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Cell contacts are required for induction by cortisol of glutamine synthetase gene transcription in the retina

(Müller glia/cell aggregation)

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ABSTRACT **In embryonic neural retina the enzyme glutamine synthetase [GS; L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] is a glia-specific differentiation marker inducible with cortisol. We show that cortisol elicits GS mRNA accumulation by stimulating transcription of the GS gene and that this stimulation requires cell contacts: in dissociated and separated retina cells GS gene transcription was not induced; when the separated cells were reassembled into multicellular aggregates, restoring cell contacts, accumulation of GS mRNA was again inducible. In cells dissociated from retina tissue that had been preinduced with cortisol, GS gene transcription rapidly declined, despite continued hormone availability. In the separated cells transcription of the histone H3.3 gene and accumulation of carbonic anhydrase II mRNA were unaffected; therefore, cell separation selectively precluded induction of the GS gene. These findings provide direct evidence for the regulatory role of cell contacts in hormonal control of gene transcription.**

Cell contact-dependent cell interactions are essential for cell organization and differentiation during embryonic development (1, 2). Changes in cell adhesion can affect differentiation and alter phenotype characteristics (1–6), vary cell growth (7, 8), and modify gene expression (9–14). The molecular basis of the effects of cell contact on cellular regulation is poorly understood. Investigation of this problem in embryonic cells requires systems in which cell associations can be controllably modified and specific gene products induced on demand. These requirements are met by the experimental system studied here: induction by cortisol of glutamine synthetase [GS; L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] in Müller glia cells of chicken embryo neural retina and the dependence of this induction on cell contacts.

In retina GS is expressed exclusively in Müller glia cells (15). GS catalyzes the formation of glutamine from glutamate; the enzyme plays a key role in recycling the neurotransmitters glutamate and γ-aminobutyrate and in supplying neurons with glutamine (16–18). In chicken embryo retina, levels of GS mRNA and GS enzyme are very low during early embryonic ages; these levels begin to rise sharply on day 15–16 (ref. 19), subsequent to adrenal cortex development, and remain high in mature retina. However, GS can be induced precociously, already at embryonic day 8, by supplying cortisol to the embryo or directly to isolated retina tissue in organ culture (20). The hormone stimulates GS mRNA accumulation (19), thereby increasing GS enzyme synthesis and its level in the glia cells (for review, see ref. 21). GS enzyme induction also requires cell contacts: in dissociated retina cells cortisol did not increase GS enzyme level, but when the cells were promptly reaggregated, GS inducibility was restored (15, 22). We report that cortisol induces GS by stimulating GS gene transcription and that induction of this transcription ultimately depends on cell contacts.

MATERIALS AND METHODS

Culture Methods and GS Induction. Neural retina tissue was isolated under sterile conditions from day-10 or day-13 embryos (White Leghorn). Retinas were organ-cultured in Erlenmeyer flasks in medium 199 with 10% fetal calf serum on a gyratory shaker at 37°C. Retina dissociation with trypsin into cell suspensions, cell aggregation, GS induction with cortisol, and culture methods followed described protocols (15, 20, 22). Monolayer cultures were prepared by plating dissociated cells in Falcon tissue culture dishes (5×10^5 cells/cm^2) pretreated with poly(L-ornithine) at 0.1 mg/ml (Sigma) and were incubated in a 5% CO_2/95% air incubator at 37°C; after 2 hr, 5% fetal calf serum was added to the medium.

Cell aggregates were prepared by dispensing 4.5×10^7 dissociated cells into 25-ml Erlenmeyer flasks with 3 ml of medium 199/10% fetal calf serum. The flasks were gassed with a 5% CO_2/95% air mixture, sealed, and rotated on a gyratory shaker (72 rpm) at 37°C.

