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Developmental control of glucocorticoid receptor transcriptional activity in embryonic retina

(glutamine synthetase/neural retina/embryonic development/Müller glia cells)

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ABSTRACT **In chicken embryo retina, competence for induction of the glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming); EC 6.3.1.2] gene by glucocorticoid hormones increases progressively with development; this competence is minimal in 6-day retina (E6) and high by day 10 (E10). Because the level of glucocorticoid receptors (GRs) in the retina does not increase during that time, we investigated whether the transcriptional activity of GR increased between days 6 and 10 of development. The glucocorticoid-inducible chloramphenicol acetyltransferase (CAT) constructs 2GRE–37TK and pΔG46TCO, which contain glucocorticoid-responsive elements attached to a TATA box and to the thymidine kinase promoter, respectively, were transfected into E6 and E10 retinas, and their inducibility was examined. CAT expression could be induced in the transfected E10 retina but was not induced in the transfected E6 retina. However, induction was obtained also in E6 retina after cotransfection with a GR expression vector. Noninducible CAT constructs (pRSV-CAT, pSV2CAT, and pBLCAT2) were expressed at both ages at similar levels. The CAT construct pGS2.1CAT, which is controlled by the upstream sequence of the chicken glutamine synthetase gene, could be induced in E10 retina but was not induced in E6 retina; however, cotransfection with the GR expression vector resulted in induction of pGS2.1CAT also in E6 retina. We interpret these results as showing that the transcriptional activity of GR in embryonic retina is developmentally controlled and suggest that its increase is causally implicated in the development of competence for glutamine synthetase induction.**

Tissue differentiation is accompanied by selective expression of genes in a temporal and cell-type-specific fashion. Control of gene transcription depends on specific regulatory factors that interact, directly or indirectly, with DNA-regulatory elements and activate transcription. For glucocorticoid-inducible genes, a functional glucocorticoid receptor (GR) is prerequisite for the induction of gene transcription. The GR–hormone complex binds to glucocorticoid response elements (GREs) in target genes, resulting in activation of a network of specific genes in a tissue-specific manner (1, 2). It has been suggested that gene induction by glucocorticoids requires interactions between GR and other transcription factors (3–7) and that such combinatorial mechanisms might account for selective gene induction in different target cells.

Expression of glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming); EC 6.3.1.2] in the neural retina of chicken embryo is an example of a glucocorticoid-inducible gene activity that is cell-type-specific and developmentally controlled (8–13). Glutamine synthetase induction in the retina is regulated at the transcriptional level (9, 13); it is always confined to Müller glia cells (10) and requires cell contact between glia and neurons (9–11). During embryonic development, glutamine synthetase is expressed in the retina at a low level until day 15, when it rises sharply (8, 11, 12), subsequent to adrenal cortex development (14), and reaches a high plateau within a few days. Glutamine synthetase can be induced precociously before the 15th day by prematurely supplying cortisol (or a related glucocorticoid) to the embryo or directly to isolated retina tissue *in vitro* (15); however, glutamine synthetase cannot be induced in retinas younger than 7 days, and its inducibility increases progressively with development (8, 12, 15). The molecular basis for this developmental control of competence for glutamine synthetase induction is not known.

Given that the induction of glutamine synthetase requires GR and, possibly, still other transcription factors, absence (or inactivity) of an essential factor could explain why early embryonic retina (E6 and earlier) is not inducible. Because glucocorticoid-binding activity in E6 retina is not lower than in E10 retina (16, 17), it is unlikely that GR level is the limiting condition in the temporal control of inducibility. However, if the transcriptional activity of GR is inhibited at early embryonic ages, the receptor would not elicit transcription of the glutamine synthetase gene, despite being able to bind the hormone. Investigating this possibility, we found that the transcriptional activity of GR is, indeed, inhibited in early embryonic retina and subsequently increases. We suggest that GR transcriptional activity in embryonic retina is controlled by a development-dependent mechanism.

