

EXPRESSION OF aFGF AND FGF9 IN THE DEVELOPING RAT RETINA

by

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To my family...

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ABSTRACT

aFGF, a member of the FGF family of growth factors, has pleiotropic effects on different cellular contexts. It has been postulated to be important in the physiology and development of vertebrate retina. The conflicting immunohistochemical data on aFGF localization within the retina led us to determine the distribution of aFGF mRNA in the two retinal layers using RT-PCR and southern hybridization techniques during rat retinal development. The external layer is composed primarily of the photoreceptors, and the internal layer contains the interneurons, Muller glial cells, and the ganglion cells. The approach provides higher specificity than immunological detection techniques, and is defined by specific primers in the PCR reactions, characterization of the PCR product by restriction digestion analysis and/or hybridization with nested oligonucleotide probes. The PCR reactions were performed at exponential phases, enabling us to compare the initial transcript levels in different samples.

aFGF was detected in both layers, which unequivocally places aFGF expression to cells other than photoreceptors. Its expression pattern suggests that it is involved in developmental events subsequent to photoreceptor differentiation, which implicates aFGF in the further maturation of the retina to attain its final physiology. Furthermore, the difference in the aFGF expression patterns in the two layers may suggest different roles of aFGF for retinal cell types, in addition to its potential role as a survival or maintenance factor.

The wide spread expression of FGF9, another member of the FGF family, in the central nervous system raises the possibility of its presence in the retina. RT-PCR strategy was performed to investigate FGF9 expression in the developing retina. We show that FGF9 is expressed, albeit at moderate levels, in the developing rodent retina. Its mRNA was detected in all postnatal days studied, as well as in embryonic days, suggesting that FGF9 is might be important as a mitogen and may function as a survival factor for the retinal cell types.

ÖZET

FGF gen ailesi üyelerinden aFGF'nin, omurgalı retinasının fizyolojisi ve gelişiminde önemli olduğu düşünülmektedir. Bu çalışmada, ters-transkriptaz polimeraz zincir reaksiyonu ve Southern Hibridizasyonu kullanılarak, aFGF mRNA'sının dış fotoreseptör katmanında, ve internöron, Müller glia hücreleri, ganglion hücrelerini kapsayan iç katmandaki dağılımı araştırılmış, gelişim sürecinde gen anlatımı düzeyi karşılaştırmalı olarak irdelenmiştir. Bu teknik, spesifik primerlerin kullanımı, PCR ürününün restriksiyon enzimleri kullanılarak veya spesifik problemlerle hibridizasyonu ile karakterizasyonuna olanak sağlamaktadır. Amplifikasyonlar, transkript düzeylerinin karşılaştırılabilmesi için, reaksiyonun logaritmik fazında gerçekleştirilmiştir.

aFGF'nin iki katmanda da gözlenmesi, aFGF anlatımını açık bir şekilde iç katmanı oluşturan hücrelere de yerleştirmiştir. aFGF'nin anlatım profili, aFGF'nin fotoreseptör doğumundan sonraki gelişim olaylarında, retinanın ileri aşamadaki olgunlaşmasında rol oynadığına işaret etmektedir. İki katmandaki aFGF ekspresyon profilindeki farklılık, bu faktörün farklı hücre tipleri için değişik roller oynadığını önermektedir.

FGF9'un merkezi sinir sistemindeki yaygın anlatımı, bu faktörün retinada varolma olasılığını güçlendirmektedir. Bu çalışmamızda, FGF9'un retinada, bütün postnatal günlerde ve onyedinci embryonik günde varlığı, ters-transkriptaz polimeraz zincir reaksiyonu kullanılarak gösterilmiştir. Böylece, bu faktörün retina hücreleri için hem mitojenik hem de yaşam faktörü olduğu savını doğrulamaktadır.

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ABBREVIATIONS

aFGF	acidic Fibroblast Growth Factor
ATP	Adenosine Triphosphate
BDNF	Brain Derived Growth Factor
bFGF	basic Fibroblast Growth Factor
cal	Calbindin
cDNA	complementary DNA
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
DNA	Deoxyribonucleic Acid
depc	Diethylpyrocarbonate
dNTP	Deoxyribonucleotide Triphosphate
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetate
EDGF	Eye-derived Growth Factor
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
GDNF	Glial cell line-derived Growth Factor
GDP	Guanosine Diphosphate
GMP	Guanosine Monophosphate
G3PDH	Glyceraldehyde-3-phosphodehydrogenase
GTC	Guanidium isothiocyanate
GTP	Guanosine Triphosphate
HSPG	Heparin Sulfate Proteoglycan
Ig	Immunoglobulin
IGF	Insulin-like Growth Factor

INL	Inner Nuclear Layer
IPL	Inner Plexiform Layer
LIF	Leukemia Inhibitory Factor
MAP	Mitogen Activating Protein
MAPK	Mitogen Activating Protein Kinase
MMLV-Rtase	Moloney Murine Leukemia Virus-Reverse Transcriptase
MOPS	3-N-morpholino propanesulfonic acid
NaOAc	Sodium Acetate
NGF	Nerve Growth Factor
NT-3	Neurotrophin-3
OD	Optical Density
ONL	Outer Nuclear Layer
op	opsin
OPL	Outer Plexiform Layer
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PLC- γ	Phospholipase C- γ
PN	Postnatal Day
PR	Photoreceptor
RCS	Royal College of Surgeons
RNA	Ribonucleic Acid
RPE	Retinal Pigment Epithelium
RT-PCR	Reverse Transcription Coupled Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SH2	Src-Homology Domain 2
TBE	Tris-Boric acid-EDTA
TGF- α	Transforming Growth Factor alpha
TGF- β	Transforming Growth Factor beta

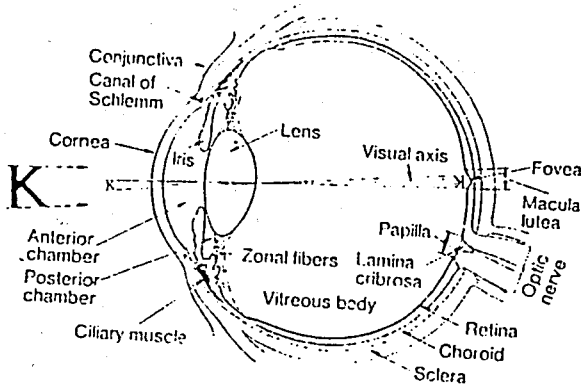
I. INTRODUCTION

A. Vertebrate Retina

The retina is a thin sheet of neural tissue that lines the back of the eye (Figure I.1.A). The retina contains a massive array of photoreceptor cells that are responsible for the transduction of light into neurochemical signals to start the visual process. The transfer of information is not passive but is a result of a complex processing of the signals. The photoreceptors synapse on a number of horizontal and bipolar cells in the outer plexiform layer. Further information is extracted through synapses between bipolar cells and another class of interneurons, the amacrine cells. Retinal ganglion cells, the output neurons of the retina, relay the integrated information arriving from the interneurons to various target locations within the brain, and visual perception is based on the cortical processing of these signals.

The different cell types that make up the mature retina are distributed in a highly ordered laminar organization where the cell bodies are located in three well defined layers (Figure I.1.B): The outer nuclear layer (ONL) is occupied exclusively by the photoreceptors; the inner nuclear layer (INL) contains the bipolar, horizontal, amacrine interneurons, and the non-neuronal Muller glial cells; and the ganglion cell layer. The synaptic contacts between the photoreceptors and the interneurons are made in the outer plexiform layer (OPL). The synaptic connections of INL neurons and ganglion cells lay in the inner plexiform layer (IPL). The axons of the ganglion cells project into the brain where the cognitive processing takes place.

A.



B.

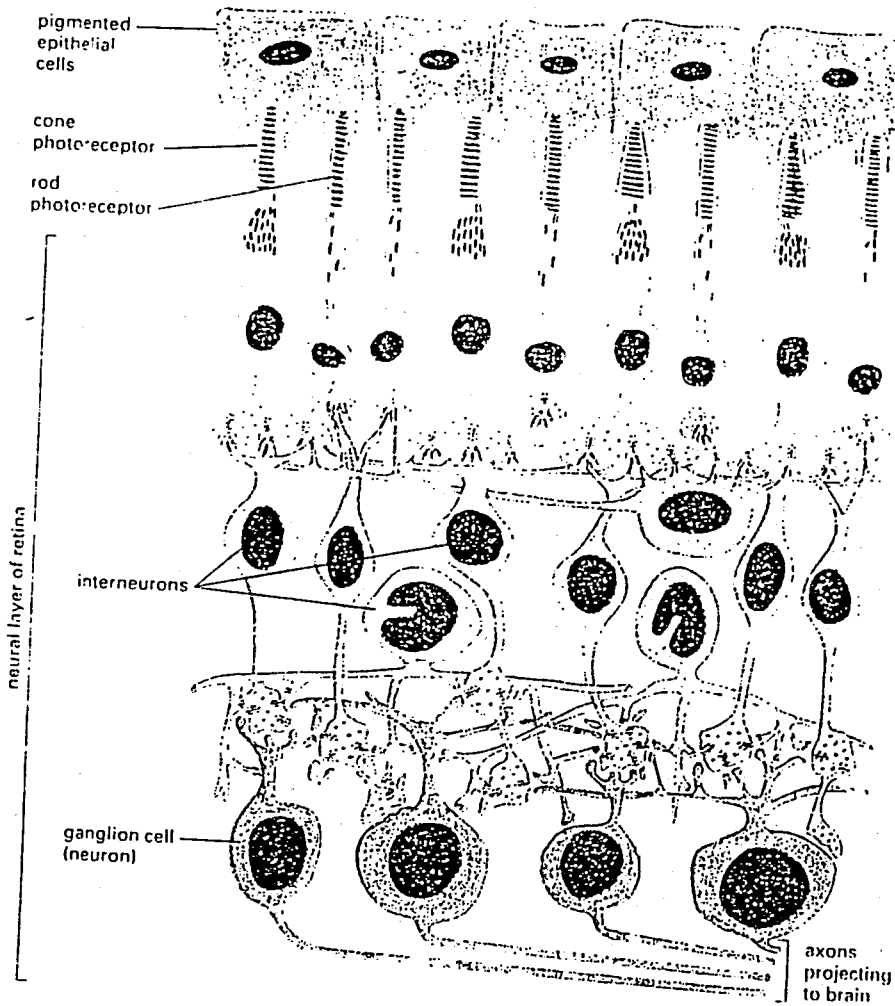


Figure 1.1. The Eye. (A) Cross section of the vertebrate eye; (B) The retinal cell layers.

1. Photoreceptor Cells and Phototransduction Cascade

The phenomenon of phototransduction is related to the structural and molecular properties of the photoreceptor cells. In vertebrates there are two major photoreceptor subtypes, the rods and the cones. Both rods and cones are elongated and polarized cells. At one end of the cell is a highly specialized synaptic terminal, connected by a short axon to a cell body occupied almost exclusively by the nucleus. The next compartment is the inner segment, which is connected by a modified cillium to the outer segment. Outer segment is a unique structure characterized by the presence of stacks of membranous disks abundantly studded with the visual pigment. This highly structural organization is reflected in the distribution of the molecular components (Deredict and Papermaster, 1991). The outer segment is highly enriched in the visual pigment opsin and components of the phototransduction cascade (Lagnado and Baylor, 1992). The outer segments of rods and cones differ not only in their shape, but also in that there is a complete separation of membranous discs from the plasma membrane in rods, but not in cones. Both photoreceptors are involved in the phototransduction, but rods are specialized for vision in dim light, whereas cones are responsible for color vision. Human beings and other primates have three subtypes of cones based on the wave length of light to which they are most sensitive: blue, green, and red. Visual pigment structure is similar in all photoreceptors, and involves an apoprotein (opsin) conjugated through a Schiff base to a vitamin A derivative, 11-cis retinaldehyde. 11-cis retinaldehyde is responsible for the light sensitivity of all the visual pigments, their characteristic spectral sensitivities are determined by the molecular differences in their respective apoproteins. Although there is great homology in the opsins present in the rods and cones, there are differences in their amino acid sequences (Nathans, 1987; Neitz *et al.*, 1991). Rods and cones also show sequence differences in other components of the phototransduction process, including transducin and phosphodiesterase subunits (Pittler and Baehr, 1991).

Phototransduction, the mechanism of visual excitation, is typically the same for all photoreceptors (Liebman *et al.*, 1987). The initial event in the visual excitation is the isomerization of 11-cis retinaldehyde to 11-trans retinaldehyde which is followed by conformational changes in the opsin molecule. Photoexcited rhodopsin triggers an

enzymatic cascade resulting in the hydrolysis of cyclic GMP. Activated rhodopsin binds to transducin, a G-protein which interconverts between its inactive GDP state and active GTP state, and catalyzes the exchange of GTP for bound GDP. The binding of GTP to transducin leads to both the release of activated rhodopsin for another round of catalysis and the dissociation of the GTP bound α subunit of transducin from $\beta\gamma$ subunits. The dissociated α subunit then activates the phosphodiesterase, which very rapidly hydrolyzes cyclic GMP. Consequently, the decrease in the level of cyclic GMP in the cytosol closes the Na^+ channels present on the rod membranes in the outer segment, which are open in dark, when the cyclic GMP levels are high. This results in the decrease of the Na^+ influx, and the plasma membrane becomes hyperpolarized. This hyperpolarization, which is the receptor potential, passively spreads from the outer segment to the synaptic terminal of the photoreceptor. Here the potential change leads to a reduction of neurotransmitter release from the synaptic terminal.

2. Interneurons and Intraretinal Integration

The input from the photoreceptor cells are received principally by horizontal and bipolar cells of the INL. Some bipolar cells respond with hyperpolarization and others with depolarization. The bipolar cells synapse on amacrine cells, ganglion cells, and they have feedback synapses on PR terminals (Figure I.1.A). Often several PR cells synapse with single bipolar cell, and several bipolar cells synapse with a single ganglion cell. Besides such convergence circuits, there is a lateral transfer of information via horizontal cells, which are electronically coupled large cells extending laterally, each of which receives input from many PRs and synapses on a number of bipolar cells and other horizontal cells. It is suspected that there are more than 20 different subtypes of amacrine cells, but very little is known about their specific functions. Amacrine cells extend laterally for considerable distances, interact with bipolars, ganglion cells and other amacrine cells. They are thought to be involved in lateral inhibitory sharpening of response acuity along with the horizontal cells. Ganglion cells are the final retinal processing sites. Each ganglion cell is

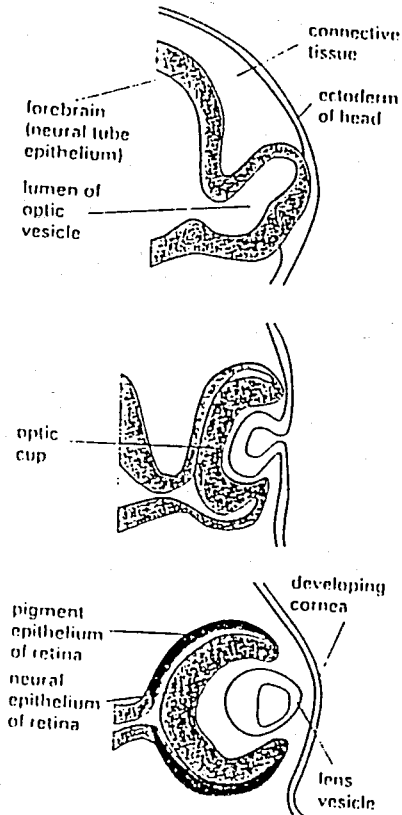
spontaneously active, receives signals from an immense number of receptors and bipolars, thus have a large receptive area. Through this complex inhibitory and excitatory synaptic network, there is summation of response over many receptors, and signal may be amplified, noise filtered, signal redundancy is reduced. As a result of this complex processing event, data on color information, shape, orientation and motion are relayed by the axons of the ganglion cells bundled into the optic nerve, to the thalamus and the visual cortex.

