Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis

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SUMMARY

We have identified Math5, a mouse basic helix-loop-helix (bHLH) gene that is closely related to Drosophila atonal and Xenopus Xath5 and is largely restricted to the developing eye. Math5 retinal expression precedes differentiation of the first neurons and persists within progenitor cells until after birth. To position Math5 in a hierarchy of retinal development, we compared Math5 and Hes1 expression in wild-type and Pax6-deficient (Sey) embryos. Math5 expression is downregulated in Sey/+ eyes and abolished in Sey/Sey eye rudiments, whereas the bHLH gene Hes1 is upregulated in a similar dose-dependent manner. These results link Pax6 to the process of retinal neurogenesis and provide the first molecular correlate for the dosage-sensitivity of the Pax6 phenotype. During retinogenesis, Math5 is expressed significantly before NeuroD, Ngn2 or Mash1. To test whether these bHLH genes influence the fates of distinct classes of retinal neurons, we ectopically expressed Math5 and Mash1 in Xenopus retinal progenitors. Unexpectedly, lipofection of either mouse gene into the frog retina caused an increase in differentiated bipolar cells. Directed expression of Math5, but not Xath5, in Xenopus blastomeres produced an expanded retinal phenotype. We propose that Math5 acts as a proneural gene, but has properties different from its most closely related vertebrate family member, Xath5.

Key words: Neuronal bHLH, Retina, Transcription, Proneural genes, Pax6, Mouse, Eye development

INTRODUCTION

Within the developing vertebrate central nervous system (CNS), cells undergo alterations in both morphology and gene expression as they differentiate. The retina is a good model for studying cell fate determination and differentiation in the CNS because it evaginates directly from the neural tube yet forms a relatively simple, laminated tissue. The retina is composed of a seven distinct cell types: retinal ganglion cells (RGCs), amacrine cells, bipolar cells, horizontal cells, rod and cone photoreceptor cells, and Müller glial cells, which originate from a uniform neuroepithelial sheet of cells. Mature retinal neurons and glia are stereotypically arranged in layers, which form as progenitor cells exit the cell cycle, migrate to the correct location and differentiate (Robinson, 1991). In vivo and in vitro studies suggest that retinal progenitor cell fate is influenced by both internal and external factors (Cepko et al., 1996). Each cell type emerges from the precursor population in an invariant temporal sequence. The differentiation status and birthdate of mature retinal cells are tightly correlated, implying that progenitors respond differently over time (Alexiades and Cepko, 1997; Wantanabe and Raff, 1990; Williams and Goldowitz, 1992). Environmental signals also affect the type of cell that a progenitor may become (Holt et al., 1988; Turner et al., 1987; Wetts and Fraser, 1988). While several intrinsic and extrinsic factors have been identified as possible differentiation cues (Furukawa et al., 1997b; Guillemot and Cepko, 1992; Park and Hollenberg, 1989; Pittack et al., 1991), the mechanism of retinal histogenesis remains largely unknown.

Because nuclear transcription factors function cell autonomously, they are good candidates for intrinsic regulators of retinal cell fate. Several classes of transcription factors are required for optic vesicle morphogenesis (Furukawa et al., 1997a; Mathers et al., 1997; Porter et al., 1997) and for differentiation of retinal cell types (Furukawa et al., 1997b). bHLH transcription factors regulate neurogenesis in both invertebrates and vertebrates (Jan and Jan, 1993; Lee, 1997; Kageyama et al., 1995). This class has been most extensively studied in the Drosophila nervous system; some family members act positively to promote neuronal cell development, whereas others antagonize the ‘proneural’ genes. The Drosophila proneural genes include the achaete-scute complex (AC-S), required for the development of external sense organs and subsets of CNS precursors, atonal (ato, necessary for chordotonal organ and photoreceptor development) and
Multiple vertebrate family members have been identified for each *Drosophila* bHLH subclass. Structurally, vertebrate proneural genes fall into two subsets, with one group, *Mash1*, *Mash2*, *Xash1* and *Xash3*, being more closely related to *Drosophila* AC-S genes (Ferreiro et al., 1992; Guillemot and Joyner, 1993; Johnson et al., 1990; Turner and Weintraub, 1994). The other, larger subset, which includes *Math1*, *Math2/Nex1*, *Math3/Xath3/Neurom*, *Math4a/Ngn2*, *Math4b/Ngn3*, *Math4c/Ngn1*, *Xath5* and *NeuroD*, are more similar to *Drosophila* ato (Akazawa et al., 1995; Ben-Arie et al., 1996; Gradwohl et al., 1996; Kanekar et al., 1997; Lee et al., 1995; Ma et al., 1996; Roztocil et al., 1997; Shimizu et al., 1995; Sommer et al., 1996; Takebayashi et al., 1997). Within and between these classes, bHLH genes are often expressed in partially overlapping patterns throughout the developing CNS.

In the developing mammalian eye, *Math1*, *Math3*, *Math4a/Ngn2* and *NeuroD* are expressed by retinal progenitor cells (Gradwohl et al., 1996; Guillemot and Joyner, 1993; Jasoni and Reh, 1996; Roztocil et al., 1997; Sommer et al., 1996; Takebayashi et al., 1997), although none appear to be expressed before RGC formation. The role of these genes during eye development is poorly understood. For example, *Mash1* is expressed by many retinal progenitors, but a targeted mutation in the *Mash1* gene produced no eye phenotype (Guillemot et al., 1993). However, explant culture of *Mash1*−/− mutant retinas demonstrated a delay in rod, horizontal and bipolar neuron differentiation (Tomita et al., 1996a). A bipolar cell number was decreased, while Müller glial cell number increased (Tomita et al., 1996a). These results suggest that *Mash1* regulates the formation of later born retinal neurons, especially bipolar cells, but has no role in early retinal neurogenesis. Instead, the formation of early-born retinal neurons in mammals may be regulated by *ato* family members, as in *Drosophila* photoreceptor cell development.

