Polysome-Dependent in Vitro Translation System Capable of Peptide Chain Reinitiation†

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ABSTRACT: A sensitive in vitro translation system has been developed which makes use of cellular polysomes as the source of mRNA and ribosomes. The soluble factors are derived from the preincubated S-30 fraction by centrifugation through a discontinuous sucrose gradient. Of the four fractions tested, fraction 1 (topmost fraction in the gradient) and fraction 2 (fraction sedimenting in 0.5 M sucrose) were stimulatory. These two fractions together yield the highest activity, corresponding to about 125 times the background incorporation. The polysome-directed system exhibits optimal activity in the range 1.8–2 mM Mg^{2+} and 125–175 mM KCl. The polysome-directed in vitro products exhibit a complexity comparable to the in vivo products resolved on the two-dimensional polyacrylamide gels of O'Farrell [O'Farrell, P. (1975) *J. Biol. Chem. 250*, 4007–4021]. The system is capable of active chain reinitiation as indicated by partial inhibition by 7-methylguanosine 5'-monophosphate and pactomycin and N-terminal end analysis of in vitro products. This system can also translate polysomes from diverse tissues such as mouse liver, rat liver, and rat brain. The levels and also the authenticity of translation of rat liver albumin and mouse liver carbamoyl phosphate synthetase I were tested by immunoprecipitation with monospecific antibodies. The results show that the major as well as the minor translation products are synthesized in this system at levels comparable to the physiological levels.

In recent years, there has been remarkable progress in the development and characterization of in vitro translation systems [for reviews, see Shafritz (1977) and Revel & Groner (1978)]. Extracts consisting of ribosomes and factors capable of peptide chain initiation, elongation, and completion have been prepared from various eukaryotic sources (Marcus et al., 1970; Lockard & Lingrel, 1969; Aviv et al., 1971; Sampson et al., 1972; Thompson et al., 1973; Pelham & Jackson, 1976). These systems can actively translate purified homogeneous mRNAs as well as heterogeneous populations of poly(A)[1]-containing mRNA species with a high degree of fidelity [for details, see Shafritz (1977)]. Such mRNA-directed systems have therefore provided invaluable methods for the analysis and characterization of gene products.

The translational efficiency of many of the in vitro systems with purified mRNAs has been reported to be very high, ranging from about 5 to 50 rounds of chain reinitiation in some cases (Pelham & Jackson, 1976). The overall efficiency with heterogeneous mRNA species such as cytoplasmic RNA or a total poly(A)-containing mRNA fraction, on the other hand, appears to be markedly lower (Pain et al., 1978; Doel & Carey, 1976), possibly because of competition (Herson et al., 1979). Furthermore, the translation products formed in these mRNA-directed systems might not provide a true and complete picture on the information being expressed in the cell at a given time, since most of the mRNA isolation methods involve the selective enrichment of a certain class of molecules. An efficient translation system directed by purified polysomes can be an excellent alternative not only because the polysomal complexes represent the true mRNA pool in the cell but also because they can provide a near physiological condition for translation.

In this paper, we describe an active polysome-directed in vitro translation system which is capable of peptide chain reinitiation. The relative extent of translation in this system appears to be very close to physiological levels as determined by immunoprecipitation of albumin and carbamoyl phosphate synthetase I (CPS I) with monospecific antibodies.

Experimental Procedures

Materials. Creatine phosphokinase and pancreatic RNase were purchased from Worthington Biochemicals Corp. Rat albumin, bovine serum albumin, and collagenase type 5 were purchased from Sigma Chemical Co. Culture media and other tissue culture reagents were purchased from Grand Island Biological Co. Electrophoretic-grade acrylamide, bisacrylamide, urea, sodium dodecyl sulfate, RNase-free sucrose, and anion-exchange resin Celex-E (Ecteola) were purchased from Bio-Rad. Cycloheximide, 7-mGMP, *N*-ethylmaleimide, sodium tetrathionate, and *p*-(chloromercuri)benzenesulfonic acid were purchased from Sigma Chemical Co. *N*-Dansyl chloride and Royal X-omatic SB-55 X-ray films were purchased from Eastman Kodak Co. Precoated polyamide thin-layer plates were purchased from J. T. Baker Co. Algal hydrolysate containing 15 ^{14}C-labeled L-amino acids and [^{35}S]methionine (650 Ci/mmol) were purchased from Amersham Co.

Preparation of Cell Extracts and Factors. Factors for in vitro translation were prepared from Ehrlich ascites hypotetraploid cells grown in the peritoneal cavity of Swiss colony mice. Freshly harvested cells from 7-day-old tumors were washed with 10 mM Tris-HCl (pH 7.5) and 0.15 M NaCl and then with a hypotonic buffer containing 10 mM Tris-HCl (pH 7.8), 40 mM KCl, and 2 mM $MgCl_2$ as described before (Lewis et al., 1976). About 10 g of packed cells was suspended in an equal volume of buffer containing 20 mM Tris-HCl (pH

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[1] Abbreviations used: poly(A), poly(adenylic acid); CPS I, carbamoyl phosphate synthetase I; EDTA, ethylenediaminetetraacetic acid; Na-$DodSO_4$, sodium dodecyl sulfate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; 7-mGMP, 7-methylguanosine 5'-monophosphate; IgG, immunoglobulin G.

