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Original Article

A Zn-finger protein, *Xfin*, is expressed during cone differentiation in the retina of the frog *Xenopus laevis*

FILIPPO M. RIJLI[1], STEFANIA DE LUCCHINI[1]*, GENNARO CILIBERTO[2] and GIUSEPPINA BARSACCHI[1]

[1]*Laboratori di Biologia Cellulare e dello Sviluppo, Dipartimento di Fisiologia e Biochimica, Universitá di Pisa and* [2]*Istituto di Ricerche di Biologia Molecolare, IRBM, Pomezia, Roma, Italy*

ABSTRACT ***Xfin* is a member of a Zn-finger multigene family that shares homology with the *Drosophila* segmentation gene, *Krüppel*. This paper reports on our identification and cellular localization, through the utilization of specific antibodies, of the expression product of *Xfin* in the *Xenopus laevis* retina, and its pattern of expression during the retinal developmental stages. By immunostaining sections of the retina, we show that the major staining is localized in the cytoplasm of the cones. The protein appears at an early differentiation stage of the cones, when they can not be univocally identified by morphological criteria, and is maintained up to the adult retina. The same antigenicity pattern is detectable in the retina of the anuran genus *Bufo*. The immunostaining data are confirmed by Western blot analysis on *Xenopus* eye protein extracts. Because of its cytoplasmic localization, and because of the ability of Zn-finger proteins to bind nucleic acids, we think that *Xfin* may be involved in the terminal differentiation of the cones through RNA-protein interactions.**

KEY WORDS: *Zn-finger proteins, retinal development, cone differentiation, antibodies*

Introduction

The vertebrate nervous system comprises a wide variety of neuronal types, but little is known about the molecular mechanisms that give rise to this vast range of cellular diversity. The neural retina is one of the neural tissues whose histogenesis has been most extensively studied. In its early developmental stages, the retina is composed of a neuroepithelial layer of homogeneous structure which gradually differentiates into multiple layers that are functionally and structurally distinct. Proliferating precursor neuroepithelial cells are determined near the time of the final mitotic division (Turner and Cepko, 1987; Holt *et al.*, 1988; Wetts and Fraser, 1988; Turner *et al.*, 1990).

The question of how different cell types, all originated from a single precursor cell, can be determined and then properly segregated and arranged to form the ordered structure of the neural retina has not yet been answered. For photoreceptors in particular, cell-type commitment is followed by a complex and delayed morphogenesis (Polley *et al.*, 1989; Watanabe and Raff, 1990), which needs to be specified by the developmental program. It is thought that environmental signals rather than lineage or birthdate determine the fate of each cell within the developing retina (Holt *et al.*, 1988). Thus, a process that directs development through cell-type identity signals is thought to exist. In addition to receptors capable of receiving these signals, other proteins capable of interpreting these signals and executing the ordered program of differentiation should conceivably exist within the cell. The nature of these putative proteins is unknown, although the generally accepted view is that they may also include regulators of gene expression. These factors are thought to be part of a network of regulatory interactions that eventually lead to cell differentiation. This network has been partially unravelled in *Drosophila*, where a genetic approach has been successfully employed (for a review see Rubin, 1989).

In vertebrates, the identification of regulatory factors is impaired by it not being feasible to perform a genetic analysis, and thus, the identity and nature of regulators required to specify cell fate in the vertebrate retina remains obscure. However, the discovery that developmental regulators share conserved motifs such as homeobox, paired box, or Zn-finger structures (see Dressler and Gruss, 1988), has made it feasible to use an indirect approach to identify developmentally important vertebrate genes.

Xfin is a Zn-finger gene, originally isolated from a *Xenopus* ovary cDNA library (Ruiz i Altaba *et al.*, 1987): it is a member of a multigene family that shares homology with the *Drosophila* segmentation gene, *Krüppel*, and it is exceptional in the fact that it possesses 37 finger motifs. In a previous work (De Lucchini *et al.*, 1991), we identified

Abbreviations used in this paper: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; DDT, 1,4-dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; NBT, 4-nitro blue tetrazolium chloride.

***Address for reprints**: Laboratori di Biologia Cellulare e dello Sviluppo, Dipartimento di Fisiologia e Biochimica, Via Carducci 13, 56010 Ghezzano, Pisa, Italy. FAX: 39-50-878486.