B. Development of the Retina

In the embryonic development, vertebrate retina begins to form with the outpocketing of the diencephalon during early neurulation (Figure I.2.A). The tip of the stalk contacts the overlying ectoderm, and that contact seems to start a series of inductive events involving both tissues (Gilbert, 1994). The ectoderm thickens and invaginates, forming the lens vesicle. The lens vesicle soon separates to form the lens, and the overlying ectoderm goes on to form the epithelium of the cornea. Most of the remaining tissues of the eyeball, including the coroid, the ciliary muscle, corneal stroma and the fibroblasts of the sclera, are primarily derived from the neural crest material.

The tip of the optic stalk itself invaginates at the same time with the ectoderm, and will form the optic cup with two layers of morphologically uniform cells (Morse and McCann, 1984). The progenitor cells of the outer layer will form the pigment epithelium (non-neuronal). All six different neuronal cell types and the glial cells of the mature retina derive from the progenitor cells of the inner layer of the optic cup. In the process of forming functional retina, the cells migrate to the proper layer following their final mitosis, complete their differentiation, and form right synaptic contacts.

A.



B.

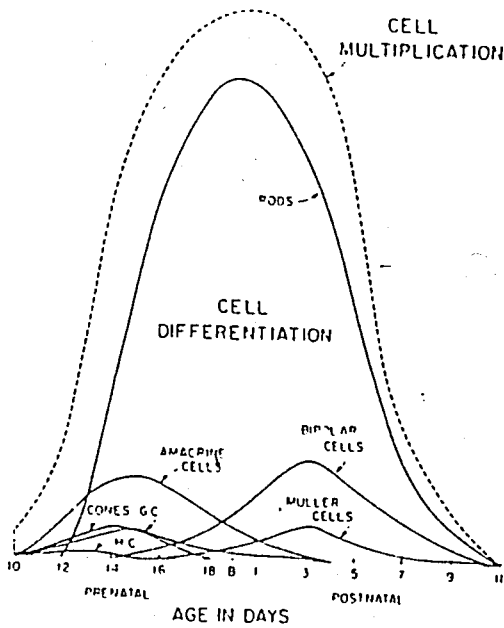


Figure 1.2. Eye Development. (A) Development of the vertebrate eye; (B) Birth schedule of retinal cells in rodents.

1. Formation of Retinal Cell Types

The birthdating experiments (Sidman, 1961; Carter-Dawson and LaVail, 1979; Young, 1985) performed with rodents revealed that the ganglion cells are the ones that become post-mitotic first and complete their differentiation before birth; cones, horizontal and amacrine cells are born at overlapping times in the embryo. Rods, bipolars and glial cells are born postnatally (Figure 1.2.B). Rods are the last cells to complete their differentiation. Cell-lineage studies using retroviral or injected tracers indicate that diverse cell types can be derived from a common progenitor cell in all the vertebrate species examined (Turner and Cepko, 1987; Wetts and Fraser, 1988; Holt et al., 1988; Turner et al., 1990). For example, among the clones of a single retinal progenitor cell rods, bipolars and glial cells can be found, indicating that the progenitor cells are multipotent. These findings emphasized the importance of environmental cues on the cell fate decisions of the progenitor cells. However, In Vitro studies and transplantation experiments suggest that competence of the progenitors do change in time, thus the intrinsic properties of these may equally be important in choosing among the possible cell fates (Wanatabe and Raff, 1990; Austin et al., 1995). For example, retinal cells from embryonic day 15 cultured in the presence of excess cells from neonatal retina were unaltered in their timing of opsin expression, suggesting that progression to the state of commitment to rod pathway controlled by intrinsic programming of the cells. Recently Cepko et al. proposed a model (1996) based on an interplay with the intrinsic programming of progenitors and the environmental signals. According to the model, the progenitor cells undergo series of changes in their intrinsic properties that enable them to respond to specific environmental cues to generate different cell types, the commitment to a particular cell type is suggested to be under the control of the environmental signals. In keeping with the current understanding of pattern formation and regional specification in animal development, the transcription factor repertoire of the progenitor cells and corresponding cis-regulatory elements will shape the progression to a specific differentiated phenotype (Cepko et al., 1996; Freund et al., 1996).

2. Growth Factors Implicated in Retinal Development

The most prominent candidate environmental signalling molecules affecting the survival and differentiation of retinal cells are the growth factors. Members of the neurotrophin family, FGF family, TGF family and other polypeptide growth factors and their receptors have been identified in retina and/or the pigment epithelium. While *In Vitro* experiments point to potential roles in proliferation, differentiation and survival, *in vivo* functions remain largely unknown. The work with transgenic animals imply the redundancy in their functions and suggests there exists an interplay of growth factors in the shaping of the nervous system (Marshall, 1995).

The neurotrophin family members are known as target derived survival and differentiation factors in the central and peripheral nervous system (Yuen et al., 1996). The members of the neurotrophin family detected in the mature and developing retina includes Nerve growth factor (NGF; Ebendal and Persson, 1988; Chakrabarti et al., 1990), Brain derived growth factor (BDNF; Maisonpierre et al., 1991; Perez and Caminos, 1995), and Neurotrophin-3 (NT-3; Bovolenta et al., 1996; Hallbook et al., 1996). Their receptors, *trk* family of receptor tyrosine kinase, are expressed in majority of retinal cells (Carmignoto et al., 1991; Jelsma et al., 1993; Rodriguez-Tebar et al., 1993; Hallbook et al., 1996; Garner et al., 1996). These neurotrophins have been shown to promote neurite outgrowth and ganglion cell survival *In Vitro* (Johnson et al., 1986; Cargmignoto et al., 1989; Rodriguez-Tebar et al., 1989; Thanos and Von Boxberg, 1994; Cohen-Cory and Fraser, 1994; De La Rosa et al., 1994).

Epidermal growth factor (EGF) and Transforming growth factor α and β (TGF- α and TGF- β , respectively; Fassio et al., 1989; Lutty et al., 1991), Platelet-derived growth factor (PDGF; Mudhar et al., 1993), insulin-like growth factor (IGF; Waldbillig et al., 1991), and Glial cell line-derived neurotrophic factor (GDNF; Choi-Lundberg and Bohn, 1995; Trupp et al., 1995) are also detected in the retina. GDNF enriched cell cultures doubles the number of photoreceptor cells and stimulate neurite extensions (Jing et al., 1996), and PDGF is postulated to mediate ganglion cell-astrocyte interactions and vascularization of the retina (Fruttiger et al., 1996). Ciliary neurotrophic factor (CNTF), Leukemia inhibitory factor (LIF); and TGF- α have been shown *In Vitro* cultures of chicken retinal progenitors

to have inhibitory effect on photoreceptor differentiation and lead to increase in bipolar cell population (Cepko et al., 1996). IGFs have been shown to stimulate proliferation of neuroepithelial cells (Hernandez-Sanchez et al., 1995) and glial cells (Ikeda et al., 1995) In Vitro.

aFGF and bFGF have been purified from the ocular tissues (Arruti and Courtois, 1978) and extensively studied in the context of retina. These results are discussed in the following section.

C. Fibroblast Growth Factors

1. Molecular Biology of FGF

FGFs constitute a family of closely related proteins of pleiotrophic functions with high affinity for heparin (Gospodarowicz et al., 1987). Acidic FGF (aFGF/FGF1), and basic FGF (bFGF/FGF2) were the first ones to be identified as mitogens for fibroblasts (Gospodarowicz et al., 1974). In addition to aFGF and bFGFs the family now includes 10 other proteins. Int-2 (FGF3), KFGF (FGF4/hst), and FGF5 were initially identified as oncogenes (Moore et al., 1986; Taira et al., 1987; Zhan et al., 1988; Wilkinson et al., 1989). FGF6, keratinocyte growth factor (FGF7/KGF), androgen-induced growth factor (AIGF/FGF8), and Glia-activating factor (FGF9) were identified by virtue of their sequence similarity to the FGF family members (Finch et al., 1989; Marics et al., 1989; Tanaka et al., 1992; Miyamoto et al., 1993). Recently, three new members, FGF10, FGF11, and FGF12, have joined the family, where FGF10 was identified from rat embryos by homology-based PCR (Yamasaki et al., 1996), and FGF11, and FGF12 were identified from humans (Coulier et al., 1996).

These heparin binding growth factors are likely to have evolved from a single ancestral gene. The family members studied so far all contain three exons in the coding region, and the 104 bp middle exon has identical 5' and 3' boundaries (Hicks et al., 1991a). They all have very long 3' untranslated regions and show diversity at the 5' non-coding sequences. The greater divergence between the introns must be the consequence of a faster evolutionary rate. The products of this gene family members show 30 to 50% amino acid homology, where aFGF and bFGF have 55% amino acid identity despite their different isoelectric points and affinities for heparin (Burgess et al., 1985). The amino acid sequences are highly conserved among different species (92%) as well.

Use of unusual initiation codons have been reported to generate different isoforms for some FGFs (Acland et al., 1990; Tanaka et al., 1992). At present the biological roles of these protein variants remain unknown. Further diversity stems from posttranslational modifications of glycosylation, phosphorylation, ribosylation, and nucleotidylation of these proteins (Mason, 1994; Borja et al, 1997).

aFGF, bFGF, and FGF9 lack the classical signal sequences found on the secreted proteins. This property of all these genes, differentiates them from the other FGF family members. How these factors are secreted by the cells in which they are synthesized is not very clear, however, evidence is accumulating to suggest that they are released from the cell by novel secretory mechanisms. Although FGF9 lacks a typical N-terminal signal sequence for secretion, it is constitutively secreted following transfection into COS cells (Miyamoto et al., 1993). Alternative Golgi/ ER independent secretion mechanism have been proposed (Florkiewicz et al., 1995), and the involvement of chaperones in the export of FGFs have been implicated (Jackson et al., 1992; Shi et al., 1997). Despite the lack of information on their secretion mechanisms, early binding and cross-linking studies have indicated the presence of high and low affinity receptors on variety of cells (Burgess and Macaig, 1989) and in ECM, thus implying that they are secreted.

2. FGF Receptors

To this date four related high affinity FGF receptors (FGFR) have been identified by molecular cloning techniques, and are referred to as FGFR1, FGFR2, FGFR3 and FGFR4 in the order of their identification (Kornbluth et al, 1988; Ruta et al, 1988; Lee et al, 1989; Dionne et al, 1990; Houssaint et al., 1990; Keegan et al, 1991; Partanen et al, 1991). These FGF receptors are members of the tyrosine kinase receptor superfamily and each has an extracellular region containing two or three immunoglobulin-like (Ig) domains and an intracellular split tyrosine kinase domain (Johnson and Williams, 1993). FGFRs show 55-72% overall amino acid identity; the most conserved domain is the kinase domain (80% amino acid identity); whereas the Ig-like domain I (19%), Juxtamembrane domain and transmembrane domain show the most divergence between receptor types. The isoforms that lack the Ig domain I are generated by exon skipping (Dionne et al, 1990; Hattori et al, 1990; Johnson et al, 1990; Mansukhani et al, 1990; Miki et al, 1991). In addition to these isoforms, another set of splice variants has been detected at the carboxyl-terminal half of the Ig domain III without altering the reading frame of the remainder of the receptor. These splice forms have been shown to exist for FGFRs 1, 2 and 3 but not 4 (Johnson et al., 1991; Vainikka et al., 1992; Werner et al., 1992; Chellaiah et al., 1994). Studies with the cloned receptors revealed that different FGF family members are able to bind to the same receptor species, albeit with different affinities. Some aspects of ligand specificity appear to involve the extracellular region encompassing the Ig-like domain II and III (Miki et al., 1992; Yayon et al., 1992; Chellaiah et al., 1994; Blunt et al., 1997).

FGFs have been known to bind with low affinity to the extracellular matrix and transmembrane heparan sulfate proteoglycans (HSPGs) (Moscatelli et al, 1987), and their presence may be essential for aFGF and bFGF activity on the target cells (Rapraeger et al, 1991; Yayon et al, 1991). Syndecan family of proteoglycans are gaining prominence as candidates of low affinity FGF receptors (Elenius et al, 1992; Salmivirta et al, 1992; Mali et al, 1993). HSPGs initially have been proposed to play a role in the accumulation of the ligand at the cell surface and/or their delivery to the signal transmitting high affinity receptors (Keifer et al, 1990; Klagsburn and Baird 1991; Yayon et al, 1991). Cross-linking studies of Kan et al. (1993) that demonstrate formation of a ternary complex with FGFR

heparan sulfate proteoglycan and FGF, supporting the hypothesis that the low affinity receptors may be an obligatory component of the signal transduction pathways that are activated by FGFs.