MATERIALS AND METHODS

Plasmids. Plasmid pGS2.1CAT contains the upstream region of the glutamine synthetase gene attached to the chloramphenicol acetyltransferase (CAT) reporter gene. The upstream region of the glutamine synthetase gene (nt +13 to −2121) was obtained from a described (12) glutamine synthetase genomic clone λGS113 and was inserted next to the coding region of the CAT gene, obtained from pSV0CAT (18), in the pBluescript vector (Stratagene). Plasmids pSV2CAT (18), pRSVCAT (19), and pBLCAT2 (20) contain the CAT reporter gene attached to viral promoter sequences of simian virus 40, Rous sarcoma virus (RSV), and the herpes simplex virus thymidine kinase gene (TK), respectively. The clone p6RGR, which contains the rat GR cDNA under transcriptional control of the RSV promoter, was provided by K. R. Yamamoto, University of California, San Francisco. The construct pΔG46TCO was derived from pG46TCO (21) and is a pUC vector containing a synthetic GRE sequence linked to TK–CAT fusion gene. To delete the transcription factor AP-1 site in pUC DNA, the pG46TCO vector was digested with *Aat* II and *Nde* I, filled in, and religated to yield

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Abbreviations: E*n*, embryonic day *n*; GR, glucocorticoid receptor; GRE, glucocorticoid response element; TK, thymidine kinase; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase.

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pΔG46TCO (−4464/183). The constructs pBLCAT2 and pΔG46TCO contain the same region of the TK promoter (nt +51 to −109). The construct 2GRE–37TK (5) harbors two copies of GRE sequences linked to the TATA box of the TK promoter (nt +51 to −37) and to the CAT gene. The RSV-L(SEL) construct, which contains the luciferase reporter gene under transcriptional control of the RSV promoter (22), was provided by S. Subramani, University of California, San Diego.

Retina Tissue and Transfection Procedure. Neural retina tissue was isolated under sterile conditions from eyes of chicken embryos (White Leghorn) at different ages. Before transfection, the tissue was cut into pieces and was organ cultured for 4 hr in Erlenmeyer flasks in medium 199/10% fetal calf serum on a gyratory shaker (72 rpm) at 38°C (10). Plasmid DNA was transfected into pieces of intact retina tissue by electroporation with a Bio-Rad gene pulser with voltage and capacitance settings of 400 V and 960 μF, as described (23). In all cases electroporation was done in cuvettes containing 1 ml of phosphate-buffered saline (PBS) (2.7 mM KCl/1.5 mM KH_2PO4/137 mM NaCl/8 mM $Na_2HPO_4 \cdot 7\ H_2O$) and a total of 1–5 μg of DNA of indicated plasmids per 8×10^6 cells. Plasmid DNA was prepared by using Qiagen plasmid preparation kit (Qiagen, Chatsworth, CA). After electroporation, retinas were maintained in PBS buffer for 10 min in ice. Retinas transfected in the same cuvette were divided into two Erlenmeyer flasks and were cultured in the presence of cortisol (0.33 μg/ml) (Sigma), or in its absence, for another 24 or 48 hr under conditions used before transfection (see above). Culture medium was changed after 24 hr.

CAT Luciferase and Glutamine Synthetase Assays. CAT luciferase and glutamine synthetase activities were determined in tissue sonicates. CAT activity was determined as described (18). Samples were heated for 10 min at 65°C before analysis. CAT activity was expressed as pmol of chloramphenicol acetylated per hr per mg of protein. In indicated experiments transfection efficiencies were controlled for by cotransfection of RSVL(SEL) (22). Luciferase activity was assayed, as described (22), and recorded by a LKB luminometer. The specific activity of glutamine synthetase was determined by the colorimetric assay (24) and expressed as μM γ-glutamylhydroxamate per hr per mg of protein.

RESULTS

GR Transcriptional Activity Is Inhibited in E6 Retina. In retina of 6-day embryos (E6 retina) glucocorticoid hormones, such as cortisol, do not induce glutamine synthetase, whereas in 10-day retina (E10 retina) there is rapid induction (8, 12, 15). Retinas at these two developmental ages were used to investigate whether GR transcriptional activity changed with development. To examine GR activity and distinguish it from that of other transcription factors, we used gene constructs under the transcriptional control of a synthetic, so called "simple GRE". These constructs do not display tissue specificity and are inducible in a wide variety of cell lines; their induction depends on the presence of functional receptor molecules (5, 25, 26). We used two such gene constructs to investigate the presence of functional GR: (*i*) pΔG46TCO, containing a synthetic GRE linked to the TK promoter–CAT gene fusion (21); (*ii*) 2GRE–37TK, containing two copies of a GRE sequence attached to the TATA box of the TK promoter and to the CAT reporter gene (5). As a control for general transcription activity and transfection efficiency, we used three gene constructs not inducible by glucocorticoids: (*i*) pRSVCAT, containing the CAT gene under the transcriptional control of RSV promoter (19); (*ii*) pSV2CAT, containing the CAT gene attached to the simian virus 40 promoter (18); (*iii*) pBLCAT2, containing the CAT gene fused to the TK promoter (20). All five constructs were transfected into E6 and E10 retinas by electroporation, and the tissue was cultured and assayed for glutamine synthetase and CAT activity. It should be explained that expression of the different constructs was examined in intact retina tissue because previous studies showed that glucocorticoids can induce glutamine synthetase only in retinal tissue and not in separated retina cells (9, 10, 27).