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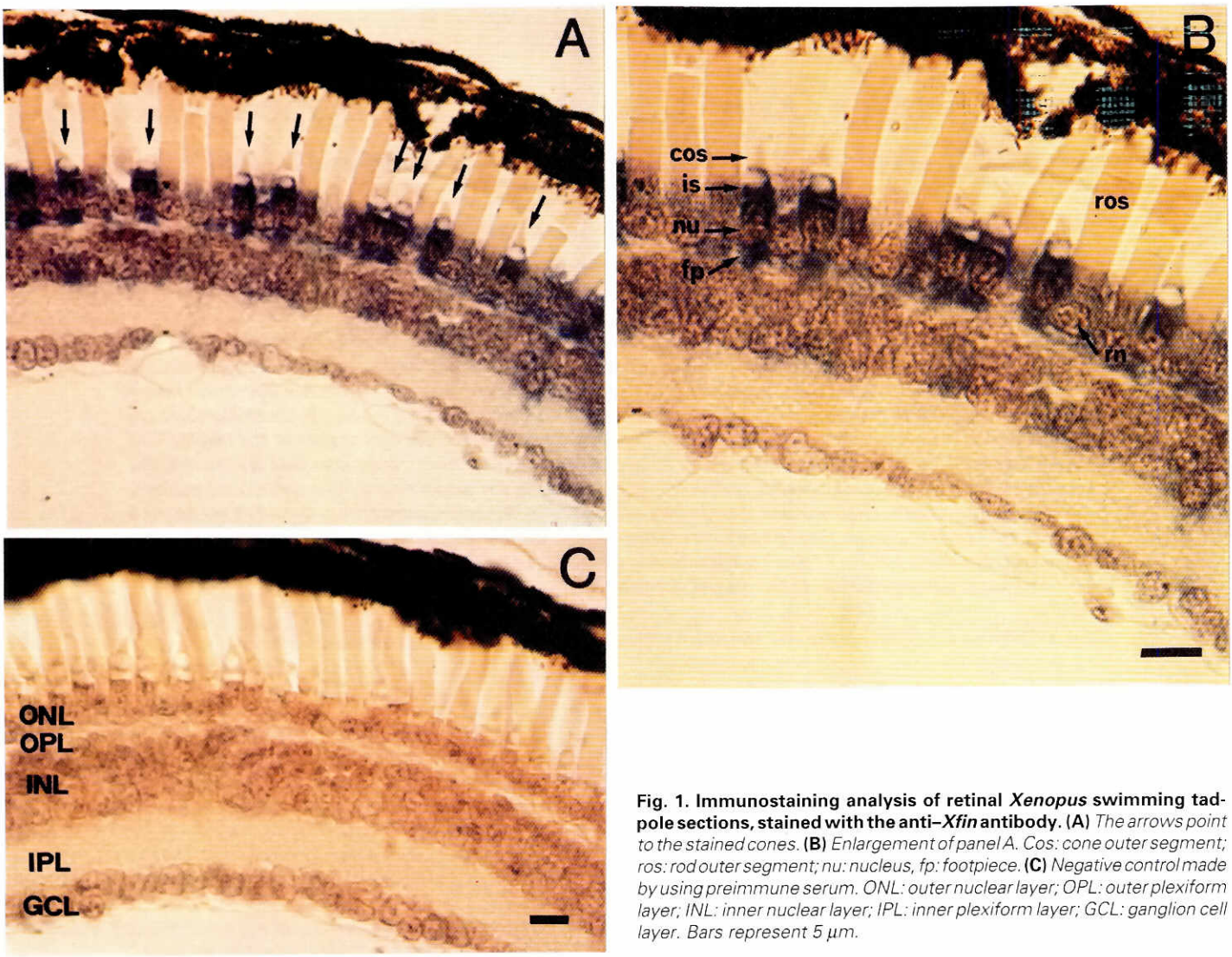

Fig. 1. Immunostaining analysis of retinal *Xenopus* swimming tadpole sections, stained with the anti–*Xfin* antibody. (A) *The arrows point to the stained cones.* **(B)** *Enlargement of panel A. Cos: cone outer segment; ros: rod outer segment; nu: nucleus, fp: footpiece.* **(C)** *Negative control made by using preimmune serum. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Bars represent 5 μm.*

the protein product of *Xfin* by use of antisera raised against two different fusion proteins, and we found that the protein expression in adult tissues is restricted to highly specialized, and embryologically unrelated, cell types. In addition, the protein localization within the cell is cytoplasmic.

