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CELL-FREE TRANSLATION AND PARTIAL PURIFICATION OF RAT LIVER FATTY ACID SYNTHETASE mRNA*

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SUMMARY

The cell-free synthesis of rat liver fatty acid synthetase has been demonstrated in a modified reticulocyte lysate translation system. The mRNA was partially purified from membrane-free polysomes by oligo (dT)-cellulose chromatography and subsequent sucrose density gradient centrifugation.

Mammalian liver fatty acid synthetase is a multifunctional enzyme complex that is regulated by dietary and hormonal factors (1,2). The molecular aspects of the regulation of this important lipogenic enzyme in rat liver have been a subject of interest to this laboratory for some time. Our recent studies have provided evidence which indicates that the dietary regulation of this enzyme occurs at the level of transcription (3,4). The purification of the fatty acid synthetase mRNA would facilitate the synthesis of a complimentary DNA probe for hybridization studies and would provide material for gene enrichment and for structural characterization studies.

In this paper we have demonstrated the cell-free synthesis of complete fatty acid synthetase polypeptide in a reticulocyte lysate translation system. We have also described a partial purification procedure for the fatty acid synthetase mRNA of rat liver. The purification procedure that we have developed involves the isolation of the membrane-free polysomes as the initial step. Subsequent purification of the mRNA utilizes affinity chromatography on oligo (dT)-cellulose and fractionation by sucrose density gradient centrifugation.

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EXPERIMENTAL PROCEDURE

Materials. The reticulocyte lysate system (mRNA-dependent by nuclease treatment and supplied as a translation kit), Aquasol, and L[^{35}S]-methionine (about 496 Ci per mmol) were obtained from New England Nuclear. Other reagents were obtained as follows: calf liver tRNA from Boehringer Mannheim, heparin from Sigma, oligo (dT)-cellulose (type 2) from Collaborative Research, Inc., fat-free diet from Nutritional Biochemicals, and goat anti-rabbit IgG from RPI Corp.

Magnesium Precipitation of Membrane-Free Polysomes from Rat Liver. Male albino rats (210 to 250 g) were fasted 48 hr and then refed a fat-free diet for 12 hr as previously described (3). At this time the amount of fatty acid synthetase synthesizing polysomes is at a maximum (3,4). The magnesium precipitated membrane-free polysomes were then isolated by the combined methods of Ramsey and Steele (5) and Palmiter (6). The livers were homogenized in 3 volumes of polysome buffer (25 mM Tris HCl, pH 7.5:25 mM NaCl: 5 mM $MgCl_2$) containing 100 μg/ml of sodium heparin. The homogenate was centrifuged at 2000 rpm for 2 min and then at 26,500 rpm for 12 min (SW-27 rotor). The supernatant solution was adjusted to 0.5 mg/ml of heparin, 2% Triton X-100 and 0.1 M $MgCl_2$, and then incubated in an ice bath for 1 hr. The precipitate was recovered by centrifugation and resuspended in buffer according to the method of Palmiter (6). The yield was about 30 A_{260} units/g liver. The ratio of $A_{260}/_{280}$ was 1.85, and that of $A_{260}/_{240}$ was 1.4.

Preparation of Poly (A)-Rich RNA from Membrane-Free Polysomes. The polysome solution (~20 A_{260} units/ml) was adjusted to 0.5% SDS,[1] 10 mM Tris HCl, pH 7.5, and 1 mM EDTA, heated at 65° for 10 min and then quickly cooled to room temperature. The treated polysome solution was centrifuged at 27,000 *g* for 10 min, the supernatant solution was adjusted to 0.5 M LiCl and then applied to an oligo (dT)-cellulose column (1 g) that was previously equilibrated with the same buffer plus LiCl. The column was washed with the high salt buffer (0.5 M LiCl, 10 mM Tris HCl, pH 7.5, 0.5% SDS and 1 mM EDTA) and then eluted with a low salt buffer (10 mM Tris, pH 7.5, 0.01% SDS and 1 mM EDTA). The eluted RNA was heat-denatured as described above and then the solution was adjusted to 0.5 M LiCl. The RNA was rechromatographed on another oligo (dT)-cellulose column. This poly (A)-rich RNA fraction was usually about 1% of the total RNA fraction. The poly (A)-rich RNA was precipitated with 2 volumes of ethanol at −20° overnight. The RNA precipitate was washed 3 times with 3 M sodium acetate, once with 65% ethanol and dried under nitrogen. The RNA was then dissolved in 20 mM Hepes buffer, pH 7.5.

