bHLH-dependent and -independent modes of *Ath5* gene regulation during retinal development

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Summary

In a wide range of vertebrate species, the bHLH transcription factor *Ath5* is tightly associated with both the initiation of neurogenesis in the retina and the genesis of retinal ganglion cells. Here, we describe at least two modes of regulating the expression of *Ath5* during retinal development. We have found that a proximal cis-regulatory region of the *Xenopus Ath5* gene (*Xath5*) is highly conserved across vertebrate species and is sufficient to drive retinal-specific reporter gene expression in transgenic *Xenopus* embryos. *Xath5* proximal transgene expression depended upon two highly conserved bHLH factor binding sites (E-boxes) as well as bHLH factor activity in vivo. However, we found that bHLH activity was not required for expression of a longer *Xath5* transgene, suggesting that additional mechanisms contribute to *Xath5* expression in vivo. Consistent with this, we showed that a more distal fragment that does not include the conserved proximal region is sufficient to promote transgene expression in the developing retina. In mouse, we found that a longer fragment of the cis-regulatory region of either the mouse or *Xenopus Ath5* gene was necessary for transgene expression, and that expression of a mouse *Math5* (*Atoh7*) transgene was not dependent upon autoregulation. Thus, despite extensive conservation in the proximal region, the importance of these elements may be species dependent.

Key words: *Ath5*, Math5 (Atoh7), Retina, Development, Regulation, Transgenic, *Xenopus*

Introduction

Transcription factors of the basic helix-loop-helix family (bHLH) are involved in the specification of a wide range of cell fates throughout the nervous system. Traditionally known for their roles in determining neural fate within the nervous system, individual bHLH factors are required for the differentiation of specific subsets of cells (Guillemot, 1999). The exact function of each bHLH factor and the type of cell that differentiates in response to their activity depends in part upon the timing and location of activity (Moore et al., 2002). Thus, proper temporal and spatial regulation of bHLH factor expression is essential for normal function.

Ath5, a vertebrate homolog of Drosophila *atonal*, is expressed in the developing retina in all vertebrates studied and plays a crucial role in regulating retinal neurogenesis (Vetter and Brown, 2001). In *Xenopus*, *Xath5* is expressed in a tightly restricted set of cells in the developing neural retina, the olfactory placodes and the pineal gland. In the retina, *Xath5* expression commences in retinal progenitors just prior to cell cycle exit and onset of differentiation, but expression is downregulated before cells become fully mature retinal neurons (Kanekar et al., 1997). In mouse, zebrafish and chick, *Ath5* is also expressed in a similar restricted manner within the retina immediately preceding the onset of retinal ganglion cell (RGC) differentiation (Brown et al., 1998; Kay et al., 2001; Matter-Sadzinski et al., 2001). *Ath5* is specifically required for the differentiation of the RGC cell type as in both mouse and zebrafish *Ath5* loss-of-function mutants, RGCs are either drastically reduced or missing altogether (Brown et al., 2001; Kay et al., 2001; Wang et al., 2001).

As *Ath5* plays such a key role in retinal development, it is important to understand how its expression is regulated. Analysis of the chicken *Ath5* (*Cath5*) promoter has suggested that it can be regulated by multiple bHLH factors in retinal cell culture, and that both Cath5 and Ngn2 are bound to the *Cath5* promoter at specific stages of retinal development (Matter-Sadzinski et al., 2001; Skowronska-Krawczyk et al., 2004). However, it is not yet known what is required for correct tissue-specific expression in vivo. Recent genome sequencing efforts have led to the development of phylogenetic footprinting strategies, whereby cross-species sequence comparison of noncoding regions from homologous genes can identify candidate enhancers that may play a role in regulating gene expression. This approach provides excellent predictive power for functional enhancer elements (Bulyk, 2003); however, all candidate elements must ultimately be tested in vivo to determine whether they can regulate gene expression and assess how they contribute to the normal pattern of expression during development (Muller et al., 2002).

In this study, we have analyzed the regulation of *Ath5* expression in *Xenopus*, as well as in mouse. First, we identified a highly conserved proximal non-coding region, and showed

that it mediates bHLH-dependent regulation of *Ath5* expression. This region was sufficient to promote transgene expression in *Xenopus*, but not in mouse. Second, we show that a longer transgene, that includes additional 5′ cis-regulatory sequence, promotes bHLH-independent transgene expression in *Xenopus*. Longer transgenes from either the mouse or *Xenopus Ath5* cis-regulatory region were also sufficient to promote expression in mouse. For the *Math5* transgene (*Atoh7* – Mouse Genome Informatics), expression did not depend upon Math5 itself. Thus, there exist both bHLH-dependent and-independent modes of *Ath5* gene regulation; however, the importance of bHLH-dependent expression mediated through the conserved proximal region may be species dependent.

Materials and methods

Molecular cloning and mutagenesis

A 14.3 kb *Xath5a* genomic clone was isolated from a *Xenopus laevis* genomic library in LambdaGEM-11 (a gift from Dr Michael King). The 3.3 kb of 5′ *Xath5* genomic sequence immediately 5′ of the coding region was cloned into the promoterless GFP reporter construct pG1 (a gift from C.-B. Chien) to create the pG1X5-3.3 kb construct. Three transgenic constructs, pG1X5Δ*Pst*I, pG1X5Δ*Hin*dIII, and pG1X5Δ*Dra*II, were made by digesting the pG1X5-3.3 kb construct with the indicated enzyme and religating. All other deletion constructs were made using PCR amplification from existing plasmids and the PCR fragments were then cloned into the pG1X5-TATAA construct. The minimal mouse *Fos* promoter was derived from the TOP:dGFP construct (a gift from R. Dorsky) (Dorsky et al., 2002) and cloned into pG1. Constructs with point mutations were made using the QuikChange II kit (Stratagene). All E-box sequences were mutated from CAnnTG to ATTnTG, which has been shown to destroy the ability of bHLH factors to bind to regulatory E-box sequences (Helms et al., 2000). The mutation introduced into the α-box site was based on a previous study (Culi and Modolell, 1998). The primer sequences used to generate constructs created with PCR amplification are available upon request. All constructs were verified by sequencing.

Transgenic procedure

The generation of transgenics was carried out as described (Kroll and Amaya, 1996), with variations (Hutcheson and Vetter, 2002). Whole-mount embryos were analyzed for GFP expression between stages 28 and 42, when the majority of retinal neurons are born. Embryos were scored as retinal positive if there was specific retinal expression that was similar in pattern and timing to endogenous *Xath5* expression. For constructs with weak or no detectable GFP expression by fluorescence, expression or lack of expression was confirmed either by in situ hybridization or by whole-mount antibody staining using a polyclonal anti-GFP antibody (Torrey Pines) and an Alexa-Fluor 488-conjugated secondary antibody (Molecular Probes). For analysis on sections, embryos were sectioned on a cryostat at a thickness of 14 μm.

In situ hybridization

Embryos were processed for in situ hybridization as described previously (Kanekar et al., 1997). Double in situ hybridization on sections was performed using a digoxigenin-labeled GFP probe and a fluorescein-labeled *Xath5* probe as previously described (Hutcheson and Vetter, 2001).

Sequence analysis

Sequence analysis was performed using VISTA (http://www-gsd.lbl.gov/vista/) (Frazer et al., 2004; Mayor et al., 2000). Alignment criteria used were 80% nucleotide identity over 30 bp blocks. Candidate transcription factor binding sites were identified using the Genomatix MatInspector module (http://www.genomatix.de/) (Quandt et al., 1995), the Transfac database (www.gene-regulation.com) (Wingender et al., 1996) or ClusterBuster (http://zlab.bu.edu/cluster-buster/) (Frith et al., 2003).