In this paper, we report on the identification and cellular localization of the *Xfin* product in the neural retina of *Xenopus* and its pattern of expression during retinal developmental stages. The aim of this work is to study regulatory genes that are presumably involved in cell-type specification within the vertebrate retina, as well as to follow their expression during the differentiation of specific cell types.

Results

The Xfin protein is localized in the cytoplasm of the cones in the neural retina

The anti–*Xfin* antibodies used for this work are described elsewhere (De Lucchini *et al.*, 1991). Briefly, one of the two antisera used (anti–*Xfin*) is directed against a large portion of the protein (395 aa), whereas the other one (anti–*Xfin* 2) is made against a smaller portion of the protein (80 aa), not comprised in the first one and containing neither fingers nor the conserved H/C link. The anti–*Xfin* serum was used to immunostain retinal sections. Fig. 1 shows functionally mature retinal sections from a swimming tadpole eye. At this stage the neural retina has attained the visual function, and photoreceptors are morphologically distinguishable, although their outer segments continue to lengthen until metamorphosis (Saxén, 1954). As shown (Fig. 1A and B), in the neural retina a major *Xfin* staining is present in the cytoplasm of the cones. Due to the limits of the method, we cannot absolutely rule out the possibility that a small proportion of the *Xfin* protein might be present within other cell types and in the cone nuclei as well; however, all other retinal structures show no comparable staining under our immunostaining conditions. Focusing under the microscope shows that the light staining corresponding to the rods may be due to underlying cones. The staining is evident in the cytoplasm of both the cone inner segment and of the cone footpiece, which protrudes into the outer

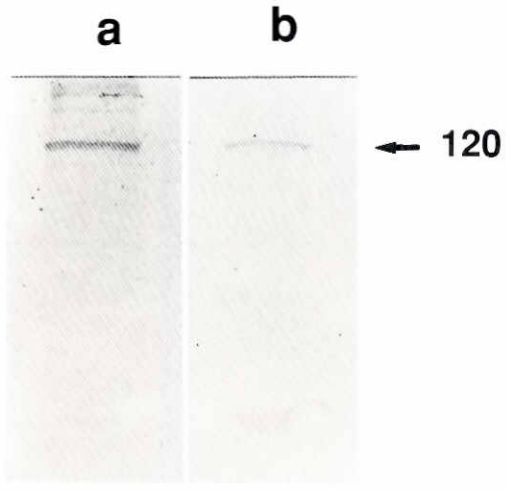

Fig. 2. Western blot analysis of *Xenopus* adult eye extracts. *Proteins were separated on a 10% SDS polyacrylamide gel and stained with the anti–Xfin2 serum, raised against a region of Xfin not containing fingers* **(a)** *and with the anti–Xfin serum* **(b)**. *Both antisera recognize the same 120 Kd antigen (arrow).*

plexiform layer. Other cone structures such as the outer segment, the oil droplet and the paraboloid do not reveal the presence of the *Xfin* protein; these structures unequivocally identify the mature cone. Retinas from adult frogs maintain the same distribution of the protein (not shown). Controls, performed using preimmune serum (Fig. 1C) or commercial anti-ß-galactosidase antibodies (not shown) do not show any staining.

Anti–Xfin antibodies recognize a 120 Kd antigen

Xfin protein expression has been previously reported in specialized cell types of unrelated embryological origin. In Western blots, the anti–*Xfin* antibodies identify an antigen with an apparent Mr of 120 Kd in SDS-PAGE (De Lucchini *et al.*, 1991). To assess the identity of the protein detected by immunostaining in the cones, we performed Western blot analysis on adult eye extracts, using the two different antibodies. Fig. 2 shows the result of such experiment. Similar to what has been found in other somatic or germinal tissues, both antibodies recognize a 120 Kd antigen. This implies that the protein detected in the cones is the same *Xfin* protein previously characterized (De Lucchini *et al.*, 1991).

Xfin is expressed during the terminal differentiation of the cones

Since the *Xfin* protein is present in functionally mature cones, we thought it would be of interest to establish its time of appearance during cone development from late embryo stages onward. To this end, embryo and larval eye sections at different developmental stages were immunostained using the anti–*Xfin* antibody. Retinal developmental stages were identified according to the classification of Grant *et al.* (1980), which is based only on retinal histology, since environmental and growth conditions may affect the rate of eye development differently from that of other organs in laboratory-reared animals.