In the current paradigm, the activation of FGFRs includes the formation of a complex between the ligand, a heparin-like molecule or a heparan sulfate proteoglycan, and a high affinity FGFR (Bernard et al., 1991; Klagsbrun and Baird, 1991; Rapraeger et al., 1991; Yayon et al., 1991). The initial binding event is followed by receptor dimerization, and autophosphorylation in the tyrosine kinase domains (Ornitz et al., 1995). Little is known about the subsequent activation of downstream signalling molecules (Ornitz et al., 1992; Spivak-Kroizman et al., 1994; Wang et al., 1994), and about the possible specificity of signal transduction by the different FGFRs. Several substrates bind to activated receptor tyrosine kinases through association between SH2 domains and autophosphorylated tyrosine residues. These include phospholipase C- γ (PLC- γ), Ras GTPase activating protein, the p-85 subunit of phosphoinositol 3-kinase, pp60^{c-src} (Anderson et al., 1990; Escobedo et al., 1991), the docking or adaptor proteins GRB2/Sem-5, SHC, Nck (Chou et al., 1992; Li et al., 1992; Lowenstein et al., 1992; Meisenhelder and Hunter, 1992; Park and Rhee, 1992), and phosphotyrosyl phosphatase Syp/PTP1D (Feng et al., 1993; Vogel et al., 1993). Thus far, only one of these proteins, PLC- γ , has been identified as a substrate of FGFR1 (Burgess et al., 1990), and its direct association with the receptor has been demonstrated (Mohammadi, et al., 1992). The extent of the phosphorylation differs according to the FGFR to be activated. For example, PLC- γ is more extensively phosphorylated by FGFR1 than by FGFR4 in NIH3T3 cells (Vainikka et al., 1992), and the pattern of protein phosphorylation indicates that independent transduction pathways are activated upon stimulation of each receptor. It has also been shown that, functional ras is required for the aFGF induced differentiation of the PC12 cells (Szeberenyi et al., 1990), and bFGF stimulation of fibroblasts leads to hyperphosphorylation of Raf-1 serine/threonine kinase, a substrate of *ras*. This finding suggests that Raf-1, which is a MAP kinase kinase kinase, has a role as one of the downstream signalling components for FGFR (Morrison et al., 1989; MacNicol et al., 1993). Indeed, MAPK pathway was found to be involved in FGF induced signalling in the astrocytes (Tournier et al., 1994). This MAPK pathway, which is activated upon FGF stimulation, results in the activation of certain transcriptional factors like Elk-1 (Marais et al., 1993). Subsequent to these sequence of events, expression of certain

transcription factors such as, c-fos and tis11(Wang et al., 1994), are induced, which presumably determines the response of the target cell.

3. Expression Patterns and Biological Activities of FGFs

3.1. aFGF and bFGF

aFGF and bFGF have wide tissue distribution and are known to have multiple roles in different physiological contexts (Folkman and Klagsburn, 1987; Burgess and Macaig, 1989). They have been shown to stimulate proliferation of a variety of cells of mesenchymal, epithelial, and neuroectodermal origin, such as fibroblasts, endothelial cells, oligodendrocytes, chondrocytes, and glia. They promote differentiation and survival neurons of cortical, hippocampal, cerebellar, hypothalamic, spinal chord, and retinal origin (Morrison et al., 1986; Walicke et al., 1986; Unsicker et al., 1987; Hatten et al., 1988; Hicks and Courtois, 1992). They may prevent death of lesioned neurons and promote regeneration (Anderson et al, 1988; Lipton et al., 1988; Cordeiro et al., 1989). They can also inhibit differentiation of skeletal muscle myoblasts (Linkhart et al., 1981; Lathrop et al., 1985). Both factors have been detected in developing embryos at low levels (Mascarelli et al., 1987; Kalcheim and Neufeld, 1993). Their role in vivo as an inducer of mesoderm formation in *Xenopus* has been well documented (Kimelman and Kirschner, 1987; Slack et al., 1987; Amaya et al., 1991). In addition to its role as an inducer of the mesoderm formation, bFGF was also reported to be influencing the anteroposterior neural pattern in *Xenopus* ectoderm (Doniach, 1995).

3.2. Other FGFs

FGF3 was initially detected as the site of integration for mouse mammary tumor viruses (Peters et al., 1983; Dickson et al., 1984). It is suggested that the most essential roles of FGF3 is restricted to embryogenesis, since its transcription appears to be virtually restricted to embryos and some germ cell tumors. FGF3 was expressed in F9 cells differentiated into parietal endoderm, but the amount of transcripts was much decreased when F9 aggregates were induced to differentiate into visceral endoderm-like cells. Studies in *Xenopus* have revealed that the expression of FGF3 was increased in the newly formed mesoderm of the gastrula (Slack et al., 1996). Also, it was demonstrated by Ozawa et al., (1996) that, during the development of the nervous system, FGF3 expression was higher in the late embryonic stages than in postnatal stages, suggesting that it was involved in early stages of brain development. FGF4 was initially isolated from human stomach tumors and Kaposi sarcomas (Delli Bovi et al., 1987; Taira et al., 1987). Subsequently, FGF4 was identified in male germ cell tumors (Yoshida et al., 1988), murine embryonic stem cells (Health et al., 1989) and embryonic carcinoma cells (Hebert et al., 1990). In developing mouse embryos, FGF4 was found to be expressed during gastrulation in primordial germ layers and later in limb and tooth buds (Niswander and Martin, 1992). FGF5 was isolated by Zhan et al. (1987, 1988), as an oncogene. It is expressed in the adult central nervous system in diverse neuronal populations (Haub et al., 1990; Gomez-Pinilla and Cotman, 1993). FGF5 has been postulated to be a survival factor for spinal motoneurons (Hughes et al., 1993). cDNAs for FGF6 were isolated on the basis of nucleic acid hybridization with a KFGF probe (Marics et al., 1989). FGF6 expression in perinatal mice was restricted to the central nervous system and skeletal muscles, with intense signals in the developing cerebrum in embryos, but observed in the cerebellum of 5-day-old neonates (Ozawa et al., 1996). FGF7 was shown to display specific patterns of expression in stromal cells from epithelial tissues, and appears to be a secreted epithelial cell-specific growth factor, which is widely expressed in epithelial tissues (Finch et al., 1989). FGF8 also shows embryonic expression, and has been suggested to play a role in gastrulation, brain development and limb formation (Ohuchi et al., 1994; Crossley et al., 1996). FGF9 is a potent mitogen for glial cells, rat primary cortical astrocytes, BALB/c3T3, and oligodendrocyte-type2 astrocyte progenitor cells.

oligodendrocyte-type2 astrocyte progenitor cells. FGF9 has weak mitogenic activity for rat PC-12 cells and no activity on human umbilical vein endothelial cells (Tagashira et al., 1995). During development, FGF9 is expressed at low levels in mid-gestation mouse embryos (Tanaka et al., 1992). It was also found to require heparin for optimal activation and that it preferentially binds to and activates FGFR2c, FGFR3b, and FGFR3c (Santos-Ocampo et al., 1996). Additionally, Seo and Noguchi (1995) suggested that FGF9 expression might be regulated by retinoic acid during neuronal differentiation in both early and late developmental processes. Another example of its developmental effects is its role in the mesoderm induction in *Xenopus*. It was shown that the FGF9 mRNA was expressed both maternally and zygotically, present at the time of natural mesoderm induction (Song and Slack, 1996; Slack et al., 1996). Thus, it is likely that FGF9 plays a role in both developmental events and in adult physiology. The biological functions of FGF10, FGF11, and FGF12, the most recently identified members of the FGF family, are yet to be studied.

D. FGFs in Retina

Major body of information on the FGFs in the retinal context is on the prototypes of the family, aFGF and bFGF. Information on the other members of the family is scarce. These studies summarized below don't demonstrate specific functions of FGFs in the retina, but suggest these factors may act as survival and differentiation factors in the retina.

The first growth factor activity was isolated from bovine retina by Arruti and Courtois (1978) and named as eye-derived growth factors (EDGF I and IDGF II). These factors were later found out to be identical to aFGF and bFGF (Baird et al., 1985). They were also purified from embryonic chick retina (Mascarelli et al., 1987) and aFGF was cloned from retinal cDNA library (Alterio et al., 1988). In situ hybridization results indicate that aFGF is expressed throughout the adult retina (Jacquemin et al., 1990; Noji et al., 1990; Elde et al.,

expressed throughout the adult retina (Jacquemin et al., 1990; Noji et al., 1990; Elde et al., 1991; Buğra et al., 1993). Immunohistochemical studies using specific aFGF antibodies done with various vertebrate species revealed conflicting localization patterns (Caruelle et al., 1989; Hanneken et al., 1989; Elde et al., 1991; Buğra et al., 1993). For example, while Elde et al. (1991) detected aFGF immunostaining only in ganglion cells, Buğra et al. (1993) localizes aFGF primarily in photoreceptors. Similarly, the bFGF, immunohistochemical data do not agree on localization patterns (Hanneken et al., 1989; Connolly et al., 1992; Raymond et al., 1992; Consigli et al., 1993; Buğra and Hicks, in press). The different results are attributed to the experimental parameters, such as fixation protocol, choice of antibody, detection method (Hanneken and Baird, 1992). Developmental expression patterns, though conflicting on cellular distribution, agree on the low FGF levels in late embryonic days, increase after birth (Connolly et al., 1992; Buğra et al., 1993; Consigli et al., 1993; Buğra and Hicks, in press). Presence of high and low affinity binding sites for these two FGFs in the rodents has been shown (Jeanny et al., 1987; Fayein et al., 1990). Developmental studies with amphibian (Launay et al., 1994) and chick retina (Heuer et al., 1990; Ohuchi et al., 1994; Tcheng et al., 1994) indicate the expression of FGFR1 and FGFR2 starting in the embryo. FGFR1 and FGFR2 knockout mice show thinning of the ONL and photoreceptor degeneration (Campochiaro et al., 1996). These studies imply that aFGF and bFGF have physiological roles in the retina formation.

Only other FGF that has been detected in the adult retina is FGF5 (Kitaoka et al., 1995). FGF5 mRNA and protein were detected in all neuronal cells, with higher levels in the ganglion cells and photoreceptors as well as the RPE cells. The ability of FGF5 to bind with high affinity to FGFR1 and FGFR2 (Clements et al., 1993), the receptor subtypes known to exist in the retina, supports the notion that this factor may also be an important trophic factor for retinal neurons. FGF9, with its wide distribution in the CNS, is a candidate protein to have roles in the retina. Other FGFs that are expressed mainly in the embryo may influence the early pattern formation.

In vitro, aFGF and bFGF can induce neurite outgrowth and stimulate opsin expression (Hicks and Courtois, 1988; 1992). Intraocular injections of bFGF retard the degeneration of photoreceptors in a rat strain (RCS) that have inherited retinal dystrophy (Faktorovich et al., 1990), and confer partial protection against phototoxicity and ischemia (Faktorovich et al., 1992; Unoki and LaVail, 1994).

aFGF and bFGF were effective in initiation of transdifferentiation of presumptive pigment epithelium cells to ganglion cells (Pittack, 1991; Guillemot and Cepko, 1992). They were also shown to enhance survival of ganglion cells in explant cultures (Bahr et al., 1989). FGFs stimulate axonal regeneration (Sievers et al., 1987; Lipton et al., 1988). Ferguson et al. (1990), demonstrated that ganglion cells contained high affinity bFGF receptors on their cell membranes, suggesting that these cells directly respond to FGF.

In the retinal Muller glial cells no bFGF (Noji et al., 1990), nor aFGF was detected (Malecaze et al., 1992). Therefore, it is unlikely that these cells act as FGF source for retinal neurons. But glia appears to express the specific receptors on their membranes (Mascereilli et al., 1991) and proliferate in response to FGFs (Hicks and Courtois, 1988). The glial cells may be indirectly involved in the action of FGFs on retinal neurons (Hicks et al., 1991a).

Even though the retinal pigment epithelium (RPE) is not part of the neural retina, it plays a vital role in maintaining the normal functioning of the photoreceptors as well as keeping the turnover rates of the outer segment in equilibrium which, if lost, results in certain retinal degenerations such as in the RCS rats (Hicks et al., 1991a). It has been reported that RPE cells contain aFGF (Hicks et al., 1991b) and bFGF (Schweigerer et al., 1987). Mitogenic effect of both factors has been shown in the primary cultures of RPE cells (Hicks et al., 1991b).

II. PURPOSE

The members of the FGF family of proteins have been found to be implicated in diverse developmental events, such as cell differentiation, survival and proliferation, mesoderm formation, limb bud development. Their In Vitro effects and in vivo tissue distribution suggest that they may play important roles during the embryonic and postnatal development of the central nervous system. In the retina, which is an extension of the central nervous system, the presence of aFGF, bFGF (Bugra et al., 1993; Bugra and Hicks, in press), and recently FGF5 (Kitaoka et al., 1994) have been reported, suggesting that they are involved in retinal development. The expression patterns of aFGF, and bFGF indicates that they are acting postnatally during the development of the retina, since their expression levels start to increase after birth (Bugra et al., 1993; Bugra and Hicks, in press). Such quantitative spatiotemporal analysis of gene expression are of great importance, providing data about the correlation between the major developmental events and the expression patterns of certain growth factors. Despite the agreement on the postnatal expression of aFGF, the data on its localization within the retina is conflicting. This led us to elucidate the distribution of aFGF expression within the retinal layers. The retina was separated into two layers, outer layer containing the photoreceptor cells and inner layer with interneurons and ganglion cells, glia. In this approach, comparative analysis of aFGF in the layers at different times after birth was performed by RT-PCR technique was performed using the total RNA extracted from these tissues.

FGF9 protein was found to be present in a wide variety of regions within the central nervous system, including hippocampus, cerebellum, and midbrain (Tagashira et al., 1995). Although FGF9 was initially isolated as a mitogen for the glial cells (Miyamoto et al., 1993), its cellular distribution was found to be confined to the neurons (Tagashira et al., 1995). It is also thought to be involved in the pattern formation in *Xenopus* embryos (Song and Slack, 1996). This distribution of FGF9 makes it a promising candidate as a diffusible factor important in the physiology of the retina and/or a factor shaping the retinal formation.

shaping the retinal formation. For this purpose, in this study, we also aimed to demonstrate the presence of FGF9 within the retina during development.

III. MATERIALS

A. Animals

Long-Evans rats used throughout the study were bred at the animal house facilities at the University of Louis Pasteur (Strasbourg). The animals were kept under diurnal lighting conditions and were fed ad libitum.

B. Chemicals

Taq DNA polymerase enzyme, deoxyribonucleotide triphosphates (dNTPs), random hexanucleotides, RNase inhibitor (RNasin), Moloney Murine Leukemia Virus reverse transcriptase (MMLV-Rtase), for the reverse transcription and PCR reactions, T4 polynucleotide kinase for the labelling of the nested oligonucleotides, and the restriction enzymes for the characterization of FGF9 were purchased from either Promega (USA) or Biolabs (USA). The [^{32}P] γ -ATP (3000mCi/mM) was purchased from Izotop (Budapest). Oligonucleotides were obtained from Genomed (Turkey), and Sephadex G-50 was obtained from Pharmacia (Sweden). All other chemicals used in this study were either from Merck (Germany) or Sigma (USA) unless stated otherwise in the text.

C. Buffers and Solutions

1. Buffers and Solutions for the extraction of RNA from the retinal tissues

All solution, except the organic solutions and guanidium thiocyanate, were treated with 0.1% diethylpyrocarbonate (depc) for 1 hour at room temperature, then autoclaved to inhibit RNase. All glass and plasticware were autoclaved before use.