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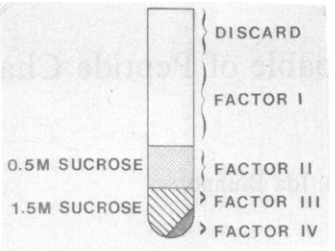

FIGURE 1: Fractionation of S-30 on sucrose gradients. The S-30 fraction was prepared from Ehrlich ascites tumor cells as described under Experimental Procedures. S-30 (13 mL) was overlaid on a discontinuous sucrose gradient containing 1.5 mL each of 1.5 and 0.5 M sucrose, both containing 20 mM Tris-HCl (pH 7.8), 120 mM KCl, 3 mM $Mg(CH_3COO)_2$, and 0.1 mM dithiothreitol, and centrifuged at 128 000*g* for 16 h at 3 °C. Factors designated I–IV were recovered as described under Experimental Procedures.

7.8), 120 mM KCl, 5 mM $Mg(CH_3COO)_2$, and 0.1 mM dithiothreitol. Cells were homogenized with 20 strokes in a tight-fitted Dounce homogenizer, and the highly active S-30 fraction was isolated as described by Aviv et al. (1971). The preincubated S-30 fraction was passed through a 1.5 × 15 cm Sephadex G-25 column. Preequilibration and elution of the column were carried out with 20 mM Tris-HCl (pH 7.8), 120 mM KCl, 3 mM $Mg(CH_3COO)_2$, and 0.1 mM dithiothreitol. Fractions containing >95 OD at 260 nm were pooled and used for preparing the factors. The S-30 fraction (13 mL) was overlaid on a discontinuous sucrose gradient containing 1.5 mL each of 1.5 and 0.5 M sucrose, both containing 20 mM Tris-HCl (pH 7.8), 120 mM KCl, 3 mM $Mg(CH_3COO)_2$, and 0.1 mM dithiothreitol, and centrifuged at 128 000*g* for 16 h at 3 °C in a Sorvall OTD-2 ultracentrifuge with an AH 627 rotor (17-mL capacity buckets). Factors designated I–III as shown in Figure 1 were collected and stored at −70 °C in small aliquots. Factor IV, mainly containing ribosomes, was suspended in 20 mM Tris-HCl (pH 7.8), 120 mM KCl, 3 mM $Mg(CH_3COO)_2$, and 0.1 mM dithiothreitol at 20–25 OD_{260}/mL and stored at −70 °C.

Preparation of Polysomes. Polysomes were prepared from freshly harvested Ehrlich ascites cells or from liver slices by a procedure modified by Palmiter (1974) and Daskal et al. (1976). Cells were washed with Tris–NaCl and with the hypotonic medium as described above. Livers were washed extensively with 0.9% NaCl to remove blood clots and minced with ice-cold scissors. Packed cells or liver slices were homogenized in 2 volumes of 20 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM $Mg(CH_3COO)_2$, 0.1 mM dithiothreitol, and 0.5% (w/v) Nonidet P40 with 10 strokes of a Dounce homogenizer. The homogenate was centrifuged at 25 000*g* (0–4 °C) for 20 min. After the top layer, mainly containing glycogen and fats, was discarded, the upper three-fourths of the supernatant was aspirated and made equal with respect to 100 mM Mg^{2+} by adding an appropriate volume of 3 M $MgCl_2$. After incubation on ice for 1 h, 12 mL of the mixture was layered over 5 mL of 0.5 M sucrose in 20 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM $Mg(CH_3COO)_2$, and 0.1 mM dithiothreitol and centrifuged at 75 000*g* for 30 min in an AH 627 rotor of a Sorvall OTD centrifuge at 4 °C. The polysome pellet was rinsed with and suspended in 20 mM Tris-HCl, 120 mM KCl, 5 mM $Mg(CH_3COO)_2$, and 0.1 mM dithiothreitol at a concentration of about 25 OD_{260} units/mL and stored in 0.1-mL aliquots at −70 °C. RNA was prepared from the polysomes by extraction with phenol–chloroform and $NaDodSO_4$ by the method of Perry et al. (1972).

In Vitro Protein Synthesis. Protein synthesis assays were run in 50–200-μL volumes as needed. In the case of the polysomal system, the reaction mixture (for a 100-μL assay) contained 20 mM Tris-HCl (pH 7.6), 140 mM KCl, 2 mM $Mg(CH_3COO)_2$, 5 mM 2-mercaptoethanol, 1 mM ATP, 0.6 mM CTP, 0.2 mM GTP, 8 mM creatine phosphate, 0.2 mM spermine, 4 μg of creatine phosphokinase, 10 μg of unfractionated mouse liver tRNA, 150 μg of polysomes, and 100 μg of factor I. Other factors when added were at 25 μg/assay. In experiments where [^{35}S]methionine was used, the assay mixture contained 80 μM 19 unlabeled L-amino acids and 20–30 pmol of [^{35}S]methionine (650 Ci/mmol). Whenever 15 ^{14}C-labeled amino acids were used for in vitro translation, the assay mixture contained 80 μM 5 unlabeled amino acids (methionine, asparagine, aspartic acid, tryptophan, and glutamine) and 0.5–5 μCi of a ^{14}C-labeled algal hydrolysate containing the 15 amino acid mixture. The polysomal RNA directed in vitro translation system was essentially similar, except that 4 mM $Mg(CH_3COO)_2$ and 100 mM KCl were used. Also, a 100-μL system contained 200 μg of the S-30 fraction and varied amounts of polysomal RNA. The reaction mixture was incubated at 35 °C for varied lengths of time up to 60 min. Aliquots of 10–50 μL were used for determining the hot CCl_3COOH-insoluble radioactivity as described by Mans & Novelli (1961).

