Partial Isolation and Translation *in vitro* of Messenger Ribonucleic Acid for Phosphoenolpyruvate Carboxykinase (Guanosine Triphosphate)

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1. mRNA was extracted from the livers of starved rats and incubated in a heterologous cell-free protein-synthesizing system derived from rabbit reticulocytes. The presence of newly synthesized phosphoenolpyruvate carboxykinase (GTP) was detected by immunoprecipitation with a specific antibody to the enzyme and analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 2. The synthesis of the enzyme was dependent on the addition of rat liver RNA, whereas RNA isolated from rat spleen was inactive. If ovalbumin and anti-ovalbumin were used to form the immunoprecipitates, no radioactivity that migrated as phosphoenolpyruvate carboxykinase was detected. 3. The optimal concentrations of magnesium acetate and KCl for phosphoenolpyruvate carboxykinase synthesis were determined. 4. When polyribosomal RNA was separated by sucrose-gradient centrifugation, phosphoenolpyruvate carboxykinase mRNA migrated between 20 and 26S, in keeping with the high mol.wt. of the protein (85000). 5. The presence of poly(A) in phosphoenolpyruvate carboxykinase mRNA was suggested by retention of mRNA activity on oligo(dT)-cellulose columns. 6. It was concluded that the cell-free synthesis of phosphoenolpyruvate carboxykinase can serve as a bioassay for intracellular phosphoenolpyruvate carboxykinase mRNA.

Phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) is one of the key regulatory enzymes in gluconeogenesis (Scrutton & Utter, 1968). The enzyme from the cytosol of rat liver is increased by starvation or alloxan-diabetes, an effect that can be overcome by re-feeding with a carbohydrate diet or by the injection of insulin respectively (Shrago *et al.*, 1963, 1967). These alterations in enzyme activity have been shown immunochemically to be due to changes in the rate of enzyme synthesis *de novo* (Hopgood *et al.*, 1973; Tilghman *et al.*, 1974). If starved rats are re-fed, the rate of phosphoenolpyruvate carboxykinase synthesis decreases 10-fold, with a half-life of approx. 40 min (Tilghman *et al.*, 1974). From studies with the intact animal (Tilghman *et al.*, 1974) as well as with Reuber H35 cells (Tilghman *et al.*, 1975), we concluded that a decrease in intracellular cyclic AMP was responsible for this rapid deinduction of specific enzyme synthesis.

The molecular mechanisms underlying the rapid cessation in phosphoenolpyruvate carboxykinase synthesis have not been identified. It was reasoned that if enzyme synthesis was being controlled by cyclic AMP at the transcriptional level, then the decrease in enzyme synthesis during deinduction must repre-

sent a rapid turnover of existing mRNA for phosphoenolpyruvate carboxykinase. Estimates of the stability of phosphoenolpyruvate carboxykinase mRNA in intact rats by using cordycepin, which specifically inhibits mRNA synthesis (Darnell *et al.*, 1971b; Singer & Penman, 1972), suggested that the mRNA for this enzyme had a short half-life of less than 1 h (Tilghman *et al.*, 1974). On the other hand, similar experiments in Reuber H35 cells under more carefully defined conditions indicated that the mRNA for the enzyme had a half-life of 4–8 h, thus implying post-transcriptional regulation of phosphoenolpyruvate carboxykinase (Tilghman *et al.*, 1975). An unequivocal answer to the question of the mechanism of deinduction must come from direct measurements of mRNA for phosphoenolpyruvate carboxykinase.

The purpose of this paper is to describe a cell-free protein-synthesizing system derived from rabbit reticulocytes which is able to synthesize phosphoenolpyruvate carboxykinase at the direction of RNA isolated from rat liver. We conclude that this bioassay will allow direct quantification of the tissue content of phosphoenolpyruvate carboxykinase mRNA.

Experimental

Materials

Creatine phosphate, creatine kinase and GTP were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The radioisotopes L-[4,5- ^3H]leucine (30–50 Ci/mmol) and [U- ^{14}C]leucine (340 mCi/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A., and ribonuclease-free sucrose and NCS solubilizer were from Amersham/Searle Corp., Arlington Heights, IL, U.S.A. Oligo(dT)–cellulose and ATP were obtained from P-L Biochemical Co., Milwaukee, WI, U.S.A., and crystalline ovalbumin and the γ -globulin fraction of anti-ovalbumin serum were obtained from ICN Pharmaceuticals, Cleveland, OH, U.S.A. Triton X-405 was a generous gift from Rohm and Haas Co., Philadelphia, PA, U.S.A.