Assays of the expression of the noninducible gene constructs (the control group) detected no major differences in the expression pattern of CAT between E6 and E10 retinas (Fig. 1 *C–E, G*). The greatest difference was found in the level of pRSVCAT expression, which was 30–40% higher in E6 than in E10 retina. CAT activity in both E6 and E10 retinas transfected with pRSVCAT was much higher than in retinas transfected with pSV2CAT or pBLCAT2 (Fig. 1), implying that the RSV promoter in the chicken retina can direct higher levels of gene expression than the simian virus 40 or TK promoters.

Examination of the two glucocorticoid-inducible constructs revealed a dramatic difference in the levels of CAT expression between the transfected E6 and E10 retinas (Fig. 1 *A, B,* and *F*). While in the transfected E6 retina cortisol induced, at best, only a slight increase in CAT expression, in the transfected E10 retina it induced a major increase: a 48-fold increase in CAT expression in E10 retina transfected with pΔG46TCO and a 5-fold increase in E10 retina transfected with 2GRE–37TK. The difference in the inducibility of the two constructs may be due to their different promoters (28): the pΔG46TCO construct contains the complete TK promoter, whereas the 2GRE–37TK construct contains only its first 37 nt (including the TATA box). As expected, expression of the endogenous glutamine synthetase gene was induced only in E10 retina (by 8.6-fold) (Fig. 1*F*). The basal levels of both CAT and glutamine synthetase were also higher in E10 than in E6 retina, possibly due to trace amounts of corticoids in the culture medium. It should be noted that these dramatic differences in CAT induction between E6 and E10 retina were obtained when the CAT assay was done with equal amounts of protein extract from the transfected retinas or when the tissue was cotransfected with the RSVL(SEL) construct (22) and the CAT assay was adjusted to include equal units of luciferase activity.

The failure of cortisol to induce CAT expression in E6 retina transfected with pΔG46TCO or 2GRE–37TK raised the possibility that the transcriptional activity of GR was inhibited in E6 retina. In this case, introduction of a large excess of GR molecules into E6 retina cells might result in CAT induction. This prediction was examined by transfecting E6 retina with the rat GR expression vector p6RGR, together with the pΔG46TCO construct. The transfected retina tissue was divided into two flasks, cultured for 24 hr with or without cortisol, and assayed for CAT activity. Transfection with p6RGR resulted in a major induction of CAT activity in E6 retina (Fig. 2, lanes 3 and 4), in sharp contrast to its low level in E6 retina, which expressed only the endogenous GR molecules (Fig. 2, lanes 1 and 2). These results indicate that the conditions prerequisite for activation of this GRE-linked TK promoter are available in E6 retina but that the endogenous GR is transcriptionally inactive at this age. This conclusion is supported by the finding that the noninducible CAT construct pBLCAT2, which contains the TK promoter without GRE, was expressed in both E10 and E6 retinas at similar levels (Fig. 1*E*). Thus, GR molecules in E6 retina, although capable of binding glucocorticoids, are transcriptionally inactive.

The Level of GR Transcriptional Activity Increases with Development. Next, we examined whether the difference in GR transcriptional activity between E6 and E10 retinas reflects a progressive increase that occurs with development.