Other vertebrate *Ath5* genomic sequences were identified as follows: *X. tropicalis* (http://genome.jgi-psf.org/xenopus0/xenopus0.home.html); mouse and human *Ath5* (*Atoh7*) have been previously isolated and aligned (Brown et al., 2002); chick (GenBank Number AJ630209) (Skowronska-Krawczyk et al., 2004); Fugu (scaffold 1775; http://genome.jgi-sf.org/fugu3/fugu3.home.html); and zebrafish (clone AL627094; http://trace.ensembl.org/perl/ssahaview). The genomic sequence for Drosophila *atonal* was derived from BAC clone #AC008094 (http://flybase.bio.indiana.edu/).

Generation of transgenic mice

0.6 kb and 2.3 kb sequences found immediately upstream from the *Math5* start ATG codon (nucleotides 2472 to 3072 and 772 to 3072 from AF418923) were cloned into the pG1 GFP reporter construct. These *Math5* (M5) enhancer-promoters and the GFP-coding sequence were purified away from vector sequences and used to generate independent mouse lines transgenic with either the M5-0.6 kb or M5-2.3 kb transgene. Similarly, the *Xenopus* pG1X5-proximal or pG1X5-3.3 kb constructs were used to generate transgenic mice. All transgenic strains are viable and fertile and maintained on a CD1 background. For some experiments the M5-2.3 transgenic line was crossed with the *Math5* mutant (Brown et al., 2001) maintained on CD-1. E12.5 or E13.5 mouse embryos from timed pregnancies were harvested, kept on ice in PBS for whole mount imaging. The genotype of M5-2.3*Tg/+* or M5-2.3*Tg/+;Math5*$^{-/-}$ embryos was determined by PCR genotyping of embryonic or adult tail DNA using primers specific for *GFP* coding sequences or as described previously (Brown et al., 2001).

Results

A 3.3 kb 5′ non-coding *Xath5* fragment can promote transgene expression in a pattern that mirrors endogenous *Xath5* expression

In order to investigate the mechanisms that regulate the expression of *Xath5*, we first sought to identify genomic sequences sufficient to drive tissue-specific expression of a transgene in vivo. Roughly 15 kb of genomic *Xath5a* sequence was isolated from a *X. laevis* genomic library. Like most genes encoding proneural bHLH factors, *Xath5* has one exon containing the coding region and no introns. In addition, the genomic clone contained 3.3 kb of 5′ and 11.8 kb of 3′ sequence flanking the *Xath5*-coding region (data not shown). Based upon analysis of several full-length *Xath5* cDNA clones (Kanekar et al., 1997), the putative transcription start site was determined to lie 205 bp 5′ of the translation start site. The highly conserved TATAA basal promoter element is 226 bp 5′ from the translation start site (Fig. 1A).

To test for regulatory activity, we fused 3.3 kb of *Xath5* 5′ non-coding sequence to a promoterless vector containing a GFP reporter gene (pG1; C.-B. Chien and D. Gilmour, unpublished) to create the pG1X5-3.3 kb construct (Fig. 1A), which was used to generated transgenic frog embryos (Kroll and Amaya, 1996). At stage 17, when *Xath5* expression first commences in the developing olfactory placodes, two faint spots of GFP were visible in the anterior end of transgenic embryos (data not shown). Like endogenous *Xath5* (Fig. 1B) (Kanekar et al., 1997), pG1X5-3.3 kb transgene expression was apparent in the neural retina, the olfactory placodes and the pineal gland at stage 28 (Fig. 1C,D).

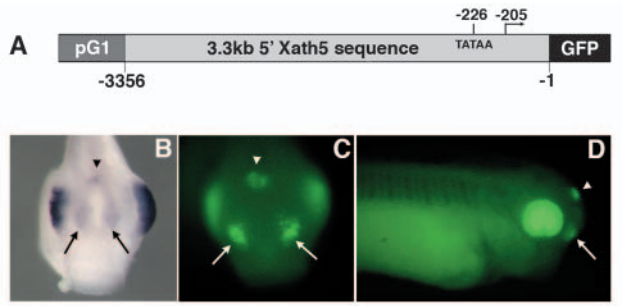


Fig. 1. Identification of a *Xath5* regulatory fragment that functions in vivo. (A) The pG1X5 construct contains 3.3 kb of 5′ *Xath5a* genomic DNA cloned into the promoterless vector pG1 with a GFP reporter. The TATAA element is at –226 bp, the putative transcription start site is at –205 bp and the translation start site is at +1 bp. (B) In situ hybridization showing that endogenous *Xath5* mRNA is expressed in the retina, the pineal gland (arrowhead), and the olfactory placodes (arrows) of a stage 28 embryo (frontal view). (C,D) Frontal and lateral views of a stage 28 transgenic pG1X5 embryo showing expression of the transgene in the same tissues as the endogenous *Xath5* mRNA (compare with B).

Isolation of a 201 bp enhancer region

To define which regions within the 3.3 kb *Xath5* cis-regulatory region are crucial for retinal expression, we generated a series of deletion constructs (pG1X5-Δ*Hin*dIII, pG1X5-Δ*Dra*II, and pG1X5-Δ*Pst*I) by restriction digest (Fig. 2A). When each construct was tested in transgenic *Xenopus* embryos, expression of the GFP transgene was comparable with that seen with the full pG1X5-3.3 kb construct (Fig. 2A). Thus, the 464 bp of sequence closest to the *Xath5*-coding region are sufficient to promote transgene expression in the retina. To verify this, the pG1X5-proximal construct containing only 427 bp of upstream sequence was used to generate transgenic embryos (Fig. 2B). These embryos consistently expressed GFP in the retina (Fig. 2C). Expression in the olfactory placodes and pineal was also observed, but less frequently (data not shown).

In the *Xath5* genomic sequence, the TATAA box, a highly conserved basal promoter element (Smale and Kadonaga, 2003), is located 226 bp upstream of the translation start site. The TATAA region alone (pG1X5-TATAA) was not sufficient to drive retinal GFP expression (Fig. 2B), suggesting that the critical elements in the *Xath5* proximal regulatory region lie between positions –427 and –226. To test this idea, the sequence from –427 to –226 was fused to a minimal mouse *Fos* promoter cloned into the pG1 vector. The *Fos* basal promoter alone did not drive retinal expression (Fig. 2B). When 201 bp of *Xath5* sequence (–427 to –226) was fused to the *Fos* basal promoter (Fig. 2B), weak but specific transgene expression in the retina was detectable (Fig. 2D). Together, these results indicate that elements sufficient for retinal expression are located between positions –427 and –226 relative to the *Xath5* translation start site.