Preparation of rat liver RNA

Male Sprague–Dawley rats weighing 150–200 g were starved for 20 h before being killed. Extreme care was taken to work under sterile conditions in all subsequent steps. Polyribosomes were isolated from livers by discontinuous sucrose-gradient centrifugation and dissociated in the presence of sodium dodecyl sulphate and EDTA (Taylor & Schimke, 1973). RNA was separated from heparin and protein by sedimentation through 12 ml 5–20% (w/v) linear sucrose gradients in the presence of sodium dodecyl sulphate and EDTA at 300 000 g in a Beckman SW40 rotor for 6 h (Taylor & Schimke, 1973). Fractions (1 ml) were collected by puncturing the bottom of the centrifuge tube.

Appropriate fractions from six tubes were pooled, adjusted to 0.2 M-potassium acetate and centrifuged for 10 min at 2000 g. Then 2 vol. of ethanol was added to the clear supernatant and the mixture was left overnight at -20°C . The RNA was collected by centrifugation for 15 min at 20 000 g and was dissolved in 20 mM-Hepes[2-(N-2-hydroxypiperazin- N' -yl)ethanesulphonic acid] (pH 7.5). If traces of sodium dodecyl sulphate remained, the solution was adjusted to 0.2 M-potassium acetate, centrifuged, reprecipitated for 1 h in 2 vol. of ethanol and resuspended in Hepes buffer. Small portions of this solution were rapidly frozen and stored in liquid N_2 .

Total cellular RNA was prepared by extraction of rat liver with phenol/chloroform/3-methylbutan-1-ol (50:50:1, by vol.) by the method of Schutz *et al.* (1973). The aqueous phase was precipitated in the presence of 0.1 M-NaCl and 2 vol. of ethanol overnight. After centrifugation of the suspension at 20 000 g for 10 min, the nucleic acid precipitate was washed twice with 3 M-sodium acetate as described by Palmiter (1974). The RNA was dissolved in 0.1 M-sodium acetate, pH 6.9, and precipitated for 1 h at -20°C with 2 vol. of ethanol. After centrifugation,

the RNA was dissolved in buffer for chromatography on oligo(dT)–cellulose columns (described below).

The procedure for chromatography of polyribosomal RNA on oligo(dT)–cellulose columns was described by Aviv & Leder (1972). The yield of poly(A)-containing RNA was generally 1–2% of the E_{260} units applied to the column. One E_{260} unit was assumed to represent 50 μg of RNA (Palmiter *et al.*, 1972).

Incubation of rabbit reticulocyte lysates

Rabbit reticulocyte lysates were prepared from phenylhydrazine-treated rabbits as described by Palmiter (1973). The lysate was stored in small batches in liquid N_2 and thawed only once.

The conditions for incorporation of radioactivity into protein by using the rabbit reticulocyte lysate were as follows: a final reaction volume of 0.5 ml contained 0.2 ml of lysate, 1 mM-ATP, 0.2 mM-GTP, 15 mM-creatine phosphate, 375 μg of creatine kinase/ μl , 20 mM-Hepes (pH 7.7), 60 μM each of 19 amino acids without L-leucine, 20 μCi of [^3H]leucine, 50–100 mM-KCl and 0.5–2 mM-magnesium acetate. The mixture was incubated at 26–28 $^\circ\text{C}$ for 60 min in the absence or presence of exogenous mRNA (5–150 μg per 0.5 ml). The reaction was terminated by adjusting the concentration of unlabelled L-leucine to 10 mM with 0.1 M-L-leucine, and the tubes were incubated at 37 $^\circ\text{C}$ for an additional 30 min. After cooling in ice, a 50 μl sample was removed for determination of total protein synthesis, and the remainder centrifuged at 100 000 g for 40 min. A 50 μl sample of the resulting supernatant fraction was used to measure the incorporation of [^3H]leucine into released protein by precipitation with 10% (w/v) trichloroacetic acid. In general, the amount of radioactivity in released protein was 85–100% of that in total protein.

