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Involvement of c-Jun in the control of glucocorticoid receptor transcriptional activity during development of chicken retinal tissue

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The ability of the glucocorticoid receptor (GR) to induce gene expression in embryonic chicken retinal tissue increases dramatically during development, although the quantity of the receptor molecules does not change greatly with age. This study examines the possible involvement of c-Jun in the developmental control of GR activity. Expression of c-Jun in retinal tissue was high at early embryonic ages and declined during development. Elevation of c-Jun expression in retina of mid-developmental ages by treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or by introduction of a c-Jun expression vector, caused a pronounced decline in the inducibility of the endogenous glutamine synthetase gene and the transiently transfected CAT constructs pΔG46TCO and pGS2.1CAT, that are controlled by a minimal consensus glucocorticoid response element (GRE) promoter and the glutamine synthetase promoter, respectively. The effect of c-Jun was dose dependent and could be reversed by overexpression of GR. C-Jun-evoked repression of GR activity could be relieved by overexpression of Jun D. Overexpression of Jun D could also elevate the responsiveness of early embryonic retina to glucocorticoids and cause a 5-fold increase in pΔG46TCO induction. The effect of Jun D could be reversed by overexpression of c-Jun. Expression of c-Jun might therefore be important for repression of GR activity at early embryonic ages.

Key words: embryonic development/glutamine synthetase/Jun D/Müller glial cells/neural retina

Introduction

Cell proliferation is often antagonistic to cell differentiation during embryonic development. In many tissues cell differentiation follows cessation of cell growth, while induction of cell proliferation, by growth factors or by oncogenes, results in loss of cell properties characteristic of the differentiated state. Cross-coupling of differentiation and proliferation pathways is illustrated by the opposing effects of steroid hormones and growth factors. Glucocorticoids, for example, have antiproliferative effects (Baxter and Forsham, 1972; Vassalli *et al.*, 1976; Allison, 1988), but can also induce differentiation processes in various target tissues by activating the expression of specific genes (Rosen *et al.*, 1963; Steinberg *et al.*, 1975; Crook *et al.*, 1978; Topper and Freeman, 1980; Doppler *et al.*, 1989). In contrast, hormone-induced differentiation can be reversed by growth factors or by oncogenes (Andres *et al.*, 1988; Narve and Ringold, 1988; Vardimon *et al.*, 1991).

A mechanistic basis for the opposing effects of glucocorticoids and growth factors was recently suggested by the finding that the glucocorticoid receptor (GR) and the transcription factor AP-1 can reciprocally alter each other's transcriptional activity. Glucocorticoids, like other steroid hormones, exert their inductive effects by activating GR molecules that bind directly to a *cis*-acting sequence, the glucocorticoid response element (GRE), and elevate expression of specific cellular genes (reviewed in Landers and Spelsberg, 1992). AP-1 is a transcription factor that consists of dimers formed between protein products of the *Jun/fos* gene families. Expression and transcriptional activity of AP-1 in the cell are activated by phorbol esters, cytokines and growth factors (reviewed in Karin, 1990). Once activated, AP-1 stimulates the transcription of a set of cellular genes involved in growth control. Recent studies have shown that AP-1 can interact with the GR and selectively activate or repress gene expression in a promoter- and cell type-specific manner (Ponta *et al.*, 1992). One mode of mutual transcriptional inhibition involves interaction between c-Jun and GR at the level of the proteins themselves (Jonat *et al.*, 1990; Schüle *et al.*, 1990a; Yang-Yen *et al.*, 1990). The interaction between the two molecules is mediated by the DNA-binding domain of GR and the leucine zipper region of c-Jun (Schüle *et al.*, 1990a; Yang-Yen *et al.*, 1990). The complex of c-Jun and GR is incapable of binding to each other's cognate DNA elements *in vitro* (Schüle *et al.*, 1990a; Yang-Yen *et al.*, 1990), although it is possible that *in vivo* the GR–Jun complex might retain DNA-binding ability and exert repression by blocking the transactivating function only (Konig *et al.*, 1992).

The ability of the transcription factors c-Jun and GR to interact with each other and to alter programs of gene expression suggests an attractive mechanism for the coordination of proliferation and differentiation processes during embryonic development. To evaluate the possible function of such a mechanism in the embryo, it is necessary to use an embryonic system that is accessible to various molecular interventions and in which responsiveness to glucocorticoids depends on tissue development. These requirements are met by the experimental system studied here, namely the development-dependent control of GR transcriptional activity and glutamine synthetase inducibility in the neural retina of chicken embryo.

Glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming); EC 6.3.1.2], a differentiation marker of retinal glial cells (Linser and Moscona, 1979), can be induced in embryonic retina by glucocorticoids which directly stimulate an increase in gene transcription (Vardimon *et al.*, 1988). The upstream sequence of the glutamine synthetase gene contains a single GRE (Zhang and Young, 1991) and confers responsiveness to glucocorticoids (Zhang and Young, 1991; Ben-Dror *et al.*, 1993). The ability to express glutamine

synthetase in response to hormonal induction increases progressively with development (Piddington and Moscona, 1967; Moscona, 1983; Vardimon *et al.*, 1986a). Glucocorticoids cannot induce a major increase in glutamine synthetase expression prior to embryonic day 8 (E8), in spite of the fact that at this early age the amount of the GR protein (Ben-Or and Okret, 1993) and the level of hormone-binding activity (Lippman *et al.*, 1974; Koehler and Moscona, 1975; Saad and Moscona, 1985) is no lower than at later embryonic ages. Using chloramphenicol acetyltransferase (CAT) gene constructs that are controlled by minimal consensus GRE promoters, we and others demonstrated that the ability of GR to stimulate gene expression increases during development: glucocorticoids can induce a marked increase in CAT expression in retina of mid-developmental ages, but not in early retina, and overexpression of GR results in CAT induction also in early retina (Pu and Young, 1990; Ben-Dror *et al.*, 1993; Zhang *et al.*, 1993).

Overexpression of GR also facilitated induction of a CAT construct that is controlled by the upstream sequence of the chicken glutamine synthetase gene in E6 retina (Ben-Dror *et al.*, 1993; Zhang and Young, 1993). The increase in GR transcriptional activity in the developing retina correlates with a decline in retinal cell growth (Vardimon *et al.*, 1993), and introduction of the oncogene v-*src*, which induces retinal cell proliferation (Calothy *et al.*, 1978), results in a marked decline in inducibility of glutamine synthetase (Vardimon *et al.*, 1991). These findings led us to postulate that component(s) of the proliferation pathway might be involved in repression of GR transcriptional activity at early embryonic ages. Results presented in this communication demonstrate that c-Jun expression is high in early embryonic retina and declines with age, and that these changes in c-Jun expression play a role in the control of GR transcriptional activity during retinal development.