To assess whether the transgene is expressed in the appropriate cells within the retina, we performed double in situ hybridization on sections from stage 42 pG1X5-proximal transgenic embryos, and found that the expression of *GFP*

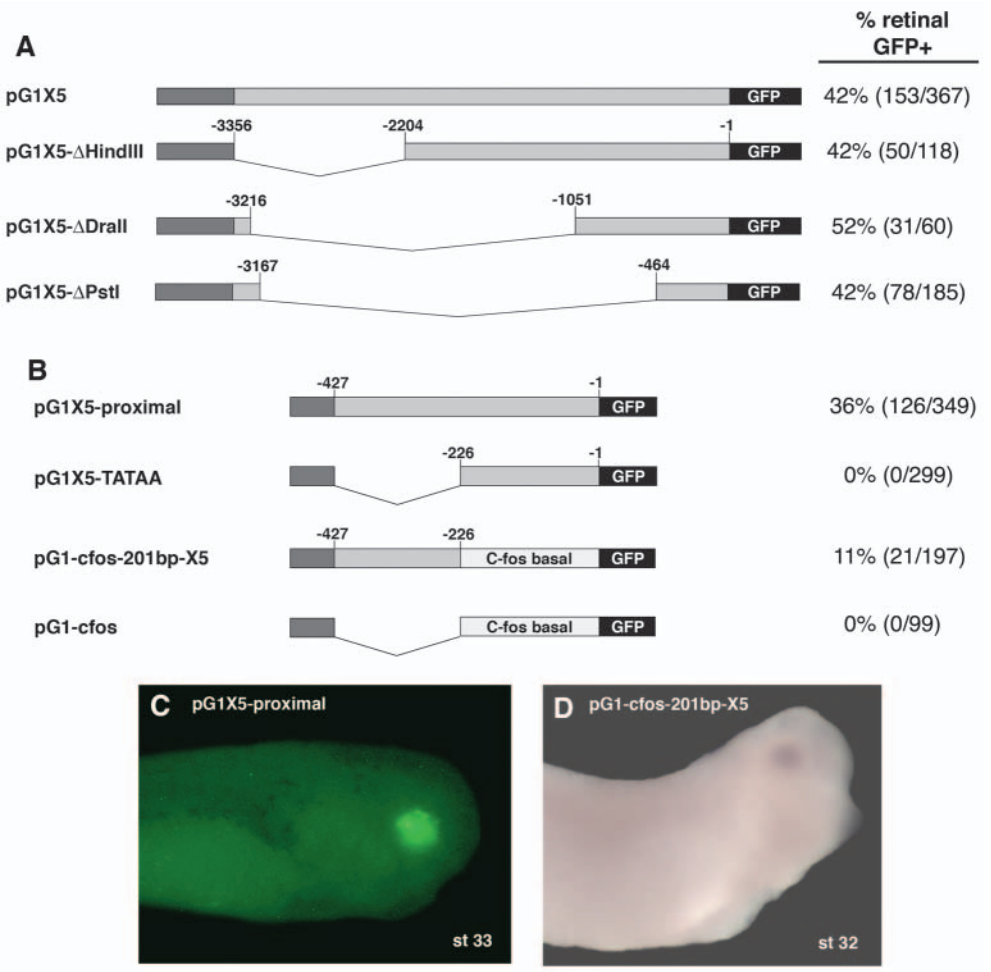


Fig. 2. Deletion analysis isolates a proximal *Xath5* cis-regulatory region. (A) *Hin*dIII, *Dra*II and *Pst*I sites were used to delete regions of *Xath5* genomic sequence in the pG1X5 construct. All three constructs drove strong transgene expression in the retina. (B) The pG1X5-proximal construct containing sequences from –427 to –1 drove robust retinal expression of the transgene. The pG1X5-TATAA construct containing sequences from –226 to –1 showed no expression. *Xath5* sequence from –427 to –226, when fused to the *Fos* heterologous basal promoter (pG1-cfos-201bp-X5), drove retinal expression, but more weakly. The basal *Fos* promoter alone (pG1-cfos) did not confer retinal GFP expression. (C) A pG1X5-proximal transgenic embryo (stage 33) shows retinal GFP expression. (D) In situ hybridization of pG1-cfos-201bp-X5 (stage 32) transgenic embryo shows weak GFP expression in the retina.

RNA (Fig. 3A,E) was restricted to the ciliary marginal zone (CMZ) in a pattern identical to endogenous *Xath5* mRNA (Fig. 3C,G) (Kanekar et al., 1997). Interestingly, at stage 42, GFP fluorescence was visible throughout the central retina in all three neuronal layers and in most neuronal cell types, probably owing to perdurance of the GFP protein (Fig. 3D). This is consistent with recent lineage analysis of *Math5*-expressing cells in the mouse retina showing that they give rise to all major classes of retinal cell types that occupy the three cell layers (Yang et al., 2003) (J. Brzezinski and T. Glaser, unpublished).

Identification of a conserved proximal regulatory region in the *Ath5* gene

All known vertebrate *Ath5* genes are expressed in the developing retina coincident with the onset of retinal ganglion cell genesis, suggesting that mechanisms regulating *Ath5* expression may be conserved as well. We therefore compared *Xath5* noncoding sequence to other *Ath5* genomic sequences to identify conserved regulatory regions. Pairwise mVISTA analysis (Frazer et al., 2004; Mayor et al., 2000) between *Xenopus laevis* and mouse identified a peak of homology in the 5′ noncoding region (Fig. 4A) within the 427 bp region that was sufficient for tissue-specific expression in *Xenopus* (Fig. 2). Alignment of this region across *X. laevis*, *X. tropicalis*, mouse, human and chick showed a 65 bp stretch with significant nucleotide identity among all of these vertebrate species. Within this sequence, two E-boxes, which are binding sites for bHLH factors (Fig. 4B, positions –270 and –260, referred to as E2 and E1), and the flanking bases were almost entirely conserved (18/21 bp identical, Fig. 4B). Additionally, both zebrafish and fugu have the conserved E1E2 cassette, although there is little conservation elsewhere in the 65 bp region (Fig. 4B).

E-boxes at –270 and –260 are required for *Xath5* proximal promoter activity

We sought to define a smaller region that still promotes transgene expression in the retina by generating sequential deletions of the pG1X5-proximal construct (Fig. 5A). pG1X5-proximal-401 showed retinal expression, as did pG1X5-proximal-389, but more weakly so. This suggests that elements necessary for robust expression, but not tissue specificity, lie between –401 and –389. Transgenic constructs containing further deletions of the proximal region up to –337 similarly promoted weak retinal expression of GFP, but the pG1X5-proximal-250 construct, in which the conserved E-boxes E1 and E2 are missing, did not (Fig. 5A).

To determine whether E1 and E2 are required elements in the proximal cis-regulatory region, we mutated each E-box individually (ΔE1 or ΔE2) or in combination (ΔE1ΔE2) in the pG1X5-proximal transgene (Fig. 5B). Single E-box mutations reduced but did not eliminate retinal expression of GFP (Fig. 5B). When both E-boxes were mutated, GFP was not expressed (Fig. 5B), indicating that these sites are critical for the activity of the proximal *Xath5* cis-regulatory region.

To further test whether expression of the pG1X5-proximal transgene depends upon bHLH activity, we generated transgenic embryos then injected RNA for *RFP* (red fluorescent protein; 1 ng) and a dominant-negative form of Xath5 (500pg) into a dorsal animal blastomere at the eight-cell stage. Dominant-negative Xath5 was created by replacing the putative Xath5 activation domain with the repressor domain from Drosophila Engrailed to create Xath5-EnR. This dominant-negative protein interferes with the proneural activity of multiple atonal-related bHLH factors, including Xath5, Xath3, NeuroD and XNgnR1 (T. VanRaay, M. Logan and M.L.V., unpublished). Expression of Xath5-EnR was able to suppress pG1X5-proximal transgene expression on the injected side (6/13; Fig. 5C-E), arguing that proneural bHLH activity is required for expression driven by the proximal *Xath5* cis-regulatory region.

