of the components listed above (under Translation of Endogenous mRNAs) plus 10 units (~0.75 μg) of micrococcal nuclease and 0.25 mM $CaCl_2$, but without radioactive leucine. After 8–10 min at 20 °C, the reaction mixture received EGTA (to 0.5 mM), [^{3}H]leucine (1.4 μM), and 0.2–0.4 μg of globin mRNA [reticulocyte poly(A$^+$) RNA]. The reaction mixtures, in a total volume of 0.05 mL, were incubated at 30 °C, and at the end of the incubation period the hot acid insoluble radioactivity was determined. The globin mRNA was prepared from the polysomes of reticulocyte lysates from acetylphenylhydrazine-treated rabbits chromatographed on oligo(dT)–cellulose columns (Krystosek et al., 1975; Aviv & Leder, 1972) twice.

(*c*) *Translation of Exogenous Synthetic mRNA.* The first incubation with micrococcal nuclease and $CaCl_2$ was carried out as described above (Translation of Exogenous Natural mRNA), except that the Mg^{2+} concentration was raised to 8.5 mM. For the second incubation, the reaction mixtures received EGTA (to 0.5 mM), [^{3}H]phenylalanine (1.4 μM, 5500 cpm/pmol), and 25 μg of poly(uridylic acid). After incubation at 30 °C for 60 min, the hot acid insoluble fractions were prepared and counted.

Sucrose Gradient Centrifugation Analyses. Some reaction mixtures containing nuclease-treated extracts and [^{35}S]-methionine (0.37 μM, 2.54 × 10^5 cpm/pmol) were analyzed by gradient centrifugation to detect intermediates in chain initiation. The equivalent of three incubations (0.15 mL) as described above, but with radioactive methionine instead of leucine or phenylalanine, was combined and incubated for 30 min, with or without globin mRNA. Formaldehyde was then added (to 0.2%), the reaction mixtures were centrifuged through linear 10–30% sucrose gradients (20000 rpm, Spinco SW 41 rotor, 14–16 h), and, after spectrophotometric analysis at 254 nm, individual gradient fractions (0.4 mL) were filtered through Millipore membranes and analyzed for radioactivity (Gasior et al., 1979). Although protein synthesis was linear for over 2 h in this system, analysis for initiation intermediates involved relatively short-term incubations in order to examine initial rates. In some cases, incubations without mRNA were analyzed for [^{35}S]methionine-containing complexes by direct filtration of the reaction (0.05 mL) through Millipore membranes (Sadnik et al., 1975); the incubations were analyzed without fixation with formaldehyde. Control experiments with and without formaldehyde treatment, in which analyses were carried out by gradient centrifugation of shorter times (40000 rpm, 3.5 h) or by filtration of the reaction mixtures directly through Millipore, indicated that formaldehyde had no quantitative or qualitative effect on the binding of [^{35}S]-methionine to translational components; its use was primarily to prevent degradation of methionine-containing complexes during long (14–16 h) centrifugations.

Analyses of Elongation Factors. Determination of elongation factor activities in cell extracts was carried out by measuring the capacity of "high-salt" cytosol to translate

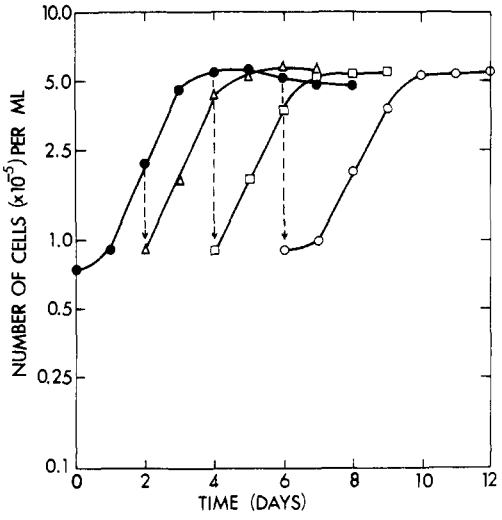

FIGURE 1: Growth of Chinese hamster ovary cells in spinner cultures. 250 mL containing ~0.8 × 10^5 cells/mL was incubated for 8 days as described in the text, and the cell density was determined daily (closed circles). A sample of exponentially growing cells (50 mL, containing ~2.5 × 10^5 cells/mL) was taken from the culture after 2 days, diluted into fresh media to a final concentration of 0.9 × 10^5 cells/mL, and incubated under similar conditions; the cell density was determined daily (open triangles). A sample of early stationary phase cells (50 mL, containing ~6 × 10^5 cells/mL) was taken from the culture after 4 days, diluted into fresh media to a final concentration of 0.9 × 10^5 cells/mL, and incubated with daily cell density analyses (open squares). A sample of late stationary phase cells was similarly diluted, incubated, and analyzed (open circles).

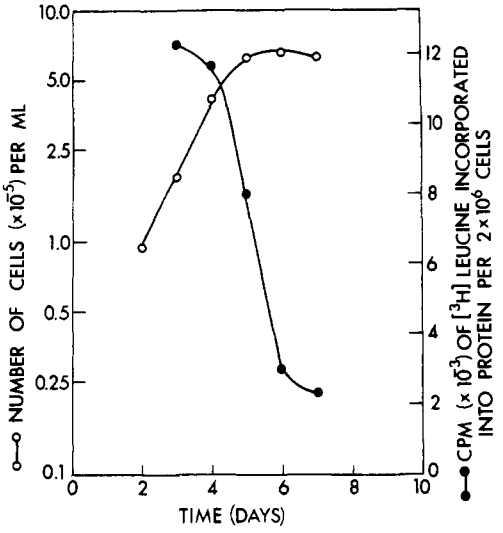

FIGURE 2: Amino acid incorporation into cultured CHO cells. Aliquots containing approximately equivalent concentrations of cells (2 × 10^6 cells/mL) obtained from the exponential phase (third and fourth day of culture) and from the stationary phase (fifth to seventh day of culture), as represented by the open circles, were incubated with [^{3}H]leucine (1.4 μM; 22 000 cpm/pmol) for 60 min at 34 °C. The hot acid insoluble protein fractions (closed circles) were then prepared and counted.

