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Molecular cloning of gefiltin (ON_1): serial expression of two new neurofilament mRNAs during optic nerve regeneration

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The goldfish visual pathway displays a remarkable capacity for continued development and plasticity. The intermediate filament proteins of this pathway do not match the intermediate filament protein composition of adult higher vertebrate neurons, which lack the capacity for growth and development. Using a goldfish retina λgt10 library we isolated cDNA clones representing the predominant goldfish optic nerve neurofilament protein, ON_1. The mRNA for this protein is abundant in retinal ganglion cells, and its level increases slowly during optic nerve regeneration. The rate of ON_1 mRNA accumulation after optic nerve crush was compared with that of plasticin, a previously described novel type III neurofilament from goldfish retinal ganglion cells. Plasticin mRNA is normally expressed at low steady state levels, but accumulates dramatically and rapidly, preceding gefiltin mRNA, in response to optic nerve crush. The predicted amino acid sequence for ON_1 indicates that it is a novel intermediate filament protein. We have named it gefiltin, for goldfish eye intermediate filament protein. The serial expression of plasticin and gefiltin is discussed with respect to the diversity of neurofilament proteins during neurogenesis [Liem,R.K.H. (1933) *Curr. Opin. Cell Biol.*, 5, 12-16].

Key words: cDNA sequence/cytoskeleton/goldfish/intermediate filament proteins/visual pathway

Introduction

The visual pathway of adult goldfish is neurogenic in that it displays continuous growth and development. Furthermore, a remarkable capacity for functional regeneration occurs after optic nerve injury (Sperry, 1963). These properties are not observed in higher vertebrates, where neurogenesis is far more restricted to early development. In the goldfish optic nerve the intermediate filament (IF) protein composition is different from that found within the more stable mammalian optic nerves (Quitschke and Schechter, 1984). The goldfish visual pathway may be viewed as somewhat unique since continued plasticity is forced by a symmetrical growth of retinal ganglion cells targeting to an asymmetrical growth of tectal cells (Johns and Easter, 1977; Meyer, 1978; Easter and Stüermer, 1984). These growth characteristics are not observed in the goldfish spinal cord. Furthermore, the complement of the IF proteins in this tissue is different from the optic nerve (Quitschke *et al.*, 1985). Thus, there may be a linkage between the expression of specific IF proteins and the special properties of the goldfish visual pathway.

Intermediate filaments are the most enigmatic of the filamentous networks which make up the eukaryotic cytoskeleton. Although the morphology of the IF network is similar in different cell types, the proteins of this network are structurally diverse. Additionally, these proteins are expressed in a cell type specific manner and are developmentally regulated (Klymkowsky *et al.*, 1989). All IF proteins have a common structural organization, consisting of a highly conserved α-helical central domain (rod) which is flanked by an amino-terminal (head) domain and a carboxy-terminal (tail) domain (Geisler *et al.*, 1982). These head and tail domains are variable in size and amino acid sequence, and impart the major portion of the heterogeneity to IF proteins (Geisler *et al.*, 1983). It is thought that the variable domains impart structural attributes that fulfill physiological and morphological requirements of specific cell types.

The persistent growth characteristics of the goldfish visual pathway have been correlated to the expression of specific proteins from two very different gene families; namely, the homeobox (Levine and Schechter, 1993) and IF gene families (Quitschke and Schechter, 1984; Giordano *et al.*, 1989; Druger *et al.*, 1992; Glasgow *et al.*, 1992). Recently, we identified a new type III neurofilament protein from goldfish retinal ganglion cells, plasticin (Glasgow *et al.*, 1992). In normal retina and the optic nerve, plasticin is in very low abundance. In contrast to plasticin, the predominant neurofilament proteins in the axons of the goldfish optic nerve consist of two 58 kDa isoelectric variants, previously designated ON_1 and ON_2 (optic nerve 58 kDa neurofilament proteins; Quitschke and Schechter, 1984). Biochemical and immunohistological studies have determined that ON_1 and ON_2 are neurofilament proteins, synthesized in retinal ganglion cells, and are transported into the optic nerve by axonal transport (Quitschke and Schechter, 1984; Jones *et al.*, 1987). Peptide mapping analysis and immunological studies have determined that ON_1 and ON_2 are similar to each other, with ON_2 possibly being a phosphorylated product of ON_1 (Jones *et al.*, 1986b; Quitschke and Schechter, 1986). The levels of ON_1/ON_2 decrease concordantly with axonal degeneration after nerve crush and then their expression increases significantly during the regrowth of the nerve (Quitschke and Schechter, 1983; Tesser *et al.*, 1986). Maximum levels of these proteins are synthesized at a time when optic axons have invaded the entire tectum (30 days after optic nerve crush), a period of intense synaptogenesis (Schmidt *et al.*, 1983; Stüermer and Easter, 1984; Hayes and Meyer, 1989).

We previously thought that ON_1/ON_2 were a goldfish form of vimentin (Quitschke and Schechter, 1984). This

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assumption was based upon immunohistological studies that examined the expression of various IF proteins in the developing nervous system of higher vertebrates (Tapscott *et al.*, 1981; Bignami *et al.*, 1982). These studies showed that vimentin is transiently expressed in neurons during neurogenesis prior to the expression of the typical neurofilament proteins of differentiated neurons. The additional observation of Cochard and Paulin (1984) that showed significant vimentin expression in the rat olfactory pathway was especially appealing, since this pathway has a robust capacity for development. The physiological parallel between this pathway and the goldfish visual pathway was striking, and we therefore expected that ON_1 would be a vimentin-type protein. Here we report the results of molecular cloning studies that show that ON_1 is a novel neurofilament protein. We name this protein gefiltin because it represents a new goldfish eye IF protein. In addition, we show that the mRNA for two novel neurofilament proteins, plasticin (Glasgow *et al.*, 1992) and gefiltin, are serially expressed in retinal ganglion cells during optic nerve regeneration.

Results

To obtain amino acid sequence data for the major neuronal IF protein of goldfish optic nerve, ON_1, cytoskeletal preparations from goldfish optic nerve were resolved by two-dimensional gel electrophoresis (Figure 1A) and electrotransferred to nitrocellulose membranes. The ON_1 protein was excised from the filter, digested with trypsin and separated by HPLC. Several optimum fractions were selected for microsequencing. Amino acid sequence was obtained for seven different peptides as shown in Figure 1B. A 44 nucleotide 'guessmer' oligonucleotide was designed from the longest contiguous ON_1 tryptic peptide sequence (peptide 6; Figure 1B).

