Expression of *Drosophila omb*-Related T-Box Genes in the Developing Human and Mouse Neural Retina

Jane C. Sowden,[1] *James K. L. Holt,*[1] *Moritz Meins,*[1,2] *Hazel K. Smith,*[3] *and Shomi S. Bhattacharya*[2]

Purpose. To examine the role of *Drosophila* optomotor blind (*omb*)–related T-box genes in development of human and mouse retina.

Methods. Mouse *Tbx2*, *Tbx3*, and *Tbx5* and human *TBX2* cDNAs were isolated from retinal cDNA libraries by hybridization to the *Drosophila omb* gene. Gene expression patterns in developing retina were analyzed by in situ hybridization.

Results. *TBX2/Tbx2*, *TBX3/Tbx3*, and *TBX5/Tbx5* were expressed asymmetrically across the embryonic neural retina with highest levels of mRNA within dorsal and peripheral retina. The dorsoventral gradient of *TBX2* expression disappeared before the ganglion cell layer (GCL) formed. Its expression then became restricted to the inner neuroblastic retina and later to the GCL and inner nuclear layer (INL). The dorsal expression domains of *TBX5/Tbx5* and *TBX3/Tbx3* were maintained during formation of the GCL. As the retina matured, *TBX3/Tbx3* expression was restricted to the INL, and *TBX5/Tbx5* was expressed within the GCL.

Conclusions. The expression pattern of *TBX2*, *TBX3*, and *TBX5* within the developing retina supports the idea that the encoded transcription factors play a role in providing positional information important for topographic mapping and in differentiation of distinct cell types across the laminar axis of the retina. (*Invest Ophthalmol Vis Sci.* 2001;42:3095–3102)

The mature visual system comprises a complex network of neuronal connections whose physiology and axonal projections are well characterized. In the human retina, more than 100 million rod and cone photoreceptors transmit visual information to some 1.2 million ganglion cells through the bipolar, amacrine, and horizontal interneurons of the inner nuclear layer (INL).[1] The ganglion cell axons project with precise topographic mapping to synaptic targets within the higher visual system. Recent progress has being made in identifying the guidance molecules that ensure the ganglion cell axons navigate to appropriate synaptic targets during development.[2–4] Less is known, however, about the genetic mechanisms by which ganglion cells and the retinal interneurons achieve their differentiated identities and make precise synaptic connections during development.

Although the visual systems of the cat and the chick have been extensively characterized, these systems are not amenable to genetic analysis. In contrast, the knowledge of the genetics of the visual system of the fruit fly, *Drosophila melanogaster*, is relatively advanced because of the ease of generating mutant flies.[5] An emerging strategy that is useful for the identification of genes regulating development of the mammalian visual system is to analyze homologues of *Drosophila* genes that are important in visual system development in the fly.[6] This strategy is based on accumulating evidence that demonstrates the conservation of genetic mechanisms underlying homologous structures in diverse species and the discovery that mutations in conserved genes underlie inherited eye malformations in flies and mammals.[7] For example, there is evolutionary conservation of the transcription factors, *Pax6* and *Sine oculis*, which are important in eye development, and of opsin proteins involved in phototransduction.[8]

The pathophysiology resulting from mutation of the *Drosophila* gene optomotor blind (*omb*) demonstrates that the *omb* gene is an important regulator of neuronal cells, which process and integrate visual information from the compound eye and transmit it to the central brain.[9] In the third larval instar of the fruit fly, *omb* is expressed in the neuroblasts of the optic lobe, which differentiate into ganglionic neurons with the arrival of axons projecting from the photoreceptor cells.[10] Several *omb* alleles are pupal lethal, and optic lobe neurons fail to differentiate. Other alleles give rise to viable flies with reduced levels of omb protein, resulting in specific behavioral and neuroanatomic defects in the visual system.[9] These mutant flies do not have large fibers in the lobula plate ganglionic complex of their visual system.

OMB is of particular interest, because it is a member of an important family of developmental regulators encoded by T-box genes.[11,12] These genes encode transcription factors that share a highly conserved and novel type of DNA-binding domain, the T-box domain. T-box genes have been characterized in a range of species, including humans, indicating the evolutionary conservation of this gene family. Phylogenetic analysis predicts that most animal species have at least five T-box genes.[12] Mutations in several T-box genes cause a variety of developmental defects in mice and humans, thus highlighting the vital role that T-box genes play in diverse developmental processes, including limb and heart morphogenesis and mesoderm formation.[13–18]

We have focused on the analysis of *omb*-related human and mouse genes to assess whether these genes play significant roles in development of the mammalian retina. Human and mouse retinal cDNA libraries were screened using the T-box region of the *omb* gene as a hybridization probe to identify *omb*-related genes expressed in the retina. We examined expression of the three T-box genes encoding the transcription factors *TBX2*, *TBX3*, and *TBX5* and found that each gene is expressed in an overlapping domain within the dorsal embryonic neural retina. As the retina differentiates, each gene is expressed in restricted and distinct subsets of retinal cells.

From the [1]Developmental Biology Unit, Institute of Child Health and the [2]Department of Molecular Genetics, Institute of Ophthalmology, University College London, United Kingdom; and [3]The Wolfson Institute for Biomedical Research, London, United Kingdom.

Supported by a Medical Research Council Career Development Award G120/191 (JCS). JKLH is supported by a Child Health Research Action Trust studentship, and MM was supported by Grant Me-1666 from the Deutsche Forschungsgemeinschaft.

Submitted for publication December 5, 2000; revised June 22, 2001; accepted July 3, 2001.

Commercial relationships policy: N.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Jane C. Sowden, Developmental Biology Unit, Institute of Child Health, University College London, 30 Guilford Street, London, WC1N 1EH, UK. j.sowden@ich.ucl.ac.uk

METHODS

In Situ Hybridization

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mouse embryos were obtained from matings of C57BL/6 x CBA mice. The day on which the vaginal plug was detected was designated embryonic day (E)0.5. Eyes from embryos at E10.5, E12.5, and E14.5 and from adult mice were analyzed. After informed consent and ethical permission had been sought and granted, human embryonic and fetal eye specimens were obtained from the Medical Research Council Tissue Bank and the Human Developmental Biology Resource, United Kingdom, according to the Polkinghorne Guidelines of the United Kingdom, which are in line with the tenets of the Declaration of Helsinki. Embryonic and fetal ages in weeks after conception were determined, either from hand and foot measurements, or, for older fetuses, by subtracting 2 weeks from the time since the last menstrual period. Tissues were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C. Standard procedures were used to embed specimens in paraffin wax and for hybridization of ^{35}S-radioisotope–labeled riboprobes to tissue sections.[19] For human specimens, optimal hybridization signal was achieved, using cryosectioned tissue and nonradioactive digoxygenin-labeled riboprobes. After fixation, human eye specimens were placed in 20% sucrose in PBS for 24 hours, oriented dorsoventrally in optimal cutting temperature (OCT) compound and then flash frozen using isopentane and dry ice. Cryosections (10 μm) were mounted onto 3-aminopropyltriethoxysilane (TESPA; Sigma, St. Louis, MO)–coated slides. In this study eyes at 6 weeks (n = 3), 8 to 9 weeks (n = 4), 12 to 13 weeks (n = 4), and 15 weeks (n = 2) were analyzed. Cryosections were hybridized with 1 ng/μl digoxygenin-labeled riboprobe, in hybridization buffer (1 mg/ml transfer [t]RNA, 50% formamide, 10% dextran sulfate, 1× Denhardt solution, 200 mM NaCl, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 5 mM EDTA, and 10 mM Tris [pH 7.5]) for 16 hours at 65°C. After hybridization, sections were washed at 65°C, in 1× SSC, 50% formamide, and 0.1% Tween-20 two times for 1 hour each, then twice for 30 minutes in maleic buffer with Tween; 0.1 M maleic acid, 0.15 M NaCl, 0.1% Tween 20 (MABT; Roche Molecular Biochemicals, Indianapolis, IN) at room temperature. To visualize the hybridized probe, sections were blocked with 1× MABT-2% blocking reagent (Roche) and 20% heat-inactivated sheep serum for 1 hour and incubated with anti digoxygenin alkaline phosphatase–conjugated antibody (1:1000 dilution) at 4°C overnight. Sections were washed four times for 5 minutes each in MABT, two times for 10 minutes each in staining buffer (2% 5 M NaCl, 5% 1 M MgCl, 10% 1 M Tris [pH 9.5], 0.1% Tween-20), and the color reaction was performed with 340 μg/ml nitroblue tetrazolium (NBT) and 170 μg/ml 5-bromo-4-chloro-3-indoyl phosphate (BCIP) in staining buffer in the dark. Sections were then mounted (Vectamount; Vector, Burlingame, CA) for microscopy.