At least one negatively acting bHLH gene, *Hes1*, is required for mammalian eye development. *Hes1* is expressed during early stages of mouse eye development and is structurally related to *Drosophila* h and *Enhancer of split* genes (Sasai et al., 1992; Tomita et al., 1996b). Loss- and gain-of-function studies demonstrate that *Hes1* regulates the timing of retinal neurogenesis, as *Hes1*-deficient mouse embryos form severely reduced optic cups by embryonic day 10.5 (E10.5) (Tomita et al., 1996b). In the *Drosophila* eye, *h* (together with *emc*) influences the timing of *ato* expression (Brown et al., 1995). *Hes1* and *h* may have similar functions during retinal neurogenesis. Like *h*, *Hes1* may repress positively acting bHLH genes such as *Mash1*, which is prematurely upregulated in the optic cup and neural tube of E10.5 *Hes1*−/− embryos (Ishibashi et al., 1995). However, as yet no clear *ato* equivalent has been identified for mammalian retinogenesis that is subject to *Hes1* regulation.

The *Pax6* transcription factor is critically required for eye formation. It contains two highly conserved DNA-binding domains, a paired box and a homeobox (Hanson and van Heyningen, 1995). *Pax6* mutations produce eye malformations in the human disease aniridia (Glaser et al., 1992; Ton et al., 1991) and, in the mouse and rat, *Small eye (Sey)* traits (Fujiwara et al., 1994; Hill et al., 1991). *Sey/+* heterozygotes have noticeably smaller eyes by midgestation (Hill et al., 1991; Hogan et al., 1986) and typically form cataracts postnatally. These malformations arise through haploinsufficiency in humans and in mice. A delay in lens induction has been postulated for *Sey/+* heterozygotes (Theiler et al., 1978), but the basis for Pax6 dosage sensitivity (Glaser et al., 1994) is unknown and no dosage-responsive downstream genes have been identified. Overexpression of human Pax6 in mice has further demonstrated that Pax6 gene dosage is critical for normal mammalian eye development (Schedl et al., 1996). *Sey*/Sey homozygotes initiate optic vesicle morphogenesis (Grindley et al., 1995), but fail to form lenses or complete retinal development. The resulting eyeless mice die at birth due to concomitant nasal and CNS defects (Grindley et al., 1995; Hill et al., 1991; Hogan et al., 1986). Because the primary eye defect in *Sey*/Sey embryos occurs prior to retinal neurogenesis, it has been difficult to understand if and how Pax6 may regulate later eye formation events such as neuron specification.

Here we report a new murine bHLH gene, *Math5*, that is closely related to *Drosophila* ato. *Math5* retinal expression begins at E11, before any other mammalian proneural gene and is spatiotemporally correlated with the appearance of RGCs, the first-born neurons. To test whether *Math5* directly regulates this process, we compared the ability of *Math5*, *Mash1* and *Xath5* to promote differentiation of *Xenopus* retinal progenitors in vivo. Surprisingly, *Math5* and *Mash1* each bias retinoblast differentiation toward a bipolar cell fate, whereas *Xath5* promotes differentiation of RGCs. Finally, we show that Pax6 is required for appropriate Math5 and Hes1 expression in the optic cup, thereby connecting Pax6 activity with retinal neuron formation.

**MATERIALS AND METHODS**

**Molecular cloning of Math5**

Reverse transcription was performed with random-primed mRNA from mouse E12.5 embryonic eye tissue and the resulting cDNA was used as a template for a degenerate polymerase chain reaction (PCR). Standard PCR conditions were used with an annealing temperature of 60°C for 40 cycles. The PCR primers corresponded to peptide sequences RRLAANA (5'-GSGCCA TCTGY AGIGTCTCRTA-3') and ETLQMAL (5'-ARGRMGKCTRGCIGMAAYGC-3'), which are completely conserved within the bHLH domains of *Xenopus* *Xath5* and *Drosophila* ato (Jarman et al., 1993; Kanekar et al., 1997). The resulting 135 bp PCR products were subcloned using a TA cloning kit (Invitrogen). Of nine plasmid subclones sequenced, six encoded a 2.8 kb HindIII fragment from one genomic clone were sequenced on both strands by automated DNA sequencing. DNA sequence
analysis was performed using MacVector and AssemblyLIGN programs, version 5.0 (Oxford Molecular Group). The GenBank accession number for Math5 is AF071223.

Math5 and Mash1 cDNAs were subcloned into the expression vector pCS2+ (Turner and Weintraub, 1994) at the EcoRI and XhoI sites (Math5) or the EcoRI site (Mash1). To create a C-terminal myc-epitope tag, the Math5-coding region was subcloned into the pCS2+MT expression vector following PCR amplification in the presence of MasterAmp PCR enhancer compound (Epicentre Technologies). The 5' primer was 5'TCGAGCGGCCAG-AAGCTTATGAATGCGCCTGCAAACC3' (Stul site underlined, ATG start codon in bold) and the 3' primer was 5'AACTCTTCTTATACTGTTGTGGAACCGGAAGAA3' (XhoI site underlined; 60 bp downstream of stop codon). Subclones generated by PCR were fully sequenced. Math5/pCS2+, Mash1/pCS2+ and Math5-MT/pCS2+ constructs produced proteins of the predicted size upon in vitro translation (TNT Coupled Reticulocyte Lysate System, Promega). No difference in functional activity was detected between pCS2-Math5 and the myc-epitope tagged version, Math5-MT/pCS2. Details of Xath5/pCS2+ constructs can be found in Kanekar et al. (1997).

Phylogenetic analysis

A total of 20 bHLH amino acid domain sequences, including Math5, were aligned by eye and subjected to parsimony analysis using PAUP, version 3.1.1 (Swofford, 1993). A heuristic search with 100 random stepwise addition replicates was performed using a step matrix for amino acid substitutions. This matrix gives the minimum number of nucleotide substitutions needed to convert one amino acid into another, based on the genetic code used by nuclear genes of most eukaryotes. 100 bootstrap replications, each representing 100 random stepwise sequences of taxon addition (Swofford, 1993) were also performed and a bootstrap majority rule consensus tree was generated. In both searches, Mash1 and Mash2 bHLH domains were designated as outgroup taxa.

Chromosomal mapping

Mouse-hamster somatic cell hybrid DNAs (Lalley et al., 1978) were tested for Math5 by PCR, using primers 5'-ACAGGAGTGGTTTTATTCCTCCC3' (forward) and 5'-GTTGGTAGCTGGCTTGAATCC3' (reverse), which amplify a 625 bp genomic DNA fragment located 800 bases 3' from the Math5 stop codon. Precise chromosome position was determined by linkage analysis of a PCR length variant between laboratory mice and Mus spretus. A set of 90 interspecific backcross progeny (Glaser et al., 1990) was typed using the Math5 primer pair and microsatellite markers D10Mit16, D10Mit109, D10Mit62, D10Mit42 and D10Mit74.