Incorporation of [^{35}S]Methionine into Whole Cells. For labeling of Ehrlich ascites cells, freshly harvested unwashed cells were diluted to (1–1.5) × 10^6 cell/mL with Hank's minimal salt medium supplemented with 5% fetal calf serum and 1% glucose. Labeling was carried out with 25 μCi/mL [^{35}S]methionine (650 Ci/mmol) for 3 h at 37 °C in a Shaker water bath.

Rat hepatocytes were prepared by the procedure of Williamson et al. (1969). The method involves the perfusion of liver with 200 mL of EDTA–heparin buffer (Hank's buffer containing 1 mM $MgCl_2$, 0.5 mM EDTA, 200 μg/mL heparin, and 0.5% bovine serum albumin) followed with 170 mL of collagenase and 0.5% bovine serum albumin through the posterior vena cava. Perfusion with the collagenase buffer was completed in about 30 min. The liver cells were released by further incubation of the digested organ with collagenase buffer at 37 °C and collected by differential centrifugation (Williamson et al., 1969). Mouse liver cells were prepared essentially by the same procedure, except that volumes of each of the buffers used for perfusion were reduced to one-fourth. The labeling was carried out in L15 medium containing 25 mM Hepes (pH 7.4) and 15% fetal calf serum for 2 h at 37 °C. The cell density was kept at (3.7–4.0) × 10^6 cells/mL.

Two-Dimensional Analysis of in Vivo and in Vitro Translation Products. The in vitro translation products were compared with the in vivo products by the high-resolution two-dimensional method of O'Farrell (1975).

The in vitro protein synthesis reaction was run in 0.1 mL at 35 °C for 60 min, treated with 10 μg of pancreatic RNase for 5 min at 35 °C, mixed with 20 μL of protease inhibitor mixture [0.1 M sodium tetrathionate, 0.1 M *N*-ethylmaleimide, and 0.1 M *p*-(chloromercuri)benzenesulfonic acid], and lyophilized to dryness. The dry powder was stored at −20 °C. Whole cells were washed twice with Hank's salt medium, lysed by sonication for 30 s in 100 μL of 10 mM Tris-HCl (pH 7.6), 100 mM KCl, and 5 mM $MgCl_2$, and incubated with 10 μg of RNase and 10 μg of DNase for 5 min at 35 °C. The lysate was mixed with 20 μL of protease inhibitor mixture and concentrated to near dryness by freeze drying. An aliquot of 50–150 μg of proteins containing about 1 × 10^6 acid-precipitable cpm was dissolved in 10 μL of O'Farrell's lysis buffer and resolved on 0.3 × 11 cm polyacrylamide–urea gels by

isoelectric focusing as described by O'Farrell (1975). The pH gradient formed in our system was consistently in the range 4.5–8.0. After equilibration for one-half h in O'Farrell's gel-equilibration buffer, the isoelectric focusing gels were overlaid on 8–14% gradient polyacrylamide gels containing 0.2% $NaDodSO_4$ (14 × 18 × 0.15 cm), fixed with 0.5% agarose in 0.065 M Tris-HCl (pH 6.8), and electrophoresed as described by O'Farrell (1975). β-Galactosidase, bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome *c* were run as markers. After the gels were stained with Coomassie blue and destained (Laemli, 1970) to determine the migration of markers, the gel was processed for flurography (Bonner & Laskey, 1974). The gel was dried under vacuum and was exposed to Royal X-omatic X-ray film at −70 °C for 4–12 days.

Isolation of Nascent Chains. The procedure of Cioli & Lennox (1973) to purify peptidyl tRNA as modified by Pestaina & Pitot (1975) was used. This method takes advantage of highly acidic charges of the tRNA component to purify the growing nascent chains by using anion-exchange chromatography. The in vitro incubation mixture (0.6–1 mL) was mixed with 40 μL of protease inhibitor mixture, dialyzed against sterile-distilled water, and lyophilized to dryness. The dry powder was dissolved in 10 mL of 0.1 M formate (pH 4.7), 6 M urea, and 0.1% Triton X-100 and passed through a 2 × 5 cm Ecteola (Celex-E) column equilibrated with low-salt buffer (0.1 M formate, pH 4.7, 0.1 M NaCl, and 6 M urea). The column was eluted with 75 mL of low-salt buffer and then with high-salt buffer (0.1 M formate, pH 4.7, 1 M NaCl, and 6 M urea) as described by Pestaina & Pitot (1975). First, 25-mL fractions of high-salt eluant corresponding to peptidyl tRNA (Pestaina & Pitot, 1975) were pooled, dialyzed against water, and concentrated to 1–2 mL by lyophilization. For release of the peptide moiety from tRNA, the concentrate was adjusted to pH 10 with NaOH and incubated at 37 °C for 25 min. The released nascent peptides were freeze-dried and used for N-terminal analysis.