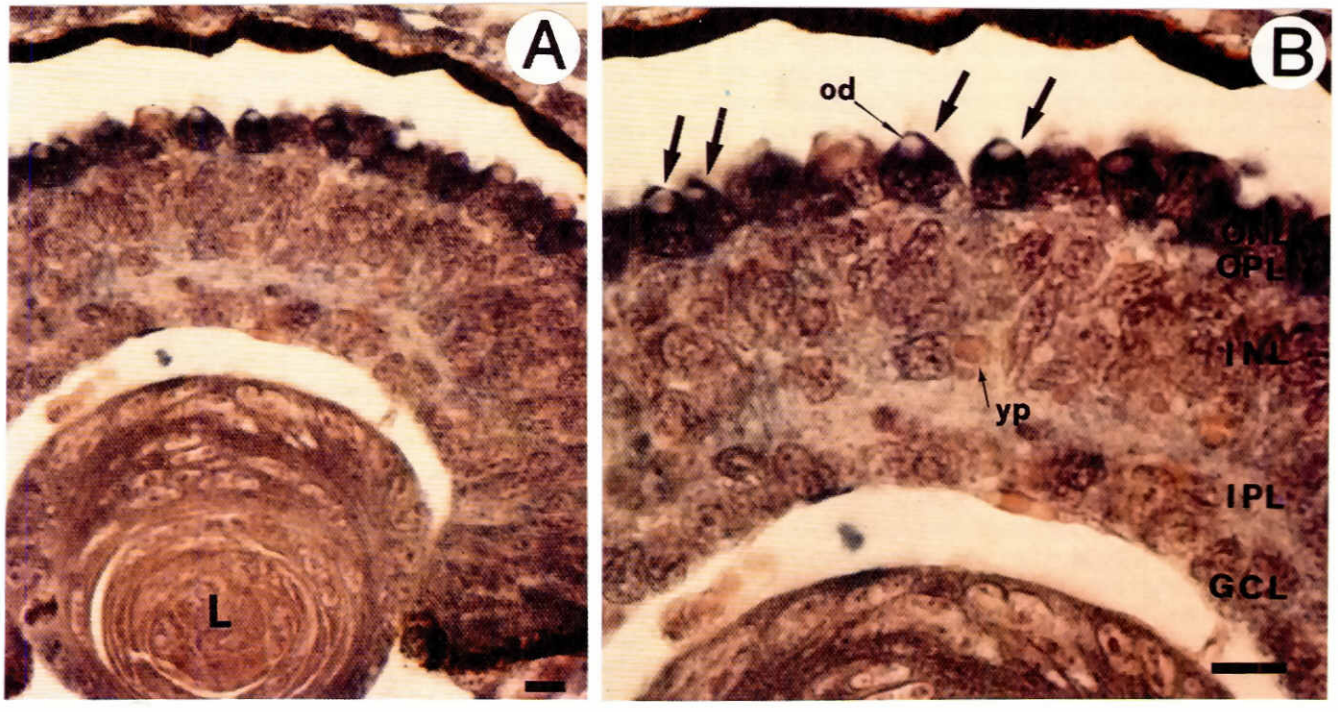

Fig. 3. Immunostaining analysis of retinal developmental stage IX. (B) *Enlargement of* **(A)**. *The arrows point to the stained cells in the photoreceptor layer. od: oil droplet; yp: yolk platelet; L: lens. Nomenclature of retinal layers as in Fig. 1. Bars represent 5 µm.*

