Isolation and Expression of *Pax6* and *atonal* Homologues in the American Horseshoe Crab, *Limulus polyphemus*

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Pax6 regulates eye development in many animals. In addition, Pax6 activates atonal transcription factors in both invertebrate and vertebrate eyes. Here, we investigate the roles of Pax6 and atonal during embryonic development of *Limulus polyphemus* rudimentary lateral, medial and ventral eyes, and the initiation of lateral ommatidial eye and medial ocelli formation. *Limulus* eye development is of particular interest because these animals hold a unique position in arthropod phylogeny and possess multiple eye types. Furthermore, the molecular underpinnings of eye development have yet to be investigated in chelicerates. We characterized a *Limulus Pax6* gene, with multiple splice products and predicted protein isoforms, and one *atonal* homologue. Unexpectedly, neither gene is expressed in the developing eye types examined, although both genes are present in the lateral sense organ, a structure of unknown function. *Developmental Dynamics 237:2209–2219, 2008.* © 2008 Wiley-Liss, Inc.

Key words: Limulus; Pax6; Atonal; chelicerate; eye development

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INTRODUCTION

Eye morphology, tissue, and cell type composition, and mechanisms of photoreception and phototransduction are features that define the diversity of eye types found in different organisms. Eye development also provides valuable data for phylogenetic analyses, particularly those concerning bilaterian relationships (reviewed in Arendt and Wittbrodt, 2001; Arendt, 2003). In recent years, evolutionary comparisons of developmental gene hierarchies have contributed to theories of eye evolution, to the extent that several long-standing views were revised (Halder et al., 1995b; Gehring, 1996; Callaerts et al., 1997; Pichaud et al., 2001). One transcription factor with a highly conserved and essential role in eye development is *Pax6*. Mutational analyses have demonstrated that *Pax6* is critical for initial eye specification and differentiation (Hill et al., 1991; Ton, 1991; Glaser et al., 1992; Quiring et al., 1994). Conversely, misexpression of *Pax6* revealed its sufficiency, because it respecifies ectodermal tissues into partial or complete ectopic eyes (Halder et al., 1995a; Tomarev et al., 1997; Chow et al., 1999). However, *Pax6* is not universally expressed during metazoan visual system development. For example, particular jellyfish, polychaete worm, myriapod, and amphioxus eyes or visual organs lack Pax6 expression (Glardon et al., 1998; Arendt et al., 2002; Piatigorsky and Kozmik, 2004; Prpic, 2005). Instead, the expression of *Pax-B* and *So* within embryonic jellyfish or adult *Platyneris* eyes respectively, point to alternative mechanisms of eye development in some species (Arendt et al., 2002; Piatigorsky and Kozmik, 2004).

Nonetheless, multiple features of the *Pax6* gene are well conserved among bilaterians. First, the amino acid sequence of the encoded protein retains high identity, particularly

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within the paired- and homeo-domains that bind DNA (reviewed in Callaerts et al., 1997). Moreover, the exon/intron structure of numerous *Pax6* genes are strongly conserved, as are distinct *cis*-regulatory elements and *trans*-acting factors that bind them (Callaerts et al., 1997; Kammandel et al., 1999; Xu et al., 1999). Some invertebrate species have duplicate *Pax6* genes (Czerny and Busslinger, 1995; Jun et al., 1998; Czerny et al., 1999; Prpic, 2005), whereas most chordates have a single *Pax6* gene (reviewed in Callaerts et al., 1997; van Heyningen and Williamson, 2002). Instead of gene duplication and functional divergence, vertebrate *Pax6* functions are divided among distinct splice products and/or protein isoforms (Carriere et al., 1993; Epstein et al., 1994; Glardon et al., 1998; Mishra et al., 2002; Lakowski et al., 2007).

Concomitant with *Pax6* gene conservation is its maintained regulation of orthologous downstream genes (reviewed in Wawersik and Maas, 2000; Hanson, 2001; Friedrich, 2006). For instance, *eya-Eya*, *so-Six*, and *atonal-Ath5* gene families all act immediately downstream of *Pax6* in both fruit fly and mouse eye development. These downstream genes also perform conserved roles in specific aspects of eye development. For example, the *atonal-Ath5* family encodes basic helix–loop–helix (bHLH) factors that specify the first retinal neuron class during fruit fly, zebrafish, chick, frog, and mouse eye development (reviewed in Vetter and Brown, 2001; Mu and Klein, 2004).