Several cDNA clones were obtained by screening two goldfish retina cDNA libraries with the oligonucleotide probe. The entire coding sequence for ON_1 was obtained by a combination of rescreening a λgt10 retina cDNA library with a 5′ cDNA probe and polymerase chain reaction (PCR) amplification of the extreme 5′ region (Figure 2A; also see Materials and methods). Figure 2B shows the nucleotide and predicted amino acid sequence of ON_1. The combined sequence is 2982 nucleotides, containing a 1483 nucleotide single long open reading frame. The first ATG codon, following a single in frame stop codon, is at position 90. The predicted protein initiating at this position is 472 amino acids long, with a calculated molecular mass of 54 472 Da. The coding region is flanked by an 89 nucleotide 5′ non-coding region and a 1477 nucleotide 3′ non-coding region. The amino acid sequences of all the microsequenced ON_1 tryptic fragments are contained within the predicted amino acid sequence of this clone, confirming that this cDNA encodes the ON_1 protein.

Comparison of the predicted amino acid sequence for ON_1 with sequences for other IF proteins indicates that the ON_1 protein is a novel IF protein. We designate this new neurofilament protein gefiltin, for goldfish eye filament protein. Gefiltin displays the characteristic structural organization of all IF proteins, consisting of head, rod and tail domains. Figure 3 shows the predicted amino acid sequence of gefiltin aligned with four neurofilament proteins; *Xenopus* XNIF, rat α-internexin, mouse NF-L and hamster vimentin. The central α-helical rod domain of gefiltin contains the typical features of IFs. The rod domain is arranged in heptad repeat units $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$ where non-polar amino acids are usually in the a and d positions and the amino acids in the other positions are either polar or charged. At least 14% of the a and d positions are charged amino acids and are found at highly conserved sites. Two small linker regions (L1 and L1−2) of variable amino acid sequence interrupt the rod domain to form three distinct α-helical tracts (coils 1, 1b and 2). Additionally, there is another small linker sequence in the amino-terminal region

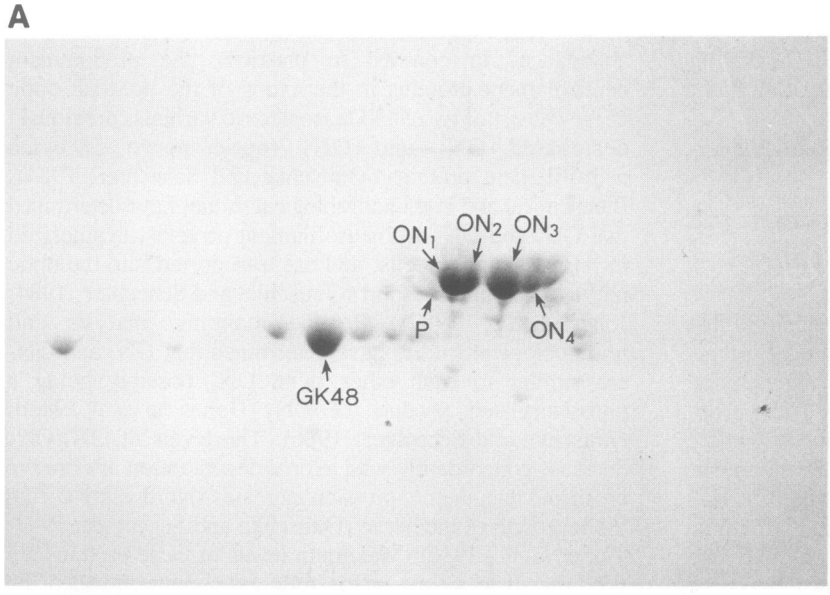

Fig. 1. **(A)** Representative Coomassie blue stained cytoskeletal preparation of optic nerve proteins separated by two-dimensional electrophoresis. The major neuronal (ON_1 and ON_2) and non-neuronal (ON_3, ON_4 and 48 kDa) IF proteins of the optic nerve are labeled. P, plasticin, is barely detectable in the normal optic nerve. Twenty two-dimensional gels were electrotransfered to nitrocellulose and the protein representing ON_1 was excised, digested with trypsin, separated by HPLC and microsequenced. **(B)** Amino acid sequence of seven ON_1 tryptic fragments. Bracketed amino acids indicate lower confidence results from the peptide microsequencing.