Mouse embryos

CD-1 embryos (Charles River) were obtained from timed matings. The day that vaginal plugs were observed was designated E0.5. Embryos were dissected in cold phosphate-buffered saline (PBS), their hindbrain and cerebral vessels were incised and they were fixed overnight in 4% paraformaldehyde/PBS at 4°C. Embryos were kept in fixative for up to 2 weeks at 4°C, dehydrated stepwise through a graded PBS/methanol series and stored in methanol at −20°C. For some experiments, fixed E11.5-E13.5 embryos were bisected sagittally in methanol before processing for in situ hybridization.

Embryos from Small eye (SeyN°/N° × SeyN°/N°) heterozygous matings were isolated in cold PBS, fixed and processed for in situ hybridization. The genotype of each embryo was determined by PCR (Xu et al., 1997), using tail or extraembryonic membrane DNA (Laird et al., 1991) as a template. Littermates with identical or nearly identical (±1) somite numbers were utilizes for analysis of Math5 (4 litters) or Hes1 (6 litters) mRNA expression. The SeyN° allele originated in a C3H × 101 F1 mouse (Hill et al., 1991) and has been maintained on a C3H/HeJ background. To facilitate gene expression studies in the developing eye, the SeyN° mutation was backcrossed to the albino FVB strain for more than five generations. No difference was observed in eye morphology (E9.5 to E13.5), pattern of gene expression (Math5 and Hes1) or Sey phenotype between these genetic backgrounds.

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was performed on mouse embryos as described by Hargrave and Koopman (1998). Sense and antisense digoxigenin-labeled RNA probes were prepared from Math5 cDNA clones in pBluescript II KS+, using a DIG RNA labeling kit (Boehringer Mannheim) and were detected by anti-digoxigenin antibody coupled to alkaline phosphatase. Hybridization and stringent posthybridization wash steps were performed at 70°C. After alkaline phosphatase development, embryos were destained for up to 4 hours in 1% Triton X-100/PBS, postfixed in 4% paraformaldehyde/PBS and photographed through a dissecting microscope using EPI 160T film and a dark-field condenser.

For in situ hybridization of tissue sections (Schraen-Weimers and Gerfin-Moser, 1993), dissected embryos were immediately embedded and cryosectioned at 14 μm. The prehybridization, hybridization and stringent wash steps were performed at 70°C using the buffers described in Hargrave and Koopman (1998). Alkaline phosphatase color development was allowed to proceed for 24 hours. Slides were postfixed in 4% paraformaldehyde and mounted in 80% glycerol/PBS. Some sections were counterstained with Neutral Red (Sigma) prior to mounting. Mounted sections were digitally captured using an Optronics 3CCD Video Camera System or were photographed using EPI 64T film and scanned with a Nikon Coolscan slide scanner.

Math5 (2 kb), Hes1 (0.9 kb), NeuroD (1 kb) and Ngn2 (1.5 kb) riboprobes were synthesized from cDNA plasmid clones. For double labels with Math5 RNA and Pax6 protein, embryos or isolated eyes were processed for whole-mount in situ hybridization, embedded and cryosectioned at 14 μm. Sections were incubated with a rabbit polyclonal antiserum reactive with the C-terminal peptide of the mouse Pax6 protein (Davis and Reed, 1996), processed and developed for peroxidase immunohistochemistry as described in Mastick et al. (1997). Double-labeled sections were mounted in 80% glycerol/PBS and digitally captured using a microscope equipped with Nomarski optics.

Microinjection of RNA into Xenopus embryos

Capped RNA was synthesized in vitro by SP6 transcription from cDNA plasmids carrying the Math5, Sey Neu or N-tubulin cDNA inserts. Several constructs were created using Message Machine kit (Ambion). For in vitro microinjection, RNA was injected into the one-cell blastomere of the following amounts: Xath5 (500 pg), Math5 or Math5-MT (50-500 pg), nfgal (60 pg). Following injection, embryos were processed as described in Kanekar et al. (1997) with one exception: embryos injected with nfgal RNA were stained with magenta-gal (Biosynth International) using an X-gal staining method (Turner and Weintraub, 1994). The digoxigenin-labeled Xenopus N-tubulin riboprobe (Richter et al., 1988) was synthesized as previously described (Harland, 1991) and whole-mount in situ hybridization for N-tubulin mRNA was performed as detailed in Kanekar et al. (1997).

For 16-cell RNA injections, Math5 RNA (50-100 pg) was injected into Xenopus blastomere D.1.1. GFP RNA (30 pg) was cojected to label cells derived from the injected blastomere. Following injection, the embryos were processed as described in Kanekar et al. (1997). Some sections of 16-cell-injected embryos were processed for NCAM immunohistochemistry using the 6F11 monoclonal antibody (Sakaguchi et al., 1989) and a rhodamine-conjugated goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch).
In vivo lipofection and BrDU analysis

In vivo lipofection of experimental and green fluorescent protein (GFP) DNA (to mark transfected cells) was performed and analyzed as previously described (Kanekar et al., 1997). The rate of cotransfection of two plasmids is very high (Holt et al., 1990). Images of labeled sections (lipofections and 16-cell RNA injections) were digitally captured by a Xillix Microimager PMI CCD camera using Openlab software. Lipofected embryos were injected with 5 μg/ml BrDU at stage 26-27 as described in Wets et al. (1993), then fixed and processed at stage 41. The embryos were cryostat-sectioned and digital images of GFP-labeled retinal sections captured as described above. BrDU labeling in these sections was then detected by immunocytochemistry (del Rio and Soriano, 1989) using a monoclonal anti-BrDU antibody (Sigma) and the rhodamine-conjugated goat anti-mouse secondary antibody described above. Images of the anti-BrDU-stained retinal sections were then acquired digitally and merged with the GFP images from the same sections to determine which cells are both GFP positive and BrDU positive.