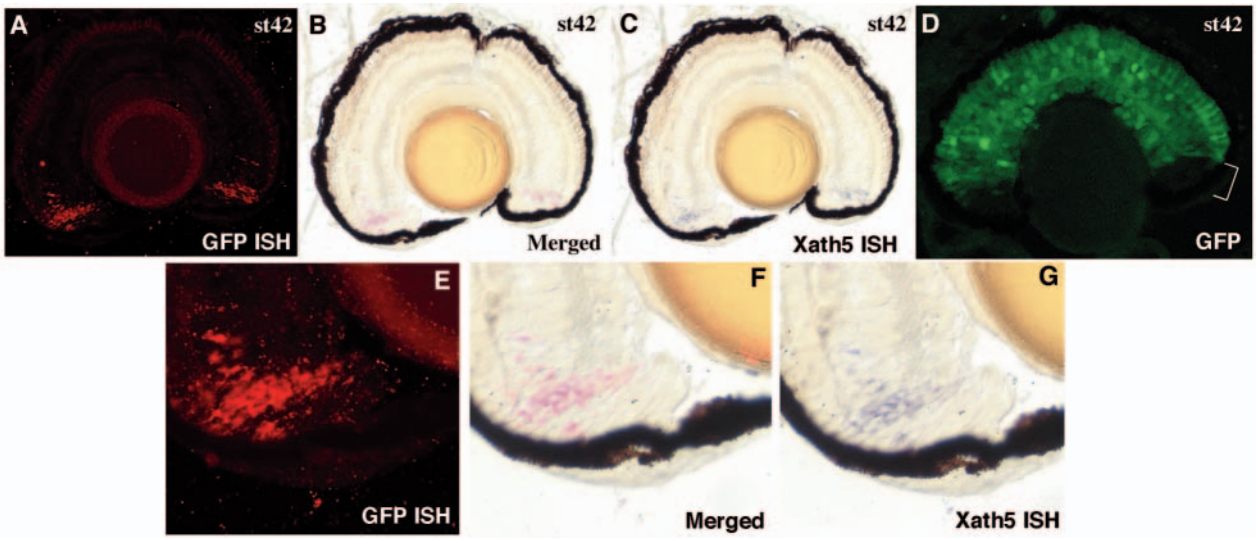


Fig. 3. The proximal regulatory region mimics endogenous *Xath5* retinal expression in the ciliary marginal zone. (A-C) Double in situ hybridization on retinal sections from stage 42 embryos comparing expression of the *GFP* transgene mRNA (red; A,B) as driven by the pG1X5-proximal construct to endogenous *Xath5* mRNA (purple; B,C). (D) Unlike the RNA, GFP protein is found throughout the central retina and in many cell types at stage 42. Bracket marks the CMZ, which lacks GFP fluorescence. (E-G) Higher magnification images of the ciliary marginal zone (CMZ) from A-C showing cell-by-cell correlation of *GFP* (red) and *Xath5* (purple) mRNAs.

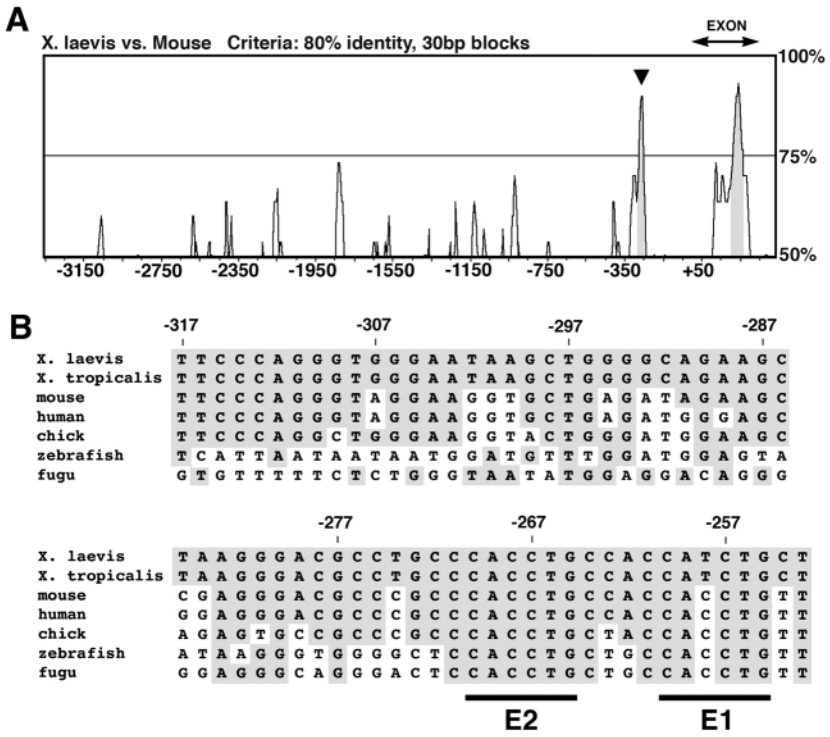


Fig. 4. Interspecies *Ath5* sequence analysis identifies a highly conserved proximal non-coding region. (A) Pairwise VISTA analysis of *X. laevis* and mouse *Ath5* 5′ noncoding sequences identifies a highly conserved region (black arrowhead) within the proximal region of *Xath5* 5′ sequence, as well as a weaker region of conservation more distally (–1597 to –1669). Basepair position relative to the *Xath5* translation start site (+1 bp) indicated at bottom of the VISTA alignment. Approximate location of *Ath5* exon indicated by double arrowed line. (B) Alignment of the conserved region from *X. laevis*, *X. tropicalis*, mouse, human, chick, zebrafish and fugu shows blocks of highly conserved nucleotides (gray). Bars mark two conserved E-box binding sites (CANNTG) found in all species.

E-boxes, together with adjacent conserved sequences, are sufficient to promote transgene expression in the retina

Although E1 and E2 are necessary elements in the proximal promoter, we sought to define the minimal set of elements sufficient to drive retinal specific expression of the transgene. To test the E-boxes alone, two copies of E1 and E2 (–272 to –253) were fused to the pG1X5-TATAA construct (Fig. 5F). The resulting transgene pG1X5-TATAA+2xE1E2 never promoted detectable GFP expression. Thus, the highly conserved E-boxes alone are insufficient for transgene expression. We then created pG1X5-TATAA+33 bp (Fig. 5F), which includes one copy of E1 and E2 as well as an adjacent motif that resembles the α-box previously identified in the *Drosophila* scute sensory mother cell (SMC) enhancer (Culi and Modolell, 1998). In *Xenopus* embryos transgenic for pG1X5-TATAA+33 bp, GFP expression was not specific and was found in a wide range of neural and muscle tissues (Fig. 5G).

Next we created pG1X5-TATAA+48 bp, which includes an additional 15 bp of conserved upstream sequence (Fig. 5F). Embryos transgenic for pG1X5-TATAA+48 bp showed GFP expression that was strikingly similar to endogenous *Xath5* expression, and also included consistent weak expression in axial muscles (Fig. 5F,H), with a small percentage of embryos showing only axial muscle expression. We conclude that addition of this 15 bp region is sufficient to confer almost complete tissue specificity to transgene expression. Interestingly, this region is highly conserved across species (11/15 nucleotides identical between *Xenopus laevis* and mouse), but contains no known transcription factor binding sites.

This 48 bp fragment contains two E boxes, the α-box and the additional 15 bp fragment required for tissue-specific expression. To assess whether the α-box contributes to the strength or specificity of expression, we mutated four residues within the α-box sequence (pG1X5-TATAA+48 bp-αmut). We found that this construct yielded a significantly reduced fraction of GFP positive transgenic embryos, but the overall pattern was unchanged (Fig. 5F). Thus, the α-box appears to contribute to the strength but not specificity of expression promoted by the short 48 bp fragment.

bHLH-independent regulation of 3.3 kb *Xath5* transgene expression

To test whether bHLH factors in *Xenopus* can regulate *Xath5* transgene expression in vivo, we overexpressed proneural bHLH factors in pG1X5-3.3 kb transgenic embryos by fertilizing eggs from an adult transgenic female and injecting RNA at the eight-cell stage encoding for RFP (1 ng) and for either Xath5, XngnR1, Xath3, XNeuroD, Xash1 or Xash3 (500 pg each). No change in transgene expression was observed with overexpression of RFP alone (21/21; Fig. 6A-C). We observed significant ectopic GFP expression with overexpression of the atonal-related bHLH factors Xath5 (30/30), XngnR1 (26/28), Xath3 (9/11) or NeuroD (12/12; Xath5 shown in Fig. 6D-F, and data not shown), but not with overexpression of either Xash1 (50/50) or Xash3 (14/15), which are Achaete-Scute-related factors (data not shown). Thus, the pG1X5-3.3 kb transgene, which includes the conserved proximal region, can be recognized and activated by Atonal-related bHLH factors.