4 M Guanidium isothiocyanate	:	4 M guanidine isothiocyanate 25 mM 1M NaCitrate (pH 7.0) 5 mg/ml sodium N-laurylsarcosine 0.1 M mercaptoethanol
Sodium Acetate	:	2 M sodium acetate (pH4-5.2) 3 M sodium acetate (pH 5.2)
Phenol	:	water saturated (pH 7.0)
Chloroform	:	49:1 chloroform:isoamylalcohol, water saturated

2. Reverse Transcriptase Buffer

5X Reverse Transcriptase Buffer	:	250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15mM MgCl ₂ , 50 mM DTT
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3. PCR Buffer

10X PCR Buffer (Mg^{2+} free) : 500 mM KCl, 100 mM Tris-HCl (pH 9.0),
1% Triton X-100

MgCl_2 stock solution : 25 mM MgCl_2

4. T4 Kinase Buffer

10X T4 Kinase Buffer : 700 mM Tris-HCl (pH 7.0); 100 mM
 MgCl_2 , and 50 mM DTT

5. Restriction Enzyme Buffers

10X Buffer for PstI (Promega) : 10 mM Tris-HCl (pH 7.4), 50 mM NaCl,
0.1 mM EDTA, 1 mM DTT, 0.15 % TritonX-
100, 0.5 mg/ml BSA, 50% Glycerol

10X Buffer for BsaJI (Biolabs) : 50 mM NaCl, 10 mM Tris-HCl, 10 mM
 MgCl_2 , 1 mM DTT (pH 7.4)

6. Electrophoresis Buffers and Gel Systems

6.1. Buffers for Agarose/Formaldehyde gel systems

10X MOPS Buffer	:	0.2 M MOPS (3-N-morpholino propanesulfonic acid), 50 mM Sodium Acetate, 10 mM EDTA (pH 7.0)
Sample Buffer	:	750 μ l Formamide, 150 μ l 10 x MOPS, 240 μ l Formaldehyde, 100 μ l depc water, 100 μ l Glycerol, 80 μ l 10% Bromophenol blue
Running Buffer	:	1X MOPS prepared in depc H ₂ O

6.2. Buffers for Non-Denaturing Gel Electrophoresis

5X TBE Buffer	:	0.9 M Trizma base, 2 mM EDTA 0.9 M Boric acid (pH 8.3)
Sample Buffer (6X)	:	0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol
Ethidium Bromide	:	10 mg/ml in H ₂ O

6.3. Gel Systems

Formaldehyde/Agarose gel	:	1% agarose, 0.66 M formaldehyde, 1X MOPS
Agarose (non-denaturing)	:	2% agarose

NuSieve (non-denaturing) : 4% GTG-NuSieve in 0.5X TBE buffer

6.4. Size Markers

pUC 18 Hae III digest : (0.445µg/µl), Sigma, BIOSCIENCES (USA);
Band sizes (bp): 587, 458, 434, 298, 267, 257, 174, 102, 80, 18, 11.

φX174 Hae III Marker : (500 ng/µl), Stratagene (USA);
Band sizes (bp): 1353, 1078, 872, 603, 310, 281-271, 234, 194, 118, 72.

φX174 Hinf I Marker : (500 ng/µl), Stratagene (USA);
Band sizes: 726, 713, 553-550, 427, 417-413, 311, 249, 200, 151, 140, 118, 100, 82, 56, 48-42-40, 24.

7. Southern Transfer and Hybridization Buffers

Alkaline transfer Buffer : 0.4 N Sodium hydroxide

20X SSC : 3 M Sodium chloride, 0.3 M Sodium citrate

SDS Stock Solution : 10% Sodium Dodecyl Sulfate

50X Denhardt's Reagent : 5 g Ficoll, 5 g Polyvinylpyrrolidone,
5 g Bovine Serum Albumin

Prehybridization Buffer : 6X SSC, 5X Denhardt's Reagent, 0.5% SDS

Hybridization Buffer : Prehybridization buffer containing
³²P γ-ATP labeled nested oligonucleotide probe

D. Equipments

Autoclave	:	Model MAC-601, Eyela, JAPAN
Balances	:	Electronic Balance Model VA124-1AAZM13AAE, Gec Avery, UK
Camera	:	DS34, Poloroid, USA
Centrifuge	:	Centrifuge 5415C, Eppendorf, USA
Deepfreezes	:	-20 °C, Arçelik, TURKEY; -70 °C , Sanyo, JAPAN
Densitometer	:	CD60, Desaga Sarstedt- Groppe, GERMANY
Documentation System	:	Biodoc, Biometra, GERMANY
Electrophoresis Tank	:	Easi-cast system, Hybaid, UK
Homogenizer	:	Bioblock, FRANCE
Hybridization Oven	:	Mini dual 14, Hybaid, UK
Incubators	:	Plus Series, Gallenkamp, GERMANY; Orbital Incubator, Gallenkamp, GERMANY
Magnetic Stirrer	:	Chiltern Hotplate Magnetic Stirrer, HS31, UK
Microwave Oven	:	Vestel, TURKEY
pH meter	:	3010, Jenway, UK
Power Supply	:	PS 250, Hybaid, UK
Refrigerator	:	Arçelik, TURKEY
Scintillation Counter	:	Tri carb 4530, Hewlett Packard, USA
Spectrophotometer	:	CE 5502, Cecil, UK
Thermocycler	:	PTC-200, MJ Research, USA
Transilluminator	:	Chromato-Vue Transilluminator, Model ITM-20UVP, USA)
Water Bath	:	Thermomix BU, BRAUN, Germany Thermomix 1441, BRAUN, Germany

E. Other

Instant Films	:	SONY, TYPE II (High Density), UPP-110HD, JAPAN
Nylon Membrane	:	Boehringer Mannheim, GERMANY
X-Ray Films	:	KODAK X-OMAT film, USA
Cassettes	:	IMS, ITALY

IV. METHODS

A. Tissue Collection

Rats were killed by CO₂ anesthesia and cervical dislocation. For whole retina RNA isolations, retinas were dissected, flash-frozen and stored at -70°C until use. Newborn (postnatal PN1), PN4, PN6, PN10, PN14, PN20, PN30 and adult retinas were collected. For PN1-PN6 whole litters were used, for later time points half-litters were sacrificed.

For vibrotome sections PN4, PN6, PN10, PN14, PN20, PN30 and adult retinas were used. Rats were sacrificed as described above. Retinas were dissected free from other ocular tissues and separated into external or internal layers using a technique originally developed for retinal transplantation studies (Silverman and Hughes, 1989). Briefly, isolated retina were flat-mounted on a gelatin block with the ganglion cell layer uppermost and sectioned along a horizontal plane, 100 µm thickness, using a vibrotome (Figure IV.1). This layer, referred as the inner layer preparation, encompasses ganglion cells, INL and IPL. Remaining 100 µm is external layer, composed of photoreceptor cells and the apical portions of the glial cells. The layer preparations were frozen at -70°C, until ready for use.

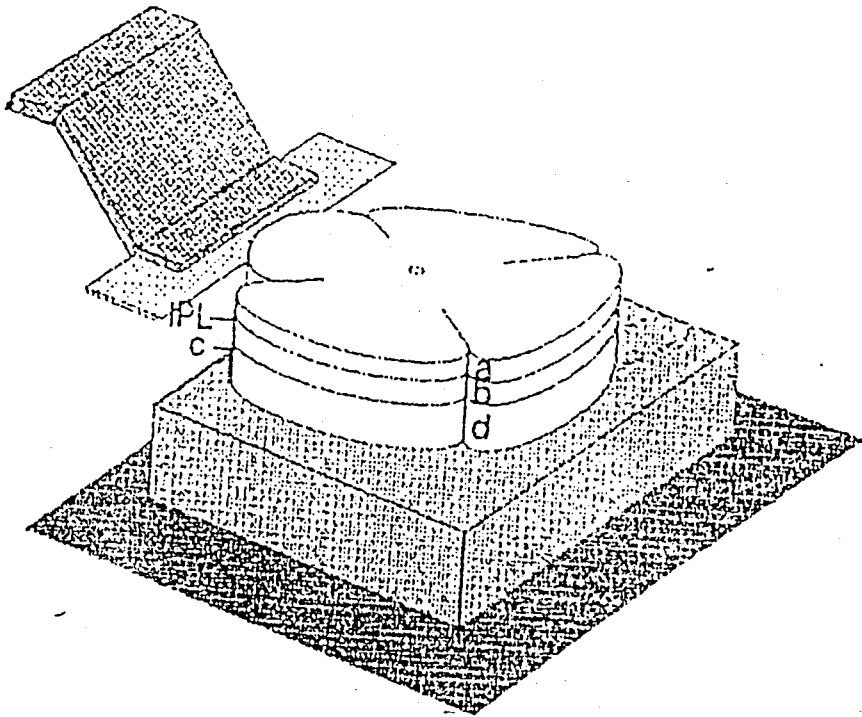


Figure IV.1. Schematic diagram representing vibratome isolation of retinal components. (a) Ganglion cell bodies; (b) Inner nuclear layer; (c) Outer plexiform layer; (d) Outer nuclear layer.

B. RNA Extraction

1. RNA Extraction from the retinal tissues

For RNA extraction, a technique previously established by Chomczynski and Sacchi (1987) was used with minor modifications. The retinal tissues from the rats of determined ages were individually homogenized in 600 μ l of GTC using a hand-held homogenizer. The volume in each tube was estimated and 1/10 volume of 2M NaOAc, equal volume of phenol, and 1/5 volume of chloroform were added in this order, vortexed and left on ice for 20 minutes to fractionate RNA from other cellular macromolecules. Subsequently, the tubes were span at 4°C for 30 minutes to separate the phases, and the aqueous phase containing the RNA was transferred into clean tubes. The new volume was estimated, and an equal volume of isopropanol was added to precipitate RNA at -20°C overnight. The pellets were collected by centrifugation at 10,000 g at 4°C, and washed twice with 500 μ l 75% ethanol. The pellets were resuspended in 600 μ l GTC and reprecipitated by adding an equal volume of isopropanol overnight at -20°C, in order to remove residual protein. The pellets were collected by centrifugation and washed twice with 75% ethanol, as before. Following the final wash, ethanol in the tubes was drained well, and the air dried pellets were resuspended in H₂O containing RNasin (40u/ μ l). In order to remove the possible contaminating DNA, the samples were treated with 2 units of RQ1 DNase at 37°C for 30 minutes. At the end of the incubation, 600 μ l GTC was added to each tube and mixed well to denature and remove DNase, which was followed by precipitation with an equal volume of isopropanol at -20°C for minimum of 2 hours. Subsequently, the pellets were collected by centrifugation, washed with ethanol, and resuspended in 50 μ l of depc treated H₂O. To remove GTC from the samples two cycles of ethanol precipitations were done by adding 1/10 volume of 3 M NaOAc, and 2.5 volume of absolute ethanol, and incubation at 20°C overnight. The final pellets were ethanol washed twice and resuspended in 30 μ l of depc treated H₂O.

C. Quantitative and Qualitative Analysis of RNA

1. Spectrophotometric Determination of the RNA concentrations

The concentration of the isolated RNA was determined by spectrophotometric analysis. Aliquots of RNA were diluted in depc treated water in a ratio of 1:1000, and optical density was measured at 260 nm and 280 nm. The samples with OD_{260}/OD_{280} ratios in the range 1.8-2 were considered pure. RNA concentrations in the sample was determined according to the formula:

$$\text{Concentration } (\mu\text{g/ml}) = 40 \mu\text{g/ml} \times OD_{260} \times \text{Dilution Factor}$$

After the calculations of the concentrations, stocks of either 200 ng/ μ l for the retinal layers or 500 ng/ μ l for the whole retina was prepared.

2. Agarose/Formaldehyde Gel Electrophoresis for the Analysis of the RNA Integrity

1% denaturing agarose gels were used to verify the integrity and the concentration of the RNA samples. The gel was prepared by boiling 1gr agarose in 87 ml depc treated water and 10 ml 10X MOPS buffer. Formaldehyde was added to the final concentration of 0.66 M after the mixture was cooled down to 50°C. The mixture was poured onto an electrophoresis plate, the comb was placed, and the gel was allowed to polymerize for one

hour at room temperature. 500 ng of the RNA samples to be analyzed was mixed with 6.5 μ l of sample buffer and the volume was brought up to 20 μ l with depc treated water, and incubated at 65°C for 5 minutes to denature the secondary structures. After the addition of 2 μ l of 1.25 mg/ml ethidium bromide, the samples were loaded to the gel. Electrophoresis was performed at 70 V for 1.5 hours. Subsequently, the gel was destained in 100 ml dH₂O for 20 minutes and photographed for documentation under the u.v. transilluminator.

D. Primer Selection

All the primers used in amplification reactions or as probes were selected from rat gene sequences published or deposited in either GENE BANK or EMBL data banks. The primers for FGF9 and aFGF were selected from regions of least homology with other members of the FGF family. Opsin oligonucleotides were checked against the beta-adrenergic receptors as they share significant homology. Calbindin primers likewise were compared with the calmodulin sequences, both having similar calcium binding domains. The sequence comparisons of appropriate gene families were done by using the multiple alignment program MULTALIN (Corpet, 1988). The stability, the secondary structures and when used as pairs the compatibility of the primers were analyzed using the OLIGO 4.04 software in Primer Analysis Software (Wojciech, Rychlik, 1992). The candidate oligonucleotide primers were further checked to ensure unique recognition of the gene under study, but not of the members of the relevant gene families using the program, ZEYNEP, which was kindly developed for our study by Çağatay Soyer (Biomedical Engineering Institute, Boğaziçi University). The sequences of the primers selected and the target sizes are listed in Table IV.1.

Table IV.1. Sequences of Oligonucleotide Primers used in RT-PCR and Hybridization Reactions.

U- Upper; L- Lower; N- nested.

GENE	PRIMER POSITION	SEQUENCE	GENE SOURCE (EMBL/ GENBANK)
aFGF	393-412	U: 5'-GATGGCACCGTGGATGGGAC-3'	#RNHBGF1
aFGF	508-527	L: 5'-AAGCCCTTCGGTGTCCATGG-3'	#RNHBGF1
aFGF	471-500	N: 5'-CTGGCCAGTCTCTGTACCCTTTATATACAC-3'	#RNHBGF1
OPSIN	434-454	U: 5'-ACTTACGCTTTGGGGAGAATC-3'	Personal (C. Barnstable)
OPSIN	731-751	L: 5'-TGACTTCCTTCTCTGCCTTCT-3'	Personal (C. Barnstable)
OPSIN	519-547	N: 5'-TTGGCTGGTCCAGGTACATCCCCGAGGGCA-3'	Personal (C. Barnstable)
CALB.	267-287	U: 5'-TCAAAGTAGCCGCTGCACCAT-3'	#M31178
CALB.	398-418	L: 5'-GCAGAAGCTCCTGGATCAAGT-3'	#M31178
CALB.	326-355	N: 5'-CACAGTTTTTTGAGATCTGGCTTCATTTCG-3'	#M31178
F9	326-346	U: 5'-CGCAGTCACGGACTTGGATC-3'	#D14839
F9	694-713	L: 5'-AGTCCTGGTCCTTCTCTTG-3'	#D14839
G3PDH	445-464	U: 5' ATGCCCCCATGTTTGTGATG 3'	#RNGADPHR
G3PDH	588-608	L: 5' ATGGCATGGACTGTGGTCAT 3'	#RNGADPHR

E. Reverse Transcription Coupled Polymerase Chain Reaction

1. Reverse Transcription

The reverse transcription reaction mixtures contained 1 μg of total RNA, 1 μl of hexanucleotide random primers (500 $\mu\text{g}/\text{ml}$), 1.2 μl of 100 mM dNTP, 12 units of RNasin 6 μl of 5X Reverse transcription buffer, and were incubated at 95 °C for 1 minute to denature the secondary structures present in the RNA samples. After this, the samples were quickly spun and 400 units of MMLV-RTase and 30 units of RNasin were added to the reaction tube, and the volume was brought to 30 μl with depc treated water. The reaction was allowed to proceed at 37°C for 2 hours. Subsequent to the reverse transcription, the enzyme was heat inactivated at 95°C for 10 minutes. The total volume of the mixture containing newly synthesized cDNA was brought up to 100 μl using depc treated water and stored at -70°C until use.