Immunoprecipitation of phosphoenolpyruvate carboxykinase from reticulocyte lysate incubations

The procedure used for the immunoprecipitation of phosphoenolpyruvate carboxykinase from reticulocyte lysate incubations was a modification of the method used for precipitating the enzyme from the cytosolic fraction of rat liver (Hopgood *et al.*, 1973). Freeze-dried antibody to phosphoenolpyruvate carboxykinase was dissolved at twice its normal strength in water and incubated with 1 vol. of undiluted rabbit reticulocyte lysate at 37 $^\circ\text{C}$ for 30 min, and then for 18 h at 0 $^\circ\text{C}$. The insoluble material was removed by centrifugation at 100 000 g for 20 min. The treated antibody was stored in small batches at -20°C . This procedure removed any γ -globulins with specificity for reticulocyte proteins.

After centrifugation of the incubated reticulocyte protein-synthesizing system for 40 min, 0.3 ml of supernatant was incubated at 37 $^\circ\text{C}$ with 90 munits

of purified unlabelled phosphoenolpyruvate carboxykinase, 125 munits* of treated antibody and Triton X-405 to 0.6%. The mixture was left at 0°C for 3 h. The immunoprecipitate was collected by sedimentation through a discontinuous gradient (0.15 ml) of 0.5 M-sucrose / 10 mM-leucine / 0.6% Triton X-405 which was layered over 0.3 ml of 1.0 M-sucrose/10 mM-leucine/0.6% Triton X-405, and was centrifuged at 10000g for 10 min as described by Taylor & Schimke (1973). The upper layers were carefully removed, and the precipitate was washed three times by repeated centrifugation at 2000g in a solution of 10 mM-L-leucine, 0.6% Triton X-405 and 0.5 M-NaCl. The precipitate was then dissolved in 150 µl of 2% (v/v) sodium dodecyl sulphate / 2% (w/v) dithiothreitol / 25% (v/v) glycerol/0.005% Bromophenol Blue in 10 mM-potassium phosphate (pH 7).

Polyacrylamide-gel electrophoresis of labelled phosphoenolpyruvate carboxykinase immunoprecipitates

The immunoprecipitates were heated at 100°C for 10 min and applied to 10% (w/v) polyacrylamide gels prepared in the presence of sodium dodecyl sulphate as previously described (Hopgood *et al.*, 1973; Ballard *et al.*, 1974). Each sample was run at the same time as a ¹⁴C-labelled antigen-antibody precipitate prepared from rat liver (Weber & Osborn, 1969). The ³H that co-migrated with authentic ¹⁴C-labelled phosphoenolpyruvate carboxykinase was calculated for each gel. The background radioactivity was determined for each experiment by preparing antigen-antibody precipitates from incubations without exogenous mRNA added. The total radioactivity migrating on sodium dodecyl sulphate/polyacrylamide gels with authentic ¹⁴C-labelled phosphoenolpyruvate carboxykinase (usually 60–150 d.p.m.) was subtracted from the total radioactivity obtained from reactions incubated in the presence of exogenous mRNA.

Results and Discussion

Synthesis of phosphoenolpyruvate carboxykinase in vitro

Livers from starved rats were used as the source of mRNA for phosphoenolpyruvate carboxykinase, since it had been previously shown that starvation increased the rate of specific enzyme synthesis to as high as 3% of total cytosol protein synthesis (Hopgood *et al.*, 1973). Polyribosomal RNA that migrated between the 28S and 18S rRNA peaks in 5–20% linear sucrose gradients was incubated in a

* A unit of enzyme activity is the amount of enzyme that catalyses the conversion of 1 µmol of substrate into product per min at 37°C. A unit of antibody is the amount of antibody required to precipitate 1 unit of phosphoenolpyruvate carboxykinase. A detailed treatment of our immunochemical techniques is provided in Hopgood *et al.* (1973).

reticulocyte protein-synthesis system at 28°C for 1 h. After termination of the reaction and centrifugation at 100000g, a portion of the supernatant was used for the immunoprecipitation of phosphoenolpyruvate carboxykinase in the presence of unlabelled carrier enzyme. To measure accurately the ³H-labelled phosphoenolpyruvate carboxykinase synthesized, the immunoprecipitates were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and only the radioactivity that co-migrated with the enzyme marker was taken as the incorporation of [³H]leucine into phosphoenolpyruvate carboxykinase.