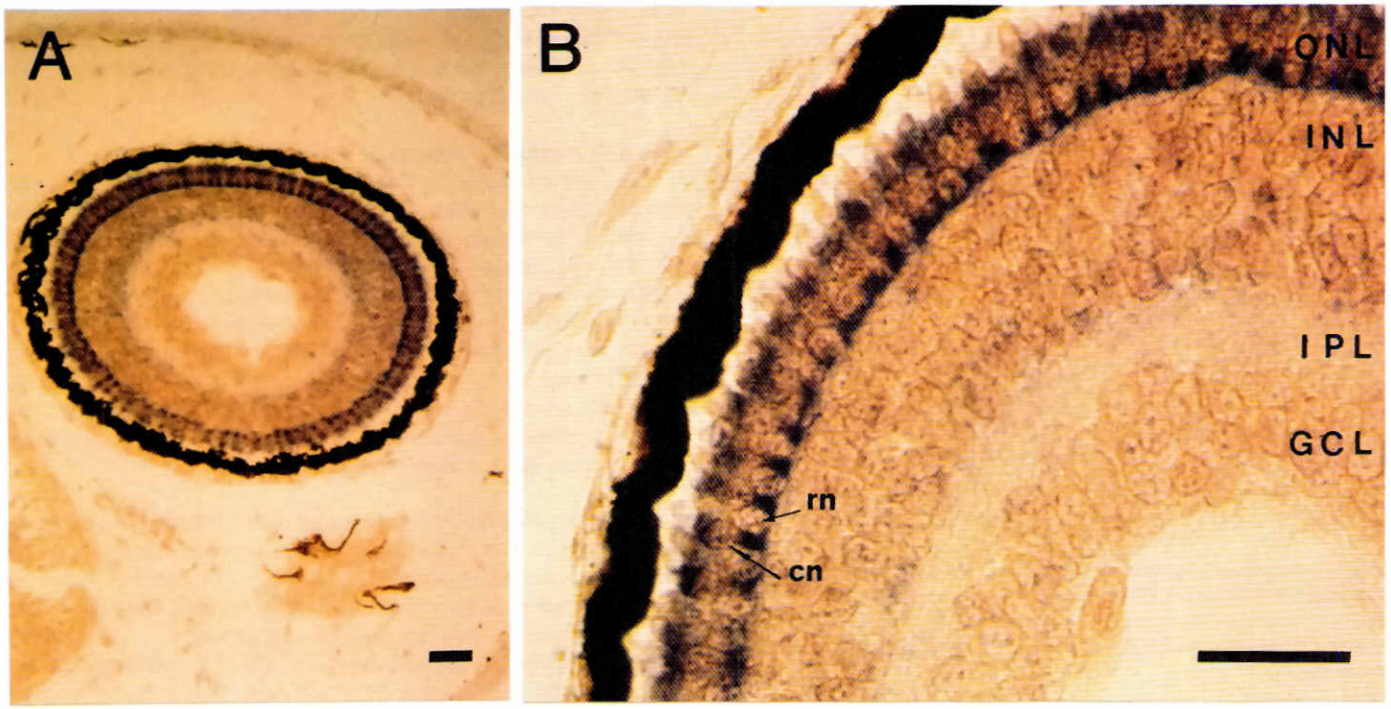

Fig. 4. Immunostaining analysis of retinal stage X. (B) *Enlargement of* **(A)**. *The nomenclature of the retinal layers is as in Fig. 1. rn: rod nucleus; cn: cone nucleus. Bars= 10 μm.*

In normal *Xenopus* embryos, the invagination of the optic vesicle to form the optic cup occurs between Nieuwkoop and Faber (1967) (NF) stages 25 and 29. All the cells of the eye cup are histologically indistinguishable until NF stage 32 o 33. Up to this stage, *Xfin* is not detectable on eye sections (data not shown). As shown in Fig. 3A and B, retinal developmental stage IX (NF 35-37) marks the very first appearance of the protein in the cytoplasm of specific cells in the outer nuclear layer (ONL).

It is not possible at this developmental stage to morphologically distinguish rods and cones since both classes of receptors possess oil droplets, which are eventually lost by rods, and the dimensions of their outer segments are similar (Witkovsky *et al.*, 1976). However, observation under the microscope shows that the staining is not uniformly distributed over the entire ONL; in addition, thereafter in organogenesis the staining is restricted to the cones in the photoreceptor layer (see below). Therefore, we think that the stained cells at this stage might represent an early step of cone differentiation. The appearance of the oil droplet indicates that putative cones have just started their terminal differentiation, since other structures characteristic of the mature cell, such as the outer segment and the footpiece are not yet developed. At retinal stage IX, lamination of the neural retina has already started with the appearance of a diffuse inner plexiform layer (IPL) and a very narrow outer plexiform layer (OPL). The size and number of yolk platelets (yp) in retinal sections has decreased, although they are still present (Fig. 3B). The lens appears spherical and still cellularized, although lens fibers begin to accumulate.

A higher density of stained cells in the ONL can be observed approaching the central portion of the retina with respect to more peripheral portions (not shown). Data reported in the literature indicate that a central-to-periphery gradient of differentiation exists in the photoreceptor layer (see Grant *et al.*, 1980). The appearance of the staining according to the same gradient would therefore suggest that the staining due to *Xfin* expression might represent an early marker for photoreceptor (possibly cone) differentiation.

At retinal stage X (NF 36-38) cone morphogenesis is more advanced, although the cell is still immature (Fig. 4A and B). At this stage, ribbon synapses between photoreceptor and horizontal cells are first detected (Witkovsky and Chen, 1977). A short outer segment and the oil droplet are visible and not stained. A distinctive feature of the photoreceptor layer is the relative position of its nuclei. Cone nuclei are in fact located on the same level above the rod nuclei.