GS was induced by adding cortisol (Sigma) to the cultures to a final concentration of 0.33 μg/ml. Culture media were changed daily. The specific enzyme activity of GS was determined in cell sonicates by the colorimetric assay (20) with some modifications as μM γ-glutamylhydroxamate per mg of protein/hr.

RNA Preparation and Analysis. RNA was prepared by the guanidinium isothiocyanate/CsCl method (23). Poly(A)$^+$ RNA was selected by using an oligo(dT)-cellulose column (type III; Collaborative Research, Waltham, MA). For RNA blot analysis, poly(A)$^+$ RNA was denatured at 60°C in 2.2 M agarose/formaldehyde gels; fractionated RNA was transferred to nitrocellulose filter (24).

For dot-blot analysis poly(A)$^+$ RNA was denatured by heating at 55°C in 0.8 M formaldehyde/1 M sodium chloride/30 mM sodium phosphate buffer, pH 7.4, and was blotted at different dilutions on nitrocellulose filters using the BioDot microfiltration apparatus (Bio-Rad). The filters were hybridized with nick-translated DNA probes. Levels of hybridization were visualized by autoradiography and measured by densitometry with a Zeineh soft laser scanner. GS mRNA levels were normalized to the amount of replacement histone H3.3 mRNA.

Recombinant Plasmids. Plasmid pGS116-9 was constructed by subcloning a 3-kilobase (kb) *Hin*dIII fragment of the genomic chicken GS clone λ-GS223 (19) into the *Hin*dIII site of pUC18 according to published protocols (24). The cloned

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Abbreviations: GS, glutamine synthetase; CA-II, carbonic anhydrase II.

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chicken carbonic anhydrase II (CA-II) cDNA pPE5-0.3 (25) was provided by J. B. Dodgson (Michigan State University). The cloned histone H3 gene pCH3dR1 (26) was provided by J. D. Engel (Northwestern University).

***In Vitro* Nuclear Run-on Transcription.** Retina tissue or retina cells were homogenized in extraction buffer (300 mM sucrose/2 mM $MgCl_2$/60 mM KCl/15 mM NaCl/14 mM mercaptoethanol/150 μM spermine/500 μM spermidine/15 mM Hepes buffer, pH 7.5), and nuclei were isolated according to published protocols (27). Isolated nuclei were incubated *in vitro* in the presence of [^{32}P]UTP (New England Nuclear), and RNA was purified (28). Linearized plasmid DNA were boiled for 5 min in 0.2 M NH_2OH/2 M NaCl and immobilized (5 μg/dot) on nitrocellulose filters. The labeled RNA (about 10^6 cpm) was hybridized [50% (vol/vol) formamide/800 mM NaCl/1 mM EDTA/0.1% $NaDodSO_4$/salmon DNA at 250 μg/ml/yeast RNA at 1 mg/ml/0.05% (wt/vol) Denhardt's solution (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/50 mM KH_2PO_4/K_2HPO_4, pH 6.5] to filters with immobilized DNA of chicken GS gene, the chicken histone H3 gene, and pBR322 for 48 hr at 42°C. Filters were washed twice in 1× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7)/0.1% $NaDodSO_4$ for 5 min at room temperature and twice in 0.1% SSC/0.1% $NaDodSO_4$ for 15 min at 60°C.

RESULTS

Cortisol Induces GS Gene Transcription. To define the role of cell contacts in GS induction we first determined whether cortisol stimulated GS gene transcription. For this experiment, we used organ cultures of retina tissue from day-13 embryos and treated them with cortisol to elicit accumulation of GS mRNA and GS enzyme to high levels (19). Using the nuclear run-on transcription assay we measured the rate of GS mRNA synthesis in nuclei isolated from cortisol-induced and uninduced retina tissue. As an internal standard for GS gene expression, we measured the synthesis of replacement histone H3.3 mRNA because it maintains a steady level in the retina and is unaffected by cortisol (29). The cloned chicken GS gene pGS116-9 (see experimental procedures) and the cloned chicken H3 gene pCH3dR1 (26) were used in these assays.

