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Biochemistry

Translation of rat liver fatty acid synthetase mRNA in a cell-free system derived from wheat germ

(mRNA translation/wheat germ cell-free system/regulation of fatty acid synthetase)

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ABSTRACT **Total liver polysomes were isolated from rats that had fasted for 48 hr and that then had been re-fed a high-carbohydrate, fat-free diet for 20–24 hr. Indirect immunoprecipitation of the polysomes with purified antibody to rat liver fatty acid synthetase and deproteination on sodium dodecyl sulfate-containing sucrose gradients gave an RNA fraction which, when translated in a cell-free system derived from wheat germ, yielded a major polypeptide of apparent molecular weight 225,000 when the translation products were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The polypeptide was specifically precipitated with antibody against rat liver fatty acid synthetase and competed with unlabeled fatty acid synthetase for binding to the antibody. It was somewhat smaller than native fatty acid synthetase subunits (molecular weight 240,000). The peptide accounted for approximately 65% of the radioactive, antibody-precipitable product, the remainder being peptides in the molecular weight range 100,000–150,000. Synthesis of the polypeptide was optimized with respect to K^+, Mg^{2+}, and spermine concentrations. The quantity of fatty acid synthetase mRNA obtained by the above procedure and measured by translation was a function of the nutritional state of the animal. The relative activity in fasting rats compared to rats that were re-fed for 12 hr was 1:12. The data suggest that rat liver fatty acid synthetase is synthesized as intact subunits from a large mRNA molecule or molecules.**

Rat liver fatty acid synthetase (referred to as the synthetase) catalyzes the *de novo* synthesis of long-chain, saturated fatty acids and has a molecular weight (M_r) of 480,000 (1). It consists of two subunits each of M_r 240,000 (2). The identity or nonidentity of the subunits of the synthetase has not been conclusively established. The quantity of synthetase protein present in liver varies with the nutritional and hormonal status of the animal (3). When fasting rats are fed a high-carbohydrate, fat-free diet, synthetase activity increases approximately 50-fold within 48 hr (4, 5). When liver polysomes are isolated at early times during the refeeding period and the nascent protein chains completed in an *in vitro* protein-synthesizing system derived from Chang liver cells or wheat germ, a peptide with M_r 240,000 and precipitable with antibody to rat liver synthetase is formed (5, 6). Moreover, the rates of synthetase synthesis measured by polysomal translation correlate well with the specific activity of the enzyme at various times after the start of refeeding (5, 6).

In this report, we describe the isolation by indirect immunoprecipitation of rat liver polysomes synthesizing the synthetase, and the translation, in a wheat germ cell-free system, of synthetase mRNA isolated from these polysomes. The principal product of translation is a peptide of M_r 225,000, somewhat smaller than the subunit peptide(s) of native synthetase. This peptide is specifically precipitated by purified antibody to rat liver synthetase and competes with purified enzyme for binding to the antibody. The quantity of synthetase mRNA that can be obtained by our procedure is a function of the nutritional state of the animal and, as expected, parallels the activity of liver polysomes in an assay of the latter for completion of synthetase nascent chains.

MATERIALS AND METHODS

Materials. Sodium heparin, ribonuclease-free sucrose, [^{3}H]leucine, fat-free diet, and rats were as described (5). ATP, GTP, spermine, and creatine phosphate were from Sigma. Creatine kinase was obtained from P-L Biochemicals. Diethyl pyrocarbonate was from Aldrich, Triton X-100 and Aquasol from New England Nuclear, and sodium deoxycholate from Schwarz-Mann. *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) and cycloheximide were obtained from Calbiochem. Raw wheat germ was from ADM Mills, Shawnee Mission, KS.