2. Polymerase Chain Reaction

We amplified the target fragment for the house-keeping gene, G3PDH to check the presence of the cDNA at the end of the reverse transcription step. Subsequently, from the cDNA products of whole retina, internal and external layers, aFGF, FGF9, opsin, and calbindin, were also amplified individually.

The PCR reaction protocol is the same for all the target amplifications, except for their magnesium concentrations and the annealing temperatures of the primer pairs. The optimal MgCl_2 concentrations and annealing temperatures for each target fragments needed to be determined experimentally. The PCR reactions were carried out in 25 μl of total volume in

1X reaction buffer containing 0.5 μ l of primer pairs for the genes under study, 1 μ l of dNTP mix (100 mM) and $MgCl_2$ as indicated in Table IV.2.

For semiquantitative comparison of transcript levels, the amplifications need to be carried out in the exponential phase of the reaction where the levels of amplified fragments reflect that of the initial transcripts (Chelly et al., 1988). The number of amplification cycles required to remain in the exponential phase were determined experimentally for each gene.

3. Analysis of the PCR Products

3.1. Agarose Gel Electrophoresis

15 μ l of the PCR products was mixed with 2 μ l loading dye and analyzed after resolving on a 2% agarose gel or a 4% NuSieve gel in 0.5X TBE buffer. Electrophoresis was carried out at 100 V. pUC Hae III, ϕ X 174 Hinf I or Hae III digests were used as molecular weight markers. The gels were stained with ethidium bromide, observed under u.v light on transilluminator and photographed for documentation.

3.2. Transfer of DNA from Agarose Gels to Positively Charged Nylon Membranes

For comparative studies, the fragments resolved on gels were transferred to nylon membranes and hybridized with appropriate nested oligonucleotide probes which were 5' end-labelled with ^{32}P . Before the transfer, the gel was soaked in 0.4N NaOH for 15 minutes for denaturation of DNA fragments. Then, the gel was placed on a glass platform, over three sheets of Watmann wicks, soaked in transfer solution, touching the bottom of the dish which contains 300 ml 0.4 N NaOH. Air bubbles between the sheets of Watmann papers were removed by rolling a glass rod over the top sheet. A membrane of the same size with

Table VI.2. PCR Amplification Conditions for aFGF, G3PDH, FGF9, Opsin, and Calbindin

GENE	AMP. CONDITIONS (°C)			[Mg] (mM)	Target size (base pairs)
	Annealing	Denaturation	Elongation		
1. aFGF	62 ⁰ C	92 ⁰ C	72 ⁰ C	3	135
2. G3PDH	55 ⁰ C	92 ⁰ C	72 ⁰ C	4	164
3. Opsin	55 ⁰ C	92 ⁰ C	72 ⁰ C	4	318
4. Calbindin	55 ⁰ C	92 ⁰ C	72 ⁰ C	4	153
5. FGF9	50 ⁰ C	92 ⁰ C	72 ⁰ C	4	388

the gel, prewetted in transfer solution, was placed on top of the gel and the wells of the gel were marked on the membrane using a pencil. After this, three wet and nine dry Watmann papers of the same size of the gel, and a stack of paper towels were placed over the membrane in that order. A glass plate was placed over this stack and weighed down with a 500 gr weight. As the transfer solution passes through the gel toward paper towels by capillary action, the possibility of short circuit was prevented by sealing the circumference of the gel with parafilm. DNA was allowed to blot overnight. After the transfer, the membrane was left at room temperature for minimum 3 hours for crosslinking of DNA to the membrane.

3.3. T4-Kinase-End Labelling of the Nested Oligonucleotide Probes

0.7 μ l of 100 ng/ μ l nested primers for aFGF, FGF9, calbindin, and opsin were individually labelled in a 20 μ l reaction mixture containing 2 μ l of 10X T4 kinase buffer, 4 μ l of 3000 Ci/mM and [32 P] γ -ATP and 1 μ l of T4 polynucleotide kinase at 37°C for one hour. At the end of the reaction, the mixture was heated at 68°C for 10 minutes to inactivate the enzyme.

3.4. Purification of the Radiolabelled Oligonucleotides

Labelled oligonucleotides were separated from the unincorporated precursor using Sephadex G-50 columns. G-50 beads were swollen in TE overnight and poured into 5 ml glass pipets plugged with sterile glass wool. After packing the column, samples were applied in 200 μ l volume. Columns were washed with TE. The radioactivity was monitored using a hand-held Geiger-Muller counter. The leading peak of radioactivity was collected and used in the hybridization reactions since it contained the oligonucleotide.

3.5. Hybridization of the Labelled Probe to the PCR Product

Prior to prehybridization and hybridization, membranes were neutralized by soaking in 6X SSC. Membranes were placed in 20 ml of the prehybridization solution, which was previously boiled at 95°C for 10 minutes to break the protein aggregates. Following prehybridization at 42°C for 1 hour in rotating glass-tubes, labelled probe, which was previously denatured by boiling for ten minutes at 95°C, was added into the tube. Hybridizations were allowed to proceed for 5 hr to overnight. The membranes were washed twice with large volumes (300 ml) of 6X SSC, 1% SDS for 20 minutes at 42°C in a shaking incubator. Subsequent washes were carried out under specific stringencies for each gene to remove any non-specific bound probe. Final washes for calbindin were with 2X SSC and 1% SDS for twenty minutes. aFGF and opsin washes were with 3X SSC and 1% SDS for twenty minutes.

After the washings, the membranes were blotted on Watmann filter paper, and wrapped in Saran wrap. The membrane and an X-OMAT AR5 film were sandwiched between two intensifying screens and placed in a film cassette. The exposures were carried out at varying times at -70°C.

3.6. Stripping the Hybridized Probe from the Membrane

This technique was applied, if it was necessary to hybridize the membrane with another probe. The membrane was placed in boiling 1% SDS for 30 minutes on top of a heating block. The solution was discarded and the same step was repeated until all the radioactivity in the membrane was gone. A Geiger-Muller counter was used to monitor the removal of the radioactivity. The blot was then rinsed with 6X SSC.

F. Restriction Enzyme Digestions

1. Digestion of the PCR Product with the Restriction Enzyme BsaJI

30 μ l of the 50 μ l PCR product was mixed with 4 μ l of 10X Reaction Buffer 2, 2 μ l of Bsa JI enzyme, and 4 μ l water and incubated at 60°C for 4 hours.

2. Digestion of the PCR Product with the Restriction Enzyme PstI

30 μ l of the 50 μ l PCR product was mixed with 4 μ l of 10X Reaction Buffer, 4 μ l of Pst I enzyme, 2 μ l water, and incubated at 37°C overnight.

3. Analysis of the Restriction Enzyme Digestions

40 μ l digested products, together with 15 μ l undigested PCR product were loaded to a 4% NuSieve gel and electrophoresed for 1 hour at 100 V. The gel was stained with ethidium bromide and observed under u.v light on a transilluminator and photographed for documentation as described before.

V. RESULTS

We used RT-PCR technique to study the steady state levels of aFGF transcripts in the retinal layers and the presence of FGF9 in the retina. Total RNA is reverse transcribed and subsequently amplified with oligonucleotides specific for the genes under study. When only the presence of the transcript was investigated, PCR reactions were done for 40 cycles and amplified fragments were detected on agarose gels stained with ethidium bromide. In contrast, for comparative analysis of steady state levels of specific transcripts, the amplifications were carried out at the exponential phase. Under these conditions, the levels of the amplified fragments reflect the initial transcript levels (Chelly et al., 1988). As a rule of thumb, the product levels at the exponential phase are too low to be detected with ethidium bromide staining of the gels. Thus, for each gene product to be analyzed, the number of amplification cycles required to be in the exponential phase of the PCR reaction was determined using Southern blotting and hybridization with radiolabelled specific oligonucleotides selected within the target fragment. The fragments were visualized by autoradiography, and the levels of amplified targets were determined by densitometric scanning.

A. RNA Preparations and cDNA Synthesis

The concentrations of RNA samples extracted from tissues of whole retinas or layers were calculated on the basis of their optical densities at 260 nm. The preparations that appeared to have no overt degradation on formaldehyde agarose gels were used further. RNA samples prepared from whole retinas obtained from rats of different ages (Figure

V.1.D), as well as the ones purified from the external and internal layers all appeared intact (Figure V.1.A and 1.B respectively). Figure V.1.C shows that the RNA concentrations of the external and internal layers were essentially the same, therefore, they could be used in comparative studies of the steady state levels of aFGF mRNA in the samples. No contaminating DNA was detected in the samples.

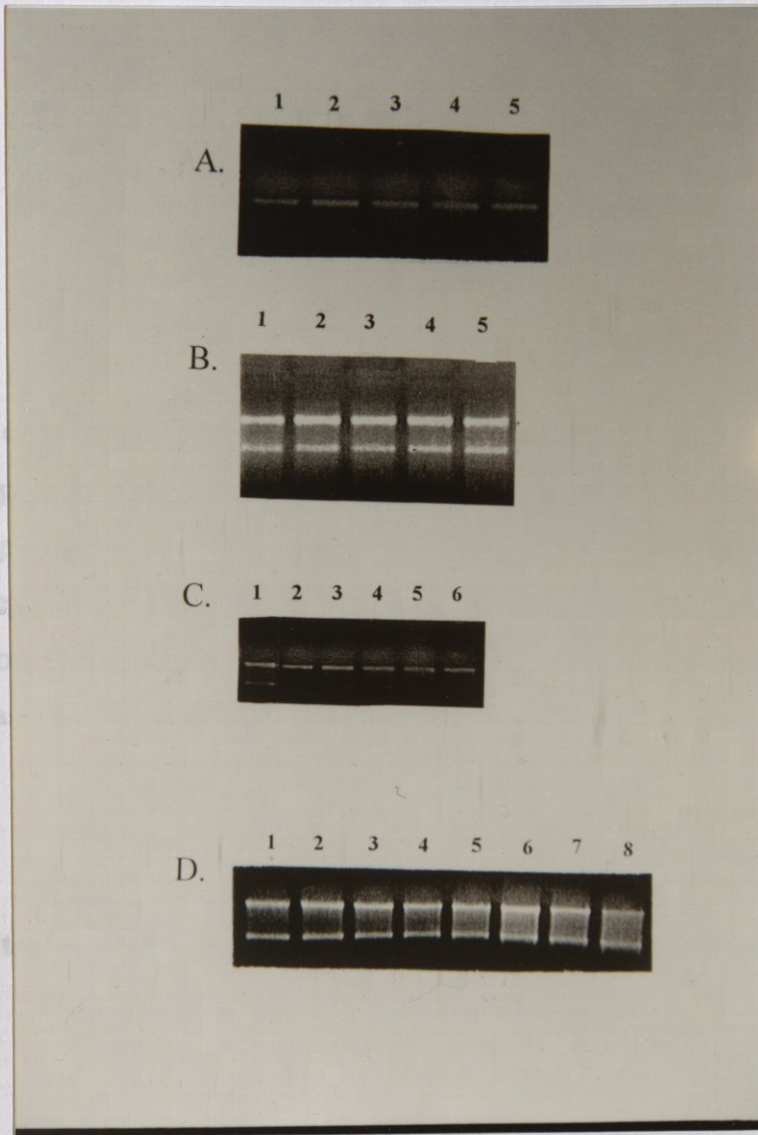


Figure V.1. RNA integrity. The integrity of the RNA extracted from (A) external layer: PN4 (lane 1), PN6 (lane 2), PN10 (lane 3), PN14 (lane 4), PN20 (lane 5); (B) internal layer: PN4 (lane 1), PN6 (lane 2), PN10 (lane 3), PN14 (lane 4), PN20 (lane 5); (D) whole retina: PN0 (lane 1), PN4 (lane 2), PN6 (lane 3), PN10 (lane 4), PN14 (lane 5), PN20 (lane 6), PN30 (lane 7), and adult (lane 8). C shows the relative concentrations of the RNA extracted from the internal and the external layers: External PN20 (lane 1), internal PN4-PN20 (lanes 2-6). The upper and lower bands correspond to 28S RNA and 18S RNA, respectively.

To detect specified transcripts in different RNA preparations, we used RT-PCR technique. A region of G3PDH, a housekeeping gene, target sequence was amplified from each cDNA preparation to verify that the reverse transcription was successful. A fragment of expected size of 164 bp was detected in all of the samples (Figure V.2).

B. Developmental Expression of aFGF in the Retinal Layers

Prior to the examination of the aFGF mRNA levels in the layers, the expression of two control genes, opsin and calbindin, were analyzed to substantiate the purity of the layers. Opsin is only expressed in the photoreceptors, thus is expected to be detected only in the external layer preparations. Calbindin, at the time of our study, was reported to be absent in the photoreceptors but detected mainly in the horizontal cells by immunohistochemical means, thus it was expected to be located only in the internal layer.

1. Developmental Expression Patterns of aFGF, Opsin, and Calbindin in Whole Retina

We determined the expression patterns in RNA samples obtained from whole retinas to serve as reference. Developmental expression of aFGF and opsin transcripts has been reported in literature (Triesman et al., 1988; Buğra et al., 1993). No reports were available on the expression of calbindin mRNA however. Figure V.3 shows the optimizations of Mg^{+2} concentrations for the amplification of all three targets in adult retinal RNA. Accordingly, subsequent amplifications of opsin, aFGF, and calbindin were carried out at 4 mM, 3 mM, and 4 mM Mg^{+2} concentrations, respectively (Figure V.3.B, 3.E, and 3.C).