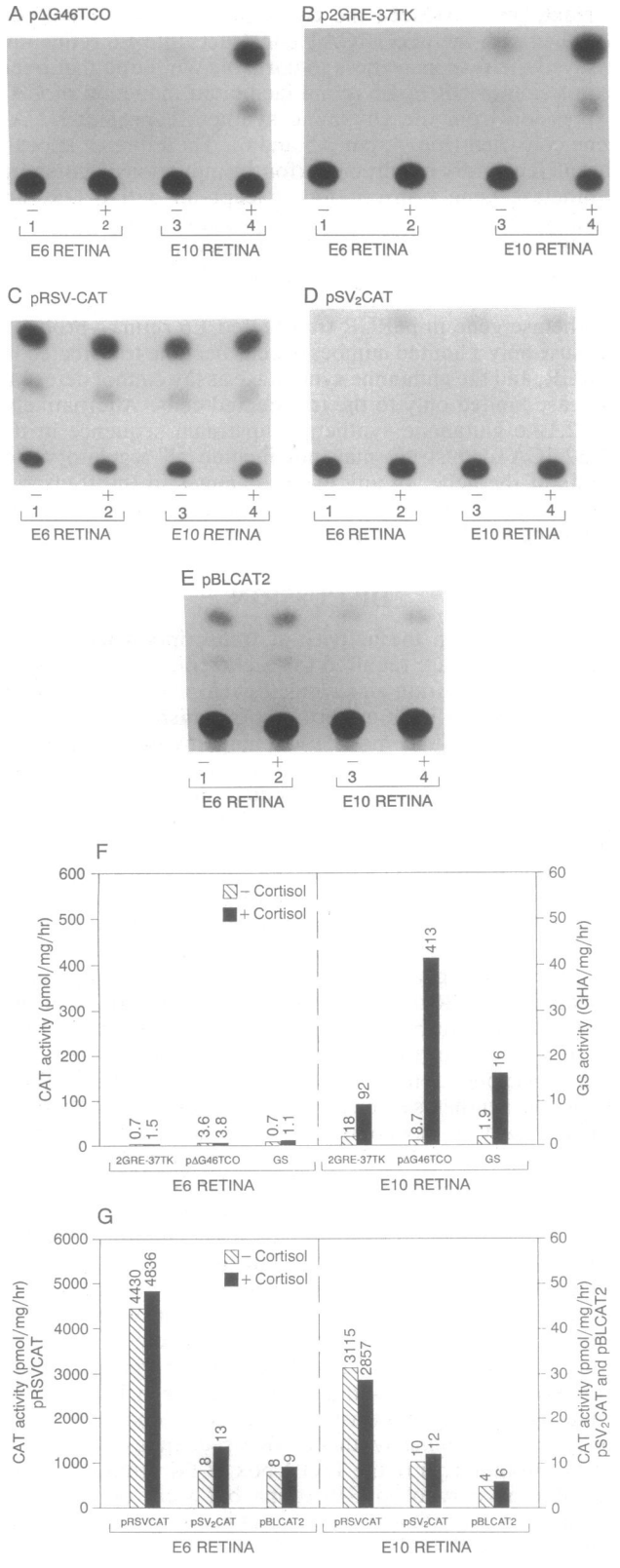

FIG. 1. Glucocorticoid-inducible CAT constructs are expressed in E10 but not in E6 retina. The glucocorticoid-inducible CAT constructs pΔG46TCO (*A*) and 2GRE-37TK (*B*) or the noninducible CAT constructs pRSVCAT (*C*), pSV2CAT (*D*), and pBLCAT2 (*E*) were transfected into E6 retina (lanes 1 and 2) and E10 retina (lanes 3 and 4) by electroporation. In all cases electroporation was done in cuvettes containing 4 E10 retinas or 14 E6 retinas and a total of 1–3 μg of DNA of indicated plasmids per 8×10^6 cells. Transfection of pΔG46TCO, pBLCAT2, and pRSVCAT was done in the presence of 1 μg of DNA per 8×10^6 cells, whereas transfection of 2GRE–37TK and pSV2CAT was done in the presence of 3 μg of DNA per 8×10^6 cells. Pieces of the transfected tissue were divided into two flasks; one was cultured for 24 hr in the presence of cortisol (lanes 2 and 4), and the other was cultured in its absence (lanes 1 and 3). CAT and glutamine synthetase (GS) activities were subsequently examined in tissue sonicates. Because pRSVCAT was found to be expressed in E6 retina at levels somewhat higher than in E10 retina (see *G*), the CAT assay in this experiment was done by using equal amounts of proteins and was not normalized according to luciferase activity originating from cotransfected RSVL(SEL). Similar results were, however, obtained in parallel experiments in which the CAT assay was adjusted to include an equal amount of luciferase activity. (*F*) Quantitative evaluation of CAT activity in retina transfected with the glucocorticoid-inducible CAT constructs. (*G*) Quantitative evaluation of CAT activity in retina transfected with the noninducible CAT constructs. Values are the means of several experiments. Results of glutamine synthetase activity in the transfected tissues are presented in *F*. Note that different scales of CAT activity are used in the diagrams for the different constructs and for glutamine synthetase activity.