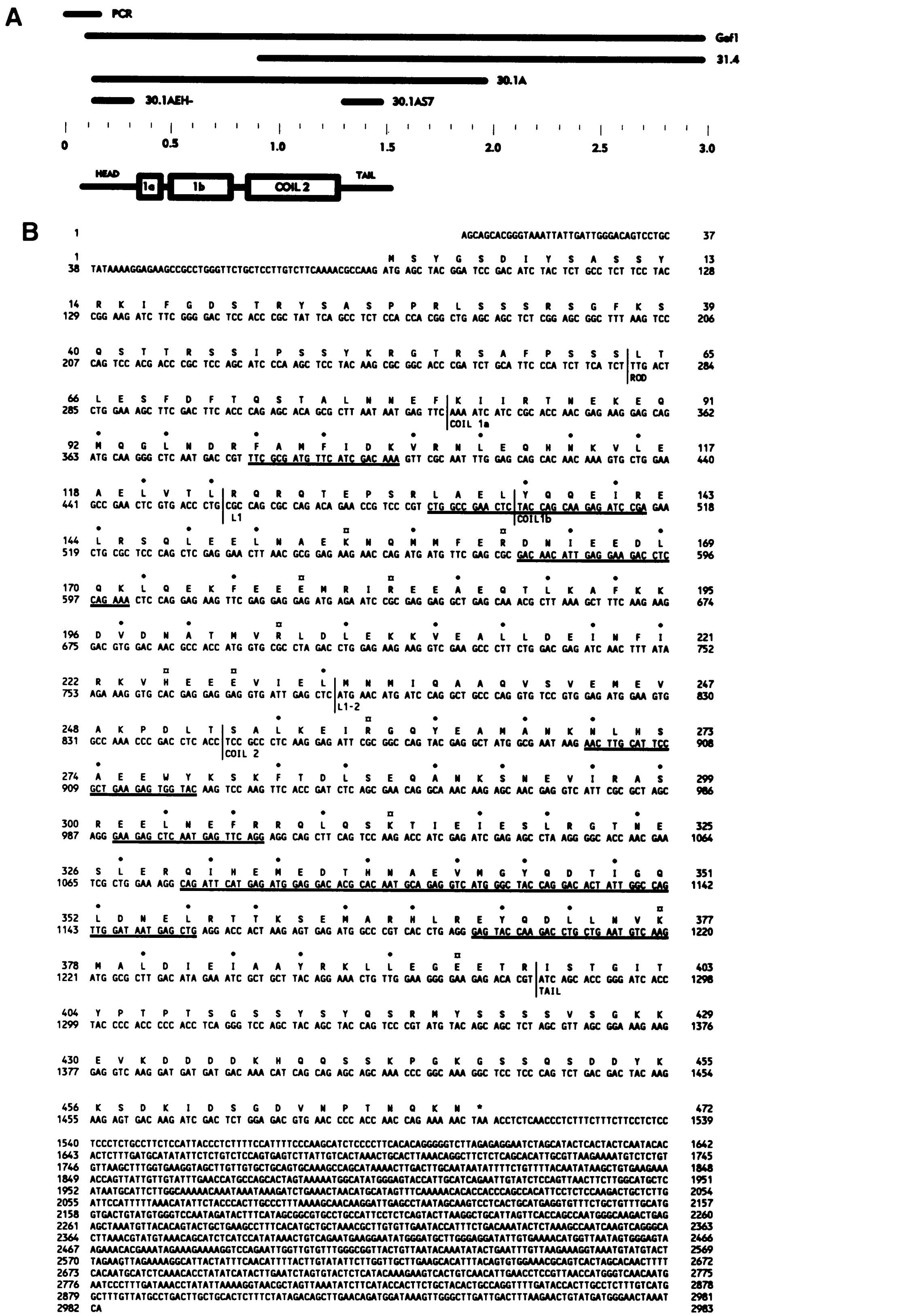

Fig. 2. (A) Schematic representation of gefiltin clones compared with the predicted gefiltin protein. The scale denotes kilobase pairs and indicates the sizes of the cDNA clones. The head and tail domains and coils 1a, 1b and 2 in the α-helical rod region are shown in the schematic diagram for the protein. Clones 30.1A and 31.4 were isolated by oligonucleotide screening of goldfish retina λgt10 and λgt11 cDNA libraries, respectively. The nearly full length cDNA clone, Gef1, was isolated from the λgt10 library by rescreening with a 5′ specific subclone of 30.1A, 30.1AEH$^-$. The extreme 5′ region of gefiltin was obtained by PCR (see Materials and methods). The subclones of 30.1A, 30.1AEH$^-$ and 30.1S7, were used for the RNase protection and *in situ* hybridization probes, respectively. **(B)** The combined nucleotide and predicted amino acid sequence of gefiltin. Microsequenced ON_1 tryptic peptide amino acid sequences are underlined. Structural regions common to all IF proteins are demarcated by vertical lines. Closed circles above the amino acids indicate the a and d residues within the repeating heptad sequence (form a-b-c-d-e-f-g). Modified open circles indicate charged amino acids that are conserved in other IF proteins. The GenBank accession number for gefiltin is L19595.

of coil 2 which contains a single highly conserved tryptophan, and a single amino acid insertion causing a stagger in the heptad repeats near the middle of this coil (Steinert and Roop, 1988). The amino-terminal head domain of gefiltin shows considerable amino acid similarity to α-internexin and XNIF. There is less similarity in this region when gefiltin is compared with vimentin or NF-L. In contrast to the head domain, the carboxy-terminal tail domain of gefiltin shows essentially no similarity to any IF protein. Conspicuously absent is the type III specific carboxy-terminal consensus sequence, IKTVETRDG. Also, the glutamate-rich regions commonly found in type IV IF proteins are absent.

Table I shows the percentage of identical amino acids at aligned positions between gefiltin and several other IF proteins in the head, rod and tail domains. The rod domain of gefiltin is most similar to type III and type IV proteins and exhibits a percentage amino acid identity that is typical for different IF proteins within the same class. The similarity of gefiltin to hamster vimentin and mouse NF-L is not appreciably different from that of *Xenopus* vimentin (Herrmann *et al.*, 1989) or *Xenopus* NF-L (Charnas *et al.*, 1992). In the head domain, gefiltin is more similar to α-internexin and XNIF. Clearly, gefiltin is most divergent in the tail domain.

Northern blot analysis of mRNA from normal retina and retina 20 days post-optic nerve crush is shown in Figure 4. A ^{32}P-labeled gefiltin cDNA probe hybridizes with a band at ~3.0 kb. This message shows a large increase in response to optic nerve crush.

RNase protection analysis was used to assay the presence of gefiltin mRNA in various tissues of the goldfish. Figure 5 shows protected gefiltin mRNA bands only in central nervous system (CNS) tissues. Gefiltin mRNA is predominantly found in the retina. Additionally, there is a small amount of gefiltin mRNA in the brain and spinal cord. No mRNA was detected in eye lens, skin, liver, oocytes or optic nerve.

The expression of gefiltin mRNA in the retina was assayed

Table I. Percent identity of gelfiltin to other IF proteins

	Head	Rod	Tail	Overall
XNIF	37	61	19	52
αI	52	66	4	54
NF-L	24	58	4	45
VIM	13	59	4	46

The head, rod and tail regions of gelfiltin were compared with the corresponding regions of the four IF proteins depicted in Figure 3.

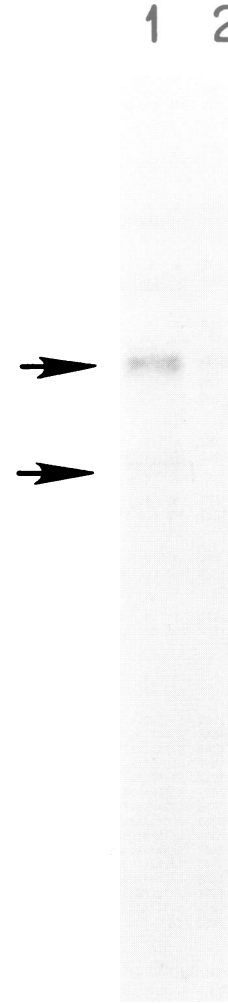

Fig. 4. Northern blot of goldfish retina mRNA hybridized with a random primed ^{32}P-labeled gefiltin cDNA probe. Lane 1, goldfish retina 20 days after optic nerve crush, 1 μg poly(A)$^+$ RNA. Lane 2, non-operated goldfish retina, 1 μg poly(A)$^+$ RNA. Arrowheads denote the position of the 28S and 18S rRNAs.