Polyacrylamide-gel electrophoresis of the immunoprecipitate formed after incubation of rat liver polyribosomal RNA in the reticulocyte system revealed the presence of a single protein that co-migrated with the ¹⁴C-labelled phosphoenolpyruvate carboxy-

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of phosphoenolpyruvate carboxykinase synthesized in reticulocyte lysates

Polyribosomal RNA from liver or spleen of starved rats was isolated by discontinuous sucrose-gradient centrifugation followed by dissociation with sodium dodecyl sulphate and centrifugation in 5–20% sucrose gradients. The RNA migrating between 28S and 18S was added to a reticulocyte lysate system of 0.5 ml total volume. After 1 h at 28°C, 0.3 ml of lysate was removed for immunoprecipitation of phosphoenolpyruvate carboxykinase. The washed immunoprecipitates were dissociated and subjected to electrophoresis on sodium dodecyl sulphate/polyacrylamide gels. (a) ○, 32 µg of rat liver 20–26S RNA; ■, ¹⁴C-labelled phosphoenolpyruvate carboxykinase marker prepared from the liver of starved rats. (b) ●, No mRNA; □, with 30 µg of rat spleen RNA; △, ovalbumin-anti-ovalbumin immunoprecipitate from a reaction mixture containing 32 µg of rat liver 20–26S RNA.

kinase marker (Fig. 1). As a control, immunoprecipitates from incubations without exogenous RNA were analysed, and no ^3H radioactivity that coincided with authentic enzyme was detected.

If immunoprecipitation was performed by using ovalbumin carrier and anti-ovalbumin serum, no radioactivity migrating on sodium dodecyl sulphate/polyacrylamide gels as the enzyme was detected. Finally, the reaction was specific for RNA obtained from liver, since RNA isolated from rat spleen, a tissue with no measureable phosphoenolpyruvate carboxykinase activity, was unable to direct the synthesis of the enzyme in the reticulocyte lysate (Fig. 1). Although the addition of exogenous RNA to reticulocyte lysates does not stimulate the incorporation of amino acids into total protein (see Fig. 2), the general efficacy of the spleen mRNA was verified independently in a wheat-germ translation system (Roberts & Patterson, 1973), where it stimulated amino acid incorporation into protein to the same extent as did mRNA from rat liver (D. Kioussis & R. W. Hanson, unpublished work).

The results of the experiments in Fig. 1 provide evidence that the reticulocyte system is capable of synthesizing phosphoenolpyruvate carboxykinase in the direction of mRNA isolated from rat liver. A more rigorous proof of specific enzyme synthesis will require either chromatography of the products after trypsin digestion and comparing the pattern

with purified liver phosphoenolpyruvate carboxykinase or demonstrating that the newly synthesized protein is co-purified with hepatic phosphoenolpyruvate carboxykinase.

The amount of phosphoenolpyruvate carboxykinase synthesized in the reticulocyte lysate depended on the amount of rat liver RNA added, up to at least $56\mu\text{g}$ of RNA/0.5 ml of incubation mixture (Fig. 2). Within this range of added RNA, there was no inhibition of protein synthesis. However, at concentrations of RNA above $80\mu\text{g}$ /0.5 ml reaction volume, inhibition of total protein synthesis was observed (not shown), an effect which has been reported by others using the reticulocyte system (Taylor & Schimke, 1973; Rhoads *et al.*, 1973). For this reason, all subsequent experiments reported used RNA concentrations within the range shown in Fig. 2.

Optimal conditions for phosphoenolpyruvate carboxykinase synthesis in reticulocyte lysates

Palmiter (1973, 1974) has reported that the optimal conditions for specific exogenous mRNA translation in rabbit reticulocyte lysates may differ from the optimal conditions for total protein synthesis, which is mostly globin. In addition, individual exogenous mRNA species can vary in their requirements for translation in this system. Therefore the effects of KCl and Mg^{2+} concentrations on phosphoenolpyruvate carboxykinase synthesis were examined to ensure that optimal conditions for enzyme synthesis were being used. The maximal synthesis of the enzyme was observed at 80–100 mM-KCl (Fig. 3a). Although the rate of total enzyme synthesis increased only 50% when the KCl concentration was increased from 20 to 80 mM, the synthesis of the enzyme increased almost 10-fold. The range for the optimal Mg^{2+} concentration was also narrow (Fig. 3b). A 3–4-fold increase in phosphoenolpyruvate carboxykinase synthesized was measured when the Mg^{2+} concentration was increased from 0.5 to 1.5 mM, whereas there was no effect on the synthesis of total soluble protein.