Fig. 1 shows that as early as 2 hr after hormone addition, the rate of GS gene transcription in nuclei from cortisol-treated retina markedly increased; the high rate was maintained for 24 hr (duration of this test). In contrast, synthesis of the H3.3 histone mRNA, the internal standard, remained nearly constant. Therefore, GS induction by cortisol involves stimulation of GS gene transcription. This conclusion is supported by the kinetics of GS mRNA accumulation in cortisol-treated retina (Fig. 2): an increase in GS mRNA level was already apparent 2 hr after cortisol addition and a maximum level was achieved within 4 hr. Histone mRNA accumulation did not change (data not shown). These results are consistent with earlier findings (30, 31) and provide strong evidence for transcriptional regulation of GS induction.

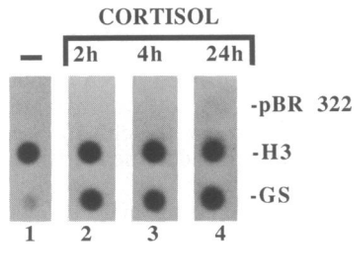

FIG. 1. Stimulation by cortisol of GS gene transcription in the retina. Linearized DNA samples (5 μg) of the cloned GS gene (pGS116-9), the cloned histone H3 gene (pCH3dR1), and the vector pBR322 were blotted on nitrocellulose filters. The filters were hybridized to [^{32}P]UTP-labeled nascent RNA synthesized *in vitro*, in isolated nuclei from day-13 embryonic retina tissue: freshly isolated tissue (lane 1), tissue cultured in medium with cortisol for 2 hr (lane 2), 4 hr (lane 3), and 24 hr (lane 4).

GS mRNA Accumulation Is Not Inducible in Separated Cells. Next we examined whether cell separation precluded GS mRNA accumulation. Measurements were made on dissociated cells from day-10 embryo retinas that were plated monodispersed in culture dishes and, for comparison, on cultures of intact retina tissue. Cortisol was added to the cultures after 24 hr; controls lacked cortisol. Poly(A)$^+$ RNA was prepared 24 hr after cortisol addition and was analyzed by RNA blotting. GS mRNA accumulation was examined by using the cloned GS gene pGS116-9, which detects the major GS transcript (≈3 kb) (19). Also assayed was the expression of two other genes that are not cortisol inducible: (*i*) replacement histone H3.3 gene—the cloned histone H3 gene pCH3dR1 (26) was used to detect the H3.3 transcript (≈1.3 kb) (29); and (*ii*) CA-II, a Müller glia cell marker, the developmental program of which differs from that of GS (19, 22); the cloned chicken CA-II cDNA pPE5-0.3 (25) was used to detect the CA-II mRNA (≈2 kb). Thus, the RNA blot filter was consecutively hybridized with ^{32}P-labeled clones of the GS gene, histone H3 gene, and CA-II cDNA. Results are shown in Fig. 3.

In cultures of intact retina tissue cortisol elicited a multifold increase in GS mRNA and of GS enzyme (Fig. 3, lanes a and b). In separated cells in monolayer culture, cortisol elicited, at best, only small increases in GS mRNA and GS enzyme levels (Fig. 3, lanes e and f)—significantly lower than in the intact tissue. The small increase in monolayer cultures correlated with spontaneous formation of cell clusters; in cultures with numerous glia/neuron clusters, cortisol elicited greater accumulation of GS mRNA than when the cells remained monodispersed. This agrees with earlier evidence that GS induction requires glia–neuron cell contacts (22).

It is significant that levels of CA-II mRNA and H3.3 mRNA were not markedly lowered by cell separation (Fig. 3, lanes a, b, e, and f). Therefore, the failure of GS mRNA to accumulate in separated cells was not due to general reduction of mRNA levels; this finding suggests that cell separation differentially precluded cortisol-dependent increase of GS mRNA level. Of course, cell separation could also affect the expression of still other, as yet unidentified, genes.

