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Cell-free translation and thyroxine induction of carbamyl phosphate synthetase I messenger RNA in tadpole liver

[mitochondrial enzyme/synthesis/regulation/reticulocyte lysate system/carbamoyl-phosphate synthase (ammonia)]

MASATAKA MORI*, SIDNEY M. MORRIS, JR.†, AND PHILIP P. COHEN‡

Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 53706

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ABSTRACT **Total RNA of tadpole and frog (*Rana catesbeiana*) liver was isolated by either 7 or 8 M guanidine·HCl extraction and translated in a cell-free protein-synthesizing system derived from rabbit reticulocytes. The identity of carbamyl phosphate synthetase I [carbamoyl-phosphate synthase (ammonia); ATP:carbamate phosphotransferase (dephosphorylating), EC 2.7.2.5] synthesized *in vitro* with the purified enzyme was established as follows: (*i*) immunoprecipitation by a specific antibody; (*ii*) comigration with purified carrier enzyme on sodium dodecyl sulfate/polyacrylamide gel electrophoresis; (*iii*) copurification with carrier enzyme by affinity chromatography on Cibacron Blue F3GA-coupled agarose; and (*iv*) formation of identical proteolytic cleavage products. Inclusion of protease inhibitors in the system resulted in no apparent change in the polypeptide molecular weight. These results indicate that carbamyl phosphate synthetase I is synthesized as a polypeptide that is indistinguishable from the mature enzyme by the analytical methods used and that it is not grossly modified during its transport into mitochondria. The level of translatable mRNA for carbamyl phosphate synthetase-I in tadpole liver was increased about 2-fold 1 day after thyroxine treatment and did not change significantly through 4 subsequent days of treatment. Thus the thyroxine-induced synthesis of carbamyl phosphate synthetase I in tadpole liver is at least partly due to an increase of translatable mRNA for this enzyme.**

Frog liver carbamyl phosphate synthetase I (CPS-I) [carbamoyl-phosphate synthase (ammonia); ATP:carbamate phosphotransferase (dephosphorylating), EC 2.7.2.5], the enzyme involved in the initial step of urea biosynthesis, has a M_r of 320,000 and consists of two identical subunits of M_r 160,000 (1–4). It is located in the mitochondrial matrix and constitutes about 20% of the total matrix protein of adult frog liver (5). The enzyme is synthesized extramitochondrially and is subsequently transported into mitochondria (5). Previous reports from this laboratory (6, 7) suggested the existence of a macromolecular enzyme precursor that is poorly reactive to the antibody against the mitochondrial CPS-I. However, further attempts to demonstrate a precursor have been unsuccessful (unpublished results). These included the administration to thyroxine (T4)-treated tadpoles of various drugs that would be expected to inhibit mitochondrial accumulation of CPS-I, such as chloramphenicol, ethidium bromide, allylisopropylacetamide (8), and microbial protease inhibitors (9). Furthermore, in labeling experiments with tadpole liver slices, the label appeared rapidly in the mitochondrial CPS-I, indicating rapid transport from ribosomes to mitochondrial matrix (unpublished experiments).

In order to clarify this problem, cell-free translation of CPS-I mRNA was undertaken. Studies on tadpole and frog liver mRNA have been hampered by high levels of ribonuclease activities (10–12). We modified an extraction method using guanidine·HCl (13) for the isolation of RNA from tadpole and frog liver that was suitable for cell-free translation. The present paper reports the translation *in vitro* of CPS-I mRNA from tadpole and frog liver to produce a polypeptide that is apparently identical to the subunit of the mature enzyme. This report also deals with the induction of translatable CPS-I mRNA during T4-induced CPS-I synthesis.

MATERIALS AND METHODS

Chemicals. The cell-free translation kit (reticulocyte lysate, L-[^{35}S]methionine) was purchased from New England Nuclear. T4 was obtained from Calbiochem. Antipain, leupeptin, chymostatin, and pepstatin, which were originally obtained from the United States–Japan Cooperative Cancer Research Program, were provided by W. Troll (New York University). Guanidine·HCl was prepared from guanidine carbonate (Matheson, Coleman & Bell) (14) and filtered through activated charcoal (Norit A, from Matheson, Coleman & Bell).