Nuclei appear unstained; staining is restricted to the cytoplasmic portion of the developing inner segment; and to the cone footpiece, which, when sectioned, appears to be intercalated by rod nuclei (Fig. 4B).

Retinal developmental stage XI (NF 39-42) is a period of synaptogenesis and retinal response to light. The pattern of staining over the ONL is similar to that described for stage X, as shown in Fig. 5. At this stage it is still difficult to distinguish between cone and rod outer segments. Ganglion cell layer thickness is reduced to two cells and the lens has a distinct epithelium with a central, concentric ring of lens fibers. Interestingly, no staining is detectable over the marginal zone (MZ), where proliferation from retinal precursor cells occurs (Fig. 5A).

The onset of visual function occurs at retinal developmental stage XII (NF 43-50). Cones and rods are morphologically distinguishable, and functionally mature. Immunostaining of larval retinal sections are shown in Fig. 1.

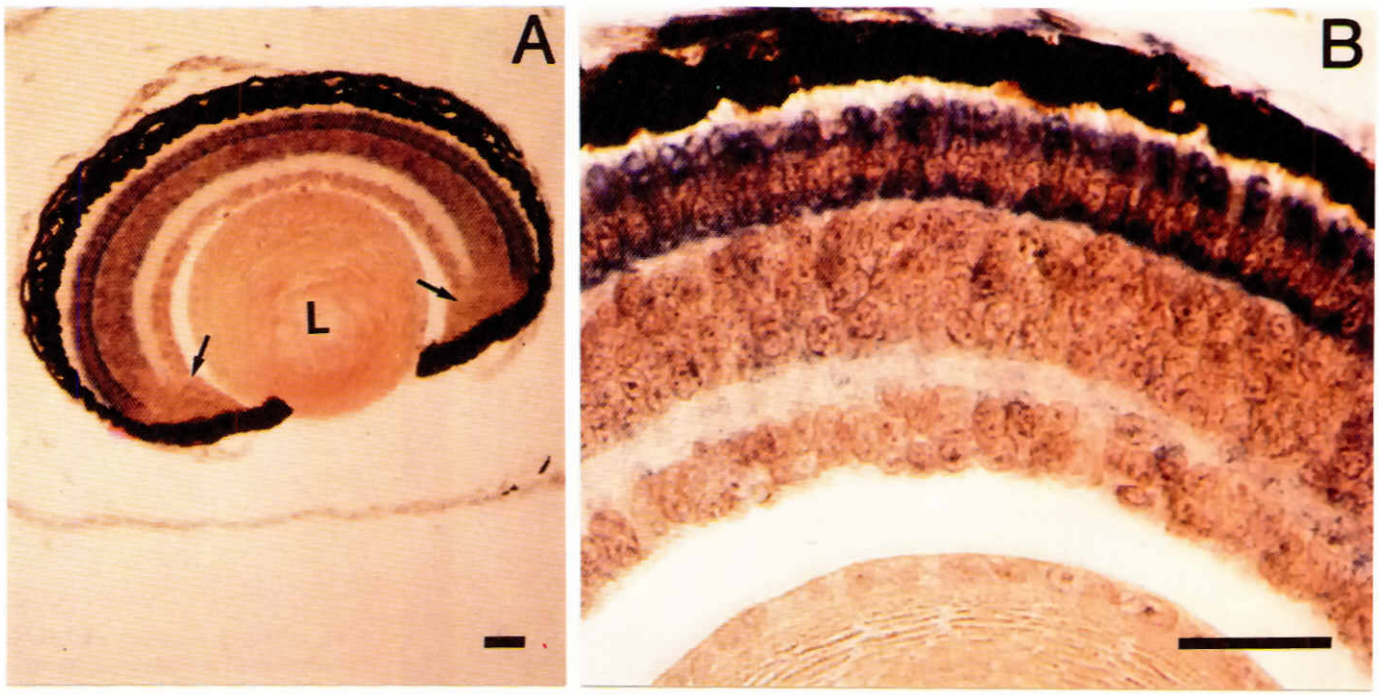

Fig. 5. Immunostaining analysis of retinal stage XI. *The arrows in* **(A)** *point to the marginal zone (MZ). L, lens. Bars= 10 μm.*

Xfin is conserved in the cones of an evolutionarily distant amphibian species

To test whether *Xfin* was conserved in the neural retina of other amphibian species we performed immunostaining on retinal sections of a tadpole belonging to the evolutionarily distant species *Bufo bufo*: we found that the protein expression pattern was conserved (Fig. 6). As in *Xenopus*, staining is restricted to the cytoplasm of the cones, both in the inner segment and in the footpiece. This finding suggests that *Xfin*, or a closely related protein, may be conserved in different species and would argue in favour of the protein playing an important role in cone differentiation.