Are cell contacts required only for the initial phase of GS induction or also for maintaining induced expression of the GS gene? Retina tissue from day-10 embryos was cultured for 48 hr in medium with cortisol to induce GS to a high level;

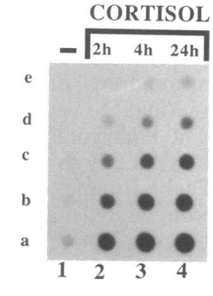

FIG. 2. Kinetics of GS mRNA accumulation induced by cortisol in retina tissue. Dot-blot analysis of total RNA (1.4 μg) from retina tissue of day-13 embryos. Freshly isolated tissue (lane 1), cultured with cortisol for 2 hr (lane 2), 4 hr (lane 3), and 24 hr (lane 4). Undiluted (row a) or diluted 1:2 (row b), 1:4 (row c), 1:8 (row d), or 1:16 (row e) RNA samples were dot blotted on nitrocellulose filters and hybridized to the ^{32}P-labeled clone of the GS gene (pGS116-9).

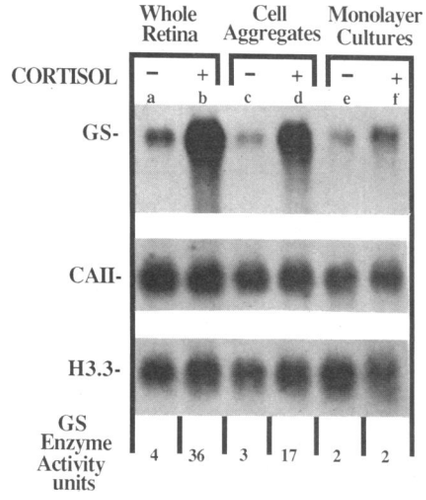

FIG. 3. Induction of GS mRNA accumulation depends on cell contacts. Retina tissue from day-10 chicken embryos was organ-cultured intact (lanes a and b) or was dissociated into single cells, which were either plated monodispersed (lanes e and f) or were immediately reaggregated into multicellular clusters (lanes c and d). All cultures were maintained for 48 hr; cortisol was added for the last 24 hr (lanes b, d, and f). Poly(A)$^+$ RNA was isolated and analyzed by RNA blotting. The filter was consecutively hybridized to the ^{32}P-labeled clones of the GS gene (pGS116-9), CA-II cDNA (pPE5-0.3), and histone H3.3 gene (pCH3dR1). At bottom is shown GS enzyme activity.

then the tissue was dissociated, and the separated cells were cultured monodispersed for 48 hr in medium with cortisol. Poly(A)$^+$ RNA was prepared and analyzed by RNA blotting, as described above. Results (Fig. 4*A*) showed that cell separation counteracted GS induction: GS mRNA accumulation and GS enzyme level declined dramatically after dispersion of the preinduced cells, irrespective of continued exposure to cortisol. The kinetics of this decline was investigated by dot-blot analysis of RNA from the preinduced cells at various times after their separation. The results (Fig. 4*B*) showed a decrease in GS mRNA level as early as 2 hr after cell separation; after 20 hr the original level of GS mRNA had declined by ≈90% despite continued cortisol exposure of the cells. Therefore, cell contacts are necessary for maintaining the induced state of GS, as well as for its initiation by cortisol.

Induction of GS Gene Transcription Depends on Cell Contact. We examined whether cortisol failed to stimulate GS gene transcription in separated cells. Using the nuclear run-on transcription assay we first measured the transcription rate of the GS gene in nuclei isolated from intact retina tissue, cortisol treated and untreated: cortisol markedly increased the rate of GS gene transcription (Fig. 5, lanes 1 and 2). Next, induced retina was dissociated, and the cells were maintained dispersed in cortisol-containing medium. As described above, there was rapid decline in GS mRNA accumulation in separated cells. The transcription assay showed that the rate of GS gene transcription in separated cells declined sharply within 2 hr (Fig. 5, lane 3) and remained low (Fig. 5, lane 4). Transcription of the histone H3.3 gene did not decline, which further indicated that cell separation selectively precluded stimulation by cortisol of GS gene activity.