RESULTS

Isolation and structural analysis of Math5

To investigate how bHLH genes regulate early retinal neurogenesis, we screened embryonic mouse eye cDNA (E12.5) for new ato-related transcription factors by degenerate RT-PCR. Primers that amplify the conserved bHLH domain were designed based upon segments of significant amino acid identity between Drosophila ato and Xenopus Xath5 (Jarman et al., 1993; Kanekar et al., 1997). The resulting 135 bp products were cloned and sequenced. Two classes of clones were identified by sequence and colony hybridization: Math1 (Akazawa et al., 1995) and a novel sequence most closely related to Xath5, which we have termed Math5. The Math5 bHLH fragment was used to probe mouse genomic and P3-P7 eye cDNA libraries. Four different cDNA clones were obtained ranging in length from 1.2 kb to 1.4 kb. All of the clones contain an identical 447 nucleotide open reading frame encoding a protein of 149 amino acids (Fig. 1A). The putative methionine start site conforms to the Kozak consensus sequence (Kozak, 1989) and is preceded by stop codons in all three frames. A 17 kb genomic clone was also isolated. The genomic and cDNA sequences are identical, indicating that Math5 contains an intronless open reading frame (Fig. 1A).

Aligned Math5 and Xath5 proteins are 91% identical within the bHLH domain (Fig. 1A,B) and exhibit limited stretches of identity outside of this domain (Fig. 1A). A phylogenetic comparison of Math5 bHLH amino acid sequence with other bHLH proteins was also performed. A heuristic search generated a consensus phylogram based on shared, derived characteristics with Mash1 and Mash2 as outgroup taxa. Bootstrap analysis also strongly supported this phylogram (Fig. 1C). In the heuristic search, 28 most parsimonious trees were produced. The length of the shortest tree is 239 steps. A strict consensus of these 28 trees, as well as the bootstrap majority rule consensus tree (Fig. 1C) demonstrate that Math5 is a derived member of the ato protein family and is most closely related to Xath5. In addition, Math5 is as closely related to Drosophila ato as it is to the lineage giving rise to Math1, Hath1 and Cath1. Finally, this analysis indicates that the C. elegans lin32 protein is a member of the ato family and does not belong to the AC-S family (Fig. 1C).

Chromosome mapping of Math5

Math5 was mapped using a mouse-hamster somatic cell hybrid panel (Lalley et al., 1978) and an interspecific mouse backcross (Glaser et al., 1990). In the somatic hybrids, Math5 segregated discordantly with every chromosome except 10. In the interspecific backcross, Math5 was further localized to a region approximately 34 cM from the centromere (Fig. 1D). No recombination was observed between Math5 and D10Mit62 among 90 N2 progeny (Fig. 1D). This position excludes a role for Math5 in the eye blebs mutation or in any of several dominant cataract mutations (Cat, Cat3, Cat5) on chromosome 10 (Loster et al., 1997). No human genetic eye diseases were found to map within the regions syntenic to this portion of mouse chromosome 10 (N. L. B., unpublished data). Math3 (Atoh3), another ato-like gene expressed in the developing mouse eye, also maps to chromosome 10 (Isaka et al., 1996). However, Math3 and Math5 are at least 40 cM apart and so do not represent a gene cluster for the bHLH protein family.

Expression of Math5 during eye development

The spatial and temporal expression of Math5 mRNA was examined during mouse embryonic development by whole-mount and sectioned in situ hybridization. Math5 was first detected at E11 (Fig. 2A-D), in a small patch of cells in the central, dorsal optic cup (Fig. 2B). From E11 to E12, the dorsal expression domain expands circumferentially (Fig. 2C). By E12.5, Math5-expressing cells are distributed throughout the developing retina (Fig. 2F,I), except for the ciliary proliferating zone (Fig. 2E,J). These cells are arranged in columnar strings or clusters, interspersed with columns of nonexpressing cells (Fig. 2D,F-H).

As retinal neurons differentiate, they exit the cell cycle and migrate toward the inner (vitreal) side of the optic cup to assume their final position (Young, 1983, 1985). Progenitor cells remain in the outer (ventricular) side. Accordingly, the retina ceases to be a uniform sheet of neuroepithelial cells. Cell division is primarily restricted to the ventricular zone and the ciliary margin of the optic cup. During later stages of development (E13.5) a single Math5-positive cell was often observed adjacent to the ciliary proliferating zone (arrow in Fig. 2E) and in columns of cells toward the central retina (arrowheads in Fig. 2E). The first neurons to differentiate are RGCs, which appear at the late E12.5/early E13 stage of development as shown by birthdating and terminal differentiation marker studies (Erkman et al., 1996; Hinds and Hinds, 1974). Math5 expression begins a full day prior to this time. Differentiated RGCs, clearly visible within a central domain and marked by their strong expression of Pax6 protein, do not express Math5 (Fig. 2J,K). The period of Math5 expression thus precedes and overlaps retinal neurogenesis. Although Math5 was detected in retinal progenitor cells from E11 through birth (Figs 2L, 4I), the number of positive cells decreased significantly at E16.5 (Fig. 4A). At birth (P0), Math5-expressing cells were primarily observed adjacent to the ciliary margin and sparsely distributed within the central retina (Figs 2L, 4I). No Math5 expression was detected in the adult retina (data not shown).

Math5 expression in other embryonic tissues

In addition to the eye, we observed Math5 expression only in the developing tenth cranial (vagal) ganglion between E13.5
and E15.5 (Fig. 2M). This contrasts with Xath5, which is found in the developing frog retina, nasal placodes and pineal gland (Kanekar et al., 1997) and with ato, which is expressed in the developing Drosophila eye, olfactory system and ocellus, a photosensitive structure that has been likened to the pineal gland (Jarman et al., 1995; Reddy et al., 1997). We were unable to detect Math5 expression, from E8.5 to birth, in the murine nasal placode, olfactory epithelium or developing pineal gland. At the same time, a comparable probe, Mash1, was easily detected in the nasal placode and olfactory epithelium (data not shown). Although Xath5 is not expressed by cells of the Xenopus vagal ganglion (M. L. V., unpublished observations), other mammalian ato-related family members, such as ngn2, are expressed in particular cranial ganglia prior to neuron formation (Ma et al., 1998).