Results

Developmental pattern of Jun expression

The ability of GR to induce gene expression in response to glucocorticoids increases progressively with retinal development (Ben-Dror *et al.*, 1993). To investigate the possibility that the Jun protein is involved in the control of GR transcriptional activity, we examined the pattern of Jun expression during retinal development. Protein extracts were prepared from retinal tissue at different developmental ages and analyzed by gel electrophoresis and Western blotting using the anti-Jun polyclonal antibody, Ab-1, as a probe. A protein band of ~40 kDa, which corresponds in size to the chicken c-Jun product (Okuno *et al.*, 1991), was detected in the retinal tissue at quantities that varied greatly with age: a high level was found in E6 and E8 retinas, and a lower level in older retinas (Figure 1A, lanes 1–5). A similar pattern of Jun expression was observed when the protein blot was reacted with the anti-c-Jun specific antibody, Ab-2. Expression of the 42–44 kDa human c-Jun protein could be detected in control cultures of HeLa cells that were induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Figure 1A, lanes 6 and 7). The relative levels of c-Jun expression at the different developmental ages were densitometrically determined and compared with previously established levels of GR transcriptional activity (Ben-Dror *et al.*, 1993), inducibility of glutamine synthetase (Vardimon

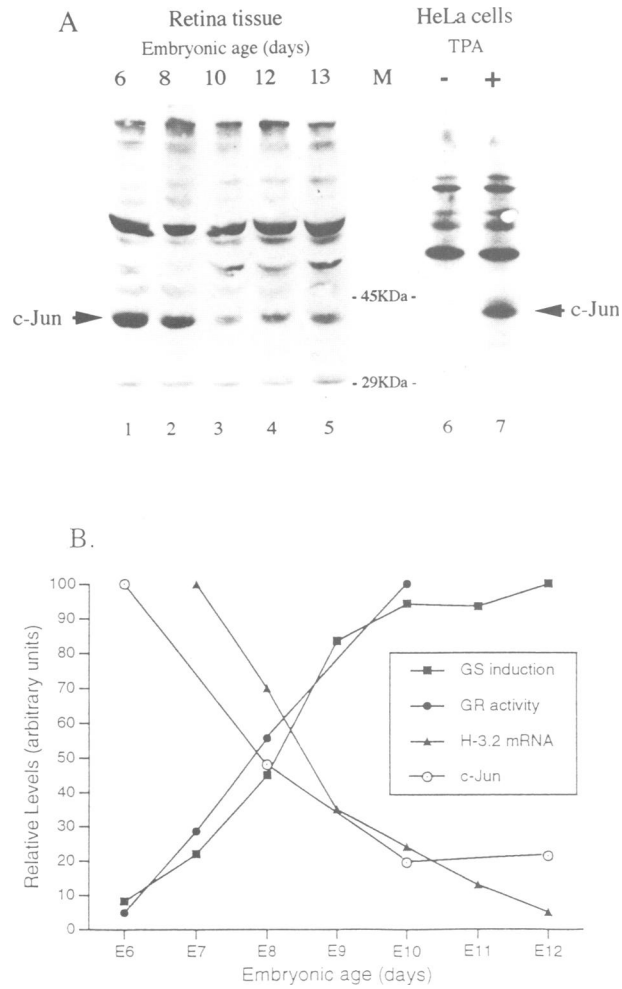

Fig. 1. Expression of c-Jun during retinal development. (**A**) Samples of cellular proteins (25 μg/lane) from retinal tissue on different days of development (lanes 1–5) and from HeLa cells, cultured for 2 h in the presence (lane 7) or absence (lane 6) of TPA, were fractionated by electrophoresis on a 10% SDS–polyacrylamide gel. The gel was electroblotted onto a nitrocellulose filter, which was reacted with the rabbit anti-Jun polyclonal antibody, Ab-1, and with horseradish peroxidase-conjugated second goat anti-rabbit antibody. Protein bands were visualized by the ECL procedure. M = size markers. (**B**) To quantitate the levels of c-Jun protein, the film was scanned by the LKB Ultrascan XL Enhanced Laser Densitometer. The highest level of c-Jun was given the arbitrary value of 100. Relative changes in accumulation of the replacement histone H3.2 mRNA (H3.2 mRNA) (Vardimon *et al.*, 1986b), inducibility of glutamine synthetase (GS induction) (Vardimon *et al.*, 1986a) and inducibility of the pΔG46TCO construct (GR activity) (Ben-Dror *et al.*, 1993) at the different developmental ages are presented.

et al., 1986a) and accumulation of the replication histone H3.2 mRNA (Vardimon *et al.*, 1986b) in the retinal tissue. The results shown in Figure 1B demonstrate that c-Jun expression is directly correlated with accumulation of the replication histone H3.2 mRNA, and inversely correlated with GR transcriptional activity and glutamine synthetase inducibility. Because accumulation of the replication histone H3.2 is directly proportional to the extent of retinal cell proliferation (Vardimon *et al.*, 1986b), these correlations suggest that at early embryonic ages proliferating retinal cells express a relatively high level of c-Jun and contain GR molecules that are incapable of activating gene transcription while at later ages, when retinal cell growth ceases, the

