

A temporally regulated, diffusible activity is required for rod photoreceptor development in vitro

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Summary

The retina is a relatively simple and well-characterized CNS structure in which cell-cell interactions have been hypothesized to influence cell type determination. By manipulating cell density in serum-free cultures we show that rat rod photoreceptor development requires a diffusible activity produced by neonatal retinal cells. This effect is not mediated by changes in cell survival or mitosis. Production of the rod promoting activity varies with developmental stage and is temporally correlated with the timing of rod generation in vivo. In low density cultures, which do not support rod develop-

ment, an increased fraction of cells stain with an antibody specific for another retinal neuron, the bipolar cell. Thus, the diffusible rod promoting activity may influence cell fate determination, and not only terminal differentiation. These results provide an approach for the molecular characterization of developmentally important signals in the vertebrate retina.

Key words: rat retina, rod photoreceptor, development, collagen gel cultures, cell type determination, differentiation.

Introduction

The vertebrate retina contains seven major cell types which have been distinguished on the basis of morphological, molecular and functional characteristics. These cell types are generated in an evolutionarily conserved order during development (reviewed by Altshuler et al., 1991), although multiple cell types are simultaneously produced at any given developmental stage. In vivo cell lineage analyses in rodent (Turner and Cepko, 1987, Turner et al., 1990), frog (Holt et al., 1988, Wetts and Fraser, 1988, Wetts et al., 1989) and chick (D.M. Fekete, J. Perez-Miguelsanz, C.L. Cepko, unpublished observations) have shown that retinal progenitor cells are multipotent: clones derived from single progenitor cells often contained multiple cell types. In particular, many two cell clones were composed of two different cell types, suggesting that progenitor cells remained uncommitted until their final mitosis. Based on these studies, as well as ablation experiments (Reh and Tully, 1986, Negishi et al., 1987) and in vitro analyses (Adler and Hatlee, 1989, Watanabe and Raff, 1990), it has been argued that cell-cell interactions contribute to the determination of retinal cell type.

Watanabe and Raff (1990) studied the development of rod photoreceptors in the rat by mixing different aged retinal cells in aggregate cultures. Their experiments exploited the observation that early in retinal

development, embryonic day 15 (E15), virtually no rods are generated, while later in development, postnatal day 1(P1), most progenitor cells produce rods. Co-culture of labelled E15 retinal cells with P1 retinal cells increased by 55-fold the fraction of E15 cells that expressed the rod-specific protein, rhodopsin, after one week in vitro. The absolute percentage of E15 cells that became opsin⁺, however, was 75-fold lower than the percentage of P1 cells that became opsin⁺, suggesting that E15 cells are limited, relative to P1 cells, in their ability to express opsin. In the converse experiment, initiation of opsin expression by P1 cells was partially inhibited by co-culture with an excess of either E15 retinal cells or non-retinal cells. These findings suggest that P1 retinal cells produce signals that promote, and are necessary for, rod determination (selection of cell type), differentiation (elaboration of mature characteristics) and/or survival. In this model, E15 cells would be unresponsive to, and also inhibit or fail to produce, the rod promoting signal.

If cell-cell signaling is required for rod development, then directly limiting interactions among neonatal retinal cells should lead to inhibition of rod generation. To test this hypothesis, and to develop a robust system with sufficient sensitivity for molecular analysis, retinal cells were separated from their neighbors in vitro using gels of purified collagen, and tested for rod differentiation. In order to follow the fates of retinal progenitor cells in collagen gel cultures, a retrovirus vector, BAG

(Price et al., 1987), encoding the gene for *E. coli* β -galactosidase, was used to infect retinal cells prior to culture. Retroviral infection specifically labels mitotic progenitor cells, since retroviral infection requires a cell to be mitotic and go through an S-phase (Varmus et al., 1977, Harel et al., 1981), and mitotic retinal cells are thought to be equivalent to uncommitted progenitors (Turner and Cepko, 1987). As BAG has been used for in situ lineage analysis in the postnatal rat retina (Turner and Cepko, 1987, Turner, et al., 1990) there exists an extensive, quantitative data set concerning the normal fates of P0 progenitor cells left in vivo to which in vitro analyses can be compared. Labelling with retrovirus vectors is indelible and non-toxic, and could be utilized in the future to transfer developmentally important genes into retinal progenitor cells.

The culture protocol used in these experiments offers a number of advantages over previous in vitro techniques for studying cell-cell interactions. First, unlike aggregate cultures, collagen gels separate cells from one another without introducing other types of cells. Second, diffusible activities freely penetrate the gel and contact the cells within, rather than cells being differentially exposed depending upon their position, as might occur in an aggregate. Third, in collagen gels, retinal cells remain disaggregated throughout the culture period. Low density monolayer cultures, which have also been used to "isolate" cells and study cell-cell interactions, allow migration that can lead to cell-cell contacts. Fourth, commitment and differentiation proceed at in vivo levels in collagen gel cultures; it has been reported by some groups that monolayer cultures do not support rod development (Sparrow et al., 1990, Watanabe and Raff, 1990, D.M.A. and C.L.C., unpublished observations). Fifth, these experiments were carried out in serum-free, defined medium, ruling out potentially confounding influence(s) of serum components.

By varying cell density in gel cultures we found that progenitor cells developed into rod photoreceptors only in the presence of threshold levels of a temporally regulated, diffusible activity produced by neonatal retinal cells. In the absence of this activity, an increased number of progenitor cells became immunoreactive with an antibody characteristic of bipolar cells, suggesting an effect on the choice of cell fate by retinal progenitor cells.

Materials and methods

Animals

C/D rats were purchased from Charles River Labs. The day of birth was considered as postnatal day 0 (P0). For embryonic rats, crown-rump length (Angulo Y Gonzales, 1932) and morphological examination of eyes, forepaws and hindpaws (Long and Burlingame, 1936) were used to determine developmental stage. Animals were killed by decapitation (postnatal pups) or CO₂ asphyxiation followed by cervical dislocation (pregnant females).

Cultures

Retinas from 2-5 neonatal rat pups, or 10-15 embryonic rats, were dissected free of other ocular tissues in Hank's Balanced Salt Solution (HBSS) and then incubated for 10 minutes (room temperature) in HBSS lacking Ca/Mg to which 1 mM EDTA had been added. Trypsin (Worthington) was added to 0.1% (from a 1% stock in HBSS/0.1 mM Hepes pH 7.3) and the retinas were incubated for 10 minutes at room temperature. Cells were washed in DME containing 10% calf serum, triturated to a single cell suspension in HBSS+20 μ g/ml DNase I (Sigma), and resuspended at 2×10^7 cells/ml in culture medium [50% DME, 50% F-12, 1 \times modified Sato's solution (Lillien et al., 1988), penicillin (100 units/ml), and streptomycin (100 units/ml)]. Cells were incubated at 37°C for 150 minutes. Polybrene (8 μ g/ml final) and 10 μ l of concentrated ($1-3 \times 10^7$ CFU/ml) BAG retrovirus stocks were then added (infection followed dissociation in order to limit cell-cell interactions during the labelling period). After 90 more minutes at 37°C cells were washed and resuspended in culture medium at 10^8 cells/ml.