Determination of N-Terminal Amino Acids. The N-terminal amino acid residues in the peptides were determined by derivation with *N*-dansyl chloride and subsequent two-dimensional separation of dansylated amino acids by thin-layer chromatography (Blackburn, 1970; Gray, 1972). Briefly, lyophilized protein samples were dissolved in 0.1–0.4 mL of 1 M $NaHCO_3$ (pH 9.0) and mixed with *N*-dansyl chloride in acetone at a molar ratio of 1:100. Dansylation was carried out at 25 °C for 16 h. The samples were neutralized by adding 6.0 N HCl and dried under vacuum. Samples were then dissolved in 6.7 N HCl and hydrolyzed in sealed vials at 110 °C for 18 h. The hydrolysates were neutralized by adding saturated $NaHCO_3$ and lyophilized again. The residues were extracted with 50 μL of acetone–acetic acid (3:2) and spotted on polyamide thin-layer plates (20 × 30 cm) for chromatography. The solvent system in the first dimension consisted of water–formic acid (200:3). For the second dimension, benzene–acetic acid (9:1) was used.

Immunoprecipitation of Albumin and Carbamoyl Phosphate Synthetase I. Rabbit antiserum against purified rat albumin was fractionated by the $(NH_4)_2SO_4$ method, and the crude IgG fraction was further purified by DEAE-cellulose chromatography (Stelos, 1964). Monospecific antialbumin IgG was purified by affinity chromatography on a Sepharose-bound albumin column (Taylor & Schimke, 1974).

Carbamoyl phosphate synthetase I was purified from mouse liver mitoplasts by the procedure of Clarke (1976) as modified by Shore et al. (1979). The purified enzyme contains a major polypeptide of 165 000 daltons and a minor polypeptide of about 45 000 daltons as reported for the rat liver enzyme (Clarke, 1976; Shore et al., 1979). The protocol for immunizing the rabbits was according to Marshall & Cohen (1961). Antibody specific for the 165 000-dalton peptide of mouse liver CPS I was purified essentially as described for the albumin system.

Immunoprecipitation of albumin and CPS I was carried out according to Palmiter et al. (1977). The optimum conditions of antigen–antibody ratios and the time were determined for both of the proteins and used for the precipitation of labeled products.

Analytical Procedures. Protein was determined by the method of Lowry et al. (1951). The polysome content was determined by the optical density reading at 260 nm with 12.5 A_{260} units = 1 mg of polysome (Avadhani & Buetow, 1972). For radioactivity determination, the filter disks or aqueous samples were counted with 10 mL of Cab-o-cil scintillation cocktail in an Intertechnique SL-400 scintillation counter. The counting efficiency for ^{14}C and ^{35}S was in the range 80–88%.

Table I: Effects of Various Factors on Polysome-Dependent Amino Acid Incorporation[a]

additions or omissions to assay mixture	amino acid incorporation	
	[^{35}S]-methionine pmol	^{14}C-labeled amino acids (cpm × 10^{-4})
none	0.02	0.20
+factor I	0.84	8.30
+factor II	0.28	2.40
+factors I and II	2.66	37.10
+factors I–III	2.41	37.60
+factors I–IV	2.45	37.50
+factors I and II–polysomes	0.02	0.28
+factors I–IV-polysomes	0.06	0.32
+S-30	2.02	27.30
+S-100	0.92	9.40

[a] Assays were run in 100-μL volumes. The assay mixture consisted of 150 μg of polysomes, 2 mM Mg^{2+}, 140 mM KCl, and all other components described under Experimental Procedures. S-30 and S-100 when added were at 175 and 205 μg, respectively. Either 20 pmol of ^{35}S- or 2 μCi of ^{14}C-labeled amino acid mixtures were added. Reaction was carried out at 35 °C for 60 min. Other details including radioactivity determinations were as described under Experimental Procedures.

Results

In Vitro Translation with Polysomes. In order to maximize the translational activity, we have fractionated the S-30 preparation from Ehrlich ascites cells (Aviv et al., 1971) on a discontinuous sucrose density gradient as described in Figure 1, and fractions designated as factors I–IV were tested for stimulation of in vitro translation. As seen in Table I, factor I yields about 40-fold stimulation over the background incorporation, and factor I together with factor II yields 120–125-fold activity. Factors III and IV do not show any additive stimulatory effect. Furthermore, factor II alone yields about 14-fold activity. Although unfractionated S-30 yields about 100-fold stimulation of incorporation, a major drawback is its high background activity. For example, in a typical incorporation experiment with [^{35}S]methionine, addition of factors I and II without added polysomes yields about 0.02 pmol of activity whereas a comparable amount of S-30 yields >0.25 pmol (results not presented). It is also seen from Table I that the postribosomal S-100 fraction prepared by conventional methods (Kruh, 1968; Aviv et al., 1971; Avadhani & Buetow,