Cell Contacts Restore Inducibility of GS Gene Expression. As stated earlier, separated cells are again inducible when they are reaggregated and cell contacts are restored. To examine the effect of cell reaggregation in further detail, retina tissue from day-10 embryos was dissociated into single cells, the cells were immediately aggregated into multicellular clusters by rotation in flasks, cortisol was added after 24 hr, and GS expression was analyzed 24 hr later. In aggregated

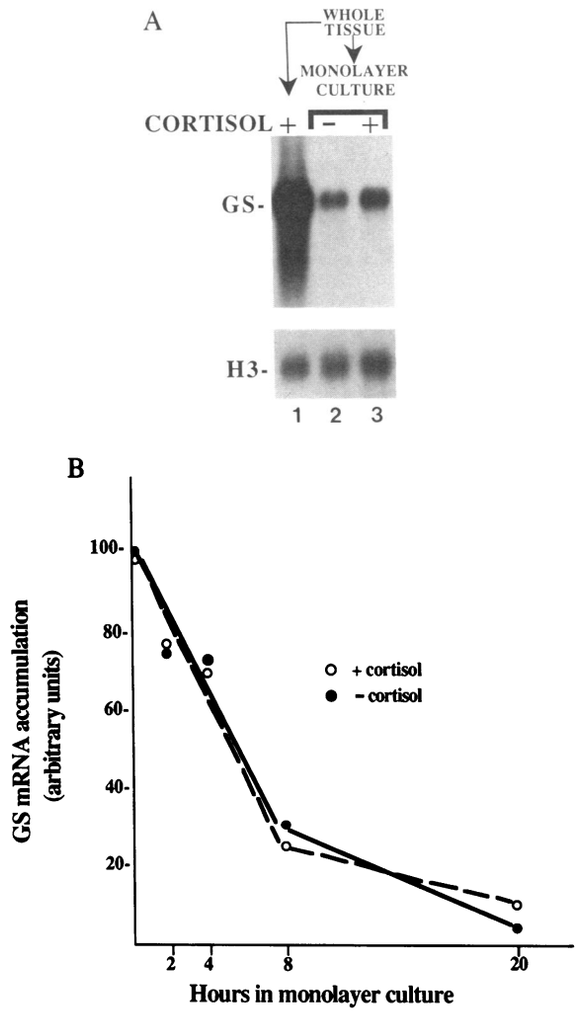

FIG. 4. Cell separation causes reversal of induction of GS expression. Retina tissue from day-10 embryos was cultured for 48 hr with cortisol to induce GS to a high level. Then the tissue was dissociated, and dispersed cells were maintained in monolayer culture for various times with or without cortisol. Poly(A)$^+$ RNA or total RNA was prepared and analyzed as follows. (*A*) RNA blot analysis of poly(A)$^+$ RNA of cortisol-induced retina tissue (lane 1) and of cells dissociated from induced retina. Cells were cultured for 2 hr without cortisol (lane 2) or in its continued presence (lane 3). The filter was consecutively hybridized to the ^{32}P-labeled clones of the GS gene and the histone H3 gene. (*B*) Decline of GS mRNA accumulation in cells separated from induced retina tissue. Cells dissociated from cortisol-induced retina were plated as a monolayer. Total RNA was isolated at 0, 2, 4, 8, or 20 hr after cell separation. The RNA was dot-blotted in duplicates and hybridized to the ^{32}P-labeled clones of the GS gene or the histone H3 gene. Autoradiograms of the blots were densitometrically scanned, and GS mRNA levels were calculated relative to histone H3 mRNA, which remained steady. Data were then plotted; highest level of GS mRNA was assigned the value of 100.

cells cortisol induced accumulation of GS mRNA and of GS enzyme, in contrast to an absence of GS induction in dispersed cells (Fig. 3, lanes c and d). In the aggregates, GS mRNA accumulation was somewhat lower than in intact retina tissue (compare lanes b and d in Fig. 3); this was to be expected because cell contacts in aggregates become gradually restored, whereas in intact retina tissue GS induction commences immediately after cortisol addition.

