EXPERIMENTAL CONSERVATION AND DELAYED EXPRESSION OF LABILE TEMPLATES FOR ENZYME SYNTHESIS IN NEURAL CELLS

S. Garfield and A. A. Moscona

Department of Biology, University of Chicago, Chicago, Illinois 60637

Received February 20,1973

Summary: Base-level synthesis of the enzyme glutamine synthetase in embryonic neural retina is mediated by labile RNA templates. Complete blocking of protein synthesis with a high dose of cycloheximide does not prevent the transcription of these templates and allows them to be conserved and to accumulate in the cells; when cycloheximide is removed and further RNA synthesis is halted, the conserved templates are expressed and the level of glutamine synthetase increases at a rate faster than basal, as determined by immunomeasurements of the nascent enzyme. Puromycin does not have this effect. It is suggested that high concentrations of cycloheximide conserve the labile templates by preventing ribosome movement along the messenger.

Analysis of the problem of mRNA stability and turnover in differentiation requires cellular systems in which mRNA specific for a given protein can exist in different states of stability. This requirement is met in the case of mRNA for glutamine synthetase (GS) in the embryonic neural retina. In this tissue, base-level synthesis of GS is mediated by short-lived templates; however, induction by hydrocortisone elicits accumulation of stable templates for GS, resulting in a several-fold increase in the rate of the enzyme synthesis (1,2,3,4).

As a step toward comparative analysis of the base-level and induced states of GS-specific mRNA, we found it feasible to experimentally conserve the labile mRNA in non-induced retina. Guided by earlier findings (5) we accomplished this by reversibly blocking protein synthesis in the retina with cycloheximide. As reported here, this agent does not prevent continuation of the base-level formation of mRNA for GS and allows it to accumulate; subsequently when translation is unblocked and simultaneously further RNA synthesis is stopped, the conserved templates mediate GS synthesis at a rate which is faster than base-level.

Materials and Methods: Retina tissue was isolated aseptically from eyes of 12-day chick embryos and cultured as before (1,2) in 25 ml Erlenmeyer flasks at 37°C on a gyratory shaker; each flask contained 3 ml culture medium (Tyrode's physiological salts solution with 20% fetal bovine serum and antibiotics). Cycloheximide (Nutritional Biochemicals Co.) was added to cultures at concentrations described in the text. Actinomycin D was added to cultures at a con-

*Abbreviations: Glutamine synthetase - GS; Cycloheximide - Cyh; Actinomycin D - ActD.

centration of 10 µg/ml. Isotopic labeling of the tissue cultures was as described previously (4). Immunomeasurements of radioactively labeled GS were carried out essentially as in previous work (4), except that here γ-globulin isolated from antiserum against purified retina GS (6) was used. Retina proteins were labeled with ^{14}C-amino acid mixture (New England Nuclear). Radioactivity was measured by scintillation counting (for further procedural details see references 1-4).

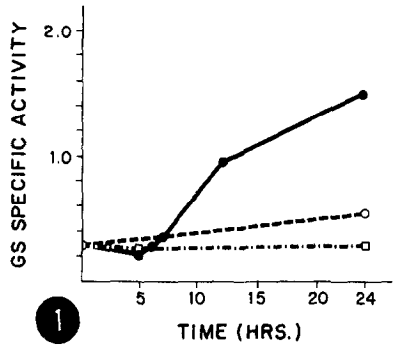
Polysomes were prepared as described before (7) by gently homogenizing the cells in TKMH buffer with Triton X; the homogenate was centrifuged at 15,000 x g and the supernatant fractionated by centrifugation (105,000 x g, for 2 hrs) in 15-40% sucrose gradients. 1 ml fractions were collected with a fractionator equipped with a UV-analyzer set at 254 nm. The specific activity of GS was determined as in previous work (1,4).