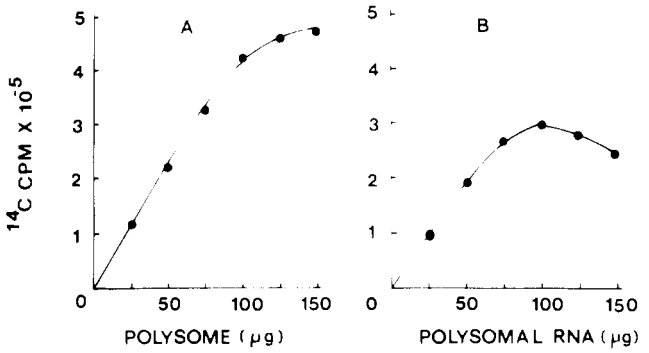

FIGURE 2: Extent of [^{35}S]methionine incorporation in the polysomal system (A) and the polysomal RNA directed S-30 system (B). Details of in vitro translation in the two systems were as described under Experimental Procedures and in Table I. In both cases, assays were run in 100-μL volume with 5 μCi of the ^{14}C-labeled amino acid mixture. The polysomal RNA was washed successively with 2 M lithium chloride, 3 M sodium acetate, and 70% ethanol to remove salts and other contaminants (Schimke et al., 1974). The reaction was run for 60 min in each case.

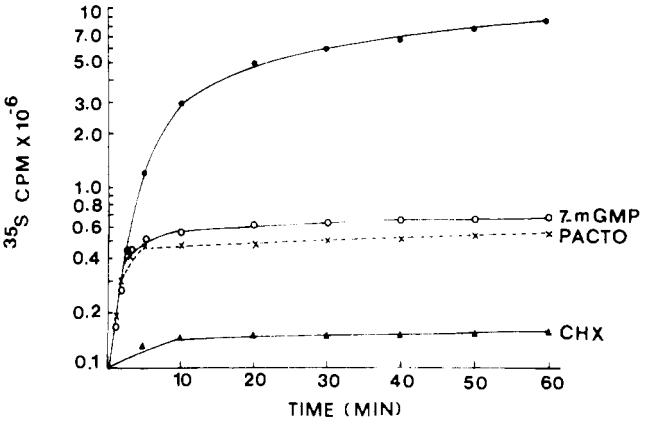

FIGURE 3: Effects of inhibitors of peptide chain initiation and elongation on the polysome-dependent translation. In vitro protein synthesis was carried out in 100-μL volumes with the optimal salt and polysome concentrations described under Experimental Procedures and in Figure 2. At intervals, samples were withdrawn, and hot CCl_3COOH-insoluble ^{35}S (cpm) was determined. The time courses of incorporation in the absence of any inhibitor (●) or in the presence of 300μg/mL cycloheximide (▲), 10^{-6} M pactomycin (×), and 0.3 M 7-mGMP (○) were determined. The values correspond to incorporation in the 100-μL assay system.

1972) yields 40–50-fold stimulation. The relatively higher stimulation obtained with the present fractionated system, therefore, appears to be due to enrichment of some high molecular weight component in factor II which may be lost from the conventional S-100 fraction under the centrifugation conditions employed.

In several in vitro systems, it is well-known that the concentrations of Mg^{2+} and K^+ ions play key roles in the fidelity as well as in the extent of translation (Shafritz, 1977). The ion concentrations required for optimal activity vary for different systems and also for different mRNAs (Shafritz, 1977). The present system yields optimal activity within an extremely narrow range, 1.8–2.2 mM Mg^{2+}. Further, the system is optimally active in the range 125–175 mM KCl (results not presented). These optimal salt concentrations yield up to 125-fold stimulation over the background activity (Table I).

The Mg^{2+} concentration needed for optimal activity in the polysomal system is markedly lower than the level of 4–5 mM needed for the in vitro translation of viral RNA and globin mRNA (Aviv et al., 1971) in the S-30 system derived from the ascites tumor cells. Although not shown here, a similar range of Mg^{2+} concentration (4.0–4.5 mM) and 100–125 mM KCl were found to be optimal for the in vitro translation of unfractionated polysomal RNA in the S-30 system.

The activity of the present polysomal system was compared with the extent of stimulation obtained with varied concentrations of polysomal RNA added to the unfractionated S-30 system. As shown in Figure 2A, the activity of the polysomal system is dependent on added polysome concentration; the activity reaches a plateau (5.2×10^5 cpm) at a polysome concentration of about 125 μg/100 μL. Under optimal salt concentrations described above, the S-30 system, on the other hand, yields about 3×10^5 cpm at a polysomal RNA concentration of about 100 μg in a 100-μL assay (Figure 2B). Higher concentrations of RNA are inhibitory to the system (Figure 2B). With the assumption that about 50% of polysomal mass is RNA, and 2–2.5% is mRNA under comparable translational conditions, the present system appears to be roughly 2–3 times more active than the unfractionated polysomal RNA directed S-30 system. Although not shown here, polysomes from diverse tissues such as mouse liver, rat liver, and rat brain yield 100–135-fold stimulation in the present system.

