*ENZYME INDUCTION IN EMBRYONIC RETINA: THE ROLE OF TRANSCRIPTION AND TRANSLATION**

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The mechanisms of induction in embryonic cells and of the timing and stability of phenotypic changes are of major importance to the understanding of differentiation. Detailed studies of these problems have been hindered by the scarcity of suitable experimental systems. What is required is an embryonic tissue in which a chemically defined inducer promptly elicits a specific, measurable response characteristic of differentiation. The induction of glutamine synthetase in embryonic neural retina meets these requirements. The normal developmental pattern of this enzyme is representative of the progress of retinal differentiation and provides a quantitative indicator of this process; moreover, this pattern can be significantly modified by defined experimental conditions.

Glutamine synthetase activity (GS) in the embryonic chick neural retina follows a characteristic developmental pattern that is typical for this tissue and is temporally and spatially correlated with other aspects of retinal development.[1-4] During early embryonic development, GS activity in the retina increases at a slow rate, then rises very sharply after the sixteenth day during the period of rapid functional differentiation and maturation of the retina. Particularly important is the fact that the rapid rise of GS activity can be induced precociously, several days before the normal time, in cultures of embryonic retina with 11β-hydroxycorticosteroids (hydrocortisone, aldosterone, or corticosterone).[5, 6] A similar precocious induction of retinal GS can be elicited also in embryos by injecting one of these steroids. Induction of GS by steroids is specific for the embryonic neural retina; it does not involve cell proliferation and therefore is not due to differential growth. The induced rise in GS activity is accompanied or followed by additional developmental changes which normally occur later in development;[2, 7, 8] thus, it is not an isolated response but one of a number of phenotypic changes accelerated by the steroid inducer. Since changes in retinal GS activity are an essential aspect of differentiation, the mode of regulation of GS activity is relevant to the mechanisms that control differentiation in this tissue.

Previous work on control mechanisms in this system was limited to long-term cultures of the retina;[4, 9-12] however, the enzyme begins to rise very shortly after exposure of the tissue to the inducer.[13] The present report is concerned with an analysis of the processes that control the early phases of the induction of retinal GS by steroid (hydrocortisone).

Materials and Methods.—Organ cultures of retina: Standard procedures for the isolation and cultivation of embryonic chick neural retina in flask organ cultures were described before.[3-7] In this study, retina tissue from 12-day chick embryos was used. Each culture contained one whole retina (approximately 10^8 cells; 2.5 mg protein) in 3 ml of culture medium in a 25-ml Erlenmeyer flask; the flasks were rotated (70 rpm) at 38°C on a gyratory shaker. The cultures were maintained for various times, as described, up to 24 hr. The medium was 80% Eagle's medium (without glutamine) with 20% fetal bovine

serum and 1% penicillin-streptomycin mixture (5000 units each/ml) (Microbiological Associates). To induce retinal GS, 3×10^{-6} gm/flask hydrocortisone (free alcohol) in Tyrode's solution was added to the culture medium.[5, 6] Radioactive materials were added in sterile, neutral salt solution. All the cultures were gassed with 5% CO_2-air mixture.

Enzyme assay: The activity of glutamine synthetase was determined by a modified glutamyl-transferase reaction[14] described in detail previously.[1, 3, 4] Tissue samples were sonicated in 0.01 *M* phosphate buffer (pH 7.1) and frozen or lyophilized, then stored at −20° or assayed immediately. Protein was determined by the method of Lowry *et al.*[15] GS specific activity was calculated as micromoles of glutamylhydroxamate formed per hour per milligram of tissue protein. Since the total protein content of the retina in culture did not change appreciably in 24 hr, changes in the specific activity of the enzyme are a measure of changes in total GS activity.