The progress of product accumulation were monitored for each gene to remain in the exponential phase in adult RNA. Amplifications were carried out for different number of cycles, products were run on agarose gels and subjected to alkaline blotting followed by hybridization with the appropriate nested oligonucleotide probes. Only 14 cycles of amplification was sufficient for opsin detection (Figure V.4.C); for calbindin and aFGF 23

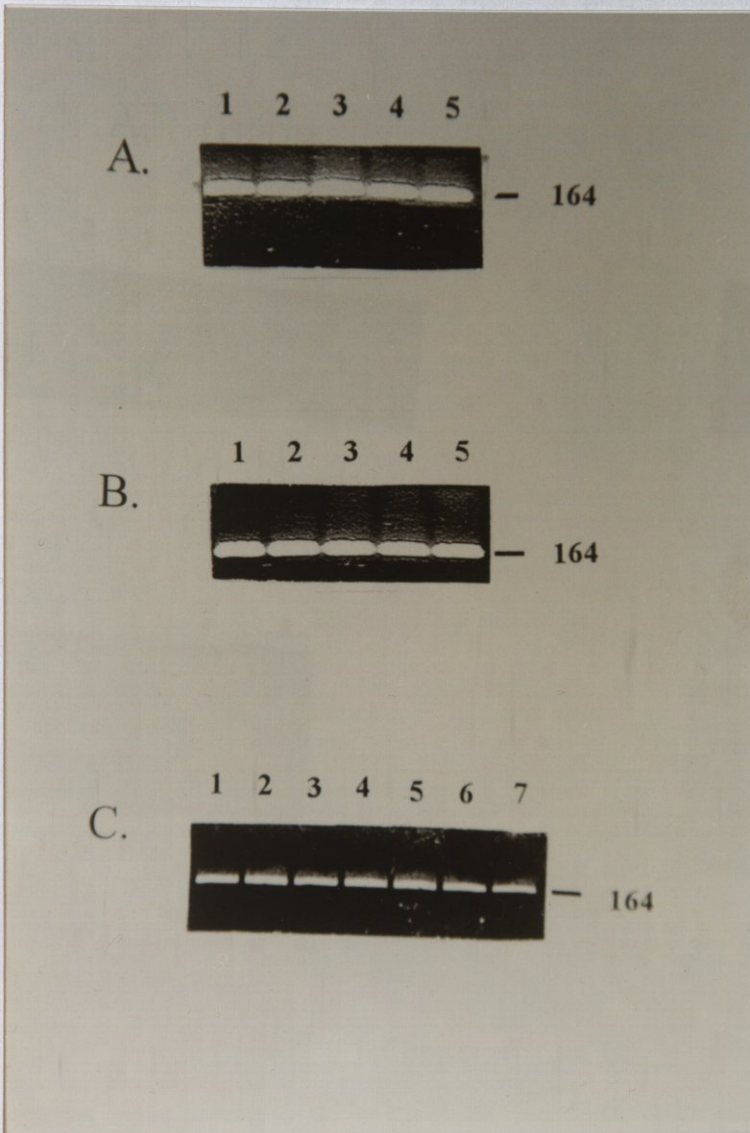


Figure V.2. Amplification of G3PDH. G3PDH is amplified from (A) internal, (B) external RNA obtained from PN4-PN20 rats (lanes 1-5), and (C) whole retina RNA obtained from PN0-adult (lanes 1-8). The amplifications were carried out for 40 cycles. The PCR products were run on 2 % agarose gels and visualized by ethidium bromide staining.

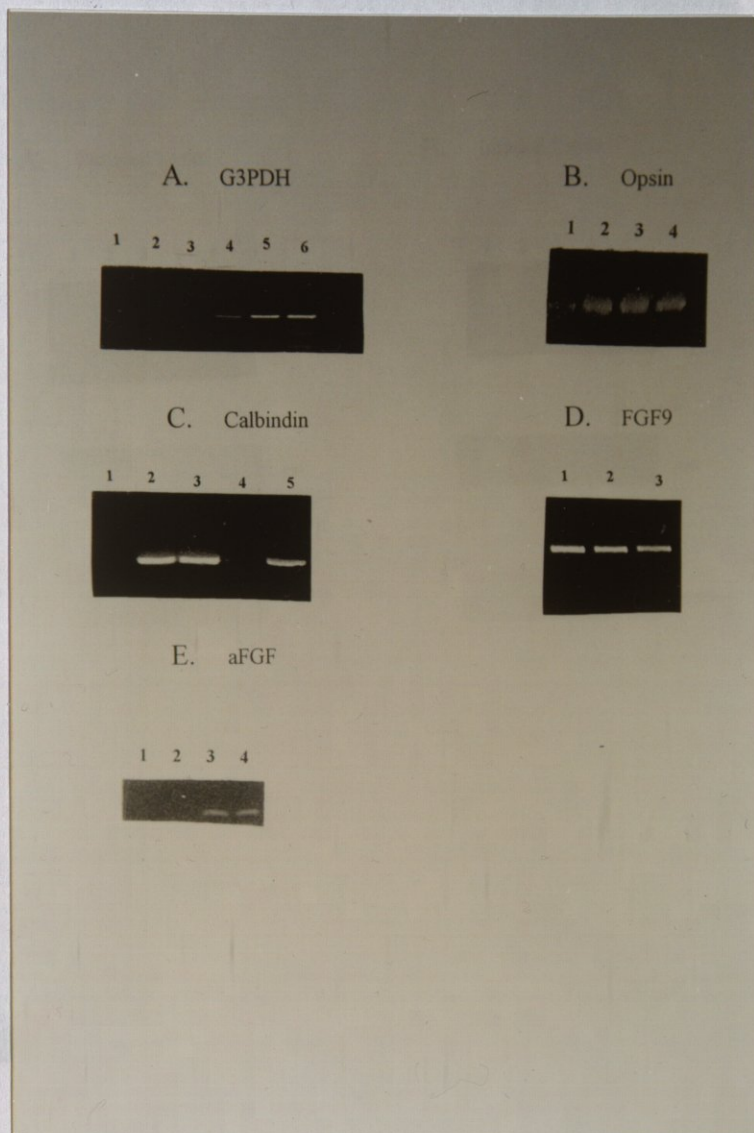


Figure V.3. Mg^{+2} titrations for PCR reactions. (A) G3PDH: 0 mM, 2 mM, 3 mM, 4 mM, 6 mM, 8 mM Mg^{+2} (lanes 1-6); (B) opsin: 2 mM, 3 mM, 4 mM, 6 mM Mg^{+2} (lanes 1-4); (C) calbindin: 2 mM, 3 mM, 4 mM, 6 mM, 8 mM Mg^{+2} (lanes 1-5); (D) FGF9: 4 mM, 6 mM, 8 mM Mg^{+2} (lanes 1-3); (E) aFGF: 0 mM, 2 mM, 3 mM, 4 mM Mg^{+2} (lanes 1-4).

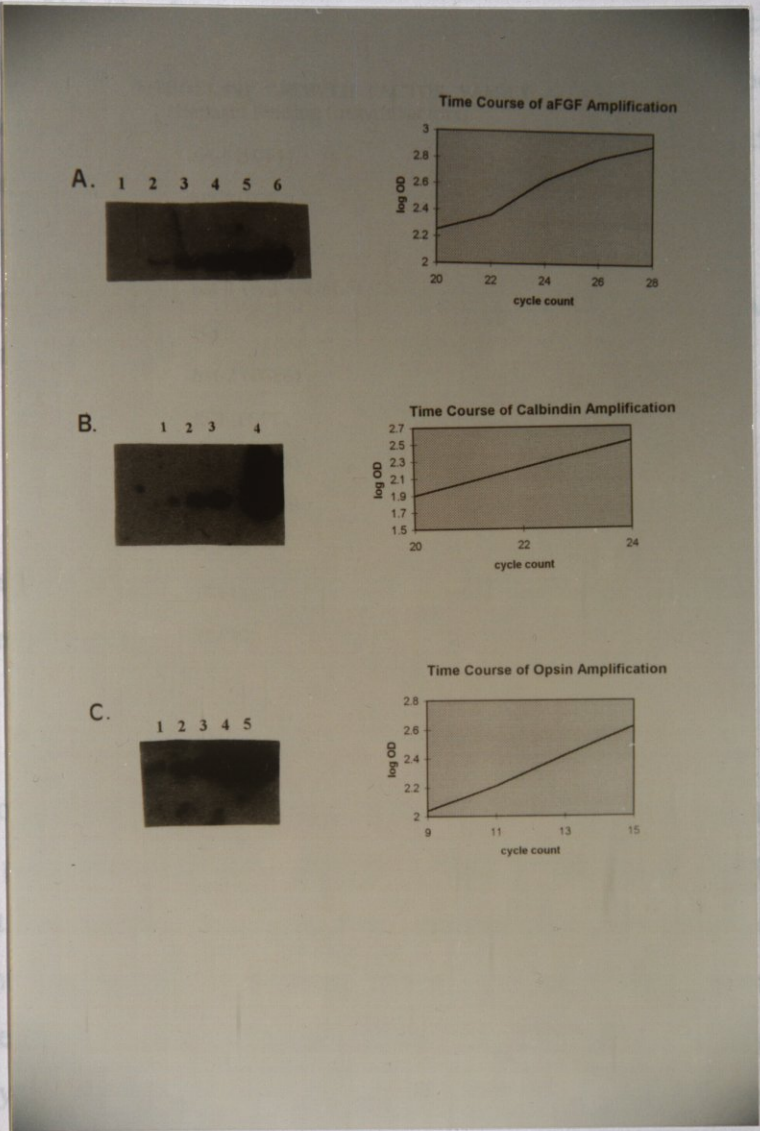


Figure V.4. Time course of amplification of aFGF, opsin, and calbindin. (A) aFGF: the amplifications are done for 20 (lane 1), 22 (lane 2), 24 (lane 3), 26 (lane 4), 28 (lane 5) cycles. (B) calbindin: the amplifications are done for 20 (lane 1), 22 (lane 2), 24 (lane 3) cycles. (C) opsin: the amplifications are done for 9 (lane 1), 11 (lane 2), 13 (lane 3), 15 (lane 4) cycles. Amplification products were detected by Southern transfer and hybridization with nested oligonucleotides. Films were exposed for various times.

cycles of amplification were chosen (Figure V.4.A, and V.4.B). As reported in literature, the steady state levels of opsin mRNA starts to increase at PN6, and it reaches adult levels by PN10 (Figure V.5.A). aFGF expression remains low until PN6, and increases three-fold by adulthood (Buğra et al., 1993) (Figure V.5.C.). We have detected a specific pattern for calbindin expression, where the level of the mRNA is essentially unchanged until PN6, then starts to increase after PN10, and reaches maximum levels by adulthood (5 fold) (Figure V.5.B and V.8.E).

For all the samples studied, no amplification products were detected when the reverse transcription step was omitted (Figure V.6.A).

2. Opsin, Calbindin, and aFGF Expression in Internal and External Retinal Layers During Development

In order to examine the distribution of opsin, calbindin and aFGF mRNA in two layers, RT-PCR was performed using RNA from PN4, PN6, PN10, PN14 and PN20 rats. The opsin fragment was amplified from internal and external layer cDNAs for 14 cycles, and the target fragment was detected by Southern blotting and hybridization with specific nested oligonucleotide probe. Autoradiograms show that 318 bp opsin target fragment is detectable only in the external layer (Figure V.7.A). This pattern of opsin expression parallels to that of the whole retina. Nested oligonucleotide probes do not detect any band in the internal layer samples (Figure V.7.B). These results indicate that the internal layer is free of photoreceptor cells.

Calbindin transcript was amplified from the cDNA of both layers for 23 cycles, and its expression was detected by Southern blotting and hybridization. Under these experimental conditions, 153 bp calbindin amplification fragment was detected in both layers. This unexpected result might either indicate the possible contamination of the external layer with the internal layer, or indicate that calbindin is expressed in the photoreceptor cells as well. The expression pattern of calbindin in the internal layer roughly paralleled the pattern

observed in the whole retinas (Figure V.7.B and V.8.D). In the external layer, uniform steady state levels of calbindin mRNA after PN6 was detected (Figure V.7.A and V.8.C).

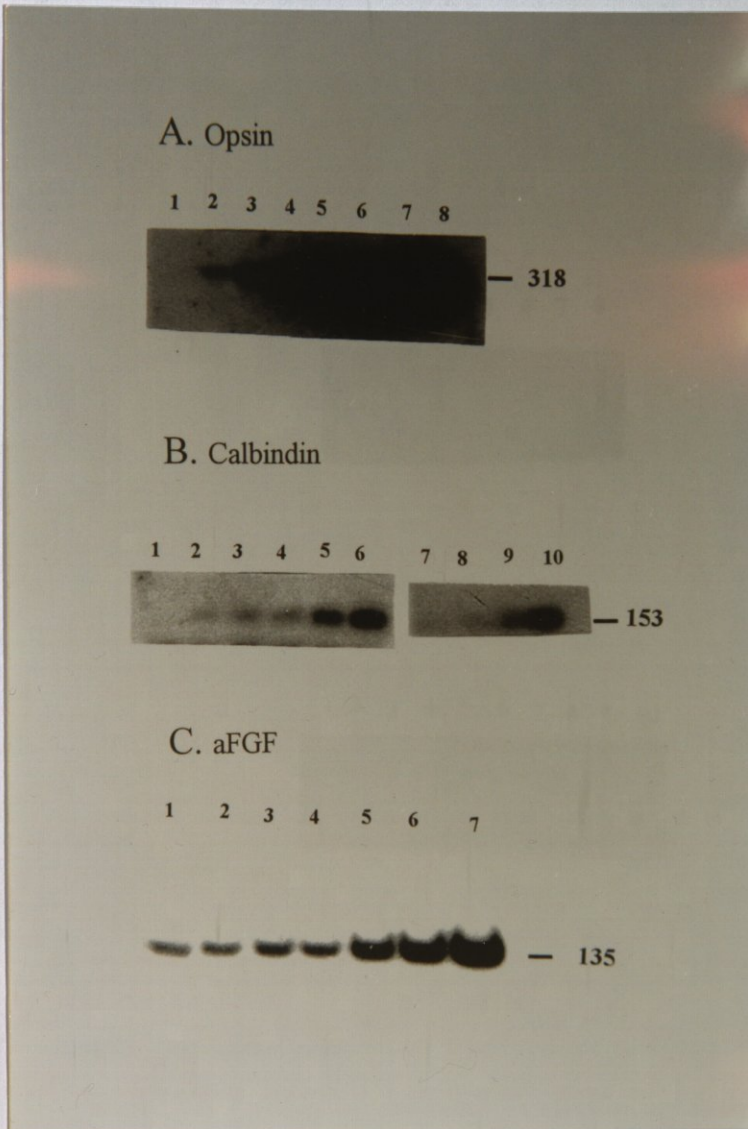


Figure V.5. Expression patterns in the whole retina. (A) opsin expression pattern in whole retina during development: PN0, PN4, PN6, PN10, PN14, PN20, PN30, and adult (lanes 1-8); (B) calbindin expression in the whole retina during development: E17, PN1, PN4, PN7, PN10, PN14 (lanes 1-6), and PN14, PN20, PN30, and adult (lanes 7-10); (C) aFGF expression in the whole retina during development (taken from Buğra et al., 1993): E17, PN0, PN4, PN7, PN10, PN13, and adult (lanes 1-7). Amplification products were detected by Southern transfer and hybridization with nested oligonucleotides. Films were exposed for various times.