poly(U) with radioactive phenylalanyl-tRNA and rat liver ribosomes (Moldave et al., 1979). The high-salt or "0.5 M KCl" cytosol, containing practically all of the elongation factor activity, was prepared from the postmitochondrial extract by adding KCl to 0.5 M and centrifuging at 100000*g* for 3 h to remove the ribonucleoprotein particles. Varying concentrations of the cytosol were incubated with 100 μg of purified rat liver ribosomes or 0.3 A_{260} unit each of derived 40S and 60S ribosomal subunits (Moldave & Sadnik, 1979), 30 mM Mops (pH 7.3), 0.2 mM GTP, 80 mM NH_4Cl, 6 mM $MgCl_2$, 2 mM dithiothreitol, 30 μg of [^{3}H]Phe-tRNA (4500 cpm/pmol of tRNA-bound phenylalanine), and 25 μg of poly(U); the total volume was 0.5 mL. After 20 min at 37 °C, the poly(phenylalanine) synthesized was determined by the hot (90 °C) trichloroacetic acid fractionation procedure. This method measured the combined activity of factors EF-1 and EF-2 in the cytosol. In some experiments, the activity of EF-1 was assayed by incubating varying concentrations of high-salt cytosol, as described above, in the presence of excess amounts of partially purified rat liver EF-2 (Moldave et al., 1971), completely resolved from EF-1. The concentration-dependent synthesis of poly(phenylalanine), obtained with varying amounts of high-salt extract (S-100), measured the EF-1 that was contributed by the cytosol, and an apparent specific activity could be estimated from the ratio of cytosol protein concentration vs. phenylalanine incorporated in the linear portion of the curve. Similarly, EF-2 activity was assayed in incubations containing varying conentrations of S-100 and excess amounts of partially purified rat liver EF-1 (Moldave et al., 1971), completely resolved from EF-2.

Results

The growth characteristics of the CHO cells used in these experiments are presented in Figure 1 (closed circles). Samples for analysis of protein synthetic activity in intact cells or in cell-free systems were obtained from the exponential phase of growth (for example, between the first and third day of culture) and from the stationary phase (for example, after the fourth day of culture). When cells that were ~24 h into the stationary phase were diluted into fresh media, as shown by the broken lines at 4 days, and incubated further, the growth characteristics (open squares) resembled those obtained when exponentially growing cells were diluted (broken lines at 2 days) and allowed to grow (open triangles). The diluted cells from these two phases of culture grew exponentially and did not exhibit a lag period, indicating that cells obtained early in the stationary phase of culture were viable and apparently physiologically normal. When cells that were 3 to 4 days in the stationary phase (6-day sample) were diluted with fresh media and incubated, a lag was observed in the growth pattern (open circles). Therefore, for most of the experiments described below, cells representative of the exponential phase were obtained from the linear portion of the growth curve and the cells representative of the stationary phase were obtained ~24 h after the growth curve had leveled off.

Cessation of growth under these conditions appeared to be due to the depletion of some essential component or components in the media supplied by serum. The addition of fresh serum to cultures whose growth had leveled off caused an increase in the number of cells. The media obtained by centrifugation from incubations in which cells had ceased to grow did not support the growth of new cells added at low densities.

The protein synthesizing activity of CHO cells, from the exponential and stationary phases of the culture described above, was compared by incubating intact cells with radioactive leucine. The results (Figure 2) indicated that the two samples representing the exponentially growing cells (third and fourth day of culture, open circles) incorporated ~12 000 cpm of radioactive leucine into protein (closed circles); the cells obtained at the beginning of the stationary phase (fifth day of culture) incorporated 60% less radioactive leucine than the exponentially growing cells, and the specific activity of cells that were 24 and 48 h into the stationary phase (sixth and seventh day of culture, respectively) was 20% of that obtained in the exponentially growing cells.

