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Purification of a membrane protein distributed in a topographic gradient in chicken retina

(neuronal differentiation/embryonic development/monoclonal antibodies/cell surface antigen)

JOSEPH R. MOSKAL*, DAVID TRISLER, MICHAEL D. SCHNEIDER†, AND MARSHALL NIRENBERG

Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

Contributed by Marshall Nirenberg, March 21, 1986

ABSTRACT **Antigenic molecules termed TOP, which are distributed in a dorsal > ventral concentration gradient in chicken retina, are expressed early in development (by 48 hr after fertilization) in the optic cup of chicken embryos and continue to be expressed in retina thereafter. ^{35}S-labeled-TOP–antibody complexes were purified by protein A-Sepharose column chromatography and subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis and autoradiography. TOP also was purified from dorsal retina by anti-TOP IgG-Affi-Gel 10 affinity column chromatography. In both cases, one major band of protein at $M_r \approx 47{,}000$ was obtained. A protein of $M_r \approx 47{,}000$ also was purified from chicken embryo brain. Cultured cells dissociated from 8-day chicken embryo retinas accumulated the amount of TOP expected of cells in the intact retina, depending on the position of the cells in the retina. TOP accumulations by cells dissociated from dorsal or ventral retina, mixed in different proportions, and cocultured were additive. These results show that TOP is a protein, that the gradient of TOP is established early in development, and that perpetuation of the gradient does not depend on the continuous presence of an extracellular gradient of diffusable molecules or on maintenance of interactions between cells.**

Previously, a monoclonal antibody was obtained that recognizes a cell surface antigen, TOP, that is distributed in a topographic dorsal > ventral concentration gradient in chicken retina (1). The gradient is established during embryonic development and also is expressed at all developmental stages thereafter, including adult retina. The antigen was detected on most or all cell types in retina and also was found in the cerebral cortex and thalamus, but little or no antigen was detected elsewhere in the nervous system or in other tissues. The antigen defines a dorsal–ventral axis of the retina and can be used to identify cell position on this axis. The concentration of TOP detected near the dorsal margin of chicken retina was at least 35-fold higher than that found near the margin of ventral retina, and the amount of TOP detected changed smoothly and continuously between the dorsal and ventral poles of the gradient (1).

In this report we describe the purification and characterization of TOP and other results that pertain to the problem of how a topographic gradient of protein is generated and perpetuated by retina cells. Preliminary accounts of some of the results have been presented (2, 3).

MATERIALS AND METHODS

Retina Cell Culture. Chicken embryo retinas were dissociated by incubation with trypsin and either collagenase or DNase I. Cells were plated on poly(L-lysine)-coated tissue culture dishes (8×10^7 cells per 100-mm dish). Culture medium was Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Cultures were maintained in an atmosphere of 5% CO_2/95% air at 100% humidity at 37°C.

TOP Radioimmunoassay. The assay has been described in detail (1). In brief, retina cell membranes were incubated either with anti-TOP monoclonal antibody or with antibody synthesized by parental P3X63 Ag8 cells. The membranes then were washed and incubated with affinity-purified ^{125}I-labeled rabbit F(ab')$_2$ directed against mouse IgG.

Purification of ^{35}S-Labeled TOP by Protein A-Sepharose Column Chromatography. ^{35}S-labeled TOP was obtained by culturing 8-day chicken embryo dorsal retina cells (8×10^7) with 1 mCi (1 Ci = 37 GBq) of L-[^{35}S]methionine per 6 ml of medium with serum (adjusted to a final concentration of 3.33 μM [^{35}S]methionine) per 100-mm Petri dish for 40 hr. [Better conditions for obtaining ^{35}S-labeled retina protein, which were found in experiments not shown here, are as follows: 8-day chicken embryo dorsal retina cells (6×10^7) were incubated in 10 ml of Eagle's minimal essential medium containing 10 μM [^{35}S]methionine (10 μCi) supplemented with 10% fetal bovine serum per 100-mm Petri dish. Cells were washed, harvested, and incubated at 4°C for 1 hr with 1 ml of solubilization buffer [Dulbecco's phosphate-buffered saline (without Ca^{2+} and Mg^{2+}) supplemented with 1% Triton X-100, 0.2% NaDodSO$_4$, 25 mM Hepes (adjusted to pH 7.4), 0.005% pancreatic DNase I, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1% ethanol]. The suspension was centrifuged for 60 min at 100,000 × *g* and the supernatant fraction was frozen. ^{35}S-labeled protein (2×10^8 cpm) in the supernatant fraction was incubated with ascites fluid containing either anti-TOP antibody or antibody synthesized by P3X63 Ag8 cells. The solutions were adjusted to 200 mM sodium phosphate (pH 8.0) and were applied to protein A-Sepharose columns (1.5-ml bed volume) that had been washed with 200 mM sodium phosphate, pH 8.0/1% Triton X-100/0.2% NaDodSO$_4$. Antibody–antigen complexes were eluted from each column with 100 mM citrate/phosphate, pH 5.9/1% Triton X-100, 0.2% NaDodSO$_4$/600 mM NaCl (4–6). Eluted protein was precipitated with 15% (wt/vol) trichloroacetic acid and subjected to NaDodSO$_4$/7% polyacrylamide gel electrophoresis.