Radioactive tracer methods: The concentration of H^3-uridine (25,500 mC/mM) was 1 μc/3 ml of culture medium. After incubation the tissue was washed three times with Tyrode's solution and sonicated in 0.01 *M* phosphate buffer, pH 7.1 (1–2 ml/retina). Portions of the sonicate (0.05–0.1 ml) were placed on 25 mm Whatman GF/A glass fiber disks, dried, and counted (see below) to determine the total uptake of the label into the tissue. To measure the incorporation of this label, the dried disks were treated with cold 10% trichloroacetic acid (TCA) and then washed with 5% TCA, ethanol-ether, and ether, according to standard procedure. The dry disks were placed in vials with a mixture of PPO-POPOP (Spectrafluor; Nuclear-Chicago) in toluene and counted in a Nuclear-Chicago Mark I liquid scintillation counter. Other portions of the same sample (0.1–0.2 ml) were used for protein and GS activity determinations.

For measuring amino acid incorporation 1 or 2 μc of a mixture of 15 amino acids (~1 mc/mg; New England Nuclear) was added to 3 ml of culture medium. The cultures were harvested and sonicated, and portions of the sonicate were placed on disks, as described above. After treatment with cold TCA, the disks were heated in 5% TCA for 30 min at 90°C and then washed, dried, and counted as above. Other portions of the same sample were used for measurements of the total uptake of the label into the tissue and for protein and GS determinations.

Results.—Induction of GS in organ cultures of embryonic retina: When retina tissue from 12-day chick embryos was cultured at 37°C in medium with hydrocortisone, there was a 1.5–2-hour "lag period," after which GS activity increased rapidly[13] (Fig. 1). In the absence of the steroid there was only a slight increase of GS activity in 24 hours, similar to that which takes place normally in embryonic retina during the corresponding period. The retina of the 12-day embryo con-

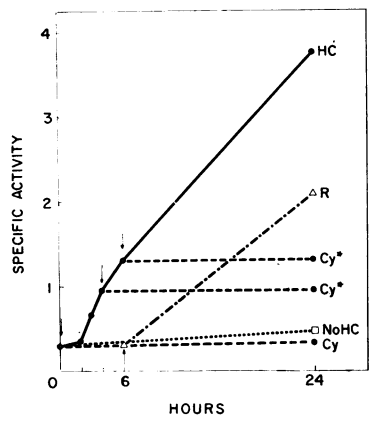

FIG. 1.—Kinetics of induction of GS activity in cultures of embryonic retina tissue by *HC* (hydrocortisone; 8.2×10^{-7} *M*) and its inhibition by cycloheximide. All cultures were with inducer except the one marked *NoHC*. Arrows pointing down mark the addition times of cycloheximide (*Cy*) (2 μg/ml) to cultures with inducer. Arrow pointing up marks transfer of tissue from medium with inducer and cycloheximide to inducing medium without the inhibitor (*R*). Similar effects were obtained with puromycin. These data represent averages from one set of experiments; the absolute values varied somewhat in different experiments. Star indicates that the same values were obtained when cultures were transferred from medium with inducer to inducer-free medium with cycloheximide.

tains very few dividing cells, and there was no measurable increase in cell number either in induced or in control cultures. Hydrocortisone did not change the total uptake or incorporation into proteins of C^{14}-amino acids. Therefore, induction by the steroid is not accompanied by any gross changes in the cellular composition of this tissue or in its over-all protein synthesis.

Requirement for protein synthesis: The induction of retinal GS was blocked by inhibiting protein synthesis. Cycloheximide, 2–5 μg/ml, added to the culture medium at the beginning of incubation inhibited 90–100 per cent of the incorporation of C^{14}-amino acids; these concentrations of the inhibitor also completely prevented GS induction (Fig. 1). Similar effects were obtained with puromycin (2–5 μg/ml). Furthermore, addition of cycloheximide at any time after GS activity began to rise stopped further increases (Fig. 1). These inhibitions could be partially reversed by washing the tissue and transferring it into inducing medium without the inhibitor. Cycloheximide caused no decrease in incorporation of H^3-uridine into RNA. Therefore, protein synthesis is essential for the initial and the continued increase in GS activity. These results and conclusion agree with findings obtained in long-term cultures of the retina[4, 9, 10, 12, 13] and suggest that the induced increase in GS activity is the result of synthesis of enzyme protein; however, other possibilities are not excluded, for example, the synthesis of proteins which regulate GS synthesis or activity.