The protein synthesizing activities of cell-free extracts from

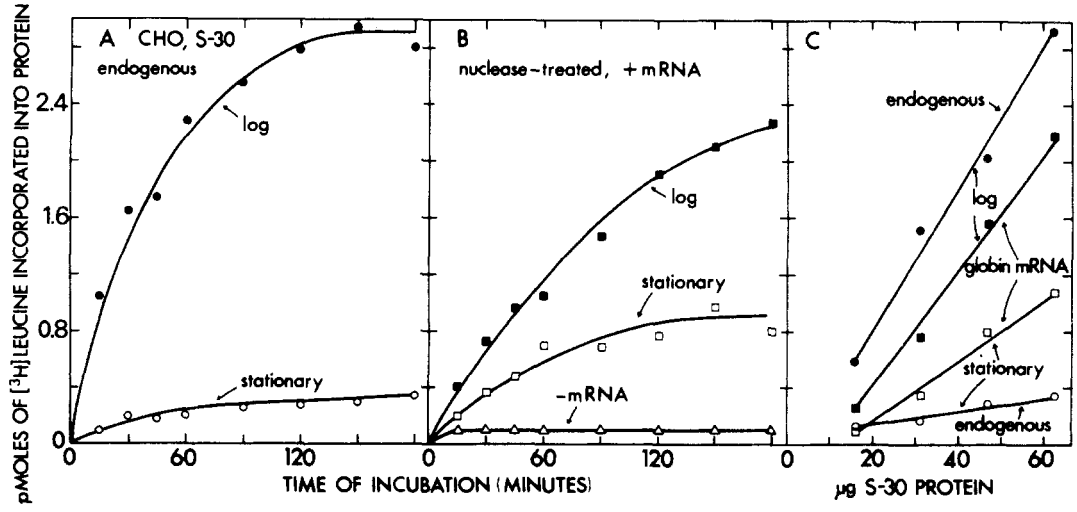

FIGURE 3: Time- and concentration-dependent translation of endogenous and exogenous mRNA in extracts from exponential and stationary phase CHO cells. (A) Postmitochondrial extracts from exponentially growing (closed circles) and stationary phase (open circles) cells, not treated with micrococcal nuclease, were incubated with radioactive leucine, without added mRNA, for varying periods of time up to 3 h, as described in the text. The incubations were analyzed for hot acid insoluble radioactivity. (B) Postmitochondrial extracts depleted of mRNA from exponentially growing (closed squares) and stationary phase (open squares) cells were incubated with radioactive leucine and globin mRNA for varying periods of time up to 3 h, as described in the text. The open triangle curve represents incubations containing extract from exponential cells, without mRNA. (C) Varying concentrations of postmitochondrial extract from exponentially growing cells (closed symbols) and from stationary phase cells (open symbols) were assayed for translation of endogenous (circles) and exogenous (squares) mRNA as described in the text and panels A and B; incubations were for 2 h.

cells in the exponential and stationary phases of growth, on endogenous templates or with exogenous mRNA, were compared (Figure 3). Panel A shows the results obtained when the postmitochondrial extracts were incubated with nucleotides, a nucleoside triphosphate generating system, a mixture of amino acids, and radioactive leucine but without exogenous mRNA; both the initial rate and the total extent of amino acid incorporation were markedly greater in the S-30 extract from exponential cells (closed circles) than from stationary cells (open circles). The initial rate of synthesis on endogenous templates was 0.07 pmol of leucine incorporated per min with extracts from exponentially growing cells and less than 0.007 pmol/min with extracts from stationary phase cells. After 3 h of incubation, exponential cell extracts had incorporated almost 3 pmol of leucine into protein while the stationary cell extracts had incorporated between 0.3 and 0.4 pmol. When the postmitochondrial extracts were treated with micrococcal nuclease to remove endogenous mRNA and then incubated as described above but in the presence of saturating concentrations of globin mRNA (panel B), the initial rate and the total extent of amino acid incorporation were still significantly higher in cells from the exponential phase (closed squares) than from the stationary phase cells (open squares). The initial rate of synthesis with exponential cell extracts was ~0.026 pmol of leucine incorporated per min, and that with stationary cell extracts was ~0.012 pmol/min. In the absence of added exogenous mRNA, amino acid incorporation in both extracts was extremely low (open triangles). Panel C shows that the translation of endogenous (circles) and exogenous (squares) templates was markedly more extensive with exponential cell extracts (closed symbols) than with stationary cell extracts (open symbols) at all concentrations of extract assayed (between about 15 and 65 μg of protein); thus, incorporation of amino acids into protein in extracts containing endogenous mRNA was six- to eightfold greater in exponential phase preparations (closed circles) than in stationary phase preparations (open circles); similarly, incorporation in mRNA-depleted extracts was two- to fourfold higher in preparations from exponential phase cells supplemented with reticulocyte mRNA (closed squares) as compared to that of stationary phase

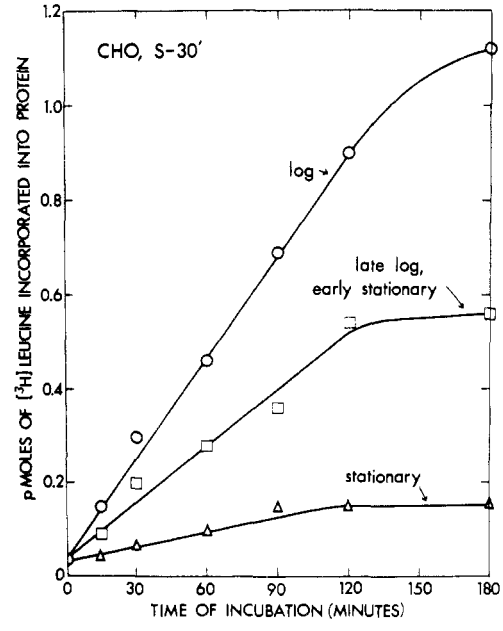

FIGURE 4: Translation of exogenous mRNA in extracts from CHO cells in various phases of growth. Postmitochondrial extracts were prepared from mid log (Figure 1, second day of culture), late log (Figure 1, third day of culture), and stationary (Figure 1, fifth day of culture) cells depleted of mRNA and incubated with radioactive leucine and globin mRNA for varying periods up to 3 h, as described in the text. The hot acid insoluble proteins from mid log (circles), late log (squares), and stationary (triangles) cells were prepared and analyzed for radioactivity.

preparations incubated with exogenous mRNA (open squares).