Translation of Poly (A)-Rich RNA in a Reticulocyte Lysate Cell-Free System. The *in vitro* assay for rat liver poly (A)-rich RNA was performed in a nuclease-treated reticulocyte lysate system (mRNA-dependent) obtained from New England Nuclear. Unless otherwise indicated, each 25 μl assay mixture contained 55 mM potassium acetate, 2.2 M magnesium acetate, 2 μg calf liver tRNA, 28 μCi of [^{35}S]methionine, 1 μg of poly (A)-rich RNA and 10 μl of reticulocyte lysate. This mixture was incubated at 30° for 2 hr and then translation was terminated by the addition of 50 μl of 3 mM methio-

[1] Abbreviations used: SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

nine and chilling in an ice bath. Total protein synthesis was determined by pipeting 3 μl of the sample onto a Whatman 3 mM filter paper, pretreated with TCA, according to the method of Hall (7).

SDS-Polyacrylamide Gel Electrophoresis Analysis of Translation Product. The combination of the 25 μl incubation mixture and 50 μl of 3 mM methionine was adjusted to 0.9% NaCl, 10 mM potassium phosphate (pH 7.0), 1% Triton X-100, 1% sodium deoxycholate, 0.02% sodium azide and 1 mM PMSF (final volume of 150 μl). Indirect immunoprecipitation was carried out as described previously (3). The radioactive immunoprecipitate was further characterized by electrophoresis on 8% SDS-polyacrylamide gel as previously reported (3). The gels were fractionated into 2 mm sections and solubilized by heating at 90° with 30% H_2O_2 for 2 hr. Radioactivity was determined in Aquasol.

Sucrose Density Gradient Fractionation of RNA. The poly (A)-rich RNA isolated from free polysomes was heat-treated in a low salt solution to eliminate aggregation as reported by Haines *et al.* (8). The heat-treated RNA was then fractionated on a linear sucrose gradient (5 to 20%) in 0.1 M sodium acetate, pH 5.5, 1 mM EDTA and 0.5% SDS. Centrifugation was at 20° for 4 hr at 40,000 rpm (SW-41 rotor). Regions of the gradient were pooled and the RNA precipitated with ethanol. The RNA was washed with 3 M sodium acetate and 65% ethanol, and then dissolved in 20 mM Hepes buffer. The fatty acid synthetase mRNA activity of each region was assayed in the reticulocyte lysate system.

RESULTS AND DISCUSSION

Cell-Free Synthesis of the Fatty Acid Synthetase. The *de novo* synthesis of full-length fatty acid synthetase polypeptide in a modified rabbit reticulocyte cell-free translation system is shown in Fig. 1. Poly (A)-rich RNA obtained from membrane-free polysomes of rat liver was fractionated on sucrose density gradients and then used to program protein synthesis in the reticulocyte lysate system. The isolation of the radioactive fatty acid synthetase polypeptide protein product was achieved by indirect immunoprecipitation (3) and the product was analyzed by SDS-polyacrylamide gel electrophoresis. A single major radioactive peak was obtained which co-migrated with authentic fatty acid synthetase protein. In contrast, immunochemical competition with unlabeled authentic fatty acid synthetase antigen produced a marked reduction in the amount of radioactive fatty acid synthetase protein. These results indicate that the synthesis of the full-length synthetase polypeptide has been achieved. This is the first report of the *in vitro* synthesis of full-length synthetase polypeptide ($M_r \simeq 250,000$). A previous study had reported the synthesis of the fatty acid synthetase in a wheat germ lysate translation system, but the product synthesized was of a smaller molecu-