The visual system of the chelicerate *Limulus polyphemus* (*Lp*) has been studied for decades, especially with respect to its circadian biology, neuroanatomy, and physiology (Hartline et al., 1956; Barlow, 1969; Snodderly and Barlow, 1970; French, 1980; Sekiguchi et al., 1982; Barlow et al., 2001). More recently, molecular approaches have been applied to studies of *Limulus* eye development (Harzsch et al., 2006). During embryogenesis three types of rudimentary eyes (lateral, median, and ventral) develop and innervate the brain (see Fig. 1 of Harzsch et al., 2006 for detailed diagrams). All are closely associated with pigmented cells termed guanophores, and these eyes seem to provide phototic input during embryonic and early larval life (French, 1980; Harzsch et al., 2006). Subsequently, the lateral compound eye and median ocellar eyes develop during larval stages and provide major visual inputs in adult animals. The lateral compound eyes, median ocelli, and lateral and ventral rudimentary eyes express visible light opsins (Smith et al., 1993; Battelle et al., 2001; Dalal et al., 2003), and median ocelli also display ultraviolet-sensitivity (Nolte and Brown, 1969). The opsins in the median rudimentary eyes are not yet known. Two *Limulus* opsin genes encoding visible light proteins have been characterized (reviewed in Battelle, 2006), and the photoreceptor cells within the various eyes classified as rhabdomeric (Arendt and Wittbrodt, 2001). In an evolutionary context, *Limulus* is interesting because it has both multiple eyes and eye types, which raises the possibility for heterogeneity in the development of different eyes and types of visual organs. In addition, regulatory genes involved in eye development have been described for all major arthropod lineages, except chelicerates.

Here, we report isolation and embryonic expression of *Lp Pax6* and *atonal* genes. Paradoxically, we found that neither gene is expressed during eye development in the *Limulus* embryo or young larvae. Instead, we found *Pax6* expression in the developing brain, ventral nerve cord, and lateral sense organ. In addition an *Lp atonal* family member was identified, but is only expressed in the developing lateral sense organ, a sensory structure whose function remains unresolved. These findings suggest that particular aspects of *Limulus* embryogenesis are divergent from other arthropods.

RESULTS

Molecular Cloning of *Limulus Pax6*

A *Limulus polyphemus* (*Lp*) *Pax6* 800-bp fragment, encoding partial paired- and homeo-domains, was isolated by low-stringency reverse transcriptase-polymerase chain reaction (RT-PCR) using the strategy, nested primers and PCR conditions previously described by Arendt et al. (2002). Multiple subclones of this fragment had identical nucleotide sequences. The 5′ and 3′ rapid amplification of cDNA ends (RACE) was then performed on mixed stage embryo cDNA. We isolated distinct 5′ RACE products (Figs. 1, 2), but only one 3′ RACE product of 1.2-kb in length. DNA sequence analysis indicated that this product contains a partial *Pax6* paired domain, homeodomain, stop codon, and 3′ UTR (Figs. 1A, 2A). Twelve different 5′ RACE subclones were DNA sequenced, their nucleotide sequences compared and then divided into five classes (Fig. 1A). Four of five classes differ only in their 5′ UTR, with all subclones converging at the same predicted splice acceptor site, 91 nucleotides upstream of the ATG start codon (asterisk in top four diagrams of Fig. 1A). The 5′ RACE products of these four classes have identical nucleotide sequences, from the putative splice site to their 3′ end within the coding region (Fig. 1A). The fifth 5′ RACE product has a shorter 5′ UTR, predicted alternative ATG start codon and four unique amino acids, which are in frame with the paired domain (bold-faced amino acids in Clone 11, Fig. 2A). This RACE product is predicted to use a separate downstream splice acceptor site (asterisk in Clone 11 in Fig. 1A). This 5′ RACE product has identical nucleotide sequence to that of the other four classes, from the first codon of the paired domain to its 3′ end.