Purification of TOP by Antibody-Affi-Gel 10 Affinity Column Chromatography. Anti-TOP IgG and control IgG were obtained by culture of hybridoma and of parental P3X63 Ag8 cells as described (1). The media were collected and the antibodies were purified by protein A-Sepharose affinity column chromatography (4). Antibodies were precipitated with ammonium sulfate (70% saturated), dialyzed against 50 mM sodium borate, pH 8.5/150 mM NaCl, and applied to protein A-Sepharose columns. The columns were washed, and antibodies were eluted with 0.1 M citrate/phosphate, pH

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*Present address: Laboratory of Cell Biology, Building 36, Room 3A-17, National Institute of Mental Health, Bethesda, MD 20892.
†Present address: Section of Cardiology, Baylor College of Medicine, 6535 Fannin, MSF905, Houston, TX 77030.

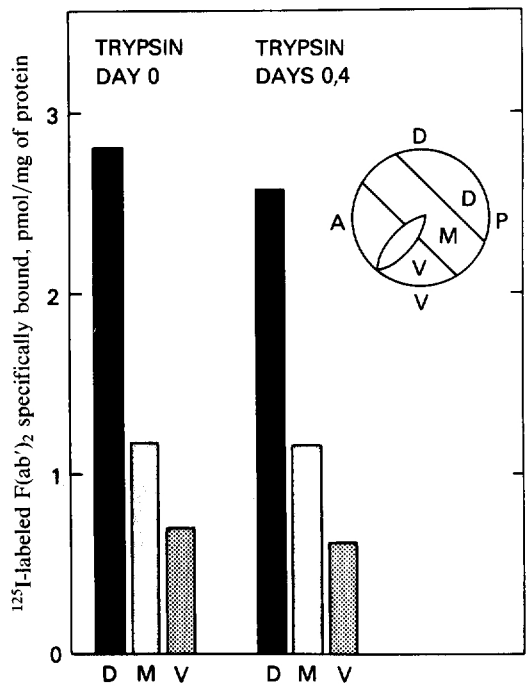

FIG. 1. Expression of TOP antigen by retina cells from dorsal, middle, or ventral 8-day chicken embryo retina that had been dissociated with 0.05% trypsin and 0.003% collagenase and cultured for 10 days. On the 4th day of culture, where indicated, cells were dissociated again with trypsin and collagenase as described above, washed, and transferred to new 100-mm dishes and incubated for an additional 6 days. Radioimmunoassay of TOP was performed as described in *Materials and Methods*. D, M, and V refer to dorsal, middle, and ventral retina, respectively; A and P refer to anterior and posterior retina, respectively.