To form gels, retinal cells prepared as above were mixed with 1 M Hepes (pH 7.3) and bovine collagen (Collaborative Research) to a final concentration of 50% culture medium, 100 mM Hepes, and 1.2 mg/ml collagen. 100 μ l of this mixture was pipetted around the edge of each well of either 24-well dishes, 96-well dishes or 35 mm dishes (Costar). In some experiments the gel was formed on a 12 mm glass coverslip to allow ease of manipulation. After 30 minutes in a 37°C, 5% CO₂ humidified incubator, 0.5-0.7 ml of culture medium was added. Explant cultures (Sparrow, et al., 1990) consisted of a small piece (approx. 1/5 of a retina) of retina cultured in one well of a 24 well dish in 0.5-0.7 ml of culture medium. Pellets (Akagawa et al., 1987, Watanabe and Raff, 1990) were formed by centrifuging 2×10^6 cells/500 μ l medium in a 0.5 ml tube for 1 minute in a Tomy HF-120 benchtop centrifuge. After 2-12 hours the contents of the tube were transferred to a 24-well dish and cultured as above.

For experiments designed to test diffusibility of rod-promoting activities, low density gels containing BAG infected retinal cells (10^6 cells/100 μ l gel) were cast on 12mm glass coverslips. High density gels (10^7 cells/500 μ l gel) containing uninfected cells were formed around the circumference of a 35 mm tissue culture dish. Low density gels were placed in the center of the dish, approx. 5 mm from the edge of the high density gel, and not moved during the seven day culture period. 2.5 ml of culture medium was carefully placed on top of the gels. In these experiments the cells/medium volume ratios were comparable to those used in the more simple density experiments (i.e. in high density conditions 4×10^6 cells per ml of medium). Only low density gels that remained in place, distant from the inducing gel, were harvested and analysed. In order to exclude from analysis of low density gels any cells that came from the high density gels, only cells in low density gels were BAG infected, and only β -gal⁺ cells were scored. The possibility of virus release from low density gels, which could potentially generate β -gal⁺ cells in the high density gels, was monitored by co-culturing BAG infected gels with monolayers of 3T3 fibroblasts and then looking for X-gal⁺ fibroblasts; negligible virus contamination was observed.

Cells of different ages were co-cultured such that 5×10^5 BAG-infected P0 retinal cells were mixed with 2×10^6 uninfected cells from various developmental stages and cultured in 100 μ l collagen gels. The possibility that virus carried over from the P0 cells could infect the inducing cells was examined by adding $>2 \times 10^6$ infected P0 cells to a sub-confluent monolayer of NIH 3T3 fibroblasts and monitoring

the appearance of X-gal⁺ fibroblasts; negligible virus contamination was observed.

At the end of the culture period (6-8 days) gels were dissociated by incubation for 20 minutes (37°C) in HBSS/0.1% collagenase (Sigma), followed by 20 more minutes at 37°C after the addition of trypsin to 0.1%. Explants and pellets were dissociated and stained in parallel with gel cultures from the same experiment, except that HBSS without Ca/Mg +1 mM EDTA was substituted for the HBSS/0.1% collagenase. Cells were triturated in medium containing 10% calf serum and 20 µg/ml DNase I and resuspended in 100-500 µl culture medium. Aliquots of this mixture were added to poly-L-lysine coated multi-well slides (Cel-line Associates, Inc.); cells were allowed to attach to the slides for two hours at 37°C.

Immunohistochemistry

For opsin staining, cells were fixed in 4% paraformaldehyde for 5 minutes followed by 5 minutes in 70% ethanol, washed in phosphate buffered saline (PBS), and incubated for 10 minutes in blocking solution (DME/10% calf serum/5% donkey serum/0.3% Triton X-100). Incubation with primary antibodies - mouse monoclonal RETP1 (Fekete and Barnstable, 1983), diluted 1:200 in blocking solution, and rabbit anti-β-galactosidase (Cappel), diluted 1:1000 in blocking solution - was for 20 minutes at room temperature in a humidified chamber. Following a 10 minute wash in blocking solution, cells were incubated for 20 minutes in secondary antibodies - AMCA-conjugated donkey anti-rabbit and Texas Red-conjugated donkey anti-mouse (both affinity purified, from Jackson Immunologicals, at 1:100 dilution in blocking solution). After a further 10 minute wash in blocking solution slides were mounted using gelvatol and observed using a Zeiss Axiophot. In some experiments the absolute percentage of RETP1⁺ cells was reduced, such that high density gels or pellets generated 10-20% positive cells after one week in vitro instead of the more usual 20-30%. In all such cases, however, the relative levels of rod generation between different conditions (i.e. high versus low density, or co-cultured versus cultured alone) were maintained. In particular, survival and development of retinal cells cultured in serum-free medium were adversely affected when the dishes were maintained outside of the incubator for significant periods of time.

For 115A10 staining (Onoda and Fujita, 1987) cells were fixed in paraformaldehyde, rinsed in PBS and incubated in block solution lacking detergent. 115A10 was used at a 1:50 dilution, followed by texas red anti-mouse at a 1:100 dilution. Cells were postfixed in 70% ethanol, washed in PBS and blocking solution containing Triton (0.3%), and incubated with rabbit anti-β-galactosidase antiserum, followed by AMCA-conjugated donkey anti-rabbit antiserum. Slides were mounted and viewed as above. Staining with 115A10 was variable, with weak signal (see Fig. 2F) and occasional high background. Thus, in some experiments it was impossible to accurately quantitate the percentage of 115A10⁺ cells, and these were excluded from analysis.

In all cases >100 β-gal⁺, or 300 total, randomly selected cells were counted per individual culture. Cells were photographed using Kodak Tri-X film.