Isolation of Polysomes. All glassware and buffers were sterilized by autoclaving. Syringes and other materials in contact with RNA-containing solutions were rinsed thoroughly with 0.1% diethyl pyrocarbonate prior to use. Total liver polysomes were isolated from rats that had fasted for 48 hr and had been re-fed a fat-free, high-carbohydrate diet for 20–24 hr (5). Polysomes were isolated by the method of Palmiter with slight modification (7). Livers were homogenized in 9 volumes of 25 mM Tris·HCl, pH 7.5, containing 25 mM NaCl, 5 mM $MgCl_2$, 0.5 mg of sodium heparin per ml, 5 μg of cycloheximide per ml, and 20% Triton X-100. The homogenate was centrifuged at 30,000 × *g* for 15 min and the pellet was discarded. To the supernatant was added an equal volume of 200 mM $MgCl_2$ in 25 mM Tris·HCl, pH 7.5/25 mM NaCl. The suspension was allowed to stand at 4° for 1 hr to precipitate the polysomes. The precipitate was collected by layering 16 ml of suspension over 8 ml of a solution of 0.2 M sucrose in 25 mM Tris·HCl, pH 7.5/25 mM NaCl/100 mM $MgCl_2$/0.2 mg of heparin per ml and centrifuging for 10 min at 30,000 × *g*. The upper layer was removed by aspiration and the top of the sucrose was washed with sterile water. The sucrose was removed by aspiration and the polysome pellet was taken up in incubation buffer (25 mM Tris·HCl, pH 7.1/5 mM $MgCl_2$/150 mM NaCl/0.5 mg of heparin per ml) with the aid of several strokes in a glass Dounce homogenizer.

Preparation of Antibodies. Purified goat antibody to rat liver synthetase and rabbit anti-goat IgG were prepared as described (5). Both antibodies were freed of contaminating ribonucleases by passing them through columns of carboxy-

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Abbreviations: synthetase, fatty acid synthetase; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; M_r, molecular weight.

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methyl-cellulose and DEAE-cellulose as described by Palacios *et al.* (8).

Indirect Immunoprecipitation of Synthetase-Synthesizing Polysomes. Antibody precipitations were carried out essentially as described by Shapiro *et al.* (9). The polysomal suspension was diluted to 10 A_{260} units/ml with incubation buffer (1 A_{260} unit is the amount of material which, when placed in a 1-cm light path, gives an absorbance at 260 nm of 1). Generally, a total of 500–1000 A_{260} units were used. To the suspension was added 40 μg of goat antibody to rat liver synthetase per ml, and the mixture was allowed to stand for 1 hr at 0° with occasional stirring. At the end of this time, 200 μg of rabbit anti-goat IgG was added per ml and the mixture was incubated further for 1 hr at 0°. The precipitated polysomes were collected by layering 10 ml of the suspension over a discontinuous sucrose gradient consisting of 12 ml of 1.0 M sucrose in incubation buffer plus 1% Triton X-100 and 1% sodium deoxycholate and 6 ml of 0.5 M sucrose in the same buffer and centrifuging at 30,000 × *g* for 20 min. The pellet was resuspended in 1.5 ml of incubation buffer and centrifuged a second time through the discontinuous sucrose gradient. The pellet was suspended in 2–3 ml of 0.1 M NaCl/50 mM EDTA/1% sodium dodecyl sulfate ($NaDodSO_4$), two volumes of absolute ethanol were added, and the material was precipitated overnight at −20°.

Deproteination of RNA. The precipitate from the ethanol precipitation was collected by centrifugation at 30,000 × *g* for 20 min and was taken up in 2 ml of 20 mM sodium acetate, pH 5.0/50 mM EDTA/0.5% $NaDodSO_4$. The solution was heated at 65° for 10 min and then rapidly cooled to 25°. One milliliter of RNA-containing solution was layered over 11 ml of a 10–30% linear sucrose gradient in 20 mM sodium acetate, pH 5.0/50 mM EDTA/0.5% $NaDodSO_4$ and centrifuged at 40,000 rpm for 6 hr in a Beckman SW-40 rotor. The gradients were pumped from the bottom through a flow cell connected to a Gilford 250 spectrophotometer and the absorbance at 260 nm was monitored continuously. The bottom 7 ml of the gradient, containing both 28S and 18S ribosomal RNA peaks, was collected and the RNA was precipitated overnight with two volumes of ethanol at −20°. The RNA was collected by centrifugation at 30,000 × *g* for 10 min, dried under nitrogen, taken up in sterile distilled water, and stored at −70°. The RNA routinely was stored at a concentration of 50–100 A_{260} units/ml and had A_{260}/A_{280} of 1.8–1.9.

