

# Regulation of lens cell growth and polarity by an embryo-specific growth factor and by inhibitors of lens cell proliferation and differentiation

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## SUMMARY

We used a double-label method, which monitors the rate at which cells enter S-phase of the cell cycle, to identify factors that control the growth of chicken embryo lens epithelial cells *in vivo*. With this assay, we identified a mitogen for lens epithelial cells in the anterior segment of the embryonic eye. When the anterior chamber was opened briefly, by tearing the cornea or displacing the lens, the growth-promoting activity was lost. None of the purified growth factors tested replaced this growth activity, including EGF, bFGF, PDGF, IGF-1, IGF-2, TGF $\beta$  and mixtures of these factors. However, chicken embryo serum or plasma did cause chicken embryo lens epithelial cells to progress through the cell cycle. The activity in serum was destroyed by heat and protease treatment. It was most active in serum from 10-day embryos, decreased with subsequent development and was undetectable from 2 days after hatching through adulthood. When embryo serum or plasma was mixed with vitreous humor or IGF-1, agents that induce lens

fiber cell formation, cell elongation was prevented. In contrast to the mitogenic activity in serum, this inhibitor of differentiation was insensitive to trypsin treatment. We also identified an activity in vitreous humor that inhibited the growth-promoting agent in embryo serum.

Plasma proteins readily enter the anterior chamber of the eye of chicken embryos. Therefore, our data imply that an activity in serum enters the anterior chamber of the embryonic eye and controls lens cell division. Furthermore, reciprocal gradients of factors from the vitreous body and the anterior chamber appear to specify lens polarity and assure normal lens morphogenesis. The activities in serum assure that lens epithelial cells divide, but do not differentiate into lens fibers. In a similar manner, factors in vitreous humor specify that cells in the posterior of the lens differentiate into fibers, but do not divide.

Key words: lens, cell growth, growth factor, chick, cell cycle

## INTRODUCTION

The vertebrate lens is made up of two populations of cells. The anterior surface is covered by a simple cuboidal epithelium, while the bulk of the lens is composed of elongated lens fibers. During lens growth, the most peripheral epithelial cells differentiate into fiber cells near the lens equator. Fiber cell differentiation is characterized by cell elongation, the synthesis and accumulation of large amounts of cytoplasmic proteins (the lens crystallins), the cessation of DNA synthesis and cell division, and the eventual degradation of most membrane-bound organelles, including the nucleus (Kuwabara, 1975; Piatigorsky, 1981).

The lens reversal experiments of Coulombre and Coulombre (1963) showed that a factor in the posterior portion of the eye could trigger chicken embryo lens epithelial cells to form lens fibers *in vivo*. Beebe et al. (1980) later identified a factor in chicken embryo vitreous humor which stimulated fiber formation *in vitro*. This factor, termed lentropin, appeared to be similar or identical to

insulin-like growth factor-1 (IGF-1; Beebe et al., 1987). Specific IGF-1 receptors were subsequently identified, localized and quantified on chicken embryo lens cells (Bassett et al., 1987; Bassett and Beebe, 1990). Other studies have shown that both basic and acidic fibroblast growth factor can stimulate the formation of fiber-like cells in rat lens epithelial cells (Chamberlain and McAvoy, 1987, 1989).

Although information has begun to accumulate concerning the control of lens fiber cell differentiation, the factor(s) responsible for the continued replication of lens epithelial cells in embryos and adults remains to be identified. A number of investigators (Modak et al., 1967; Modak and Perdue, 1970; Zwann and Kenyon, 1984) have determined that, as the peripheral lens epithelial cells duplicate their DNA, they undergo a final division and are displaced toward the lens equator. However, most of the epithelial cells in the adult lens are not dividing. A few studies have shown that known growth factors can cause quiescent epithelial cells from the center of the newborn or adult lens epithelium to re-enter the cell cycle (Rothstein et al., 1980;

Reddan and Dziedzic, 1982; Reddan et al., 1982; Reddan and Wilson-Dziedzic, 1983; Arruti et al., 1985; McAvoy and Chamberlain 1989). It is not known whether any of these agents are lens mitogens in the embryo.

To investigate the relationship between DNA synthesis and lens fiber cell differentiation, we developed and tested a double-label technique that measures the rate at which cells progress from G<sub>1</sub> into S-phase of the cell cycle (Hyatt and Beebe, 1992). In the current study, we used this technique to identify a mitogen for lens epithelial cells in the anterior segment of the eye and in serum and plasma from chicken embryos. Similar activity was not present in serum from young or adult chickens and could not be replaced by any of several defined growth factors, mixtures of these factors or extracts of tissues known to be abundant sources for growth factors. In addition, we identified an activity in serum that antagonized the differentiation-promoting effects of vitreous humor and an activity in vitreous humor that blocked the mitogenic activity in embryonic serum. We suggest that these inhibitory factors establish reciprocal gradients which, in concert with lentropin and the lens mitogen(s), establish and maintain the polarity of the lens.

## MATERIALS AND METHODS

### Explantation and culture of lens epithelia

Fertile White Leghorn chicken eggs were obtained from Truslow Farms, Chestertown, MD. Lenses were removed from 6-day-old embryos (E6) and the lens epithelium was separated from the fiber mass by microdissection. A square explant containing approximately  $2 \times 10^4$  cells was cut from the central region of the lens epithelium and simultaneously attached to the bottom of a 35 mm Petri dish, as previously described (Beebe and Feagans, 1981). For each datum point, lens explants were dissected from 6-8 embryos.