The decrease in the cell-free protein synthetic activity detected when the CHO cultures went from the exponential to the stationary phase appeared to be time dependent, as presented in Figure 4. The postmitochondrial S-30 extract was prepared from exponentially growing (at mid log) cells, from cells approximately just prior to entering or just entering the stationary phase, and from cells ~24–48 h into the stationary phase and the extracts were depleted of endogenous mRNA. On incubation in the complete system, in the presence

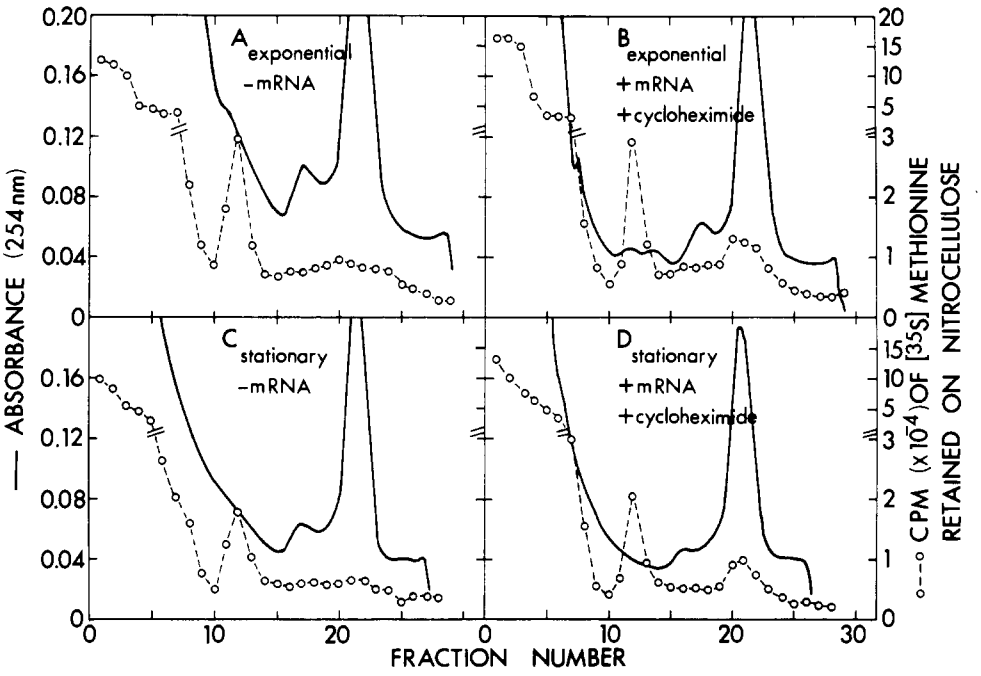

FIGURE 5: Gradient centrifugation pattern of [^{35}S]methionine incubated with mRNA-depleted postmitochondrial extracts from exponential phase cells in the absence of mRNA (A) and in the presence of globin mRNA plus cycloheximide (B) and extracts from stationary phase cells in the absence of mRNA (C) and in the presence of mRNA plus cycloheximide (D). Incubations at 30 °C for 60 min, components, and centrifugation and analytical procedures were as described in the text. The solid lines represent the optical density pattern obtained automatically at 254 nm with a recording spectrophotometer. The broken lines (open circles) represent the radioactive methionine in each gradient fraction retained on Millipore membranes.

of globin mRNA, amino acid incorporation with all three extracts was linear for ~2 h. However, the initial rate and total extent of mRNA translating activity of the "late log" phase cell extracts (squares) were ~50% lower, and those from the stationary phase (triangles) were ~90% lower, as compared to extracts from exponentially growing cells (circles). For example, the initial rates calculated from the data with exponential, late log, and stationary cell preparations were 0.011, 0.006, and 0.001 pmol of leucine incorporated per min, respectively.

The results presented above suggested that, in addition to a decrease in the amount of protein synthesis dependent on endogenous polysome-associated mRNA, the activity or the concentration of a component or components of the translational apparatus was lower in stationary phase cells than in exponentially growing cells. The difference in translational activity could reflect a specific change in the initiation, elongation, or termination reactions in protein synthesis or a more generalized effect involving more than one step. Therefore, a number of activities involved in translation were assayed in mRNA-depleted extracts from exponentially growing and stationary phase cells. The early steps in initiation, up to the formation of the 40S·ternary complex intermediate, were assayed by incubating nuclease-treated extracts for 40 min with [^{35}S]methionine, in the absence of mRNA, followed by direct filtration of the reaction mixtures through Millipore membranes. When varying concentrations of mRNA-depleted extracts from exponential and stationary phase cells were analyzed, not shown in detail here, the concentration-dependent radioactivity retained on nitrocellulose appeared to be essentially the same with both preparations. The radioactive components retained on nitrocellulose membranes included complexes of Met-tRNA$_f$ with Met-tRNA ligase, eIF-2 plus GTP, and 40S·eIF-2·GTP. The resolution and extent of formation of the 40S·ternary complex intermediate, in mRNA-depleted extracts, are described in Figure 5. Extracts from exponentially growing (panel A) and stationary phase (panel C) cells were incubated with [^{35}S]methionine in the absence of exogenous mRNA and then centrifuged through sucrose gradients (Gasior et al., 1979). Two methionine-containing complexes were detected by filtration of gradient fractions on Millipore membranes: one of them was obtained in the region of the cytosol proteins toward the top of the gradient and the other was in the 40S ribosomal subunit region, in fractions numbered 10–15. The peak in the absorbance pattern usually seen in the region of the 40S subunits (Gasior et al., 1979) is obscured by the high concentration of cytosol in these preparations and ultraviolet-absorbing components in the incubation. In this experiment, the amount of [^{35}S]methionine associated with 40S subunits appeared to be ~30% lower with stationary phase extract (panel C) than with exponentially growing extract (panel A); several such experiments revealed that the differences between the two preparations were less marked, within 20%, and in one case the amount of 40S intermediate with stationary cell extracts was 15–20% higher than that from exponential cells.

Incubations and analyses similar to those described above were carried out with extracts from exponential and stationary phase cells in the presence of globin mRNA and cycloheximide to allow formation of the 80S initiation complex but to prevent its utilization (Figure 5, panels B and D). An additional methionine-containing complex was detected in the 80S ribosome region, in fractions numbered 20–25. Comparison of the sedimentation patterns obtained with exponential (panel B) and stationary (panel D) phase extracts revealed that the amounts of radioactive methionine associated with 80S ribosomes, as a proportion of the total amount of methionine bound to ribonucleoprotein particles (40 S plus 80 S), were very similar; in each case, ~50% of the total particle-bound radioactivity was recovered in the 80S initiation complex.