Animals. Adult frogs (*Rana catesbeiana*) and premetamorphic tadpoles (weighing 8–12 g) were obtained from Mogul-Ed (Oshkosh, WI). Tadpoles were kept without food for a few days in dechlorinated water at 15°C before use. T4 solution (0.12 mM in 1 mM NaOH) was injected intraperitoneally (0.6 nmol/g of body weight), and the tadpoles were kept in 26 nM T4 at 23°C. Immersion fluid was changed daily.

Isolation of RNA. Total RNA of tadpole and frog liver was isolated by the following two modifications of the method described in detail by Deeley *et al.* (13). *Method A.* Immediately after excision and removal of the gall bladder, livers (2–4 g) were homogenized in 20 vol of 20 mM NaOAc (pH 5.0 at 4°C)/7 M guanidine·HCl/1 mM dithiothreitol for 3 min in a Waring Blendor jacketed in dry ice. Cellular debris was removed by centrifugation at 11,000 × *g* for 10 min at −10°C and the supernatant fluid was filtered through a sterile gauze pad. Prechilled ethanol (−20°C, 0.5 vol) was added and the solution was kept at −20°C for 15 min. The precipitated RNA was collected by centrifugation and quickly dissolved in a minimal volume of 20 mM NaOAc (pH 7.0 at 4°C)/7 M guanidine·HCl/1 mM dithiothreitol/20 mM Na_2EDTA. The ethanol precipitation was repeated twice and the RNA was dissolved in a minimal volume of the buffer (pH 7.0) containing 7 M guanidine·HCl, used above, and then extracted with an equal volume of $CHCl_3$/isoamyl alcohol (24:1, vol/vol). An

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Abbreviations: CPS-I, carbamyl phosphate synthetase I [carbamoyl-phosphate synthase (ammonia)]; T4, L-thyroxine; $NaDodSO_4$, sodium dodecyl sulfate.

* Present address: Department of Biochemistry, Chiba University School of Medicine, Inohana, Chiba 280, Japan.
† Present address: Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, OH 44106.
‡ To whom reprint requests should be addressed.

equal volume of ethanol was added to the aqueous layer and the solution was kept at −20°C for 20 min. The precipitated RNA was collected by centrifugation and washed twice with 66% (vol/vol) ethanol/0.1 M NaOAc (pH 5.0) and once with ethanol. The RNA was then dried under reduced pressure and stored at −80°C. *Method B.* RNA was prepared as in method A, except that 8 M guanidine·HCl was used. After the final ethanol precipitation step, the RNA was dissolved in a minimal volume of 20 mM Na_2EDTA (pH 7.0 at 4°C), followed by addition of an equal volume of 6 M NaOAc, pH 5.2. After storage at −20°C for 20 min, RNA was collected by centrifugation. Precipitation from 3 M NaOAc was repeated once. Final washing and drying of RNA was performed as in method A. Method A permitted significantly better differential precipitation of RNA and protein in the initial ethanol treatment steps.

Analysis of RNA. Integrity of the RNA preparations was evaluated by electrophoresis in polyacrylamide gels containing formamide (15), followed by staining with Stains-All (Eastman) (16). Only those RNA preparations yielding sharp 18S and 28S ribosomal RNA bands in the absence of low M_r degradation products were used for translation assays. RNA was estimated by using an extinction coefficient at 260 nm of 20 cm^{-1} ml mg^{-1}.

Cell-Free Translation. RNA was translated by using a commercial cell-free system derived from rabbit reticulocytes in accordance with the protocol provided (New England Nuclear, catalog no. NEK-001). Incubation was at 37°C or 25°C in either a 25- or a 50-μl system. Incorporation of [^{35}S]methionine into protein was determined. Each experiment included as a blank the complete reaction mixture without added RNA.

Antibody Preparation. Frog liver CPS-I was purified to homogeneity by affinity chromatography with Affi-Gel Blue (Cibacron Blue F3GA-coupled agarose, Bio-Rad) (4). Antibody against frog liver CPS-I was produced in rabbits and partially purified by $(NH_4)_2SO_4$ precipitation (17).