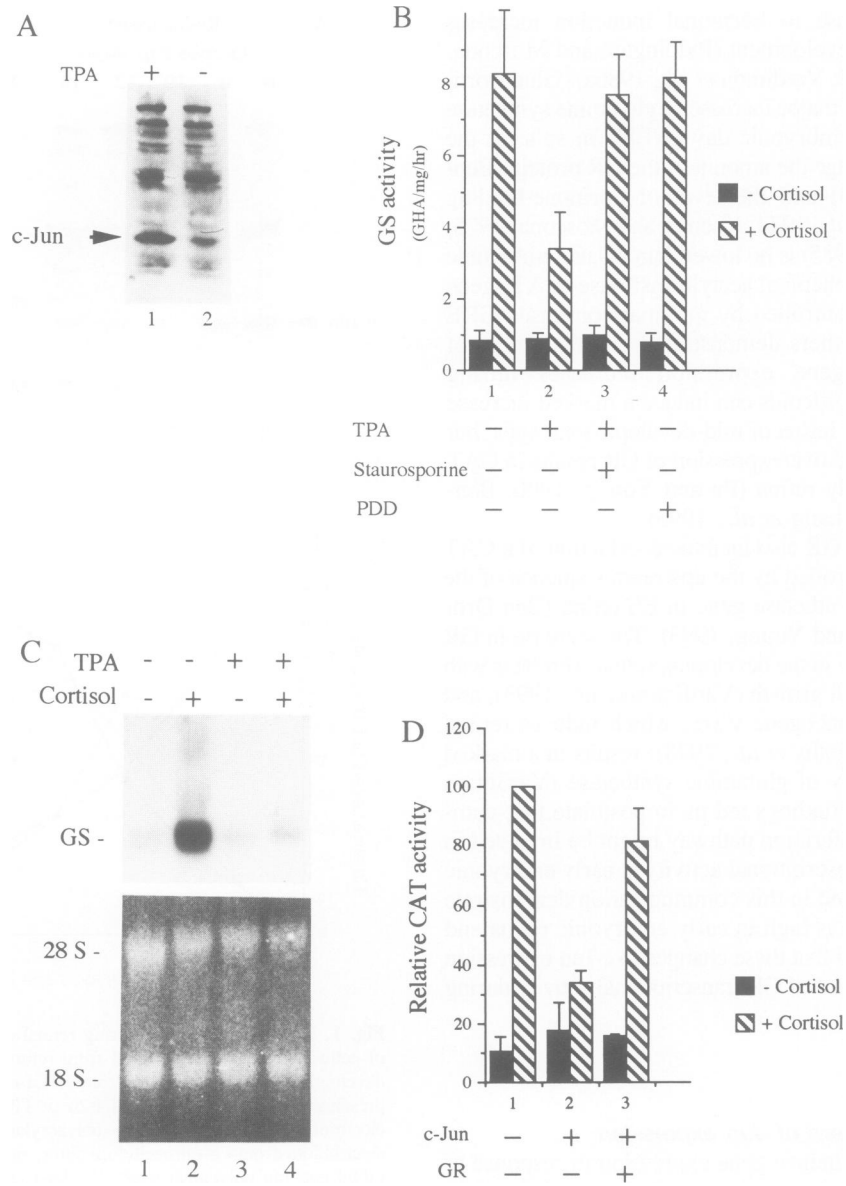

Fig. 2. Overexpression of c-Jun represses hormonal induction of glutamine synthetase. (**A**) c-Jun induction in retinal tissue: E13 retina was cultured for 2 h in the presence (lane 1) or absence (lane 2) of TPA. Cellular proteins were prepared and samples (25 μg/lane) were fractionated by electrophoresis on a 10% SDS–polyacrylamide gel. Electroblotting, reaction with antibodies and visualization of protein bands were as in Figure 1. Similar results were obtained with E11 and E12 retina. (**B**) Inhibition of glutamine synthetase (GS) expression by TPA: E11 retina was cultured for 24 h in the presence (striped bars) or absence (black bars) of cortisol. TPA (lane 2), TPA and staurosporine (lane 3) or PDD (lanes 4) were added to the medium. Glutamine synthetase activity was determined in tissue sonicates by the colorimetric assay (Linser and Moscona, 1979). The results shown represent the average values for three experiments. (**C**) Inhibition by TPA of hormonal induction of GS mRNA accumulation: E11 retina was cultured for 24 h in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of cortisol. TPA was added at the beginning of culture (lanes 3 and 4). Total RNA was prepared, size-fractionated (30 μg/lane) by electrophoresis on 0.8% agarose gel and stained by ethidium bromide (lower panel). Fractionated RNA was transferred to nitrocellulose filter, probed with the ^{32}P-labeled clone of the GS gene, pGS116–9, and visualized by autoradiography (upper panel). (**D**) Overexpression of c-Jun inhibits induction of the glutamine synthetase promoter: The CAT construct pGS2.1CAT, which is controlled by the glutamine synthetase promoter, was transfected into E11 retina (1.5 μg/8 × 10^6 cells) (lane 1) together with the c-Jun expression vector, RSV c-Jun (2 μg/8 × 10^6 cells) (lane 2) or with RSV c-Jun (2 μg/8 × 10^6 cells) and the GR expression vector p6RGR (2 μg/8 × 10^6 cells) (lane 3). The transfected tissues were cultured in the presence (striped bars) or absence (solid bars) of cortisol. CAT assay was adjusted to include an equal amount of luciferase activity, originating from co-transfected RSVL (SEL). The percentage of CAT conversion was calculated by scanning the TLC plates with the PhosphorImager™ V 5.25 (Molecular Dynamics). In each experiment the value of CAT conversion in the cortisol-treated control (lanes 1) was used to normalize all other results. The data shown are the means of three separate experiments.

expression of c-Jun declines and inducibility increases. The finding that c-Jun expression in the retinal tissue is inversely correlated with GR transcriptional activity raised the possibility that c-Jun is involved in inhibition of receptor activity at early embryonic ages.

Overexpression of c-Jun inhibits glutamine synthetase induction and suppresses GR transcriptional activity

A high level of c-Jun does not always result in inhibition of GR transcriptional activity (Shemshedini *et al.*, 1991; Ponta *et al.*, 1992). It was, therefore, important to determine

whether a high level of c-Jun can inhibit glutamine synthetase induction and receptor transcriptional activity in the embryonic retinal tissue. TPA treatment, which can elevate c-Jun expression in different cell systems, was found to induce c-Jun expression in the retinal tissue as well. Incubation of retinal tissues at mid-embryonic ages (E10−E13) for 2 h in the presence of TPA resulted in a marked increase in c-Jun expression (Figure 2A). TPA also caused a decline in hormonal induction of glutamine synthetase expression (Figure 2B): in the absence of TPA, cortisol induced an increase of ~8-fold in glutamine synthetase expression, while in its presence the increase was only 3-fold (Figure 2B, lanes 1 and 2). The effect of TPA on glutamine synthetase induction could be reversed by staurosporine, an inhibitor of protein kinase C (PKC) (Hidaka *et al.*, 1984) (Figure 2B, lane 3), while 4-α-phorbol-12-13-didecanoate (PDD), a phorbol ester that does not activate PKC and does not induce an increase in Jun expression, did not repress hormonal induction of glutamine synthetase (Figure 2B, lane 4). Repression of glutamine synthetase induction by TPA was due to inhibition of glutamine synthetase mRNA accumulation, as indicated by Northern blot analysis of RNA from TPA-treated and untreated retinal tissue (Figure 2C).

Because TPA induces both an increase in c-Jun expression and repression of glutamine synthetase induction, we considered the possibility that glutamine synthetase repression is mediated by c-Jun. This was examined by introduction of the c-Jun expression vector pRSVc-Jun (Ryseck *et al.*, 1988) into cells of the retinal tissue together with the CAT construct pGS2.1CAT, which is controlled by the upstream region of the glutamine synthetase gene [nucleotides (nt) +13 to −2121]. Inducibility of this construct in transfected retinal tissue is similar to that of the endogenous glutamine synthetase gene: it can be induced by glucocorticoids in retina of mid-developmental ages, but not in early retina (Zhang and Young, 1991, 1993; Ben-Dror *et al.*, 1993), 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. The constructs pRSVc-Jun and pGS2.1CAT were transfected by electroporation into E11 retina cells, and the tissue was cultured for 48 h in the presence or absence of cortisol and assayed for CAT activity. Transfection of pRSVc-Jun caused a 3-fold decline in CAT induction (Figure 2D, lanes 1 and 2). This decline could be prevented by co-transfection of the GR expression vector p6RGR (Figure 2D, lane 3). Thus, c-Jun can repress the hormone-dependent increase in transcription of a CAT construct that is controlled by the glutamine synthetase promoter, and overexpression of GR can reverse this effect. The glutamine synthetase promoter is rendered responsive to glucocorticoids by a single GRE that is juxtaposed to an AP-1/ATF/CRE-like site (Zhang and Young, 1991). This site could be a potential candidate for mediating c-Jun repression of pGS2.1CAT induction. However, deletion analysis of the AP-1/ATF/CRE-like site has indicated that this site is probably involved in enhancing the hormonal response, rather than repression (Zhang and Young, 1991, 1993). We therefore considered the possibility that the c-Jun protein inhibits the transcription activity of the GR protein directly, without binding to a specific regulatory site, and thus represses the inducibility of glutamine synthetase.

