Molecular Vision 2003; 9:301-7 <http://www.molvis.org/molvis/v9/a42>
Received 21 April 2003 | Accepted 13 June 2003 | Published 3 July 2003

Elovl4 mRNA distribution in the developing mouse retina and phylogenetic conservation of *Elovl4* genes

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Purpose: Stargardt-like macular dystrophy (STGD3) is an autosomal dominant form of early onset macular degeneration. The disease causing gene *ELOVL4* encodes a protein that belongs to a family of proteins functioning in elongation of long chain fatty acids. The purpose of this study is to characterize cross-species conservation of *ELOVL4* and investigate its mRNA distribution in the developing mouse eye.
Methods: Bovine and porcine orthologs of the human *ELOVL4* gene were cloned using RT-PCR method. EST and HTGS databases were searched for orthologs of *ELOVL4*. Cross-species alignments were performed using ClustalW. In situ hybridizations using murine Elovl4 probes were performed on frozen sections of mouse eyes.
Results: Elovl4 orthologs from mammalian to invertebrate species share strong sequence homology with human ELOVL4 at the amino acid level, suggesting functional conservation of Elovl4 during evolution. Expression of *Elovl4* in mouse retina begins at E15 during embryogenesis and persists in postnatal stages. However, *Elovl4* is predominantly expressed in the retinal ganglion cells at P1-P3, followed by predominant expression in the outer nuclear layer at P7, with its final expression enriched in inner segments of photoreceptors.
Conclusions: *Elovl4* expression in developing retina follows a dynamic pattern. It switches from predominant ganglion cell expression in embryonic and early postnatal development to predominant expression in the photoreceptor inner segments in later stages. Phylogenetic analysis reveals strong conservation of Elovl4 among different species throughout the vertebrate subphylum consistent with our hypothesis that ELOVL4 performs a fundamentally important function.

Stargardt macular dystrophy (STGD) is a juvenile onset macular dystrophy characterized by decreased visual acuity, macular atrophy, and extensive flecks [1]. Stargardt-like macular dystrophy (STGD3, 600110) is an autosomal dominant form of the disease. The disease shares some similarity with age-related macular degeneration (AMD) including abnormal accumulation of lipofuscin in retinal pigment epithelium (RPE), and atrophy of RPE and photoreceptors in the macula. The gene responsible for STGD3 on chromosome 6q14, identified recently by positional cloning [2] as *ELOVL4*, encodes an enzyme presumably involved in the elongation of very long chain fatty acids. Sequence analysis of human *ELOVL4* cDNA predicts a protein of 314 amino acids that shared 35% identity with members of the yeast ELO family and 38% identity with the human ELO1 homolog (HELO1, or ELOVL1) involved in elongation of long chain fatty acids [3]. Members of the yeast ELO family and HELO1 share common structural features, including multiple putative membrane-spanning domains and a single histidine cluster motif (HXXHH). This motif is characteristic of diiron-oxo proteins such as fatty acid desaturases, in which the histidine residues are involved in coordination of the reception of electrons in reduction reactions [4,5]. Other motifs are putative dilysine motifs (KKXX or KXKXX, at the C-terminus) thought to signal ER retention [6,7]. The ELO family members and HELO1 possess biochemical features that would allow them to participate in the catalysis of reduction reactions occurring during fatty acid elongation [8]. Each gene in the *ELO* family is thought to encode a single enzyme component of complex enzymatic systems that function in the synthesis of long chain fatty acids [9].

Mutation analysis of the *ELOVL4* gene in one Stargardt-like macular dystrophy pedigree revealed a five base pair deletion starting at position 790 of the open reading frame (790-794delAACTT; [2]). This mutation results in a frame-shift and a premature stop codon, causing the loss of a 51 amino acid fragment at the protein's C-terminus that includes the dilysine targeting signal thought to be responsible for localization to the endoplasmic reticulum. Subsequently, two 1-base pair deletions (789delT and 794delT) were identified in an independent large Utah pedigree confirming the role of the *ELOVL4* gene in a subset of dominant macular dystrophies[10]. Northern blot studies revealed a high level of *ELOVL4* mRNA expression in adult human retina, and RT-PCR detected lower

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levels in brain and testis [2]. In situ hybridization studies of adult rhesus monkey and mouse retinal sections demonstrated strong signals in the inner segments of both rod and cone photoreceptors [2].