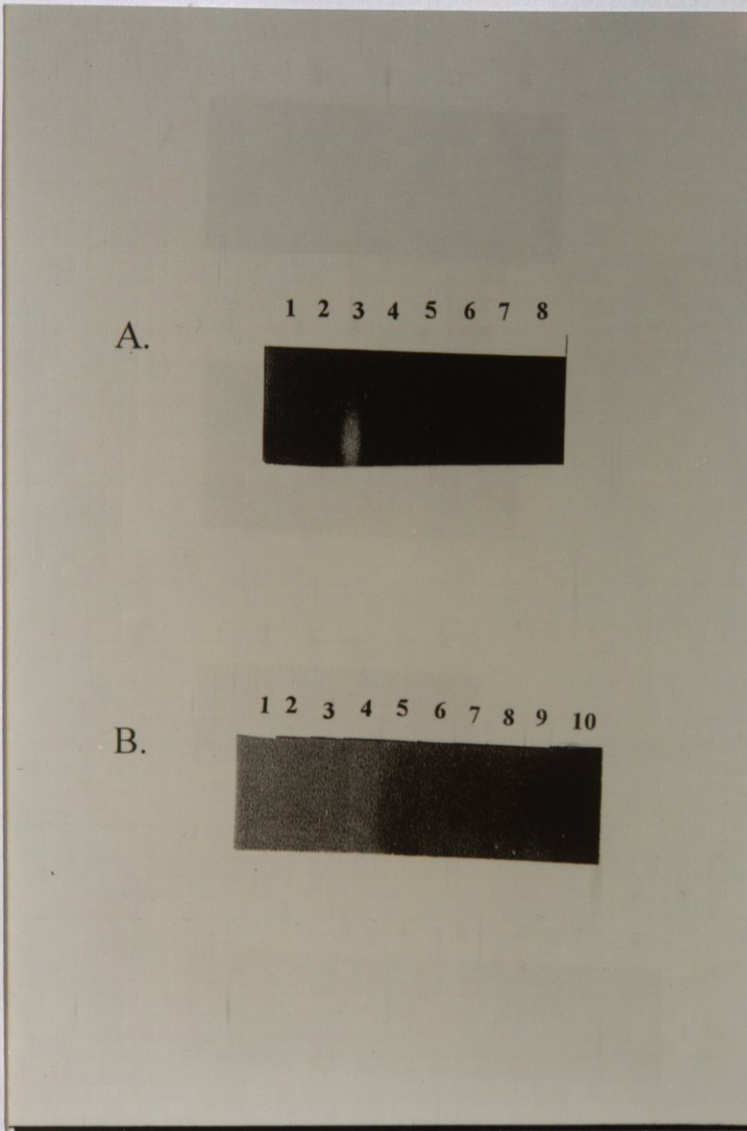


Figure V.6. The dependence of PCR amplification on reverse transcription. PCR reactions were carried out without reverse transcription step. (A) PN0-adult whole retina RNA samples (lanes 1-8). (B) in the external (lanes 1-5) and internal (lanes 6-10) retinal RNA from PN4, PN6, PN10, PN14, and PN20 rats. The amplifications are carried out for 40 cycles and the PCR products are run on 4 % NuSieve agarose gels and visualized by ethidium bromide staining.

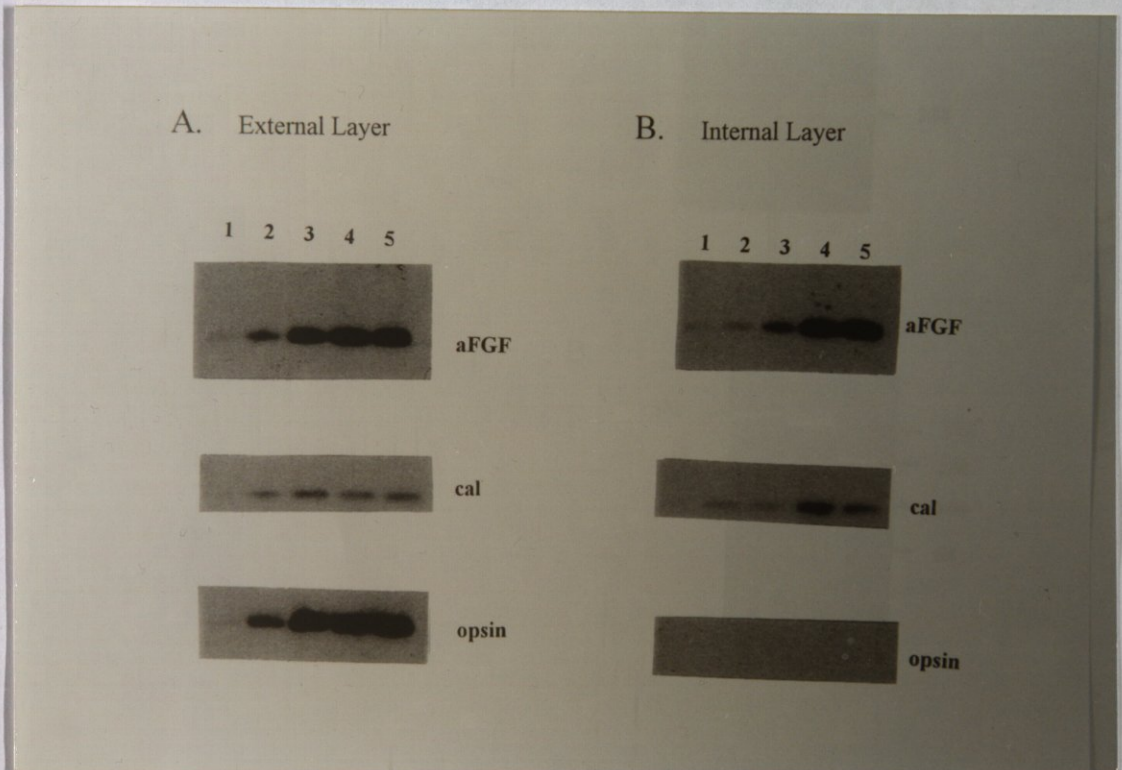
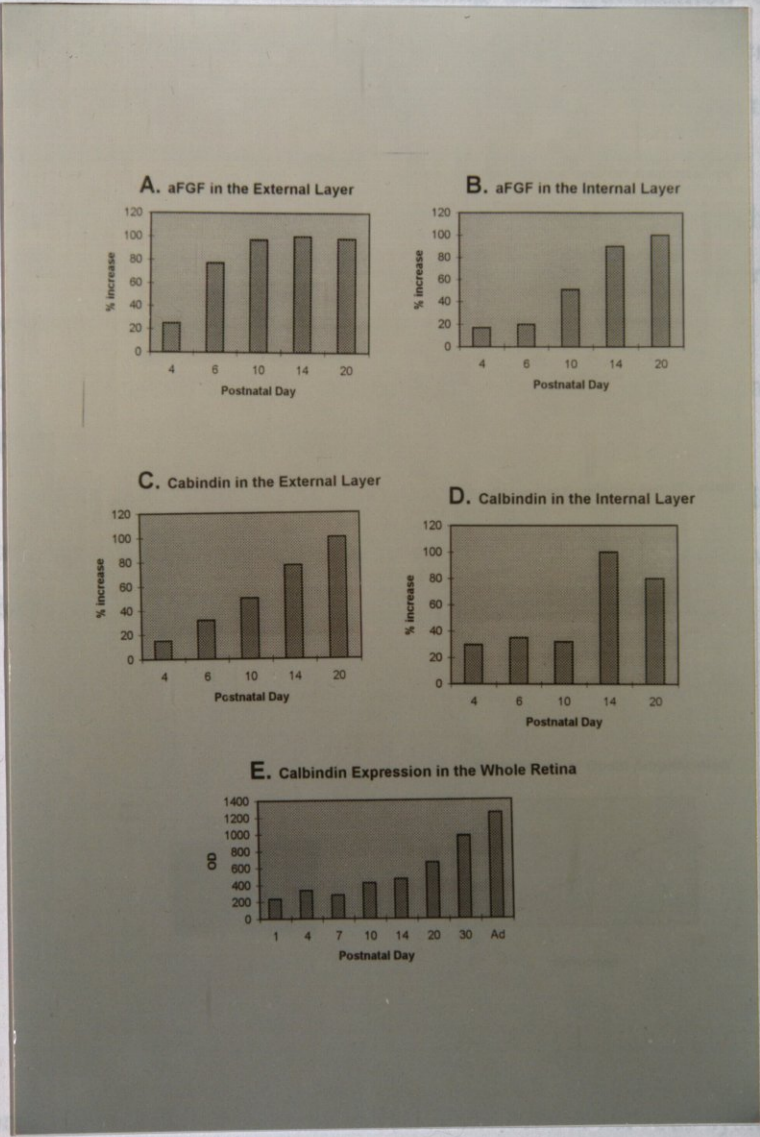


Figure V.7. Expression in the retinal layers. aFGF, calbindin, and opsin expression in the external (A) and internal (B) retinal layers from PN4, PN6, PN10, PN14, and PN20 rats (lanes 1-5). Amplification products were detected by Southern transfer and hybridization with nested oligonucleotides. Films were exposed for various times.

In order to examine the distribution of aFGF in the internal and external layers, aFGF gene was amplified for 24 cycles from the PN4, PN6, PN10, PN14 and PN20 cDNAs, and its expression was analyzed by Southern blotting and hybridization to aFGF nested oligonucleotide. aFGF expression increases at PN6 and reaches its maximum at PN14 and PN20 in the external layer, while in the internal layer, the expression is delayed onset and reaches its maximum at PN20. However, with a higher magnification, it is observed that aFGF expression in the internal layer reaches a level that is approximately 10% of the external layer. This result, unequal expression of aFGF in the internal and external layers, suggests that aFGF is more abundantly expressed in the external layer.

As in the case of aFGF, calbindin expression in the external layer increases with age, but the reverse transcriptase-PCR results show that calbindin mRNA amplification is delayed onset and reaches its maximum at PN20 in the internal layer.



FGF9 expression in the developing retina (Tagashira et al., 1995); therefore, we used hippocampal RNA to optimize RT-PCR conditions for the FGF9 target. The amplifications resulted in a single product of expected size of 388 bp when Mg^{+2} concentration was 4 mM, and the annealing temperature was 50°C (Figure V.3.D). A same size product was also obtained when a retinal RNA sample was used (Figure V.9.A). The identity of this amplified fragment was confirmed by digestion with

Figure V.8. Quantification of aFGF and calbindin expression in the developing retina. Histograms of aFGF expression in the external (A) and internal layer (B), calbindin expression in the external (C), and internal layer (D), calbindin expression in whole retina (E) are drawn according to their densitometric readings.

In order to examine the distribution of aFGF in the internal and external layers, aFGF gene was amplified for 24 cycles from the PN4, PN6, PN10, PN14, and PN20 cDNAs, and its expression was analyzed by Southern blotting and hybridization to aFGF nested oligonucleotide probes. In the external layer, aFGF expression starts to increase at PN6 and reaches its maximum level at PN20 (3 fold) (Figure V.7.A and V.8.A). In the internal layer, the expression pattern of aFGF was similar to the one in the whole retina, however, with a delayed onset (Figure V.7.B and V.8.B). By PN14, aFGF expression reaches a level that is approximately 4 fold of PN4. Since, there was no opsin expression in the internal layer, this result, unequivocally, places the aFGF expression to the cells comprising the inner retina. The comparison of the relative levels of aFGF in two layers suggest that aFGF is more abundantly expressed in the external layer.

As in the case of the whole retina studies, the amplifications were dependent on the reverse transcription step (Figure V.6.B), indicating the PCR products were due to the mRNA amplifications and not due to the contaminating DNA.

C. The Expression of FGF9 in Developing Rat Retina

FGF9 expression was shown to be localized to the central nervous system (Tagashira et al., 1995); therefore, we used hippocampal RNA to optimize RT-PCR conditions for the FGF9 target. The amplifications resulted in a single product of expected size of 388 bp when Mg^{+2} concentration was 4 mM, and the annealing temperature was 50°C (Figure V.3.D). A same size product was also obtained when a retinal RNA sample was used (Figure V.9.A). The identity of this amplified fragment was confirmed by digestion with two different restriction enzymes. Pst I digestion produced 334 and 54 bp fragments, and Bsa II digestion resulted in 288 and 100 bp fragments (Figure V.9.B), as predicted from the restriction map of FGF9 (Miyamoto et al., 1993). When RT-PCR was carried out using PN0, PN4, PN6, PN10, PN14, PN20, PN30, and adult RNA, the FGF9 amplification

product was detected in all postnatal days (Figure V.10.A). The expression of the FGF9 target was not restricted to the postnatal developmental days, since it was also observed in the neural retina of 17 day old embryo (Figure V.10.B). Thus, FGF9 is expressed in the retina at all developmental stages.