Evidence for Peptide Chain Reinitiation. Several studies in the past have shown that polysomes stimulate amino acid incorporation in vitro in the presence of soluble factors (Avadhani & Buetow, 1972; Shires et al., 1973; Ramsey & Steele, 1976). There is, however, no information on the possibility of peptide chain reinitiation in these systems. Available evidence does suggest that the polysomal systems are noninitiating and their activity is restricted to peptide chain elongation (Schnaitman, 1969; Baliga et al., 1974). We present evidence which demonstrates that the system described in this paper is indeed capable of efficient chain initiation. Two different approaches have been used to demonstrate chain initiation.

In the first approach, specific inhibitors of peptide chain initiation and elongation were used. Cycloheximide is known to inhibit peptide chain elongation by interfering with GTP-dependent translocation (Stewart-Blair et al., 1971). Pactomycin, at a low concentration (10^{-6} M), specifically inhibits peptide chain initiation (Stewart-Blair et al., 1971) without affecting the completion of existing peptide chains. In addition, several 5′-cap analogues such as 7-mGMP are also known to inhibit the translation initiation, possibly due to competition with methylated cap structures on the 5′ end of mRNA (Muthukrishnan et al., 1975; Both et al., 1975). As shown in Figure 3, addition of cycloheximide results in almost complete inhibition (>96%) of the activity. The extent of inhibition with 10^{-6} M pactomycin is significantly lower (about 83–86%). Similarly, 7-mGMP at 0.3 M concentration inhibits the [^{35}S]methionine incorporation by about 80%. It is also seen that up to about 5 min of incubation the rates of incorporation in the control system without added inhibitors and in systems with the inhibitors are identical, suggesting that this period may mostly represent elongation and completion of existing chains. With the assumption that the activity in the presence of pactomycin and 7-mGMP is mostly due to peptide chain elongation, it appears that on an average each mRNA molecule is reinitiated 5–8 times.

The second approach involved N-terminal amino acid sequencing by using the N-dansylation method. As shown in Figure 4, when the product formed in vitro by using [^{35}S]-methionine was subjected to acid hydrolysis following dansylation, significant ^{35}S counts were found in the dansyl–amino products. These results were further verified by isolating the nascent chains as peptidyl-tRNA by the ion-exchange method

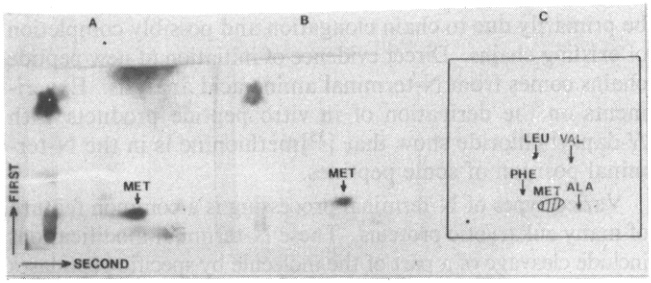

FIGURE 4: N-Terminal amino acid analysis of in vitro products and the nascent chains. The dansylamino derivatives of ^{35}S-labeled in vitro products or ^{35}S-labeled nascent chains were subjected to two-dimensional thin-layer chromatography as described under Experimental Procedures. The dansylamino derivatives were detected either by autoradiography or by UV illumination. (A) Autoradiogram showing [^{35}S]dansylmethionine in the 60-min incubated in vitro product; (B) autoradiogram showing [^{35}S]dansylmethionine in the nascent chains isolated after 20-min in vitro reaction; (C) same as (B), except that the dansyl derivative was detected by UV illumination. Arrows in (C) indicate the position of markers.

(Cioli & Lennox, 1973; Pestaina & Pitot, 1975). Nascent chains isolated at 20 min of in vitro reaction contain N-terminal [^{35}S]methionine. It is interesting to see that the thin-layer plates also show only one fluorescent spot in the position of *N*-dansylmethionine upon illumination with UV light. Nascent chains isolated within the first 5 min of incubation do not contain [^{35}S]methionine in the N-terminal position. These results support the observation that with chain initiation inhibitor no new chains may be initiated until about 5–10 min of incubation. N-Terminal analysis of nascent chains also suggests that almost all of the peptide chains in this system are initiated with methionine.

Level of Translation and Physiological Relevance. Although the extent of stimulation of amino acid incorporation is an important factor in determining the usefulness of the in vitro translation system, the relative levels of translation of varied mRNAs and also the fidelity of translation are probably more important factors. We have, therefore, compared the ^{35}S-labeled products synthesized in the present polysomal system and in the polysomal RNA directed S-30 system with the in vivo products by using the high-resolution two-dimensional method of O'Farrell (1975). The polysome fraction reflects the total mRNA pool being actively translated in the cell. An accurate and complete translation of the polysomes or polysomal RNA is therefore expected to result in the synthesis of a large number of peptides. The autoradiograms presented in Figure 5 show that the products formed in the polysome-directed in vitro system are nearly comparable to the in vivo products, with respect to both total number and relative levels (Figure 5A,B). The minor differences between these two patterns might reflect the degree of cleavage of N-terminal signal peptides under these two experimental conditions (Schechter, 1973; Blobel & Doberstein, 1975). The two-dimensional pattern of polysomal RNA directed in vitro products, however, appears to lack several very large as well as small molecular weight components seen in both the polysomal system and the in vivo system (see Figure 5C).