To characterize the 5′ end of *Lp Pax6* further, we sequentially probed a genomic Southern blot at high stringency, with three cDNA probes (Fig. 1A,B). When the hybridization patterns of Probes 1 and 2 were compared, a subset of bands are shared between these probes (white asterisks), while some bands were hybridized strictly by Probe 2 (yellow asterisks or a white arrow). To understand whether Probe 2-specific bands represent restriction fragments containing 3′ nucleotides beyond the end of probe 1, we next determined the genomic Southern blot pattern of probe 3 (Fig. 1A, probe diagram). Comparison of Probe 2 and 3 hybridization patterns (Fig. 1B) indicates that all bands, except one (white arrow in middle blot), contain *Lp Pax6* 3′-specific sequences. Bands denoted with orange asterisks are common to all three probes. To explore the possibility that the *Lp* genome contains more than one *Pax6* gene, we probed a genomic Southern blot with Probe 2 at low stringency, and observed the identical hybridization pattern found at high stringency in Figure 1B (data not shown). We conclude

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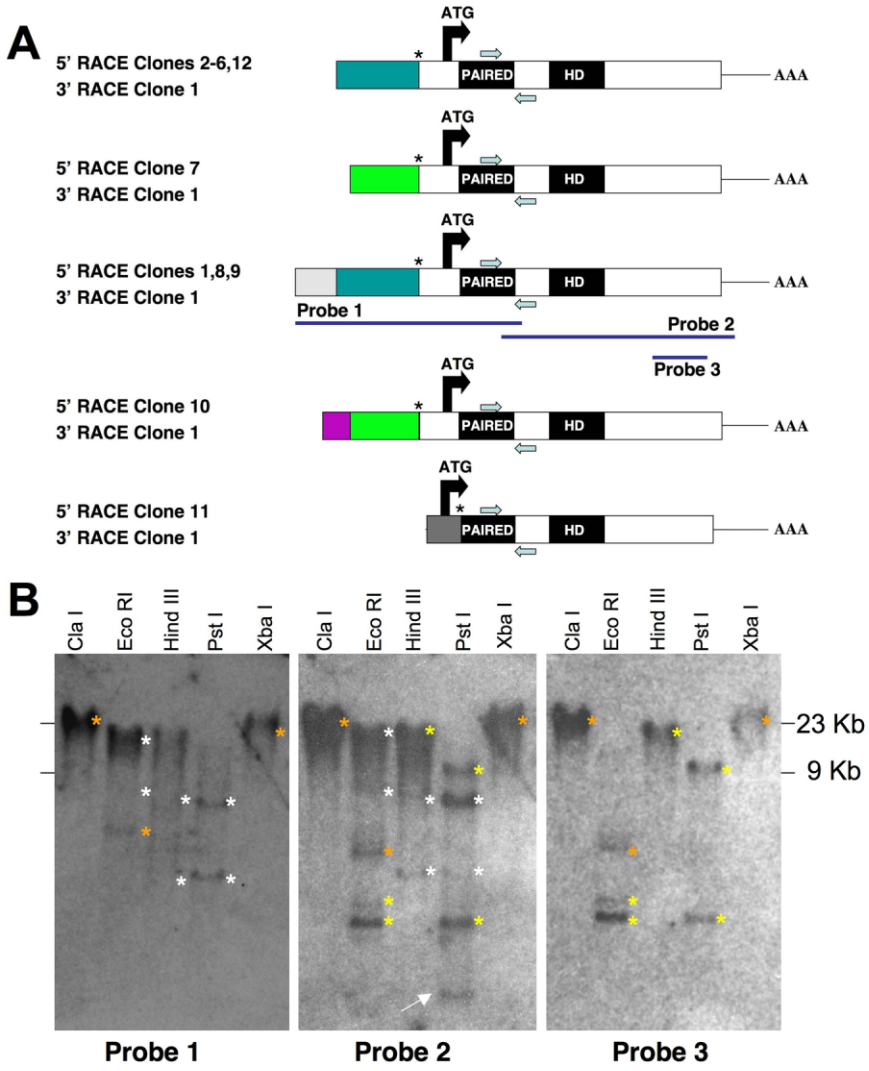