Epithelia were exposed to growth factors or other supplements dissolved in basal medium (Ham's F-10, GIBCO, Grand Island, NY) at the concentrations indicated in each experiment. Human recombinant PDGF and purified porcine TGF (R&D Systems, Minneapolis, MN), human recombinant EGF (Imcera Bioproducts, Inc., Terre Haute, IN), human recombinant basic FGF (generously donated by Chiron Corp., Emeryville, CA) and human recombinant IGF-2 (Bachem Bioscience, Inc., Philadelphia, PA) were reconstituted and stored following the manufacturers' guidelines. The bovine pituitary extract, P-Neurex, came from Upstate Biotechnology, Inc., Lake Placid, NY. All other reagents were purchased from Sigma Chemical Co., St Louis, MO. Embryonic chicken serum was obtained by drawing whole blood from the vitelline artery through glass micropipettes. Adult chicken serum was prepared from blood collected by syringe from the jugular veins of adult male chickens. Whole blood was allowed to clot for two hours at room temperature, then centrifuged for 20 minutes at 3000 g. Plasma was prepared from E15 embryos by drawing blood into heparinized capillary tubes. Cells were pelleted by centrifugation as above. Serum and plasma were stored at  $-20^\circ\text{C}$ . Vitreous humor was prepared from E15 chicken embryos according to methods previously described (Beebe and Feagans, 1981).

Anterior half eyes were prepared by cutting around the circumference of E6 eyes at the equator of the eye cup with iris scissors. Anterior half eyes were then separated from the vitreous body and the posterior of the eye using no. 5 forceps. Anterior eye segments were cultured as described after two washes, each in 3 ml of basal medium. Lens explants and other tissues were

incubated in a total of 2 ml of medium for the times indicated at  $37^\circ\text{C}$  in 95% air/5% CO<sub>2</sub>.

### Labeling and detection procedures

Validation of the methods for double-labeling lens cells is presented in Hyatt and Beebe (1992). This approach uses a short pulse of [<sup>3</sup>H]thymidine to label all cells in S-phase at the beginning of the labeling protocol. This is followed by a longer incubation in BrdU, a thymidine analog. During this period, cells that enter S-phase will be singly labeled with BrdU, cells remaining in S-phase will be doubly labeled and cells that have departed S-phase for G<sub>2</sub> will be singly labeled with [<sup>3</sup>H]thymidine. This is a sensitive method to measure the rate at which cells are entering the S-phase over a short period. It is particularly suited to detecting changes in the rate at which cells enter S-phase after treatments that alter cell cycle time or the percentage of cycling cells.

### Serum treatments

Aliquots of 20% embryonic serum in Ham's F-10 medium were incubated at  $65^\circ\text{C}$  for 60 minutes, while control serum was incubated on ice for the same time interval. Trypsin treatment was carried out following the procedures of Smith and McLachlan (1990). Trypsin was added to embryonic serum to a concentration of 100  $\mu\text{g/ml}$  and incubated at  $37^\circ\text{C}$  for 2 hours. Soybean trypsin inhibitor (Sigma) was then added to 100  $\mu\text{g/ml}$  and the mixture incubated for an additional 30 minutes at room temperature. For controls, trypsin was preincubated with soybean trypsin inhibitor in basal medium for 30 minutes before addition to test serum, or trypsin inhibitor alone was added to serum for 30 minutes after preincubation of serum for 2 hours at  $37^\circ\text{C}$ . Treated serum was diluted to a final concentration of 20% (v/v) in basal medium prior to use.

### Measurement of cell length

Cell length was measured with a Zeiss inverted microscope equipped with differential interference contrast optics by focusing on the upper and lower surfaces of the lens epithelial monolayer. The distance between these focal planes was determined with a micrometer built into the focusing mechanism (Baltimore Instrument Co.; Beebe and Feagans, 1981).

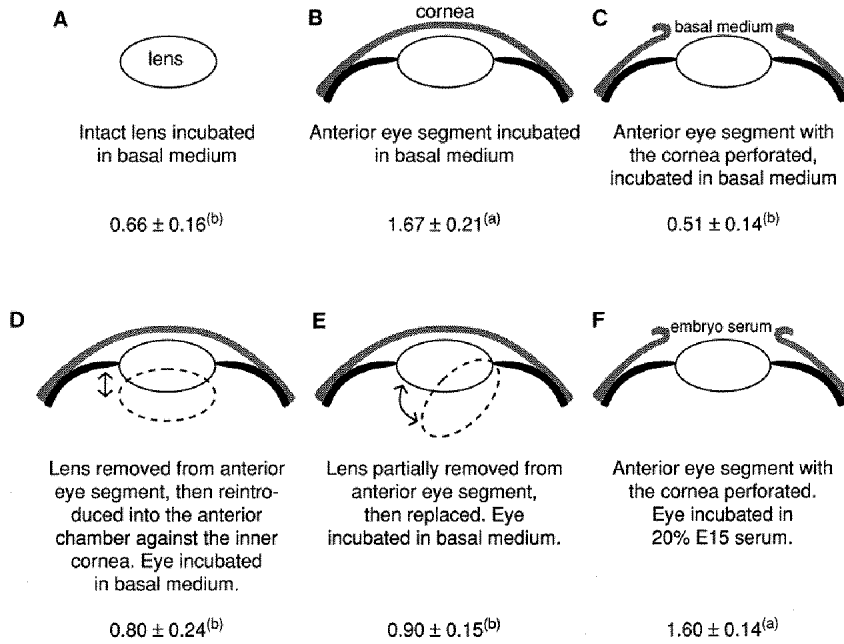
### Statistical methods

Unpaired one-tailed *t*-tests were utilized to compare labeling values between treatment groups. Treatment groups were considered to differ significantly if  $P < 0.05$ .