RT-PCR analysis

RNA was prepared from 150,000 cells (25,000 cells from each of six duplicate cultures) by the acid-phenol method (Chomczynski and Sacchi, 1987), and made into cDNA using conditions as described by the manufacturer of the Reverse Transcriptase (BRL). PCR was performed using 2% of each sample in a standard (Perkin-Elmer) 50 µl reaction containing

0.9-1.2 mM MgCl₂ and 1 µCi of [³²P] dCTP (3000 Ci/mmol). The oligonucleotides used span intron-exon boundaries, do not amplify genomic DNA, and (for rod specific transcripts) detected much less signal from cDNA of degenerated (10 week old) rd/rd mouse retina (which specifically lacks rods) than from comparable amounts of cDNA of normal mouse or rat retina (data not shown). The amplified products were the correct size (monitored on ethidium bromide stained gels) and restriction digestion confirmed the identity of each fragment (D.M.A., Karin Sorra, and C.L.C., unpublished observations). The amount of cDNA and number of PCR cycles were titrated for each oligonucleotide pair such that PCR amplification had not yet begun to plateau: in all cases little or no product was detectable on ethidium-bromide stained gels, although intensely staining product could be generated after 5-10 additional cycles. PCR cycles consisted of 30 seconds at 93°C, 30 seconds at 60°C, and 60 seconds at 72°C. The nucleotide sequence and number of cycles used for each oligonucleotide were as follows: mouse rhodopsin (Baehr et al., 1988) nucleotides 312-338 and 723-698, 30 cycles; mouse transducin-α (Rapport et al., 1989), nucleotides 38-64 and 495-469, 34 cycles; mouse S-antigen (Tsuda et al., 1988), nucleotides 352-375 and 851-826, 25 cycles; mouse retinoblastoma (Bernards et al., 1989), nucleotides 292-315 and 797-794, 30 cycles. Products were separated on 3% sieving agarose gels and dried; autoradiography film was exposed for 6-22 hours without an intensifying screen. The radioactive products were quantitated using a beta-scanner (Betagen), confirming that the expression of rod specific genes was diminished >90% in low density gels, while that of retinoblastoma was comparable regardless of density.

BrdU treatment

Gel cultures were incubated in the presence of 2 µM BrdU for the first 24 hours of culture. After three more days in vitro cells were dissociated and stained as above, except that incubation with 25 µg/ml of DNase I (Sigma) for 1 hour at 37°C was used after fixation to unmask the BrdU epitope. The antibody staining protocol was as for opsin immunohistochemistry except for the substitution of anti-BrdU antibody (Bu20a, 1:1 dilution) in place of RETP1.

Results

Rod development occurs in high cell density gel cultures at levels comparable to that observed in vivo and in other culture preparations

Approximately 1% of neonatal (P0) rat retinal cells express detectable levels of rhodopsin (Watanabe and Raff, 1990, D.M.A. and C.L.C., unpublished observations); none of these opsin⁺ cells are mitotic, since a delay of at least 48-54 hours exists between a cell's last S-phase and induction of rhodopsin immunoreactivity (Watanabe and Raff, 1990). In vivo, approximately one quarter of the cells in a rat retina will become opsin⁺ during the first postnatal week (Watanabe and Raff, 1990; Fig. 3). To see if collagen gel cultures could support this normal induction of rhodopsin expression, neonatal (P0) retinal cells were infected with BAG (to label mitotic progenitor cells), suspended in collagen gels (2×10⁶ cells/100 µl gel) and grown in serum-free defined medium. Microscopic examination of gels revealed no apparent cell aggregation (Fig. 1A,B) or migration out of the gels (data not shown). After one

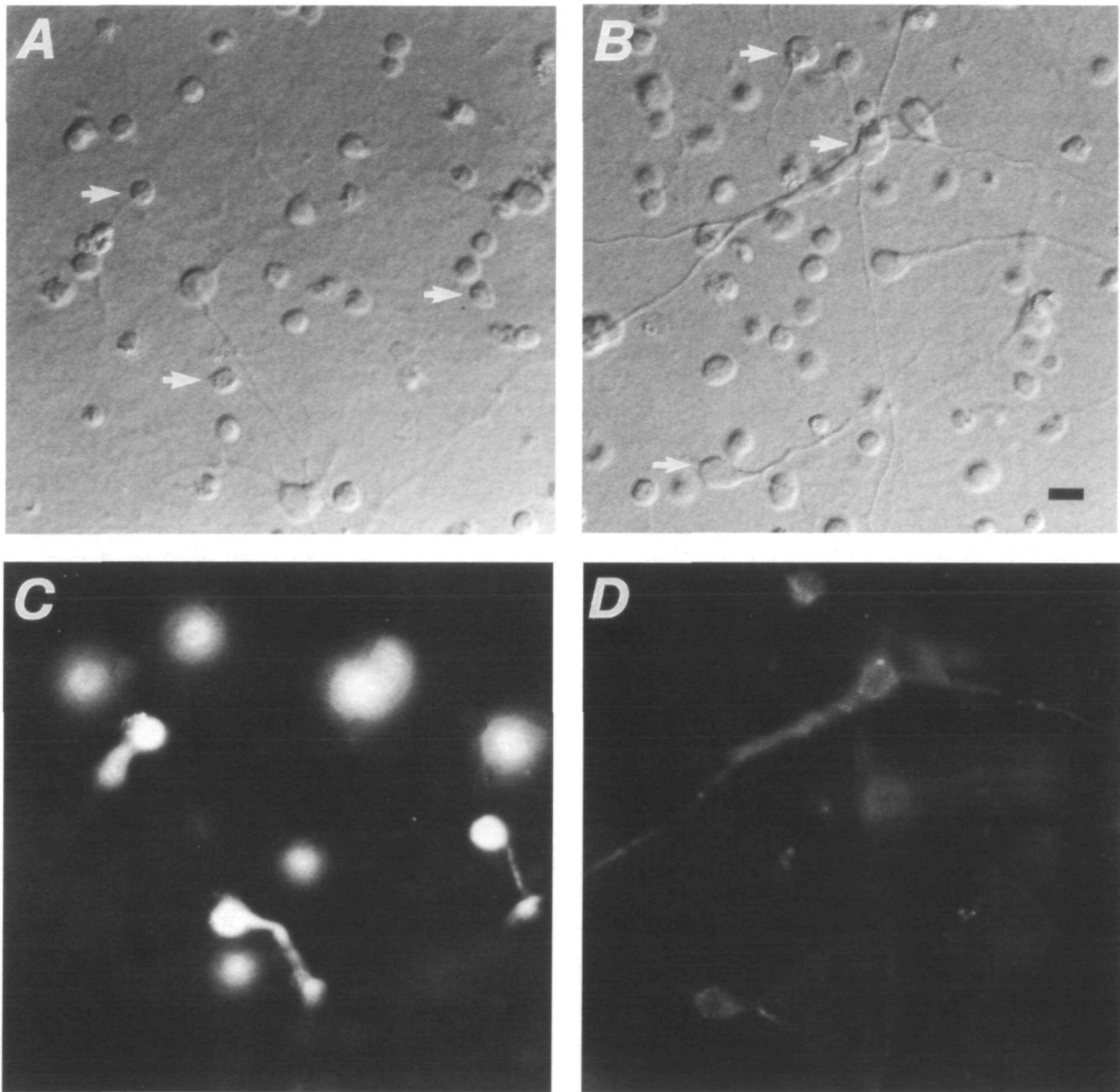


Fig. 1. Immunohistochemical staining of retinal cells grown in collagen gel cultures. P0 retinal cells were grown for seven days in serum free, collagen gel cultures at 10^6 cells/100 μ l gel and stained with cell-type specific antibodies in situ. (A,C) Cultures stained with anti-rhodopsin monoclonal antibody (RETP1) and photographed using Nomarski (A) or Texas red immunofluorescence (C) optics. Morphologically, RETP1⁺ cells tended to be either monopolar neurons, or spherical cells without any process. (B,D) Cultures stained with 115A10, a monoclonal antibody specific to bipolar cells, photographed using Nomarski (B) or Texas-red immunofluorescence (D) optics. Many 115A10⁺ cells were multipolar neurons, while others lacked processes. Arrows (A,B) indicate immunostained cells. Since the gels are three-dimensional, many cells in each field are out of the plane of focus. Scale bar=10 microns.