The procedure used for wholemount in situ hybridization was as previously described,[19] except a modified hybridization buffer was used: 50% formamide, 5× SSC, 2% blocking powder, 0.1% Triton X-100, 0.5% 3-([3-cholamidopropyl] dimethylammonio)-2-hydroxy-1-propanesulfonate (CHAPS), 1 mg/ml yeast RNA, 5 mM EDTA, 50 μg/ml heparin. Hybridizations were performed at 80°C and posthybridization washes were as follows: 5 minutes in solution 1 (50% formamide, 5× SSC, 0.1% Triton X-100, 0.5% CHAPS); 5 minutes in three parts solution 1 to one part 2× SSC; 5 minutes in one part solution 1 to one part 2× SSC; 5 minutes in 1 part solution 1 to three parts 2× SSC; two times for 30 minutes each in 0.1% CHAPS and 2× SSC; and two times for 30 minutes each in 0.1% CHAPS and 0.2× SSC. All washes were performed at 65°C. Four E10.5 embryos were hybridized with each antisense and sense mouse probe in wholemount experiments.

The following plasmids were used to synthesize riboprobes for in situ experiments: (1) 498 bp of human *TBX2* cDNA downstream of the T domain in pGEM3Zf(+) (Promega, Southampton, UK); (2) 1.4 kb human *TBX3* cDNA in pBluescript II-SK(−) (Stratagene, La Jolla, CA); (3) 2.4 kb human *TBX5* cDNA in pBluescript II-SK(−); (4) 255 bp of the T-box region of mouse *Tbx2* cDNA in pBluescript II-KS(−); (5) 255 bp of the T-box region of the mouse *Tbx3* cDNA in pBluescript II-KS(−); (6) 1.1 kb of *Tbx5* cDNA in pBluescript II-KS(−). Sense and antisense RNA transcripts were synthesized, using either SP6 polymerase (Roche) or T7 polymerase (Roche), and labeled with ^{35}S-uridine triphosphate (UTP) or digoxygenin-UTP for hybridization to 8-μm tissue sections or wholemount embryos. To compare expression patterns of *TBX2/Tbx2*, *TBX3/Tbx3*, and *TBX5/Tbx5*, serial sections from human and mouse eye specimens were hybridized with respective probes. No signal was detected with the RNA sense probes. Embryos and sections were photographed with a photomicroscope (Diaplan; Leica, Cambridge, UK) or a stereomicroscope (MZ12; Leica), respectively (Ektachrome 64T film; Eastman Kodak, Rochester, NY), and the images were digitized on a scanner (FilmScan 200; Epson Seiko, Nagano, Japan) and assembled into figures on computer (Photoshop ver. 5.0; Adobe, San Diego, CA, and Powerpoint; Microsoft Corp. Redmond, WA).

Isolation of Mouse T-Box cDNAs

A ^{32}P-labeled 1.8-kb *Bam*HI fragment from the *Drosophila omb* cDNA, including the 600-bp T-box domain was used as a hybridization probe to screen a cDNA library in λZAP Express vector (Stratagene) prepared from neural retina and lens dissected from E15.5 mouse embryos; 5×10^5 plaque-forming units were hybridized overnight at reduced stringency at 52°C in a standard hybridization solution. Filters were washed twice in 0.5× SSC and 0.5% SDS at 52°C for 20 minutes before exposure to x-ray film at −80°C. pBK-CMV plasmids were excised from positive hybridizing plaques identified in tertiary screens and sequenced directly using plasmid primers flanking the insertion site and a dye terminator kit (Big Dye; Applied Biosystems, Foster City, CA). Of 14 clones analyzed, three contained T-box cDNAs. The other 10 clones that hybridized to the 1.8-kb *omb* probe did not hybridize to a smaller 300-bp *omb* probe, covering only the central T-box sequence, and encoded cDNAs for unrelated proteins, mouse lens α-crystallin, a protein of the major histocompatibility class II complex, and a protein-elongation factor.

Degenerate PCR amplifications were performed using 1 μg mouse E15.5 retina and lens cDNA library in plasmid pBK-CMV. The degenerate primers span nucleotides encoding two conserved amino acid sequences, YIHPDSP and AVTAYQN, in the T-box of *Drosophila omb* and other T-box genes. PCR fragments of approximately 255 bp obtained after two 30-cycle rounds of amplification were gel purified using a gel extraction kit (QiaQuick; Qiagen, Crawley, UK) and subcloned into the pGEM-T vector (Promega) according to the manufacturer's instructions. Recombinant plasmids were sequenced directly.

RESULTS

Identification of T-Box Genes Expressed in the E15.5 Mouse Retina

To isolate T-box genes, which are expressed in the developing retina and are related by sequence to the *Drosophila omb* gene, we used both conventional cDNA library screening and degenerate PCR amplifications. At E15.5 in the mouse, all six types of neuronal cell (rod, cone, horizontal, amacrine, bipolar, and ganglion) and Müller glial cells, which are characteristic of the mature neural retina, are developing and differentiating.[1] A ^{32}P-labeled cDNA probe that spans the T-box domain of the *omb* gene was used to screen a cDNA library prepared from E15.5 mouse neural retina and lens at low stringency. Four positive clones were identified and sequenced. Sequence comparisons indicated that three clones contained sequences identical with the *Tbx2* cDNA previously isolated from E11.5 mouse embryos,[11] and one contained sequence identical with *Tbx5* cDNAs isolated from E8.5 mouse embryos and embryonic mouse limb cDNA libraries (GenBank

Accession No. U57330, AF140427[20,21]). The 5′ ends of the retinal *Tbx2* cDNAs lie within a trinucleotide repeat sequence $(CGG)_5$ that encodes a string of alanine residues (starting at position 228 bp of the previously reported mouse *Tbx2* cDNA sequence, U15566. The retinal *Tbx5* cDNA contains a 129-bp 5′ untranslated leader sequence (compared with 418 bp in the limb *Tbx5* cDNA) and a 1005-bp 3′ untranslated region.