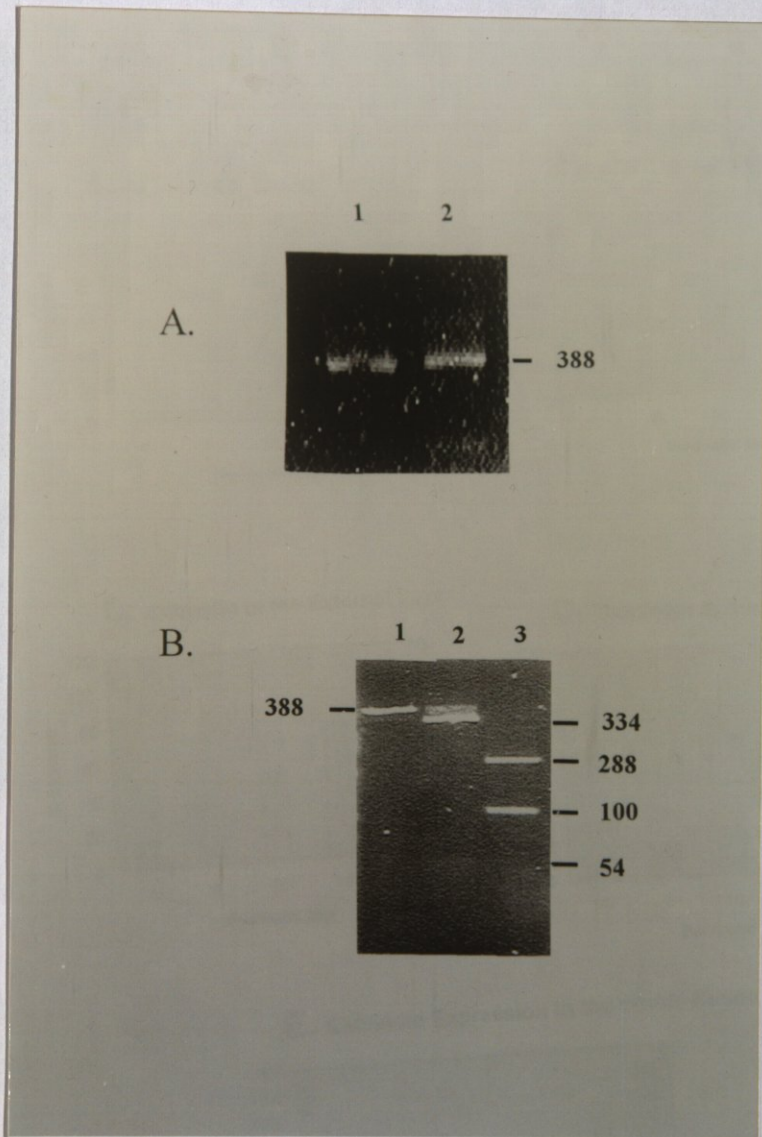


Figure V.9. FGF9 expression. (A) Amplification of FGF9 from hippocampus (lane 1) and whole retina PN20 (lane 2). (B) Restriction digestion analysis of FGF9 amplification product: Undigested FGF9 (lane 1), Pst I digestion (lane 2), Bsa JI digestion (lane 3). The amplifications are done for 40 cycles, and the PCR products are run on a 4% NuSieve gel and visualized after ethidium bromide staining.

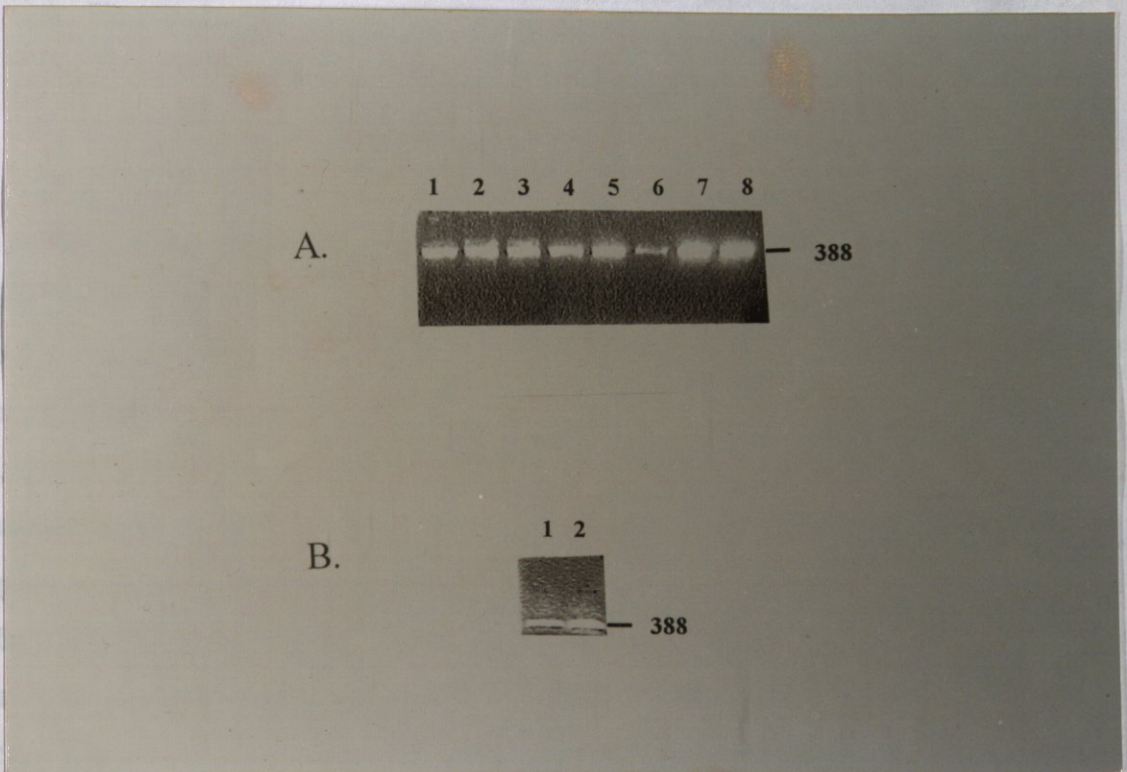


Figure V.10. FGF9 expression in whole retina. (A) FGF9 is amplified from whole retina RNA obtained from PN0 (lane 1), PN4 (lane 2), PN6 (lane 3), PN10 (lane 4), PN14 (lane 5), PN20 (lane 6), PN30 (lane 7) and adult (lane 8) rats. (B) FGF9 is amplified from E17 (lane 1) and P1 (lane 2) rat whole retina. The amplifications are done for 40 cycles, and the PCR products are run on a 4% NuSieve gel and visualized by ethidium bromide staining.

VI. DISCUSSION

A. The Regional Distribution of aFGF mRNA within the Developing Retina

Wide spread interest has been shown in the role of FGFs in the development, differentiation and physiopathology of the retina. Two growth factor activities isolated from bovine retina, named as eye-derived growth factor I and II (EDGF) (Arruti and Courtois, 1978), were later shown to be identical to aFGF and bFGF, respectively (Baird et al., 1985). These factors were shown to stimulate opsin expression and induce neurite outgrowth (Hicks and Courtois, 1988; 1992), and enhance survival of ganglion cells in explant cultures (Bahr et al., 1989). Muller glial cells proliferate in response to FGFs (Hicks and Courtois, 1988). aFGF and bFGF were effective in transdifferentiation of presumptive pigment epithelium cells to ganglion cells (Pittack, 1991; Guillemot and Cepko, 1992). They were also shown to stimulate axonal regeneration (Sievers et al., 1987; Lipton et al., 1988), and retard the degeneration of photoreceptors in rat with inherited retinal dystrophy (Faktorovich et al., 1990). However, *in vivo* functions of these factors are not clear.

Spatial and temporal expression of these factors were studied in order to help understand their potential roles in the retina. However, there is discrepancy in the literature on the localization of aFGF within the adult retina. Caruelle et al. (1989) and Fu et al. (1991) reported ubiquitous presence in all retinal layers by immunohistochemical staining. The presence of aFGF protein only in the ganglion cells was reported by Elde et al. (1991), while immunostaining primarily in the photoreceptors was detected by Buğra et al. (1993). These conflicting results in the localization data are attributed to the experimental parameters such as the differential epitope specificity of the antibodies, the detection method, and the treatment of tissues. *In situ* hybridization studies generally localize the message in all cell layers (Noji et al., 1990; Buğra et al., 1993; Jaquemin et al., 1993),

except Elde et al. (1991) detects the transcript only in the ganglion cells. The developmental expression patterns of aFGF, studied by RT-PCR and immunochemical staining, indicated that it is present throughout the retina and are acting postnatally (Buğra et al., 1993; Buğra and Hicks, in press). Developmental in situ studies were inconclusive due to the low levels of the aFGF transcripts in young animals. In this study, we used RT-PCR and Southern hybridization approach to elucidate the localization of the aFGF expression within the retinal layers. Two retinal layers were used in these experiments: the outer layer of retina, containing primarily the photoreceptors, and the inner layer which is comprised of the interneurons, cell bodies of the Muller glial cells and the ganglion cells. PCR reactions were performed at different exponential phases for different genes where the levels of amplification products reflect the initial transcript levels. The technique provides higher specificity than immunological detection techniques, defined by specific primers in the PCR reactions, characterization of the PCR product by restriction digestion analysis and hybridization with nested oligonucleotide probes.

Before the analysis of aFGF expression in the inner and the outer layers, the purity of the layers were tested by using two control genes. Rod specific opsin was chosen as the photoreceptor layer specific gene; while calbindin was chosen as the marker for the internal layer as it had been reported to be expressed in horizontal and amacrine cells (Hamano et al., 1990; Pasteels et al., 1990; Pochet et al., 1991; Mitchell et al., 1995). The opsin target fragment was only detected in the outer layer, suggesting that there is no significant carry over of the external layer cells in the inner layer preparations. However, we have detected calbindin transcripts in both the inner and the outer layers. This result suggests either the contamination of the outer layer with a portion of the inner layer, or the expression of calbindin both in the inner and the outer layer cells. The contamination possibility, though cannot be ruled out entirely, is unlikely due to the fact that the technique is well established for transplantation experiments (Silverman and Hughes, 1989) and has allowed quantitative compositional analysis of phospholipids (Dreyfus et al., 1996). Two recent publications also lend support to the latter possibility. Enz et al. (1996) detected strong immunolabelling in both cones and bipolar cells of the macaque retina, Yan (1997) reported immunostaining in the photoreceptor layer in humans as well as in the INL and ganglion cells. At any rate, since the main interest lies in the expression of aFGF in the cells other than photoreceptors, the absence of opsin in the inner retina is sufficient for the interpretation of the results

obtained in the internal layer. The developmental expression data obtained from whole retinal RNA samples showed that the calbindin expression levels increase after PN10 and reaching maximum in the adult. Calbindin expression increases significantly around PN14 in both layers, which coincides with period of active synapse formation in the rat retina. Calcium binding proteins at the axon terminals of the postsynaptic neurons are known to be important in the calcium dependent signalling stimulated upon interaction of the receptors at the axon terminal of the postsynaptic cell with the neurotransmitter. The bipolars and the horizontal cells are known to be the postsynaptic cells of the photoreceptors. The increase in the calbindin expression in the internal layer at PN14 may suggest a specific event in the maturation of synapses to gain their physiological functions in the visual cascade, by means of synthesizing the required proteins.

The aFGF expression was detected in both of the layers, with more intense signals in the outer layer. This result shows that aFGF is synthesized by some of the cells comprising the internal layer in addition to the photoreceptors. The expression pattern was similar to the previous reports in the whole retina (Buğra et al., 1993), when the results obtained in the internal and external layers are taken together. In the internal layer, aFGF expression remained at low levels at PN4 and PN6, then started increasing and reached three-fold of the basal levels at PN20, whereas in the external layer, the increase in the level of expression at PN20 is four-fold of PN4. Another result being in agreement with the previous findings in the whole retina obtained by in situ hybridizations using aFGF antisense riboprobes is the localization of aFGF expression predominantly to the photoreceptors (Buğra et al., 1993). We have detected higher expression of aFGF in the outer retina. The relative abundance of the amplification product in the outer layer with respect to inner layer indicates that the steady state levels are higher in the photoreceptors, assuming that the presence of calbindin in photoreceptor layers (Enz et al., 1996; Yan, 1997) is not artifactual. Rod photoreceptors are the last cells to be differentiated in the vertebrate retina and their time of differentiation overlaps with the increase in the opsin expression after PN6 in rats (Triesman et al., 1988). The postnatal increase in the aFGF levels both in the photoreceptor cells and in the cells making up the internal layer after PN6, suggests that this trophic factor could play a role in further maturation of retinal structure, such as the establishment of synapses between differentiated photoreceptors and their postsynaptic cells, the bipolars and horizontal cells. It is known that aFGF stimulates sprouting in various neuronal cell types including

photoreceptors. The increase in the aFGF expression in both the photoreceptor cells and other cells making up the retina can suggest the possibility that this factor could be involved in either the survival of these cell types, or that it acts as a diffusible factor which plays a role in the maturation of the retina to attain its final shape after the differentiation of the retinal cell types.

FGFs use autocrine or paracrine mechanisms to exert their effects on differentiation, survival, and proliferation. However, in order to modulate such responses, coordination with their specific cell surface receptors should be established. It is known that aFGF binds with high affinity to FGFR1, FGFR2, and FGFR3 (Johnson and Williams, 1993). It will be interesting to study the cellular distribution of these two FGF receptors within the retina to get a better understanding of the possible routes of aFGF action during retinal development.

B. The Presence of FGF9 mRNA in the Developing Rat Retina

FGF9 was initially isolated from glial cells and was named as glia-activating factor (Miyamoto et al., 1993) due to its potent mitogenic activity on these cells. Tagashira (1995) detected FGF9 expression in the neurons of different regions of the central nervous system, rather than non-neuronal glia cells. As the neural retina is part of the CNS, the possibility of FGF9 being important in the physiology of the retina is raised. Thus, in this study, we aimed to investigate the presence of FGF9 in the rat retina by RT-PCR. Our results indicate that FGF9 is expressed in this tissue, albeit with moderate levels. Initially, we amplified FGF9 from the hippocampus RNA, where it was previously reported to be expressed. The PCR amplification of the adult whole retinal RNA yielded the same 388 bp fragment. This fragment resulted in an expected 200 and 188 bp restriction pattern after Bsa II digestion, as well as 234 and 54 bp restriction fragments after Pst I digestion. The presence of FGF9 in the whole retina obtained from different ages was confirmed by amplifying this 388 bp fragment from PN0, PN4, PN6, PN10, PN14, PN20, PN30 RNAs.

The same results were obtained when another set of cDNAs from E13, PN1, PN4, PN7, PN10, and PN13 rats were amplified for FGF9. The quantity of the PCR products appeared almost the same for all ages after amplification, but more elaborate quantitative analysis should be performed to investigate the differential expression of this factor in the retina during development. In our experiments, we have detected the presence of FGF9 in E17 RNA, indicating that this factor is present at times of early and active cell differentiation within the retina where there is extensive proliferation and cell migration. This might suggest the mitogenic activity of FGF9 on the retinal cell types of neuronal origin as well as the retinal glial cells. Thus, this factor may be involved in keeping the proliferative capacity of the multicellular retinal progenitor cells until they become postmitotic and afterwards may exert its effect on their survival. Since FGF9 is an embryonically expressed FGF in other species and we have also identified FGF9 expression in E17 RNA, subsequent studies should focus on its expression in earlier developmental stages in the retina, to be able to propose a mechanism for its mode of action in this tissue.

FGF9 was found to be preferentially binding and activating FGFR2c, FGFR3b, and FGFR3c (Santos-Ocampo et al., 1996). The elucidation of the cell types that express FGF9 and the localization of the receptor subtypes for FGF9 in the retina will provide information on the target cells responsive to this factor.

To summarize, it can be concluded that different members of the FGF family have different roles in retinal development. The diversity in the responses of the target cells to different FGFs are primarily a result of both the history of the cell and the presence of specific plasma membrane receptors. Although aFGF seems to be involved in the differentiation of certain cell types and the maturation of the retina, FGF9, with the present data, cannot infer such a developmental role. But the studies on the relative abundance and the distribution of these two factors, as well as their specific high affinity receptors during development, and the correlation of the results with the status of the retinal structure at specific developmental times will provide more information about its possible roles in retinal development.

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