In addition to the analyses of chain initiation reactions in extracts from exponential and stationary phase cells described above, elongation reactions were also analyzed. Polypeptide chain elongation activity, as reflected by the poly(U)-dependent

Table I: Effect of Cytosol from Exponentially Growing Cells on the Poly(U)-Dependent Synthesis of Poly(phenylalanine) with Postmitochondrial Extracts from Stationary Phase CHO Cells

incubation components[a]	pmol of [^{3}H]Phe incorpd into protein
S-30′ (exponential) + poly(U)	18.5
S-30′ (stationary) + poly(U)	5.4
S-30′ (stationary) + poly(U) + S-100 (exponential)	14.9

[a] Incubation mixtures contained micrococcal nuclease treated CHO S-30′ extract from exponentially growing or stationary phase cells and all the other components described in the text [plus $Mg(OAc)_2$ to 8.5 mM], including 1.4 μM [^{3}H]phenylalanine and 25 μg of poly(uridylic acid); one reaction also contained 50 μg of 100000g supernatant protein (S-100), obtained by ultracentrifugation of S-30′ from exponentially growing cells after addition of KCl to 0.5 M. Incubations were at 30 °C for 60 min. In the absence of poly(U), less than 0.4 pmol of [^{3}H]phenylalanine was obtained in the acid-insoluble precipitate.

synthesis of poly(phenylalanine), was examined in nuclease-treated extracts from exponential and stationary phase cells (Table I). The activity of postmitochondrial extracts from exponentially growing cells (line 1) was over 3 times greater than that from stationary phase cells (line 2). As shown in line 3, when ribosome-free cytosol from exponentially growing cells was added to incubations containing stationary phase postmitochondrial extract, incorporation was markedly stimulated and approached that obtained with exponential cell S-30′ extract.

Figure 6 describes the results of experiments designed to assay elongation factor activities in the cytosol of a number of samples taken from different phases of the culture. Cells were obtained from the stationary phase at the end of the first growth cycle (panel A, triangle, 4 days; designated as stationary phase no. 1), 3 h after dilution of stationary phase no. 1 cells into fresh media (square, lag phase), 24 h after dilution (open circle, exponential phase), and at the end of the second growth cycle (diamond, 8 days, stationary phase no. 2). Varying concentrations of the high-salt cytosol extract prepared as described above (Experimental Procedures) were assayed for their ability to catalyze chain elongation (Figure 6, panel B) as measured by the poly(U)-dependent synthesis of poly(phenylalanine) with [^{3}H]Phe-tRNA in the presence of purified rat liver 40S and 60S subunits. The concentration-dependent EF-1 plus EF-2 activity in the cytosol from the exponentially growing cells (circles) was higher than that of the stationary phase no. 1 cells (triangles) or the lag phase cells (squares). The specific activities calculated from these data indicated that ~0.33 pmol of [^{3}H]phenylalanine was incorporated per μg of "exponential" cytosol protein, as compared to 0.13 pmol for the stationary phase no. 1 and 0.14 pmol for the lag phase cytosol. When incubations contained excess amounts of completely resolved, partially purified, rat liver EF-2, they measured the EF-1 activity in the cytosol (panel C). The concentration-dependent activity of EF-1 in exponentially growing cells (circles) was greater than that of lag phase (squares) or stationary phase (triangles and diamonds) cells; the specific activity of the exponential phase EF-1 was ~0.51 pmol of [^{3}H]phenylalanine incorporated from radioactive Phe-tRNA into protein per μg of cytosol protein, while the specific activity of the EF-1 in the other cytosols was ~0.22 pmol/μg. In contrast, incubations in the presence of excess amounts of EF-1, to measure EF-2 activity (panel D), indicated that the cytosols from the exponential, lag, and