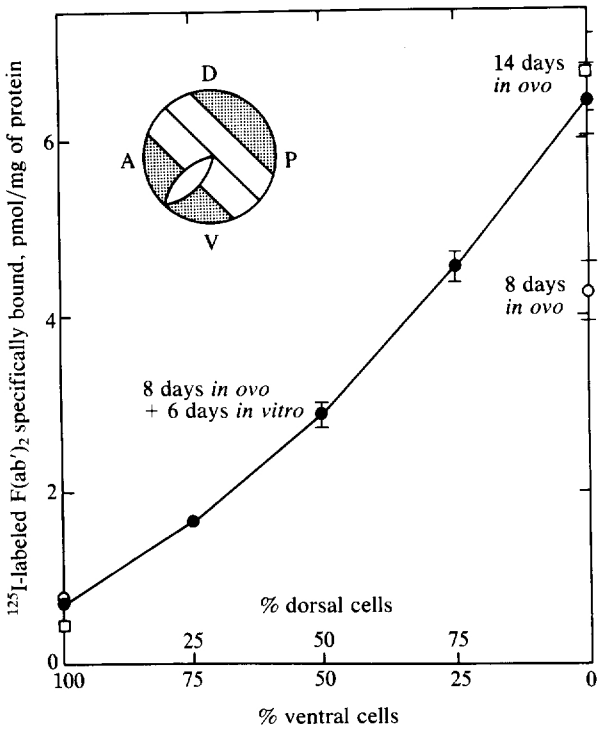

FIG. 2. TOP antigen expression in monolayer cultures containing varying proportions of cells dissociated with 0.05% trypsin in the presence of 0.005% pancreatic DNase I from dorsal or ventral 8-day chicken embryo retina. Each 100-mm Petri dish contained 8×10^7 retina cells and 15 ml of medium. After 6 days of culture, TOP was quantitated by radioimmunoassay. The open circle and open square on the left ordinate refer to pmol of ^{125}I-labeled F(ab')$_2$ specifically bound per mg of protein in freshly dissected 8-day embryonic ventral retina and 14-day embryonic ventral retina, respectively. The open circle and open square on the right ordinate refer to pmol of ^{125}I-labeled F(ab')$_2$ specifically bound per mg of protein in freshly dissected 8-day chicken embryo dorsal retina and 14-day embryo dorsal retina, respectively. D, V, A, and P refer to dorsal, ventral, anterior, and posterior, respectively.

3.5/1 M NaCl, dialyzed against phosphate-buffered saline, and coupled to Affi-Gel 10 (Bio-Rad). Each antibody-Affi-Gel 10 column was adjusted so that 1 mg of IgG was covalently coupled to 1 ml of Affi-Gel 10. One hundred freshly dissected dorsal halves of 14-day chicken embryo retinas or 20 brains minus the cerebella were homogenized with 20 strokes in a Dounce homogenizer and incubated at 4°C for 60 min in solubilization buffer supplemented with 5 mM EGTA (5 mg of protein per ml). The suspensions were centrifuged for 30 min at 30,000 × *g*, and equal portions of the supernatant fractions containing solubilized protein were applied to the antibody-Affi-Gel 10 columns at flow rates of ≈5 ml/hr. Each effluent was cycled through the appropriate column two additional times. Each column was washed with 100 ml of solubilization buffer and then with 100 ml of solubilization buffer with 0.1%, rather than 1%, Triton X-100 and without $NaDodSO_4$. Protein was eluted with 200 mM acetic acid, pH 3.0/0.1% Triton X-100/150 mM NaCl (7). Eluates were neutralized with sodium carbonate, and dithiothreitol and $NaDodSO_4$ were added to portions (1.5 ml) of the eluate (total eluate collected, 4 ml) at final concentrations of 5 mM and 2%, respectively. The samples were diluted 1:10 with Laemmli's tank buffer (8) and were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis as described by Neville (9).

RESULTS

TOP is expressed early in embryonic development in the optic cup, approximately 48 hr after fertilization. As determined by TOP radioimmunoassay, tissue from the optic cup, head, and trunk of 48-hr embryos specifically bound 3.78, 0.88, and 0.59 pmol of ^{125}I-labeled F(ab')$_2$ per mg of protein, respectively. The number of TOP molecules detected in the optic cup approached the levels detected in the adult retina. Low levels of TOP were detected in optic vesicles, 33 hr after fertilization (data not shown).

We next asked whether dissociated chicken embryo retina cells from dorsal, middle, or ventral retina that were cultured *in vitro* express the amount of TOP that would be expected of cells in those regions of the intact retina *in ovo* (Fig. 1). Little or no TOP was detected immediately after 8-day chicken embryo retina cells were dissociated to single cells with trypsin, because trypsin destroys the antigenicity of TOP (1). However, after 10 days of culture, TOP accumulations by cultured cells from dorsal, middle, or ventral retina were similar to the amounts of TOP detected in the appropriate regions of the intact retina. Similar results were obtained when retina cells were trypsinized, cultured for 4 days, trypsinized again, cultured for an additional 6 days, and then assayed for TOP. These results show that TOP is synthesized by cultured retina cells, and that the amount of TOP detected is dependent on the original position of the cells in the intact retina.