Analysis of *In Vitro* Translation Products. After cell-free translation, the reaction mixture was brought to 0.5 ml by the addition of 17.5 mM Na phosphate (pH 7.4)/0.15 M NaCl/1 mM methionine/0.2 mM antipain/0.2 mM leupeptin/0.2 mM chymostatin/0.2 mM pepstatin and centrifuged at 105,000 × *g* for 40 min to remove ribosomes. Purified CPS-I (10 μg) was added as carrier to the supernatant, followed by an excess of antibody. The immunoprecipitation mixture was incubated for 15 min at 37°C and overnight at 4°C. The immunoprecipitate was then collected by centrifugation in a Beckman Microfuge and washed three times with 0.5 ml of 0.15 M NaCl/1% Triton X-100/1% deoxycholate/1 mM methionine.

The immunoprecipitate was dissolved in sodium dodecyl sulfate ($NaDodSO_4$) sample buffer [75 mM Tris·HCl, pH 6.8/10% (wt/vol) glycerol/5% (vol/vol) 2-mercaptoethanol/2.3% (wt/vol) $NaDodSO_4$/0.001% bromophenol blue] by heating at 100°C for 3 min and electrophoresed on 0.1% $NaDodSO_4$/polyacrylamide (5 or 6%) slab gels in a discontinuous buffer system (18). The gels were stained with 0.1% Coomassie blue/25% (vol/vol) isopropanol/10% (vol/vol) acetic acid and destained in 5% isopropanol/10% acetic acid. After gel electrophoresis, [^{35}S]methionine incorporation into CPS-I was quantitated by scintillation counting of serial 5-mm gel slices. Gel slices were solubilized according to Mahin and Lofberg (19) and assayed by scintillation counting in 18 ml of Scintisol (Isolab, Akron, OH). Polyacrylamide gel slabs were fluorographed according to Bonner and Laskey (20) with exposures of 1–7 days on either Kodak X-Omat R or SB-5 film. In several experiments, the dried gels used for fluorography were cut into 5-mm strips and assayed in 5 ml of Scintisol in order to quantitate [^{35}S]-methionine incorporation into CPS-I.

RESULTS

Total RNA was successfully isolated from tadpole and frog liver by either 7 or 8 M guanidine·HCl extraction. The incorporation of [^{35}S]methionine into total protein increased as a function of the amount of RNA added to the protein-synthesizing system up to 10 μg/25 μl and then decreased with higher amounts of RNA. Total protein synthesis at 37°C increased up to 30 min and then slightly decreased by 60 min. When assayed at 25°C, protein synthesis proceeded at a slower rate and reached a higher level than at 37°C. Large polypeptides of M_r up to about 140,000 were synthesized by using control mRNA isolated from adenovirus-infected HeLa cells (Fig. 1, lane 1). The total translation products of RNA from T4-treated tadpole liver included a faint band at the position corresponding to the CPS-I subunit (Fig. 1, lane 2). A similar band was observed with liver RNA of adult frog (Fig. 1, lane 3).

Immunoprecipitation of the incubation mixture with antibody to frog liver CPS-I and analysis of the immunoprecipitate on $NaDodSO_4$/polyacrylamide gels gave the result shown in Fig. 2A. The major band of radioactivity was associated with a peptide of M_r 160,000 that comigrated with carrier CPS-I. A control experiment, in which bovine liver ornithine transcarbamylase (EC 2.1.3.3) and antibody against that enzyme were added instead of carrier CPS-I and CPS-I antibody, showed no band of radioactivity at the position corresponding to CPS-I. Identification of the newly synthesized peptide as