week in vitro the cells were dissociated from the gels, double-immunohistochemically stained using antibodies to rhodopsin (RETP1) and the retrovirally encoded β -galactosidase protein, and scored for the percentage of opsin⁺ cells among β -gal⁺ cells and for the percentage of opsin⁺ cells among the total population. Many retrovirally infected cells (β -gal⁺), as well as uninfected cells, became opsin⁺ during the week in gel culture (Fig. 2A,C,E). In addition, cells were stained in situ without dissociation of the gel matrix in order to examine the morphology and arrangement of

opsin⁺ cells. Numerous opsin⁺ cells were observed for which no contacts with other cells could be seen under microscopic examination (Fig. 1C).

Mitotic cells are thought to be uncommitted, and thus for retrovirally infected cells to become opsin⁺ both commitment and differentiation must have taken place in vitro. When cultured in collagen gels at high cell density, $23 \pm 5\%$ (mean \pm s.e.m.) of β -gal⁺ cells became opsin⁺ after one week in vitro. This value is indistinguishable from that observed in retinal explants (Sparrow, et al., 1990) and "pellets" (Akagawa, et al.,

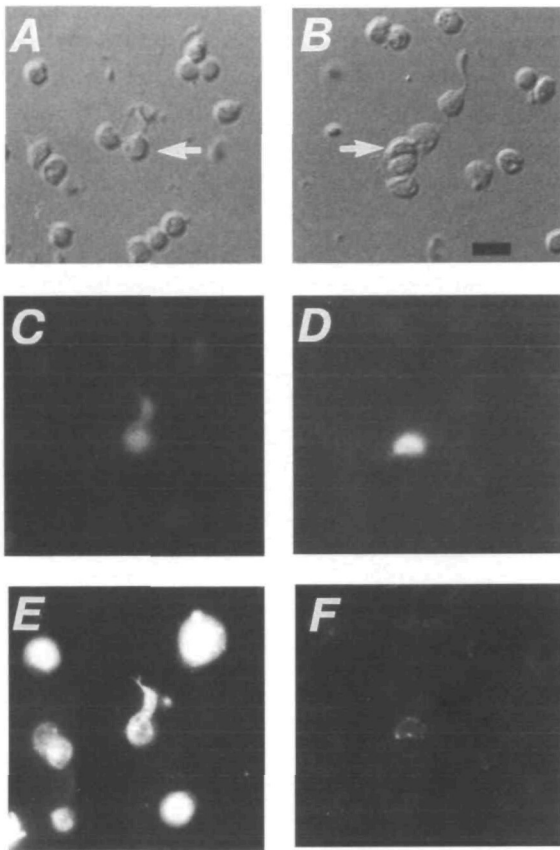


Fig. 2. Double immunohistochemical staining of retinal cells dissociated from a gel culture. P0 retinal cells were grown for seven days in serum free, collagen gel cultures at 2×10^6 cells/100 μ l gel. The cells were then dissociated from the gel, replated on slides for two hours, and double-stained with antibodies to the retrovirally encoded β -galactosidase protein and with cell-type specific antibodies. (A,C,E) Cells stained with anti-rhodopsin monoclonal antibody (RETPI) and anti- β -galactosidase antiserum photographed using Nomarski optics (A); AMCA-immunofluorescence (β -gal; C); and texas-red immunofluorescence (RETPI; E). (B,D,F) Cells stained with anti-bipolar cell monoclonal antibody (115A10) and anti- β -galactosidase antiserum photographed using Nomarski optics (B), AMCA-immunofluorescence (β -gal; D) and texas-red immunofluorescence (115A10; F). Arrows point to double-positive cells under Nomarski optics. Scale bar=10 microns.

1987, Watanabe and Raff, 1990) cultured in parallel to the gels (Fig. 3A). The fraction of *total* cells that expressed opsin ($21 \pm 4\%$) in high-density gels was comparable to that seen in the retina of a litter-mate whose retina developed in vivo for the same period ($24 \pm 1\%$), or in explants or pellets (Fig. 3B). Thus, high density gel cultures grown in serum-free medium support physiologic levels of opsin induction during the first week in vitro.

Development of opsin⁺ cells is dependent upon high cell density

The appearance of opsin expressing cells in gel cultures

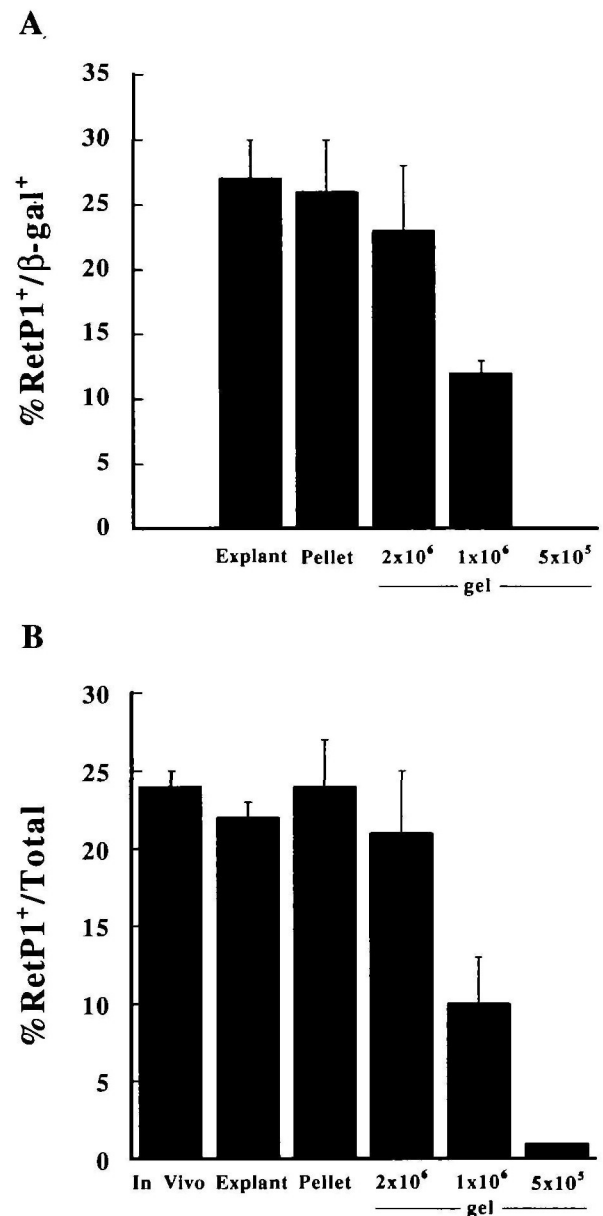


Fig. 3. Differentiation of neonatal retinal cells into rod photoreceptors is density dependent. (A) The percentage of retrovirally infected cells (β -gal⁺) that expressed rhodopsin after one week in vitro. (B) The percentage of total cells that expressed rhodopsin after one week in vitro or in vivo. The conditions under which cells were grown are indicated under each bar. Data are expressed as the mean \pm s.e.m. of triplicate cultures from a representative experiment.