Trypsinized cells from 8-day chicken embryo dorsal retina and ventral retina were mixed in different proportions, and cells then were cultured for 6 days to determine whether interactions between cells from different topographic regions of retina regulate the expression of TOP. The results (Fig. 2) show that TOP accumulations by cocultured dorsal and ventral retina cells were approximately additive. Thus, in-

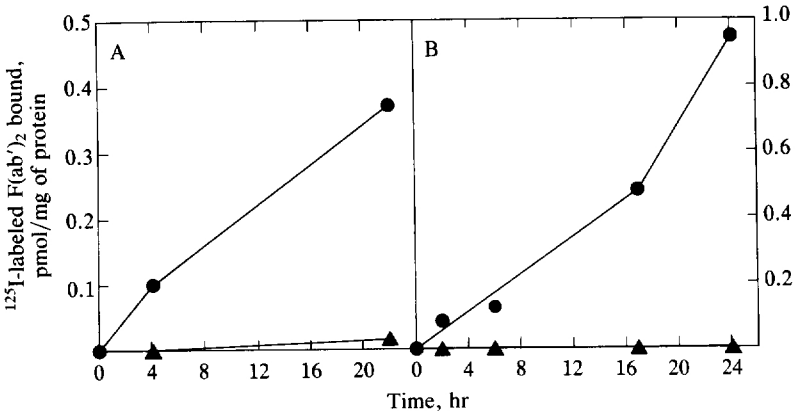

FIG. 3. Inhibition of TOP synthesis in cultured retina cells by cycloheximide (*A*) or actinomycin D (*B*). Cells were dissociated from dorsal halves of 8-day chicken embryo retina with 0.05% trypsin (which destroys the antigenicity of TOP) and 0.005% pancreatic DNase I in phosphate-buffered saline without Ca^{2+} or Mg^{2+} and were washed with medium containing 1 mM phenylmethylsulfonyl fluoride. Each 100-mm Petri dish coated with poly(L-lysine) was innoculated with 8×10^7 cells. Cells were cultured in the absence (●) or presence (▲) of cycloheximide (2 μg/ml of medium) or actinomycin D (1 μg/ml of medium). Cells were harvested at the indicated times and frozen until assayed for TOP as described in *Materials and Methods*.

teractions between cocultured dorsal and ventral retina cells had little or no effect on TOP accumulation.

Cycloheximide (2 μg/ml) or actinomycin D (1 μg/ml) almost completely inhibited the synthesis of TOP by cultured cells dissociated from dorsal 8-day chicken embryo retina with trypsin (Fig. 3). These results suggest that the expression of TOP by cultured retina cells is dependent on *de novo* protein synthesis, rather than on posttranslational modification of precursor protein molecules.

Incubation of 8-day chicken embryo retina cells with 50 ng of tunicamycin per ml of medium for 24 hr resulted in a decrease in the amount of TOP detected, to 32% of the value found with cells cultured without tunicamycin (Table 1). This suggests that TOP may be a glycoprotein synthesized via a dolichol-oligosaccharide intermediate.

Incubation of dorsal 8-day chicken embryo retina cells with wheat germ agglutinin decreased antibody binding to retina cell membranes, whereas concanavalin A or *Ulex europaeus* I lectins increased antibody binding to retina cell membranes 204% and 130%, respectively (Table 1). In contrast, incubation of retina cells with bovine brain gangliosides had little or no effect on the binding of anti-TOP antibody to retina cell membranes (1). These results show that the amount of antibody specifically bound to TOP can be increased or decreased by the binding of a lectin to an appropriate receptor.

Table 1. Characterization of TOP

Treatment	^{125}I-labeled F(ab')$_2$ specifically bound to retina membranes, % control
*Experiment 1**	
None (control)	100 (467 cpm per well)
Wheat germ agglutinin	58
Concanavalin A	204
Ulex europaeus lectin	130
Experiment 2†	
None (control)	100 (1390 cpm per well)
Tunicamycin 50 ng/ml	32
Tunicamycin 100 ng/ml	19

^{125}I-labeled F(ab')$_2$ concentration was 440 nM.