## RESULTS

### Evidence for a mitogen in the anterior segment of the embryonic eye

When chicken embryo lens epithelia were removed from the eye, their cells rapidly decreased the rate at which they entered S-phase of the cell cycle (Hyatt and Beebe, 1992). This suggested that, while they were in the eye, these cells were under the influence of an endogenous mitogen. To test this possibility, we cultured whole anterior eye segments from 6-day-old embryos in unsupplemented Ham's F-10 medium. Anterior eye segments consisted of the lens, cornea and prospective ciliary epithelium-iris complex. After 3 hours of culture under these conditions, the rate at which lens epithelial cells initiated DNA synthesis was similar to that of lenses that were freshly removed from the eye (Fig. 1B; Hyatt and Beebe, 1992). If the lens was cul-



**Fig. 1.** Evidence for growth-promoting activity in the anterior chamber of the embryonic eye. Anterior half-eyes or isolated lenses were cultured in basal medium or medium supplemented with 20% E15 serum for 3 hours. In some experiments, the anterior chamber was opened to the medium by tearing the cornea or by briefly removing or displacing the lens. After incubation, central lens epithelia were explanted and double labeled. Indicated values are the percentage of single-labeled nuclei from the central lens epithelium representing the mean  $\pm$  s.e.m. of three experiments. <sup>(a)</sup>Significantly different from lens incubated in basal medium ( $P < 0.05$ ). <sup>(b)</sup>Significantly different from intact anterior eye segment ( $P < 0.05$ ).

tured by itself, the lens epithelial cells reduced their rate of entry into S-phase nearly three-fold (Fig. 1A).

In some of the anterior half eyes, the cornea was perforated at the beginning of the incubation period, exposing the lens epithelium to the culture medium. In these cases, labeling decreased, approximating the levels seen in isolated lenses (Fig. 1C).

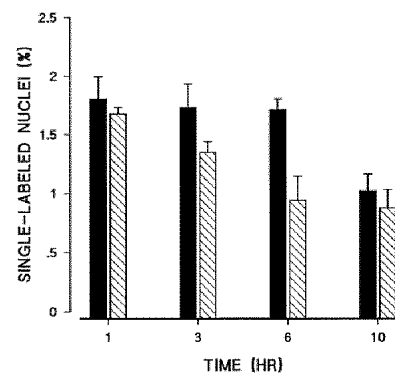
Additional studies tested whether the loss of proliferative activity in this experiment was due to the destruction of the corneal tissue overlying the lens epithelium or to the dilution of a soluble, growth-promoting activity normally present in the anterior chamber of the eye. Two dissection techniques were used to replace the fluids in the anterior chamber, while causing minimal damage to the cornea. In the first, lenses were removed from the anterior half eye by grasping the fragments of the vitreous body adhering to posterior lens capsule. Lenses were then replaced in the anterior segment in close proximity to the inner surface of the cornea (Fig. 1D). In the second experiment, lenses were separated from the ciliary epithelium over approximately half their circumference. This opened the anterior chamber to the surrounding culture medium. The lenses were then returned to their original position (Fig. 1E). This second dissection procedure maintained the shape of the anterior segment and the proximity of the lens to the cornea better than the first. After three hours of incubation, lenses were removed from the anterior eye segments and epithelial explants were prepared and double labeled. After both procedures, the rate at which lens cells entered S-phase was not significantly different from isolated lenses cultured in basal medium (Fig. 1A,D,E). The results of these experiments suggested that a soluble activity was present in the anterior chamber of the embryonic eye.

#### Evidence for a lens mitogen in chicken embryo serum

Beebe et al. (1986) showed that plasma proteins entered the

anterior segment of the eye throughout the first two-thirds of embryonic development. Therefore, the lens cell mitogen(s), identified above, might have been derived from embryo serum. To investigate this possibility, serum from chicken embryos and adults was assayed for its ability to sustain cell division in cultured chicken embryo lens epithelia. Serum from E15 chicken embryos diluted to 20% (v/v) in Ham's F-10 medium had mitogenic activity comparable to that found in the anterior segment of the eye (1.42% single-labeled cells after 3 hours). No mitogenic activity was detected in serum from adult chickens. When anterior segments with the cornea perforated to release aqueous humor were cultured in 20% embryo serum, the percentage of single-labeled nuclei was similar to the values obtained from undamaged anterior segments (Fig. 1E).

Fig. 2 compares the rates at which lens epithelial cells entered S-phase when cultured for increasing times as part



**Fig. 2.** Percentage of single-labeled nuclei in the central epithelia of day-6 embryos from lenses incubated within anterior eye segments (solid bars), or from explants cultured in E15 serum (diagonal hatched bars) for different time periods. Values represent means  $\pm$  s.e.m. of triplicate experiments.