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          HEAD
GEF       MSYGSDIYS--ASSYRKIFGDSTRYSASPPRLS---SSRSGFKSQS--------TTRSS
XNIF      MTSRELYT----SSYKKIFGDSPRSSSLLYTTNS-SSSRSQSYRPR--------EAYTS
αI        MSFGSEHYLCSASSYRKVFGDGSRLSA---RLSGPGASGS-FRSQSLSRSNVASTAACS
NF-L      MSSFSYEPYFS-TSYKRRYVETPRVHI--------SSVRSGYSTARSAYSSY-SAPVSS
VIM       MSTRSVS----SSSYRRMFGGPGTSNQRSSNRSYVTTSTRTYSLGSLRPSTSRSLYSS
                              |ROD                        |COIL 1a
GEF       IPSSYKRGTRSAF-----PSSS|LTLESFDFTQSTALNNEF |KIIRTNEKEQMQGLNDR
XNIF      NISSYRKVSRSPG------HLS|SAQDHFDLSQSTALSNEL |KIVRTNEKEQLQGLNDR
αI        SASSLGLGLAYR--------RL|PASDGLDLSQAAARTNEY |KIIRTNEKEQLQGLNDR
NF-L      SLSVRRSYSSSSG------SLM|PSLENLDLSQV-AISNDL |KSIRTQEKAQLQDLNDR
VIM       PGGAYVTRSSAVRLRSSMPGVR|LLQDSVDFSLADAINTEF |KNTRTNEKVELQELNDR
                                  |L1                 |COIL 1b
GEF       FAMFIDKVRNLEQHNKVLEAELVTL|RQRQTEPSRLAEL|YQQEIRELRSQLEELNAEK
XNIF      FVTYIEKVHHLEQQNKLLESEVTLL|RQKHSEPSRLSHI|YEQEIRELRSKLEEQEQDK
αI        FAVPIEKVHQLETQNRALEAELAAL|RQRHAEPSRVGEL|FQRELRELRAQLEEASSAR
NF-L      FASFIERVHELEQQNKVLEAELLVL|RQKHSEPSRFRAL|YEQEIRDLRLAAEDATNEK
VIM       FANYIDKVRFLEQQNKILLAELEQL|KGQGK--SRLGDL|YEEEMRELRRQVDQLTNDK

GEF       NQMMFERDNIEEDLQKLQEKFEEEMRIREEAEQTLKAFKKDVDNATMVRLDLEKKVEAL
XNIF      DQAQLVYEHLGACLEQLKLKLEQESARREEAEDVMKNYRKDLDQATLNRLQLEKKVESL
αI        AQALLERDGLAEEVQRLRARCEEESRGREGAERALKAQQRDVDGATLARLDLEKKVESL
NF-L      QALQGEREGLEETLRNLQARYEEEVLSREDAEGRLMEARKGADEAALARAELEKRIDSL
VIM       ARVEVERDNLAEDIMRLREKLQEEMLQREEAESTLQSFRQDVDNASLARLDLERKVESL
                              |L1-2                      |COIL 2
GEF       LDEINFIRKVHEEEVIEL|MNMIQAAQ--VSVEMEV-AKPDLT|SALKEIRGQYEAMAN
XNIF      LDEIAFLRKVHEEEIAEL|QASVQEAQ--ISVEMDVVSKPDLT|AALKEIRMQYEVLSA
αI        LDELAFVRQVHDEEVAEL|LATLQASSQAAAEVDVAVAKPDLT|SALREIRAQYESLAA
NF-L      MDEIAFLKKVHEEEIAEL|QAQIQYAQ--ISVEMDVSSKPDLS|AALKDIRAQYEKLAA
VIM       QEEIAFLKKLHDEEIQEL|QAQIQEQH--VQIDVDV-SKPDLT|AALRDVRQQYESVAA

GEF       KNLHSAEEWYKSKFTDLSEQANKSNEVIRASREELNEFRRQLQSKTIEIESLRSTNESL
XNIF      RNQQSSEEWYQAKIANVSLEASRNNDSVRQAKEEITEYRRQLQARTLEIDALRSANESL
αI        KNLQSAEEWYKSKFANLNEQAARSTEAIRASREEIHEYRRQLQARTIEIEGLRGANESL
NF-L      KNMQNAEEWFKSRFTVLTESAAKNTDAVRAAKDEVSESRRLLKAKTLEIEACRGMNEAL
VIM       KNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESNEYRRQVQSLTCEVDALKGTNESL

GEF       ERQIHEMEDTHNAEVMGYQDTIGQLDNELRTTKSEMARHLREYQDLLNVKMALDIEIAA
XNIF      ERQLQEAEDRSNEEMSHLQDTIGQLDNALRTTKEEMARHLREYQDLLNVKMALDIEIAA
αI        ERQILELEERESAEVAGYQDSIGQLESDLRNTKSEMARHLREYQDLLNVKMALDIEIAA
NF-L      EKQLQELEDKQNADISAMQDTINKLENELRSTKSEMARYLKEYQDLLNVKMALDIEIAA
VIM       ERQMREMEENFALEAANYQDTIGRLQDEIQNMKEEMARHLREYQDLLNVKMALDIEIAT
                              |TAIL
GEF       YRKLLEGEETR|ISTGITYPTPTSGSSYSYQSRMYSSSSVSGKKEVKDDDDKHQQSSKP
XNIF      YRKLLEGEETR|LTSVGGGSMFGIGYPYSSGSYSGGRSSTTSTISIRKEEKKESPEGGK
αI        YRKLLEGEETR|FSTSGLSISGLNPLPNPSYLLLPPRILSTTSKVSSAGLSLKKEEEEE
NF-L      YRKLLEGEETR|LSFTSVGSITSGYSQSSQVFGRSAYSGLQSSSYLMSARAFPAYYTSH
VIM       YRKLLEGEESR|ISLPLPNFSSLNLRETNLESLPLVDTHSKRTLLIKTVETRDGQVINE

GEF       GKGSSQSDDYKKSDKIDSGDVNPTNQKN*
XNIF      GGSSGQPKTSKPGDQEKISQKAAAN*
αI        EEEEEGASKEVTKKTSKVGESFEETLEETVVSTKKTEKSTIEEITTSSSQKM*
NF-L      VQEEQSEVEETIEATKAEEAKDEPPSEGEAEEEEKEKEEGEEEEGAEEEEAAKDES...
VIM       TSQHHDDLE*
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Fig. 3. The predicted amino acid sequence of gefiltin (GEF) manually aligned with those of *Xenopus* XNIF (XNIF; Charnas *et al.*, 1992), rat α-internexin (αI; Ching and Liem, 1991), rat NF-L (NF-L; Chin and Liem, 1989) and hamster vimentin (Quax *et al.*, 1983). Vertical lines demarcate structural regions common to all IF proteins. Amino acids in bold represent sequences that are identical to the gefiltin sequence. Dashes are inserted to optimize alignment.

by RNase protection at several time-points after optic nerve crush. Retina RNA was assayed from the left non-operated eye and the right operated eye at 0, 5, 10, 20, 27, 37 and 120 days after optic nerve crush as seen in Figure 6A. The day 0 time-point and the left non-operated retina time-points remain relatively unchanged and serve as a baseline control. The operated right retina show a large increase in gefiltin mRNA levels during regeneration.