Fig. 1. *Lp Pax6* encodes multiple isoforms. **A:** Diagram of five different types *Lp Pax6* 5′ rapid amplification of cDNA ends (RACE) products with a single 3′ RACE product. Color-coding indicates different 5′ untranslated region (UTR) sequences. Use of the same color among different classes of clones indicates stretches of exact nucleotide identity. The positions of gene-specific RACE primers are indicated by blue arrows, below (5′ RACE) or above (3′ RACE) each diagram. 11 of 12 5′ RACE products have identical sequences beginning at a putative splice acceptor site (asterisk) upstream of the start ATG codon. One 5′ RACE subclone (#11) has an alternative in frame ATG start, plus 4 unique amino acids immediately upstream of the paired domain (bold-faced amino acids in Fig. 2A). The longest 5′ RACE product (probe 1) was used for Southern blot analyses. **B:** Autoradiographs of replicate *Limulus* genomic Southern blots hybridized with the cDNA probes shown in A. Enzymes used to digest *Limulus* genomic DNA are indicated above each lane. White asterisks to the right of bands indicate those in common for Probes 1 and 2, yellow asterisks mark bands in common between Probes 2 and 3, and orange asterisks indicate bands common to all three probes. Several of the bands marked by white asterisks gave a weaker signal on the Southern blot autoradiogram. The white arrow in the middle blot points to the only band that uniquely hybridizes with Probe 2.

that *Lp* embryos possess at least one *Pax6* gene, with multiple 5′ splice products, which are predicted to produce two distinct protein isoforms (Figs. 1A, 2A).

Evolutionary Comparison of *Pax6* Paired Domains

Overall *Lp* Pax6 shares significant amino acid identity in the paired domain with all Pax6 proteins aligned (Fig. 2B). The *Lp* Pax6 paired domain has highest amino acid identity (92%) with *Drosophila* toy, and lowest (78%) with the flatworm *Dugesia* (Fig. 2B). Both parsimony and Bayesian analyses provide strong support that among the Pax6 paired domains analyzed, *Lp Pax6* is closely related to *Drosophila toy*. Moreover, within the paired domains of toy and ey, there is a critical difference of one amino acid, which has been previously demonstrated as critical for DNA binding affinity (Czerny et al., 1999). In both *Lp* Pax6 and *Drosophila* toy this residue is an asparagine, which is typical of most nonarthropod *Pax6* genes. However, *Drosophila* ey and amphioxus Pax6 encode a glycine at this position. This further suggests that *Lp Pax6* is more closely related to *toy* than it is to *ey*. The paired domains of these two genes may also be closely related to *Pax6.2* of the myriapod *Glomeris*. However, this interpretation is tentative because the complete amino acid sequences of the *Glomeris Pax6* paired domains may demonstrate that *Pax6.2* is not closely related to *Lp Pax6* and *toy* (Fig. 3).

Although our data suggest a monophyly of *Pax6*-eye genes among ecdyzosoans, the relationship of *Drosophila* paired, to that of *Ciona* Pax6 and all Pax6 sequences analyzed, is puzzling (Fig. 3). The positions of *Drosophila* paired and ey are not well supported by our analyses, because the bootstrap values were low. Because bootstrap values >70% and Bayesian posterior probabilities >0.95 are needed to show strong support in phylogenetic analyses (Hillis and Bull, 1993; Wilcox et al., 2002), an expansion of the Pax6 paired domain dataset and its outgroups might resolve these discrepancies. Of interest, there is strong support from both phylogenetic analyses for the close relationship among hemichordate and mollusk Pax6-eye paired domains (Fig. 3).

Cloning and Evolutionary Comparison of an *Lp atonal* Homologue

To isolate *Lp Atonal* bHLH homologues, degenerate bHLH domain primers and standard PCR conditions were used with *Lp* genomic DNA (Brown et al., 1998), because *atonal* gene orthologues contain no introns. Ten subclones containing the predicted 135-bp product were DNA sequenced and the nucleotide sequences compared. We found three highly related, but distinct, bHLH domains encoded (Fig. 4A). The nested RT-PCR strategy of Arendt et al. (2002) was tried several times on mixed embryo cDNA, but no PCR products recov-

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A. *Limulus polyphemus Pax6* amino acid sequences