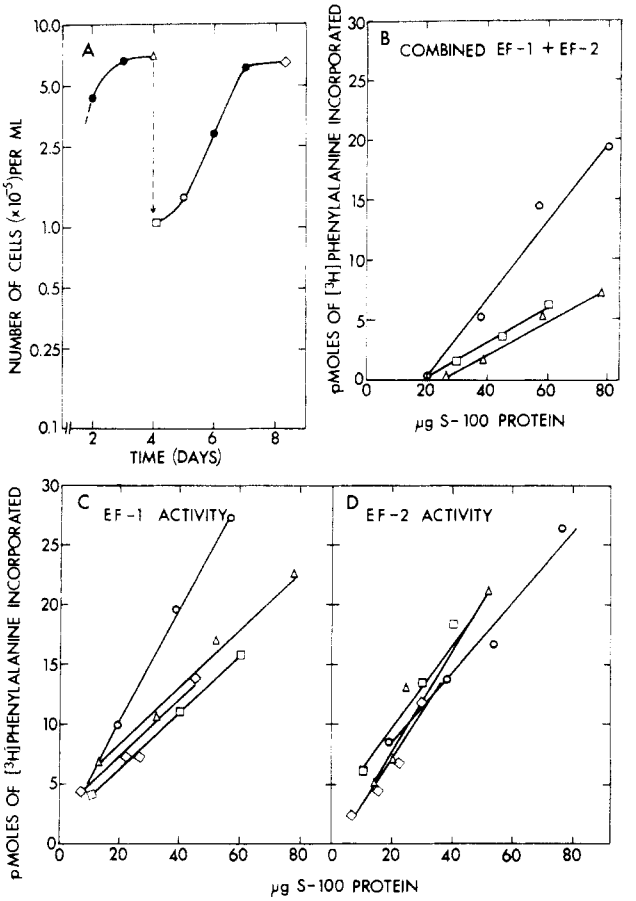

FIGURE 6: Analysis of elongation factor activities in the cytosol fraction of CHO cells from various growth phases. (A) Growth pattern of CHO cells in spinner cultures. Samples for elongation factor analyses were taken at the first stationary phase (triangle, fourth day of culture), at the lag phase after dilution of the first stationary phase cells into fresh media (square), during the exponential phase (open circle, fifth day of culture), and at the second stationary phase (diamond, eighth day of culture). The cytosols were prepared from each, and varying concentrations were assayed in the poly(U)-dependent poly(phenylalanine) synthesis system with rat liver ribosomes, as described in the text, for total elongation factor activity (B), EF-1 activity, in the presence of excess EF-2 (C), and EF-2 activity, in the presence of excess EF-1 (D).

stationary phases of the culture exhibited essentially the same EF-2 activity.

Control experiments with rat liver ribosomes, poly(U), and Phe-tRNA revealed that the synthesis of poly(phenylalanine) with either EF-1 or EF-2, individually, was less than 1% of that obtained with EF-1 plus EF-2. Also, the addition of cytosol from stationary cells to incubations containing rat liver ribosomes and EF-1 plus EF-2 did not inhibit poly(phenylalanine) formation and the addition of cytosol from exponential cells did not stimulate poly(phenylalanine) formation. These findings suggested that stationary cells did not contain an inhibitor of elongation and that the EF-1 and EF-2 were added in saturating concentrations. Other control experiments indicated that although EF-1 and EF-2 were added in saturating concentrations, they were not present in vast excess.

When 3 μg of highly purified rat liver EF-1 was added to incubations containing mRNA-depleted postmitochondrial extract from stationary phase cells, poly(U), [^{3}H]phenylalanine, etc., the synthesis of poly(phenylalanine) was stimulated over 70%; thus, in the presence of purified EF-1, the activity of stationary cell S-30′ extract in chain elongation was restored almost to the level (within 20%) of that obtained with exponential cell extracts.

Table II: Comparison of Polypeptide Chain Elongation Activity in Postmitochondrial Extracts from Exponentially Growing and Late Stationary Phase CHO Cells

	sp act. of cytosol[b]		
incubation components[a]	combined EF-1 and EF-2	EF-1	EF-2
S-100 (exponential)	0.51	0.49	0.76
S-100 (stationary, 3 days)	0.31	0.20	0.71

[a] Incubation mixtures contained 100000*g* 0.5 M KCl supernatant protein (S-100) obtained by ultracentrifugation from exponentially growing cells or from cells 3 days into the stationary phase, rat liver derived 40S and 60S ribosomal subunits, [^{3}H] phenylalanyl-tRNA, poly(U), and other components as described in the text. [b] The specific activities, calculated from the linear portion of the concentration curves, are expressed as picomoles of radioactive phenylalanine incorporated from [^{3}H] Phe-tRNA into protein per microgram of cytosol protein. The incubation mixtures contained cytosol (for assaying combined EF-1 and EF-2), cytosol plus 20 μg (Moldave et al., 1971) of partially purified, resolved EF-2 (for assaying EF-1 activity), or cytosol plus 30 μg (Moldave et al., 1971) of partially purified, resolved EF-1 (for assaying EF-2 activity).

An experiment was carried out to determine whether the change in the activity of elongation factor EF-1 between exponential and stationary phases was specific and not merely an early reflection of changes in cell viability (Table II). The activities of elongation factors in exponentially growing cells were compared with those in cells taken 3 days after onset of the stationary phase. The specific activities of the combined elongation factors and of EF-1 were markedly higher in the exponentially growing cells (line 1) than in "3-day stationary" phase cells (line 2); however, even after 3 days, the specific activity of EF-2 in the stationary phase cytosol remained almost the same as that in the exponential phase cytosol.

The termination sequence of reactions in protein sythesis was measured by the release of completed polypeptide chains from polysomes to the incubation media. Large-scale incubations of nuclease-treated extracts, from exponential and stationary cells, were carried out with [^{3}H]leucine, globin mRNA, and the other components required for translation of mRNA; the ribonucleoprotein fraction was separated from the soluble supernatant fraction by ultracentrifugation, and the acid-insoluble proteins were isolated and counted. The results, summarized in Table III, indicated that the total amount of radioactive protein (combined ribonucleoprotein and cytosol fractions) synthesized with exponential cell extracts was significantly greater than that synthesized with extracts from stationary phase cells; however, the proportion of radioactivity released into the cytosol in both preparations was the same, between 85 and 90%.

Table III: Release of Radioactive Protein from Ribonucleoprotein Particles in Postmitochondrial Extracts from Exponentially Growing and Stationary Phase CHO Cells[a]

	pmol of [^{3}H] Leu recovered in protein	
cell fraction	exponential	stationary
ribonucleoprotein	0.57	0.14
cytosol	3.27	1.14

[a] The incubation mixtures containing [^{3}H] leucine, globin mRNA, and nuclease-treated S-30′ extract from log or stationary phase cells, as described in the text, were incubated for 2.5 h and then diluted with an equal amount of buffered-salt solution and centrifuged at 100000*g* for 3 h. The hot (90 °C) trichloroacetic acid insoluble fractions from the supernatant cytosols and from the sedimented ribonucleoproteins were prepared and counted.