To estimate the relative rates of gefiltin versus plasticin mRNA accumulation during regeneration, a standard curve was prepared by densitometric scanning of RNase protection assays on known amounts of *in vitro* transcribed gefiltin and plasticin sense RNAs. Densitometric scannings of the gefiltin and plasticin RNase protection time-course experiments were compared with standard curves to estimate the absolute amounts of gefiltin and plasticin mRNA present in 20 μg of total retinal RNA at each time-point during regeneration. These qualitative estimates of the absolute levels of mRNA are plotted graphically in Figure 6B. It is clear from these experiments that plasticin mRNA is normally expressed at very low levels. After optic nerve crush, plasticin mRNA levels increase dramatically, peaking at ~20 days. In contrast, gefiltin mRNA is moderately abundant in the normal adult retina. After optic nerve crush, gefiltin mRNA levels slowly increase, peaking at ~40 days.

The site of gefiltin mRNA synthesis in the retina was examined by *in situ* hybridization using an ^{35}S-labeled antisense RNA probe to the variable tail region of gefiltin. Hybridization is observed as discontinuous clusters in the retinal ganglion cell layer (Figure 7). In retinas at 11, 16 and 20 days after optic nerve crush, there is a significant increase in signal hybridization (Figure 7C, D and E). In contrast, retinas from the left non-operated (control) eyes, show no increase in signal hybridization (Figure 7F). Gefiltin mRNA could not be detected when the sections were incubated in pancreatic RNase A prior to hybridization or when the tissue was hybridized with the sense RNA probe.

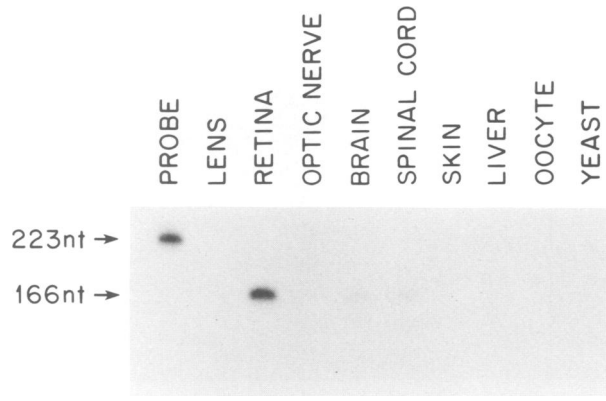

Fig. 5. RNase protection analysis of gefiltin RNA levels in goldfish tissues. A 223 nucleotide, ^{32}P-labeled antisense RNA probe was incubated with 20 μg of total RNA from various goldfish tissues (labeled), digested with RNase A and run on an 6% polyacrylamide–urea gel. The protected fragment is 166 nucleotides.

Discussion

We report the primary structure of the predominant neurofilament protein of the goldfish visual pathway that we have been investigating for some time (ON_1; Quitschke and Schechter, 1983). Previous biochemical and immunohistochemical studies show that this protein is synthesized in goldfish retinal ganglion cells, induced after optic nerve injury and is transported into the optic nerve within the slow phase of axonal transport. (Quitschke and Schechter, 1984; Jones *et al.*, 1986b, 1989; Jones and Schechter, 1987). Here

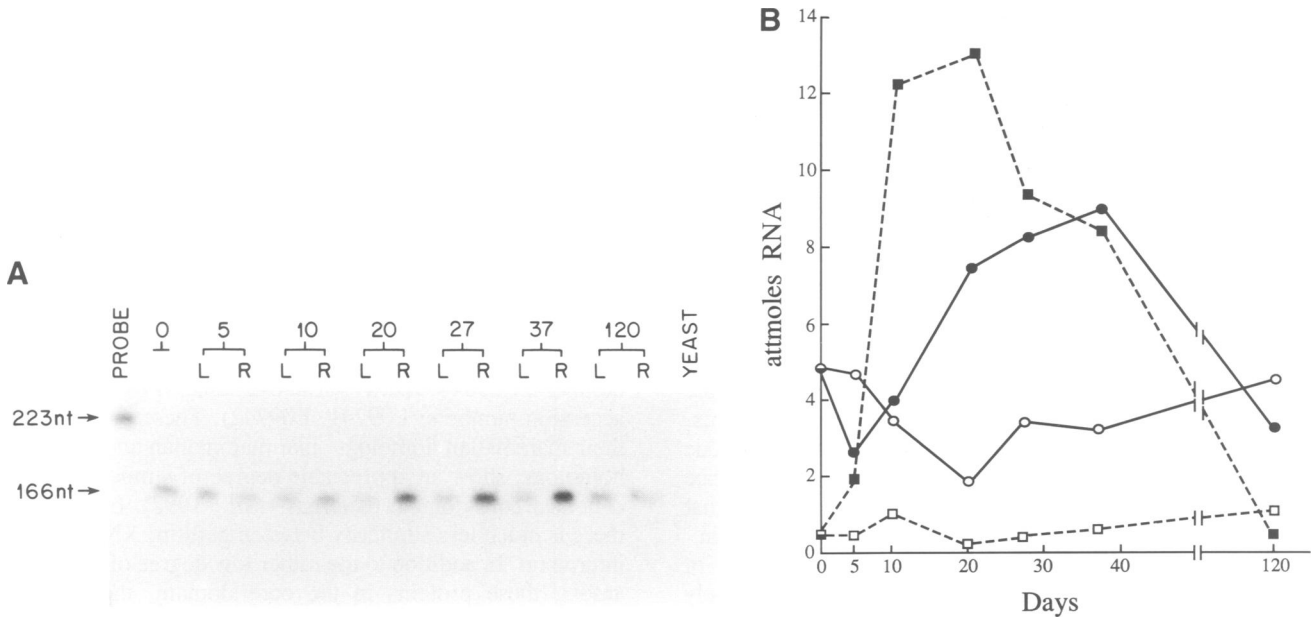

Fig. 6. Response of gefiltin and plasticin mRNA levels in the retina to optic nerve crush. (**A**) RNase protection analysis of gefiltin mRNA levels in the retina during optic nerve regeneration. A 223 nucleotide, ^{32}P-labeled antisense RNA probe was incubated with 20 μg total RNA from retina which either innervate the crushed or the opposite non-crushed (control) optic nerve. The non-hybridized RNA was digested with RNase A and separated on an 6% polyacrylamide–urea gel. The protected fragment is 166 nucleotides. L, left, non-operated (control) retina; R, right, operated retina. The number of days after optic nerve crush is shown above the lanes. (**B**) Graph of the estimated molar amounts of plasticin and gefiltin mRNA in 20 μg total retina RNA at various times after optic nerve crush. Solid squares, plasticin mRNA in operated retina; open squares, plasticin mRNA in non-operated retina; solid circles, gefiltin mRNA in operated retina; open circles, gefiltin mRNA in non-operated retina.