Isoform predicted by sequence of 5′ RACE Clones 1-10,12

```
MPHKGHSGVNQLGGVYVNGRPLPDSTRQKIVELAHSGARPCDISRILQVSNGCVSKILGRYYETGSIKPRTIGGSKPRVATSAVVNKIADYKRECPSVFAWEIRDRLLADGVCNSENIPSVSSINRVLRN
LTSQKDQQEPSPHHVPMTGPESVYDKLRLLNGSQPWPWYPTGPTHHFHGIPPTSSPVALGQNAGLVGPVHGGNGHPSLAACESHRQDHPSVKKDSIDATSDGNSENNSSADEDSQLRMRLKRKLXRNRTS
FTNEQIEALEKEFERTHYPDVFARERLAEKITLPEARIQVWFSNRRAKWRREEKLRNQRRVVEQPTGTTVLAPPNGRLPINGTFYNSMYPSLGQPMGGMGDSYSMPPSSSMASNHCLQQRDPTSYTYMFH
DPLSLGSYSRSSCTPSQSINGHPSYSVNGGNAHGAGVISPGVTVPLQVPSQGPDLSMATNYWHRIQ
```

Isoform predicted by sequence of 5′ RACE Clone 11

```
MKPARHSGVNQLGGVYVNGRPLPDSTRQKIVELAHSGARPCDISRILQVSNGCVSKILGRYYETGSIKPRTIGGSKPRVATSAVVNKIADYKRECPSVFAWEIRDRLLADGVCNSENIPSVSSINRVLRN...
```