Discussion

As Chinese hamster ovary cells in culture pass from the exponential phase to the stationary phase, the protein synthetic activity as determined by amino acid incorporation with whole cells decreases drastically, between 80 and 90% within 24 h. A marked difference in biosynthetic activity is also obtained when postmitochondrial cell-free systems from exponential and stationary cells are tested for amino acid incorporation on endogenous mRNA templates; translation of endogenous mRNAs is 80–90% lower with stationary cell extracts than with exponential cell extracts. Other observations, not presented in detail here, reveal that the polysome patterns obtained when exponential and stationary cell extracts are analyzed by gradient centrifugation are distinctly different; whereas S-30 from exponential cells reveals polysomes containing up to at least 8 ribosomes/polynucleotide template, the S-30 from stationary cells reveals only 80S ribosomes and 40S plus 60S ribosomal subunits. Indeed, the ribosome pattern exhibited by stationary cell extracts is similar to that obtained after treatment of exponential cell S-30 with micrococcal nuclease to degrade endogenous mRNA. Thus, the amount of mRNA in stationary phase cells or the ability to translate endogenous mRNAs appears to be decreased. This finding is in agreement with previous reports (Johnson et al., 1974; Rudland et al., 1975; Levis et al., 1976; Meedel & Levine, 1978) and may be the consequence of a change in the rate of production of mRNA, increased turnover, or a change in a posttranscriptional step. The quantitative analysis of polyadenylated and/or functional mRNAs in exponential and stationary cells is in progress.

In addition to the decrease in protein synthesis that could reflect changes in the amount of endogenous mRNAs, however, evidence that changes occur in the activity of the translational system itself is obtained when the translation of exogenous messenger RNAs is examined. When mRNA-depleted (nuclease-treated) extracts are assayed with amounts of globin mRNA that are not limiting, the initial rate and the total extent of translation are ~70% lower in stationary cell S-30′ as compared to exponential cell S-30′. These findings suggest that the activity of a component or components of the translational machinery of the cell is altered in the stationary phase; these alterations could involve one or more steps at the level of chain initiation, elongation, or termination.

The formation of the 80S initiation complex requires a number of intermediary steps such as the formation of Met-tRNA$_f$, synthesis of the ternary complex eIF-2·Met-tRNA$_f$·GTP, interaction of the ternary complex with 40S ribosomal subunits, binding of mRNA, and joining of 60S subunits. When mRNA-depleted extracts from exponential or stationary cells are incubated with [^{35}S]methionine in the absence of mRNA, the total amount of radioactive methionine-containing complexes retained on nitrocellulose membranes is the same. In the absence of mRNA, the radioactivity retained on the filters represents Met-tRNA$_f$ bound to methionyl-tRNA ligase, ternary complex, and 40S·ternary complex intermediate, but complexes beyond the 40S intermediate are not formed; the 40S intermediate, however, constitutes only a small percent of the methionine-containing complexes formed without mRNA. Sucrose gradient analyses of these incubations indicate that the formation of the 40S·methionine complex in extracts from exponential and stationary cells is not markedly different. In most cases, the values for 40S-bound [^{35}S]methionine are within 20% or at most 30%, which would not appear to account for the two-

to threefold change in translation observed in this system. When exogenous mRNA is added to incubations containing either exponential or stationary phase S-30′ and cycloheximide is present to prevent utilization of the 80S initiation complex, ~40–50% of the radioactive methionine is transferred from the 40S to the 80S complex. This observation indicates that the activities of components required for binding of mRNA to the 40S·ternary complex intermediate and for the joining of 60S subunits to form the 80S initiation complex are similar in S-30′ extracts from exponentially growing and stationary phase cells. Thus, analyses of the intermediary steps in polypeptide chain initiation, from the aminoacylation of $tRNA_f^{Met}$ to the formation of 80S initiation complex, suggest that this phase of protein synthesis is not significantly affected when cells transit from the exponential to the stationary phase and does not account for the shift in the polysome pattern or the decrease in translational activity.

Polypeptide chain elongation, with the 80S initiation complex associated with natural mRNA or with ribosomes and synthetic polynucleotide templates that do not require the initiation sequence of reactions, involves elongation factors EF-1 and EF-2. Elongation factor EF-1 and GTP catalyze the binding of aminoacyl-tRNAs to the A site of posttranslocated ribosomes, ribosomal peptidyltransferase catalyzes the formation of a peptide bond between the peptidyl moiety at the P site and the aminoacyl moiety at the A site, and EF-2 and GTP then catalyze the translocation of newly formed peptidyl-tRNA from the A to the P site. The poly(U)-dependent formation of poly(phenylalanine) in mRNA-depleted S-30′ extracts reflects peptide chain elongation reactions. By use of extracts from stationary phase cells, translation of poly(U) is ~70% lower than with exponential cell extracts. Cytosol free of ribonucleoprotein particles from exponentially growing cells stimulates poly(phenylalanine) synthesis with stationary cell postmitochondrial extracts; this finding and other data presented here indicate that the lower activity in stationary cells is not due to the presence of an elongation inhibitor or to defective ribosomes but to a change in the activity of one or both elongation factors. A number of experimental findings are consistent with the interpretation that the decrease in peptide chain elongation obtained with stationary cells is due specifically to a decrease in EF-1 activity. When the ribonucleoprotein-free cytosols are analyzed for individual elongation factor activities, using the poly(U) assay with exogenous ribosomes and Phe-tRNA, EF-1 activity in stationary cells is markedly lower than that in exponential cells, while the activity of EF-2 is the same (within 10%) in both extracts; the addition of EF-1 that is completely free of EF-2 to incubations containing stationary cell cytosol restores poly(U) translation with Phe-tRNA almost to the level observed with exponential extracts; also, the addition of highly purified EF-1 to postmitochondrial extracts (S-30′) from stationary cells restores poly(U) translation with phenylalanine almost to the level observed with exponential extracts.