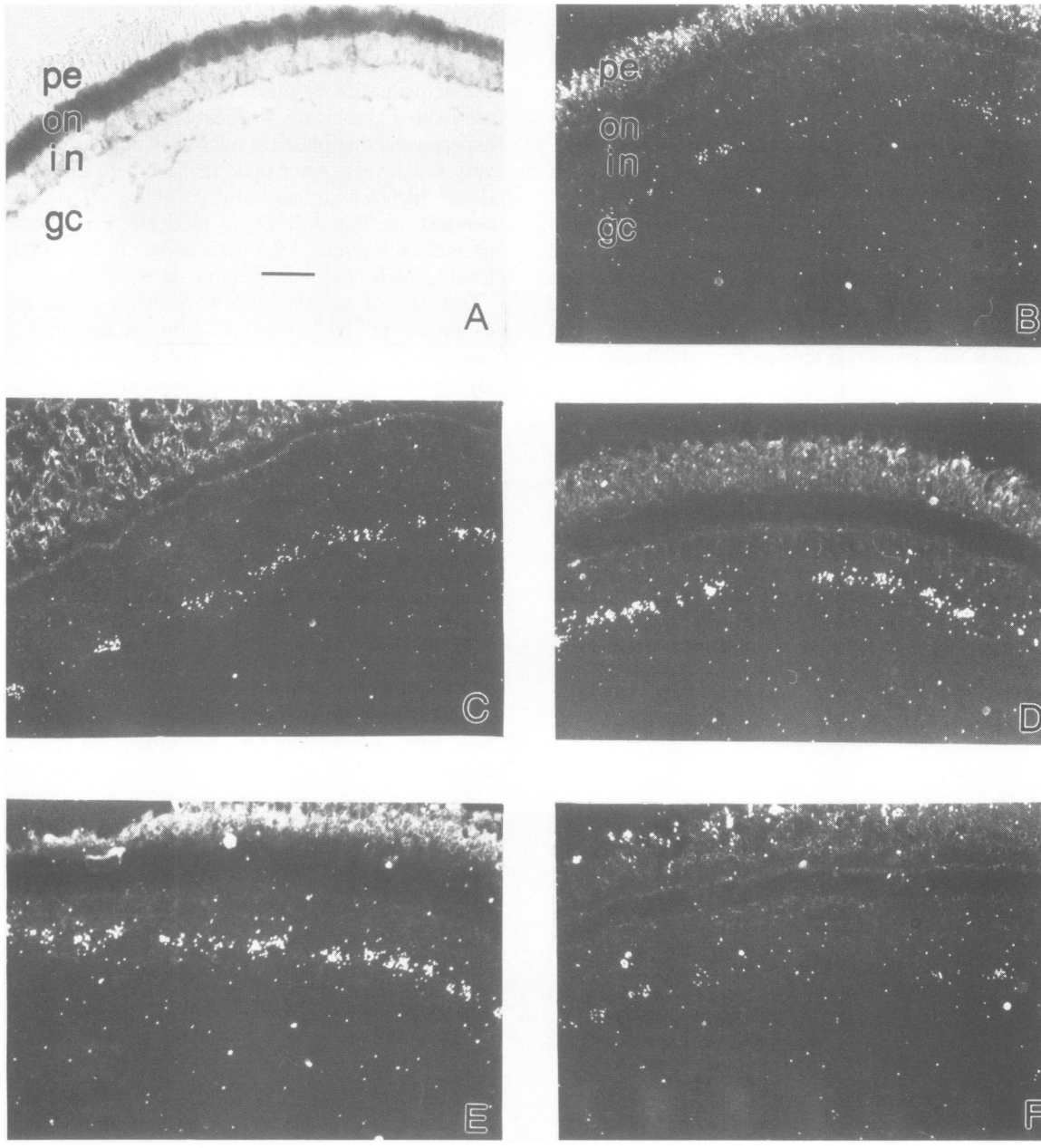

Fig. 7. Localization and expression of gefiltin mRNA in goldfish retina, detected by *in situ* hybridization. (**A**) Bright field. (**B**) Dark field. (**C**) Dark field, 11 days after optic nerve crush. (**D**) 16 days post-crush. (**E**) 20 days post-crush. (**F**) 20 days post-crush, non-operated (control) eye. GC, retinal ganglion cell layer; IN, inner nuclear layer; ON, outer nuclear layer; PE, pigment epithelium. Scale bar = 67 μm.

we isolated ON_1 from two-dimensional gels and directly sequenced seven peptide fragments. Using a degenerate oligonucleotide probe representing one of these fragments, a cDNA was isolated for the ON_1 protein. All seven peptide fragments overlapped the predicted amino acid sequence from the cDNA. The amino acid sequence indicates that ON_1 is a new IF protein which we have named gefiltin.

Comparison of the predicted amino acid sequence of gefiltin with other IF proteins indicates that it is most closely related to the type IV IF proteins, with highest homology to XNIF and α-internexin. Although gefiltin is most similar to XNIF and α-internexin, the degree of homology to these proteins is much lower than that typically found between other goldfish IF proteins and their mammalian homologs. We have recently isolated goldfish cDNAs representing homologs of mammalian keratins K8 (Giordano *et al.*, 1989) and K18 (Druger *et al.*, 1993), vimentin (GenBank accession numbers; L23840, L23841, L23842) and NF-M (GenBank accession numbers; L09741, L09742). These proteins and their mammalian homologs, like mammalian and *Xenopus* homologs, show an appreciable degree of similarity in the α-helical core domain (Charnas *et al.*, 1992). In contrast, there is much less similarity between gefiltin, XNIF and α-internexin. In addition to the rather low degree of similarity among these proteins in the core domain, the variable carboxy-tail domains are quite divergent. This argues for a type IV subclass of distinct, yet related, α-internexin-like proteins, presently consisting of α-internexin, XNIF and gefiltin.

Although the amino acid sequence of gefiltin shows a somewhat equal divergence from vimentin and NF-L, examination of well conserved specific sequences supports

the view that gefiltin is a type IV IF protein. In the amino-terminal head domain of gefiltin, the sequence SSYRKIFGD is clearly homologous to the functionally important nonapeptide motif SSYRRIFGG from *Xenopus* vimentin (Herrmann *et al.*, 1992). This motif is seen in all type III IF proteins, with the notable exception of goldfish plasticin (Glasgow *et al.* 1992). Additionally, this motif is highly conserved in α-internexin and XNIF, while less conserved versions are seen in NF-L and NF-M. However, two hallmark features of type III IF protein rod domains, from fish to mammals, are the presence of a single cysteine roughly in the middle of coil 2, and the sequence TYRKLLEGEE at the highly conserved carboxy-terminal end of coil 2. Gefiltin, like all type IV IF proteins, lacks this cysteine and has the sequence AYRKLLEGEE at the end of coil 2. It is interesting to note, however, that the sequence of a cytoplasmic IF protein from the cephalochordate *Branchiostoma lanceolatum* also lacks this cysteine and contains the sequence AYRKLLEGEE, despite having a clear type III genomic organization (Riemer *et al.*, 1992). All of this, taken together, along with our preliminary observations of the gene organization of gefiltin, supports the classification of gefiltin as a type IV IF protein.