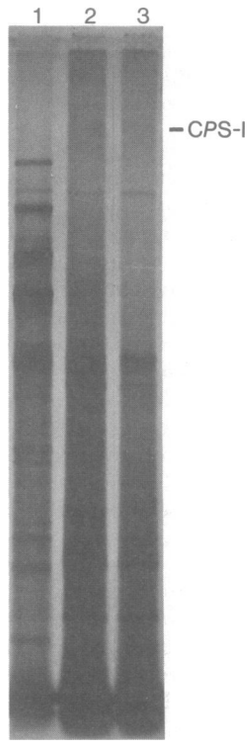

FIG. 1. Analysis of the polypeptides synthesized *in vitro*. Translation was carried out at 37°C for 60 min in a volume of 25 μl with adenovirus-infected HeLa cell mRNA (lane 1), 10 μg of total RNA of T4-treated (4 days) tadpole liver (lane 2), or 10 μg of total RNA of frog liver (lane 3). RNA was isolated by method A. The proteins synthesized were precipitated by acetone, solubilized by heating in $NaDodSO_4$ sample buffer, and analyzed by $NaDodSO_4$/10% polyacrylamide gel electrophoresis followed by fluorography. Migration is toward the bottom of the figure.

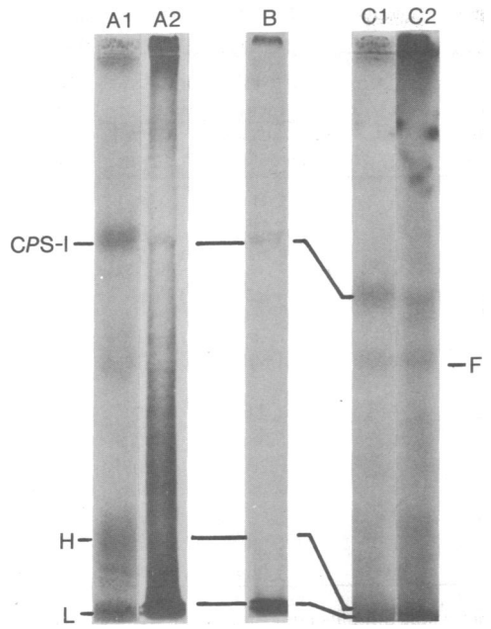

FIG. 2. Identification of CPS-I in the translation products. Protein synthesis was conducted at 37°C for 60 min in a volume of 25 μl with 10 μg of RNA prepared by method A from T4-treated (4 days) tadpoles. To the 105,000 × *g* supernatant of the reaction mixture was added 10 μg of purified frog liver CPS-I (*A* and *B*) or an extract of crude mitochondrial fraction from frog liver containing about 10 μg of endogenous CPS-I (*C*). In *B*, supernatant supplemented with purified CPS-I was applied to a column (0.5 × 1.0 cm) of Affi-Gel Blue and CPS-I was eluted by Mg^{2+}-free buffer (4). Immunoprecipitation was performed in the presence (*A* and *B*) and absence (*C*) of the protease inhibitors. The immunoprecipitate was run on a 6% (*A* and *B*) or 5% (*C*) polyacrylamide/$NaDodSO_4$ gel and the gel was stained and fluorographed. *A1* and *C1*, stain; *A2*, *B*, and *C2*, fluorography. H and L are heavy and light chains, respectively, of immunoglobulin; F is the proteolytic fragment of CPS-I.