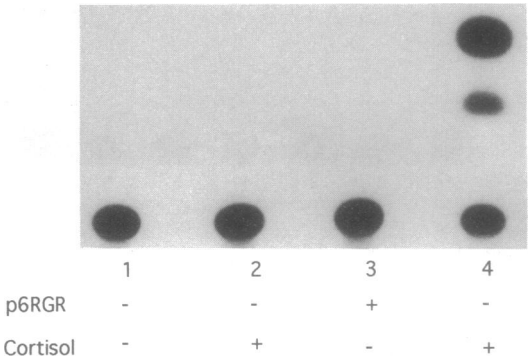

FIG. 2. Overexpression of GR facilitates induction of CAT expression in E6 retina. E6 retina was transfected with the glucocorticoid-inducible CAT construct pΔG46TCO with (lanes 3 and 4) or without (lanes 1 and 2) the GR expression vector p6RGR. Transfection was done in the presence of 1 μg of pΔG46TCO DNA and 4 μg of p6RGR DNA per 8×10^6 cells. DNA of the pBluescript vector was used to adjust the amount of transfected DNA to a total of 5 μg. The transfected tissue was divided into two flasks and cultured for 24 hr with (lanes 2 and 4) or without (lanes 1 and 3) cortisol. CAT assay was done by using equal amounts of protein.

The glucocorticoid-inducible CAT construct, pΔG46TCO, was transfected into E6, E7, E8, and E10 retinas together with the RSVL(SEL) construct. The transfected retinas were divided into two flasks, cultured for 24 hr with or without cortisol, and assayed for CAT luciferase and glutamine synthetase activity. The CAT assay was adjusted to include equal units of luciferase activity. We found that the level of CAT induction increased with development, similar to the increase in inducibility of glutamine synthetase (Fig. 3). These results indicate that the transcriptional activity of GR increases progressively with development; they suggest a causal relationship between GR activation and the increase in the competence for glutamine synthetase induction in the retina.

Overexpression of GR in E6 Retina Renders the Glutamine Synthetase Promoter Inducible. Absence of transcriptionally active GR in early embryonic retina would preclude induction of glutamine synthetase at early embryonic ages. However, it is also conceivable that, still other regulatory factor(s) required for this induction may be absent or inactive at early ages. We recently cloned the upstream region of the chicken glutamine synthetase gene and found that responsiveness to glucocorticoids is conferred by a sequence located between nt 1727 and 2121 upstream of the initiation site (unpublished data), in close agreement with the findings of Zhang and Young (29). We used the pGS2.1CAT construct, which

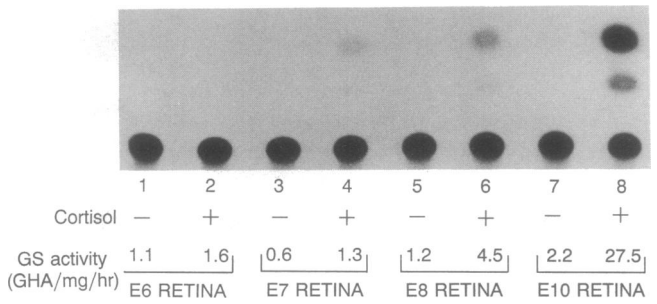

FIG. 3. Expression of the glucocorticoid-inducible CAT construct increases with development. The glucocorticoid-inducible CAT constructs, pΔG46TCO, and the luciferase construct RSVL-(SEL) were cotransfected into E6 (lanes 1 and 2), E7 (lanes 3 and 4), E8 (lanes 5 and 6), and E10 (lanes 7 and 8) retina by electroporation. Transfection was done in the presence of 1 μg of pΔG46TCO DNA and 1 μg of RSVL(SEL) DNA per 8×10^6 cells. Pieces of the transfected tissue were divided into two flasks—one cultured for 24 hr with cortisol (lanes 2, 4, 6, and 8) and the other without cortisol (lanes 1, 3, 5, and 7). CAT, luciferase, and glutamine synthetase (GS) activities were subsequently examined in tissue sonicates. The CAT assay was adjusted to include an equal amount of luciferase activity. Similar results were obtained in parallel experiments in which the CAT assay was done with equal amounts of protein. GHA, γ-glutamylhydroxamate.