was exquisitely sensitive to the concentration of neighboring retinal cells. A four-fold reduction in cell density (to 5×10^5 cells/100 μ l gel) reduced generation of opsin⁺/ β -gal⁺ cells to an undetectable level (Fig. 3A). The response was graded, as a density reduction between these extremes (10^6 cells/100 μ l gel) supported an intermediate level ($12 \pm 1\%$) of opsin expression. In addition, the overall percentage of opsin⁺ cells (of total), compared to that in the starting population, did

not increase in low density gels (Fig. 3B); this is in contrast to the situation in high density gels, wherein the proportion of total cells that are opsin⁺ rises from 1% to 21% during the week in vitro. When retinal cells from older postnatal rats, such as P4 and P6, were cultured in low density gels they also maintained, but did not increase, their overall percentage of opsin⁺ cells (data not shown). Thus, high density culture was required in order for additional cells to become opsin⁺ during the culture period.

Loss of rod development is not correlated with rod cell death or effects on mitosis

Two measurements were performed to investigate the possibility that low density culture inhibited rod survival, rather than rod development. First, cell survival, averaged over 51 cultures, was found to be $33.8 \pm 1.4\%$ in high density cultures, and $34.4 \pm 1.4\%$ in low density cultures (quantification of survival immediately after plating in collagen resulted in 40%–60% cell yields, suggesting that much of the decreased cell number was attributable to loss during dissociation of the gel and the counting procedure). These values were indistinguishable using a two-tailed paired sample *t*-test ($P \geq 0.5$). In addition, the hypothesis that there were 20% fewer cells in low density gels than in high density gels (the percentage of rods in high density gels) can be rejected at $P \leq 0.001$. Second, if rod survival was density dependent, pre-existing opsin⁺ cells placed in vitro should die after a week in low density culture. P6 retinal cells, of which 23% were opsin⁺, were cultured at low density; the percentage of opsin⁺ cells at the end of one week ($23 \pm 1\%$) was indistinguishable from that of the starting population. Cells from other postnatal ages, when cultured at low density, also maintained but did not increase the total percentage of opsin⁺ cells (Fig. 3B; data not shown). These observations confirm that rods did not die in low density cultures.

Effects of density on cell division could conceivably underlie the diminution in rod generation observed in low density gels. This possibility was investigated by culturing gels in the presence of BrdU, a thymidine analog incorporated into cells synthesizing DNA, which can be detected immunohistochemically. The mitotic index of the assay population (β -gal⁺ cells) during the first 24 hours of culture was the same in gels of different densities (Table 1). The percentage of total cells that were mitotic during the first 24 hours of culture was

Table 1. Cell division in gel cultures

cells/gel	% BrdU ⁺ (of total)	% BrdU ⁺ (of β gal ⁺)
2×10^6	25 ± 0	74 ± 3
1×10^6	21 ± 1	75 ± 2
5×10^5	19 ± 1	77 ± 4

Collagen gels containing various cell densities were cultured in the presence of BrdU (2 μ M) for the first 24 hours in vitro. After 72 more hours in vitro gels were dissociated and the cells were replated on slides and immunostained for β -galactosidase and BrdU. Data are expressed as the means \pm s.e.m. of triplicate cultures from a representative experiment.

slightly lower in low density, as compared to high density, cultures (Table 1). Little cell division was observed beyond the first day in vitro, however, in all gel cultures (data not shown). Retinal cells from younger animals, such as E15 embryos, also stopped dividing prematurely when cultured in collagen gels, despite their extensive proliferation in vivo or in pellet cultures (Watanabe and Raff, 1990; D.M.A. and C.C., unpublished observations). It has been shown recently that cerebellar progenitors also stop dividing when cultured in collagen gels (Gao et al., 1991). Although normal levels of cell division were not maintained in collagen gels, the observation that mitosis is comparable in high and low density cultures suggests that effects on mitosis are unlikely as an explanation of the differential loss of rod development in low density gels.

Multiple rod specific transcripts fail to be induced in low density cultures

To confirm that the program of rod commitment and/or differentiation, and not specifically the expression of rhodopsin protein, was affected by low density culture, the polymerase chain reaction (PCR) was used to study gene expression in gel cultures. Expression of the rod-specific genes rhodopsin, α -transducin, and S-antigen was diminished in low density cultures (Fig. 4). A ubiquitously expressed gene (retinoblastoma) showed comparable signal across all densities, confirming that each sample contained similar amounts of amplifiable cDNA. These results indicate that the coordinated program of rod differentiation, as reflected by the induction of rod-specific transcription, required cell-cell interactions.

Expression of a marker characteristic of bipolar cells is increased in low density gels

The data presented above are consistent with the model that the terminal differentiation of rods requires influences from other retinal cells. An additional possibility is that low density culture might block rod determination, the process whereby a progenitor cell becomes restricted to the rod fate, as opposed to that of another cell type. If so, β -gal⁺ cells that normally develop as rods might become another retinal cell type if cultured at low cell density. In the rat, lineage mapping has shown that postnatal progenitor cells left in vivo give rise to rods, bipolar cells, Müller glia, and amacrine cells, with many clones containing mixtures of these cell types (Turner and Cepko, 1987). Rods (87%) and bipolars (9%) were the most abundant cell types generated by infection of P0 progenitor cells (calculated from Turner and Cepko, 1987).