Math5 and Hes1 retinal expression require Pax6 function in a dose-dependent manner

The Pax6 gene is expressed throughout vertebrate eye development and controls many aspects of ocular formation. Pax6 expression begins at mouse E8.0 within the neural plate and optic vesicle and persists throughout eye development into adulthood (Koroma et al., 1997; Walther and Gruss, 1991). Early Pax6 expression is ubiquitous within the optic cup (Walther and Gruss, 1991) while later it becomes restricted to differentiating RGCs and amacrine cells (Belecky-Adams et al., 1997). To test whether Pax6 function regulates Math5 transcription, we examined Math5 mRNA expression in Sey/+ × Sey/+ littermates at E11.5. Optic vesicle development in Sey/Sey embryos arrests at the E9.5 stage of morphogenesis but optic vesicle and stalk structures are present at E11.5 (Grindley et al., 1995). We saw no expression of Math5 in Sey/Sey embryos (Fig. 3C,F), even at the earliest time of Math5 expression, the 46 somite (E11) stage (data not shown). We consistently observed a marked reduction in the size of the Math5 expression domain in Sey/+ embryos (compare Fig. 3B to A and E to D), strongly suggesting that Math5 expression depends upon Pax6 function. As yet, no other downstream targets of Pax6 regulation are known to display a similar gene dosage dependence.

The mouse bHLH gene Hes1 is

![Diagram](image-url)
expressed in the optic cup at E11.5 and functions in retinal formation (Tomita et al., 1996b). To explore the relationship between Hes1, Pax6 and Math5, we examined Hes1 expression in Sey/+ × Sey/+ litters at the identical somite ages as for Math5 (Fig. 3G-L). In these experiments, Hes1 expression also depended upon Pax6 gene dosage, but in a reciprocal manner to Math5. Hes1 mRNA expression was increased in Sey/+ embryos in comparison to wild-type (compare Fig. 3H to G and K to J), and was abundant within the optic vesicle remnant of Sey/Sey embryos at E11.5 (Fig. 3I, L). Pax6 function is similarly required for proper Hes1 expression at E10.5, when the increase in Hes1 expression in Sey/Sey optic vesicles is more striking (Fig. 3M-O). The size of the Hes1 expression domain in E10.5 Sey/Sey optic vesicles reflects abnormal morphology of this structure (Grindley et al., 1995) rather than an increased number of cells expressing Hes1 (N. L. B., unpublished observations). These findings suggest that Pax6 may negatively regulate Hes1 expression. The effects of Pax6 deficiency upon Hes1 are eye-specific, since no changes in Hes1 mRNA were noted in other expression domains such as the tailbud, somites and spinal cord (data not shown). We conclude that Pax6 function is required to properly regulate the expression of Math5 and Hes1 in the developing optic cup.

**Math5 precedes NeuroD, Ngn2 and Mash1 in the developing retina**

Several studies of vertebrate proneural genes have suggested a hierarchy of regulatory relationships within the bHLH family (Cau et al., 1997; Kanekar et al., 1997). For example, during mammalian olfactory neurogenesis, Mash1 activates a bHLH gene cascade within neural progenitor cells (Cau et al., 1997). In this study the AC-S-related gene Mash1 was shown to precede and act upon ato-like genes. To evaluate this relationship in the murine eye, we compared the spatial and temporal expression of Math5 to that of Mash1, the only known AC-S family member expressed in the mammalian retina (Guillemot and Joyner, 1993; Jasoni and Reh, 1996), and to NeuroD and Ngn2, ato-like genes with retinal expression (Gradwohl et al., 1996; Lee et al., 1995; Sommer et al., 1996).

Math5 expression initiates at E11 in the central optic cup (Fig. 2B) and Ngn2 and NeuroD mRNA are not detected at this time (data not shown). Instead, NeuroD and Ngn2 initiate expression at E13.5 (Fig. 4B-D), 2 days after Math5 (Figs 2L, 3A). Interestingly, NeuroD is expressed in two domains at all ages examined (Fig. 4B, C, F, J), within the outermost cells of the ventricular zone and differentiated RGCs. This suggests NeuroD may function in progenitors as well as in terminally differentiated neurons. Mash1 retinal expression initiates at E14.5 (Guillemot and Joyner, 1993; Jasoni and Reh, 1996), 3 days after birth (Fig. 2E, F, H, I). These data strongly suggest that Math5 expression precedes NeuroD and Ngn2.

**Fig. 2. Math5 expression during embryogenesis.** In situ hybridization of whole-mount (A-L, K) and sectioned (J-L, M) embryos with Math5 digoxigenin-labeled riboprobes. Rostral is at the left in B-F, I, M and at the top in G, H, L. (A-C) Lateral views of E11.5 embryos showing Math5 expression exclusively in the optic cup (arrow in A). Math5 optic cup expression begins at E11 (46-somite stage) in a dorsal patch of cells. This domain expands throughout the next 24 hours to encompass the entire optic cup. (D) Horizontal section of E11.5 optic cup, as indicated in C. Math5-expressing cells span the retinal epithelium and are interspersed with groups of nonexpressing cells. (E) Horizontal section of an E13.5 retina, oriented as indicated in I, labeled for Math5 mRNA (blue cells) and Pax6 protein (brown nuclei), and viewed at high magnification. Undifferentiated cells at the ciliary margin (lower left) and differentiated RGCs (lower right) express Pax6. An isolated Math5-expressing cell (arrow) is adjacent to the undifferentiated region. Toward the central retina (right), columnar clusters of Math5-expressing cells (arrowheads) are separated by columns of nonexpressing cells. (F-H) Sagittal section of E12.5 optic cup showing the interspersion of Math5-positive and -negative cells. (G-H) High magnification views of the area marked by a bracket in F, at two different focal planes. Two strongly Math5-positive cells at the ventricular surface of the neuroretina (large arrow) appear to have recently divided. The columnar arrangement of Math5-positive cells, spanning the width of the optic cup, is indicated (small arrows). (I) Lateral view of an E13.5 eye showing strong Math5 expression throughout the retina. (J) In situ hybridization at E13.5, sectioned as indicated in I and counterstained with neutral red. Differentiated RGCs (arrows) do not express Math5 (blue cells). (K) Transverse section of E14.5 eye labeled for Math5 mRNA (blue cells) and Pax6 protein (brown nuclei). Pax6 protein is abundant in RGCs, located on the vitreal side of the retina, while undifferentiated cells in the ventricular zone are Math5-positive. (L) At birth, Math5 expression is restricted to the undifferentiated region of the retina, closest to the ciliary margin (lower left). (M) Vagal (tenth) cranial ganglion cells express Math5 (blue cells) at E13.5 (counterstained with neutral red). Scale bars: 500 μm in A-C, I; 100 μm in F, J-M; and 50 μm in D, E, G, H. Abbreviations: L, lens; c, cochlea.
days after the onset of *Math5*. All four genes are strongly expressed by retinal progenitors from E14.5 to E16.5, as judged by in situ hybridization to sections of embryonic eyes (Fig. 2K and Gradwohl et al., 1996; Sommer et al., 1996).