We have examined the expression of gefiltin mRNA in retinal ganglion cells during optic nerve regeneration by RNase protection assays and by *in situ* hybridization. There is a moderate steady state level of gefiltin mRNA in normal retinal ganglion cells. After optic nerve crush, gefiltin mRNA slowly accumulates during regeneration. The increase in gefiltin mRNA begins at ~20 days and peaks at ~40 days after optic nerve crush, corresponding to a period of intense synaptogenesis which occurs 15–40 days following nerve crush. This is in agreement with our previous observations of an increase in ON_1 protein synthesis during optic nerve regeneration (Quitschke and Schechter, 1983). In contrast, plasticin mRNA accumulation is rapid and dramatic. The different rates of plasticin versus gefiltin mRNA accumulation during optic nerve regeneration suggest that these two mRNAs are regulated by distinct mechanisms. The initial increase in plasticin mRNA correlates with growing neurites prior to reaching the optic tectum, suggesting a response to growth signals. In contrast, the increase in gefiltin mRNA does not occur until after the neurites have reached the tectum, suggesting that gefiltin mRNA accumulation is responding to factors from the tectum. This idea is supported by results from our previous studies on the effect of tectal ablation on expression of ON_1 protein. These studies showed that tectal ablation dramatically reduces the synthesis of ON_1 in retinal explants cultured 23 days after induction of regeneration by optic nerve crush (Hall and Schechter, 1991).

Goldfish retinal ganglion cells are heterogeneous with respect to their morphology, in that four major types have been described (Hitchcock and Easter, 1986). Additionally, the continuous development of new retinal ganglion cells provides a range of birth dates for these cells (Johns and Easter, 1977; Meyer, 1978). Our experiments do not distinguish whether plasticin and gefiltin are serially expressed in different retinal ganglion cell populations, or whether they are serially expressed in the same cells.

Isolation of several new mammalian neurofilament proteins by molecular cloning has revealed an orchestrated pattern of IF expression during higher vertebrate neurogenesis (Fliegner and Liem, 1991). Superimposed on this changing expression pattern is a neurofilament network composed of varying IF proteins in different neuronal cell types. During the earliest stages of this process, the type I and type II keratins, K8 and K18, are expressed in neuroepithelial cells (Jackson *et al.*, 1980). As neurogenesis proceeds, vimentin and nestin are coexpressed in neuronal stem cells (Bignami *et al.*, 1982; Cochard and Paulin 1984; Lendahl *et al.*, 1990). As the neuronal stem cells become post-mitotic, nestin expression is suppressed. Coincident with the end of migration and the beginning of neuronal differentiation, α-internexin expression begins, soon to be followed by NF-L and NF-M expression in most CNS neuronal cell populations (Chiu *et al.*, 1989; Kaplan *et al*, 1990). Alternatively, peripherin, NF-L and NF-M expression begins in peripheral nervous system (PNS) cells and some CNS neuronal cell populations (Escurat *et al.*, 1990; Gorham *et al.*, 1990; Troy *et al.*, 1990). The expression of NF-H lags somewhat behind the expression of NF-L and NF-M as the neurons mature (Shaw and Weber, 1982; Pachter and Liem, 1984). This picture is somewhat more complex now that several new neurofilament proteins have recently been isolated from lower vertebrates. These include plasticin from goldfish retinal ganglion cells (Glasgow *et al.*, 1992), XNIF from developing *Xenopus* neurons (Charnas *et al.*, 1992) and tanabin from *Xenopus* neuronal growth cones (Hemmati-Brivanlou *et al.*, 1992). Gefiltin is the latest member of a growing list of neurofilament proteins. The isolation of these additional neurofilament proteins from lower vertebrates, where developmentally specific molecules are particularly accessible, suggests that there is a more complex developmental expression of neurofilaments than is currently recognized. Considering the large variation in morphology of developing and mature neurons, it is not surprising to find that there is a large variation in neurofilament protein composition in the nervous system.

Historically, the goldfish visual pathway has been central to neurobiological issues concerning development, plasticity and specificity. Our analysis of the neurofilament proteins in this pathway suggests that particular neurofilament proteins support physiological functions, both in specific neuronal cell types, and at specific stages of neuronal differentiation. It is interesting to note that new evidence indicates that certain neurofilament proteins may also be a component in spatially regulating axon position (Kaprielian and Patterson, 1993).

Materials and methods

Animals

Common goldfish (*Carassius auratus*, 8–12 cm) were obtained from Mt Parnell Fisheries, Mercersburg, PA and maintained in 40 gallon tanks at ~18°C. Intraorbital nerve crush was performed on the right optic nerve after anesthetization by immersion in 0.05% tricaine methanesulfonate. The left optic nerve was left intact to serve as a control.

Cytoskeletal preparation and two-dimensional electrophoresis

Cytoskeletal proteins were isolated from goldfish optic nerve as described previously (Jones *et al.*, 1986b). Two-dimensional electrophoresis was performed essentially as described by O'Farrell (1975), with slight modifications (Quitschke and Schechter, 1980).

Trypsin digestion, HPLC separation and protein microsequencing

After electrophoresis by two-dimensional gel SDS–PAGE, protein was electrotransfered to nitrocellulose (Towbin *et al.*, 1979) and visualized by staining with Ponceau S. Nitrocellulose containing the ON_1 protein was excised and submitted to *in situ* digestion with trypsin (Aebersold *et al.*,

1987), omitting the NaOH wash. The resulting peptide mixture was separated by narrow-bore high performance liquid chromatography using a Vydac C18 2.1 mm × 150 mm reverse-phase column on a Hewlett-Packard 1090 HPLC with 1040 diode array detector. Optimum fractions from this chromatogram were chosen based on differential UV absorbance at 210 nm, 277 nm and 292 nm peak symmetry resolution. Selected fractions were submitted to automated Edman degradation on an applied Biosystems 477A protein sequencer using a microcartridge and cycles optimized for a 30 min cycle time. Details of strategies for the selection of peptide fractions and their microsequencing have been previously described (Lane *et al.*, 1991).