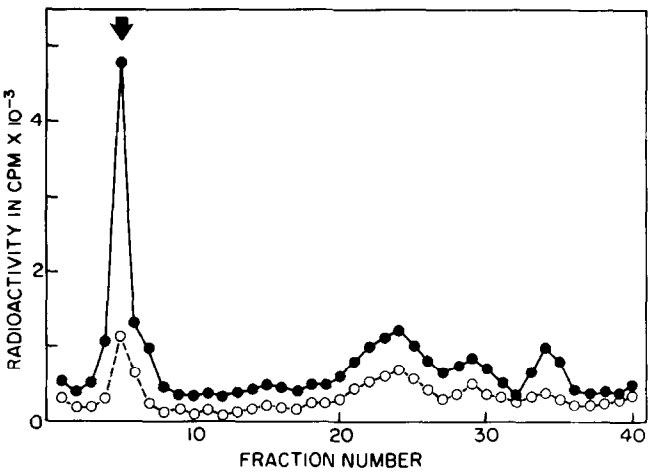

Fig. 1. Synthesis of fatty acid synthetase polypeptide in the modified rabbit reticulocyte lysate cell-free translation system. The indirect immunoprecipitated radioactive protein products were analyzed by SDS polyacrylamide gel electrophoresis. Protein synthesis was directed by the addition of sucrose density gradient-purified poly (A)-rich RNA (1 μg per reaction mixture) obtained from membrane-free polysomes (•——•). Immunochemical competition was performed on duplicate reaction products by the addition of unlabeled authentic fatty acid synthetase protein (10 μg) prior to indirect immunoprecipitation (o——o). Co-electrophoresis of unlabeled authentic fatty acid synthetase polypeptide is indicated by the arrow.

lar weight and probably represented incomplete polypeptide of fatty acid synthetase (9). The reticulocyte lysate, regarded as the most efficient cell-free translation system (10,11), along with our specific modifications in the system (described below) and our method of mRNA purification may account for our success in the synthesis of full-length fatty acid synthetase polypeptide. Our starting source of RNA was membrane-free polysomes. These are rapidly isolated from the membrane fraction of rat liver which has a high nuclease activity. Presumably, this procedure provides more intact, undegraded mRNA.

Optimization of the Reticulocyte Cell-Free Translation System for the Synthesis of Fatty Acid Synthetase Polypeptide. The general characteristics of the reticulocyte cell-free protein-synthesizing system were examined in order to optimize the synthesis of the fatty acid synthetase polypeptide (Fig. 2). The optimum concentrations of magnesium and potassium ions required for the synthesis of the fatty acid synthetase polypeptide were 2.2 mM and 60 mM, respectively (Fig.

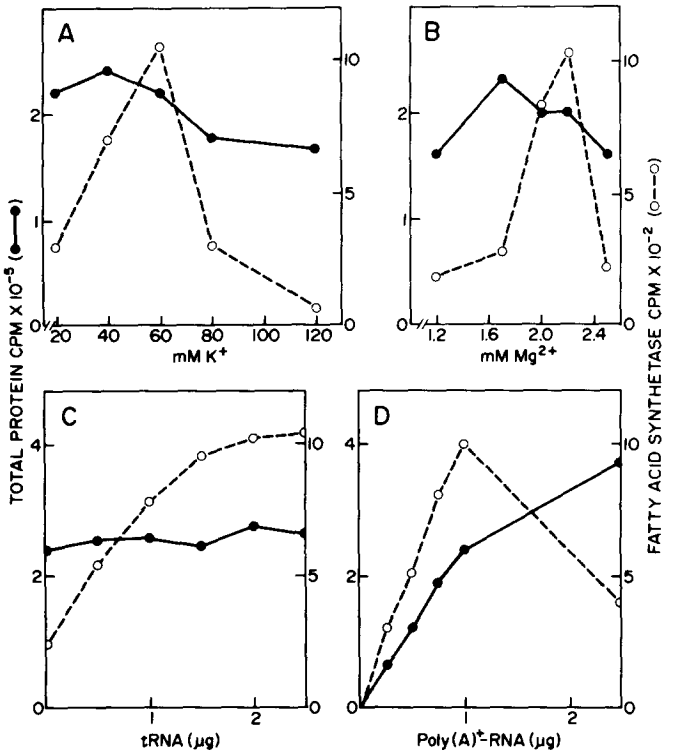

Fig. 2. Optimization of conditions for the synthesis of fatty acid synthetase and total protein in the reticulocyte cell-free translation system. Sucrose density gradient-purified poly (A)-rich RNA (1 μg RNA per reaction) was used to direct the synthesis of the fatty acid synthetase polypeptide. The conditions tested for the cell-free translation of protein are as indicated on this figure and the incubation was for 120 min at 30°.