Termination of protein synthesis involves the interaction of a protein factor (RF) and GTP at the A sites of ribosomes whose P sites are occupied by the completed polypeptide chain in the form of peptidyl-tRNA; this leads to the hydrolysis and release of the peptidyl moiety. The assay used here, which measures the amount of radioactive protein released into the cytosol in the presence of all of the components required for translation, reflects this sequence of reactions. Although postmitochondrial extracts from stationary cells translate globin mRNA to a lesser extent than similar extracts from exponential cells, the proportion of radioactive protein that is released from the polysomes is exactly the same, between 85 and 90%. This finding indicates that the RF-dependent recognition of the termination codon and the hydrolysis of the completed polypeptide chain from peptidyl-tRNA, which requires the ribosomal peptidyltransferase reaction, are not affected in the transition from the exponential to the stationary phase.

The results described above indicate that at least two processes required for translation are significantly altered in stationary cell extracts as compared to exponentially growing cells. One is the availability of mRNA, which is reflected in polysome disaggregation and the decrease in endogenous protein synthesis dependent on polysome-associated mRNA; the other is the activity of EF-1, which is required for the binding of aminoacyl-tRNAs in chain elongation and is markedly decreased in stationary cells. The other reactions involved in protein synthesis, such as the formation of ternary complex with eIF-2, interaction with 40S subunits, binding of mRNA, joining of 60S subunits, peptidyltransferase, translocation, termination, and release, do not appear to be significantly different in the two culture phases examined. Changes in elongation factor activity, particularly EF-1, have been reported in a number of systems. For example, increases in EF-1 activity have been observed in cold-adapting fish (Haschemeyer, 1969) and in response to immune challenge (Willis & Starr, 1971) or laser irradiation injury (Nicholls, 1973) in rats; decreases in EF-1 activity have been obtained after thyroidectomy in rats (Nielsen et al., 1976) and in cultured cells whose growth has been arrested by serum deprivation or high cell density (Engelhardt & Sarnoski, 1975; Hassell & Engelhardt, 1976). These results and the findings presented in this report that EF-1 activity varies during cell growth while other reactions involved in translation do not suggest that this factor may play a central role in the regulation of translation; it is possible, however, that this role may be limited to some cells under certain conditions and that regulation under different circumstances may involve other components of translation. The change in EF-1 reported above could reflect a decrease in its synthesis, or an increase in its degradation, or the inactivation of the factor. Studies designed to determine whether metabolic turnover can account for these results or the nature of the inactivation of this factor, which interacts with a number of other components of the translation system, are in progress.

Cell division and growth are also markedly restricted at very high cell density, even when cells are presented continuously with fresh media. Experiments similar to those described here, to determine whether the pattern of growth observed when fresh media is added frequently to the cells affects translation, are also under investigation.

Acknowledgments

The assistance of Eva Mack and Wayne Sabo is gratefully acknowledged.

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Group Fractionation and Determination of the Number of Ribosomal Subunit Proteins from *Drosophila melanogaster* Embryos†

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ABSTRACT: Proteins were extracted from ribosomes and (for the first time) from ribosomal subunits of *Drosophila melanogaster* embryos. The ribosomal proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis. The electrophoretograms displayed 78 spots for the 80S monomers, 35 spots for the 60S subunits, and 31 spots for the 40S subunits. On the basis of present information, we propose what we believe to be a reliable and convenient nomenclature for the proteins of the ribosomes and each of the subunits. A pair of acidic proteins from *D. melanogaster* appears to be very similar in electrophoretic mobility to the acidic proteins L7/L12 from *Escherichia coli* and L40/L41 from rat liver. The electrophoretogram of proteins from embryonic ribosomes shows both qualitative and quantitative differences from those of larvae, pupae, and adults previously reported by others. The proteins of the 40S subunit range in molecular weight from approximately 10000 to 50000, and those from the 60S subunit range from approximately 11000 to 50000.

The proteins of the 80S ribosomes were fractionated by stepwise elution from carboxymethylcellulose (CMC) with lithium chloride. The proteins were separated into seven groups (A–G) containing between 9 and 23 proteins each. Small samples were removed from every fifth fraction of the CMC column and analyzed by one-dimensional electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate ($NaDodSO_4$). Further fractionation of the proteins of the 80S ribosome was achieved by pooling adjacent fractions from the CMC column that shared similarities in their $NaDodSO_4$ profiles. Twenty-four pools were obtained. From the analysis of these pools by two-dimensional polyacrylamide gel electrophoresis, it was found that each pool contained between 1 and 12 major proteins. Most proteins occurred in only one pool each. This fractionation procedure has proved valuable as an initial step in the isolation and characterization in *Drosophila* proteins.

The purification and characterization of eucaryotic ribosomal proteins are important requisites for an understanding of the structure and function of ribosomes at the molecular level. Although much is known about the morphological, chemical, and immunological characteristics of procaryotic ribosomes, little is known of those from eucaryotes [see Wool & Stöffler (1974), Wittmann (1974), and Wool (1979) for reviews and references]. *Drosophila* nucleic acids have been extensively studied (Tartof, 1975; White & Hogness, 1977; Laird & Chooi, 1976), but far less information is available about their ribosomal proteins. Large differences have been detected between the protein patterns of *Drosophila* larval,

† From the Department of Biology, Molecular, Cellular and Developmental Biology Program, Indiana University, Bloomington, Indiana 47401. *Received July 27, 1979.* This work was supported by grants from the National Science Foundation (PCM 77-17654) and the National Institutes of Health (PHS R01 GM23410-02) awarded to W.Y.C.
‡ Recipient of a predoctoral fellowship from the Molecular, Cellular and Developmental Biology Training Grant (PHS T32 CMO7227-04).

0006-2960/80/0419-1425$01.00/0