MESSENGER RNA FOR GLUTAMINE SYNTHETASE: ITS PARTIAL PURIFICATION, TRANSLATION IN A CELL-FREE SYSTEM AND ITS REGULATION BY HYDROCORTISONE[1,2]

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SUMMARY: Messenger RNA for glutamine synthetase (GS), extracted from polysomes of embryonic chick retina cells, was partially purified by chromatography on oligo (dT)-cellulose on the basis of its poly (A)-content and translated in a cell-free protein synthesizing system derived from wheat germ. Identification and quantitation of GS in the products of cell-free synthesis was achieved by immunoprecipitation of the enzyme and electrophoresis in sodium-dodecyl-sulfate polyacrylamide gels. Comparison of the amount of GS synthesized in vitro by equal amounts of mRNA purified from polysomes of control or hydrocortisone induced retina indicate that the hormone elevates the level of GS mRNA on the polysomes from induced cells.

INTRODUCTION: During the ontogeny of the chick embryo, adrenal steroids elicit the induction of glutamine synthetase (GS) in the neural retina in ovo on or about the 16th day of development (1). In cultures of isolated retinas from younger (8-14 day) embryos, the enzyme can be induced precociously by HC or several other 11 β-OH-corticosteroids (2), thus providing a convenient system for studying hormone action. The biochemical events during the induction of GS by HC have been studied extensively (3,4). In particular, it has been shown that the induction requires transcription (5-7) and that the higher levels of enzyme in the induced retina are a result of increased rate of its synthesis (8,9). However, unlike the effect of estradiol (10) or of HC in other target tissues (11,12), total RNA or total protein synthesis in the retina is not altered by HC. Using antiserum against purified retinal GS (13), we have shown (7) that the amount of immunoprecipitable nascent enzyme in the polysomes from induced retina is about 3-fold that from controls. These studies were indicative of an higher quantity of functional mRNA in the induced retina. To provide direct evidence for this, an effective method of purification and assay of GS mRNA was needed. Here, we report the development of a cell-free system from wheat germ that allows faithful translation and assay of partially purified mRNA for GS and demonstrate directly that HC elevates the level of mRNA for GS.

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[1] Abbreviations: Glutamine synthetase-GS; Hydrocortisone-HC; Sodium dodecyl sulfate-SDS

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MATERIALS AND METHODS: Organ Culture. Neural retinas from 11-day chick embryos were isolated aseptically and cultured as described before (7). Following a 10 hour incubation in the absence of hormone, cultures in which induction of GS was desired were incubated for an additional 10 hours in the presence of HC (0.9 μM). Controls were incubated identically without inducer. All cultures were harvested after a total of 20 hours of incubation.

Extraction of Polysomal RNA. The harvested retinas were washed with ice-cold Tyrode's solution and lysed in 0.5% triton-X-100 made up in TKMH buffer (50 mM tris-HCl, 100 mM KCl, 10 mM magnesium acetate, and 100 μg/ml heparin, pH 7.4) as described (7). The lysates were spun at 27,000 x g for 5 minutes. The supernatants were used for the isolation of polysomes by Mg^{++} precipitation and extraction of polysomal RNA by phenol-chloroform mixture as described by Palmiter (14).

Oligo (dT)-Cellulose Chromatography. The procedure of Leder et al (15,19) was used except that 40-50 $O.D._{260}$ units of RNA was applied to 200 mg columns of oligo (dT)-cellulose. Over 95% of the RNA was unadsorbed (Fraction I) and appeared with the application buffer (0.01M tris, 0.5M KCl, pH 7.4). Poly (A)-containing mRNA was eluted with 0.01M tris, 0.1M KCl (Fraction II) and 0.01M tris (Fraction III) respectively. The yield of mRNA in Fraction II was very low (about 0.5% of the total); Fraction III represented 2-3% of the total RNA applied RNA Fractions I and III were precipitated with ethanol and stored overnight at -20°C. They were spun, washed, dried with N_2 and dissolved in water.