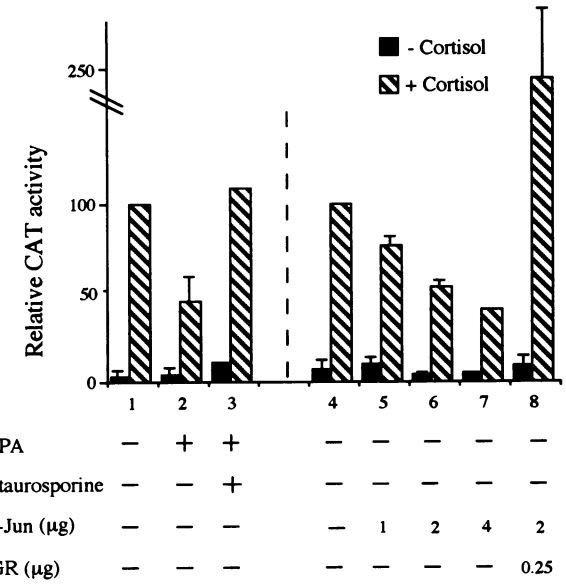

Fig. 3. GR transcription activity is repressed by TPA and by overexpression of c-Jun. The glucocorticoid-inducible CAT construct, pΔG46TCO, was transfected into E11 retina (1 μg/8 × 10^6 cells) by electroporation. Pieces of the transfected tissue were divided into Erlenmayer flasks and cultured in the presence of TPA (lane 2), TPA and staurosporine (lane 3) or in their absence (lane 1). Various amounts of the c-Jun expression vector pRSVc-jun were co-transfected into E11 retina without (lanes 5−7) or with (lane 8) the GR expression vector p6RGR. The cultures were either untreated (solid bars) or treated (striped bars) with cortisol. Numbers indicate the amount of co-transfected plasmid DNA in μg/8 × 10^6 cells. The percentage of CAT conversion was calculated as in Figure 2D. In each experiment the value of CAT conversion in the cortisol-treated control (lanes 1 and 4) was used to normalize all other results. The data shown (except for lanes 3 and 7, which present a single result) are the means of three separate experiments.

This was examined by using the CAT construct pΔG46TCO, which is under the transcriptional control of a minimal consensus GRE promoter and does not contain an AP-1 site (Sakai *et al.*, 1988; Ben-Dror *et al.*, 1993). The construct was transfected into E11 retina cells and the tissue was divided into several flasks and cultured for 24 h in the presence or absence of cortisol, TPA and staurosporine (Figure 3). As in the case of the endogenous glutamine synthetase gene, CAT induction was repressed by TPA treatment (Figure 3, lane 2), which caused a 2-fold decline in hormonal induction of CAT expression; with the addition of staurosporine, this decline was reversed (Figure 3, lane 3). A decline in CAT induction was also observed upon co-transfection of pRSVc-Jun. This decline was dose dependent (Figure 3, lanes 5−7) and could be reversed by introduction of the GR expression vector, p6RGR (Figure 3, lane 8). Taken together, our results clearly demonstrated that elevation of c-Jun expression in E11 retina can repress the ability of the endogenous GR molecules to induce gene expression and suggest that the high level of the endogenous c-Jun protein in early embryonic retina might be related to inhibition of GR activity.

Jun D antagonizes the action of c-Jun and facilitates CAT induction

Involvement of c-Jun in repression of GR transcriptional activity *in vivo*, in early embryonic retina, could best be

demonstrated if blocking of c-Jun relieves repression and facilitates hormonal induction of gene expression. Because members of the *jun/fos* gene family have been shown, under certain conditions, to form non-functional heterodimers (Chiu *et al.*, 1989; Kobierski *et al.*, 1991; Okuno *et al.*, 1991), we decided to examine whether c-Fos, Jun B or Jun D could be used as 'dominant negative repressors' with the ability to sequester the inhibitory activity of c-Jun and restore GR transcriptional activity. First we examined whether these proteins were able, by themselves, to inhibit GR activity when overexpressed in the retinal tissue. E11 retina was transfected with the glucocorticoid-inducible CAT construct, pΔG46TCO, together with the expression vectors pRSVc-fos (Sassone-Corsi *et al.*, 1988), pRSVjun B (Ryder *et al.*, 1988) or pRSVjun D (Hirai *et al.*, 1989), cultured for 24 h in the presence or absence of cortisol, and assayed for CAT activity. No significant decline in CAT induction was observed in the c-*fos*, *jun B* or *jun D* transfected tissues (Figure 4, lanes 3–5), in sharp contrast to the reduced level of CAT induction in the c-*jun* transfected tissue (Figure 4, lane 2). In fact, Jun D induced a small increase in CAT induction that might reflect, as explained below, an interplay between Jun D and the endogenous c-Jun.

Next we examined whether overexpression of either of these proteins can prevent the inhibitory effect of c-Jun. E11 retina was co-transfected with the glucocorticoid-inducible CAT construct, pΔG46TCO, with the c-Jun expression vector pRSVc-jun, and with pRSVc-fos, pRSVjun B or pRSVjun D. The transfected tissues were cultured in the presence or absence of cortisol and assayed for CAT activity. The results clearly demonstrated that while c-Jun-evoked repression of CAT activity was not affected by c-Fos (Figure 4, lane 7), and only slightly relieved by Jun B (Figure 4, lane 8), overexpression of Jun D resulted in a 3-fold increase in the inducibility of CAT (Figure 4, lane 9). In view of the negative results with c-Fos and Jun B, we examined whether these proteins were properly expressed in the transfected retina by using an AP-1-controlled CAT construct (Angel *et al.*, 1987). As in other cell systems (Chiu *et al.*, 1989; Hirai *et al.*, 1989; Doucas *et al.*, 1991; Tiliang and Karin, 1993), in E11 retina overexpression of c-Fos enhanced the c-Jun-evoked increase in CAT expression, while overexpression of Jun B repressed CAT expression (not shown). It should also be noted that overexpression of Jun D had no effect on the pRSVL(SEL) plasmid, which was co-transfected as an internal control (not shown). This makes it unlikely that the observed effect of Jun D is due to the titration of factors required by the Rous sarcoma virus (RSV) promoter present in the c-Jun expression vector. Because the pΔG46TCO construct does not contain an AP-1 site, repression of the inductive activity of GR by c-Jun and inhibition of this repression by Jun D probably occur by a mechanism that is independent of binding to specific DNA sites. It is possible that Jun D can sequester the inhibitory effect of c-Jun and thereby facilitate GR transcriptional activity.