Results and Discussion: The basic experimental results are summarized in Fig. 1. Retinas were cultured for 5 hrs in the presence of 500 µg/ml cycloheximide (Cyh); this concentration of Cyh stops protein synthesis completely. The tissue was then thoroughly washed to remove Cyh and transferred for further incubation into medium containing actinomycin D (ActD; 10 µg/ml) to stop all transcription. Immediately after the transfer GS specific activity began to increase and by 24 hrs reached levels 5 to 6 times higher than initially present. Cultures not treated with Cyh showed no significant increase in GS activity after transfer to ActD. Addition of ActD at zero hr together with Cyh, prevented the subsequent increase of GS activity. Thus, RNA synthesis during the period of Cyh treatment is an essential condition for the rise of the enzyme level after Cyh removal.

The magnitude of the increase of GS level elicited by the Cyh pre-treatment was dependent on Cyh concentration (Fig 2): maximum effects were obtained with 150-500 µg/ml Cyh. These doses blocked protein synthesis in these cells 90-100%, but the inhibition was, at least partially reversible. The magnitude of the GS-related effect of Cyh was also directly correlated with the duration of treatment (Fig 3), indicating a time-dependent accumulation of templates for the synthesis of GS.

Contrary to Cyh, puromycin elicited no such increases of GS in this system, at any concentration tested. This is of interest because these agents inhibit protein synthesis by quite different modes of action (8). It suggests that the Cyh effect in this case is causally related to the particular mechanism by which it prevents translation.

The following experiments demonstrated immunochemically that the increase of GS activity described above resulted, in fact, from de novo enzyme synthesis (Fig. 4). Retinas were treated with Cyh for 5 hrs, washed, transferred to me-



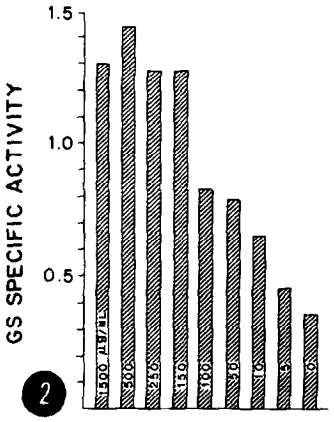


Fig 1. Retinas from 12-day chick embryos were cultured for 5 hrs in the presence of 500 µg/ml cycloheximide (Cyh); after thorough washing they were transferred (arrow) to medium containing 10 µg/ml actinomycin D (ActD) and further incubated for additional 19 hrs (total 24 hrs). The resulting increase in glutamine synthetase (GS) specific activity is shown by: ●——●; GS specific activity in controls: o---o. If RNA synthesis was stopped (with ActD) simultaneously with the treatment with Cyh, this prevented the subsequent increase of GS: □---□

Fig 2. The effect of different concentrations of Cyh on the subsequent increase of GS activity. Retinas were cultured in the presence of Cyh for 5 hrs, then washed and transferred to medium containing 10 µg/ml ActD. GS specific activity was determined after a total of 24 hrs of incubation. Control tissue was cultured without inhibitors.

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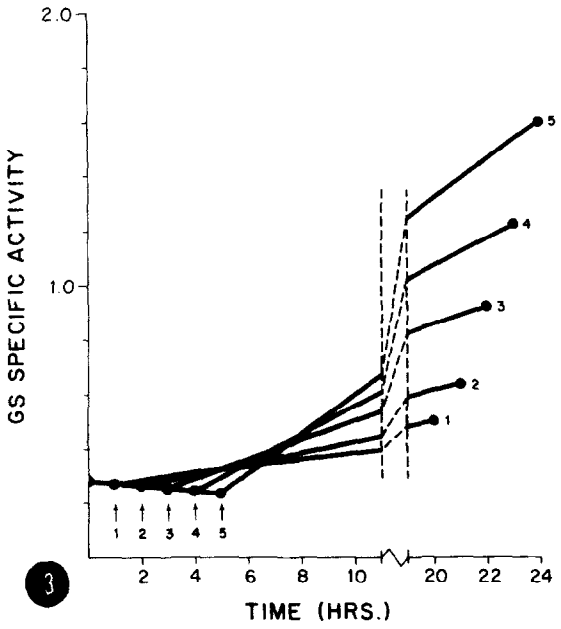
dium with ActD and cultured in the presence of ^{14}C-amino acid mixture for 19 hrs (total of 24 hrs); controls were not treated with inhibitors. The tissues were then washed, sonicated and centrifuged at 105,000 x g for 30 minutes. The supernatants were reacted with anti-GS γ-globulin under conditions ensuring maximal precipitation of the enzyme (90% of the enzyme activity). The amount of immunoprecipitated radioactivity, determined as DPM/mg protein, provided a measure of the amount of GS synthesized during the period of continuous labeling. The results, summarized in Fig. 4, showed that in the tissue pre-treated with Cyh, both GS specific activity and the amount of newly made, immunoprecipitable enzyme increased approximately 2.5 times above the control levels.