As a first step in characterization of the potential function of Elovl4 in the retina, we cloned the porcine and bovine ortholog of *Elovl4* and compiled Elovl4 orthologous sequences across vertebrate species. We investigated the mRNA distribution pattern of *Elovl4* in the mouse retina during development. Our results are consistent with the notion that Elovl4 represents a component of a fatty acid biosynthetic system that has been strongly conserved during evolution.

METHODS

Nomenclature: In this paper we refer to the human gene as *ELOVL4* and the predicted protein as ELOVL4; genes encoding protein orthologs of ELOVL4 in other species as *Elovl4* and their predicted proteins as Elovl4.

Materials: Porcine and bovine retina tissues were obtained from a local slaughterhouse. All experimental procedures involving the use of laboratory mice complied with NIH guidelines as approved by the Institutional Animal Care and Use Committee of the University of Utah. Mouse retina tissues were collected from BALB/c and CD1 mouse strains. For histological analysis, the mouse tissues were fixed with 4% (w/v) paraformaldehyde (PFA) in 1X PBS solution. We used a stock solution of 16% PFA (w/v; Electron Microscopy Science, Fort Washington, PA) and diluted it with 10X PBS solution and water to a final concentration of 4% PFA (w/v) and 1X PBS. Alternatively, we used 2.5% (w/v) glutaraldehyde/2% (w/v) PFA as a fixative.

*Molecular cloning of porcine (*Sus scrofa*) and bovine (*Bos taurus*) Elovl4 cDNA:* We designed PCR primer sequences that correspond to a well conserved region among human, monkey and mouse *Elovl4* sequences. Total RNA from porcine and bovine retina was isolated using Trizol (Invitrogen, Carsbad, CA, USA) according to the manufacturer's instructions. Total RNA (5 µg) was used to generate cDNA by reverse transcription in a total volume of 20 µl. We used 2 µl of the cDNA solution for a 20 µl PCR reaction. To obtain a full-length porcine *Elovl4* cDNA sequence, we performed rapid amplification of cDNA ends (RACE). Primers 5' TTG GGG AAG GGG CAG TC 3', 5' AAA CAC TGT GTC CAA ATA CTC A 3' and 5' ATG ATC CCA TGA ATA ACT CTC 3' were used as 3' end primer in conjunction with 5'end Invitrogen adapter primer in 5' RACE (Invitrogen, Carsbad, CA, USA) according to the manufacturer's instructions. For 3'end RACE, we used 5' ATG GGG CTC CTG GAC TCG GAG 3' as a forward primer, and 5' CAG TTA AGG CCC AGT TC 3' as a reverse primer. In the case of bovine *Elovl4*, we identified an EST (BE665768) from the GenBank which showed strong homology with human *ELOVL4*. Conceptual translation of this EST revealed that it lacks N terminal and C-terminal portions of the protein. Primer pair 5' GAT GGG GCT CCT GGA CTC 3' and 5' CAA CAG TTA AGG CCC AG 3' corresponding to well-conserved regions of human, monkey, porcine and mouse Elovl4 was used for amplification in a bovine retinal cDNA library. Amplified DNA fragments were subject to DNA sequencing with the Taq Dyedeoxy Terminator Cycle Sequencing Kit (Beckman-Coulter, Fullerton, CA) according to the manufacturer's instructions.

Retrieval of ELOVL4 orthologous sequences: The rat Elovl4 sequence was conceptually translated from exon sequences derived from large genomic contigs AC113258 (contig lacking exon 3) and AC126517 (independent contig lacking exon 5). Similarly, the pufferfish (*Fugu rubripes*) and zebrafish (*Danio rerio*) Elovl4 amino acid sequences were derived from GenBank sequences AC095019 and AL929535, respectively. The sea squirt (*Ciona intestinalis*) Elovl4 sequence was from GenBank accession number AK112719. The Macaque sequence (*Macaca fascicularis*) was derived from AB063100. Other sequences are from EAA07022 (*Anopheles gambia*), NP_648436 (*Drosophila melanogaster*), and NP_729666 (*Drosophila melanogaster*). The origins of other Elovl sequences are indicated in figure legends.