Although either E1 or E2 are required for expression of the pG1X5-proximal transgene, we found that they are not required for expression of the full pG1X5-3.3 kb transgene (Fig. 6G), suggesting that additional more distal elements may also contribute to expression. We noticed another weaker region of homology between *Xenopus*, mouse, human and chick (–1597 to –1669, Fig. 4A) that included two well-conserved E-boxes at –1600 and –1629 (E3 and E4, respectively; data not shown). However, mutation of all four conserved E boxes (ΔE1-4) did not abolish pG1X5-3.3 kb transgene expression (Fig. 6G).

As there are additional non-conserved E-boxes, we wanted to further test whether bHLH activity is required for pG1X5-3.3 kb transgene expression using Xath5-EnR. We fertilized eggs from an adult *Xenopus laevis* female transgenic for the pG1X5-3.3 kb transgene, and injected RNA at the eight-cell

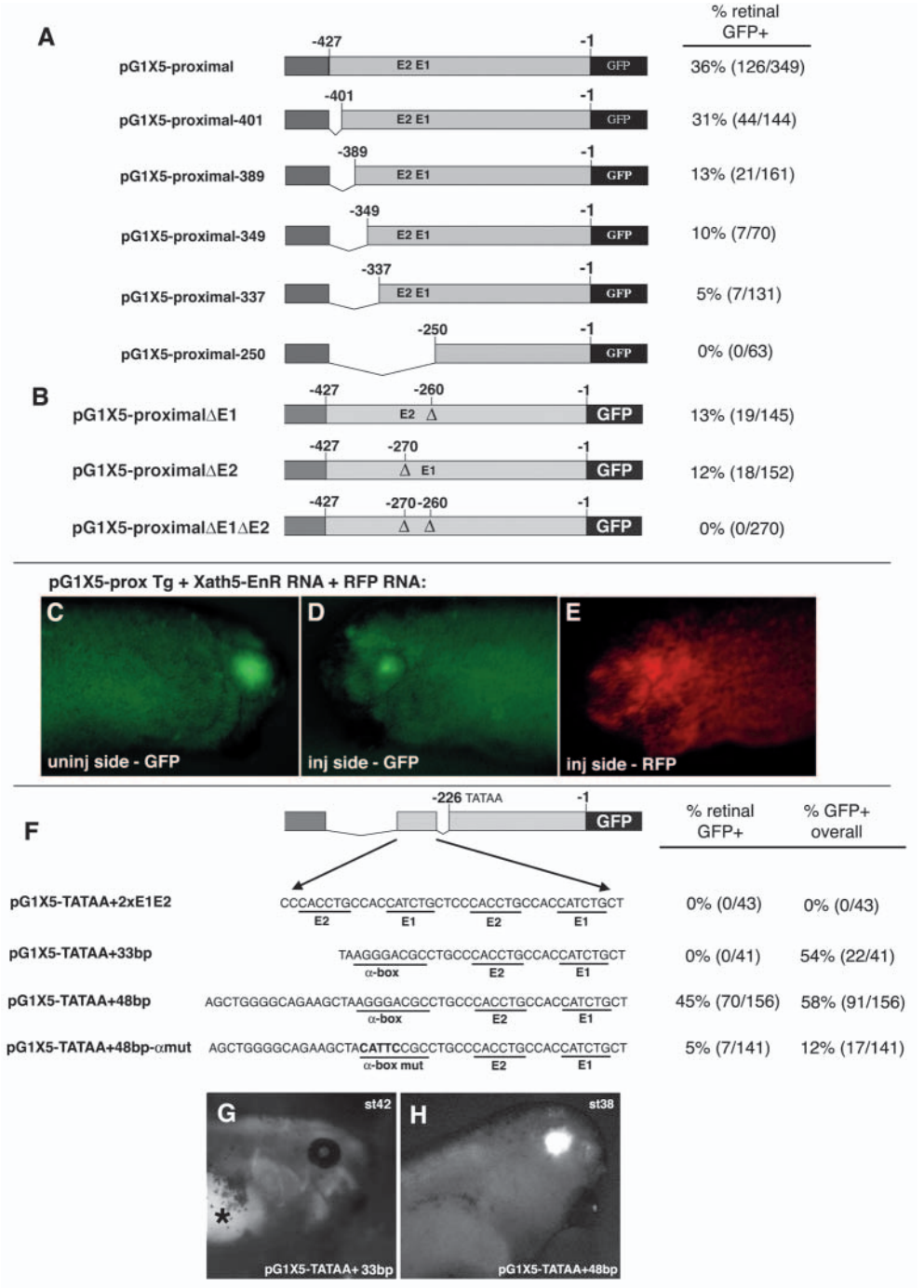


Fig. 5. Proximal transgene expression is dependent upon conserved E-boxes and bHLH activity, but E-boxes alone are not sufficient for retinal expression. (A) Sequential deletions of the *Xath5* proximal region: pG1X5-proximal-401 provided robust retinal GFP expression while pG1X5-proximal-389, -349 and -337 drove weaker retinal expression and at reduced frequency. (B) Mutation of either E1 or E2 (ΔE) reduced the percentage of embryos expressing GFP while mutation of both eliminated all transgene expression. (C-E) Injection of RNA for the dominant-negative Xath5-EnR suppressed expression of the pG1X5-proximal GFP transgene on the injected side (D) compared with the uninjected side (C). RNA encoding RFP (red) was co-injected to mark the injected side (E). (F) Multimerized E-boxes (pG1X5-TATAA+2xE1E2) were not sufficient to promote expression, while the E-boxes and adjacent α-box (pG1X5-TATAA+33 bp) promoted non-specific GFP expression throughout the CNS and head musculature (GFP+ overall), but did not promote specific retinal expression. The pG1X5-TATAA+48 bp transgenic construct promoted GFP expression in a *Xath5*-like pattern, but also in the axial somites, with a small percentage only showing expression in axial somites (contributing to the increase in overall GFP+ embryos). The robustness of transgene expression was reduced by mutation of the α-box with the pG1X5-TATAA+48 bp transgenic construct. (G) pG1X5-TATAA+33 bp is expressed non-specifically in CNS and muscle (H) pG1X5-TATAA+48 bp transgene is expressed in *Xath5*-like pattern and in axial somites. Asterisk indicates gut autofluorescence.

stage encoding for Xath5-EnR (500 pg) and for RFP (1 ng). Overexpression of Xath5-EnR did not suppress pG1X5-3.3 kb transgene expression (63/63; Fig. 6H-J), and also did not suppress expression of endogenous *Xath5* (15/16; data not shown). Together, these data argue for a bHLH-independent component to Xath5 gene regulation in vivo, probably mediated by more distal cis-regulatory sequences.