contains the CAT reporter gene attached to the upstream sequence (nt +13 to −2121) of the glutamine synthetase gene, to investigate the involvement of GR in the developmental control of glutamine synthetase inducibility. The construct was transfected into E6 and E10 retinas, which were then cultured for 48 hr in the presence or absence of cortisol. Glutamine synthetase and CAT activity (Fig. 4, lanes 1 to 4) could be induced only in E10 retina, indicating that the sequence located within the 2121 nt upstream of the glutamine synthetase gene not only confers responsiveness to glucocorticoids but also mediates developmental control of inducibility.

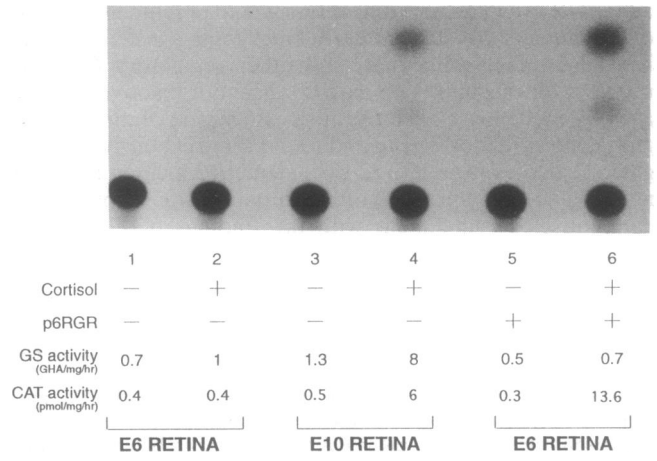

FIG. 4. The glutamine synthetase promoter–CAT gene construct is inducible in E10 retina but is not inducible in E6 retina; induction in E6 retina is facilitated by overexpression of GR. The glutamine synthetase promoter–CAT gene construct pGS2.1CAT was transfected into E6 retina (lanes 1 and 2) and E10 retina (lanes 3 and 4); it was also transfected into E6 retina together with the GR expression vector p6RGR (lanes 5 and 6). Transfection was done in the presence of 3 μg of DNA per 8×10^6 cells and included, where indicated, 0.75 μg of p6RGR plasmid DNA. The transfected retina was divided into two flasks and cultured for 48 hr with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) cortisol. Tissue sonicates were assayed for CAT and glutamine synthetase (GS) activities. CAT assay was done by using equal amounts of protein.

Next, we introduced the GR expression vector p6RGR, together with the pGS2.1CAT construct, into E6 retina and assayed the responsiveness to cortisol. We found that overexpression of GR in E6 retina facilitated induction of CAT expression from the glutamine synthetase promoter–CAT gene construct (Fig. 4, lanes 5 and 6). Therefore, it appears that all regulatory requirements for the induction of glutamine synthetase gene transcription, except an active GR, are available in E6 retina, although the possibility that high levels of GR can compensate for the absence of other required regulatory factor(s) cannot be excluded. There was no detectable increase in expression of the endogenous glutamine synthetase gene in p6RGR-transfected E6 retina—probably because only a limited number of cells become transfected by p6RGR, and the glutamine synthetase assay cannot detect an increase limited only to the transfected cells. Alternatively, the 2.1-kb glutamine synthetase upstream sequence in the pGS2.1CAT construct may not include all regulatory elements of the gene. In any event, changes in the transcriptional activity of GR play a major role in the increase of glutamine synthetase inducibility in the developing retina.

DISCUSSION

Timed changes in the activity of transcription factors, including the GR, are required for orchestrated expression of gene networks during embryonic development. Modulation of factor activity is an important mechanism for control of gene function. Such modulation may involve changes in synthesis and level of a particular transcription factor, post-translational modifications, and induction of antagonists that specifically repress the factor's transcriptional activity. How such changes are elicited is unclear; however, it has been shown that in embryonic retina, cell contacts between Müller glia and neurons influence levels of glucocorticoid-binding activity (8, 10, 17), suggesting that signals generated by these cell contacts regulate GR levels in Müller cells (11, 30). Whether these signals play a role also in the developmental modulation of GR transcriptional activity, described in this paper, remains to be determined, and the present findings provide a basis for future investigation of this problem.