In situ hybridization: Mouse tissues were collected from different developmental stages and fixed in 4% (w/v) PFA overnight at 4 °C. Cryosections of 14 µm thickness were used in all hybridization experiments. A mouse *Elovl4* cDNA containing 60 bp of the 5' UTR, 939 bp coding sequence, and 83 bp of the 3' UTR was subcloned into the *Eco*RI site of pBluescript II KS (+) vector (Stratagene, La Jolla, CA, USA) and used to generate pmElovl4-T2. The DNA sequence of the insert was confirmed by sequencing. Digoxigenin-UTP labeled mouse *Elovl4* sense and antisense RNA probes were transcribed with T7 and T3 RNA polymerases, respectively. In situ hybridization conditions were as previously described [11,12]. In situ hybridization signals were visualized using a Nikon E800 microscope and captured with a SPOTII digital camera.

RESULTS

Identification of Elovl4 orthologs: Porcine and bovine *Elovl4* cDNAs were cloned by RT-PCR using bovine and porcine retinal cDNAs. Additional orthologs and Elovl4-like sequences, including rat, macaque, chicken, pufferfish, zebrafish, sea squirt (*Ciona intestinalis*), mosquito (*Anopheles gambiae*), and fruitfly (*Drosophila melanogaster*) were retrieved from the GenBank, or were identified from EST/genomic contigs through search of the NCBI EST/HTGS databases (see Methods). As shown in Figure 1, the mammalian Elovl4 amino acid sequences show more than 90% similarity with human ELOVL4, suggesting that they are indeed orthologs. Sequence comparison of mammalian Elovl4's with other vertebrate and invertebrate sequences shows a high conservation of the central region, the putative active enzymatic site of the elongase. All Elovl4 polypeptide sequences share several common features: (1) the Elovl4 proteins are mostly hydrophobic consistent with an integral membrane protein with at least five or six transmembrane domains. (2) they possess a diiron-oxo binding HXXHH motif in the central part of the protein, presumably the active site of the elongase. (3) the C-terminal regions