The level of GS activity attained at the time of cycloheximide addition did not decrease rapidly in the presence of the inhibitor (Fig. 1) and, under these conditions the enzyme had a half life longer than 20 hours. The stability of GS in the presence of cycloheximide was not dependent on the simultaneous presence of the steroid inducer in the culture medium; in cultures transferred after four or six hours from inducing medium into inducer-free medium with cycloheximide, GS activity leveled off but did not decline appreciably (Fig. 1). It remains to be determined if the stability of GS under these conditions depends on the intracellular pool of inducer that might have accumulated in the cells in the first four hours.[13]

RNA synthesis and GS induction: Actinomycin D (Act D) added at the beginning of incubation to cultures in inducing medium at concentrations from 0.2 to 10 μg/ml blocked H^3-uridine incorporation into RNA by 75–99 per cent and completely prevented GS induction (Fig. 2); 0.2 μg/ml Act D was a borderline concentration that occasionally allowed some increase in GS activity. Therefore, RNA synthesis is essential for the induction of GS by hydrocortisone. We next sought to determine whether the increase in GS activity was continuously dependent on new RNA synthesis. Act D (10 μg/ml) was added to cultures at various times between zero and six hours; enzyme activities were measured over a period of 24 hours. The results (Fig. 2) showed that in the first three to four hours of cultivation the induction of GS became progressively less dependent on new RNA synthesis; if RNA synthesis was blocked after four hours, GS activity continued to rise to values close to those of the controls. Act D (10 μg/ml) added at four hours blocked H^3-uridine incorporation as efficiently as at zero hours (Table 1). Thus, the later Act D was added between zero and four hours, the greater was the subsequent increase of GS activity; this suggests that in the first

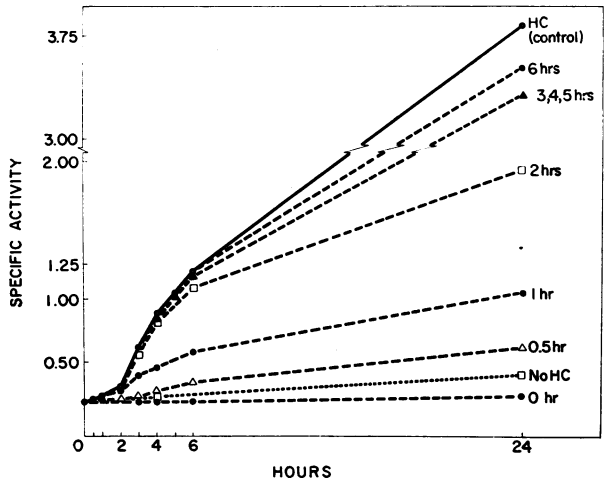

FIG. 2.—Effects of Act D (10 μg/ml) added at various times on the induced increase in GS specific activity. All cultures (except control—*No HC*) were with the inducer (*HC*). Act D was added at the times listed on the right, next to the specific activity values at 24 hr. In this series of experiments, the results for 3, 4, 5, and 6 hr were below the control (*HC*) average but fell within the general range of control values. The absolute values varied somewhat in different experiments.

four hours of induction there is an accumulation in the cells of relatively stable RNA which mediates the increase of GS activity either directly, as templates for enzyme synthesis, or indirectly by controlling the rate of translational processes. If the increase in GS activity is the result of enzyme synthesis, then the RNA templates for this enzyme are relatively stable and after four hours of induction can continue to function under these conditions for at least 20 hours independently of further transcription.