The following experiments demonstrated that the increased accumulation of newly made GS was due to an increase in the rate of GS synthesis over the control level. Retinas were treated with Cyh (500 µg/ml) for periods from 2 to 5 hrs, then washed and transferred to medium with ActD; 2 3/4 hrs later they were pulsed for 15 min with ^{14}C-amino acid mixture. The tissues were collected, washed, sonicated, and the amount of newly made GS was determined radioimmunochemically as described above; the DPM/mg protein of immunoprecipitated radio-



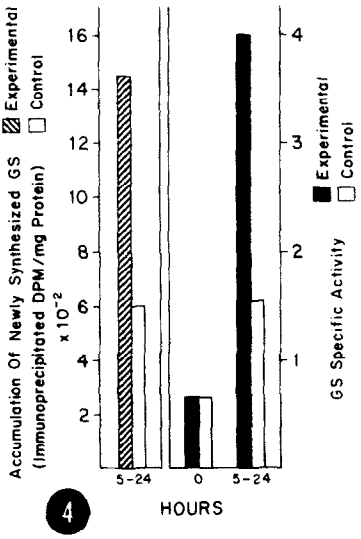
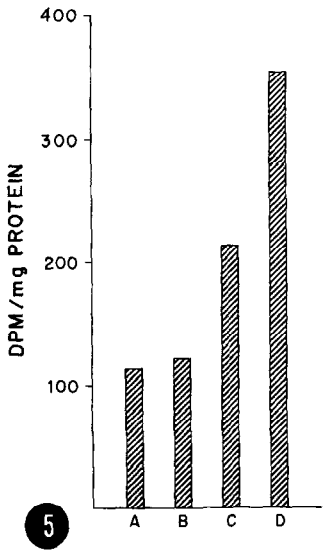


Fig 3. Time-dependence of treatment with Cyh (500 µg/ml) on the subsequent increase of GS specific activity. Retinas were treated with Cyh for 1-5 hrs, then washed (arrows) and immediately transferred to medium with ActD (10 µg/ml) for further 19 hrs of incubation. GS specific activity was then determined. Numbers on the right and under the arrows: hrs of treatment with Cyh.

Fig 4. Immunochemical measurements of the accumulation of newly made GS in the retina, subsequent to pre-treatment for 5 hrs with 500 µg/ml Cyh, and after transfer to ActD (10 µg/ml), as described in the text. ▨ - tissue treated with Cyh; □ - untreated. Accumulation of the newly made, isotopically labeled (1 µC/ml ^{14}C-amino acid mixture) enzyme is expressed as corrected DPM in immunoprecipitated enzyme, per mg protein. Corrected DPM: DPM precipitated by anti-GS γ-globulin, minus DPM precipitated by normal γ-globulin. Normal γ-globulin did not precipitate measurable amounts of GS activity. GS specific activity in these experiments is shown on the right. ■ -tissue treated with Cyh; □ -untreated. GS specific activity measurements were made on aliquots of the 105,000 x g supernatants used for immunoprecipitation.

activity provided a measure of the 15 min rate of GS synthesis. The results (Fig 5) showed that longer treatments with Cyh resulted in greater rates of enzyme synthesis (Fig. 5). This together with the preceding results suggests compellingly that Cyh treatment caused a progressive accumulation of RNA templates for the synthesis of GS. It is conceivable that also other kinds of labile mRNA in these cells are conserved by this treatment.