Discussion

In this paper we show that the *Xfin* gene product is mainly, and perhaps exclusively, expressed in the neural retina in a specific cell type, namely in the cones of the photoreceptor layer. Analyzing the *Xfin* protein expression pattern through the retinal developmental stages – from the embryo to the adult – allowed us to establish what the relationship is between the onset of antigenicity and the appearance of the cone phenotype. *Xfin* protein can first be detected in the developing eye, when the photoreceptor system is still largely immature (Fig. 3A and B), both from a morphological and a functional point of view (retinal stage IX). At this stage, although the photoreceptor layer has already been formed, it is not possible to identify cones by morphological criteria (Witkovsky *et al.*, 1976).

Our analyses show that not all the photoreceptor cells are stained at retinal stage IX, and that the major *Xfin* staining is restricted to the cones in the differentiated retina. These findings make it reasonable to assume that the stained cells represent developing cones; should this assumption be correct, the onset of antigenicity would precede the onset of an identifiable cone phenotype.

It is generally believed that in the frog retina cell fate is determined at or after the final mitosis of a neuroepithelial precursor.

The timing of *Xfin* expression and the fact that the protein is present throughout maturation of the cones up to the adult stage suggest that *Xfin* is involved in the terminal differentiation and phenotypic maintenance of the differentiated cell, rather than in determination events. In fact, the onset of *Xfin* expression occurs several hours after the final mitosis; more importantly, although cone phenotype is not yet established when *Xfin* is first detected, the cell has already occupied its proper position within retinal layers and started its morphological differentiation, as shown by the appearance of the oil droplet. It is worthy of note that the protein is not detectable in the ciliary margin of the retina (Fig. 5); this region produces new retinal cells throughout most of the frog's life (Hollyfield, 1968; Straznicky and Gaze, 1971; Beach and Jacobson, 1979). In this proliferative zone there are no determined mother cells, since clones generated in this area give rise to cells of all retinal types (Wetts and Fraser, 1988).

The regulatory factors involved in cell type specification are difficult to characterize without more specific knowledge of the molecular mechanism involved (see Introduction). Zn-finger motifs are amongst the most highly conserved DNA-binding domains that regulate gene expression during development (for a review, see Dressler and Gruss, 1988), and a Zn-finger gene, *glass*, has been found to be responsive to positional signals in the developing *Drosophila* eye (Moses and Rubin, 1991).

The discovery that the same antigenicity pattern found in *Xenopus* is present in the retina of an evolutionarily distant anuran species *(Bufo)* argues in favour of the *Xfin* protein playing an important role