A distal regulatory region in the *Ath5* gene is sufficient for retinal expression

To test whether distal cis-regulatory sequences alone have enhancer activity, we fused a distal *Pst*I fragment that lacks the conserved proximal region (–3162 to –464) to pG1X5-TATAA. We observed specific retinal GFP expression in 39% of transgenic embryos (Fig. 7A,B), with much less frequent olfactory and/or pineal expression as well (data not shown). This distal region also promoted weak but specific retinal expression when coupled to the mouse Fos basal promoter (Fig. 7A). Thus, the distal *Xath5* cis-regulatory region contains elements sufficient for retinal expression that are distinct from the conserved proximal region.

Proximal *Ath5* regulatory sequences do not promote retinal transgene expression in mouse, but longer cis-regulatory fragments do

As sequence alignments revealed conservation in the *Ath5* cis-regulatory region between *Xenopus* and mammals, we tested 0.6 kb and 2.3 kb pieces of 5′ *Atoh7* genomic sequence in transgenic *Xenopus* embryos. Tailbud stage embryos were analyzed for GFP mRNA expression by in situ hybridization. The 0.6 kb *Atoh7* cis-regulatory region (pG1M5-0.6 kb) promoted retinal expression in *Xenopus*, although expression was much weaker and at a lower rate than for the pG1X5-proximal construct (8/144 embryos retinal GFP-positive; Fig. 8A). The weak activity of the pG1M5-0.6 transgene indicates that despite high sequence conservation, crucial elements may have diverged between *Xenopus* and mouse. By contrast, the pG1M5-2.3 kb construct promoted robust expression of GFP in the retina (19/56 embryos retinal GFP-positive; Fig. 8B), but also in other neural tissues such as the midbrain, hindbrain and cranial ganglia. Within the mature *Xenopus* retina, GFP mRNA was restricted to the CMZ, similar to that seen in pG1X5-proximal embryos (Fig. 8C, compare with Fig. 3).

Based on our results in *Xenopus*, we sought to analyze the activity of these regulatory regions in the developing mouse retina. We determined that the pG1M5-0.6 kb transgene exhibited no GFP fluorescence or mRNA in mice (0/3 independent lines, 0/31 embryos), which is consistent with the low frequency of transgenics and weak expression seen with this transgene in *Xenopus*. The 2.3 kb region of *Atoh7* regulatory DNA conferred strong optic cup and retinal GFP expression in E11-13 mouse embryos (four out of four independent lines, Fig. 8D,F) along with ectopic expression in brain and spinal cord, consistent with expression of this transgene in *Xenopus* (not shown). When the activity of the *Xenopus* cis-regulatory region was tested in mice, we found that the 427 bp pG1X5-proximal transgene exhibited no GFP fluorescence or mRNA expression (0/4 independent lines, 0/78 embryos), in contrast to the robust expression seen with this transgene in *Xenopus*. Interestingly the longer 3.3 kb pG1X5 construct showed specific optic cup and retinal expression in E11-13 mouse embryos (1/6 lines, 29/29 embryos; Fig. 8E). Thus, the short proximal region from either mouse or *Xenopus* is not sufficient for transgene expression in mouse, but longer transgenes from either species gave robust retinal expression.

In the developing chick retina, Cath5 is bound to its own promoter (Skowronska-Krawczyk et al., 2004), suggesting that autoregulation may play a role in *Ath5* gene regulation. In addition both Atonal and Math1 (Atoh1) regulate their own expression (Baker et al., 1996; Helms et al., 2000). Therefore, we tested for autoregulation of *Math5* by crossing two independent 2.3 kb M5-GFP transgenic lines to mice carrying the *Math5* targeted deletion (Brown et al., 2001). Mice heterozygous for both the transgene and mutant allele were intercrossed and the resulting embryos examined for GFP fluorescence in the optic cup at E13.5, when endogenous *Math5* (Brown et al., 1998) and the ~2.3 kb M5-*GFP* transgene are maximally expressed. This experiment was performed four independent times, using two litters for each transgene (n=56 embryos total). In every mutant embryo that possessed the transgene (n=12), robust GFP fluorescence was observed (compare Fig. 8F with 8G). Thus, Math5 is not required for transgene expression, suggesting that autoregulation is not an essential mechanism for regulating *Math5* expression during mouse retinal development.

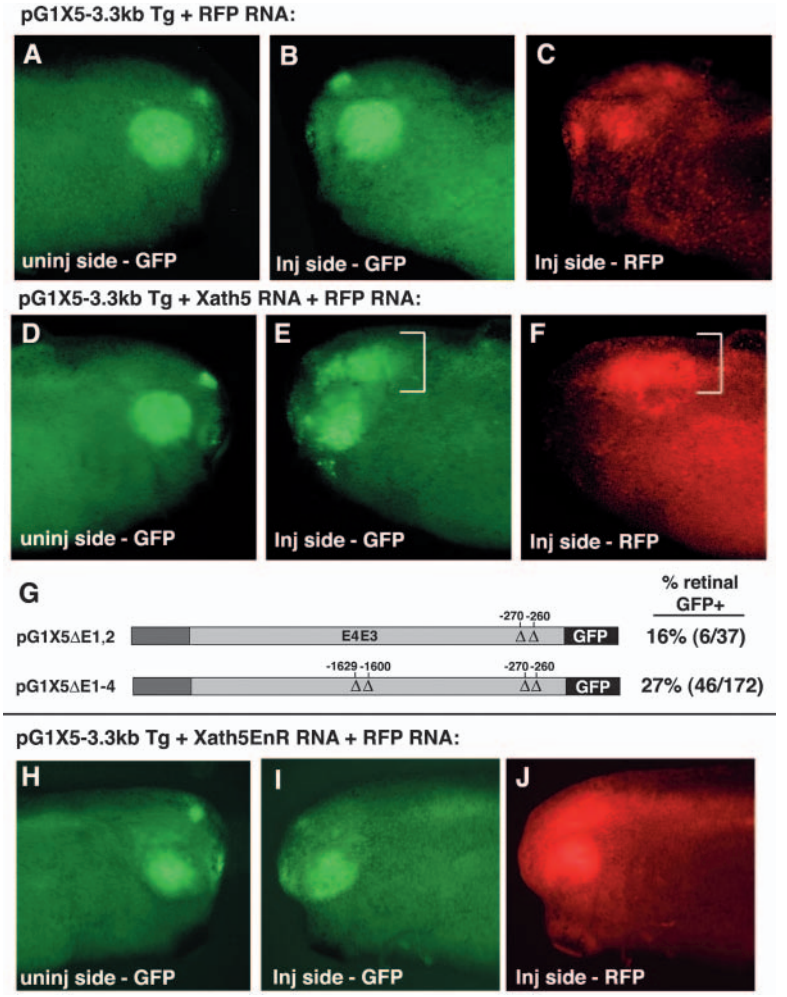
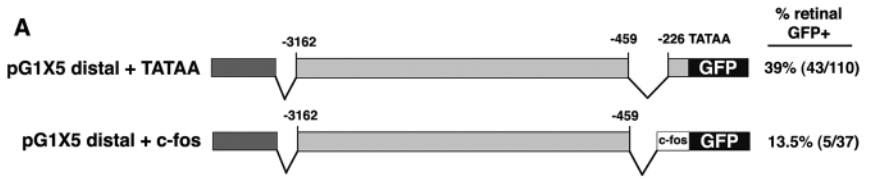


Fig. 6. 3.3 kb *Xath5* transgene expression can be activated by bHLH factors, but is not dependent upon conserved E-boxes or bHLH activity. (A-C) Injection of RNA encoding RFP alone did not alter transgene expression on the injected side. (D-E) Injection of RNA for *Xath5* ectopically activated transgene expression on the injected side (bracket in E) compared with the uninjected side (D). RFP marks the region targeted on the injected side (bracket in F). (G) Mutation of E1 and E2 (ΔE1,2) in the pG1X5-3.3 kb transgene did not eliminate retinal transgene expression. Mutation of two additional conserved E-boxes, E3 and E4, also did not abolish transgene activity. (H-J) Injection of RNA for the dominant-negative Xath5-EnR did not suppress expression of the pG1X5-3.3 kb transgene on the injected side (I) compared with the uninjected side (H). RNA encoding RFP (red) was co-injected to mark the injected side (J).