In attempting to further define the mechanism by which Cyh conserves labile templates for GS, we examined the effects of several Cyh concentrations on retina polysomal profiles. It has been suggested that Cyh inhibits protein syn-



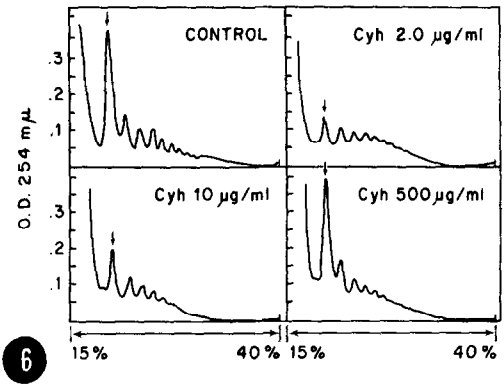


Fig 5. Immunochemical measurements of the rates of GS synthesis subsequent to treatment of the retina for different times with 500 µg/ml Cyh. The cultures were pulsed for 15 min with ^{14}C-amino acid mixture (10 µC/ml) 2 3/4 hrs after removal of Cyh and addition of ActD. Bars represent the rates of enzyme synthesis per mg protein (see legend Fig 4). Duration of treatment with Cyh: A, 2 hrs; B, 3 hrs; C, 4 hrs; D, 5 hrs. Further explanations in text.

Fig 6. Polysomal profiles prepared from retinas cultured for 5 hrs in the presence of different concentrations of Cyh. The control profile was prepared from tissue cultured for 5 hrs without Cyh. Arrow points to monosome peak.

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thesis by preventing movement of ribosomes along mRNA, and that its effectiveness in this respect is concentration dependent (9). Results of our preliminary experiments (Fig. 6) showed that treatment for 5 hrs with 2 µg/ml Cyh (a concentration which does not elicit a subsequent rise in GS level) caused a marked decrease in the optical density (O.D.) of the monosome peak (arrow) and an increase in the O.D. in the region of the heavy polysomes. Presumably, this concentration of Cyh did not prevent attachment of ribosomes to mRNA, but slowed down ribosome movement along the messenger. In contrast, treatment with 500 µg/ml Cyh (which totally blocks translation processes and elicits a subsequent rise in GS level) yielded a polysomal profile which closely resembled that of the controls; this is interpreted as due to the complete arrest of ribosome movement (9) and the consequent "freezing" of the ribosomal profile. Consistent with these interpretations, treatment with 10 µg/ml Cyh yielded polysomal profiles of intermediate appearance.

The above results suggest the following working hypothesis. It has been reported that decay of mRNA is related to translation (9,10,11). Accordingly, the high doses of Cyh prevent the decay of the normally labile mRNA in this

system by blocking movement and, presumably, attachment of ribosomes. Consequently, the pool of free GS templates increases, due to their continued base-level synthesis. When protein synthesis is allowed to resume, the conserved templates are translated and the level of GS increases at a rate faster than the basal rate. In this connection it is important that puromycin does not elicit in this system an effect on GS similar to that of Cyh, since this drug inhibits protein synthesis by an entirely different mechanism than Cyh.

The fact that continued expression of the conserved templates, after Cyh removal, requires suppression of further RNA synthesis indicates that their persistence or function is subject to control by other gene products. This situation was anticipated on the basis of earlier results (1,4,5) and is entirely consistent with the postulated involvement of regulatory gene products in the control of GS translation (1,4,13).

Work is in progress to compare the properties of such experimentally conserved labile templates for base-level GS synthesis with those that accumulate and function in the hydrocortisone-mediated induction of GS in the retina; in this connection, it should be mentioned that synthesis of poly-adenylate (poly-A) is involved in the hormonal induction of GS in the retina (14) and that poly-A may have a role in mRNA stability (15,16).

Acknowledgments: This work was supported by National Institutes of Health Research Grant HD-01253, and a predoctoral stipend to S. Garfield from Training Grant T01-HD 00297. We thank Dr. P. K. Sarkar for valuable advice, and Barbara Goldman for critical review of the manuscript.

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