B. Paired domain amino acid alignment

```
                                                                                                                                  %
Limulus          HSGVNQLGGVYVNGRPLPDSTRQKIVELAHSGARPCDISRILQ--------------VSNGCVSKILGRYYETGSIKPRTIGGSKPRVATSAVVNKIADYKRECPSVFAWEIRDRLLADGVCNSENIPSVSSINRVLRN   Iden.

Drosophila toy   HSGINQLGGVYVNGRPLPDSTRQKIVELAHSGARPCDISRILQ--------------VSNGCVSKILGRYYETGSIKPRAIGGSKPRVATTPVVQKIADYKRECPSIFAWEIRDRLISEQVCNSDNIPSVSSINRVLRN    92%
Drosophila ey    HSGVNQLGGVFVGGRPLPDSTRQKIVELAHSGARPCDISRILQ--------------VSNGCVSKILGRYYETGSIRPRAIGGSKPRVATAEVVSKISQYKRECPSIFAWEIRDRLLQENVCTNDNIPSVSSINRVLRN    88%

Platynereis      HSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQ--------------VSNGCVSKILGRYYETGSIRPRAIGGSKPRVATPEVVSKIAQYKRECPSIFAWEIRDRLISEGVCTQDNIPSVSSINRVLRN    90%
Lineus           HSGVNQLGGVFVNGRPLPDSTRQRIVELAHSGARPCDISRILQ--------------VSNGCVTKILGRYYETGSIRPRAIGGSKPRVATPEVVGKIAHYKRECPSIFAWEIRDRLISDAVCNQDNIPSVSSINRVLRN    89%
Lethenteron      HSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQ--------------VSNGCVSKILGRYYETGSIRPRAIGGSKPRVATPEVVGKIAQYKRECPSIFAWEIRDRLISEGACTEDNIPSVSSINRVLRN    90%
Branchiostoma    HSGVNQLGGVFVGGRPLPDSTRRKIVELAHQGARPCDISRILQ--------------VSNGCVSKILGRYYETGSIRPRAIGGSKPRVATPEVVAKIAQFKRECPSIFAWEIRDRLISEGICTNENIPSVSSINRVLRN    87%
Saccoglossus     HSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQ--------------VSNGCVSKILGRYYETGSIRPRAIGGSKPRVATPPVVGKIAQFKRECPSIFAWEIRDRLLQEQVCTQDNIPSVSSINRVLRT    88%
Paracentrotus    HSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQ--------------VSNGCVSKILGRYYETGSIRPRAIGGSKPRVATPHVVTRIAHYKRECPSIFAWEIRDRLLAEKICNQENIPSVSSINRVLRN    90%
Caenorhabditis   HTGVNQLGGVFVNGRPLPDATRQRIVDLAHKGCRPCDISRILQ--------------VSNGCVSKILCRYYESGTIRPRAIGGSKPRVATSDVVEKIEDYKRDQPSIFAWEIRDRKLLADNICNNETIPSVSSINRVLRN    82%
Dugesia          HSGINQLGGMFVNGRPLPDVTRQRIIELSQSGARPCDISRILQ--------------VSNGCVSKILCRYYGTGSIKPRAIGGSKPRVATNTVVRKVTIKKQESPGMFAWEIRDRLLQDGVCNQDNLPSISSINRILRS    78%
Girardia Pax6a   ------------------------------------------------------------GCVSKILCRYYETGSIRPRAIGGSKPTVATSSVVSKTLAYKRECPSIFSWEIRDRLLQEGVCNQDNIPSVSSINRVLRS    83%
Loligo           HSGVNQLGGVFVNGRPLPDSTRQRIVELAHSGARPCDISRILQ--------------VSNGCVSKILGRYYETGSIRPRAIGGSKPRVATPEVVQKIAQFKRECPSIFAWEIRDRLISEGVCTQDNIPSVSSINRVLRN    88%
Euprymna         HSGVNQLGGVFVNGRPLPDSTRQRIVELAHSGARPCDISRILQ--------------VSNGCVSKILGRYYETGSIRPRAIGGSKPRVATPEVVQKIAQFKRECPSIFAWEIRDRLISEGVCTQDNIPSVSSINRVLRN    88%
Ciona            HSGMNQLGGMFVNGRPLPDSIRQKIVEFAHNGARPCDISRILQ--------------VSNGCVSKILARYYETGTIRPRAIGGSKPRVATPEVVNKIASYKRECPSIFAWEIRDRLINEGICNNDNIPSVSSINRVLRN    86%
Danio            HSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQ--------------VSNGCVSKILGRYYETGSIRPRAIGGSKPRVATPEVVGKIAQYKRECPSIFAWEIRDRLISEGVCTNDNIPSVSSINRVLRN    90%
Oryzias          HSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQ--------------VSNGCVSKILGRYYETGSIRPRAIGGSKPRVATPEVVAKIAQYKRECPSIFAWEIRDRLISEGICTNDNIPSVSSINRVLRN    89%
Takifugu         HSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQTHD--AVQVLDSEKVSNGCVSKILGRYYETGSIRPRAIGGSKPRVATPEVVAKIAQYKRECPSIFAWEIRDRLISEGICTNDNIPSVSSINRVLRN    81%
Gallus           HSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQTHADAKVQVLDNQNVSNGCVSKILGRYYETGSIRPRAIGGSKPRVATPEVVSKIAQYKRECPSIFAWEIRDRLISEGVCTNDNIPSVSSINRVLRN    81%