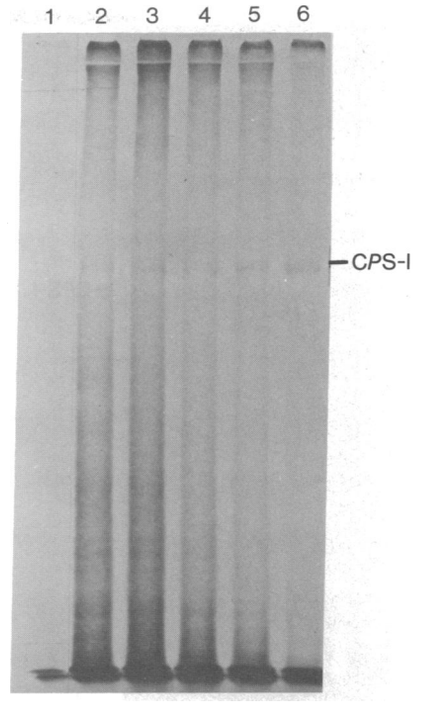

FIG. 3. Effect of RNA amount and protease inhibitors on CPS-I synthesis. Protein synthesis was conducted at 37°C for 60 min in a volume of 25 μl in the absence (lanes 1–5) and presence (lane 6) of antipain, leupeptin, chymostatin, and pepstatin (0.2 mM each). The amount of RNA added was: none (lane 1); 2.5 μg (lane 2); 5 μg (lane 3); 10 μg (lanes 4 and 6); and 20 μg (lane 5). RNA was isolated from T4-treated (4 days) tadpoles by method A. Immunoprecipitate was run on $NaDodSO_4$/6% polyacrylamide gel and processed for fluorography.

CPS-I was further confirmed by copurification with the authentic enzyme by affinity chromatography on Affi-Gel Blue; it was adsorbed to the gel at pH 8.3 in the presence of 5 mM Mg^{2+} and eluted by a Mg^{2+}-free buffer (4) (Fig. 2*B*). We have previously shown that frog CPS-I is cleaved to a fragment of M_r 139,000 by a protease that is present in a crude mitochondrial fraction of frog liver, and the fragment is fully reactive with antibody against the native enzyme (4). Fig. 2*C2*, band F, shows that the radioactive product synthesized *in vitro* was cleaved by the protease in the same manner as the authentic enzyme.

The level of CPS-I synthesis showed a dependence on added RNA similar to that seen for total incorporation, but reached a maximal value at a lower amount of added RNA (5 μg/25 μl) (Fig. 3, lanes 1–5). Inclusion of protease inhibitors resulted in less radioactivity in the lower M_r region of the $NaDodSO_4$ gels, suggesting that the protease inhibitors are effective in preventing premature termination or endogenous proteolysis of CPS-I during translation *in vitro* (Fig. 3, lane 6). Preliminary experiments with avian mRNAs in this system indicate that these antiproteases can also selectively alter the translational efficiency of specific mRNAs (unpublished experiments). However, addition of the protease inhibitors resulted in no apparent change in the molecular weight of CPS-I synthesized *in vitro*.

The synthesis of CPS-I increases severalfold in tadpole liver during a few days of immersion in dilute T4 solution after injection of T4 (21, 22). In order to see whether the enzyme induction was due to corresponding increases in amount of translatable mRNA for CPS-I, liver RNA isolated from tadpoles treated with T4 for various periods was translated and the immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by fluorography (Fig. 4). While synthesis of CPS-I was seen with RNA from untreated tadpoles, there was an approximately 2-fold increase after T4 treatment for 1 day; synthesis did not increase further after 4 days of treatment and was essentially equivalent to that seen with frog liver RNA. For quantitation, the gels were sliced, solubilized, and assayed for radioactivity (Table 1). Increase of CPS-I synthesis *in vitro* after T4 treatment was nearly 2-fold at every amount of added RNA. A slight decrease was seen 2 days after T4 treatment; the reason for this is not known at present. No significant difference was observed in total trichloracetic acid-precipitable radioactivity with RNA preparations from control and T4-treated tadpoles.

DISCUSSION

Our modifications of the procedure of Deeley *et al.* (13) permitted the preparation of undegraded RNA fractions from tadpole and frog liver that were suitable for translation studies.

Whereas earlier studies from this laboratory were interpreted as providing evidence for extramitochondrial synthesis of low molecular weight precursors that were assembled into active enzyme after rapid transport into the mitochondria (see ref. 5), the present results clearly demonstrate that CPS-I is synthesized as a large polypeptide with a molecular weight essentially identical or very close to that of the monomeric subunit of the mature enzyme. Because the analytical methods used here are incapable of distinguishing proteins that differ in size by less