Transcription-independent increase of GS activity requires protein synthesis: Actinomycin D (10 μg/ml) added to cultures after four hours of induction inhibited incorporation of amino acid into protein during the first hour by approximately 20 per cent, compared with controls without the antibiotic (findings to be published); thus, protein synthesis continues in these cultures in the absence of RNA synthesis, presumably on stable messenger RNA. We sought to determine whether the continued increase of GS activity in the absence of RNA synthesis required protein synthesis. Cycloheximide (2 μg/ml) and Act D (10 μg/ml) were added after four hours to cultures in medium with the steroid inducer. The effect of cycloheximide was monitored by measuring the incorporation of C^{14}-amino acids into proteins and by determinations of GS activity at the end of the 24-hour culture period. There was no increase in GS activity in cultures with cycloheximide (Fig. 3). Thus, at the time when transcription was no longer required for the increase in GS activity, protein synthesis continued to be essential. This supports the possibility that the accumulation of GS activity in the absence of transcription may also be due to enzyme synthesis or require the synthesis of other proteins involved in this process, or both.

Transition to independence from transcription does not require protein synthesis: As shown above, RNA synthesis is essential in the initial phases of GS induction, but after four hours GS activity can continue to increase in the absence of further transcription. We next sought to determine whether during those first four hours continuous protein synthesis was required in order that the increase might become independent of RNA synthesis. Cycloheximide (2 μg/ml) was added at the

beginning of incubation to cultures in medium with the steroid inducers. After four hours the tissues were washed free of cycloheximide, transferred to new medium with inducer and with Act D (10 μg/ml), and incubated for the remainder of the 24-hour culture period. Controls were without Act D. The results (Fig. 3) showed that GS activity increased in each case after removal of cycloheximide. Therefore, continuous protein synthesis during the first four hours of induction is not essential for causing the rise in GS activity to become potentially independent of further transcription. Since RNA synthesis is essential, the conclusion that this independence is due to accumulation of stable RNA during the first four hours of induction seems inevitable. Furthermore, these results indicate that the function of the steroid inducer in this system is not dependent on continuous protein synthesis and that it affects, directly or indirectly, transcriptional processes.

The increase of GS activity after four hours of induction in the absence of transcription does not depend on the presence of the steroid inducer in the culture medium. Retinas maintained for four hours in medium with hydrocortisone were washed as much as possible to reduce the external pool of the steroid; they were divided into two groups and transferred to the following media: (1) with inducer and Act D (10 μg/ml); (2) without inducer, with Act D. At the end of the 24-hour culture period, GS activity was measured (Fig. 3). GS activity continued to rise in the absence, as well as in the presence, of the inducer in the culture medium. Therefore, the transcription-independent increase in GS activity in the presence of Act D does not require a continuous supply of exogenous inducer and is, therefore, due to the internal pool of steroid that accumulates earlier in the cells, to the effects of the steroid during the initial phases of induction, or to Act D which at this concentration may exert effects that in their final outcome equal those of the inducer.

Effect of a low dose of Act D at four hours: Act D (0.2 μg/ml) was added to cultures in medium with the steroid inducer four hours after the start of incubation;

TABLE 1. *Effect of different concentrations of Act D* on incorporation of H^3-uridine into RNA and on the increase of GS specific activity.*

Act D added at:	Dose of Act D (μg/ml)	Dpm/mg protein	Inhibition of H^3-uridine incorporation (%)	GS specific activity at 24 hr
—	—	72,573	—	3.84–5.14
0 hr	10.0	660	99.10	0.28–0.30
"	2.0	2,040	97.19	0.23–0.32
"	0.6	15,200	79.06	0.27–0.55
"	0.2	18,420	74.62	0.48–1.00
—	—	65,473	—	4.00–6.00
4 hr	10.0	433	99.34	3.00–6.14
"	2.0	4,406	93.28	2.05–2.00
"	0.6	14,720	77.52	1.79–2.21
"	0.2	27,113	58.59	1.03–2.20

H^3-uridine (1 μc/3 ml medium) was added 0.5 hr after addition of Act D; the cultures were harvested and processed 1 hr later. The range of GS specific activities at 0 hr is 0.21–0.34; after 4 hr in inducing medium, the range is 0.80–1.21.