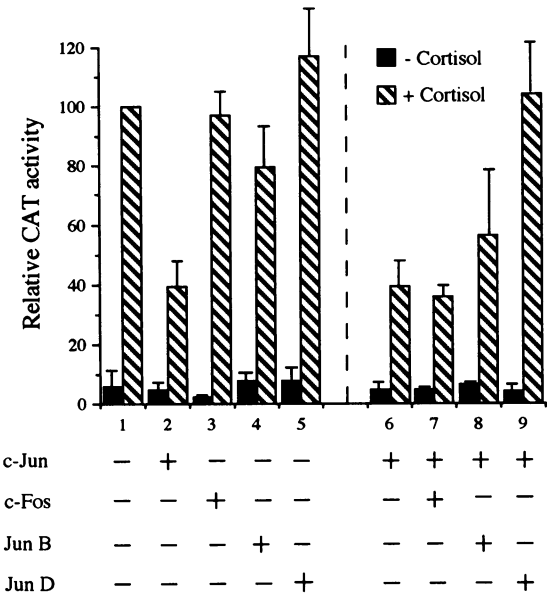

Fig. 4. Jun D relieves c-Jun-evoked repression of GR activity. The glucocorticoid-inducible CAT construct, pΔG46TCO, was transfected into E11 retina (1 μg/8 × 10^6) without (lanes 1, 3, 4 and 5) or with 2 μg/8 × 10^6 cells of pRSVc-jun (lanes 2 and 6–9) and together with 2 μg/8 × 10^6 cells of pRSVc-fos (lanes 3 and 7), pRSVjun B (lanes 4 and 8) or pRSVjun D (lanes 5 and 9). The transfected tissues were untreated (black bars) or treated (striped bars) with cortisol. The percentage of CAT conversion was calculated as in Figure 2D. In each experiment the value of CAT conversion in the cortisol-treated control (lane 1) was used to normalize all other results. CAT conversion values are the means of three separate experiments.

Gene induction in early embryonic retina

The ability of Jun D to antagonize the action of c-Jun in E11 retina offered a potential tool to examine the possible involvement of c-Jun in GR repression at early embryonic ages. If c-Jun is involved in inhibition of GR activity, then introduction of a large excess of Jun D into early retinal cells should result in an increased responsiveness to glucocorticoids. This prediction was examined by transfection of E6 retina with the pΔG46TCO construct, together with the Jun D expression vector, pRSVjun D. Control cultures of E6 retina were co-transfected with the pΔG46TCO construct and the pRSVc-fos or pRSVjun B construct. The transfected retinal tissues were cultured for 48 h with or without cortisol and assayed for CAT activity. Introduction of 2 μg pRSVJun D DNA/8 × 10^6 E6 retina cells, an amount found to abolish the effect of the overexpressed c-Jun in E11 retina (Figure 4, lane 9), caused only a minor increase in CAT induction in E6 retina (Figure 5A, lane 2). Doubling the amount of the transfected Jun D plasmid resulted in an increase of almost 5-fold in the inducibility of the CAT construct in E6 retina (Figure 5A, lane 3). The effect of Jun D could be completely reversed by overexpression of c-Jun (Figure 5A, lane 4). Transfection of pRSVjun B in the same increased amount caused a slight increase in CAT induction (Figure 5A, lane 5), while overexpression of c-Fos had no effect on the inducibility of the CAT construct (Figure 5A, lane 6). The finding that overexpression of Jun D facilitates GR activity in E6 retina suggests that c-Jun is indeed involved in inhibition of GR transcriptional activity at early embryonic ages.

In agreement with previous data (Ben-Dror *et al.*, 1993; Zhang and Young, 1993), we found that the glutamine synthetase promoter-controlled construct pGS2.1CAT, which is responsive to glucocorticoids in E10 (Figure 2D, lane 1), was not inducible in E6 retina (Figure 5A, lane 7), but that overexpression of GR rendered it inducible also at this early age (Figure 5A, lane 9). However, overexpression