***In Vitro* Protein-Synthesizing System.** Each 100-μl assay contained 15 mM Hepes (pH 7.4), 100 mM KCl, 2 mM MgOAc, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 16 μg of creatine phosphokinase, 20 μM each of 19 nonradioactive amino acids minus leucine, 20 μCi of [^{3}H]leucine (60 Ci/mmol), 2.0 A_{260} units of wheat germ extract prepared according to the procedure of Roberts and Paterson (10) but excluding the pre-incubation step, 1 μg of rat liver tRNA, 75 μM spermine, and varying amounts of RNA. Incubations were carried out at 25° for 2 hr and terminated by the addition of 10 μl of 50 mM leucine and Triton X-100 and sodium deoxycholate to a final concentration of 0.5% each. For the measurement of total protein synthesis, a 5-μl aliquot of each incubation mixture was added to 1 ml of 10% trichloroacetic acid and heated at 90° for 10 min. The precipitated protein was collected on 0.45 μm Millipore filters, 10 ml of Aquasol scintillation fluid was added, and the radioactivity on the filters was measured in a Packard Tri-Carb scintillation spectrometer model B2450.

Analysis of Translation Mixtures. Indirect immunoprecipitation of incubation mixtures with purified goat antibody to rat liver synthetase and rabbit anti-goat IgG were carried out as described by Strauss *et al.* (6) with minor modifications. The immunoprecipitates were analyzed on 6% $NaDodSO_4$/polyacrylamide slab gels as described (6). The dried gels were cut into 2-mm slices and dissolved with 0.5 ml of 30% H_2O_2 at 90° for 1 hr. Radioactivity was measured in 10 ml of Aquasol.

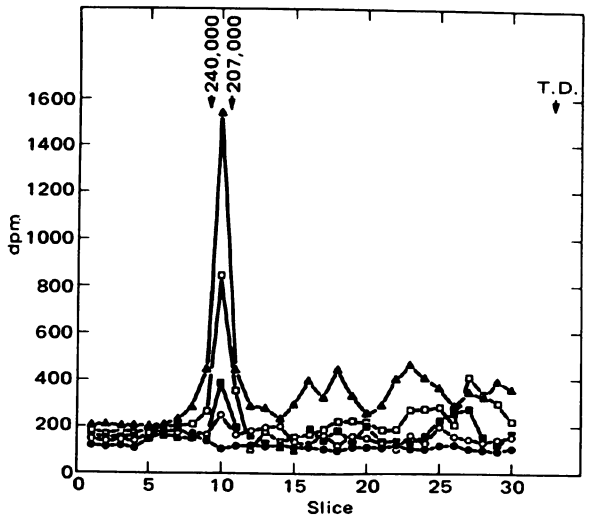

FIG. 1. Translation of rat liver synthetase mRNA in a wheat germ cell-free system. Liver polysomes were prepared and immunoprecipitated with purified goat antibody to rat liver synthetase and the RNA was extracted and translated. The reaction mixtures were treated with goat anti-rat synthetase followed by rabbit anti-goat IgG, and the precipitates were analyzed on 6% $NaDodSO_4$/polyacrylamide gels. Gels were dried and cut into 2-mm slices; the slices were dissolved in 0.5 ml of 30% H_2O_2 by heating for 1 hr at 90°. Radioactivity was determined in 10 ml of Aquasol scintillation fluid. ●, No RNA added; ○, 0.2 A_{260} unit of RNA; ■, 0.4 A_{260} unit of RNA; □, 1.0 A_{260} unit of RNA; ▲, 2.0 A_{260} units of RNA. The M_r standards run in parallel slots of the same gel are rat liver synthetase (240,000) and myosin (207,000). T.D., tracking dye.