Conventional library screening was complemented by performing PCR amplifications of aliquots of the same E15.5 eye cDNA library using degenerate primers that encode two conserved hexapeptide stretches within the T-box region of *Drosophila omb* (M81796) and other T-box genes. PCR products of approximately 255 bp were subcloned, and 33 recombinants were sequenced. Of these, four were identical with mouse *Tbx3* cDNA (U57328) previously isolated from E11.5 embryos,[11] and 29 were identical with mouse *Tbx2*.

We concluded that three *omb*-related T-box genes, *Tbx2*, *Tbx3*, and *Tbx5*, are expressed in the developing eye (neural retina and lens). No other T-box cDNAs were detected. Across the 180-amino-acid (aa) region of the *omb* T-box domain (nucleotides [nt] 868-1408; Y16899), the mouse *Tbx2*, *Tbx3*, and *Tbx5* cDNAs share 66.9% nt (72.8% aa), 64.0% nt (72.6% aa) and 64.6% nt (70.2% aa) identity, respectively. Of these, the *Tbx2* gene is most highly related at the amino acid and nucleotide level to the *Drosophila omb* gene.

Identification of Human Homologues of *omb*

Expressed sequence tag (EST) databases were also searched for human cDNAs sharing high levels of sequence identity with the *Drosophila omb* cDNA. A human cDNA clone (IMAGE ID:223216; available from IMAGE Consortium at http://info@image.llnl.gov) which shares sequence similarity with *omb*, was identified from the Soares adult retinal cDNA library (Washington University School of Medicine; N2b5HR, prepared from a 55-year-old male retina). The clone contains a 2.6-kb insert, and sequence analysis confirmed that it represents the human *TBX2* mRNA (NM005994). The 5′ end of the human retinal *TBX2* cDNA, like the mouse *Tbx2* retinal cDNAs, lies within a conserved trinucleotide repeat encoding alanine residues. Because these human and mouse retinal *TBX2/Tbx2* cDNAs terminate at their 5′ ends within a guanosine cytosine (GC)-rich segment interrupting the T-box domain and are shorter than cDNAs isolated from other sources, it is likely that they do not represent full-length mRNAs. No other human T-box cDNA sequences derived from retinal RNA were identified within the EST databases.

In Situ Hybridization Analysis of *TBX2*, *TBX3*, and *TBX5* Expression in the Neural Retina

To assess whether T-box genes are involved in the differentiation of retinal neurons, we used in situ hybridization techniques to examine gene expression during development of the mammalian retina. This study focused on the analysis of human *TBX2* and its mouse homologue *Tbx2* during retinal development, because the cDNA library screening experiments identified *TBX2/Tbx2* cDNA clones within the developing and mature retina. Human cDNA probes for *TBX3* and *TBX5*, the human homologues of mouse *Tbx3* and *Tbx5* (kindly provided by David Law, University of Michigan, Ann Arbor, MI and J. David Brook, Queen's Medical Centre, University of Nottingham, UK, respectively) were obtained for a comparative expression study. Expression of *TBX2/Tbx2* was compared with expression of *TBX3* and *TBX5* in human embryonic and fetal retina and with *Tbx3* and *Tbx5* in the differentiating and adult mouse retina. No previous studies have been performed to analyze expression of these genes within the human eye. Expression of *Tbx2*, *Tbx3*, and *Tbx5* has been reported previously within the mouse optic cup,[22] but expression of these genes has not been analyzed during retinal differentiation and lamination.

Expression of *TBX2*, *TBX3*, and *TBX5* Expression across the Optic Cup

To examine patterns of gene expression across the developing optic cup, human embryonic and fetal eyes were sectioned either sagittally through the dorsal (superior) and ventral (inferior) retina (Figs. 1A, 1E, 1H) or coronally, revealing all four quadrants of the retina (nasal, temporal, dorsal, and ventral; data not shown). At 6 weeks after fertilization the embryonic neural retina was immature, consisting almost entirely of neuroblastic cells. Most of the characteristic cell types of the mature retina were as yet unborn; this period marks the beginning of retinal ganglion cell genesis. At this stage *TBX2* was expressed in a dorsoventral gradient across the optic cup with the highest expression in the dorsal hemisphere (Fig. 1B). Similar gradients of expression were seen for *TBX3* and *TBX5* (Fig. 1C, 1D). The *TBX2* expression domain extended more widely across the dorsal hemisphere (Fig. 1B) and encompassed the smaller *TBX5* domain, which is most abundant in the dorsal peripheral retina (Fig. 1D). *TBX2* expression subsequently lost dorsoventral asymmetry and was expressed throughout the inner neuroblastic (INB) retina from 8 to 9 weeks onward (Fig. 1F), whereas both *TBX3* and *TBX5* expression continued to be restricted to the dorsal retina until at least 12 weeks (Figs. 1G, 1I–K).

Expression of *TBX2*, *TBX3*, and *TBX5* across the Laminar Axis of the Developing Neural Retina

By the end of the embryonic period of development (8 weeks) the INB and outer neuroblastic (ONB) layers could be clearly distinguished (Figs. 1E, 1L), *TBX2* expression became restricted to the INB layer at this stage (Figs. 1F, 1M). *TBX2* mRNA was also detected outside the retina within the developing cornea (Fig. 1F). Neither *TBX3* nor *TBX5* showed any restriction across the laminar axis at 8 to 9 weeks, and both were expressed across the width of the dorsal peripheral retina (Fig. 1G and data not shown).

By 12 to 13 weeks, the process of stratification of the neural retina was under way and the inner plexiform layer (IPL) had formed (Fig. 1N). *TBX2* mRNA was most abundant in the developing ganglion cell layer (GCL; Fig. 1O). *TBX3* mRNA, by contrast, was expressed exclusively in cells of the inner aspect of the dorsal neuroblastic retina (Fig. 1P, and low-magnification view, 1I). The *TBX5* expression domain began to narrow across the laminar axis at this stage and became restricted to newly born ganglion cells in the dorsal retina (Fig. 1J).

At 15 weeks, the GCL was clearly established, and the presumptive INL was visible because of the increasing stratification of the outer retina (Fig. 1Q). *TBX2* mRNA was restricted to the GCL and to the developing INL, including cells at the inner aspect of the neuroblastic layer (Fig. 1R). *TBX3* expression was no longer restricted across the dorsoventral retinal axis and instead showed restriction across the laminar axis to cells of the nascent INL (Fig. 1S). At this stage, *TBX5* mRNA could not be detected within the neural retina.

In the adult retina (Fig. 1T), expression of *TBX2* was maintained in the GCL and could be detected within cells of the INL (Fig. 1U). No *TBX2* expression was found in photoreceptor cells (Fig. 1U). Neither *TBX3* nor *TBX5* was detectable within the adult retina by in situ hybridization. However, RNA PCR amplification of adult neural retina samples indicated that low levels of *TBX3* and *TBX5* mRNA were in fact present (Fig. 2), suggesting that a small number of retinal cells may express these genes.