2A and B). The addition of calf liver tRNA to the reticulocyte lysate was essential for the *in vitro* synthesis of fatty acid synthetase polypeptide; tRNA (80 μg/ml) increased the synthesis of fatty acid synthetase by 4-fold without significantly affecting total protein synthesis (Fig. 2C). The time-course of cell-free protein synthesis was also examined (data not presented). A linear synthesis of fatty acid synthetase polypeptide and total protein occurred for 120 min at 30°. Translation activity leveled off over the next 30 min for both total and fatty acid synthetase protein synthesis. Synthesis of full-length fatty acid synthetase polypeptide was linear with mRNA concentrations up to 40 μg/ml (Fig. 2D). In all subsequent studies, as well as the one reported for Fig. 1, the optimum conditions were employed.

Partial Purification of the Fatty Acid Synthetase mRNA. The rationale for the purification of the fatty acid synthetase mRNA is based on the unique properties of the mammalian fatty acid synthetase protein. The evidence from antibody binding studies (4,12) indicated that the fatty acid synthetase mRNA is localized on the membrane-free polysomes. Furthermore, we had previously identified the fatty acid synthetase mRNA as a large species ($M_r \cong 2.4 \times 10^6$) which contains a polyadenylate region (3). This mRNA is of sufficient size to code for the 250,000 molecular weight fatty acid synthetase polypeptide. Thus, isolation of RNA from membrane-free polysomes and subsequent size fractionation of poly (A)-rich RNA on sucrose gradients were logical procedures for the purification of the fatty acid synthetase mRNA. In addition, the source of rat liver RNA was from animals which were conditioned by fasting and fat-free diet refeeding for 12 hr to maximize the level of fatty acid synthetase mRNA (3,4).

The results of studies on the partial purification of fatty acid synthetase mRNA from rat liver are summarized in Table I. Enrichment for the fatty acid synthetase mRNA was achieved by the

TABLE I

PARTIAL PURIFICATION OF FATTY ACID SYNTHETASE mRNA FROM RAT LIVER

RNA fraction	Cell-free protein synthesis		Relative mRNA activity
	Total polypeptides	Fatty acid synthetase	
	(cpm/μg RNA)		
	cpm x 10^{-5}	cpm	%
Unfractionated total polysomal RNA	0.6	---	---
Poly (A)-rich RNA from total polysomes	6.9	410	0.06
Poly (A)-rich RNA from membrane-free polysomes	7.1	1300	0.18
Sucrose density gradient RNA (30-37S region)	2.7	6800	2.5

The conditions for assays are described under "Experimental Procedure."

isolation of membrane-free polysomes. The poly (A)-rich RNA prepared from the membrane-free polysomes produced 3 times more translation of the complete fatty acid synthetase polypeptide than did the poly (A)-rich RNA obtained from the total polysome fraction. The enrichment for fatty acid synthetase mRNA activity by the isolation of the rat liver membrane-free polysomes provides further evidence for the localization of the synthesis of the fatty acid synthetase in liver. In addition, the removal of the membrane fraction at an early stage in mRNA purification is beneficial, since the high level of RNase associated with this particulate fraction of the liver would be quickly eliminated (5). Primarily complete fatty acid synthetase polypeptide was synthesized, which indicates that the mRNA is relatively undegraded.

The poly (A)-rich RNA fraction was further purified by sucrose density gradient fractionation. The separation of the large size RNA by sucrose density gradient centrifugation produced a 17-fold enrichment of fatty acid synthetase mRNA (Table I). The poly (A)-rich RNA was fractionated into 4 regions on a preparative sucrose density gradient and the fatty acid synthetase mRNA activity was located in the 30S to 37S region of the RNA profile. This result confirms our earlier study in which we first reported the large size of the fatty acid synthetase mRNA as approximately 33S (3). The amount of fatty acid synthetase mRNA present in the most active template RNA fraction obtained by size separation represents 2.5% of the total RNA fraction. However, this estimate is based on *in vitro* polypeptide translation, not on RNA mass, and represents a minimum value. The purity of mRNA can not be determined precisely in a cell-free translation system since other mRNAs, as well as rRNA, which are probably present in this fraction, would interfere in the translation assay (11,13).

Studies on the further purification of fatty acid synthetase mRNA by additional size separation procedures are in progress. When purified mRNA is obtained, studies on the synthesis of complimentary DNA will be carried out.

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