The relative extent of translation of highly abundant and less abundant proteins in this in vitro system was further investigated since it is directly related to the usefulness of the system for various metabolic and biological studies. Albumin is a major translation product in the liver, ranging up to about 11% of the total proteins synthesized (Taylor & Schimke, 1973). CPS I, a urea cycle enzyme, was also chosen because it consists of a very large polypeptide of 165 000 daltons. In addition, this cytoplasmically translated enzyme is under

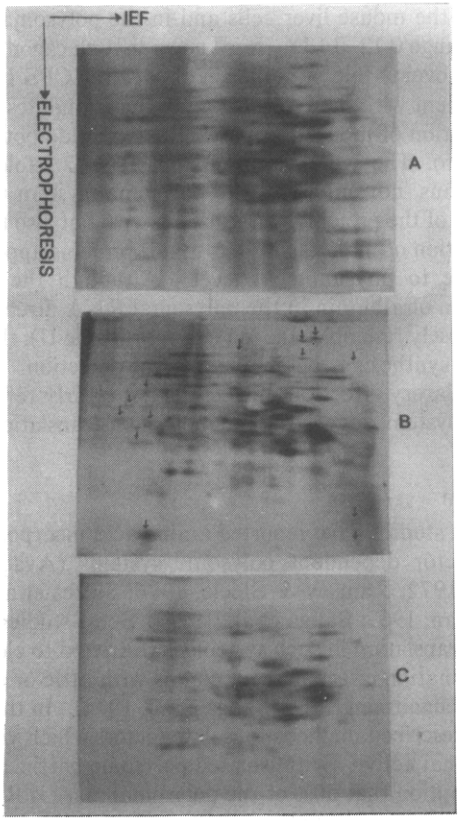

FIGURE 5: Comparison of the in vitro translation products with the in vivo products. Ehrlich ascites cells were labeled with 25 μCi/mL [^{35}S]methionine for 3 h. The syntheses of Ehrlich ascites polysome and polysomal RNA dependent in vitro products were carried out for 60 min with 30 pmol of [^{35}S]methionine/100-μL assay as described under Experimental Procedures and in Figure 2. Polypeptides synthesized in whole cells (A), in the polysomal system (B), and in the polysomal RNA directed S-30 system (C) were subjected to two-dimensional analysis as described by O'Farrell (1975). In each case, (0.9–1.0) $\times$ 10^6 acid-insoluble ^{35}S-labeled proteins were layered on the isoelectric focusing gels.

Table II: Extent of Translation of Rat Albumin and Mouse Liver Carbamoyl Phosphate Synthetase I in Vivo and in Vitro[a]

system	type of translation	acid-precipitable ^{35}S (cpm) precipitated by monospecific antibody (%) albumin	CPS I
rat liver	hepatocytes	9.7	
	in vitro polysome	9.8	
	in vitro polysomal RNA	9.6	
mouse liver	hepatocytes		0.13
	in vitro polysome		0.12
	in vitro polysomal RNA		<0.04
glucagon-stimulated mouse liver	hepatocytes		0.27
	in vitro polysome		0.28
	in vitro polysomal RNA		<0.04

[a] Experimental details of in vivo and in vitro incorporation and immunoprecipitation were as described under Experimental Procedures and in Figure 1. Zinc glucagon was injected subcutaneously for 2 days at a dose rate of 4.0 mg kg^{-1} day^{-1} following the schedule described by Snodgrass et al. (1978).

hormonal regulation (Snodgrass et al., 1978). Results of immunoprecipitation with monospecific antialbumin IgG show that albumin accounts for about 9.8% of the total in vitro products synthesized in the polysome-dependent system. This level is almost identical with the level synthesized in the intact liver cells (Table II). Further, the level of translation of CPS

I both in the mouse liver cells and in the polysomal system is in the range 0.12–0.13%. It is known that glucagon injection results in over 2-fold stimulation of rat liver CPS I activity. In agreement with these results, glucagon stimulates the level of translation of mouse liver CPS I polypeptide both in vivo and in vitro. The overall stimulation is about 2.2-fold in both cases. Thus, not only does the polysomal system translate almost all of the proteins synthesized in vivo but also the levels of translation of major as well as minor proteins appear to be very close to physiological level. Although the level of translation of albumin in the polysomal RNA directed S-30 system closely resembles the in vivo level (Table II), the extent of CPS I synthesis is below the level of detection. Further, the stimulatory effect of glucagon is not clearly reflected in the S-30 system, suggesting the inefficient translation of this mRNA.

Discussion

Several studies have reported amino acid incorporation in S-100 factor dependent polysome systems (Avadhani & Buetow, 1972; Ramsey & Steele, 1976; Shires et al., 1973; Schnaitman, 1969; Baliga et al., 1974). Some studies suggest that the translation in such systems is restricted to elongation and termination of the nascent chains with little or no reinitiation (Schnaitman, 1969; Baliga et al., 1974). In this paper, we have described methods to isolate factors which yield high translational activity with isolated polysome particles. With the assumption that 50% of the polysomal mass is RNA and that roughly 5% of the polysome-associated RNA is mRNA, about 4 μg of polysomal mRNA yields over 120-fold stimulation. Under almost identical conditions, the S-30 system yields only about 30% activity. These results on polysomal RNA directed translation are in agreement with reports showing 30–60-fold stimulation of incorporation by unfractionated polysomal RNA or heterogeneous poly(A)-containing mRNA in varied S-30 systems (Doel & Carey, 1976; Pain et al., 1978; Rudensey & Infante, 1979). In this respect, activity obtained in the present system appears to be significantly higher than that reported in varied S-30 systems.