RESULTS

When liver polysomes derived from rats fed a fat-free, high-carbohydrate diet for 20–24 hr after a 48-hr fast were reacted with antibody to rat liver synthetase, approximately 2–3% were precipitated when 40 μg/10 A_{260} units of antibody was used. The use of higher amounts of antibody resulted in a precipitate that did not deproteinate efficiently on $NaDodSO_4$-containing sucrose gradients. When the precipitated polysomes were dissociated in acetate/$NaDodSO_4$/EDTA buffer and the RNA was isolated on sucrose gradients and translated in a wheat germ cell-free system, a 2- to 3-fold stimulation of [^{3}H]leucine incorporation into total protein was observed. Indirect immunoprecipitation of the incubation mixture with antibody to rat liver synthetase and analysis of the precipitate on $NaDodSO_4$/polyacrylamide gels gave the result shown in Fig. 1. The major peak of radioactivity was associated with a peptide of M_r 225,000 which migrated slightly ahead of the synthetase peptide(s), M_r 240,000. This band accounted for approximately 65% of the total radioactivity on the gel, the remainder being found primarily in smaller peptides in the M_r range 100,000–150,000. Synthesis of the major peptide was linear with RNA concentration up to the highest level tested, 2.0 A_{260} units/100 μl.

The time course of synthesis of the major peptide precipitable with anti-synthetase antibody, M_r 225,000, is shown in Fig. 2 and yields an S-shaped curve with a lag phase of 10–15 min. Such a lag is to be expected for a peptide of this size translated from a large mRNA molecule.

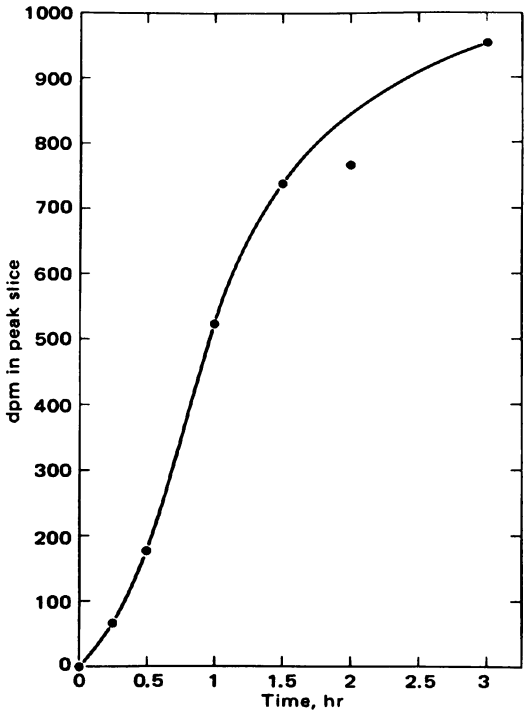

FIG. 2. Time course of translation of synthetase mRNA. Translation assays were carried out as described in the legend of Fig. 1 with 2.0 A_{260} units of RNA. Reactions were terminated at the indicated times by cooling in ice and adding 10 μl of 50 mM unlabeled L-leucine. Gel electrophoresis was carried out on the immunoprecipitated products as in Fig. 1. The total counts in the major peak of M_r 225,000 are plotted as a function of time of incubation.

To investigate further the nature of the large peptide synthesized *in vitro*, competition studies were carried out in which increasing amounts of purified rat liver synthetase were added to translation mixtures subsequent to the incubation and prior to antibody precipitation of product. The effect on the amount of product recovered is given in Fig. 3 and indicates that unlabeled synthetase effectively competes with the large synthetic peptide for binding to anti-synthetase antibody. This result suggests that the peptide is specifically recognized by the antibody and is either the enzyme subunit or a major part of the subunit as found in the intact enzyme. The smaller peptides

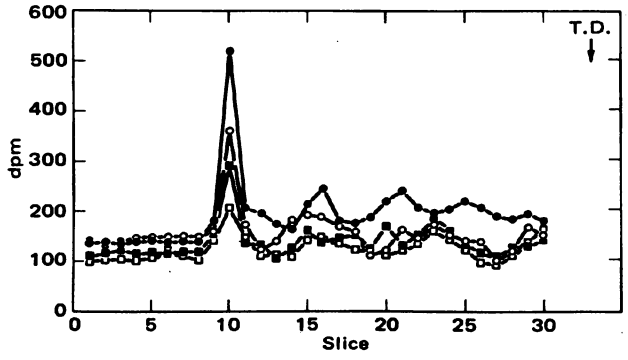

FIG. 3. Effect of rat liver synthetase on the immunoprecipitation of products from the translation of synthetase mRNA. Assays were carried out as described in the legend of Fig. 1 with 0.5 A_{260} unit of RNA. At the end of the incubation period, various amounts of purified rat liver synthetase were added and the reaction mixtures were immunoprecipitated with anti-synthetase antibody and analyzed as in Fig. 1. ●, No synthetase added; ○, 10 μg of enzyme added; ■, 25 μg of enzyme added; □, 100 μg of enzyme added. T.D., tracking dye.