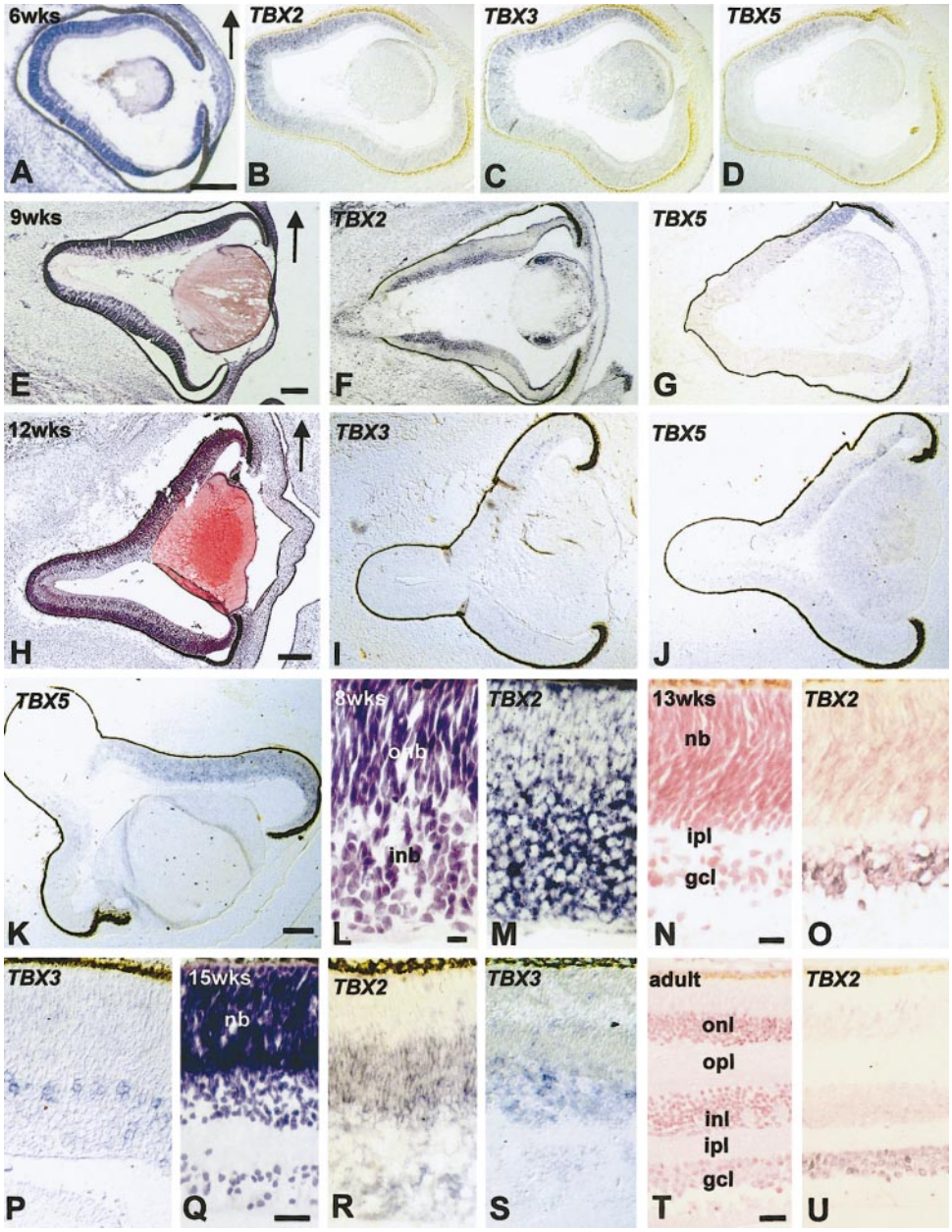

FIGURE 1. *TBX2*, *TBX3*, and *TBX5* expression in the human retina. In situ hybridization using digoxygenin-labeled riboprobes for *TBX2*, *TBX3*, and *TBX5* as indicated (**B–D**, **F**, **G**, **I–K**, **M**, **O**, **P**, **R**, **S**, **U**). Sections adjacent to the experimental sections were stained with hematoxylin and eosin (**A**, **E**, **H**, **L**, **N**, **Q**, **T**). (**A–J**) Sections are oriented so the dorsal–superior retinal hemisphere is *uppermost*. *Arrow*: dorsal orientation. (**A–D**) Ocular sections, sagittal plane from human embryonic eyes at 6 weeks. *TBX2*, *TBX3*, and *TBX5* were expressed in the dorsal and peripheral neural retina (**B–D**). (**E–G**) Nine-week fetal eye, sagittal plane. *TBX2* was expressed in the INB retina (**F**), whereas *TBX5* was restricted to the dorsal peripheral retina (**G**). (**H–K**) Twelve-week fetal eye, sagittal plane, plane of section in (**H–J**) is nasal to the optic nerve, and (**K**, dorsal is to the *right*) is through the optic nerve. *TBX3* (**I**) and *TBX5* (**J**, **K**) showed dorsally restricted expression within the neural retina. *TBX5* expression was becoming restricted to the developing GCL (**J**). (**L–U**) High-power view of sections through retina showing *TBX2* and *TBX3* expression across the laminar axis. At 8 weeks, *TBX2* expression was highest in the INB retina (**M**). At 12 to 13 weeks *TBX2* was detected in the developing GCL (**O**), whereas *TBX3* was restricted to the innermost cells of the neuroblastic layer (**P** and low-power magnification, **I**). At 15 weeks, *TBX2* mRNA was detected in the GCL, and the developing INL (**R**), whereas *TBX3* expression was restricted to the innermost cells of the developing INL (**S**). In the adult, *TBX2* mRNA was detected in the INL and the GCL (**U**). Scale bar, (**A–K**) 200 μm; (**L–S**) 25 μm; (**T**, **U**) 10 μm.

Expression of *Tbx2*, *Tbx3*, and *Tbx5* in the Developing Mouse Retina

In situ hybridization of wholemount E10.5 mouse embryos (equivalent to ~4 weeks' human gestation) was performed using digoxygenin-labeled *Tbx2*, *Tbx3*, and *Tbx5* riboprobes. *Tbx2* mRNA was detected in the dorsal hemisphere of the optic cup (Fig. 3A), consistent with previous reports.[22] At this stage *Tbx3* was also expressed within the dorsal portion of the optic cup, but at relatively low levels (Fig. 3B). *Tbx5* mRNA localized to a narrow band in the dorsal portion of the optic cup within the *Tbx2* expression domain (Fig. 3C). To compare these expression domains, adjacent coronal sections through the optic cup of E10.5 embryos were hybridized with the *Tbx2*, *Tbx3*, and *Tbx5* ^{35}S-labeled riboprobes. The plane of section cut simultaneously through the dorsal (superior) and ventral (inferior) retina, which at this stage consisted almost entirely of undifferentiated progenitor cells. The expression domains of the three genes were coincident (Figs. 3D–F). The *Tbx5* domain was tightly demarcated and lay within the broader *Tbx2* domain, which extended more ventrally, whereas *Tbx3* expression was indistinguishable at this stage from that of *Tbx2* (Figs. 3D–F).

To examine variation in T-box gene expression across the retinal axes as retinal ganglion cells develop, transverse sections through the eye at E12.5 and E14.5 were analyzed by in situ hybridization (Fig. 4). By E12.5 in the mouse (equivalent to approximately 6 weeks of human development), cell differentiation in the neural retina was under way, and nerve fibers originating in the primitive ganglion cells were projecting toward the optic disc (Fig. 4A).[23] *Tbx2* and *Tbx3* mRNAs were abundant throughout the dorsal hemisphere (Figs. 4B, 4C), but could not be detected in ventral retina (data not shown). *Tbx5* mRNA was restricted to the dorsalmost third of the optic cup and was particularly abundant peripherally (Fig. 4D). The continued restriction of T-box gene expression to the dorsal retina at this stage of development and the tightly restricted domain of *Tbx5* expression was consistent with the pattern of expression of the orthologous genes in the human retina.