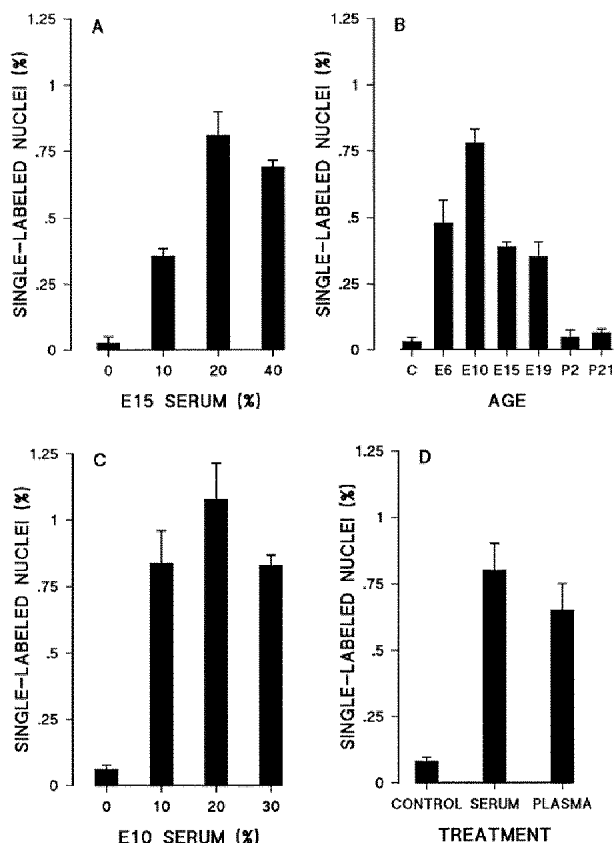
of anterior eye segments in Ham's F-10 medium, or as isolated central lens epithelia in 20% E15 serum. In both conditions, comparable levels of DNA synthesis were maintained in the lens. In comparison, when lens explants were cultured for 10 hours in vitreous humor, which promotes lens fiber differentiation, or in basal medium, single-labeled nuclei were nearly undetectable (Hyatt and Beebe, 1992).

Previous studies showed that lens epithelia cultured in another embryo serum, fetal bovine serum (FBS), elongated, accumulated large amounts of lens crystallin mRNAs and increased the rate of synthesis of crystallin proteins (Philpott and Coulombre, 1965; Milstone and Piatigorsky, 1975; Milstone et al., 1976). These are characteristics of lens fiber cell differentiation. However, E6 lens epithelial cells did not elongate when cultured for 5 hours in medium supplemented with 20% chicken embryo serum. These cells averaged  $10.7 \pm 0.4 \mu\text{m}$  in length after culture for 5 hours. This was similar to the length of cells in freshly isolated epithelia ( $10.2 \pm 0.4 \mu\text{m}$ ) or in epithelia cultured in basal medium for 5 hours ( $10.1 \pm 0.4 \mu\text{m}$ ). However, lens epithelia treated with either vitreous humor or IGF-2 did elongate in these experiments ( $18.8 \pm 1.2 \mu\text{m}$  and  $16.3 \pm 0.9 \mu\text{m}$ , respectively). These findings are consistent with a previous report (Beebe et al., 1987), which also showed that E15 serum did not stimulate lens fiber formation.

We then determined the optimal concentration of E15 serum needed to sustain maximal entry into S-phase in explanted lens epithelia. Both 10% and 40% E15 serum in Ham's F-10 medium were less effective than 20% serum after 6 hours of culture (Fig. 3A).

DNA synthetic activity decreases steadily in the central region of the lens epithelium during embryonic development (Persons and Modak, 1970). To see whether the mitogenic activity of the serum correlated with this decline in lens cell division, we tested serum from embryos of different ages and from hatched chicks. Since E15 serum had maximal activity at 20% and lower activity at 10% (v/v), we chose 10% as a test concentration. Fig. 3B shows that maximal activity was present in serum from embryos at 10 days of embryonic development. After hatching, serum had no detectable mitogenic activity for embryonic lens epithelial cells, when compared to controls with no serum supplement. The absence of mitogenic activity in serum from hatched embryos was not due to an inhibitor, since the mitogenic activity of E15 serum was not reduced when it was mixed with an equal amount of serum from hatched chicks. In this experiment,  $0.81 \pm 0.14\%$  of cells cultured for 10 hours in 20% E15 serum were singly labeled, compared to  $0.24 \pm 0.05\%$  of cells cultured in 20% P2 serum. When serum from embryos of the two ages were mixed, the percentage of single-labeled cells was  $0.85 \pm 0.12$ . The dose-response relationship for E10 serum generally resembled that obtained with E15 serum, except for its greater potency at the 10% concentration (Fig. 3C).