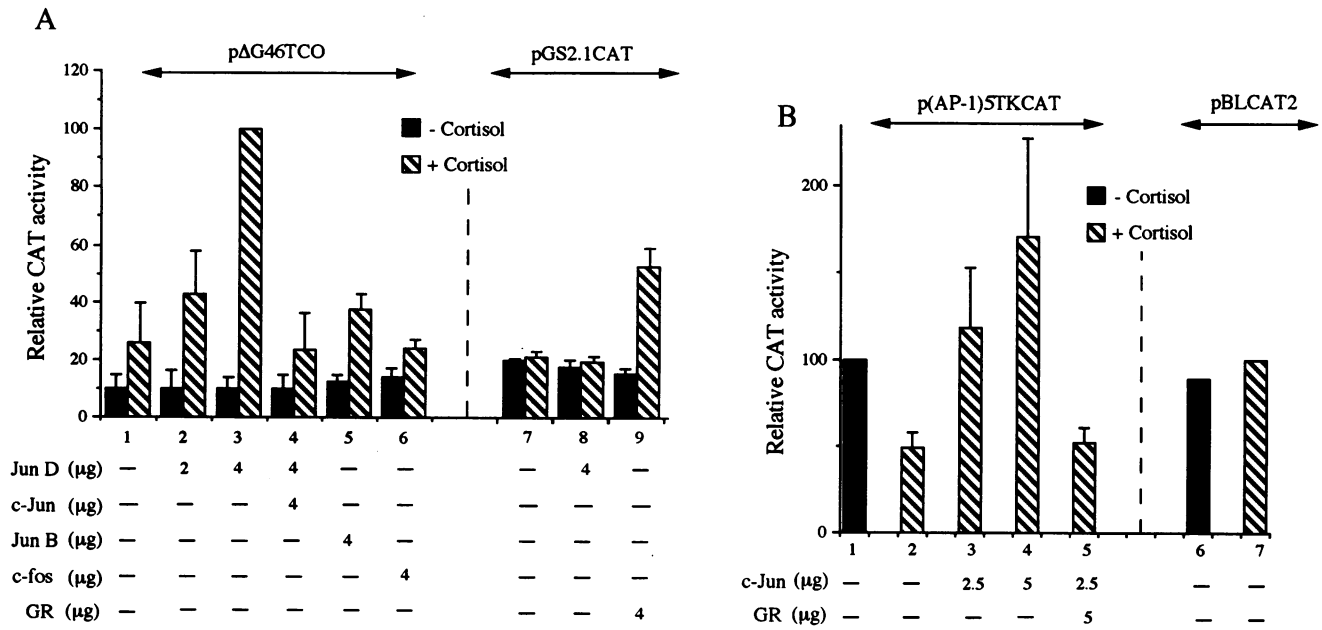

Fig. 5. Gene expression in E6 retina. (**A**) The CAT constructs pΔG46TCO or pGS2.1CAT were transfected into E6 retina (2.5 μg/8 × 10^6 cells) without (lanes 1 and 7) or with pRSVjun D (lanes 2, 3 and 8), pRSVjun D and pRSVc-jun (lane 4), pRSVjun B (lane 5), pRSVc-fos (lane 6) or p6RGR (lane 9). The transfected tissues were untreated (black bars) or treated (striped bars) with cortisol. Numbers indicate the amount of co-transfected plasmid DNA in μg/8 × 10^6 cells. The percentage of CAT conversion was determined as in Figure 2D and in each experiment the highest value of CAT conversion (which in all cases was the sample of lane 3) was used to normalize all other results. CAT activity values for lanes 1–6 are the means of four separate experiments and for lanes 7–9 are the means of two separate experiments. (**B**) The CAT constructs $p(AP\text{-}1)_5$TKCAT and pBLCAT2 were transfected into E6 retina (2.5 μg/8 × 10^6 cells) without (lanes 1, 2, 6 and 7) or with pRSVc-jun (lanes 3 and 4) or pRSVc-jun and p6RGR (lane 5). The transfected tissues were untreated (black bars) or treated (striped bars) with cortisol. Numbers indicate the amount of co-transfected plasmid DNA in μg/8 × 10^6 cells. The percentage of CAT conversion was determined as in Figure 2D, and in each experiment the value of CAT conversion in the samples of lanes 4 and 7 was used to normalize all other results. The data shown (except for lanes 6 and 7, which present a single result) are the means of four separate experiments.

of Jun D had no effect on either basal or glucocorticoid-inducible levels of pGS2.1CAT (Figure 5A, lane 8). This is in contrast to the 5-fold increase in induction of pΔG46TCO (Figure 5A, lane 3) that we observed under similar transfection conditions. Thus, Jun D-mediated relief of GR repression that can activate the pΔG46TCO construct in E6 retina is not sufficient to render the glutamine synthetase promoter responsive to glucocorticoids.

In reciprocal transfection experiments, we used an AP-1-controlled CAT construct to investigate whether endogenous GR molecules in E6 retina can repress c-Jun-mediated activation of gene expression. Addition of cortisol caused repression of CAT expression (Figure 5B, lanes 1 and 2) in $p(AP\text{-}1)_5$TKCAT transfected retina, but not in retina transfected with the control plasmid pBLCAT2, which contains the TK promoter without AP-1 (Figure 5B, lanes 6 and 7). Repression could be relieved by overexpression of c-Jun (Figure 5B, lanes 3 and 4), but not by over-expression of both c-Jun and GR (Figure 5B, lane 5). The ability of c-Jun and GR proteins to repress each other's activity in E6 retina suggests that mutual interference in E6 retina might be due to direct interactions between the two endogenous proteins.

Discussion

The results presented in this study suggest that the developmental control of tissue responsiveness to glucocorticoids involves an inhibitory interaction between c-Jun and GR. We demonstrated that in the retina c-Jun was expressed at a particularly high level at early embryonic ages when retinal cells are still proliferating and GR is transcriptionally inactive. With development, as cell proliferation declines and ceases and the inductive activity of GR becomes high, the level of c-Jun expression decreased (Figure 1B). By day 11 of development, the level of c-Jun was already low and glucocorticoids could induce a marked increase in expression of the transiently transfected CAT construct, pΔG46TCO, that is controlled by a minimal consensus GRE promoter. Elevation of c-Jun expression by TPA treatment or by introduction of a c-Jun expression vector resulted in a pronounced decline in CAT induction. The effect of c-Jun was dose dependent and could be reversed by overexpression of GR. Therefore, a high level of c-Jun expression in embryonic retinal tissue can repress the ability of the endogenous GR molecules to stimulate gene expression in response to glucocorticoids. The fact that the pΔG46TCO construct does not contain an AP-1 binding site suggests that in the cellular context of retinal cells c-Jun can render GR inactive by directly interacting with the GR protein in a mode previously described (Jonat *et al.*, 1990; Schüle *et al.*, 1990a; Yang-Yen *et al.*, 1990), or by interacting with a factor in the transcription machinery that is essential for hormonally stimulated transcription. Another possible mode for repression of GR activity involves downregulation of receptor expression. If c-Jun can, for example, inhibit the transcription of the GR gene, this would lead to a decline in receptor activity. In contrast to this possibility is our finding that TPA treatment, which induces a marked increase in c-Jun expression and a decline in GR transcriptional activity, does not cause a detectable change in the level of the GR protein (data not shown).

Overexpression of c-Jun in E11 retina could also repress the inducibility of both the endogenous glutamine synthetase gene and the transiently transfected pGS2.1CAT construct, that is controlled by the glutamine synthetase promoter. This repression might reflect the inhibitory effect of c-Jun on GR transcriptional activity. However, excess c-Jun might also prevent hormone-dependent activation of the glutamine synthetase promoter by binding to a putative repression site in the control region of the gene. This mode of inhibition has been described for several genes that contain overlapping binding sequences for both the steroid hormone receptors and the transcription factor AP-1 (Guertin *et al.*, 1988; Mordacq and Linzer, 1989; Diamond *et al.*, 1990; Schüle *et al.*, 1990b; Zhang *et al.*, 1991). The enhancer region of the glutamine synthetase gene does not appear to contain overlapping binding sequences for GR and AP-1, but does contain an AP-1/ATF/CRE-like site immediately upstream of the GRE. Deletion of this AP-1/ATF/CRE-like site or point mutations within it result in loss of inducibility, while overexpression of GR restores the responsiveness of the deleted promoter to glucocorticoids (Zhang and Young, 1991, 1993). It has therefore been suggested that this site binds to an essential ancillary transcription factor that acts co-operatively with the GR molecules to stimulate gene expression. Nuclear extracts obtained from retina of early or late embryonic ages have been found, by gel shift assays, to contain similar levels of proteins that interact with the AP-1/ATF/CRE-like site, suggesting that the developmental control of inducibility does not reflect the timed appearance of an ancillary factor (Zhang and Young, 1993). In contrast, Ben-Or and Okret (1993) demonstrated that a retinal C/EBP-like protein, which recognizes the AP-1/ATF/CRE-like site, is expressed in E12 retina at a higher level than in E7 retina and suggested that this protein might be involved in the temporal control of glutamine synthetase induction. Because the AP-1/ATF/CRE-like site appears to be involved in enhancement of the hormonal response, the c-Jun-mediated repression of GR activity might be the principal cause for loss of inducibility.