Cell-Free Protein Synthesis. The procedures used for the preparation of wheat germ extracts and other conditions for protein synthesis were modified from Roberts and Paterson (16). The final assay mixture contained in a total volume of 0.2 ml: 1 mM ATP; 0.2 mM GTP; 8 mM creatine phosphate; 0.05 mg creatine phosphokinase; 0.05 mM of 19 amino acids; 2 mM dithiothreitol; 20 mM N-2-

TABLE I

Efficiency of immunoprecipitation of GS by anti-GS γ-globulin[a].

Amount of ^{14}C-labeled retina supernatant taken for immunoprecipitation		CPM precipitated by		CPM in GS[b]
CPM	Enzyme units (GHA)	Normal γ- globulin	Anti-GS γ-globulin	
44,450	0.5	210	461	250 (0.56%)
88,900	1.0	434	920	486 (0.55%)
177,800	2.0	1336	2436	1100 (0.61%)
222,250	2.5	1660	2930	1270 (0.57%)

[a]Aliquots of 100,000 g supernatants from sonicates of retinas labeled with ^{14}C-amino acid mixture (1 μc/ml) for 24 hours were adjusted to a total of 3 GHA content by adding supernatants from unlabeled retina and immunoprecipitated with 300 μgs of normal-or anti-GS-γ-globulin in a total volume of 1.5 ml as described in 'Methods'.

[b]Figures in parenthesis represent counts specifically precipitated by the anti-GS γ-globulin as percent of total radioactivity.

hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES); 5 μc ^{3}H-leucine; 2.25 mM magnesium acetate; 55 mM KCl; and 0.05 ml of preincubated S-30 from wheat germ extract. Spermine and RNA were added as indicated. Incubations were at 25°C for 90 minutes. To determine the radioactivity incorporated into total proteins, aliquots were precipitated with 7.5% TCA containing 10 mM leucine. The precipitates were collected in millipore filters, washed, and counted in a Beckman LS200 counter using toluene containing 4 g/l of omnifluor (New England Nuclear).

Preparation of GS and Anti-GS γ-globulin. Chick retinal GS was purified to homogeneity as described (13) and antibodies were prepared in rabbits. Gamma-globulins were prepared as described before (7).

Identification of GS Synthesized in Vitro. Following the incorporation period, the assay mixture was centrifuged at 140,000 x g for one hour to sediment polysomes. The supernatants containing newly synthesized released proteins were divided into two halves. Carrier GS (0.05 ml of 100,000 x g supernatant from unlabeled retina sonicates containing 3 GHA units of enzyme) was added and the fractions were brought to 10 mM sodium phosphate pH 7.1, 0.85% saline, and 2% triton-X-100 in a total volume of 1.5 ml by adding concentrated stock solutions. Immunoprecipitation was carried out with equivalent amounts (300 μg) of anti-GS γ-globulin or normal γ-globulin and incubating the mixtures at 37°C for 30 minutes and at 4° for another 30 minutes. The immunoprecipitates were washed, assayed for radioactivity and electrophoresed in 10% SDS-gels (17) as described by Schutz, et al (25). In separate gels, which were stained with coomassie blue, authentic retinal GS (13) was run as marker.

Enzyme Assay. GS activity was determined by the glutamyl-transferase reaction (19) and expressed as μmoles of γ-glutamyl hydroxamate (GHA) formed per hour per ml assay solution.