Serum and plasma from E15 animals were compared to determine whether the growth-promoting activity in serum was derived from thrombocytes or other components of the clotting reaction. The percentage of single-labeled cells from explants incubated in plasma were slightly lower than in serum. However, these differences were not statistically



**Fig. 3.** (A) Dose-response of embryonic lens epithelia to E15 serum. Embryonic central lens epithelia (E6) were cultured for 6 hours, then double-labeled to measure the rate of entry into S-phase, as described in Materials and methods. (B) Analysis of the growth-promoting activity of serum from embryos and hatched chicks of different ages. Central lens epithelia from day-6 embryos were cultured for 6 hours in 10% serum. (C) Response of embryonic lens epithelia to different concentrations of E10 serum. Embryonic central lens epithelia were cultured for 6 hours. (D) Comparison of the growth-promoting activity of serum and plasma. Embryonic central lens epithelia were cultured for 6 hours in 20% E15 serum or plasma. Data points for all figures are means  $\pm$  s.e.m. of three independent experiments.

significant (Fig. 3D). Thus, it did not appear that products of the clotting reaction were primarily responsible for the mitogenic activity in serum.

### Characterization of the lens mitogen in embryonic serum

As an initial step in characterizing the growth-promoting factor(s) present in embryonic serum, we heated E15 serum to  $65^\circ\text{C}$  for 1 hour or exposed it to trypsin for 2 hours. Controls for these experiments, which were exposed to untreated E15 serum, had  $1.04 \pm 0.12$  and  $0.93 \pm 0.06$  percent single-labeled nuclei, respectively. Lens epithelia cultured without serum supplementation had  $0.09 \pm 0.04$  and  $0.09 \pm 0.10$  percent single-labeled nuclei in the two studies. Heat treatment eliminated the ability of serum to maintain mitogenic activity ( $0.14 \pm 0.03$  % single-labeled nuclei). Activity was also destroyed by trypsin treatment. In this case, cells cultured in serum that had been treated with

**Table 1. The effect of growth factors on lens epithelial DNA synthesis**

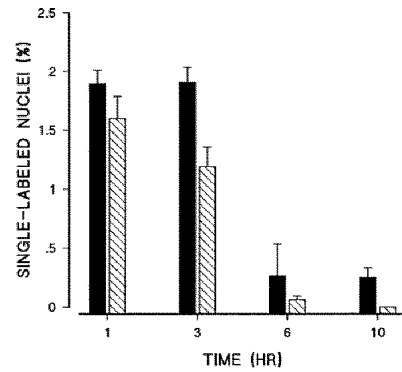
Treatment	Concentration	% Single-labeled nuclei
Basal medium	—	0.25
EGF	100 ng/ml	0.17
	1 ng/ml	0.19
bFGF	100 ng/ml	0.56
	1 ng/ml	0.40
IGF-1	1 ng/ml	0.33
IGF-2	100 ng/ml	1.79
	1 ng/ml	1.03
PDGF	100 ng/ml	0.56
	1 ng/ml	0.44
TGF <sub>1</sub>	100 ng/ml	0.47
	1 ng/ml	0.65
TGF <sub>2</sub>	1 ng/ml	0.44
P-neurext	30 µg/ml	0.19

Embryonic central lens epithelia were cultured in the indicated concentrations of growth factors for 3 hours. Values for percentage of single-labeled nuclei are the means of two experiments.

trypsin for 2 hours, then with soybean trypsin inhibitor, had  $0.08 \pm 0.02\%$  single-labeled cells. When soybean trypsin inhibitor was added to serum during trypsin treatment, the percentage of single-labeled cells was  $0.91 \pm 0.13$ . In each case, values for the percentage of single-labeled cells are the means of three separate experiments. These tests suggested that the mitogenic activity in embryo serum is due to the presence of a protein or proteins.

We performed several studies to determine whether known mitogens or combinations of these growth factors could substitute for the activity in embryonic serum. Epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) have previously been shown to be mitogenic for cultured postnatal and adult lens epithelia of other species (Reddan and Dziedzic, 1982; and Reddan and Wilson-Dziedzic, 1983; Brewitt and Clark, 1988; McAvoy and Chamberlain, 1989). TGF<sub>1</sub>, TGF<sub>2</sub>, IGF-2 and a commercially available bovine pituitary extract, P-Neurext, were also screened. Of the growth factors tested singly (Table 1), only IGF-2 at 1 and 100 ng/ml maintained DNA synthesis above 1.0% through the first 3 hours of culture. However, when IGF-2 was tested at 6 and 10 hours, it failed to maintain DNA synthesis at levels comparable to those seen with serum treatments (Fig. 4).

The capability of IGF-2 to maintain DNA synthesis only through the first three hours of culture could be due to its known properties as a 'progression factor' (Stiles et al., 1979; Campisi and Pardee, 1981). The control of eukaryotic cell proliferation is governed by a series of regulatory events that occur in the G<sub>1</sub>-phase of the cell cycle (Pardee, 1989). Each of these events requires a growth factor or adequate supply of essential nutrients before cells can progress to the next control point (Pledger et al., 1977; and Stiles et al., 1979; Leof et al., 1982; Campisi and Pardee, 1981). 'Progression factors' act primarily on cells that are in the latter part of G<sub>1</sub>, stimulating them to initiate DNA synthesis. If IGF-2 only acted on a subpopulation of cells near



**Fig. 4.** Effects of IGF-2 on the initiation of lens cell DNA synthesis. Entry into S-phase of the cell cycle decreased sharply after culture for greater than 3 hours in 100 ng/ml (solid bars) or 1 ng/ml (diagonal hatched bars) of IGF-2. Values represent means  $\pm$  s.e.m. of three experiments.

the end of G<sub>1</sub> that were competent to progress to S-phase, its action would have been limited to only a few hours, after which the number of 'competent' cells would have been depleted.