Homo, Mus        HSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQ--------------VSNGCVSKILGRYYETGSIRPRAIGGSKPRVATPEVVSKIAQYKRECPSIFAWEIRDRLISEGVCTNDNIPSVSSINRVLRN    90%

Drosophila prd   QGRVNQLGGVFINGRPLPNNIRLKIVEMAADGIRPCVISRQLR--------------VSHGCVSKILNRYQETGSIRPGVIGGSKPRIATPEIENRIEEYKRSSPGMFSWEIREKLIREGVCDRSTAPSVSAISRLVRG    60%
Rattus Pax4      LSSVNQLGGLFVNGRPLPLDTRQQIVQLAIRGMRPCDISRSLK--------------VSNGCVSKILGRYYRTGVLEPKGIGGSKPRLATPAVVARIAQLKDEYPALFAWEIQRQLCAEGLCTQDKAPSVSSINRVLRA    68%
Homo Pax4        ---MNQLGGLFVNGRPLPLDTRQQIVRLAVSGMRPCDISRILK--------------VSNGCVSKILGRYYRTGVLEPKGIGGSKPRLATPPVVARIAQLKGECPALFAWEIQRQLCAEGLCTQDKTPSVSSINRVLRA    69%
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Fig. 2. Amino acid sequence of *Lp Pax6*. **A:** Deduced amino acid sequence of two predicted protein isoforms of *Limulus Pax6* that only differ by four amino acids (bold-faced in clone 11). The paired domain is underlined and the homeodomain shaded. **B:** Amino acid sequence comparison of the paired domain for *Lp* Pax6 with various invertebrate and vertebrate Pax6 proteins in the NIH/NCBI database. Dark shading indicates identical amino acids, light shading similar amino acids to *Lp* Pax6. Percent identities (uncorrected pairwise sequence divergences) are listed at the right. The *Lp* Pax6 paired domain shares the highest percent identity with that of *Drosophila* toy.

ered contained bHLH domains. To obtain additional *Lp atonal* coding sequences, gene-specific primers were designed from the bHLH domain isolated in 6 of 10 subclones (Fig. 4A) and 5′ and 3′ RACE performed with mixed stage embryo cDNA. For 3′ RACE one 450 bp product was obtained that is predicted to encode the 3′ end of a bHLH domain, plus two additional amino acids (Fig. 4B), immediately followed by a stop codon and 3′ UTR. We were unable to isolate any 5′ RACE products, despite repeated attempts with several different gene-specific primers and a variety of PCR conditions. The partial coding region obtained is highly similar to *Drosophila* Atonal (69%) Cato (71%) and Amos (73%), but shares the highest amino acid identity with *Anopheles* Atonal (81%; Fig. 4C). Interestingly, this *Lp atonal* partial cDNA encodes a polypeptide with higher amino acid identity to mouse Math1 (78%) than to Math5 (71%). This finding suggests that *Limulus* Atonal is more related to Math1.

Both parsimony and Bayesian analyses provide strong support that among bHLH domains, *Lp atonal* is most closely related to *atonal*, *Ath1*, and *Ath5* (Fig. 5) While there is strong support for the monophyly of *Ath1* genes, there is only very weak support for the inclusion of *Lp atonal* in a lineage comprising both fruit fly *atonal* and vertebrate *Ath5* genes. Interestingly, there is strong support for a lineage each of *atonal*, *Ath1*, and *Ath5* (the latter is found only in deuterostomes), as well as a lineage for arthropod *amos* and *atonal*, excluding the *Limulus* gene described here. *Lp* Atonal does not share any of the autapomorphic amino acids found in the loop region of *Drosophila* Cato, which suggests that these proteins are not closely related. Likewise, the first amino acid in *Lp* Atonal Helix1 is a histidine, whereas in *Drosophila* Cato and the arthropod *Amos-Atonal* lineage this residue is an asparagine (although in *Drosophila* Atonal it is a glutamine). Therefore, the phylogenetic affinities of this *Lp* Atonal bHLH domain remain obscure.