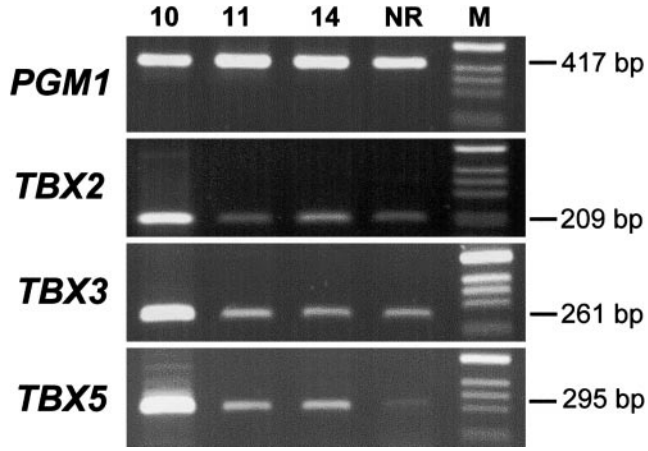

FIGURE 2. PCR amplification of *TBX2*, *TBX5*, and *TBX5* mRNA from human retina. RNA PCR amplification of *TBX2*, *TBX3*, and *TBX5* mRNA from 10-, 11-, and 14-week human fetal eyes and adult neural retina (NR). *TBX2*, *TBX5*, and *TBX5* and ubiquitously expressed phosphoglucomutase, *PGM1*–specific primers, were used to amplify fragments of 209, 261, 295, and 417 bp, respectively. All primers are from exon sequences and PCR products span exon–intron boundaries. Thirty cycles of amplification were performed for each primer set to amplify aliquots of oligo dT-primed cDNA, under standard conditions. *Lane M*: molecular weight standard.

By E14.5 (approximately 7–9 weeks of human development) the external and internal neuroblastic layers of the neural retina could be distinguished histologically (Fig. 4I). This time point is around the peak birth date of ganglion and amacrine cells.[1] *Tbx2* expression extended throughout the dorsal hemisphere. In transverse sections through the optic cup at the level of the optic nerve *Tbx2* mRNA was concentrated within the INB retina (Fig. 4E). The restriction to the INB retina was more marked dorsally (Fig. 4H) and was not apparent ventral to the optic nerve (Fig. 4K). In contrast, *Tbx3* mRNA levels were low within the central retina (Fig. 4F and data not shown). Strong *Tbx3* signal was only detected within the peripheral retina (Fig. 4F) in a dorsotemporal location. Here, its expression overlapped with *Tbx5* (Fig. 4G). Expression of *Tbx3* and *Tbx5* in the peripheral edge or ciliary margin of the retina extended across the width of the retina (Figs. 4F, 4G).

In addition to expression at the periphery, *Tbx5*, similar to *Tbx2*, was abundantly expressed at the location of the newly forming ganglion cells in the inner retina (Fig. 4G). The *Tbx5* expression pattern had a marked punctate appearance, suggesting only subsets of ganglion cells were labeled (Fig. 4G). *Tbx5* labeling was detected in developing ganglion cells throughout the dorsal retinal hemisphere (Figs. 4G, 4J, 4M). The *Tbx5* expression domain had thus extended ventrally, compared with earlier stages (Fig. 4D).

At E14.5, *Tbx2* expression (Figs. 4E, 4H, 4K), but not *Tbx3* or *Tbx5* expression, was also abundant in the developing cornea and throughout the margins of the optic cup. At the optic cup margins, *Tbx2* expression was found both within the retina and in the overlying neural crest–derived mesenchyme that gives rise to the anterior segment of the mature eye. This observation is consistent with expression of *TBX2* mRNA within the developing cornea during human ocular development (Fig. 1F). *Tbx2*, but not *Tbx3* or *Tbx5* was also detected within the mesenchyme of the developing upper eyelids (Fig. 4L).

In the mature mouse retina (Fig. 4N), as in the human retina, *Tbx2* mRNA was most abundant within the GCL and INL, whereas the photoreceptor layer did not express *Tbx2* at significant levels (Fig. 4O). In comparison, *Tbx5* mRNA was not detected in the mature retina. *Tbx3* expression was de-

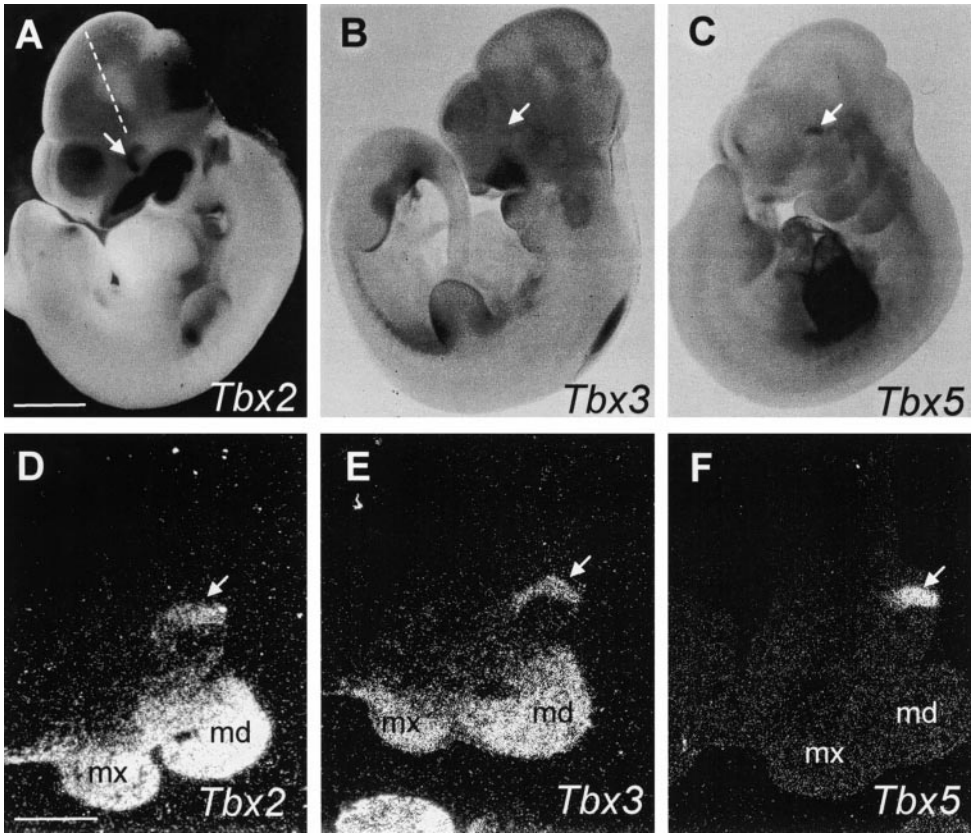

FIGURE 3. T-box gene expression in the embryonic mouse optic cup. In situ hybridization of wholemount E10.5 embryos hybridized with *Tbx2* (**A**), *Tbx3* (**B**), and *Tbx5* (**C**) digoxygenin-labeled riboprobes. *Dotted line*: plane of sections (**D**), (**E**), and (**F**). In situ hybridization of ocular sections from E10.5 embryos hybridized with *Tbx2* (**D**), *Tbx3* (**E**), and *Tbx5* (**F**) using radioactively labeled riboprobes. *Arrows*: expression of *Tbx2*, *Tbx3*, and *Tbx5* in dorsal hemisphere of the optic cup. Expression of *Tbx2* and *Tbx3* in the maxillary process (mx) and mandibular process (md) is also indicated. Scale bar, (**A–C**) 500 μm; (**D–F**) 200 μm.