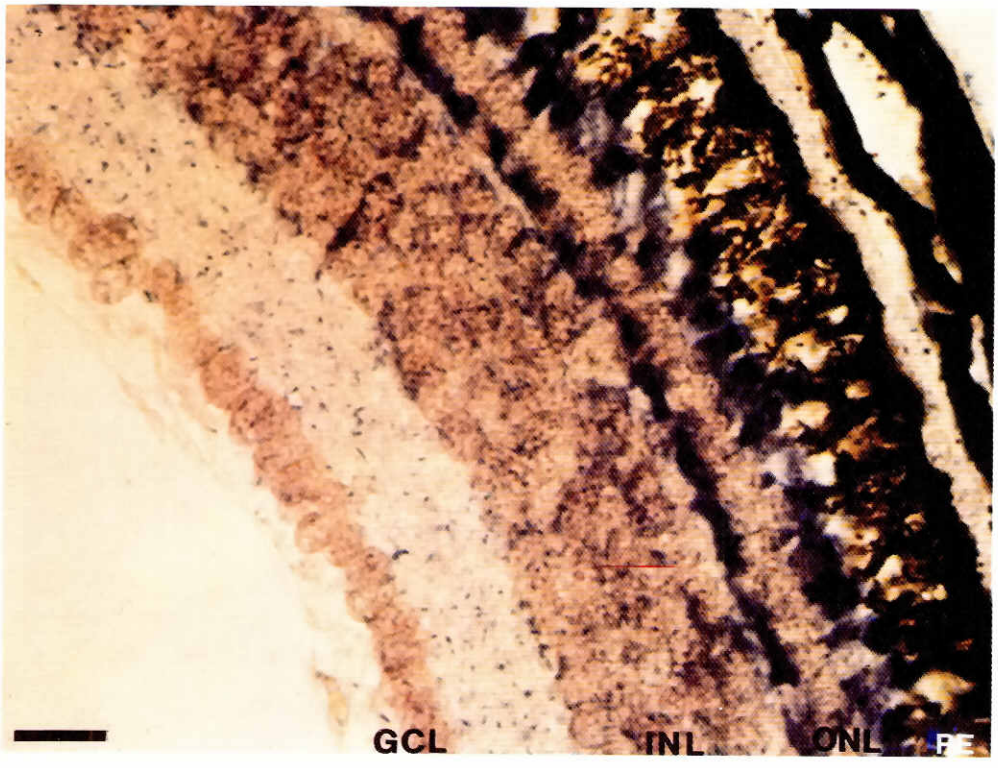

Fig. 6. Immunostaining analysis of a retinal *Bufo* tadpole section, showing the same antigenicity pattern as in *Xenopus*. *Nomenclature of the retinal layers as in Fig. 1. PE: pigmented epithelium. Bar= 5 μm.*

in the establishing and/or maintaining cone phenotype. We are presently investigating the extent of *Xfin* conservation.

The main, and perhaps the exclusive, subcellular localization of the *Xfin* protein is cytoplasmic in cones. This finding is consistent with the previously reported cytoplasmic localization of the *Xfin* protein in highly specialized cell types, such as male germ cells and the epithelial cells of a specific portion of the nephric tubule (De Lucchini *et al.*, 1991).

Within the limits of the method, our immunostaining assay revealed no detectable *Xfin* protein in the nucleus during the various developmental stages of cones: we therefore hypothesize that the *Xfin* protein could have a specific cytoplasmic role. As Zn-finger proteins are able to bind specific RNA sequences, as well as DNA (see Klug and Rhodes, 1987), we can speculate that the role played by the *Xfin* protein involves protein-RNA interactions. These interactions might interfere with RNA stability or translational activity of individual messages or sets of mRNAs, possibly representing a novel regulatory mechanism of differentiation.

Materials and Methods

Embryos

Xenopus laevis specimens were obtained from NASCO (Wisconsin, USA). Embryos were obtained by gonadotrophin stimulation and mating. Embryos were staged according to Nieuwkoop and Faber's (1967) external criteria.

Bufo tadpoles were collected in the wild from the vicinity of Carrara (Italy).

Preparation of protein extracts and Western blot analysis

Eyes from adult specimens were homogenized in 2-4 ml/g of 15 mM HEPES (pH 7.6), 100 mM KCl, 6 mM $MgCl_2$, 1mM EDTA (pH 8.0), 250 mM sucrose, 1mM DTT, 0.5 mM PMSF. The homogenate was cleared by centrifugation at 4,000 rpm for 10 minutes, at 4°C. Supernatant was recovered and protein concentration determined against a BSA standard by the Bradford method (Bradford, 1976).

SDS-PAGE electrophoresis and Western blot analysis using alkaline phosphatase conjugated secondary antibodies were performed by standard methods (see Harlow and Lane, 1989). The anti–*Xfin* antibody was used at a dilution of 1:1000, while the anti–*Xfin* 2 antibody was diluted 1:500.

Immunostaining analysis

Embryos, tadpoles and adult eyes were fixed in 2% formaldehyde, 5% acetic acid and 250 mM NaCl for 3-4 hours. After fixation, they were dehydrated, embedded in paraffin (m.p. 57°C) (Fisher) and serially sectioned at 6-8 μm through their transverse or longitudinal planes.

For immunostaining analysis using the alkaline phosphatase detection system, sections were incubated essentially as described by Oliver *et al.* (1988) with minor modifications. Antibody dilution was 1:200 and the incubation was carried on overnight at 4°C. The background was decreased by adding in the incubation mixture and during all the washes 5% normal goat serum, as the secondary antibody was a goat-anti rabbit alkaline phosphatase conjugated (Promega). Color development (substrate BCIP and NBT) reaction was done for 10-30 min. The reaction was stopped with 20 mM Tris, 5 mM EDTA (pH 8.0). Sections were dehydrated and mounted in Permount (Fisher).

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