GS inducibility was restored by cell reaggregation also in cells that had remained separated for a longer time. Dissociated retina cells were monolayer-cultured for 48 hr; then they were dispersed by mild trypsinization and aggregated by

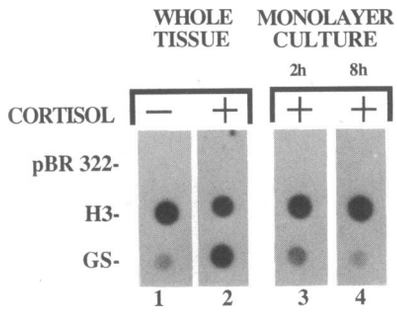

FIG. 5. GS gene transcription declines in separated cells: nuclear run-on transcription assay. Linearized DNA samples (5 μg) of the cloned GS gene, the cloned H3 gene, and the vector pBR322 were blotted on nitrocellulose filters. The filters were hybridized to [^{32}P]UTP-labeled nascent RNA synthesized in isolated nuclei (run-on assay). The nuclei were obtained from day-10 embryo retina tissue (lane 1), retina tissue cultured for 48 hr with cortisol (lane 2), and cells dissociated from cortisol-induced retina and maintained separated for 2 hr (lane 3) and 8 hr (lane 4) in medium with cortisol.

rotation for 48 hr in medium with cortisol; control cells remained in monolayer culture and were treated with cortisol. The results, summarized in Fig. 6, showed that in the aggregated cells cortisol induced a marked increase in GS mRNA and GS enzyme levels (Fig. 6, lanes 3 and 4); in the separated, monolayer-cultured cells only minor increases occurred (Fig. 6, lanes 1, 2, 5, and 6).

DISCUSSION

The importance of cell contacts and contact interactions in regulation of cell differentiation is a basic tenet of developmental biology. Our results demonstrate that the induction by cortisol of GS gene expression in embryonic neural retina (in Müller glia cells) requires cell contacts. Cortisol stimulates GS gene transcription in intact retina tissue, but not in dissociated and separated retina cells; however, the separated cells become again inducible if they are reassembled into multicellular aggregates and reestablish cell contacts. Persistence of the induced state also requires cell contacts, for when cortisol-induced retina tissue is separated into cells, there is rapid "deinduction" of GS and loss of inducibility.

We found no evidence that the failure of cortisol to stimulate GS gene transcription in separated cells was due to general suppression of transcription in these cells or to decreased stability of mRNAs: there was no measurable decline in the transcription of histone H3.3 gene or in accumulation of H3.3 mRNA and CA-II mRNA. Hence, cell contacts in the retina are essential for the stimulation by cortisol of GS gene transcription; disruption of cell contacts renders the cells noninducible.

Two previous observations are significant to the present results: (*i*) In the retina, GS is induced and expressed only in Müller glia cells, not in the neurons (15). (*ii*) Immunohistochemical studies (22) suggest that GS induction requires glia interaction with neurons, and, furthermore, that direct cell contact, and not a diffusible factor, is involved in this interaction. Our working hypothesis (2) postulates that contacts with neurons generate "signals" in the glia cells that capacitate them for GS gene induction; cell separation abrogates these signals and precludes induction.