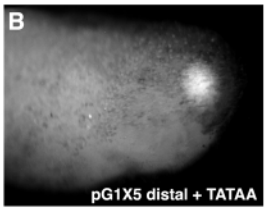


Fig. 7. The distal cis-regulatory region of *Xath5* alone is sufficient to promote transgene expression in the developing retina. (A) A *Pst*I fragment that lacks the conserved proximal region promotes retinal transgene expression when coupled to either the pG1X5-TATAA basal promoter (pG1X5 distal + TATAA) or to the *Fos* basal promoter (pG1X5 distal + Fos). (B) Fluorescent image of a stage 30 pG1X5distal+TATAA transgenic embryo showing expression of the transgene in the retina.

Discussion

Two modes of *Ath5* gene regulation in *Xenopus*

Our analysis has identified two modes of *Ath5* gene regulation in the developing *Xenopus* retina. First, we found a conserved proximal cis-regulatory region in the *Ath5* gene and showed that this region was sufficient to promote bHLH-dependent transgene expression in the developing *Xenopus* but not mouse retina. Proximal transgene expression in *Xenopus* required two highly conserved E-boxes that function together with adjacent conserved sequences to promote retinal expression. We also revealed a bHLH-independent mode of *Xath5* gene expression mediated through more distal sequences and showed that the distal cis-regulatory region alone was sufficient to promote retinal transgene expression in *Xenopus*. Furthermore, transgenes that included these more distal sequences were efficiently expressed in both *Xenopus* and mouse. Thus, we found both conserved and non-conserved aspects to *Ath5* gene regulation in the developing retina. It remains to be determined whether these two regulatory regions contribute to different spatial or temporal aspects of *Ath5* gene expression.

Activity of the *Xath5* proximal cis-regulatory region is bHLH-dependent

bHLH factors probably play a role in regulating the retinal expression of *Xath5* in vivo through the conserved proximal region. There is ample precedence for vertebrate bHLH factor cross-regulation and autoregulation. For example, in *Xenopus* during primary neurogenesis at open neural plate stages, Xngnr-1 activates the expression of *Xath3* and *NeuroD*, while Xath3 and NeuroD crossactivate each other's expression (Ma et al., 1996; Perron et al., 1999). Expression of a 1 kb *Neurod2* promoter in mouse brain was also recently shown to depend upon two E boxes in the proximal promoter region (Lin et al., 2004).

We found that overexpression of multiple atonal-related bHLH factors, including Xath5, Xath3, XNeuroD and X-ngnr-1, could ectopically activate pG1X5-3.3 kb transgene expression in *Xenopus* embryos. Which of these are likely to play a role in regulating *Xath5* expression in vivo? In the CMZ, a number of bHLH factors are expressed in an overlapping but sequential manner in a peripheral to central spatial pattern that reflects the sequence of gene activation during early eye development (Perron et al., 1998). *XNeuroD* and *Xath3* (*NeuroM*) do not precede *Xath5* (Kanekar et al., 1997; Perron et al., 1999), so they are unlikely to initiate *Xath5* expression, but could help maintain retinal expression. *X-ngnr-1* is expressed in the CMZ in an earlier yet overlapping pattern, so it may help initiate and/or maintain *Xath5* expression. In chick, the related gene *Ngn2* is co-expressed with *Cath5* in early retinal progenitors, and Ngn2 protein is bound to the *Cath5* promoter during retinal development, and activates expression of a *Cath5* transgene in cell culture (Skowronska-Krawczyk et al., 2004; Matter-Sadzinski et al., 2001). Thus, X-ngnr-1 (or Ngn2 in chick) is a strong candidate for regulating *Ath5* expression in vivo.

We did not observe ectopic pG1X5-3.3 kb transgene expression with overexpression of either Xash1 or Xash3, suggesting that these factors do not positively regulate *Xath5* gene expression. In chick, *Ath5* and *Ash1* are expressed in mutually exclusive domains in the retinal neuroepithelium, and *Cash1* expression inhibited activity of the chick *Ath5* regulatory region in transfected retinal cells in culture (Matter-Sadzinski et al., 2001). It remains possible that in *Xenopus*, Xash1 or Xash3 could have similar inhibitory activity on bHLH-dependent expression from the pG1-X5 proximal region alone.

E boxes alone are not sufficient for retina-specific expression

We determined that either E1 or E2 are necessary in the context of the proximal *Xath5* promoter to drive retinal expression of a GFP transgene. However these two highly conserved and essential E-boxes alone were insufficient for retinal-specific expression. A 33 bp fragment that included E1 and E2 plus adjacent conserved 5′ sequence drove GFP expression throughout the CNS as well as in muscle, suggesting it may be non-specifically recognized by multiple bHLH factors. This 33 bp sequence includes a motif that is similar to the consensus for an α-box, which was first identified as a critical element in the *scute* sensory mother cell (SMC) enhancer (Culi and Modolell, 1998), and may be a candidate binding sequence for a winged-helix zinc finger transcription factor (Genomatix MatInspector). In *Xenopus*, this sequence played a role in robustness but not specificity of transgene expression.

Inclusion of an additional 15 bp of 5′ sequence constrained transgene expression to tissues where endogenous *Xath5* is expressed, along with weak expression in the axial muscles. The additional 15 bp of sequence included in this construct are highly conserved, with 11 out of 15 nucleotides identical between *Xenopus laevis* and mouse; however, no candidate transcription factor binding sites were apparent. It is intriguing to speculate that some novel factor interacts with this sequence and participates in bHLH-dependent *Ath5* gene regulation.

Proximal *Ath5* sequences are not sufficient for gene expression in mouse

In the mouse retina, bHLH factors are unlikely to initiate *Math5* expression. *Ngn2* commences expression at E13, 2 days later than *Math5* (Brown et al., 1998), and *Math5* is not preceded by expression of genes encoding any other known proneural bHLH factor. Thus, *Math5* must be regulated by