To test this possibility, we combined known 'competence factors' with IGF-2, then measured the rate of entry of lens cells into S-phase after culture for 6 hours. Of the combinations tested (Table 2), only those containing bFGF, PDGF and IGF-2 increased the number of cells entering S-phase above the levels seen when lens epithelia were cultured in basal medium. However, the values obtained in these experiments were about 5 times lower than those observed when lens explants were cultured in embryo serum.

#### Antagonistic effects of embryo serum and vitreous humor on lens cell differentiation and division

We have previously shown that, in chicken embryos, plasma proteins rapidly enter the eye from the blood and that plasma proteins are abundant in embryonic vitreous humor (Beebe et al., 1986). We therefore mixed chicken embryo serum or plasma with vitreous humor and cultured lens epithelia in these mixtures. Chicken embryo vitreous humor or IGF-1 caused lens epithelial cells to elongate, which is an early measure of lens fiber formation (Beebe et al., 1980, 1987; Table 3). Chicken embryo serum or plasma did not stimulate lens cell elongation (Beebe et al.,

**Table 2. The effect of combinations of growth factors on lens cell DNA synthesis**

Supplements	Concentrations (ng/ml)	% Single-labeled
IGF-2, EGF	1, 10	0.07
IGF-2, PDGF	1, 10	0.09
IGF-2, PDGF, EGF	1, 10, 10	0.10
IGF-2, bFGF	1, 10	0.10
IGF-2, bFGF, EGF	1, 10, 10	0.07
IGF-2, bFGF, PDGF	1, 10, 10	0.29
IGF-2, bFGF, EGF, PDGF	1, 10, 10, 10	0.24

Embryonic lens epithelia were cultured for 6 hours in medium supplemented with the indicated combinations of growth factors. Values for percentage of single-labeled nuclei are the means of two experiments.

**Table 3. Effects of serum, plasma and vitreous humor on lens cell growth and differentiation**

Supplement	% Single-labeled nuclei	Cell length ( $\mu\text{m}$ )
Vitreous humor (E15)	0.17 $\pm$ 0.04	18.6 $\pm$ 0.6
IGF-1	0.03 $\pm$ 0.05	21.6 $\pm$ 1.2
Embryo serum (E15)	0.80 $\pm$ 0.11	11.3 $\pm$ 0.5
Embryo plasma (E15)	0.45 $\pm$ 0.03	11.5 $\pm$ 0.4
Vitreous humor + serum	0.22 $\pm$ 0.08	11.4 $\pm$ 0.2
Vitreous humor + plasma	0.11 $\pm$ 0.01	11.2 $\pm$ 0.6
IGF-1 + serum	0.82 $\pm$ 0.02	11.9 $\pm$ 0.4
None	0.00 $\pm$ 0.00	10.5 $\pm$ 0.5

Vitreous humor inhibited the mitogenic activity of serum or plasma. Conversely, serum and plasma prevented the cell elongation stimulated by vitreous humor or IGF-1. Embryo serum, plasma or vitreous humor (all 20% v/v) were used independently or in combination. IGF-1 was used at 100 ng/ml. Lens epithelia were double labeled after culture for 10 hours, lens cell length was measured after culture for 24 hours. All values are means  $\pm$  s.e.m. for three experiments.

1987; Table 3). However, serum or plasma (20%, v/v) completely blocked the cell elongation stimulated by 20% vitreous humor. This inhibition persisted for at least 24 hours in culture. Similarly, serum prevented cell elongation when mixed with IGF-1 (Table 3). In these tests, IGF-1 was used at levels that were ten times greater than those needed to stimulate lens cell elongation (Beebe et al., 1987).

Conversely, vitreous humor suppressed the mitogenic activity in embryonic serum or plasma. After 10 hours of culture in chicken embryo serum or plasma, the rate at which lens cells entered S-phase remained high (Fig. 2; Table 3). Addition of 20% chicken embryo vitreous humor to serum or plasma reduced the rate of entry into S-phase at least four-fold. Unlike vitreous humor, IGF-1 (100 ng/ml) did not reduce the progression of lens epithelial cells through the cell cycle. This suggested that the reduction of lens cell division seen when lens epithelial cells are treated with vitreous humor or IGF-1 was not simply a consequence of lens fiber cell differentiation.

As with the mitogen found in embryo serum, we performed tests to determine the chemical nature of the serum inhibitor of lens cell elongation (Table 4). Heating E15 serum at 65°C for 1 hour partially reduced the ability of E15 serum to inhibit lens cell elongation. Treatment of E15 serum with trypsin had no detectable effect on its ability to inhibit cell elongation. This contrasted with the effect of heat and trypsin on the mitogen in embryo serum, which was completely destroyed by similar treatments. These data suggested that the mitogenic and the differentiation-inhibit-

**Table 4. Treatment of the serum inhibitor of lens cell elongation with heat and trypsin**

Treatment	Cell length ( $\mu\text{m}$ )
E15 serum, untreated	10.1 $\pm$ 0.9
E15 serum, 65°C, 1 hour	15.1 $\pm$ 3.3
E15 serum + trypsin (+inhibitor after 2 hours)	9.2 $\pm$ 0.3
E15 serum + trypsin (+inhibitor simultaneously)	9.5 $\pm$ 0.9
No additions (20% E15 vitreous humor only)	17.7 $\pm$ 1.5

Serum (E15) was heated to 65°C for 1 hour or treated with trypsin for 2 hours. The treated serum was mixed with E15 vitreous humor and added to lens epithelial explants. Cell lengths were recorded 5 hours after the beginning of treatment. Values are means  $\pm$  s.e.m. of three experiments.

ing activities found in chicken embryo serum were due to the action of different molecules.