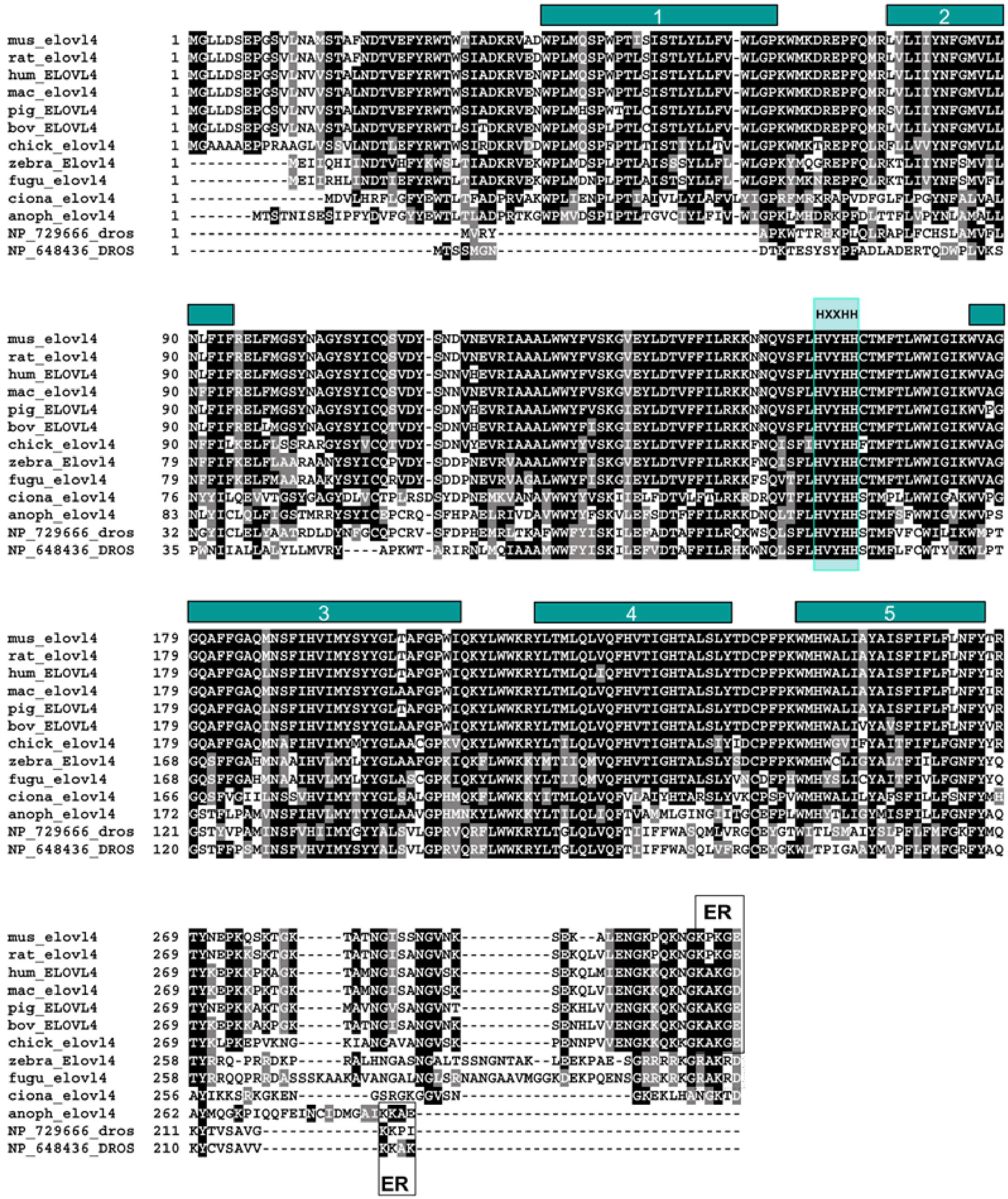

Figure 1. Comparison of Elovl4 amino acid sequences. Five putative transmembrane domains are indicated by bars 1-5. Carboxy-terminal dilysine signal KXKXX (vertebrates) or KKXX (invertebrates) presumably responsible for endoplasmic reticulum retention and the diiron-oxo binding HXXHH motif (His158-His162) are boxed. Residues printed in white on a black background are perfectly conserved in seven or more of the sequences shown. Residues printed in white on a gray background represent conservative substitutions (M=V=L=I, K=R=H, D=E, W=Y) in seven or more sequences shown. All other residues are printed in black on a white background. Note that in *fugu* and zebrafish C-termini, the ER motif is RXKXX (boxed with a broken line). Conserved residues are printed in white on black background. The sequences were derived from the following GenBank accession numbers: AF277093, mus_elovl4 (*Mus musculus*); the rat sequence rat_elovl4 (*Rattus norvegicus*) was derived from genomic contigs AC113258 (lacking exon 3) and AC126517 (lacking exon 5); NP_073563, hum_ELOVL4 (STGD3; *Homo sapiens*); AF461182, AB063100, mac_elovl4 (*Macaca fascicularis*); BU353103, chick_elovl4 (*Gallus gallus*); AK112719, ciona_elovl4 (*Ciona intestinalis*, sea squirt); the zebrafish (*Danio rerio*) sequence zebra_Elovl4 was derived from ESTs (BI706328, BI428673); EAA07022, anoph_Elovl4 (*Anopheles gambiae* str. PEST); NP_729666_dros and NP_648436_dros are two *Drosophila melanogaster* sequences, presumably elovl4 homologues.

are hydrophilic and contain a dilysin motif (KXKXX in vertebrates, KKXX in invertebrates Figure 1) possibly responsible for endoplasmic reticulum retention.