*Each 50-μl reaction mixture (containing 10^6 dissociated 8-day chicken embryo retinal cells) was incubated for 15 min with or without the indicated lectin (50 μg) before quantitation of TOP by radioimmunoassay (1).

†Cells were exposed to tunicamycin for 24 hr before TOP was assayed. At these concentrations, tunicamycin inhibited [^{3}H]mannose incorporation into trichloroacetic acid-precipitable protein but did not significantly reduce incorporation of [^{14}C]leucine. Leupeptin (50 μM) had little or no effect on the amount of TOP detected in the absence or presence of tunicamycin.

In addition, each well of 96-well multiwell plates was coated with 0.1–100 ng of a glycosaminoglycan, either heparin, chondroitin sulfate, or hyaluronic acid. Wells were washed, incubated with antibody to TOP, washed, and then incubated with ^{125}I-labeled F(ab')$_2$ (440 nM) directed against mouse immunoglobulin, produced in rabbits. No specific binding of anti-TOP antibody to the glycosaminoglycans tested was detected. [In this experiment, in the absence of glycosaminoglycans, 1390 cpm of ^{125}I-labeled F(ab')$_2$ was bound per well.]

^{35}S-labeled TOP was obtained by culturing dissociated 8-day chicken embryo retina cells with [^{35}S]methionine for 40 hr. Cells were washed, and ^{35}S-labeled protein was solubilized with 1% Triton X-100/0.2% $NaDodSO_4$ and incubated either with antibody to TOP or with antibody synthesized by parental P3X63 Ag8 cells. ^{35}S-labeled protein–IgG complexes and free IgG were retained by protein A-Sepharose at pH 8.0. The columns were washed and the labeled protein–IgG complexes and free IgG were eluted at pH 5.9. The eluted ^{35}S-labeled protein then was subjected to nonreducing $NaDodSO_4$/polyacrylamide gel electrophoresis. Under these conditions anti-TOP IgG–TOP complexes dissociate, whereas protein subunits of IgG do not dissociate from one another. ^{35}S-labeled proteins were visualized by staining with Coomassie blue and by autoradiography (Fig. 4). Only one major band of protein was detected by Coomassie blue staining, at $M_r \approx 150{,}000$, which corresponds to IgG directed against TOP or IgG synthesized by P3X63 Ag8 cells. Autoradiography of the same gel revealed only one band of

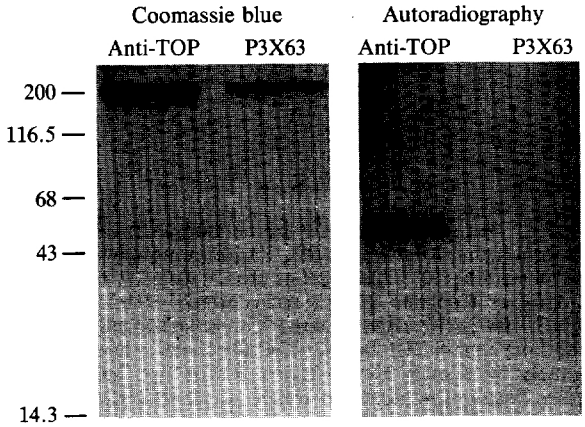

FIG. 4. Purification of ^{35}S-labeled TOP by protein A-Sepharose column chromatography. Cell extracts were incubated with ascites containing either anti-TOP or antibody synthesized by P3X63 Ag8 cells. Antigen–antibody complexes were isolated by chromatography on protein A-Sepharose, precipitated with 15% trichloroacetic acid, and analyzed by $NaDodSO_4$/PAGE followed by staining with Coomassie blue and then by autoradiography. Positions of molecular weight standards ($M_r \times 10^{-3}$) are at left.

^{35}S-labeled protein dissociated from anti-TOP IgG–antigen complexes, at $M_r \approx 47{,}000$. No band of ^{35}S-labeled protein was detected with P3X63 Ag8 IgG.