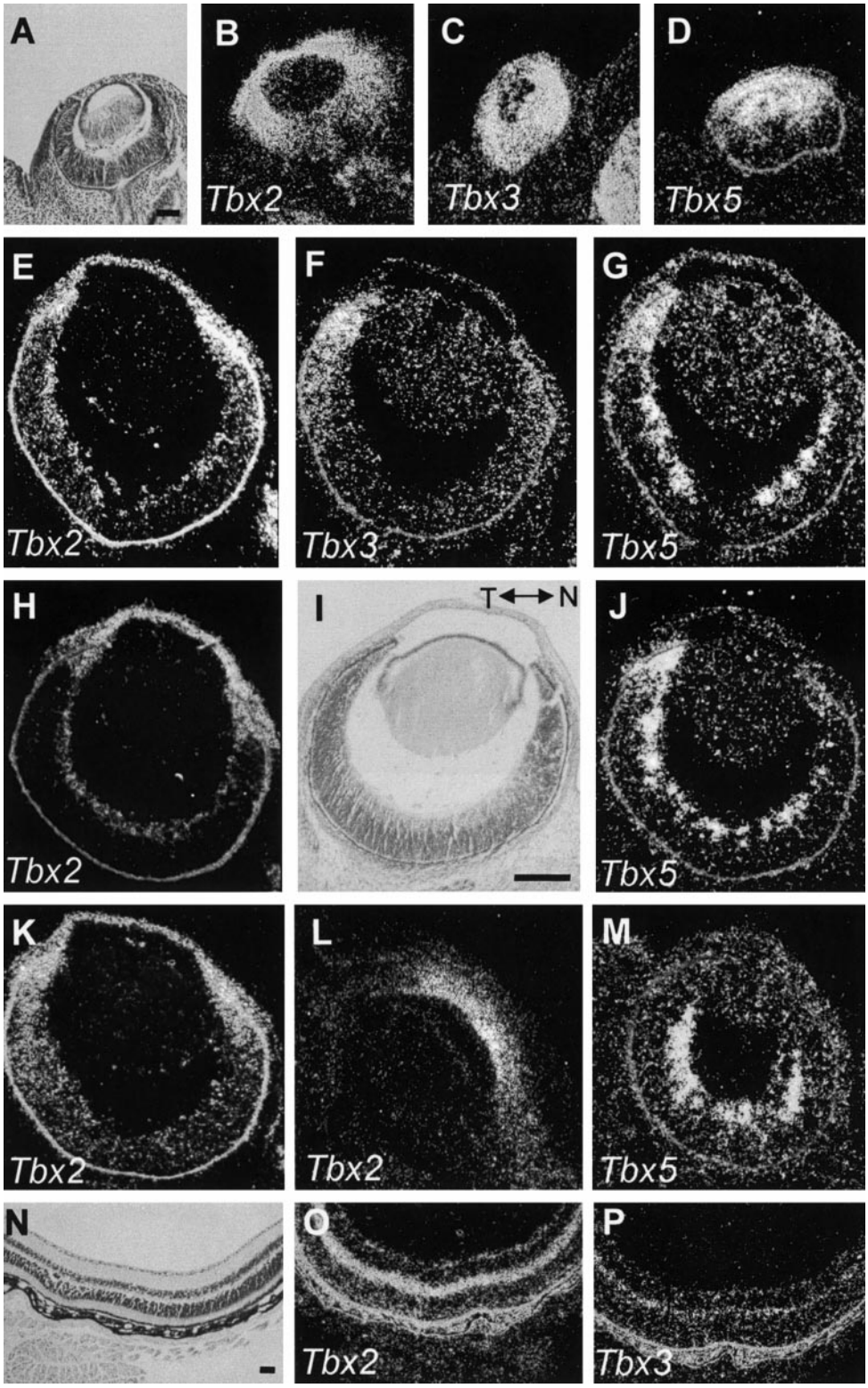

FIGURE 4. T-box gene expression in developing and adult mouse retinas. In situ hybridization analysis using ^{35}S-labeled riboprobes showing *Tbx2*, *Tbx3*, and *Tbx5* expression in the E12.5 (**B–D**) and E14.5 (**E–H**, **J–M**) embryonic retina and adult mouse eye (**O**, **P**). (**A**), (**I**), and (**N**) are stained with hematoxylin and eosin. *Arrow*: nasotemporal axis. (**A–D**) Eye sections from E12.5 mouse, transverse plane. (**A**, **B**) Through the optic nerve; (**C**, **D**) through the dorsal retinal hemisphere. *Tbx2* and *Tbx3* were expressed in the dorsal retinal hemisphere (**B**, **C**). *Tbx5* expression was restricted to the dorsalmost third of the optic cup (**D**). (**E–M**) Eye sections from E14.5 mouse, transverse plane; (**E–G**) through the optic nerve; (**H–J**) and (**L–M**) dorsal; (**K**) ventral to the optic nerve. *Tbx2* was expressed in the INB of the dorsal retinal hemisphere (**E**, **H**) but not in the ventral hemisphere (**K**). *Tbx2* was also expressed in the developing cornea (**E**, **H**, **K**) and the upper eyelid (**L**). *Tbx3* was expressed only in the peripheral temporal retina (**F**). *Tbx5* was expressed at high levels in a punctate pattern within the developing GCL throughout the dorsal hemisphere (**G**, **J**, **M**). (**N–P**) Adult eye sections of central retina close to the optic nerve. *Tbx2* was expressed within the INL and the GCL (**O**). *Tbx3* was expressed within the INL (**P**). Scale bar, 200 μm.

tected within only a subset of cells of the INL (Fig. 4P), the location of these cells was consistent with the sites of expression of human *TBX3* at 15 weeks.

DISCUSSION

In the present study at early stages of human and mouse development, three T-box genes, *TBX2/Tbx2*, *TBX3/Tbx3*, and *TBX5/Tbx5*, that are closely related to the *Drosophila omb* gene, were expressed in overlapping domains within the dorsal neural retina of the embryonic optic cup (Table 1). Recent reports of expression of the orthologous *Tbx2*, *Tbx3*, and *Tbx5* genes within the dorsal optic cup of chick,[24] *Xenopus* frog,[25] and zebrafish embryos[26,27] confirm a high level of evolutionary conservation of these spatial patterns of gene expression across the dorsoventral axis of the vertebrate eye.

TABLE 1. T-Box Gene Expression in the Developing Human and Mouse Retina

	Weeks after Conception			
Human Gene	**6**	**9**	**12**	**15**
TBX2	D	inb	GCL, INL	GCL, INL
TBX3	D	D	D, INL	INL
TBX5	D	D	D, GCL	—
	Embryonic Day			
Mouse Gene	**10.5**	**12.5**	**14.5**	**Adult**
Tbx2	D	D	D, INB	GCL, INL
Tbx3	D	D	D	INL
Tbx5	D	D	D, GCL	—

D, expression within dorsal neural retina. Where expression is restricted to specific retinal layers, this is indicated as follows: INB, inner neuroblastic retina; GCL, ganglion cell layer; INL, inner nuclear layer.