The inhibitory interaction between c-Jun and GR was found to be greatly reduced by overexpression of Jun D. Co-transfection of the Jun D expression vector into E11 retina could relieve c-Jun-evoked repression and restore the ability of GR to induce gene expression in response to glucocorticoids. In contrast, GR activity was not affected by overexpression of c-Fos and repression was only slightly relieved by overexpression of Jun B. The effect of Jun D on GR activity could best be explained if Jun D competitively inhibits GR−c-Jun complex formation by recruiting c-Jun molecules into a Jun D−c-Jun heterodimer that is incapable of repressing GR activity. Formation of non-functional heterodimers between products of members of the *jun/fos* gene family has been previously implicated in several other cellular effects (Chiu *et al.*, 1989; Schütte *et al.*, 1989; Kobierski *et al.*, 1991; Okuno *et al.*, 1991; Tiliang and Karin, 1993). Jun D has been characterized in man, mouse and chicken (Hirai *et al.*, 1989; Ryder *et al.*, 1989; Hartl *et al.*, 1991), and found to differ from c-Jun and Jun B in several respects. *jun D,* unlike c-*jun* and *jun B,* is not considered an 'immediate early gene' because it does not respond to growth stimuli with a sharp transient increase in transcription (Hirai *et al.*, 1989; Ryder *et al.*, 1989). When overexpressed, Jun D does not alter chicken fibroblast cell growth (Castellazzi *et al.*, 1991; Hartl *et al.*, 1991), while c-Jun and to a lesser extent Jun B can transform the cells (Bos *et al.*, 1990; Castellazzi *et al.*, 1990, 1991; Hirai *et al.*, 1990). In addition, Jun D does not affect estrogen-dependent transcriptional activation, although this activity is strongly suppressed by c-Jun and partially by Jun B (Doucas *et al.*, 1991), and while c-Jun and Jun B are potent repressors of muscle-specific gene transcription, Jun D has no effect on these proteins (Li *et al.*, 1990). The protein products of the three different *jun* genes are very similar in their C-terminal parts, but differ in their N-terminal regions. Definition of the structural requirements for the differential effects of c-Jun and Jun D on GR activity should shed light on the mechanistic basis for the interplay between these proteins in the embryonic retina.

We took advantage of the fact that Jun D can sequester the inhibitory activity of c-Jun to examine the possible involvement of c-Jun in GR repression at early ages. Overexpression of Jun D had a marked effect on GR activity in E6 retina. At this developmental age, the transcriptional activity of the GR molecules is greatly inhibited and the endogenous glutamine synthetase gene cannot be induced by glucocorticoids (Ben-Dror *et al.*, 1993; Zhang *et al.*, 1993). Responsiveness of E6 retina can be restored by overexpression of GR which facilitates the induction of CAT constructs that are controlled by minimal consensus GRE promoters or by the glutamine synthetase promoter (Ben-Dror *et al.*, 1993; Zhang *et al.*, 1993). Worth noting in this connection is a recent study by Ben-Or and Okret (1993) which, in apparent contrast to results obtained by others and by us (Ben-Dror *et al.*, 1993; Zhang *et al.*, 1993), suggests that GR activity in early embryonic retina is similar to that in retina of mid-developmental ages. In that study, however, GR activity was examined in separated E7 and E10 retinal cells that were aggregated and maintained in culture for 48 h. Because transition from the non-responsive to the glucocorticoid-inducible state occurs between day 7 and day 9 of retinal development (Ben-Dror *et al.*, 1993; Zhang and Young, 1993), and since inducibility is ultimately dependent on appropriate contact interactions between retinal cells (Linser and Moscona, 1979; Vardimon *et al.*, 1988), under these experimental conditions there is no major difference in the inducibility of the endogenous glutamine synthetase gene (Vardimon *et al.*, 1988; S.Reisfeld and L.Vardimon, unpublished data). It is therefore not surprising to find no great difference in GR activity between E7 and E10 retinal cell aggregates, even though in the intact E10 retina both GR activity and glutamine synthetase inducibility are many times higher than in the intact E7 retina (Figure 1B and Vardimon *et al.*, 1986a; Ben-Dror *et al.*, 1993; Zhang and Young, 1993).

Transfection of Jun D into E6 retina caused an increase of almost 5-fold in hormone-dependent expression of the glucocorticoid-inducible CAT construct, pΔG46TCO. The effect of Jun D was dose dependent and could be reversed by overexpression of c-Jun. As in E11 retina, overexpression of c-Fos did not affect induction of CAT expression, while Jun B caused a small increase in inducibility. The dramatic effect of Jun D on GR activity in E6 retina resembles its effect on GR activity in c-Jun-transfected E11 retina and suggests that the high level of c-Jun in early embryonic ages might be at least partly responsible for GR repression. As postulated above, Jun D might titrate the inhibitory activity

of the endogenous c-Jun by forming non-functional heterodimers with the c-Jun molecules and thus prevent the inhibitory interaction between GR and c-Jun. Although GR and c-Jun could reciprocally inhibit each other's transcriptional activity (Figure 5B), further studies are required in order to determine whether the c-Jun molecules form a direct physical association with the GR molecules in early embryonic retina. The differential effects of Jun D, Jun B and c-Fos on c-Jun-mediated repression of GR activity might reflect a difference in the functional activity and/or stability of the heterodimers formed with c-Jun. It would be of interest to determine if Jun D has a physiological role in modulating c-Jun activity in growing cells and during development.

While overexpression of Jun D facilitated a marked increase in CAT induction in the pΔG46TCO transfected E6 retina, no increase was observed in the pGS2.1CAT transfected tissue. Overexpression of Jun D might yield a sufficiently high level of active GR molecules for stimulation of the pΔG46TCO construct, which contains two GREs immediately upstream of the TK promoter, but not for activation of the glutamine synthetase promoter which contains a single GRE, ~2 kb upstream of the initiation site. Different promoters might require different thresholds of GR for hormonal induction of gene activity (Zhang and Young, 1993). Indeed, overexpression of GR, by transfection of the GR expression vector p6RGR, facilitated induction of the pGS2.1CAT construct in E6 retina. This construct can also be rendered inducible in early retina tissue by increasing the activity of protein kinase A (PKA) (Zhang *et al.*, 1993). PKA can enhance the ability of the GR molecules to induce gene expression, but it does not appear to play a role in the developmental control of GR activity; unlike c-Jun, its expression does not alter with age (Zhang *et al.*, 1993). Nevertheless, the control of GR activity might not be restricted to the inhibitory interaction with c-Jun, but might include other mechanisms, such as programmed changes in the amount of the receptor molecules. Preliminary results suggest that although the total amount of GR molecules in the retina tissue does not alter greatly with age, the amount of receptor molecules per cell might change: at early embryonic ages GR may be present in all cells at a relatively low level, while at later ages GR expression may decline in the differentiating neurons, but increase in Müller glia cells (where glutamine synthetase is expressed). This possibility is consistent with the finding that the level of GR in glia-depleted retina is many times lower than that in the intact retinal tissue (Grossman *et al.*, 1994). A relatively low level of receptor expression at early embryonic ages, in conjunction with a high level of c-Jun expression, might prevent responsiveness of proliferating early retina cells to glucocorticoids.