At E16.5 the number of *Math5* - and *Ngn2*-expressing cells begins to noticeably diminish (Fig. 4E,H), whereas *Mash1* is expressed by most, if not all, progenitors (Fig. 4G). In similar whole-mount experiments, Jasoni and Reh (1996) observed that *Mash1* is expressed by 10-30% of the progenitor population at a given time. By birth, *Math5* and *Ngn2* expression are nearly absent from the central retina (Fig. 4I,L), while *Mash1* and *NeuroD* mRNA is present in the adult retina (Acharya et al., 1997). Birthdating studies have shown that retinal histogenesis can be divided into an early phase (from E10.5 to birth), when most RGCs, cones and horizontal cells are born, and a later phase (from E15 to postnatal day 9) when the majority of bipolar, amacrine, rod and Müller glial cells are generated (Carter-Dawson and LaVail, 1979; Young, 1985). Our findings correlate *Math5* and *Ngn2* expression with the appearance of early-born neurons, *Mash1* expression with late phase neurogenesis and *NeuroD* expression with both phases of retinal neuron formation.

**Math5 promotes ectopic neurogenesis and retinal expansion in Xenopus embryos**

Since Math5 and Xath5 share a high degree of sequence identity within the bHLH domain, we tested whether they have similar functional activity. Xath5, like other ato-related bHLH proteins, is able to promote the formation of ectopic *N-tubulin*-positive cells when overexpressed in *Xenopus* embryos by 2-cell RNA injection (Kanekar et al., 1997). We therefore injected *Math5* RNA into one cell of a 2-cell-stage embryo, collected the embryos at the open neural plate stage and probed by whole-mount in situ hybridization for *N-tubulin* expression, a neuron-specific marker (Richter et al., 1988). Coinjected nβgal RNA was detected by staining with magenta-gal, which marked the injected side of the embryo (Fig. 5Ai). Overexpression of *Math5* caused the appearance of ectopic *N-tubulin*-positive cells throughout the neural plate and lateral ectoderm on the injected side with a very punctate pattern of staining similar to what is seen with overexpression of Xath5 (30/30 embryos, Fig. 5Aii) (Kanekar et al., 1997). This suggests that *Math5* can promote neuronal differentiation when overexpressed.

Targeted expression of Xath5 in retinal progenitor cells using both 16-cell RNA injection and in vivo lipofection caused a strong bias toward the RGC fate (Kanekar et al., 1997 and Fig. 3).
To determine whether Math5 has an effect on progenitor cell formation, Math5 expression was targeted to the developing retina by injecting a mixture of Math5 and GFP RNA into blastomere D.1.1 of 16-cell Xenopus embryos. This blastomere contributes to over 50% of the cells in the ipsilateral retina (Huang and Moody, 1993; Moody, 1987). The GFP signal was then used to identify clones of cells derived from the injected blastomere in sections of stage 41 embryos. In control embryos injected with GFP RNA alone, clusters of GFP-positive retinal cells spanned the neural and pigmented cell layers (n = 17 retinae, Fig. 5Bii). Embryos co-injected with Math5 and GFP RNA also contained clusters of GFP-positive cells within the retina (Fig. 5Bv) but the cell layers were severely disrupted within these clusters (n = 17/19 retinae, Fig. 5Biv). This marked disruption in retinal structure suggested a block in the differentiation of cells overexpressing Math5.

We therefore stained the sections with antibodies against NCAM to determine whether the labeled cells had any features of differentiated neurons. We found that clones of GFP-positive cells in Math5-overexpressing embryos (Fig. 5Bvi) were weakly NCAM-positive, indicating that these cells showed some evidence of neuronal differentiation. We also observed a continuous population of GFP-positive cells extending from the back of the retina to the neural tube and, in some cases, relatively large clones of GFP-positive clones were also identifiable within the neural tube (data not shown). This might indicate either hyperplasia of neural retinal cells expressing Math5 causing expansion towards the neural tube, or a failure of the evaginating optic cup to resolve from the neural tube when Math5 is overexpressed. This expanded retinal phenotype and disruption of retinal organization was never observed when Xath5 was overexpressed by 16-cell RNA injection (Kanekar et al., 1997). This suggests that not all aspects of Xath5 function are conserved in Math5.

In vivo lipofection of Math5 and Mash1 promotes retinal bipolar cell fates

Because 16-cell injection of Math5 and Xath5 RNA had different effects, we used in vivo lipofection to further analyze their functional activities in Xenopus retinal progenitors. Stage 18 embryonic optic vesicles were injected with GFP DNA alone or in combination with Xath5, Math5 or Mash1 DNA, accompanied by a lipid transfection reagent (Holt et al., 1990). The transected GFP-positive cells were scored by morphology at stage 41 in retinal sections. In most cases, GFP fluorescence in transected cells allowed unambiguous identification of retinal cell type based upon laminar position and morphology (Holt et al., 1990; Kanekar et al., 1997).