It has been shown that purified homogeneous mRNA such as globin mRNA is translated with high efficiency in rabbit reticulocyte as well as in wheat germ extracts (Pelham & Jackson, 1976), yielding about 5–50 rounds of translation/mRNA molecule. Comparable data for the translation of heterogeneous mRNA populations in these systems are not yet available. It is, however, a general experience that polysomal RNA is translated with significantly lower efficiency than the purified mRNAs. On the basis of the effects of chain initiation inhibitors, it appears that the present system translates the heterogeneous species of mRNA with an efficiency equivalent to 5–8 rounds on an average. The high activity obtained in this system may be attributed to the component sedimenting in 0.5 M sucrose in our fractionation procedure, designated as factor II. Although the precise nature and its role in translational activity are unknown, factor II may represent some high molecular weight initiation and regulatory factors known to be present in several eukaryotic cells (Shafritz, 1977; Revel & Groner, 1978; Benne & Hershey, 1976). Use of optimal Mg^{2+} and K^+ ions is an additional factor responsible for high translational activity in this system.

The present polysome system can actively initiate new peptide chains as demonstrated by two independent approaches. Pactomycin and 7-mGMP inhibit the activity up to about 80–86% as compared to almost 96–98% inhibition caused by cycloheximide. Our results also suggest that the incorporation obtained up to about 5–10 min of reaction may be primarily due to chain elongation and possibly completion of existing chains. Direct evidence of initiation of new peptide chains comes from N-terminal amino acid analysis. Experiments on the derivation of in vitro peptide products with *N*-dansyl chloride show that [^{35}]methionine is in the N-terminal position of some peptides.

Varied types of N-terminal processing is a common feature of many eukaryotic proteins. These N-terminal modifications include cleavage of a part of the molecule by specific peptidases (Schechter, 1973; Blobel & Doberstein, 1975) and chemical modifications such as acetylation (Pestaina & Pitot, 1975; Jornvall, 1975; Brown & Roberts, 1976) and methylation (Uy & Wold, 1977). In Ehrlich ascites cells, it has been reported that over 80% of the nascent chains are N-acetylated (Brown & Roberts, 1976). In agreement with these observations, nascent chains purified as peptidyl-tRNA from steady-state polysomes as well as those isolated from in vitro reaction up to 10 min of incubation are very poorly dansylated, possibly because of their blocked N-terminal residues. Nascent chains isolated at 20, 30, and 60 min dansylate easily and yield [^{35}S]dansylmethionine.

Involvement of Met-tRNA in eukaryotic peptide chain initiation is well documented (Shafritz, 1977; Housman et al., 1970; Oberg & Shatkin, 1972). Similarly, in vitro products dictated by varied monospecific mRNAs contain N-terminal methionine (Schechter et al., 1977; Palmiter et al., 1977). Nevertheless, it remains unclear if methionine is the only initiator in the eukaryotes. Our results showing methionine as the only dansylamino product in the form of both a fluorescent spot after UV irradiation and a radioactive spot on the autoradiogram indicate that almost all of the peptide chains in this polysome-directed system are initiated with methionine. Further, since the polysome preparation contains both membrane-bound and free polysome populations, it is likely that these results directly reflect the in vivo situation.

Two-dimensional analysis of in vitro products shows that the system actively translates several hundred proteins (Figure 5). Although the precise authenticity of all of the in vitro translation products is not known, the results suggest that the same multitude of mRNAs may be involved in the polysome-directed in vitro translation as in the intact cells. The polysomal RNA dependent S-30 system, on the other hand, appears to be less efficient in the synthesis of larger polypeptides, and the two-dimensional pattern (Figure 5C) shows fewer products.

Besides being relatively more active, the polysomal system appears to offer additional advantages for metabolic and regulatory studies. In the case of the myosin system, it has been shown that different sets of proteins are associated with the mRNA, depending on whether it is in active translation in the form of polysomes or in the nontranslational state as free mRNA–protein particles (Jain & Sarkar, 1979). These mRNA-associated proteins are believed to be involved in translational regulation [for details, see Shafritz (1977) and Revel & Groner (1978)]. In this respect, the polysome-directed system might provide translational conditions very close to in vivo conditions.

This possibility was verified by comparing the relative extents of albumin and CPS I synthesized in vivo in the whole cells and in the in vitro system by immunoprecipitation with monospecific antibody. The results of immunoprecipitation (Table II) show that the levels of translation of both major products like albumin and minor products like CPS I in the present polysomal system are almost identical with the in vivo level. Any variation from the normal level of translation such

as under hormonal influence is accurately reflected in the polysomal system. This system can, therefore, provide a useful tool for comparison and evaluation of even minor gene products in varied cell types and also under different experimental conditions.

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