Materials and methods

Plasmids

The plasmid pΔG46TCO (Ben-Dror *et al.*, 1993) was derived from pG46TCO (Sakai *et al.*, 1988) and is a pUC vector containing two copies of a synthetic GRE sequence linked to the herpes thymidine kinase (TK) promoter−CAT fusion gene. A potent AP-1 site in the pUC backbone was deleted in the pΔG46TCO construct (Ben-Dror *et al.*, 1993). The plasmid pG46TCO was kindly provided by Dr K.R.Yamamoto, University of California, San Francisco. The RSVL(SEL) construct, which contains the luciferase reporter gene under the transcriptional control of the RSV promoter (de Wet *et al.*, 1987), was kindly provided by Dr S.Subramani, University of California, San Diego. Plasmid pGS116-9 is a subclone of the chicken GS gene (Vardimon *et al.*, 1988), and the plasmid pGS2.1CAT contains the upstream region of the glutamine synthetase gene (nt +13 to −2121) attached to the CAT reporter gene (Ben-Dror *et al.*, 1993). The construct $p(AP\text{-}1)_5$TKCAT contains five copies of a synthetic AP-1 site [TPA response element (TRE) binding site] upstream of the TK promoter and the CAT gene (Angel *et al.*, 1978), while pBLCAT2 contains the TK promoter attached to the CAT gene (Luckow and Schütz, 1987). The plasmids pRSVc-jun (Ryseck *et al.*, 1988), pRSVjun B (Ryder *et al.*, 1988), pRSVjun D (Hirai *et al.*, 1989) and pRSVc-fos (Sassone-Corsi *et al.*, 1988) were kindly provided by Dr M.Yaniv, Institut Pasteur, Paris. The clone p6RGR, which contains the rat GR cDNA under the transcriptional control of the RSV promoter, was kindly provided by Dr K.R.Yamamoto, University of California, San Francisco. Plasmid DNA was prepared by use of the QIAGEN plasmid preparation kit (Qiagen).

Culture methods and glutamine synthetase induction

Neural retinal tissue was isolated under sterile conditions from eyes of chicken embryos (White Leghorn) at day 6 (E6) or day 11 (E11) of development. The retina was cut into small pieces and placed in 50 ml Erlenmeyer flasks in medium 199 with 10% fetal bovine serum on a gyratory shaker (65 r.p.m.) at 38°C. Glutamine synthetase was induced with cortisol (0.33 μg/ml) (Sigma) added at the beginning of culture time. The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (0.1 μg/ml) (Sigma) or PDD (0.1 μg/ml) (Sigma) was added at the beginning of culture with or without cortisol. Staurosporine (25 nM) (Sigma) was added 30 min before the addition of TPA and cortisol. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Prior to TPA treatment, cells were incubated in DMEM containing 0.5% fetal calf serum for 24 h and then treated with TPA (0.1 μg/ml) for 2 h and harvested.

Transfection procedure

Transfection was carried out as described previously (Ben-Dror *et al.*, 1993). Neural retinal tissue was isolated and organ cultured for 4 h, as described above. Plasmid DNA was transfected into pieces of intact retinal tissue by electroporation using a Bio-Rad Gene Pulser with voltage and capacitance settings of 400 V and 960 μF, as described previously (Pu and Young, 1990). In all cases, electroporation was performed in cuvettes containing 1 ml phosphate-buffered saline (PBS) (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM $Na_2HPO_4 \cdot 7H_2O$) and a total of 1−10 μg DNA of indicated plasmids/8 × 10^6 cells. Transfection efficiencies were controlled by the co-transfection of RSVL(SEL) (1 μg/8 × 10^6 cells) (de Wet *et al.*, 1987). To account for possible non-specific effects of variable amounts of DNA in the transfection assays, the amounts of DNA in each set of transfection experiments were made equivalent by addition of vector DNA. Following electroporation, retinas were maintained in PBS buffer for 10 min in ice. Retinas, transfected in the same cuvette, were divided into Erlenmeyer flasks and cultured for another 24 or 48 h in the presence or absence of the indicated drugs under the conditions employed prior to transfection. Fresh medium was substituted after 24 h.

CAT luciferase and glutamine synthetase assays

CAT luciferase and glutamine synthetase activities were determined in tissue sonicates. CAT activity was determined as described by Gorman *et al.* (1982). Samples were heated for 10 min at 65°C prior to analysis. CAT activity in E6 or E11 retina was determined with 10^3 or 2 × 10^3 pmol ^{14}C-labeled chloramphenicol per assay, respectively. In all experiments the CAT assay was adjusted to include an equal amount of luciferase activity, originating from co-transfected RSVL(SEL). The percentage of CAT conversion was calculated by scanning of the TLC plates with the PhosphorImager™ V 5.25 (Molecular Dynamics). Luciferase activity was assayed as described previously (de Wet *et al.*, 1987) and recorded by an LKB luminometer. The specific activity of glutamine synthetase was determined by the colorimetric assay (Linser and Moscona, 1979) and expressed as μM γ-glutamylhydroxamate (GHA)/h/mg protein.

RNA preparation and analysis

Cellular RNA was prepared by use of the RNAzolTM₁ B RNA isolation solvent (Biotecx Laboratories Inc., Houston, TX). For Northern blot analysis, RNA was denatured by heating at 60°C for 15 min in 2.2 M formaldehyde/50% formamide, and was fractionated by electrophoresis in 0.8% agarose gels containing 2.2 M formaldehyde and MOPS buffer. The fractionated RNA was transferred to nitrocellulose filter and hybridized with pGS116-9 DNA (Vardimon *et al.*, 1988) labeled with ^{32}P by nick translation. The levels of hybridization were visualized by autoradiography.

Protein preparation and immunoblotting

Retinal tissue or HeLa cells were washed in CMF buffer (137 mM NaCl/2.7 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4/5.5 mM glucose). Tissue or a cell pellet was resuspended in RIPA buffer [150 mM NaCl/1% Triton

X-100/0.5% deoxycholate/0.1% sodium dodecyl sulfate (SDS)/50 mM Tris (pH 8)/5 mM disodium ethylenediaminetetraacetate (EDTA)/5 μg/ml leupeptin/5 μg/ml aprotonin/2 μg/ml antipaine/2 μg/ml chemostatin and 0.25 mM phenylmethyl sulfonyl fluoride (PMSF)] on ice and homogenized with 10 strokes of pestle A and 10 strokes of pestle B in a Dounce homogenizer. Cell lysates were clarified by centrifugation at 90 000 *g* for 60 min at 4°C. Protein concentration was determined by the Bio-Rad protein assay. Equal amounts of protein (25 μg/lane) in sample buffer [60 mM Tris (pH 6.8)/5% glycerol/1% SDS/0.2% bromophenol blue/140 mM β-mercaptoethanol] were separated on 10% SDS–polyacrylamide gels and electroblotted onto nitrocellulose filters in Tris–glycine buffer [48 mM Tris (pH 8.5)/39 mM glycine/0.037% SDS/20% methanol]. To identify the Jun protein, the filters were incubated for 1 h in a blocking solution of 3% milk and 0.01% Thimerosal (Sigma) in TBS buffer [10 mM Tris (pH 8)/150 mM NaCl]. The filters were then reacted with rabbit polyclonal anti-Jun antibody (Ab-1) or anti-c-Jun antibody (Ab-2) (Oncogene Science) in TBST buffer [10 mM Tris (pH 8)/150 mM NaCl/0.025% Tween 20] and subsequently with horseradish peroxidase-conjugated second goat anti-rabbit antibody (Cappel), and visualized by the enhanced chemiluminescence (ECL) procedure (Amersham). Autoradiograms were densitometrically scanned using the LKB Ultrascan XL Enhanced Laser Densitometer.

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