In a previous study (Kanekar et al., 1997), in vivo lipofection of Xath5 and GFP DNA was shown to cause a dramatic bias towards the RGC fate (Fig. 6B,D), compared with control lipofection of GFP DNA alone (Fig. 6A,D). Increased representation of early-born RGCs caused by Xath5 overexpression was at the expense of later-born cell types such as amacrine, bipolar and Müller glial cells. In contrast, in vivo lipofection of Math5 and GFP DNA produced a dramatic
increase in bipolar cells, one of the last neuronal cell types to be born in the retina (Fig. 6C,D). However, both Xath5 and Math5 overexpression suppressed Müller glial cell differentiation (Fig. 6D). We also noted larger clusters of labeled cells in retinai transected with Math5 as compared with Xath5 or GFP alone (data not shown), suggesting that Math5 might delay the differentiation of retinal progenitor cells and allow them to continue to proliferate. This property would be consistent with the observed bias toward late-born retinal cell types. To test this, we labeled embryos with BrDU soon after lipofection then collected the embryos at stage 41 and scored the number of transfected cells that were BrDU positive. We found that a similar percentage of transfected cells were

**Fig. 5.** Overexpression of Math5 in Xenopus embryos by RNA injection. (A) Injection into one cell of a 2-cell Xenopus embryo with RNA for n\(\beta\)-gal alone (i) or in combination with Math5 (ii). Stage 14-15 embryos were stained to detect \(\beta\)-galactosidase activity (magenta) and probed by whole-mount in situ hybridization for N-tubulin expression (purple). Embryos are oriented in a dorsal view with anterior at the top and injected side on the right. (i) Control embryo expressing \(\beta\)-gal alone demonstrating the normal pattern of N-tubulin expression in the neural plate. (ii) Embryo expressing both Math5 and \(\beta\)-gal with ectopic N-tubulin on the injected side. (B) Injection into blastomere D.1.1 of a 16-cell Xenopus embryo with RNA for GFP alone (i-iii) or with RNA for Math5 plus GFP (iv-vi). The embryos were fixed and cryostat sectioned at stage 41. Sections were immunostained for NCAM and labeled with Hoechst to visualize the retinal cell layers. (i-iii) Retinal section from a control embryo injected with GFP RNA alone. Hoechst staining reveals normal lamination (i). Within the retina, a large cluster of cells derived from the injected blastomere is labeled by GFP (ii) and robust NCAM staining is observed (iii). (iv-vi) A retinal section from an embryo injected with GFP and Math5 RNAs. Hoechst staining highlights a disruption in the normal arrangement of retinal cell layers (iv). This disrupted region corresponds to a cluster of GFP-labeled cells within the retina (v). The GFP-positive cells, including those extruding from the back of the retina, are NCAM-positive (vi). Scale bar 1 \(\mu\)m.

**Fig. 6.** In vivo lipofection of Math5 promotes retinal bipolar cell fates. Embryos were transfected at stage 18 in the region of the optic vesicle with DNA for GFP alone (A) or GFP in combination with Xath5 (B), Math5 (C) or Mash1 (not shown). At stage 41 these embryos were fixed, cryosectioned and stained with Hoechst dye to visualize the retinal cell layers. GFP-labeled cells were scored in retinal sections by laminar position and morphology. In section of embryos transfected with Xath5, there are more labeled cells in the RGC layer (B), while sections of embryos transfected with Math5 show both a larger number of GFP-labeled cells overall and more labeled bipolar cells (C). Bipolar cells were scored as cells with distinct cell bodies in the INL and with thin processes extending radially but not extending into the ONL or GCL. These can be distinguished from Müller glial cells, which are radially oriented but have a more complex morphology (see Dorsky et al., 1995). (D) Percentage of retinal cell types labeled following transfection with DNA for GFP (green, \(n=432\) cells from 5 eyes), GFP plus Xath5 (red, \(n=439\) cells from 5 eyes), Math5 (navy, \(n=866\) cells from 7 eyes) or Mash1 (light blue, \(n=440\) cells from 6 eyes). Xath5 data are derived from Kanekar et al. (1997) and are included here for comparison. Expression of Xath5 biases progenitors toward RGC fate, while both Math5 and Mash1 promote the formation of bipolar cells. The per cent representation of each cell type was calculated as a weighted average. Error bars indicate SEM; asterisk, \(P<0.01\) by Student’s t-test. Cell types on the graph are listed from left to right in rough order of birth. Abbreviations: G, retinal ganglion cell; B, bipolar cell; P, photoreceptor; A, amacrine cell; H, horizontal cell. Scale bar 0.5 \(\mu\)m.

BrDU-positive for both Math5 (49\%, \(n=83\) cells) and control embryos (47\%, \(n=79\) cells) arguing against a specific effect on proliferation of retinal precursor cells. As a control, the function of a second mammalian retinal bHLH gene, Mash1 was tested in this assay. In vivo lipofection of Mash1 DNA caused a significant bias toward bipolar cell fate although less dramatically than Math5 overexpression (Fig. 6D). This result is consistent with an observed delay in mammalian bipolar cell development in the absence of Mash1 (Tomita et al., 1996a).
DISCUSSION

We have characterized a new mouse bHLH gene, Math5, that is closely related to Xenopus Xath5 and Drosophila ato. Math5 is expressed in the developing optic cup, from E11 through birth and in the tenth cranial ganglion. In the retina, Math5 expression precedes the differentiation of all neurons and is closely associated with RGC birthdates. When ectopically expressed in Xenopus embryos, Math5 exhibits proneural activity and can alter retinal cell fates. We have also demonstrated that, in the developing mouse optic cup, Math5 and Hes1 are reciprocally regulated by Pax6.

Math5 and Hes1 are targets of Pax6 regulation

Pax6 has been proposed to act at or near the top of a regulatory hierarchy of transcription factors controlling invertebrate and vertebrate eye formation (Desplan, 1997; Halder et al., 1995). However, very few Pax6 target genes have been identified during vertebrate eye development (Cvekl and Piatigorsky, 1996; Xu et al., 1997). We have identified two murine bHLH transcription factors, Math5 and Hes1, whose expression relies upon Pax6. Interestingly, both genes require Pax6 in a dose-dependent manner, but we do not yet know whether Math5 and/or Hes1 are direct transcriptional targets. Their strict dependence upon Pax6 gene dosage suggests that this may be the case and provides a molecular entry point for understanding the haploinsufficiency of Pax6 ocular phenotypes.