## DISCUSSION

The results of this study show that a lens mitogen is present in the anterior chamber of the embryonic eye. The experiments that identified this factor were possible because we used a method that measures rapid changes in cell proliferation (Hyatt and Beebe, 1992). Previous studies of lens cell mitotic or proliferative activity relied on methods that measured the total number of cells in S-phase (Modak and Perdue, 1970; Zwann and Kenyon, 1984; Nath et al., 1987). Because S-phase is of long duration (5-6 hours) in comparison to the time course of the experiments performed in the current study, rapid changes in proliferative activity could have been masked by the large number of cells progressing through S-phase. By identifying only those cells entering S-phase during a short time period, changes in proliferative activity that occurred in as little as 1 hour were readily identified.

### Is the mitogenic activity in the anterior chamber the same as the activity in embryo serum or plasma?

The source of the lens growth activity in the anterior segment of the eye is not known with certainty. It may be produced locally or diffuse into the eye from the vascular system. There are several findings that strongly suggest that the activity in the anterior chamber originates in the blood. Several well-known, purified growth factors and one tissue extract, known to be rich in growth factors, failed to support lens cell division. The only lens mitogen(s) detected in our studies were derived from the embryonic vascular system, either FBS or chicken embryo serum or plasma. Plasma proteins, including proteins of relatively high molecular weight, readily enter the anterior chamber of the embryonic eye from the blood (Beebe et al., 1986). Therefore, the serum mitogen would have to be selectively excluded or inactivated to prevent it from acting in the anterior chamber of the eye.

If the mitogenic activity was made locally, it was not produced in sufficient quantity by cultured anterior segments to be restored after the anterior chambers of cultured half eyes were briefly opened to the medium (Fig. 1). In other experiments, basal medium was conditioned for 12 hours with the ciliary epithelium-iris complex of E10 embryos. No mitogenic activity was detected in these tests (data not shown). Based on these data, it is our working hypothesis that the mitogen found in the anterior chamber of the embryonic eye is derived from the vascular system of the embryo.

If the lens mitogen activity that we detected in chicken embryo serum is responsible for lens cell division *in vivo*, its level in the blood and its access to the anterior chamber of the eye could determine the rate of growth of the lens. Our results show that the mitogenic activity of serum was highest in blood from younger embryos, especially at E10, and that this activity was not detectable within two days after hatching. The concentration of plasma proteins

in the eye declines during the latter third of development, suggesting that access of plasma proteins to the intraocular fluids decreases at this time (Beebe et al., 1986). The labeling index of central lens epithelial cells also declines throughout embryonic and early postembryonic life (Persons and Modak, 1970). Thus the activity of the serum mitogen and its presumed access to the anterior segment of the eye correlate with the mitotic activity in the lens epithelium.

### The potential importance an embryo-specific mitogen in the blood

We have found no previous examples in which autologous embryonic serum was used to culture embryonic cells or tissues. Although fetal bovine serum is often used to culture cells from adults and embryos of other species, tissues or cells from bovine embryo are rarely studied.

The mitogen that we have described may be important in the development of other tissues in the embryo. This could be easily tested using the approaches outlined in this study. In addition, serum from embryos may contain some of the growth-promoting agents found in 'embryo extract,' a supplement often used to culture tissues or cells obtained from chicken embryos. Embryo extract is a crude homogenate of 10-day-old chicken embryos, clarified by centrifugation. Coon and Cahn (1966) fractionated embryo extract and reported that the low molecular weight fraction stimulated cell proliferation while the high molecular weight fraction promoted pigment and cartilage cell differentiation. Based on the wide use of FBS for cell culture, a growth-promoting activity similar to that found in chicken embryos is likely to be present in mammalian embryos.

### Antagonistic stimulatory and inhibitory gradients may determine lens polarity

The discovery that embryo serum blocked the ability of vitreous humor to stimulate lens cell elongation was surprising. Cell elongation is one of the first responses seen when lens epithelial cells are stimulated to differentiate into lens fibers (Piatigorsky et al., 1972). We do not know yet whether other measures of fiber differentiation, like increased crystallin synthesis or crystallin mRNA accumulation, are similarly blocked.

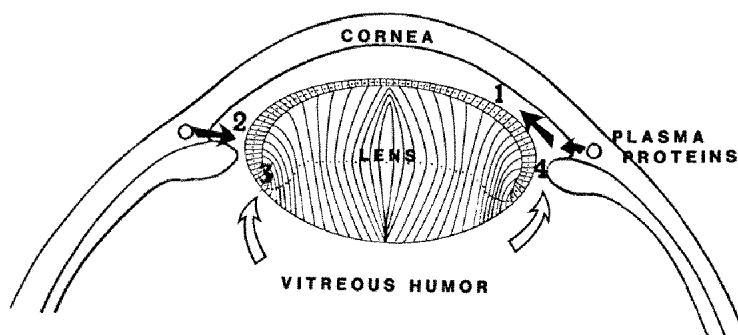
In a recent study (Hyatt and Beebe, 1992), we showed that fetal bovine serum, which has long been known to cause lens epithelial cells to differentiate into fiber-like cells (Philpott and Coulombre, 1965; Milstone and Piatigorsky,

1975), also maintained a high rate of epithelial proliferation for at least 10 hours. Therefore, since lens cells cultured in FBS both elongated and continued to proliferate, FBS may lack an inhibitor of lens cell elongation like that found in chicken embryo serum.