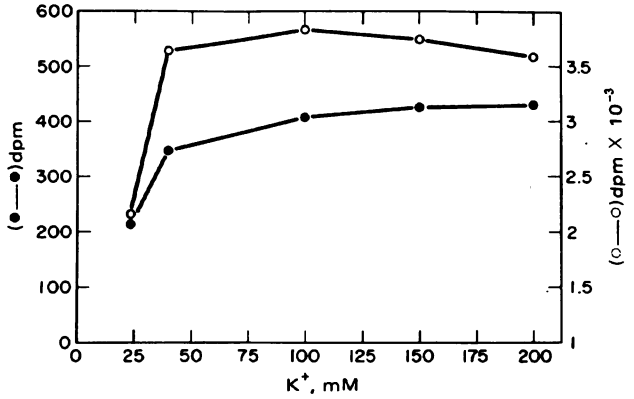

FIG. 4. Dependence of total protein synthesis and synthetase synthesis on the concentration of KCl in the wheat germ incubation mixture. Assays were carried out as in the legend of Fig. 1 with 0.5 A_{260} unit of RNA and the indicated concentrations of KCl in a total volume of 100 μl. The Mg^{2+} and spermine concentrations were 2 mM and 75 μM, respectively. ●, Synthetase synthesis measured as for Fig. 1 and expressed as dpm in slice 10; ○, total protein synthesis measured as in *Materials and Methods*.

synthesized *in vitro* were also effectively competed out by unlabeled synthetase, suggesting that they may be partially completed translation products of synthetase mRNA.

In order to define optimal conditions for the translation of synthetase mRNA, we varied the concentrations of K^+, Mg^{2+}, and spermine in the incubation mixture. The optimal potassium ion concentration for total protein synthesis in our translation system was near 100 mM, while the dependence of the synthesis of synthetase peptide on potassium ion concentration was relatively flat in the vicinity of 100 mM KCl (Fig. 4). The magnesium optimum for synthetase synthesis measured at 75 μM spermine was substantially higher (5 mM) than the optimum for total protein synthesis (2 mM) (Fig. 5). However, this could be lowered by using higher spermine concentrations. Spermine at a concentration of 500 μM, when assayed with 2 mM Mg^{2+}, gave maximal stimulation of synthetase synthesis (Fig. 6). A relatively high spermine optimum was anticipated for the

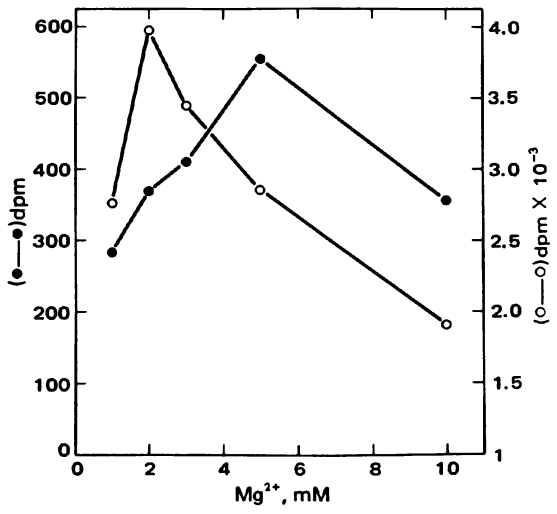

FIG. 5. Determination of the optimum Mg^{2+} concentrations for synthetase synthesis and total protein synthesis. Assay mixtures contained 0.5 A_{260} unit of RNA and the indicated concentrations of magnesium acetate. The K^+ and spermine concentrations were 100 mM and 75 μM, respectively. ●, Synthetase synthesis measured as for Fig. 4; ○, total protein synthesis measured as in *Materials and Methods*.