Anti-TOP IgG or IgG synthesized by parental P3X63 Ag8 cells was purified from medium harvested from hybridoma or P3X63 Ag8 cells by protein A-Sepharose affinity column chromatography. Each antibody preparation then was coupled to Affi-Gel 10 (agarose with a 10-atom-spacer arm terminating in a reactive *N*-hydroxysuccinimide ester). Solubilized protein samples from 14-day chicken embryo retina or brain were applied to an anti-TOP IgG-Affi-Gel 10 column and a P3X63 Ag8 IgG-Affi-Gel 10 column. Columns were washed, antigens were eluted and then were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions, and proteins were visualized by silver staining. Only one band of protein ($M_r \approx 47{,}000$) from retina or brain was detected in eluates from the anti-TOP IgG-Affi-Gel 10 column (Fig. 5). Little or no protein was detected in the eluates from the P3X63 Ag8 IgG-Affi-Gel 10 column. Protein eluted from the anti-TOP IgG-Affi-Gel 10 column also was subjected to isoelectric focusing. Only one band of protein from retina or brain was detected, with a pI $\approx$ 4.1 (data not shown).

DISCUSSION

TOP, a plasma membrane protein that is distributed in a large dorsal > ventral gradient in chicken retina (1), was solubilized and purified extensively. The apparent M_r of TOP is $\approx$47,000 and the apparent pI is 4.1. A protein with the same M_r and pI also was purified from chicken embryo brain. We estimate that TOP is a moderately abundant membrane glycoprotein in 14-day chicken embryo dorsal retina, comprising perhaps 0.2% of the plasma membrane protein but only about 0.01% of total retina protein.

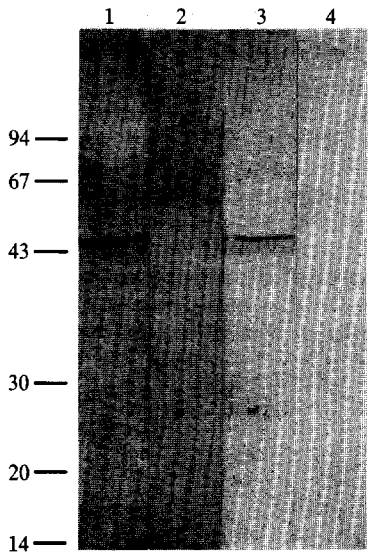

FIG. 5. Purification of TOP by antibody-Affi-Gel 10 column chromatography. After $NaDodSO_4$/PAGE, proteins were visualized by silver staining (10). Lane 1: retina protein eluted from an anti-TOP IgG-Affi-Gel 10 column. Lane 2: retina protein eluted from a P3X63 Ag8 IgG-Affi-Gel 10 column. Lane 3: Cerebrum and thalamus protein eluted from an anti-TOP IgG-Affi-Gel 10 column. Lane 4: Cerebrum and thalamus protein eluted from a P3X63 Ag8 IgG-Affi-Gel 10 column. Positions of molecular weight standards ($M_r \times 10^{-3}$) are at left.

Cells dissociated from 8-day chicken embryo retina with trypsin, which destroys TOP antigenicity, synthesize and accumulate the amount of TOP during subsequent culture that would be expected of cells in the intact retina, dependent upon the original position of the cells in the retina. Thus, populations of single cells that were dissociated from retina and cultured continue to synthesize and accumulate the amounts of TOP that would constitute a gradient in the intact retina. Coculture of cells dissociated from dorsal or ventral retina and mixed in different proportions did not affect TOP accumulation by the cells; hence, no regulation of TOP expression was detected that could be attributed to interactions between cells from dorsal and ventral retina.

TOP is expressed early in development, approximately 33 hr after fertilization, by cells in the optic vesicles of chicken embryos before the retina is formed. At approximately 48 hr after fertilization, as the optic cup is formed, the number of TOP molecules detected per milligram of total protein is similar to the levels that are found in adult retina. These results suggest that TOP is expressed by dividing cells that give rise to retinal cells and that the gradient of TOP is established as retinal cells are generated. The results also suggest that the gradient of TOP is established early in development by transient events that regulate either the expression or the accumulation of TOP in each cell. The gradient, once formed, is relatively stable. Perpetuation of the gradient does not depend on the continuous presence of an extracellular gradient of diffusable molecules or on the maintenance of interactions between cells.

We assume that cell progeny inherit the parental ability to express the TOP gene or genes. The possibility that multiple rounds of cell division are required to establish a gradient of TOP expression (for example, by differential DNA methylation) requires further study. Our working hypothesis is that the gradient in cellular accumulation of TOP reflects a gradient in the rate of expression of the TOP gene or genes, but other possibilities, such as regulation of turnover of TOP mRNA or protein, are not ruled out.

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