This observation points to an advantage of using autologous embryo serum for culturing developing tissues. Serum or plasma from distantly related species (or even from embryos of the same species at different stages) may have quite different activities or potencies when used to treat embryonic cells.

The inhibitor of lens fiber cell differentiation found in serum may provide the answer to a potential paradox. We have shown that at least one of the factors in chicken embryo vitreous humor that promotes lens fiber differentiation is functionally and immunologically related to the insulin-like growth factors (Beebe et al., 1987). Recently, Robcis and co-workers (1991) showed that IGF-1 is present in chicken embryo serum by E6. Assuming that plasma proteins have access to the anterior chamber of the eye, IGF in the blood should stimulate the epithelial cells in the anterior of the lens to form fibers. This does not happen. If it did, lens polarity would be destroyed and, presumably, a non-functional lens would result. Based on our data, it seems likely that the inhibitor of lens cell elongation in embryo serum prevents serum IGF-1 from causing the anterior lens cells to form fibers, thereby preserving lens polarity.

We were equally surprised to find that chicken embryo vitreous humor inhibited the ability of embryo serum to maintain the proliferation of lens epithelial cells. The reduction in cell cycle progression seen in these experiments was not simply a consequence of activating a 'fiber differentiation program,' because lens epithelial cells cultured in FBS initiate fiber differentiation without substantially reducing their rate of cell division (Hyatt and Beebe, 1992). In addition, exposure of lens epithelia to human IGF-1, a potent inducer of fiber differentiation (Beebe et al., 1987), did not reduce the rate at which lens cells entered S-phase when they were also treated with embryo serum. It should be pointed out that Piatigorsky and Rothschild (1972) showed that lens epithelial cells cultured in FBS for three days substantially reduce the rate at which they incorporate thymidine into DNA. This longer term reduction in lens cell replication may have been caused by the activation of a 'fiber differentiation program' or to the loss of mitogenic activity from the medium.



**Fig. 5.** Diagram of the mechanism proposed to account for the establishment and maintenance of lens polarity in the chicken embryo eye. Plasma proteins leak from the blood into the anterior chamber where they (1) stimulate cell division in the lens epithelium and (2) prevent lens fiber differentiation by factors diffusing from the vitreous humor. Factors in vitreous humor, at the posterior pole of the lens, (3) stimulate lens fiber differentiation, while (4) inhibiting the growth-promoting activity of serum proteins that may diffuse from the anterior chamber.

Several groups have described inhibitors of mitosis in vitreous humor from adult animals (Raymond and Jacobsen, 1982; Luty et al., 1983, 1985; Jacobson et al., 1983, 1985; Singh et al., 1990). To our knowledge, no such activity has previously been identified in the embryonic eye.

The presence of an inhibitor of proliferation in the vitreous humor and the presumed presence of an inhibitor of fiber differentiation in the anterior segment provides an effective mechanism to establish and maintain the polarity of the lens. A monolayer of epithelial cells covers the anterior lens surface, while elongated, terminally differentiated fiber cells are found exclusively in the posterior portion of the structure (Fig. 5). Previous investigators rotated the lens of chicken embryos 90° and replaced it in the eye. This caused the epithelial monolayer to extend into the anterior and the vitreous chambers. A sharp boundary was quickly established between epithelial cells in the anterior half of the lens, which continued to divide, and epithelial cells in the posterior half, which differentiated into lens fibers (Coulombre and Coulombre, 1969; Zwaan and Kenyon, 1984). The ability of the lens to form a new boundary between epithelial cells and differentiating fiber cells reveals the continued plasticity of the lens epithelium in the embryo. It may also reflect the interaction between mitogenic activity in the anterior chamber and the anti-proliferative activity in vitreous humor, as well as between lentropin in the vitreous body and differentiation-inhibiting activity in the anterior chamber.

Recently, McAvoy and Chamberlain (1989) postulated a mechanism for the establishment and maintenance of lens polarity, based on variations in FGF concentrations in the ocular media. They showed that, as the concentration of FGF in culture medium was increased, epithelial cells from 3-day-old rat lenses first divided, then migrated and, finally, accumulated  $\alpha$ -crystallin (a marker for lens fiber cell formation). McAvoy and Chamberlain suggested that variations in FGF concentration in the anterior chamber, posterior chamber, and vitreous body could regulate the activities of the cells that bordered on those compartments and, thereby, maintain lens polarity.

Our data suggest that a more complex mechanism may be operating. The opposition of stimulatory and inhibitory factors in different compartments of the eye might result in a more precise and stable determination of lens cell position and polarity than would be obtained from a single gradient, or even from opposing gradients of purely stimulatory agents. Even if a single-gradient model is sufficient to explain the spatial organization of the lens, the role of agents that stimulate or inhibit lens cell proliferation or differentiation, identified in this study, remain to be explained. More information is needed about the identity and distribution of these molecules, as well as a more complete view of their effects on the lens.

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The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Ser-

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