The narrow range of optimal concentrations for Mg^{2+} and KCl for phosphoenolpyruvate carboxykinase synthesis relative to that for total protein synthesis is probably explained by the absolute requirement of initiation for specific enzyme synthesis. Total protein synthesis, which represents mainly globin synthesis, would proceed for some time at salt concentrations which would not permit initiation (Miller & Schweet, 1968; Gilbert & Anderson, 1970). It should be emphasized that the absolute values for the optimal conditions were not identical for every lysate preparation, although the general shape of the concentration curves remained constant. This confirms the results of Palmiter (1973, 1974) and emphasizes the importance of optimizing reticulocyte translation systems for each individual mRNA.

Fig. 2. Effect of exogenous RNA on the synthesis of phosphoenolpyruvate carboxykinase in reticulocyte lysates

Reticulocyte lysates were incubated with increasing amounts of 20–26S RNA isolated from the liver of starved rats. The radioactivity incorporated into released protein (○) and immunoprecipitated phosphoenolpyruvate carboxykinase (●) was determined and corrected in 0.5 ml incubation volume. The values are duplicate determinations of a representative experiment.

Fig. 3. Effect of salt concentrations on the synthesis of phosphoenolpyruvate carboxykinase in reticulocyte lysates

(a) A 40 μg portion of RNA from the liver of starved rats, prepared as described in the legend to Fig. 1 was used in reticulocyte lysates, total volume 0.5 ml. The concentration of magnesium acetate added to each incubation was 1.5 mM. The radioactivity in protein (○, d.p.m.) and in phosphoenolpyruvate carboxykinase (●, d.p.m.) was obtained from duplicate determinations of a representative experiment and was corrected to 0.5 ml reaction volume. (b) The concentration of KCl was maintained at 80 mM, and the concentration of Mg²⁺ varied by adding magnesium acetate. The values are the radioactivity in protein (○; c.p.m.) and in phosphoenolpyruvate carboxykinase (●; d.p.m.) per 0.5 ml reaction volume containing 56 μg of RNA, for duplicate determinations of a representative experiment.

The time-course of protein synthesis in the reticulocyte lysate is shown in Fig. 4. The incorporation of radioactivity into total released proteins increased rapidly for the first 10 min of incubation, followed by a lower rate of increase for up to 60 min. Phosphoenolpyruvate carboxykinase synthesis was linear with time for up to 20 min, and then increased at approximately the same rate as total protein synthesis. Extrapolation of the curve in Fig. 4 to zero labelling does not yield an estimate of the transit time for phosphoenolpyruvate carboxykinase mRNA translation, because of the way in which the experiments were performed. After the appropriate incubation period at 28°C, the radioactive leucine was diluted with a large excess of unlabelled leucine and the tubes were incubated for an additional 30 min at 37°C. This procedure was required to prevent the immunoprecipitation of proteins migrating on sodium dodecyl sulphate/polyacrylamide gels with molecular

weights less than phosphoenolpyruvate carboxykinase, which were presumed to be incomplete peptide chains. Alternatively, these proteins could have been denatured at 37°C during the immunoprecipitation reaction, and were sedimented during the following centrifugation. In either case, the preincubation of the reaction mixture at 37°C before ultracentrifugation eliminated the presence of low-molecular-weight proteins in the immunoprecipitate.