In vitro, 115A10, a monoclonal antibody which labels bipolar cells in vivo (Onoda and Fujita, 1987), stained $21 \pm 3\%$ of β -gal⁺ cells in high density cultures (Figs 1 B,D, 2 B,D,F, 5). A small number of β -gal⁺ cells were also observed to stain with markers to Müller glia and amacrine cells (data not shown). These results suggest that, in high density gels, progenitor cells developed into the full range of cell types observed in vivo. When

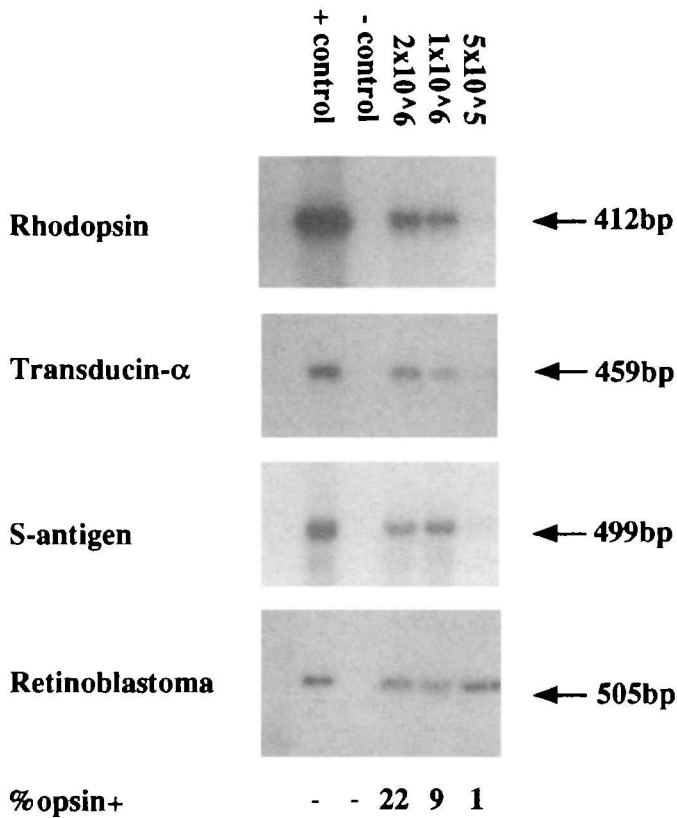


Fig. 4. RT-PCR analysis of retinal cells cultured in gels for one week. Lane 1: P9 retinal RNA (+control). Lane 2: negative control in which no cells were added to the RNA extraction (-control). Lanes 3,4,5: gel cultures grown at 2×10^6 cells, 1×10^6 cells and 5×10^5 cells/100 μ l gel, respectively. For each culture sample the percentage of total cells that were RETP1⁺ is indicated at the bottom of the figure. The size in base pairs of each PCR product is indicated on the right. Data is from a representative experiment.

cell density was lowered such that rod development was diminished, however, there was a dramatic increase in the fraction of 115A10⁺/ β -gal⁺ cells (Fig. 5). In addition, monolayer cultures that failed to support rod development also contained a high proportion of 115A10⁺ cells (Laura Lillien and C.C., unpublished data). These data suggest that manipulating cell-cell interactions through changes in cell density may influence the choice of newly generated retinal cells between the rod and bipolar cell fates.

High density gels can stimulate rod development without direct cell contact

To begin to molecularly characterize the interactions required for rod development, high density gels were tested for the ability to stimulate rod generation in low density gels without direct physical contact. BAG-infected cells were dispersed in low density gels and cultured alone, or together with a high density gel containing uninfected cells, such that the two gels were separated by approx. 5mm. The ratio of cells to medium volume used were equivalent to those used in the

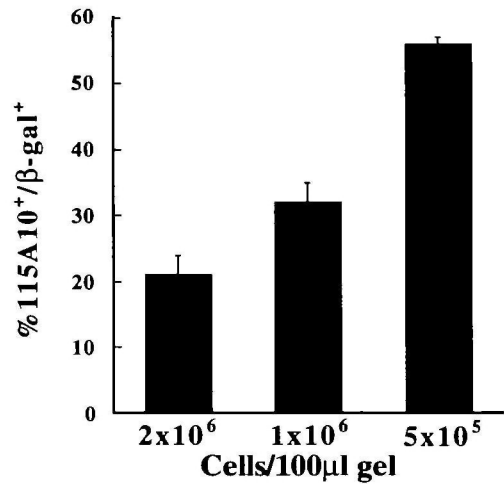


Fig. 5. Decreasing cell density leads to an increased fraction of β -gal⁺ cells staining with the bipolar cell marker, 115A10. Cells were grown as described in Fig. 1 except staining with 115A10 was used in place of RETP1. Data are expressed as the mean \pm s.e.m. of triplicate cultures from a representative experiment.

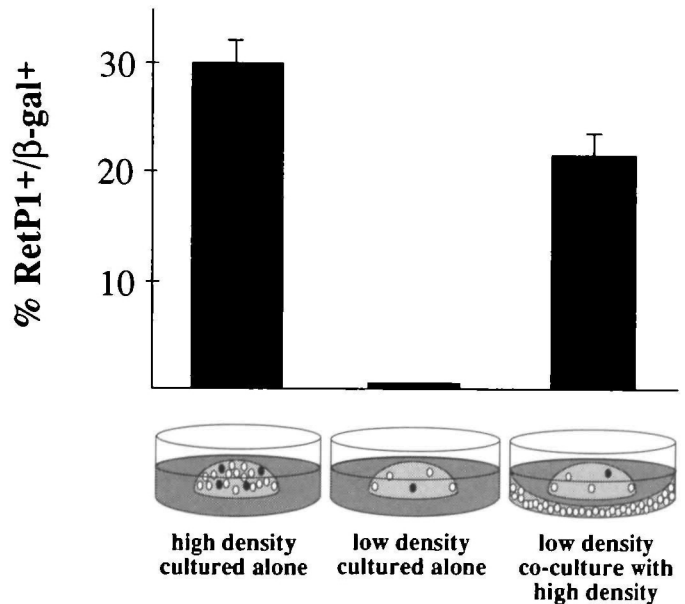


Fig. 6. A diffusible activity(s) can restore rod development to low density cultures. BAG infected P0 retinal cells were cultured alone, or co-cultured with gels containing a high density of uninfected P0 cells. The percentage of β -gal⁺ cells in the low density gel that became opsin⁺ were counted. Data are expressed as mean \pm s.e.m. of triplicate cultures from a representative experiment.

simpler density experiments (high density, 4×10^6 cells/ml medium; low density, $< 10^6$ cells/ml medium). After one week, low density gels cultured alone contained $1 \pm 0\%$ opsin⁺/ β -gal⁺ cells. Low density gels co-cultured with a high density gel contained $22 \pm 2\%$ opsin⁺ cells. This value is similar to that observed in high density gels in this experiment ($30 \pm 2\%$) (Fig. 6). Cells or processes were never observed growing out of

any gels, arguing against the possibility that cells or parts of cells physically connected the two gels. Gel cultures were not unique in their release of the diffusible activity, since pellet and monolayer cultures, when co-cultured with low density gels, induced opsin expression in low density gel cultures (data not shown). Co-culture with a confluent monolayer of 3T3 fibroblasts, on the other hand, did not increase the proportion of rods in low density cultures (data not shown). These data suggest that retinal cells can stimulate rod development in gel cultures without direct cell-cell contact.