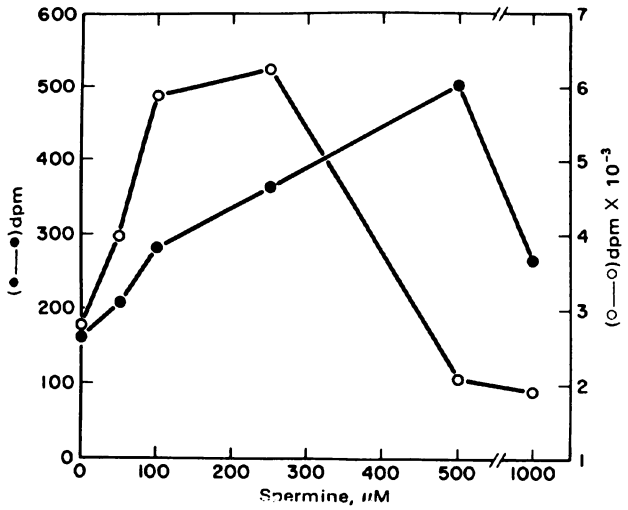

FIG. 6. Effect of spermine concentration on synthetase and total protein synthesis. Assays contained 0.5 A_{260} unit of RNA and the indicated concentrations of spermine in a total volume of 100 μl. The K^+ and Mg^{2+} concentrations were 100 mM and 2 mM, respectively. ●, Synthetase synthesis measured as for Fig. 4; ○, total protein synthesis measured as in *Materials and Methods*.

translation of synthetase mRNA because of the large size of the product and the postulated role of spermine in maintaining the integrity of the ribosome–mRNA complex so that larger polypeptides can be synthesized (11). We have found, however, that as long as the potassium ion concentration is maintained at 100 mM, lowering the spermine concentration below the optimum does not result in an increase in the proportion of smaller polypeptides at the expense of the major product with M_r 225,000 (data not shown).

It has been shown that the induction of synthetase activity in rat liver after a fast and refeeding is closely paralleled by a greatly increased incorporation of [^{3}H]leucine into polysomal nascent chains of synthetase measured in a cell-free protein-synthesizing system (5, 6). It would be expected that the amount of synthetase mRNA isolated from antibody-precipitated polysomes would increase in parallel with the induction of enzyme activity and the increase in polysomal translation rate of the enzyme. To test this, we fasted rats for 48 hr and re-fed them the fat-free diet beginning at zero time and continuing up to 24 hr. Rats were sacrificed at 0, 2, 4, 8, 12, and 24 hr after the start of refeeding and polysomes were isolated and immunoprecipitated with anti-synthetase antibody. Very low yields of RNA were obtained at the early time points, but yields increased throughout as expected. The mRNA was translated in the wheat germ system using the optimal conditions described above. Results presented in Table 1 show that synthetase synthetic activities using polysomes and mRNA run closely parallel with each other. Maximal incorporation of [^{3}H]leucine into the enzyme occurred at or near 12 hr of refeeding in both cases and preceded the rise in enzyme activity.

DISCUSSION

In this report we have shown that fatty acid synthetase mRNA is translated in a wheat germ cell-free protein-synthesizing system to give a large polypeptide, M_r 225,000, which is very near the size of native enzyme subunits, M_r 240,000 (2). Under optimal conditions, approximately 10% of the total labeled amino acid that is precipitated by trichloroacetic acid is associated with this polypeptide. A polypeptide of similar size can

Table 1. Comparison of the effect of nutritional state on synthetase synthesis from polysomes and mRNA

Time of refeeding, hr	Polysomes,* dpm/enzyme peak	mRNA,† dpm/enzyme peak	Enzyme spec. act.,‡ units/mg of protein
0 (fasting)	673	41	1.1
2	1034	98	1.1
4	1121	140	1.3
8	2438	236	4.3
12	8280	475	7.1
24	6389	382	24.0

* Polysomes were isolated as described in *Materials and Methods*. They were assayed in a "run-off" system with wheat germ extract and the reaction mixtures were subjected to indirect immunoprecipitation with anti-synthetase antibody and analysis on Na-DodSO$_4$/polyacrylamide gels as described (5). Each translation assay contained 1 A_{260} unit of polysomes per 100 μl.