The length of time during which the synthesis of protein in reticulocyte lysates is linear is partially a function of the temperature of incubation (Palmiter, 1973; Gross & Rabinovitz, 1973), and is different for each lysate preparation. It has been shown that haemin prolongs this time-period by preventing the formation of an inhibitory complex which interferes with polypeptide initiation (Gross & Rabinovitz, 1972, 1973; Adamson *et al.*, 1972). However, in the two lysate preparations used in the present work,

Fig. 4. Time-course of synthesis of phosphoenolpyruvate carboxykinase in reticulocyte lysates

Reticulocyte lysates were incubated in the presence of $43 \mu\text{g}$ of 20–26S RNA isolated from the liver of starved rats. The incubations were terminated after various times by adjusting the concentration of L-leucine to 10mM. The radioactivity incorporated into released protein ($\circ$; c.p.m.) and phosphoenolpyruvate carboxykinase ($\bullet$; d.p.m.) were determined and corrected to a reaction volume of 0.5ml. The values represent duplicate determinations of a representative experiment.

neither haemin nor additional rabbit reticulocyte initiation factors obtained from washing ribosomes in 0.5M-KCl stimulated the rate of total protein synthesis or specific enzyme synthesis. Since the incorporation of radioactivity into total protein in our experiments was within the range reported by Palmiter (1973) for haemin-stimulated protein synthesis using reticulocyte lysates, it is likely that the lysates that we have used were already saturated with haemin.

The data in Figs. 2–4 were obtained with six different preparations of 20–26S RNA and indicate the variations in the efficiency of the RNA for phosphoenolpyruvate carboxykinase synthesis. If the optimal specific radioactivities for the experiments are averaged, a value of 31.8 ± 2.4 (S.E.M.) d.p.m. in phosphoenolpyruvate carboxykinase/ μg of 20–26S RNA is obtained.

Characteristics of phosphoenolpyruvate carboxykinase mRNA

Sephadex G-100 chromatography of rat liver cytosolic phosphoenolpyruvate carboxykinase gave an estimate of 74000 for the mol.wt. of its single polypeptide chain (Ballard & Hanson, 1969), and by

sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purified enzyme, a value between 85000 and 90000 was determined (Tilghman *et al.*, 1976). If 80000 is used as an approximation of the mol.wt., this would represent a molecule of about 660 amino acids. The minimum mol.wt. for a mRNA coding for phosphoenolpyruvate carboxykinase would be 660000 and would have a sedimentation coefficient of about 22S (Taylor & Schimke, 1973; McConkey, 1967). To determine whether this prediction holds, the distribution of phosphoenolpyruvate carboxykinase mRNA in sodium dodecyl sulphate/sucrose gradients was examined (Fig. 5). After centrifugation, 1 ml fractions were collected and equal amounts of RNA from each fraction were added to reticulocyte lysate incubations. Phosphoenolpyruvate carboxykinase mRNA activity migrated with an average mobility of 23S, distributed between the 28S and 18S rRNA species. Thus the size of the mRNA as estimated by this technique is in agreement with its molecular weight extrapolated from the molecular weight of the enzyme. This method par-

Fig. 5. Sucrose-gradient centrifugation of phosphoenolpyruvate carboxykinase mRNA activity

Polyribosomes were prepared from starved-rat liver and dissociated with sodium dodecyl sulphate as described in the Experimental section. The RNA was layered in 12ml 5–20% sucrose density gradients, was centrifuged at $300000g$ for 6h, and 1ml fractions were collected. The RNA from six gradients was pooled into eight fractions, precipitated and collected by centrifugation; $30 \mu\text{g}$ of each fraction was added to reticulocyte lysates (total volume 0.5ml), and the ^3H -labelled phosphoenolpyruvate carboxykinase synthesized at 26°C for 1h was isolated by immunoprecipitation and quantified on sodium dodecyl sulphate/polyacrylamide gels. The d.p.m. of phosphoenolpyruvate carboxykinase (bars) represent the amount of enzyme synthesized in 0.5ml reaction volume. $\bullet$, E_{260} per ml for a representative gradient.

tially separated phosphoenolpyruvate carboxykinase mRNA from most cellular mRNA species, which sediment with a mean coefficient of 16–18S (Garrett *et al.*, 1973; Tweedie & Pitot, 1974).

It is generally accepted that most eukaryotic mRNA species, except for histone mRNA (Adesnik *et al.*, 1972), contain poly(A) sequences at their 3' end (Darnell *et al.*, 1971a; Edmonds *et al.*, 1971; Lee *et al.*, 1971). To determine whether the mRNA for phosphoenolpyruvate carboxykinase also contains a poly(A) segment, total polyribosomal RNA, which included the bottom 10ml of sodium dodecyl sulphate/sucrose gradients (Fig. 5), was chromatographed on a column of oligo(dT)–cellulose, which preferentially binds RNA containing poly(A) sequences.