Other observations are consistent with the idea that the rod promoting activity does not require cell-cell contact, and diffuses into the medium in gel cultures. For example, 2×10^6 cells, when packed together (as in a retina), occupy a volume of 1-2 μl , whereas in "high" density gels this same number of cells is dispersed over 100 μl ; culturing cells in either manner supports comparable rod development (Fig. 3). In addition, the density dependence of rod formation was more accurately reflected in the ratio of cells to medium volume, rather than that of cells to gel volume. For example, rod generation in high density gel cultures was diminished if the medium was changed frequently, or if a larger volume of medium was used (data not shown).

Production of rod promoting activity is temporally correlated with the birthdays of rods in vivo

In rats, *in vivo* rod generation (birthdays) begins at E15, is maximal around P0, and declines by the end of the first postnatal week (M.M. LaVail, D. Yasumura, and D.H. Rapaport, personal communication, see Fig. 7). To compare the temporal sequence of *in vivo* rod birthdays to the production of rod promoting activity *in vitro*, retinal cells from various developmental stages were tested for the ability to restore rod generation to a low density collagen gel culture of P0 cells. The P0 cells assayed for rod development were distinguished from the inducing population as they were infected with the BAG retrovirus. 100 μl gels contained 5×10^5 P0 infected "assay" cells, and 2×10^6 "inducing" cells from different aged rats, and were cultured in 0.5 ml medium for one week.

The ability to restore rod development to P0 cells *in vitro* was temporally regulated in parallel to *in vivo* birthdating (Fig. 7). E15 cells did not support rod development of the infected P0 cells, whereas rod development was maximal when P0 cells were mixed with retinal cells from E19, P0 or P4. Interpretation of data from the latest time point, P8, which supported a lower level of rod development, is confounded by the observation that cell survival was 50% less in P8 co-cultures than in those of younger cells (data not shown); the decreased level of opsin⁺/ β -gal⁺ cells in P8 co-cultures thus could be due to lower levels of rod promoting activity released from P8 cells, or to a lower effective density of cells due to cell death. In either case, the correlation between *in vitro* mixing experiments and *in vivo* rod production suggests that the cell-cell interaction revealed by low density culture may

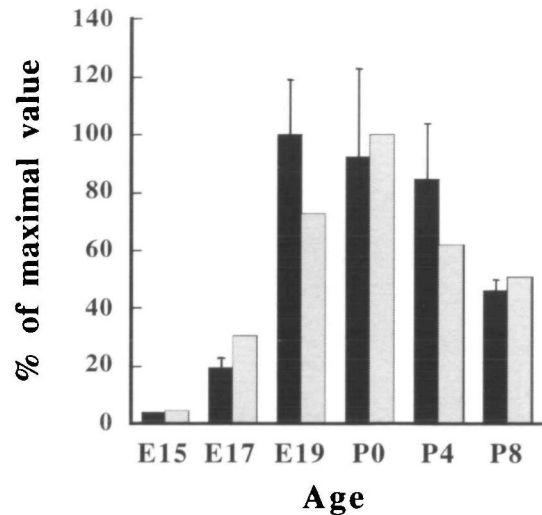


Fig. 7. Ability to support differentiation of neonatal cells into rod photoreceptors is temporally correlated with rod birthdays *in vivo*. [³H]thymidine birthdating of the rat retina is represented by the hatched bars; the fraction of heavily labelled nuclei that were identified as rods (for each date of thymidine injection) is expressed as a percentage of the maximal value (P0=77%) (M.M. LaVail, D. Yasumura, and D.H. Rapaport, personal communication). The solid bars represent the percentage of β -gal⁺ P0 cells that expressed rhodopsin after one week *in vitro* when co-cultured with cells from the ages described. The data are expressed as a percentage of the maximal value (E19=26%); each data point is the mean \pm s.e.m. of triplicate cultures from a representative experiment.

play a role in rod commitment and/or differentiation *in vivo*. In addition, these results, taken together with the NIH 3T3 cell co-culture experiment and the recent experiments of Watanabe and Raff (this issue), indicate that the rod promoting activity is specifically produced by retinal cells from rod-generating developmental stages, and not as a non-specific product of all cells.

Discussion

We set out to study the role of cell-cell interactions in determining retinal cell type, with a long term goal of biochemically and/or molecularly characterizing the underlying signals involved. In this report we show that rod development, as measured by the expression of multiple markers of rod differentiation, is dependent upon the influence of other neonatal retinal cells. This effect is not due to selective death of rods in low density gel cultures, or to effects of cell density on the proliferation of progenitor cells. When this rod promoting activity is at low levels (in low density gels), a greater proportion of cells stain for an antigen characteristic of bipolar cells, suggesting that the choice of cell type is influenced by this activity. The activity can act without direct cell contact, and is temporally regulated in parallel to rod generation *in vivo*.

Cell type determination and differentiation can be

modeled as two separate processes: the choice of cell type, and maturation along a program specific to that cell type. It is possible that an activity required for opsin expression could influence the choice of cell type, in which case it would act as an instructive developmental signal. If, on the other hand, the activity was required by committed rod photoreceptors to continue differentiation, then it would act as a permissive signal. A third possibility is that a signal could be required both for rod determination, and then subsequently for progress along the differentiation pathway. We believe that the results described in this report are most consistent with the third possibility.

Lowering cell density leads to compensatory changes in the proportion of cells expressing antigens characteristic of rod (RETP1) and bipolar (115A10) cells

If rod determination was the process that could not occur at low density, one might expect uncommitted P0 progenitor cells, which otherwise would become rods, to be diverted into another pathway. This prediction was fulfilled, in that the proportion of progenitor cells becoming positive for a bipolar cell marker, 115A10⁺, showed a density dependence inverse to that observed for rods. Assuming that the 115A10 antigen was expressed on the same cell population in vitro as in vivo, the excess 115A10⁺ cells in this experiment represent bipolar cells. In this case, lowering cell density would create compensatory changes in the proportions of rod and bipolar cells, consistent with a change in cell fate. Bipolar cell development could then either represent a default pathway of development for postnatal retinal progenitor cells, or be stimulated by a signal that can act at a lower cell density than that required for rod development.