† Synthetase mRNA was isolated from polysomes of rats re-fed for the indicated times by indirect immunoprecipitation and deproteination on NaDodSO$_4$/sucrose gradients. The mRNA was translated and the products were analyzed as described in *Materials and Methods* except that the concentration of spermine was 500 μM. Numbers are dpm in the major polypeptide band with M_r 225,000 and have been normalized to 0.5 A_{260} unit of RNA.

‡ Synthetase was assayed as described (12).

also be translated from synthetase mRNA in the reticulocyte lysate cell-free system (data not shown).

The RNA preparation used in this study was derived from liver polysomes by indirect immunoprecipitation with purified goat antibody to rat liver synthetase followed by dissociation of the polysomes in NaDodSO$_4$/EDTA buffer and deproteination on sucrose gradients. Previous attempts in our laboratory to isolate and translate synthetase mRNA by a variety of other techniques have been unsuccessful. These have included isolation of total liver polysomes followed by dissociation and chromatography on columns of oligo(dT)-cellulose, phenol/chloroform extraction of total polysomes, and dissociation of polysomes in NaDodSO$_4$-containing buffers followed by isolation of RNA on sucrose gradients. Only a relatively small proportion of total liver mRNA is expected to be synthetase mRNA, based on the amount of synthetase protein present in liver in the fully induced state (5), and this, along with the large size expected of this RNA, would most likely account for the isolation problems. Other large eukaryotic mRNAs have been purified and translated in cell-free systems, notably, vitellogenin mRNA from chicken liver, which codes for a polypeptide of M_r 240,000 (13, 14). However, vitellogenin synthesis can be induced to very high levels by administration of estradiol with a corresponding increase in the mRNA content, thereby facilitating isolation.

Rat liver synthetase is known to be present at very low levels in fasting animals, but the synthetic rate increases dramatically within a few hours after the start of refeeding a high-carbohydrate, fat-free diet (6). This change may be followed by measuring the completion of polysomal nascent chains of the enzyme in a cell-free system (5, 6). It follows that when mRNA derived from polysomes that have been reacted with antibody against rat liver synthetase is translated *in vitro*, the amount of product made as a function of time of refeeding should correspond closely to the amount made from polysomes isolated at the same times, assuming that the RNA remains intact and the translation system responds faithfully to increasing levels of mRNA. This was found to be the case in our system, as illustrated in Table 1.

The apparent size of the major polypeptide synthesized by the wheat germ system in response to synthetase mRNA is somewhat smaller than intact subunits of the enzyme. This could be due to a number of factors, such as premature termination of translation, cleavage of the mRNA either during isolation or in the translation assay by nucleases present in the wheat germ preparation (11), or partial degradation of the primary translation product during isolation.

The large size of the translation product of synthetase mRNA suggests that the enzyme subunits are synthesized intact from a large mRNA and not as a series of smaller fragments which are linked together some time during or after translation. Furthermore, there was no evidence for increased amounts of immunoprecipitable peptides smaller than the putative synthetase peptide at the early times after induction (data not shown). However, direct evidence that the synthetase is synthesized as a single polypeptide chain awaits the isolation of a single species of mRNA of the correct size that is capable of directing the synthesis of the enzyme.

Since the isolation procedure described here can detect only mRNA incorporated into polysomes actively synthesizing synthetase, it cannot be used to answer the question whether "adaptive synthesis" of the enzyme is due to an increase in the level of synthetase mRNA or an increased efficiency of translation of existing mRNA. This will require further purification of the mRNA and the preparation of a complementary DNA copy to probe for synthetase mRNA sequences in total liver mRNA (15) or the development of a method for detecting synthetase mRNA directly by translation of total liver mRNA. Efforts directed toward the latter have been unsuccessful thus far, perhaps because of the relatively small quantity of synthetase mRNA expected in liver as well as degradation of the mRNA during isolation. Additional studies will be necessary to solve these problems.

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