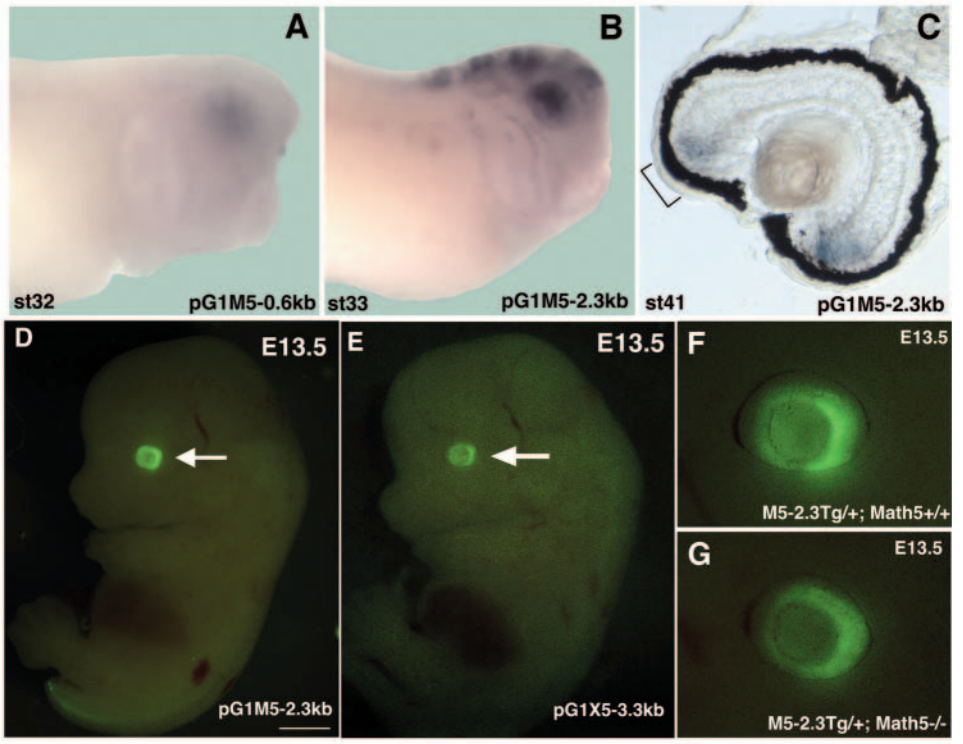


Fig. 8. Cross-species analysis of *Ath5* transgene expression. (A) The proximal 600 bp of the *Math5* cis-regulatory region drives weak GFP expression in the retina of transgenic frog embryos, as shown by in situ hybridization on a pG1M5-0.6 kb transgenic *Xenopus* embryo. (B) GFP in situ hybridization in a pG1M5-2.3 kb transgenic embryo. The 2.3 kb *Math5* fragment drives strong transgene expression in the retina, cranial ganglia, midbrain and hindbrain regions in transgenic *Xenopus* embryos. (C) In situ hybridization on retinal sections from stage 41 pG1M5-2.3 kb transgenic embryos shows that the domain of *GFP* mRNA is restricted to the CMZ (bracket). (D) E13.5 whole embryo image demonstrating retinal expression of the pG1M5-2.3 kb transgene in mouse embryos at E13.5 (arrow). (E) The *Xenopus* 3.3 kb *Xath5* transgene (pG1X5-3.3 kb) also shows retinal expression at E13.5. (F,G) *Math5* is not required for expression of the pG1M5-2.3 kb transgene as equivalent fluorescence from the transgene was observed between wild-type (F) and *Math5*$^{-/-}$ (G) embryos. Scale bar: 500 μm in D.

other factors present in the developing optic vesicle and cup. Consistent with this idea, the short proximal region from either mouse or *Xenopus*, which contains the conserved E-boxes, was insufficient to promote transgene expression in mouse. Thus, despite strong sequence homology in the proximal cis-regulatory region, the role bHLH factors play in regulating the expression of *Ath5* appears to be species specific. This may in part depend upon whether other bHLH factors precede *Ath5*, as in frog and chick, or whether it is the first retinal bHLH gene expressed, as in the mouse. We also cannot rule out a role for bHLH factors in some other aspect of *Ath5* gene regulation, potentially at later stages of development in mouse. It also remains possible that in *Xenopus* or chick, bHLH factors do not play an essential role in regulating *Ath5* gene expression as we identified a bHLH-independent distal cis-regulatory region that alone was sufficient to promote retinal transgene expression.

Is there autoregulation of *Ath5* gene expression?

Autoregulation may also play a role in regulating *Xath5* expression. In *Drosophila*, Atonal function is crucial for its own expression (Sun et al., 1998), and in vertebrates Math1 has autoregulatory activity in the developing spinal cord, but not in the cerebellum (Gazit et al., 2004; Helms et al., 2000). In chick retinal cultures, *Cath5* transgene expression could be activated by chick Ath5 itself and Cath5 was found bound to its promoter in vivo, suggesting autoregulation (Skowronska-Krawczyk et al., 2004; Matter-Sadzinski et al., 2001). Consistent with this, we showed that overexpression of Xath5 could activate ectopic transgene expression in *Xenopus*. However, in mouse expression of the pG1M5-2.3 kb transgene did not depend upon Math5, arguing that autoregulation is not essential for *Ath5* gene expression in the developing mouse retina. This fits with the observation that expression of a β-galactosidase reporter introduced into the *Math5* locus by homologous recombination is expressed in *Math5*$^{-/-}$ optic cup and retina (E11-birth) (Brown et al., 2001) (data not shown). Consistent with this, expression of the longer pG1X5-3.3 kb transgene in *Xenopus*, as well as expression of endogenous *Xath5*, was not blocked by the dominant-negative Xath5-EnR. Thus, there are clearly additional sequences that can mediate *Ath5* gene expression in both species, as discussed below. This outcome does not preclude the possibility that autoregulation contributes to some aspect of *Ath5* expression during retinal development.

bHLH-independent gene regulation mediated through distal cis-regulatory sequences

We found that that unlike the proximal *Xath5* transgene, expression driven by the full 3.3 kb transgene was not bHLH dependent, as it did not require four conserved E-boxes and could not be suppressed by overexpression of Xath5-EnR. Thus, we conclude that more distal sequences mediate bHLH-independent expression. We found that a distal fragment from the *Xath5* cis-regulatory region alone was sufficient to promote retinal transgene expression in *Xenopus*. Future analysis will focus on defining which elements within this region are required for expression. Interestingly, longer 5′ cis-regulatory regions from either mouse (2.3 kb) or *Xenopus* (3.3 kb) were sufficient to promote appropriate transgene expression in mouse, consistent with our conclusion that *Ath5* gene expression in mouse is largely bHLH-independent. However, there is little sequence homology between mouse and *Xenopus* in the distal region other than E3 and E4, so it is unclear whether the mechanisms governing *Math5* and *Xath5* gene regulation through more distal sequences will be conserved.

What are the signals that regulate bHLH-independent *Ath5* gene regulation? In *Drosophila*, the initiation and propagation

of *atonal* expression directly depends upon signals such as hedgehog (Dominguez, 1999; Sun et al., 1998). However in the 3.3 kb *Xath5* cis-regulatory sequence, we found no clear binding sites for Gli zinc finger transcription factors, which mediate hedgehog signaling, although it remains possible that regulation is indirect. In the fish retina, *Ath5* expression is initiated by a signal from the optic stalk (Masai et al., 2000), and it is possible that this signal acts through the cis-regulatory sequences that we have identified. In support of this idea, the 3.3 kb pG1X5 transgene was expressed in the developing retina in transgenic zebrafish embryos, suggesting that the 3.3 kb *Xath5* regulatory sequences are appropriately recognized in zebrafish (A. Pittman and C.-B. Chien, personal communication).

bHLH-dependent and -independent *Ath5* gene regulation

We have identified bHLH-dependent and -independent modes of *Ath5* gene regulation in *Xenopus*, raising the issue of how they contribute to endogenous *Xath5* expression. It is possible that the distal and proximal cis-regulatory sequences serve overlapping or redundant functions. Alternatively, the distal and proximal regions may regulate different phases of *Xath5* expression. For example, during *Drosophila* eye development *atonal* gene expression is initiated in a bHLH-independent fashion by factors such as hedgehog, then expression becomes dependent upon Atonal itself (Hsiung and Moses, 2002). It is therefore possible that *Xath5* gene regulation is similar, with initiation of gene expression being bHLH-independent and maintenance of expression requiring Xath5 and/or other bHLH factors such as X-Ngnr-1. In chick, bHLH factors are clearly involved in regulation of Cath5 (Skowronska-Krawczyk et al., 2004; Matter-Sadzinski et al., 2001), but it remains to be determined whether there is bHLH-independent regulation as well. In mouse, we found no evidence for bHLH-dependent *Atoh7* gene regulation, demonstrating that *Math5* expression is bHLH independent. Thus, we have shown that although some mechanisms of *Ath5* gene regulation are conserved, there are intriguing species-specific differences that remain to be explored.

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