Phylogenetic conservation of Elovl4 genes: Computer modeling revealed that all Elovl4 orthologs evolved from a common ancestor (Figure 2). Within the last decade, the yeast *ELO1*, *ELO2* (FEN1), and *ELO3* (SUR4) genes were shown to encode integral membrane proteins involved in elongation of fatty acids [13,14]. Since the discovery of the yeast ELO subgroups, human, mouse and many other vertebrate homo-

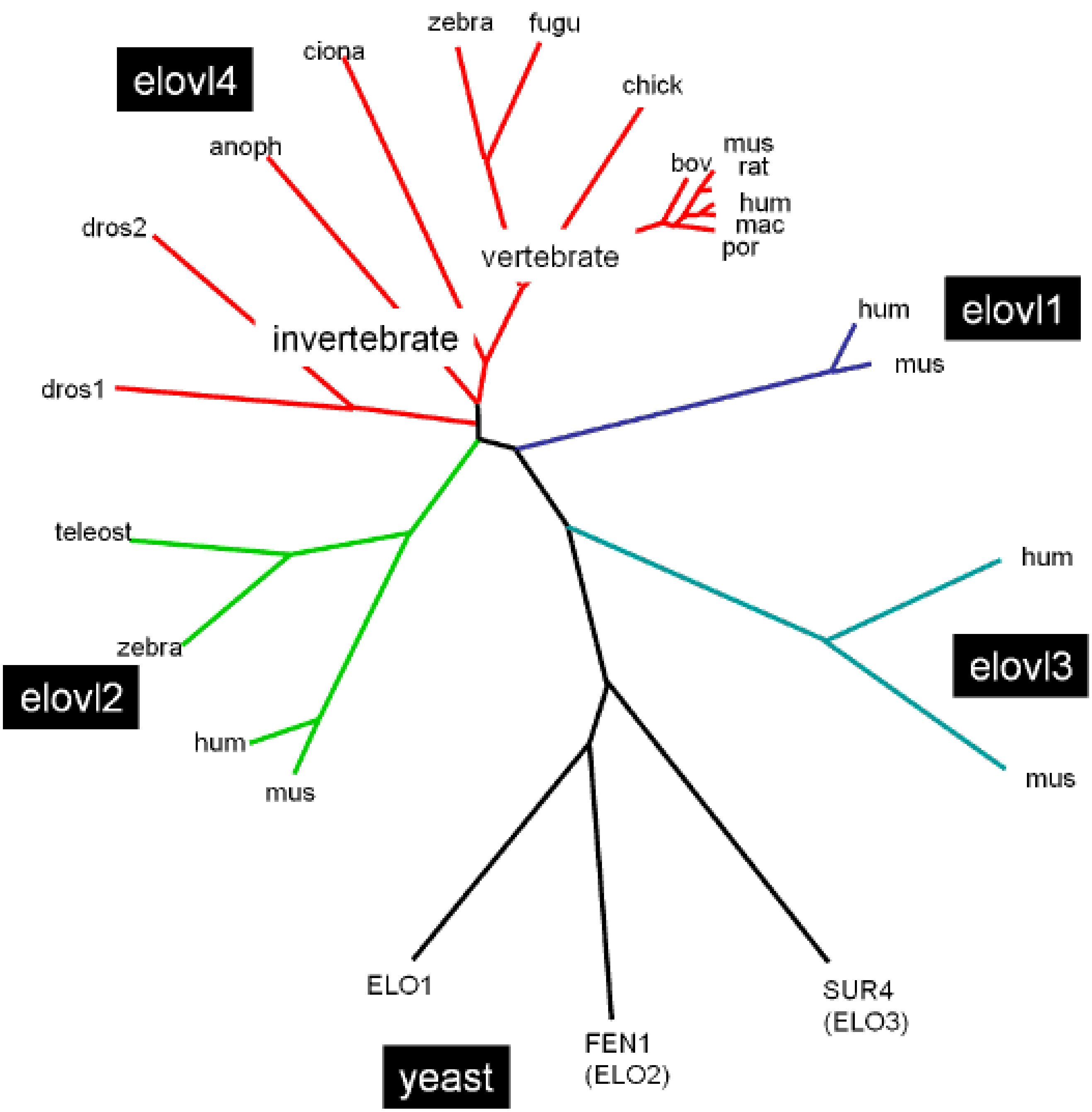

Figure 2. Phylogenetic tree of human ELOVL related polypeptides. The three yeast sequences ELO1-ELO3 are shown to group independently of Elovl sequences of other species. Of vertebrate Elovl1-Elovl3 sequences only mouse, human and selected teleost sequences are shown. The large Elovl4 group contains mammalian, other vertebrate, and invertebrate sequences retrieved from the GenBank or "cloned in silico" using the BLAST algorithm [22]. The sequences were aligned with ClustalW (Version 1.82) and viewed with Treeview (Version 1.6.6). The Elovl4 sequences are identical to those in Figure 1. Yeast group: NP_012339, yeast_elo1 (*Saccharomyces cerevisiae*); NP_009963, yeast_fen1 (ELO2); NP_013476, yeast_sur4 (ELO3). Elovl1 group: AAH06735, mus_elovl1; BAA91813, hum_elovl1. Elovl2 group: AAN77156, zebra_elovl2 (*Danio rerio*); AF465520.1, teleost_elovl2 (*Scophthalmus maximus*); AF170908, mus_Elovl2 (SSC2); 20138035, hum_Elovl2. Elovl3 group: 26006738, hum_elovl3 (ELO3); NP_031729, mus_elovl3 (gp30, cig30).