The bound fraction of RNA was eluted by washing the column with a buffer of low ionic strength, and the abilities of the unbound and bound RNA fractions to direct the synthesis of phosphoenolpyruvate carboxykinase in reticulocyte lysates were compared with the activity of the polyribosomal RNA applied to the column (Table 1). The specific radioactivity of total polyribosomal RNA (expressed as d.p.m. in phosphoenolpyruvate carboxykinase/10 μ g of RNA) was approximately 5-fold less than the specific radioactivity of RNA migrating in sodium dodecyl sulphate/sucrose gradients between the 18S and 28S species (see Fig. 2). It is possible therefore to enrich for phosphoenolpyruvate carboxykinase mRNA by using this fraction. However, when the total polyribosomal RNA was enriched for poly(A)-containing RNA by adsorption on oligo(dT)–cellulose, there was a 20-fold increase in the specific radioactivity of RNA, accompanied by a decrease in the specific radioactivity of the RNA that did not bind to the column. Thus phosphoenolpyruvate carboxykinase mRNA probably contains a poly(A) sequence, a finding that is in accord with the inhibition in the synthesis of this enzyme in intact animals (Tilghman *et al.*, 1974) and Reuber H35 cells (Tilghman *et al.*, 1975; Gunn *et al.*, 1975) by cordycepin.

When total polyribosomal RNA or RNA purified

on oligo(dT)–cellulose was used to synthesize phosphoenolpyruvate carboxykinase, a single labelled polypeptide chain, migrating with authentic enzyme on sodium dodecyl sulphate/polyacrylamide gels was always observed. In addition, mRNA prepared by oligo(dT)–cellulose chromatography of total cellular RNA also directed the synthesis of the enzyme (Table 1). In these experiments the RNA was not enriched for mRNA coding for high-molecular-weight peptides as it was when 20–26S RNA was used as a template.

The ability of rabbit reticulocyte lysates to synthesize phosphoenolpyruvate carboxykinase in the direction of rat liver RNA, and the linearity of the response to exogenous RNA, should provide an excellent system for investigating possible alterations in intracellular mRNA during induction and deinduction of the enzyme *in vivo* by cyclic AMP. The fact that the system is sensitive to enrichment of phosphoenolpyruvate carboxykinase mRNA by differential centrifugation or extraction of poly(A)-containing mRNA indicates that differences in specific mRNA content of RNA, if present, should be detectable. These experiments also indicate that the use of heterologous cell-free systems is feasible for mRNA species that are minor components of total cellular mRNA. Phosphoenolpyruvate carboxykinase represents about 3–5% of total cytosol proteins synthesized during starvation (Hopgood *et al.*, 1973). This rather low percentage of total cellular protein synthesis, together with the relatively large size of the enzyme (85000 daltons), complicates its synthesis in a cell-free translation system. Most other mRNA translated *in vitro* code for proteins present in significantly greater concentrations than hepatic phosphoenolpyruvate carboxykinase (Brawerman, 1974). Also, an assay for mRNA for this enzyme could offer a unique opportunity to study the effect of cyclic AMP on enzyme synthesis. The only other inducible hepatic enzyme that has been studied at this level is tryptophan oxygenase (Schutz *et al.*, 1973, 1975), but this enzyme is not regulated by cyclic AMP.

Table 1. *Oligo(dT)–cellulose chromatography of total rat liver RNA*

Polyribosomal RNA obtained after sodium dodecyl sulphate/sucrose-gradient centrifugation, or total cellular RNA, was applied to a 4 ml oligo(dT)–cellulose column. The unbound fraction of RNA and the bound RNA eluted at low ionic strength were collected and 5–100 μ g amounts of RNA were incubated in reticulocyte lysates. The values represent means $\pm$ s.e.m. of seven to nine determinations, each using two preparations of RNA.

Fraction of RNA	Total E_{260} units	Radioactivity in phosphoenolpyruvate carboxykinase (d.p.m./10 μ g of RNA)
Total polyribosomal RNA	180	68 $\pm$ 26
RNA not bound to oligo(dT)–cellulose	—	29 $\pm$ 9
RNA bound to oligo(dT)–cellulose	1.7	1362 $\pm$ 88
Total cellular RNA bound to oligo(dT)–cellulose	—	793 $\pm$ 43

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