RESULTS AND DISCUSSION: Immunochemical Precipitation of GS. In view of our final goal - to identify and quantitate GS in the products of the cell-free protein synthesis, the efficiency of immunoprecipitation of GS by the anti-GS γ-globulin was examined. Preliminary experiments of the determination of equivalence point in the reaction of anti-GS γ-globulin and the enzyme showed that

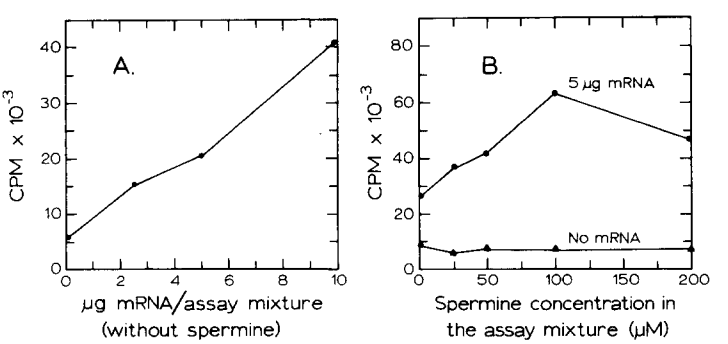

Fig. 1. Effect of spermine on the incorporation of ^{3}H-leucine into total protein in the wheat germ cell-free system. Indicated amounts of mRNA (Fraction III from oligo-dT-cellulose column) were translated in the absence (A) or presence (B) of various concentrations of spermine.

300 µg of the anti-GS γ-globulin could precipitate 2-4 GS activity units (GHA) with 90-95% efficiency. To further test the efficiency of immunopricipitation of radioactive GS, retinas from 11-day embryos were labeled for 24 hours in the presence of inducing dose of HC with 1 µc/ml of ^{14}C-amino acid mixture. Increasing aliquots of 100,000 x g supernatants from sonicates of these labeled retinas, containing 0.5 to 2.5 GHA units of enzyme activity, were adjusted to 3 GHA content by addition of nonradioactive enzyme from unlabeled retina sonicate supernatants. One set of these mixtures was reacted with 300 µg of anti-GS γ-globulin and the other with 300 µg of normal γ-globulin. Table I shows that the amount of radioactivity specifically precipitated (difference between radioactivity precipitated by anti-GS γ-globulin and normal γ-globulin) is a linear function of the amount of enzyme in the radioactive supernatant taken for immunoprecipitation, thus, reinforcing the quantitative precipitation of the enzyme by the anti-GS γ-globulin.

Translation of RNA in the Wheat Germ Cell-Free System. Fig. 1A shows the pattern of incorporation of ^{3}H-leucine into total protein as a function of the amount of mRNA added. A linear increase in incorporation was observed up to at least 10 µg of mRNA per 0.2 ml of assay mixture. The stimulation in protein synthesis was about 3-fold with 5 µg mRNA and 6-fold with 10 µg mRNA. In view of several reports (20,21) on the stimulatory effects of spermine in protein synthesis in vitro, we tested the effects of various concentrations of spermine on protein synthesis directed by 5 µg of retina mRNA in the wheat germ cell-free system (Fig. 1B). The presence of 100 µM spermine in the assay mixture gave an additional 2-3 fold increase in protein synthesis without increasing the background incorporation, thus giving a net stimulation of 6-10 fold by 5 µg mRNA. In all subsequent experiments 100 µM spermine was included in the assay mixture.

Identification and Quantitation of GS Synthesized in Vitro. Equal amounts of mRNA purified from polysomes of control of induced retinas were translated in the in vitro system. The newly synthesized released proteins were separated and immunoprecipitated as described in 'Methods'. The results (Table II) show that incorporation of ^{3}H-leucine into total protein did not differ significantly for control or induced mRNA. In both cases about 40% of the total incorporation could be accounted for in the released chains. The amount of radioactivity specifically precipitated by anti-GS γ-globulin was about three times higher for the HC-induced mRNA than that in the control indicating the presence of elevated quantity of mRNA for GS in the polysomes from induced retina. No such difference was observed in the immunoprecipitates from the products of cell-free synthesis directed by the unadsorbed RNA (Fraction I) from oligo