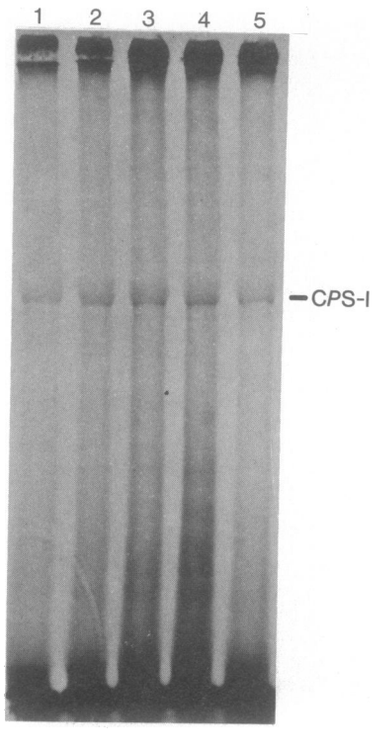

FIG. 4. Effect of T4 treatment on [^{35}S]methionine incorporation into immunoprecipitated CPS-I. Tadpoles (nine animals per group) were treated with T4 for 0 (lane 1), 1 (lane 2), 2 (lane 3), and 4 (lane 4) days and RNA was isolated by method B. The RNA (20 μg) was translated at 25°C for 2 hr in a volume of 50 μl in the presence of the protease inhibitors. The immunoprecipitate was run on NaDodSO$_4$/6% polyacrylamide gel and the gel was fluorographed. Lane 5 represents translation of adult frog RNA (20 μg).

than 2000 daltons, the possibility cannot be excluded that a "pre-CPS-I" is initially synthesized containing a "signal" peptide of less than 2000 daltons that is cleaved off in conjunction with its transport into the mitochondrion, analogous to the processing of the extramitochondrially synthesized subunits of yeast F_1-ATPase (23).

The incorporation into CPS-I in the translation assays, relative to total incorporation, ranged from approximately 0.003 to 0.01%. The percentage incorporation levels for CPS-I reported here undoubtedly underestimate the actual amount of mRNA for CPS-I within the total mRNA population because the translation assay only scores intact mRNA for CPS-I, and optimal conditions for translation of mRNA for CPS-I in this system have not yet been achieved. Although the assay conditions used were sufficient to determine relative changes in levels of mRNA for CPS-I, further studies using more highly purified mRNA preparations and optimal translation conditions will be required for quantitative correlations of CPS-I and mRNA levels.

The marked increase in CPS-I during the early stages of T4-induced metamorphosis (24) as a result of increased *de novo* synthesis (6, 7, 22, 25, 26) raises the question as to the primary regulatory mechanism involved. The present study indicates that regulation of the rate of CPS-I synthesis by T4 is determined by the level of mRNA for the enzyme, strongly suggesting that this regulation occurs, at least in part, at the transcriptional level. Additional supporting evidence for this interpretation is provided from the following earlier findings: inhibition of CPS-I induction by actinomycin D (21); increased chromatin template capacity *in vitro* (27); increased histone acetylation (28, 29) and phosphorylation (30, 31); increased RNA polymerase activities (32, 33); and changes in RNA profiles associated with synthesis of CPS-I (10, 34).

Table 1. Effect of T4 treatment on [^{35}S]methionine incorporation into total protein and immunoprecipitated CPS-I

T4 treatment, days	RNA added, μg	[^{35}S]Methionine incorporation: Total protein, cpm × 10^{-5}	[^{35}S]Methionine incorporation: CPS-I, cpm	Relative increase in % CPS-I synthesis*
0	5	5.01	28	1.0
	10	7.61	25	1.0
	20	6.30	17†	1.0
1	5	4.62	45	1.7
	10	7.76	41	1.6
	20	4.60	31†	2.5
2	5	4.92	37	1.3
	10	5.88	37	1.9
	20	6.25	20†	1.2
4	5	5.76	44	1.4
	10	7.63	56	2.2
	20	7.06	34†	1.8

Tadpoles (nine animals per group) were treated with T4 for 0 to 4 days and RNA was isolated by method B. Translation was at 25°C for 2 hr in a volume of 50 μl in the presence of antipain, leupeptin, chymostatin, and pepstatin (0.2 mM each).

* Comparisons between different time points are made only at the same level of added RNA.

† 2,5-Diphenyloxazole-impregnated and dried gels were cut into 0.5-mm strips and assayed for radioactivity.

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