The interpretation described above requires confirmation with independent markers of bipolar cells, since an alternative explanation is that the 115A10 antigen is expressed in vitro on a non-bipolar class of cells that express it at undetectable levels, or not at all, in vivo. In order to provide independent confirmation that the observed 115A10⁺ cells represent bipolar cells, a number of other bipolar cell markers were investigated. An antibody to protein kinase C that labels rod bipolar cells (Negishi et al., 1988, Wood et al., 1988) did not stain either fresh dissociated retinal cells, or retinal cells in gel cultures, from the developmental stage (postnatal day 7) used in this study (data not shown). On the other hand, RETB1, which labels both rods and bipolar cells in vivo (Barnstable et al., 1983), stained the majority of cells in cultures of both high and low density (data not shown), supporting the model that the 115A10⁺ cells were either rods or bipolar cells, and not a third cell type. Since every other rod marker examined was diminished in low density gels (see Fig. 4), it seems likely that the RETB1 staining is due to the presence of bipolar cells in the culture. Finally, expression of *L7*, a gene expressed in the retina only in bipolar neurons, appeared grossly comparable in high and low density

cultures analysed using RT-PCR (data not shown); since the technique as used herein was insufficiently quantitative to show small differences in magnitude, this result confirms that there are bipolar cells present in both high and low density cultures, but does not resolve whether the number of bipolar cells is increased in low density gels.

In sum, the results discussed above suggest, but do not prove, that the cell-cell interaction described in this report acts as an instructive signal, influencing the determination of progenitor cells as either rod or bipolar cells.

Continued cell-cell interactions are required for postmitotic cells to differentiate as rod photoreceptors

As discussed, ³H-birthdating and retroviral lineage mapping both suggest that retinal cell type is determined during or soon after a progenitor cell's final mitosis. In addition, a recent report suggests that neocortical progenitor cells become committed to a laminar fate within hours of their final S-phase (McConnell and Kaznowski, 1991). In the retina, a long delay (>48-54 hours) exists between a presumptive rod's final mitosis and the first detectable expression of rhodopsin (Knight and Raymond, 1990, Watanabe and Raff, 1990). Thus, if determination occurred within hours of final mitosis, cells that have been determined as rods must exist in an opsin⁻ state for a significant period of time.

In low density gel cultures, the percentage of total cells that were opsin⁺ did not increase during the culture period. Many of the cells in these cultures, however, were postmitotic at the start of the experiment, and would normally become opsin⁺ if cultured in high density gels or left in vivo. This can be inferred from the observation that 23% of mitotic P0 cells become opsin⁺ after one week in vitro (Fig. 3B), and only 25% of total cells are mitotic in vitro (Table 1). Thus, the minority of new opsin⁺ cells that appear in high density cultures were mitotic during the culture period. More direct evidence that postmitotic cells require high density for opsin induction comes from experiments using retinal cells from P4 and P6 animals. In these cultures, virtually all cells were postmitotic at the start of the experiment. Many of these postmitotic, opsin⁻ cells would become opsin⁺ under normal circumstances, but no increase in opsin⁺ cells was observed. Thus, cells poised between terminal mitosis and the onset of detectable opsin expression were dependent upon high density culture, and continued cell-cell interactions, to initiate opsin expression. In addition, since cells taken from all developmental stages required high density culture in order to induce novel opsin expression, it would appear that the this influence was continuously required for this process to occur. More mature, opsin⁺ photoreceptors did not appear to require this cell-cell interaction to maintain opsin expression, however, since the overall percentage of opsin⁺ cells did not decrease in low density gel cultures.

These results show that postmitotic cells required high density culture in order to become opsin⁺. If these cells had already been determined as rods soon after mitosis, then they would appear to require a permissive developmental signal in order to continue differentiation.

Two models of rod photoreceptor development

Two models that can explain the apparent effects on cell type determination and differentiation are suggested on the basis of these observations. In the first, rod promoting activity is required only for rod determination, and cells that become determined immediately initiate opsin expression. The lag between birthday and opsin expression would, in this case, reflect a delay between a cell becoming postmitotic and being determined to become a rod. No increase in the number of rods would be observed in low density cultures, since all committed rods would already be opsin⁺ and the remaining postmitotic cells would not have been determined to become rods. This model fails to explain why retinal cell type *in vivo* would be tightly correlated with the time of cell birth, however, if cell type determination is occurring many days after final mitosis. In the second model, initiation of rod determination occurs around the time of the cell's final mitosis, but continued cell-cell interactions are required for maintenance of the decision and further differentiation. This would explain the correlation of cell type with birthday, as well as why no opsin⁻ cells became opsin⁺ in low density culture. Cells that were exposed to rod promoting signals for only a short period of time might either remain as committed rods whose differentiation could not continue, or might regress to an uncommitted state and again become responsive to influences that determine cell type.

Rod-promoting activity can act without cell-cell contact

Our experiments demonstrate that the rod promoting activity produced by neonatal retinal cells can act without direct cell contact. Recent work by Watanabe and Raff confirms this observation, demonstrating that neonatal retinal cells, but not cells from other sources, can stimulate rod differentiation (in pellets of E15 retinal cells) across a 0.01 micron filter (Watanabe and Raff, this issue). In their experiments, however, the activity could only influence cells placed a short distance (<<0.5 mm) from the source. The difference between this limited diffusion, and the greater diffusibility that we observe, may reflect differences in the release of activity from pellet cultures as compared to gels, or the binding of the activity to the filters used in their assay. Despite the activity's diffusibility *in vitro*, it seems likely that it may be highly localized *in vivo*, since progenitors can produce cell types other than rods (i.e. bipolars) even when rod generation is maximal. Binding of the activity to an extracellular matrix molecule is one possible mechanism for such localization. In addition, modulation of responsiveness to the

activity could play a role in determining where and when rod development occurs *in vivo*. Consistent with this idea, E15 cells were limited in their response to opsin promoting signals, relative to that of P1 cells (Watanabe and Raff, 1990).

Other developmental systems may offer clues as to the molecular nature of the activity described in this report. Many similarities exist between the vertebrate retina and that of *Drosophila* (e.g. cell-cell interactions, rather than lineage, are crucial for cell type determination; the phototransduction cascades use similar proteins). However, genetic and molecular studies have shown that the development of a *Drosophila* photoreceptor (R7) requires a direct cell-cell contact, mediated by two integral membrane proteins, the *boss* and *sevenless* gene products (Banerjee and Zipursky, 1990, Krämer et al., 1991). This mechanism for selecting cell type may be somewhat different from the diffusible interaction described here. An alternative possibility is that the rod-promoting signal is mediated by a peptide growth factor, examples of which apparently mediate cell-cell interactions in many other developmental systems [for example, *Xenopus* mesoderm induction (reviewed by Melton, 1991) and the development of the rat O2-A glial progenitor cell (reviewed by Raff, 1989; Lillien and Raff, 1990)]. In a preliminary screen, a number of well-characterized growth factors (including TGF- β 2, TGF- α , acidic and basic FGF, NGF, PDGF, and IGF-1) were tested for the ability to stimulate rod development in low density gel cultures: no increase in the number of rods was observed (D.M.A. and C.L.C., unpublished observations). Whether the rod promoting activity is a member of a previously identified protein family or a completely novel factor, the high level of rod induction in the culture system described above, and the diffusible nature of the activity, should facilitate its molecular characterization, and ultimately lead to a fuller understanding of vertebrate neural development *in vivo*.

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