(dT)-cellulose chromatography (Table II). The GS content of immunoprecipitates from the mRNA-directed proteins was further confirmed by SDS- polyacrylamide gel electrophoresis. It was previously demonstrated that retinal GS is composed of eight identical subunits of molecular weight of 42,000 and it migrates as a single band in SDS-gels (13). Fig. 2A shows that polysomal mRNA from induced retina, purified on oligo (dT)-cellulose, directs the synthesis of a product that migrates identically with authentic retinal GS (arrow) or ^{14}C-labeled GS immunoprecipitated from 100,000 x g supernatants of sonicates of retinas labeled with ^{14}C-amino acid mixture (Fig. 2B). Analysis of the immunoprecipitated products of synthesis directed by the mRNA from control retina also showed a small peak corresponding to the position of the marker GS (Fig. 2A). For the mRNA from induced retina, the amount of radioactivity in this position is about 5-fold that of the control mRNA. These results are consistent with the low rate of enzyme synthesis in the non-induced retina and a 5-fold increase in the rate seen after eight hours of induction (9). They

TABLE II

Immunoprecipitation of GS from products of cell-free synthesis directed by RNA preparations from control or HC-induced retina

Type[a] and amount of RNA assayed	CPM in total protein	CPM in released chains	CPM precipitated by Normal γ-globulin	CPM precipitated by Anti-GS γ-globulin	CPM in GS
Fraction III from control retina (15 µg)	250,000	104,000	276	396	120 (40)[b]
Fraction III from induced retina (15 µg)	300,000	124,000	247	572	325 (210)
Fraction I from control retina (25 µg)	130,000	58,000	150	144	0
Fraction I from induced retina (25 µg)	110,000	55,000	148	144	0

[a]Fraction III represents the poly (A)-containing mRNA fraction and Fraction I represents the unabsorbed RNA from the oligo (dT)-cellulose column which is primarily ribosomal.

[b]Figures in parenthesis show the differences in the radioactivity precipitated by anti-GS γ-globulin and normal γ-globulin in the peak region corresponding to subunits of GS on SDS-polyacrylamide gels as calculated from Fig. 2A.

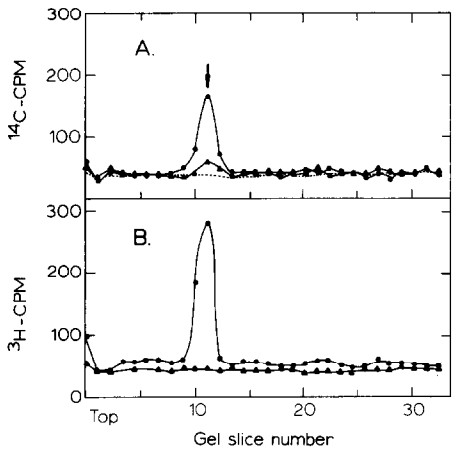

Fig. 2. SDS-polyacrylamide gel electrophoresis of the products of cell-free synthesis after immunoprecipitation. A: Proteins directed by 15 µg of mRNA from control (▲-▲-▲) or HC-induced (●-●-●) retina and immunoprecipitated by anti-GS γ-globulin. Radioactivity precipitated by normal γ-globulin from the products of cell-free synthesis directed by 15 µg of mRNA from control or induced retina gave virtually identical electrophoretic patterns (-----). The arrow indicates the position of authentic retinal GS used as marker. B: As an additional marker, ^{14}C-labeled GS from 100,000 g supernatants of sonicates of retinas labeled with ^{14}C-amino acid mixture was precipitated by anti-GS γ-globulin (●-●-●) or normal γ-globulin (▲-▲-▲) and electrophoresed simultaneously.

suggest that the enhanced rate of GS synthesis in the induced retina is correlated to an increased amount of GS mRNA in the polysomes.

Hormonal regulation of cellular mRNA has been reported for several other sex-steroid-dependent proteins (ovalbumin, conalbumin, avidin) in the chick oviduct (22-24) and for tryptophan oxygenase in the rat liver (25). It remains unknown whether the control by the steroid is at the level of synthesis of mRNA or its degredation. Since the mRNA fractions used in the present study were obtained from isolated polysomes, the effect of HC on the storage or stability of GS mRNA in other compartments of the retina cell is also uncertain. These possibilities are now open to investigation.

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