In embryonic retina, competence for induction of the glutamine synthetase gene by glucocorticoid hormones is minimal early in development and progressively increases (9). We found that this increase was related to GR activity, in that the transcriptional activity of GR increased with embryonic age—i.e., was developmentally controlled. GR can mediate induction with cortisol of the glutamine synthetase gene in 10-day retina (E10) but not in E6 retina, even though the total level of cortisol-binding activity in E6 is not lower than in E10 retina. There is no general suppression of transcription in E6 retina because transfected CAT constructs controlled by the viral RSV, TK, or simian virus 40 promoters were expressed at similar levels in E6 and E10 retinas (Fig. 1). In contrast, glucocorticoid-inducible CAT constructs containing synthetic GRE sequences attached to the TK promoter, or to the TATA box of TK promoter, were inducible by cortisol in E10 retina but were only slightly inducible in E6 retina. However, induction of CAT in E6 retina was greatly enhanced by cotransfecting it with a GR expression vector. Absence of induction in E6 retina of genes controlled by a "simple GRE" promoter and the fact that overexpression of GR promoted inducibility strongly suggest that the endogenous GR in E6 retina is transcriptionally inactive and that its activity increases with development.

That the developmental increase in GR transcriptional activity is causally correlated with the increase in inducibility of glutamine synthetase (10–12) was further shown by transfecting E6 and E10 retinas with the CAT construct pGS2.1CAT, which is controlled by the upstream sequence

of the glutamine synthetase gene. Expression of this construct could be induced in E10, but could not be induced in E6 retina; however, overexpression of GR in E6 retina resulted in induction of this construct. Our findings strongly suggest that the temporal control of glutamine synthetase inducibility in developing retina is determined, at least in part, by changes in GR transcriptional activity. Zhang and Young have shown that a single GRE element juxtaposed to an AP-1/ATF/CRE-like site in the upstream sequence of the glutamine synthetase gene confers responsiveness to glucocorticoids (29). This raises the possibility that proteins interacting at the AP-1/ATF/CRE-like site act in collaboration with GR in the hormonal induction of glutamine synthetase and are involved in the developmental control of inducibility.

Stimulation of gene transcription is a multistep process involving binding of GR to GREs and, possibly, other regulatory factors. Responsiveness to glucocorticoids requires a critical threshold of transcriptionally active GR molecules in the cells (25, 26, 31–33). When the receptor level is below that threshold or when some step essential for receptor function is inhibited, activation of target gene transcription will not occur. As mentioned above, although the total hormone-binding activity in E6 retina is not lower than in E10 retina (16, 17), glucocorticoids do not induce the glutamine synthetase gene in E6 retina. A probable reason for this is the difference in the transcriptional activity of GR described in this paper. An additional possibility relates to changes in the GR amount per cell: in early embryonic retina GR may be present in all the cells but at a lower level per cell than required for the induction; as development progresses, GR level may decline in differentiating neurons, whereas in Müller glia cells [where glutamine synthetase is induced (10)] the level of transcriptionally active GR increases. Thus, progressive compartmentalization of GR in Müller glia (24) and its transcriptional activation could explain the increase in glutamine synthetase inducibility between days 6 and 10 of development.

Modulation of GR activity might involve modification by phosphorylation/dephosphorylation (34–37) and/or oxidation/reduction (38), which could inhibit GR transcriptional activity at early embryonic ages. Other candidate modulators are jun and fos because their overexpression can antagonize glucocorticoid action and block GR transcriptional activity (39–42). Indeed, we found that overexpression of c-jun inhibited GR activity in embryonic retina cells (L.V. and Y. Berko-Flint unpublished data); thus, high levels of c-jun in early retina and their subsequent decline may be implicated in developmental modulation of GR transcriptional activity. In this context it is noteworthy that both GR activity and glutamine synthetase inducibility increase at the time when cell proliferation in the retina is declining; retina growth in chicken embryo ceases by day 12 (43, 44), at which age GR is transcriptionally active and glutamine synthetase inducibility is high. Assuming that proliferating retinal cells express high levels of c-jun and/or c-fos, inhibition of GR transcriptional activity would prevent induction by glucocorticoids of differentiation products, such as glutamine synthetase, in cells that are still growing. Thus, regulation of GR transcriptional activity in embryonic retina may represent a cross-talk between cell proliferation and differentiation pathways in a developing tissue.

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