Phylogenetic comparisons assign the vertebrate *Tbx2*, *Tbx3*, and *Tbx5* genes to the same T-box gene subfamily as the invertebrate *omb* gene and suggest that these vertebrate genes arose by duplication of an ancient vertebrate *omb*-like gene sequence.[12,20] In the eye imaginal disc of *Drosophila*, the *omb* gene does not display asymmetric expression.[10] However, within leg and wing imaginal discs, *omb* expression is restricted to a dorsal compartment,[28] suggesting some conservation of function for *omb*-related genes in patterning of invertebrates and vertebrates. Other embryologic studies have demonstrated that *Tbx2*, *Tbx3*, and *Tbx5* play important roles in vertebrate limb morphogenesis,[29,30] and mutation of the human *TBX3* and *TBX5* genes both affect formation of the upper limbs.[13–15]

Defining the dorsoventral and nasotemporal axes of the neural retina is an essential step in development of the visual system. The most critical requirement for positional information across the developing retina is to provide coordinate identities to retinal ganglion cells and activate appropriate guidance molecules, because their axons project to topographic targets within the superior colliculus (optic tectum) and lateral geniculate nucleus.[31] The early asymmetric expression patterns of the T-box genes reported here, *TBX2*, *TBX3*, and *TBX5,* and by others in lower vertebrates, implicate all three genes in dorsoventral patterning of the optic cup and in controlling ganglion cell axon guidance. Human retinal axons grow into the brain during the eighth week after fertilization,[32] and asymmetric expression of *TBX3* and *TBX5* is maintained beyond this stage; hence, the time frame of expression is appropriate for activating ganglion cell surface markers needed for axon guidance. Other types of retinal cells are also distributed asymmetrically across the retina, and their development is likely to respond to positional information across the retinal axes. For instance, the dorsal retinal hemisphere has relatively high numbers of middle-wavelength cone photoreceptor cells compared with the ventral retina.[33]

Until recently, little was known about the genetic pathways that demarcate retinal territories and influence neuronal differentiation across the dorsoventral and nasotemporal axes of the neural retina. There are now a number of molecules that have been identified showing asymmetric patterns of expression across the nasotemporal axis and a smaller number showing asymmetric expression across the dorsoventral axis. The latter include Eph receptors and their ephrin ligands[34,35] and retinoic acid–synthesizing enzymes,[36] in addition to transcription factors such as the T-box genes examined in this study. Only a handful of dorsally expressed transcription factors have been described previously.[37–39] Understanding the interactions between these different classes of molecules is key to understanding the genetic control of development of retinal connections. In this respect it is interesting to note that in vitro both Tbx2 and TBX3 function as transcriptional repressors, whereas other T-box proteins have transcriptional activator properties.[40–42]

In the past year, two important studies have demonstrated the major role that asymmetrically expressed transcription factors play in activating the guidance molecules necessary for retinotopic projections. In chick embryos, misexpression of *Tbx5* in the ventral retina leads to dorsalization of the ventral hemisphere and aberrant routing of the ventral projections,[43] whereas misexpression of the *emx*-related homeobox gene *Vax2* within the dorsal retina represses *Tbx5* expression and causes targeting errors in dorsal projections.[44] Ectopic *Tbx5* expression represses expression of members of the family of receptor tyrosine kinases (Eph receptors), *EphB2* and *EphB3*, and induces expression of the membrane-bound ligands (ephrins), *ephrinB1* and *ephrinB2*.[43] Other Eph molecules have already been shown to be critical for guidance and mapping of ganglion cell axons along the nasotemporal retinal axis.[2–4] It seems likely that the T-box proteins in combination with other transcription factors such as *Vax2*, may establish retinal projections along the dorsoventral axis through regulation of expression of Eph/ephrin molecules.

As the retina matured, all three T-box genes examined showed distinctive spatial patterns of expression across the laminar axis of the retina. None of the genes was expressed within the photoreceptor cells; instead, expression was limited to cells of the forming INL and GCL. Expression patterns of the three genes in mouse and human were consistent at all stages examined, during the period when retinal cell differentiation, lamination, and ganglion cell axonogenesis occur. *TBX5* was expressed within ganglion cells, whereas *TBX3* labeled subsets of cells within the forming INL, and *TBX2* was expressed within both the GCL and the INL.

The similarity between mouse and human expression patterns supports the use of the mouse as a model system for future functional studies. A number of transcription factors have been identified that show restricted patterns of expression across the laminar retinal axis during mouse development.[45,46] Some of these factors have recently been shown to play critical roles in determining cell commitment and differentiation.[47–49] The T-box gene expression is complex, in that it showed graduated expression in two dimensions, both across the retina and across the retinal layers (summarized in Table 1). These findings suggest a dual role for *omb*-related T-box genes in the human and mouse developing neural retina, in early dorsoventral patterning, and in the process of lamination that accompanies the differentiation of retinal neuroblasts into the many cell types of the inner retina.

Acknowledgments

The authors thank Gert Pflugfelder for the *omb* cDNA, Virginia Papaioannou for *Tbx2*, *Tbx3*, and *Tbx5* cDNAs, Christine Campbell for *TBX2* primers, and Angela Gouge for retinal cDNA; and Lesley Wong, Medical Research Council Tissue Bank, London, Philip Luthert, and Adam Rutherford for invaluable discussions and technical assistance.

References

1. Young RW. Cell differentiation in the retina of the mouse. *Anat Rec.* 1985;212:199–205.
2. Drescher U, Kremoser C, Handwerker C, Loschinger J, Noda M, Bonhoeffer F. In vitro guidance of retinal ganglion cell axons by

RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. *Cell.* 1995;82:359–370.
3. Cheng HJ, Nakamoto M, Bergemann AD, Flanagan JG. Complementary gradients in expression and binding of elf-1 and mek4 in development of the topographical retinotectal projection map. *Cell.* 1995;82:371–381.
4. Winslow JW, Moran P, Valverde J, et al. Cloning of AL-1, a ligand for an Eph-related tyrosine kinase receptor involved in axon bundle formation. *Neuron.* 1995;14:973–981.
5. Martin KA, Poeck B, Roth H, Ebens AJ, Ballard LC, Zipursky SL. Mutations disrupting neuronal connectivity in the Drosophila visual system. *Neuron.* 1995;14:229–240.
6. Wawersik S, Maas RL. Vertebrate eye development as modeled in Drosophila. *Hum Mol Genet.* 2000;9:917–925.
7. Halder G, Callaerts P, Gehring WJ. New perspectives on eye evolution. *Curr Opin Genet Dev.* 1995;5:602–609.
8. Hill RE, Davidson DR. Comparative development: seeing eye to eye. *Curr Biol.* 1994;4:1155–1157.
9. Pflugfelder GO, Heisenberg M. Optomotor-blind of Drosophila melanogaster: a neurogenetic approach to optic lobe development and optomotor behaviour. *Comp Biochem Physiol.* 1995;110A: 185–202.
10. Poeck B, Hofbauer A, Pflugfelder GO. Expression of the Drosophila optomotor-blind gene transcript in neuronal and glial cells of the developing nervous system. *Development.* 1993;117:1017–1029.
11. Bollag RJ, Siegfried Z, Cebra-Thomas JA, Garvey N, Davison EM, Silver LM. An ancient family of embryonically expressed mouse genes sharing a conserved protein motif with the T locus. *Nat Genet.* 1994;7:383–389.
12. Papaioannou VE, Silver LM. The T-box gene family. *Bioessays.* 1998;20:9–19.
13. Bamshad M, Lin RC, Law DJ, et al. Mutations in human TBX3 alter limb, apocrine and genital development in ulnar-mammary syndrome (published correction appears in *Nat Genet.* 1998;19:102). *Nat Genet.* 1997;16:311–5.
14. Basson CT, Bachinsky DR, Lin RC, et al. Mutations in human TBX5 cause limb and cardiac malformations in Holt-Oram syndrome. *Nat Genet.* 1997;15:30–35.
15. Li QY, Newbury-Ecob RA, Terrett JA, et al. Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachyurany (T) gene family. *Nat Genet.* 1997;15:21–29.
16. Russ AP, Wattler S, Colledge WH, et al. Eomesodermin is required for mouse trophoblast development and mesoderm formation. *Nature.* 2000;404:95–99.
17. Chapman DL, Papaioannou VE. Three neural tubes in mouse embryos with mutations in the T-box gene Tbx6. *Nature.* 1998;391: 695–697.
18. Herrmann BG, Labeit S, Poustka A, King TR, Lehrach H. Cloning of the T gene required in mesoderm formation in the mouse (see comments). *Nature.* 1990;343:617–622.
19. Wilkinson DG. *In Situ Hybridisation*. Oxford, UK: IRL Press at Oxford University Press; 1992.
20. Agulnik SI, Garvey N, Hancock S, et al. Evolution of mouse T-box genes by tandem duplication and cluster dispersion. *Genetics.* 1996;144:249–254.
21. Bruneau BG, Logan M, Davis N, et al. Chamber-specific cardiac expression of Tbx5 and heart defects in Holt-Oram syndrome. *Dev Biol.* 1999;211:100–108.
22. Chapman DL, Garvey N, Hancock S, et al. Expression of the T-box family genes, Tbx1-Tbx5, during early mouse development. *Dev Dyn.* 1996;206:3793–90.
23. Kaufman MH. *The Atlas of Mouse Development*. London: Academic Press Ltd.; 1992.
24. Gibson-Brown JJ, Agulnik SI, Silver LM, Papaioannou VE. Expression of T-box genes Tbx2-Tbx5 during chick organogenesis. *Mech Dev.* 1998;74:165–169.
25. Takabatake Y, Takabatake T, Takeshima K. Conserved and divergent expression of T-box genes Tbx2-Tbx5 in *Xenopus*. *Mech Dev.* 2000;91:433–437.
26. Begemann G, Ingham PW. Developmental regulation of Tbx5 in zebrafish embryogenesis. *Mech Dev.* 2000;90:299–304.
27. Ruvinsky I, Oates AC, Silver LM, Ho RK. The evolution of paired appendages in vertebrates: T-box genes in the zebrafish. *Dev Genes Evol.* 2000;210:82–91.
28. Grimm S, Pflugfelder GO. Control of the gene optomotor-blind in wing development by decapentaplegic and wingless. *Science.* 1996;271:1601–1604.
29. Rodriguez-Esteban C, Tsukui T, Yonei S, Magallon J, Tamura K, Belmonte JCL. The T-box genes Tbx4 and Tbx5 regulate limb outgrowth and identity. *Nature.* 1999;398:814–818.
30. Takeuchi JK, Koshiba-Takeuchi K, Matsumoto K, et al. Tbx5 and Tbx4 genes determine the wing/leg identity of limb buds. *Nature.* 1999;398:810–814.
31. Godement P, Salaun J, Imbert M. Prenatal and postnatal development of retinogeniculate and retinocollicular projections in the mouse. *J Comp Neurol.* 1984;230:552–575.
32. O'Rahilly R. The prenatal development of the human eye. *Exp Eye Res.* 1975;21:93–112.
33. Szel A, Lukats A, Fekete T, Szepessy Z, Rohlich P. Photoreceptor distribution in the retinas of subprimate mammals. *J Opt Soc Am A Opt Image Sci Vis.* 2000;17:568–579.
34. Connor RJ, Menzel P, Pasquale EB. Expression and tyrosine phosphorylation of Eph receptors suggest multiple mechanisms in patterning of the visual system. *Dev Biol.* 1998;193:21–35.
35. Braisted JE, McLaughlin T, Wang HU, Friedman C, Anderson DJ, O'Leary DDM. Graded and lamina-specific distributions of ligands of EphB receptor tyrosine kinases in the developing retinotectal system. *Dev Biol.* 1997;191:14–28.
36. Wagner E, McCaffery P, Drager UC. Retinoic acid in the formation of the dorsoventral retina and its central projections. *Dev Biol.* 2000;222:460–470.
37. Yoshiura KO, Leysens NJ, Reiter RS, Murray JC. Cloning, characterisation and mapping of the mouse homeobox gene Hmx1. *Genomics.* 1998;50:61–68.
38. Monaghan AP, Davidson DR, Sime C, et al. The Msh-like homeobox genes define domains in the developing vertebrate eye. *Development.* 1991;112:1053–1061.
39. Papalopulu N, Kintner C. A *Xenopus* gene, Xbr-1, defines a novel class of homeobox genes and is expressed in the dorsal ciliary margin of the eye. *Dev Biol.* 1996;174:104–114.
40. Carreira S, Liu B, Goding CR. The gene encoding the T-box factor Tbx2 is a target for the microphthalmia-associated transcription factor in melanocytes. *J Biol Chem.* 2000;275:21920–21927.
41. He M, Wen L, Campbell CE, Wu JY, Rao Y. Transcription repression by *Xenopus* ET and its human ortholog TBX3, a gene involved in ulnar-mammary syndrome. *Proc Natl Acad Sci USA.* 1999;96: 10212–10217.
42. Kispert A, Koschorz B, Herrmann BG. The T protein encoded by Brachyurany is a tissue-specific transcription factor. *EMBO J.* 1995; 14:4763–4772.
43. Koshiba-Takeuchi K, Takeuchi JK, Matsumoto K, et al. Tbx5 and the retinotectum projection. *Science.* 2000;287:134–137.
44. Schulte D, Furukawa T, Peters MA, Kozak CA, Cepko CL. Misexpression of the Emx-related homeobox genes cVax and mVax2 ventralizes the retina and perturbs the retinotectal map. *Neuron.* 1999;24:541–553.
45. Freund C, Horsford DJ, McInnes RR. Transcription factor genes and the developing eye: a genetic perspective. *Hum Mol Genet.* 1996;5:1471–1488.
46. Harris WA. Cellular diversification in the vertebrate retina. *Curr Opin Genet Dev.* 1997;7:651–658.
47. Gan L, Xiang M, Zhou L, Wagner DS, Klein WH, Nathans J. POU domain factor Brn-3b is required for the development of a large set of retinal ganglion cells. *Proc Natl Acad Sci USA.* 1996;93:3920–3925.
48. Erkman L, McEvilly RJ, Luo L, et al. Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. *Nature.* 1996;381:603–606.
49. Burmeister M, Novak J, Liang MY, et al. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. *Nat Genet.* 1996; 12:376–384.