It is noteworthy that the role of GS in retina physiology also involves glia–neuron cooperation (18). Glutamate released from neurons is converted by glial GS into glutamine, which neurons use to make glutamate (16). Thus, GS induction and cell contacts may relate to this metabolic cycle.

Regulation of gene transcription by glucocorticoid hormones involves a series of processes, which include hormone

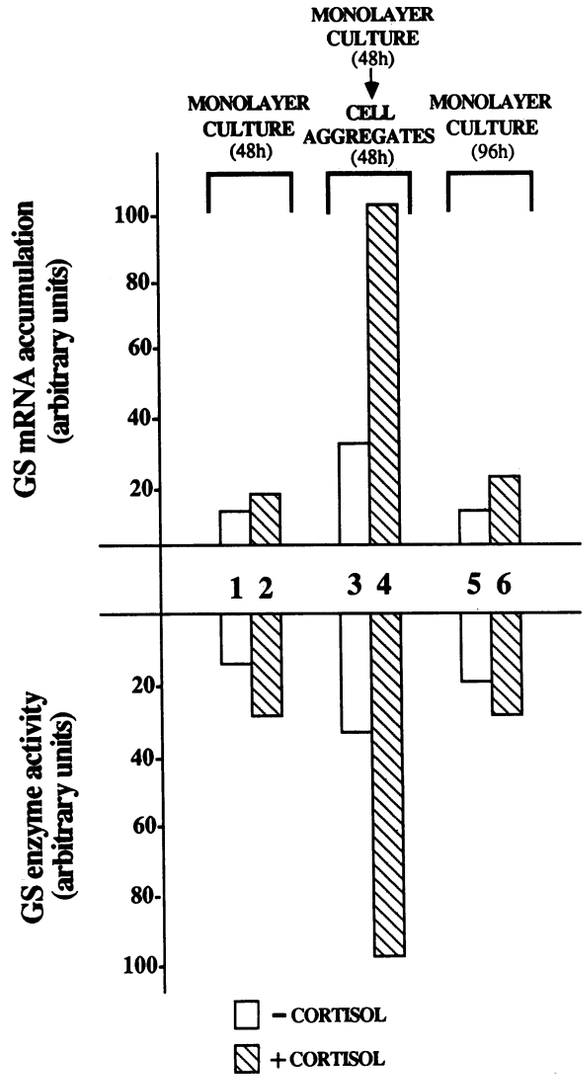

FIG. 6. GS inducibility is restored in cell aggregates. Retina tissue (day-10 embryos) was dissociated, and the cells were plated in monolayer culture for 48 hr (lanes 1 and 2); then cells were collected and aggregated by rotation in flasks for 48 hr (lanes 3 and 4). Control monolayer cultures were left for an additional 48 hr in medium with or without cortisol (lanes 5 and 6). Total RNA was prepared and was dot-blotted in duplicate. One filter was hybridized to the ^{32}P-labeled clone of the GS gene; the other was hybridized to the labeled clone of the histone H3 gene. Filters were scanned, and GS mRNA levels were calculated relative to histone mRNA (upper graph); the lower graph shows corresponding GS enzyme activities. Highest levels were assigned a value of 100.

complexing with intracellular receptors and modulation by the complexes of specific gene transcription (for review, see ref. 32). Our data that cortisol induces GS in the retina by stimulating GS gene transcription confirm earlier suggestions (20, 30, 31). The finding that cell separation precludes GS gene induction raises questions about which step in the induction process is regulated by cell-contact dependence. Among candidates are the cortisol-binding receptors. As reported (33), their level (activity) partially declines in separated retina cells and increases again when the cells are reaggregated. Attempts to establish a consistent and direct causal correlation between changes in the level of these receptors and GS inducibility in the retina were unsuccessful (34). Nevertheless, the possibility remains that partial loss of receptors in separated cells prevents GS induction. However, it is equally likely that some other process, obligatory for increasing the rate of GS gene transcription, is cell-

contact dependent, and that its preclusion prevents GS induction.

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