Remarkably, Pax6 deficiency in mice affects Math5 and Hes1 opposite directions. Math5 expression is downregulated and Hes1 expression is upregulated in the absence of Pax6 (Fig. 3). There are two possible mechanisms for such regulation, which are not mutually exclusive. Pax6 may independently regulate Hes1 and Math5, or it may do so in a linear, sequential manner. In the latter case, Pax6 represses Hes1, which represses Math5. We favor the second possibility because changes in Pax6 gene dosage affect Math5 and Hes1 expression differently (Fig. 3). Decreases in Pax6 expression increase Hes1 mRNA levels (Fig. 3G-L) but lower the number of Math5-expressing cells (Fig. 3A-F). Pax6 may directly regulate Hes1 transcription. In turn, Hes1 would regulate Math5 expression thereby influencing the timing or extent of retinal neurogenesis. This pathway can be further tested by examining Math5 expression in Hes1 mutant embryos and analyzing Math5 and Hes1 genomic DNA for the presence of eye enhancers with Hes1- or Pax6-binding sites.

Our proposed pathway is consistent with the proneural activity of Math5 (Fig. 5B), the relative timing of Hes1 and Math5 expression in the optic cup and observations involving related genes. Hes1 represses Mash1 in the developing mouse eye and nervous system (Ishibashi et al., 1995), and h represses AC-S proneural genes in the Drosophila nervous system (Ohsako et al., 1994; Van Doren et al., 1994). In addition, h and emc are required for proper ato expression in the developing fly eye (Brown et al., 1995). Interestingly, the expression of h and ato appear to overlap with Pax6 (eyeless) expression (Brown et al., 1995; Halder et al., 1998). Whether ato and h are eyeless target genes and can be integrated into the network of early eye genes remains to be determined. Finally, a similar pathway has emerged for sensory nerve cell determination in Caenorhabditis elegans, involving vab-3 (Pax6), lin-22 (h) and lin-32 (ato) (Wrischnik and Kenyon, 1997; Chisolm and Horvitz, 1995; Zhang and Emmons, 1995; Zhao and Emmons, 1995), suggesting that this regulatory hierarchy may be very widely conserved.

The evolutionary relationship of Math, Xath5 and ato

We have used three criteria, gene structure, expression and function, to test the orthology of Math5, Xath5 and ato. These three proteins share 100% amino acid identity of their basic DNA-binding domains. Domain swapping experiments between Drosophila ato and scute have demonstrated that basic domain amino acid composition confers neuron-type specificity (Chien et al., 1996). Therefore, complete conservation of Math5, Xath5 and ato basic domains implies that they function analogously. In the Drosophila compound eye, ato is expressed prior to R8 cell differentiation (Jarman et al., 1994). Similarly, Math5 and Xath5 are expressed prior to RGC formation. In each case, expression precedes differentiation of the first neuronal cell type, and it is tempting to speculate that these genes function orthologously in eye formation. However, it is important to be cautious (Abouheif, 1997) since, outside the eye, the expression patterns diverge.

Another test of orthology is gene function. In the fly eye, ectopic ato expression causes an increase in neuronal differentiation, with an excess of R8 photoreceptors (Dokucu et al., 1996). Ectopic expression of Math5, Xath5 and ato in Xenopus embryos promotes neurogenesis (Fig. 5A; Kanekar et al., 1997; S. K. and M. L. V., unpublished observations). Lipofection of ato and Xath5 into the Xenopus eye causes a significant bias towards RGC fate (Kanekar et al., 1997 and S. K. and M. L. V., unpublished observations). However, overexpression of Math5 by lipofection in the frog retina results in a bias towards bipolar cell formation. Therefore, more comparisons of gene function are required to resolve the orthology issue. We are currently removing Math5 function in mice by gene knockout to assess whether RGCs or other retinal neurons are affected. It will also be important to test whether Math5 and/or Xath5 rescue the ato mutant phenotype, and to compare Math5, Xath5 and ato overexpression in mammalian tissues or cells.

The role of Math5 in retinal development

Math5 expression coincides with critical cellular events in the mammalian optic cup. The spread of Math5 expression from the dorsal cup circumferentially toward ventral matches the progression of RGC layer formation between E12.5 and E13.5 (Hinds and Hinds, 1974; Young, 1983, 1985). However, the columns of Math5-expressing cells (Fig. 2F-H) do not correlate with previously described retinal structures. These cells may be clonally related (Turner et al., 1987) and/or represent ‘proneural’ clusters, analogous to groups of retinal precursors that express ato in the morphogenetic furrow (Jarman et al., 1993). Other vertebrate genes with a similar expression pattern are Notch and Delta (Dorsky et al., 1995, 1997; Henrique et al., 1997). It will be interesting to compare Math5, Notch and Delta expression domains to test whether they are complementary and to test for Notch or Delta regulation of Math5. Lastly, Math5 expression is not observed in differentiated retinal neurons but instead, Math5 mRNA is restricted to dividing progenitors (Figs 5, 6), suggests that Math5 functions as a determination
factor, possibly to specify a particular retinal neuron class like RGCs, rather than during terminal differentiation. Paradigmatically, Math5 expression is excluded from the presumptive ciliary body, known to be highly proliferative (Young, 1985). However, cell division within the ciliary proliferative and the retinal ventricular zones may not be equivalent. The proliferating, undifferentiated margins of the vertebrate eye (ciliary marginal zone or presumptive ciliary body) are not unlike the region ahead of and within the morphogenetic furrow of a Drosophila eye imaginal disc. The presence of single Math5-positive cells adjacent to this region (Fig. 2E) and the correlation of several classes of transcription factors in the frog ciliary margin (Perron et al., 1998) circumstantially support this idea.

Given the sequence conservation, evolutionary relationship and similarity of retinal expression patterns for Math5 and Xath5, our finding that ectopic Math5 expression does not promote RGC fate in the frog retina was unexpected. There are several possible explanations for this result. First, Math5 may play a role in regulating bipolar cell differentiation, although the timing of Math5 expression relative to bipolar cell genesis argues against this. Second, Math5 and Xath5 may function analogously, but Math5 protein is incompletely active or relatively less stable in Xenopus retinal progenitor cells. For example, differences in stability, nuclear localization and functional difference between Xath5 to promote RGC formation. In spite of this apparent may antagonize the action of endogenous Math5 as an intrinsic factor guiding the commitment of mammalian retinal progenitors and raise the possibility that mechanistic aspects of retinogenesis, like eye pattern formation in general may be evolutionarily conserved.

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Note added in proof
A new member of the atonal bHLH family highly related to Math5 and Xath5 has recently been identified in chicken and deposited in GenBank (accession number 2760443).

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