Isolation and sequencing of clones

A 44 nucleotide 'guessmer' oligonucleotide (CAI ATI CAT GAI ATG GAI GAI ACI CAC AAT GCI GAI GTI ATG GG) was synthesized (Operon Technologies) which corresponds to the microsequenced ON_1 amino acid sequence, QIHEMEDTHNAEVMG. The procedure for guessing the best nucleotides at degenerate codons are outlined in Sambrook *et al.* (1989). This oligonucleotide was ^{32}P-end labeled with T_4 polynucleotide kinase and used to screen two goldfish retinal cDNA libraries by standard methods (Sambrook *et al.*, 1989). Filters were hybridized overnight at 37°C. The final wash was in 0.2 × SSC at 50°C for 20 min.

Twenty positively hybridizing clones were isolated from ~2.5 × 10^5 plaques of our 20 day post-optic nerve crush retina λgt11 cDNA library (Glasgow *et al.*, 1992) Five clones were isolated from ~2.5 × 10^5 plaques of a 3 day post-optic nerve crush λgt10 cDNA library (gift of Dr Dan Goldman). The five largest clones were characterized further. Two overlapping partial cDNA clones, 30.1A and 31.4, were completely sequenced in both directions with the TN 1000 transposon mediated sequencing system (Gold BioTech.) To obtain longer clones, ~8 × 10^5 plaques of the λgt10 cDNA library were screened at high stringency with a ^{32}P-labeled 5′ *Eco*RI−*Hin*dIII fragment of clone 30.1A, 30.1AEH$^-$ (Figure 2A). The final wash was in 0.2 × SSC, 0.1% SDS at 68°C for 30 min. Five positively hybridizing clones were isolated, the longest of these (as determined by Southern hybridization), Gef1, was subcloned into pBS KS$^-$ (Stratagene). Internal restriction sites and synthesized primers where utilized to partially sequence this clone using the Sequenase II kit (USB). All overlapping sequences between clones Gef1, 30.1A and 31.4 are identical. Sequences were analyzed by computer using DNASIS and the GCG programs (Devereux *et al.*, 1984).

Polymerase chain reaction

The extreme 5′ 103 nucleotides of gefiltin were obtained by PCR amplification from genomic DNA. The 5′ primer, 5′-GGA AGC TTG AAT GCG AGG, was designed from the 5′ non-coding region of a variant full length gefiltin-like clone, Gef3 (unpublished results). A *Hin*dIII site was created in this primer by changing one nucleotide (bold face) at the 5′ end of the primer. The 3′ primer, 5′-GA ATA GCG GGT GGA GTC C, represents nucleotides 142−159 of the combined gefiltin sequence (Figure 2B) and lies within clone 30.1A. The 3′ primer was chosen so that it would be specific for gefiltin, while excluding the variant Gef3. The PCR reaction consisted of: 7.0 mM $MgCl_2$, 20 mM Tris pH 8.75, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1 mg/ml BSA, 200 μM dNTPs, 25 pmol of each primer, 2.5 U cloned Pfu polymerase (Stratagene) and 0.5 U Perfect Match polymerase enhancer (Stratagene) in a volume of 100 μl. Goldfish genomic DNA (100 ng) was used as a template. Forty-five cycles were performed with denaturing at 95°C for 40 s, annealing at 50°C for 40 s and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min.

The reaction products were analyzed on a 2.0% agarose gel and a 177 bp band was observed. This DNA band was purified from the gel by the QIAEX gel purification protocol (QIAGEN) and digested with *Hin*dIII and *Bgl*II. The digestion reaction was separated on a 2.0% agarose gel, the DNA band was purified as above and cloned into pBS (Stratagene). Five independent clones were sequenced in both directions with the Sequenase II kit (USB). The sequence of all five clones was identical.

RNA isolation, Northern blot and RNase protection assays

RNA was isolated as previously described (Glasgow *et al.*, 1992). The Northern blot used for plasticin (Glasgow *et al.*, 1992) was stripped and reprobed with 2 × 10^6 c.p.m./ml random primed (Amersham) ^{32}P-labeled clone 30.1A insert. The final wash was in 0.1 × SSC, 0.1% SDS, at 68°C for 1 h.

RNase protection assays were performed as described for plasticin (Glasgow *et al.*, 1992). The gefiltin RNase protection probe corresponds to an *Eco*RI−*Hin*dIII fragment of clone 30.1A subcloned into pBS (Stratagene), p30.1AEH$^-$ (Figure 2A). A 223 nucleotide antisense ^{32}P-labeled riboprobe was synthesized by *in vitro* transcription from the T_3 promoter. All RNAs used for the plasticin and gefiltin RNase protection assays were aliquots from the same RNA isolations.

The amounts of plasticin and gefiltin mRNA in 20 μg retina RNA at various time-points during optic nerve regeneration were estimated by comparison with standard curves. RNase protection standard curves were created as follows. Plasticin and gefiltin sense RNAs were *in vitro* transcribed from the pCD2 (Glasgow *et al.*, 1992) and p30.1AEH$^-$ subclones, respectively. The amount of *in vitro* transcribed RNA was measured by absorbance at 260 nm minus the absorbance at 260 nm of parallel mock reactions without polymerase. Sense RNAs were diluted to 0.01, 0.1, 1.0 and 10 pg per reaction and used for RNase protection assays. Relative optic density readings were taken from the resulting autoradiograph and plotted against the known molar quantities of plasticin and gefiltin sense RNA for the standard curves. RNase protections with non-operated retina RNA were run concurrently to establish the level of baseline plasticin and gefiltin expression. The increase in plasticin and gefiltin mRNA levels was estimated by comparison with the these standard curves and plotted graphically (Figure 6B).

In situ hybridizations

Goldfish were dark adapted and anesthetized in Tricaine prior to tissue removal. Retinas were rinsed in PBS and fixed with 4% paraformaldehyde in PBS for 2 h. Regenerating and control retinas were cryoprotected overnight in 30% sucrose and embedded in a 1:1 mixture of OCT (Miles Labs) and Aquamount (Lerner Labs), as previously described (Jones *et al.*, 1986a). Cryostat sections (10 μm) were collected on Superfrost plus slides (Fisher Scientific). Slides were processed as previously published (Sternini *et al.*, 1989), with the following modifications. Retinas were deproteinized with proteinase K for 7.5 min at room temperature.

A 229 nucleotide gefiltin riboprobe was labeled with ^{35}S-UTP by *in vitro* transcription of the gefiltin subclone, p30.1AS7 (Figure 2A), using the T_7 (antisense RNA) or T_3 (sense RNA) promoters. Slides were incubated overnight at 60°C in 40 μl hybridization buffer containing 4 ng probe. Additional controls were incubated in pancreatic RNase A (50 μg/ml) for 30 min at 37°C prior to prehybridization.

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