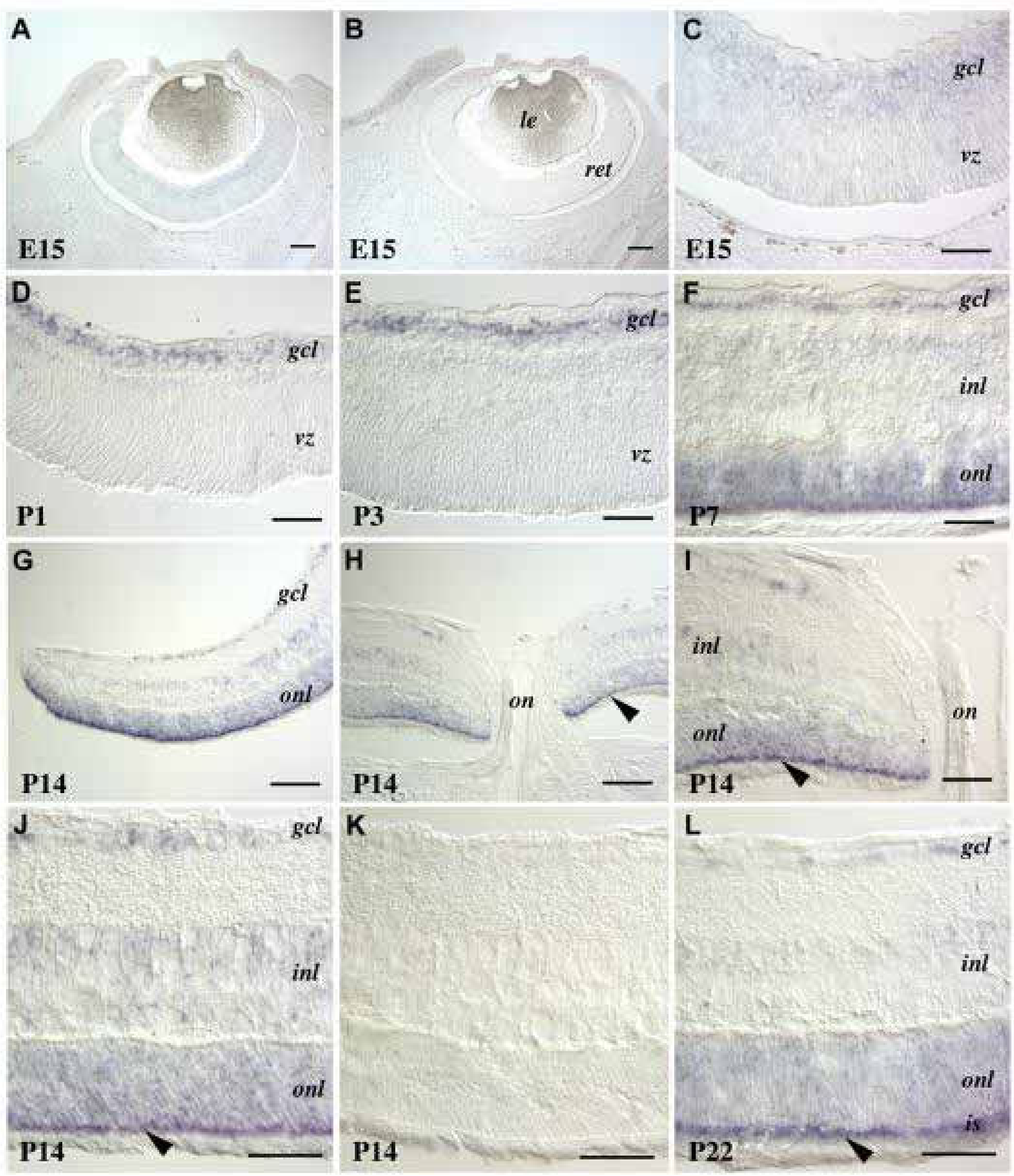

Figure 3. Expression patterns of Elovl4 mRNA in developing mouse retinas. Mouse eye tissue sections hybridized with Digoxigenin-labeled Elovl4 RNA probes are shown. Panels **A**-**C** and **D**-**L** show sections derived from embryonic day 15 (E15) and different postnatal days (with the first 24 h after birth defined as postnatal day 0, P0), respectively. Panels **B** and **K** were hybridized to the sense probe; the other sections were hybridized to antisense probes. Arrowheads point to the inner segment. Abbreviations: gcl, ganglion cell layer; inl, inner nuclear layer; is, photoreceptor inner segment; le, lens; on, optic nerve; onl, outer nuclear layer; ret, retina; vz, ventricular zone; P1, P3, P7, P14, P22, postnatal day 1, 3, 7, 14, and 22, respectively. Scale bars represent 100 μm in **A**, **B**, **G**, and **H** and 50 μm in the other panels.

logues of fatty acid elongation factors have been identified. The sequence similarity among the yeast ELO proteins is 43-55%, while their sequence similarity to the mammalian Elovl4 polypeptides is only 17-18% (18-22% to the human ELOVL1-ELOVL3 proteins). Sequence similarity of mammalian Elovl4s to fugu/zebrafish homologues is 61-64%, and among the mammalian *Elovl4* gene products is >89%. Interestingly, the sequence similarity of human ELOVL4 to invertebrate homologues is as high as 40-43%, much higher than between *Drosophila* rhodopsin R1-6 and human rhodopsin (22%), the photoreceptor molecules in the invertebrate and human retina, respectively. The high sequence conservation in invertebrate and vertebrate species is consistent with an important metabolic function, such as elongation of fatty acids.

Localization of Elovl4 mRNA in the developing mouse retina: To further understand the potential role of Elovl4 in the vertebrate retina, we investigated mRNA distribution of *Elovl4* during mouse eye development using in situ hybridization (Figure 3). As early as mouse embryonic day 15 (E15; Figure 3A-C), *Elovl4* mRNA was detected in the inner portion of the retina occupied by postmitotic ganglion cells. A low level of hybridization signal was also observed in the ventricular zone, where the proliferating progenitor cells and post mitotic cone precursor cells resided. This pattern of expression persisted in the early postnatal period until postnatal day 3 (P3; Figure 3D,E). At P7, when differentiation of photoreceptors was underway, high levels of *Elovl4* transcripts appeared in the outer nuclear layer (Figure 3F). By P14, the most intense Elovl4 hybridization signals were located in the outer portion of the retina (Figure 3G-J). However, *Elovl4* mRNA was continuously present in the inner nuclear layer and the ganglion cell layer two weeks after birth. In the mature retina (P22), the *Elovl4* mRNA was mostly restricted to the outer nuclear layer, especially the inner segment of photoreceptors (Figure 3L).