* Added at 0 or 4 hr to cultures with inducer.

GS activity was assayed at the end of the 24-hour culture period (Fig. 4). In marked contrast to the noninhibitory effect of the higher concentration of Act D added at the same time, 0.2 μg/ml suppressed further increase in GS activity by 70–90 per cent. Measurements of uridine incorporation showed that 0.2 μg of Act D/ml was less effective in inhibiting RNA synthesis than 10 μg/ml was (Table 1). Therefore, after four hours of induction complete inhibition of transcription does not interfere with the continued increase in GS activity, whereas partial inhibition of RNA synthesis does. An explanation for these seemingly paradoxical results is suggested below.

Discussion and Summary.—A simple explanation of these findings requires the assumption that the induced increase in GS activity is the result of enzyme synthesis and accumulation; it applies to the possibilities that the induction involves either an increase in the rate of GS synthesis or a decrease in its rate of degradation. This assumption is supported by the observations that the increase in GS activity requires at all times continuous protein synthesis; however, its confirmation must await data from immunological precipitation of radioactively labeled enzyme. Granted the assumption of enzyme synthesis, we suggest that the following sequence of events may be involved in the induction of retinal GS:

(*a*) Synthesis of RNA is required for GS induction by the steroid. Tran-

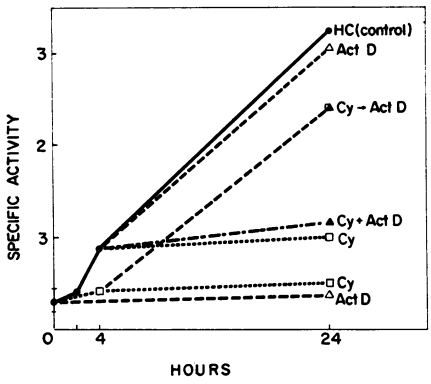

FIG. 3.—Summary of results showing that (1) after 4 hr of induction GS activity continues to increase although RNA synthesis is halted (10 μg/ml Act D); (2) inhibition of protein synthesis by cycloheximide (2 μg/ml) during the first 4 hr of induction does not prevent the subsequent transcription-independent rise in GS activity; (3) protein synthesis is essential for the transcription-independent increase of GS activity after 4 hr. All cultures were in medium with inducer. *HC (control)*, cycloheximide (*Cy*) was added at 0 or 4 hr, as indicated. *Cy → Act D*, GS activity in cultures transferred after 4 hr in cycloheximide into medium with Act D.

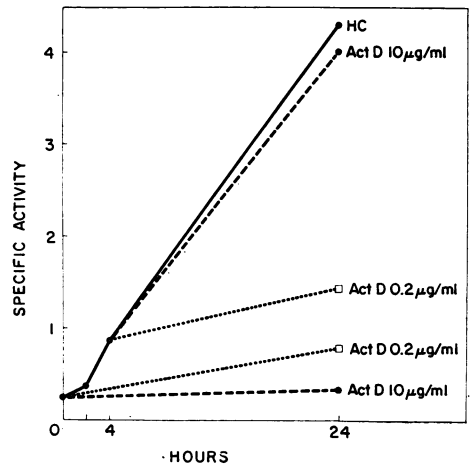

FIG. 4.—Reverse effects of high and low doses of Act D on the increase of GS activity when the inhibitors were added to cultures at 0 or 4 hr. All cultures were in medium with the steroid inducer. (Table 1 shows the inhibition of H^3-uridine incorporation under these conditions.) Added to the cultures at 0 hr, both doses of Act D inhibited GS induction; the lower dose sometimes inhibited incompletely, as in the experiments represented here. Added at 4 hr, the high dose was not inhibitory, whereas the low dose suppressed approximately 70–90% of GS increase, compared with control cultures.

scription in the initial phases of induction leads to the accumulation of stable RNA that is essential for the increase of GS. The evidence suggests that the steroid affects, directly or indirectly, transcriptive processes; it may initiate the synthesis of new transcripts or modify the rate of existing transcription.

(*b*) The effects of the high and low concentrations of Act D suggest that the synthesis of GS after four hours of induction (and possibly earlier) is subject to control by two endogenous regulators, both of which are RNA or require RNA synthesis and have different susceptibilities to Act D: (1) A translational *repressor* of GS synthesis (or degrader of GS, if GS induction is due to a decrease in its rate of degradation) with its formation blocked by a high, but not by a low, concentration of Act D; and (2) a *derepressor*, which counteracts the translational repressor, with its formation suppressed by a low dose of Act D. Thus, the blocking of all RNA synthesis in four-hour cultures by a high dose of Act D stops also the transcription of the repressor (degrader) RNA and allows the continued increase of GS because by that time the RNA species directly involved in enzyme synthesis has already accumulated and can function in the absence of further transcription. On the other hand, a low dose of Act D does not stop the synthesis of the repressor (degrader) RNA but blocks the synthesis of the derepressor RNA and thus decreases the accumulation of GS. Thus, the persistent need for the derepressor RNA under normal conditions is masked by the repressor RNA-blocking effect of the high dose of Act D.

The continued increase of the induced GS when all transcription is stopped recalls similar observations in other eukaryont cells in which protein synthesis can go on after arrest of RNA synthesis.[10, 12, 16–23] However, the significant and distinct point that emerges from our data is that, under normal conditions the GS system does not actually cease to be under genomic control, in that the continued increase in GS activity after four hours remains dependent on transcription of the postulated derepressor RNA. Thus, in reality, the accumulation of GS appears to be continuously controlled through transcription-dependent processes. Whether this control circuitry persists in later stages of retinal development remains to be determined.

It was suggested above that the steroid inducer may be directly or indirectly responsible, during the initial four hours, for the formation of RNA required for the increase of GS. This may be messenger RNA (mRNA) for GS, as suggested by Reif-Lehrer and Amos.[12] However, there are other possibilities. For example, mRNA for GS may be present in these cells at the time of induction, but it may not be active or it may function at a low rate; thus, induction could be primarily an increase in the translational efficiency of pre-existing GS templates through provision of RNA species that are limiting (transfer or ribosomal RNA). In the first four hours of induction, sufficient GS templates become activated or accumulate *de novo*, and GS formation can then continue without further transcription of the RNA species that function directly in the synthesis of the enzyme; however, its rate of synthesis or accumulation remains indirectly subject to genomic control through the dual effects of the translational controllers, as discussed above.

The above interpretation is, in general, consistent with the original models of

Jacob and Monod in which translational as well as transcriptional controls were envisaged.[24] The possible role of translational repressors in the control of inducible enzymes has been postulated for tryptophan pyrrolase and tyrosine transaminase in rat liver[19] and for tyrosine transaminase in cultured Morris hepatoma cells.[20] Control at the level of translation and the activation of pre-existing RNA templates have been suggested for postfertilization protein synthesis in sea-urchin eggs,[25-27] for hemoglobin synthesis,[28] for the increased protein synthesis by ribosomes from insulin-treated muscle,[29] and for other systems.[30] We are aware of alternative interpretations for the facts described here, possibly along the concepts proposed for other systems by Monod *et al.*[31] Our present interpretations are intended primarily as working guidelines; their testing should help in clarifying further the regulatory events involved in the induction of GS in the embryonic retina and might provide more detailed insights into the mechanisms of differentiation in other embryonic cells.

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