DISCUSSION

Stargardt-like macular dystrophy (STGD3), an autosomal dominant form of macular degeneration, is characterized by bilateral atrophic changes in the macula, degeneration of the underlying RPE, and the presence of prominent flecks in the posterior pole [1,15]. The disease-causing gene *ELOVL4* encodes a protein with similarities to a family of proteins involved in the elongation of long chain fatty acids. Elucidating the normal role of ELOVL4 and determining how mutations in *ELOVL4* lead to macular degeneration would benefit from further characterization of this gene and its expression profile in the retina. Therefore, we conducted an investigation of localization of *Elovl4* mRNA in developing mouse retina and phylogenetic analysis of Elovl4 in mammalian and vertebrate species.

We cloned the porcine and bovine orthologs of the human *ELOVL4* gene and sequence analysis of these cDNAs show more than 90% amino acid similarity with human ELOVL4, monkey Elovl4, and mouse Elovl4. Additionally, we identified Elovl4 orthologs in chicken, rat, pufferfish, and zebrafish with database searches. All orthologs share a high sequence identity at the amino acid level with human ELOVL4. Like its human counterpart, each Elovl4 ortholog possesses all essential domains consistent with an enzyme involved in elongation of long chain fatty acids. Each ortholog also showed significant percentage in amino acid identity to members of the *ELO* gene family in human and yeast which encode components of the membrane-bound fatty acid elongation system [3,14]. Thus, evolutionary constraint to maintain high sequence similarity in this gene among vertebrates strongly suggests that Elovl4 performs an essential function, which is consistent with a role for Elovl4 in elongation of long chain fatty acids.

Docosahexaenoic acid (DHA) is the most abundant form of long chain polyunsaturated fatty acid (PUFA) in the retina. DHA contains 22 carbon atoms and 6 double bonds and represents about 50% of the fatty acids in phospholipids of outer segments of photoreceptors [16]. Biosynthesis of PUFAs in retinal photoreceptors, such as DHA, requires dietary consumption of the essential α-linolenic acid and a subsequent series of elongation steps [17,18]. Elovl4 may be involved in one or several of the elongation steps required for DHA biosynthesis, however further biochemical analysis is necessary to determine the role of Elovl4 in lipid metabolism specifically in the retina.

Previous in situ hybridization studies showed that in the adult monkey and mouse retina, Elovl4 is expressed in photoreceptor cells [2]. Here we demonstrated that *Elovl4* mRNA expression during mouse retinal development follows a more dynamic pattern, extending its role in retinal development to ganglion cells in addition to photoreceptor cells. During early retinal neurogenesis, *Elovl4* is expressed by postmitotic neurons occupying the ganglion cell layer. Within the first postnatal week differentiating photoreceptors also begin to express *Elovl4*. As the retina matures, the *Elovl4* transcript becomes more restricted to the inner segment as previously reported. The significance of *Elovl4* expression in the ganglion cells of the mouse retina is unclear although this expression coinsides with the period of ganglion cell axonal growth during retinotectal connection. Examination of human patients with Stargardt macular dystrophy bearing an *ELOVL4* mutation has not yet revealed any abnormalities of ganglion cells (Kang Zhang, unpublished data).

Mutations in genes involved in long chain fatty acid metabolism have been implicated in several central nervous system diseases in human, including adrenoleukodystrophy [19], adrenomyeloneuropathy [20], and Canavan disease [21]. However, *Elovl4* is the first gene possibly involved in this biosynthetic pathway linked to retinal photoreceptor degeneration. Identification of Elovl4 orthologs in other species, especially in zebrafish, as well as characterization of the expression of Elovl4 in the mouse retina during development should allow exploitation of powerful genetic tools in dissecting Elovl4 functions and delineating causative mechanisms leading to this retinal disease.

ACKNOWLEDGEMENTS

We thank Kim Howes for advice and critical reading of the manuscript. KZ is supported by grants from the National Eye Institute (EY13868), American Health Assistance Foundation, the Karl Kirchgessner Foundation, The Ruth and Milton Steinbach Fund, Ronald McDonald House Charities, the Val and Edith Green Foundation, and the Simmons Foundation. Z. Yang is supported by grants from Fight for Sight and the Knights Templar Eye Research Foundation. X-J Yang is supported by grants from the Research to Prevent Blindness Foundation, the Foundation Fighting Blindness, the Karl Kirchgessner Foundation, and the National Eye Institute (EY12270).

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The print version of this article was created on 8 Jul 2003. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.