Lp Pax6 and Atonal Expression During Embryogenesis

By RT-PCR, both *Lp Pax6* and *atonal* mRNAs are expressed during early (st 0–12), mid (st 13–17), and late (st 18–20) horseshoe crab embryogenesis (Fig. 6A). To determine the tissue localization of these transcripts, we also performed whole mount in situ hybridization. Unfortunately, we were unable to analyze embryos younger than st 13 due to their fragility during riboprobe hybridization at stringent temperatures. Beginning around stages 16/17, we observed *Lp Pax6* mRNA expression in the forming brain (white arrows in Fig. 6B,D,F,H), ventral nerve cord (black arrows in Fig. 6B,D,F,H) and lateral sense organs (Fig. 7K,L; n = 25 experiments). All three expression domains were absent in age-matched sense controls

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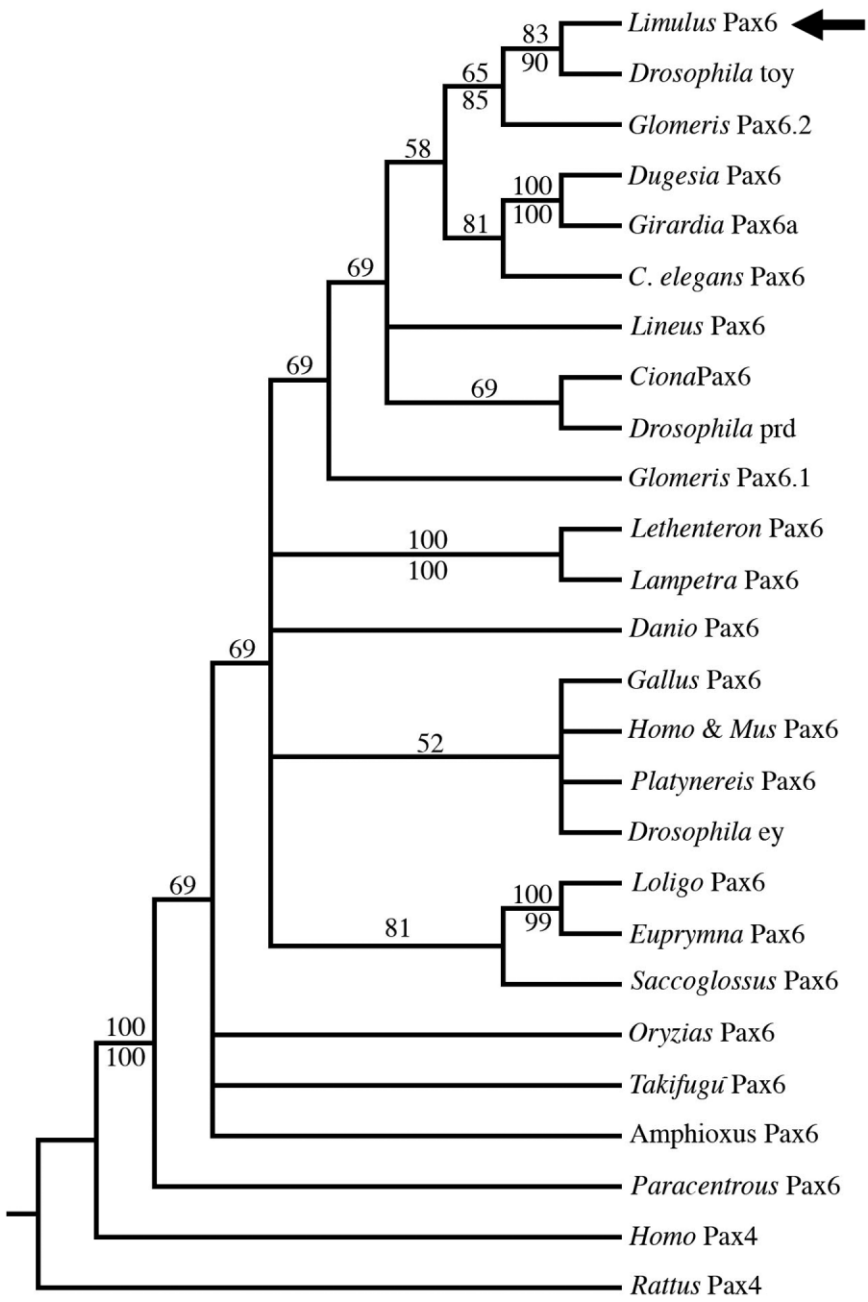

Fig. 3. Phylogenetic analyses of amino acids in the *Lp Pax6* paired domain. Majority rule consensus tree of the 114,805 most parsimonious trees resulting from a heuristic search using maximum parsimony; numbers above branches are the percentage of trees in which the topology appears. Numbers below branches are the posterior probabilities for that topology found through a Bayesian phylogenetic analysis using a mixed amino acid model. *Pax6* paired domains form a clade that is strongly supported by both analyses. Among the *Pax6* paired domains, there is strong support that the *Lp Pax6* paired domain (black arrow) is most similar to that of *Drosophila toy*. There is also strong support from both analyses for clades comprising the paired domains of planarian flatworms (*Dugesia* and *Girardia*), lampreys (*Lethenteron* and *Lampetra*) and mollusks (*Loligo* and *Euprymna*).

(Fig. 6C,E,G,I and data not shown). Among the different *Pax6* expression domains, the lateral sense organ exhibited the most robust expression (Fig. 7K). However, the alkaline phosphatase color reaction product that appeared in evaginating appendages from stage 14 to trilobite larvae was not real *Pax6* mRNA expression, because sense control embryos had the same staining (asterisks in Fig. 6B–I). We also analyzed *Lp atonal* expression in parallel to that of *Pax6*. By contrast, *atonal* is only expressed in the forming lateral sense organ, from st 17/18 to hatching trilobite larvae (Fig. 7I,J and data not shown; n ≥ 25 experiments). For both *Lp Pax6* and *atonal*, no expression was found in any of the diverse eyes developing from stage 13 to trilobite larvae hatching (Figs. 6, 7 and data not shown).

Comparison of *Lp Pax6*, *Atonal*, and *MyoIII* Expression During Eye Formation

Lp MyoIII protein expression has been described for the embryonic lateral, median, and ventral rudimentary photoreceptors, and larval and adult compound lateral eye ommatidial and medial ocellar photoreceptors (Battelle et al., 2001; Harzsch et al., 2006). The *Lp MyoIII* gene encodes a photoreceptor cell-specific, unconventional myosin that is under circadian regulation (Battelle et al., 1998). To establish conditions for *Limulus* whole-mount in situ hybridization and to follow embryonic and larval eye development, we compared *MyoIII* mRNA and protein expression from st 13 through newly hatched larval development (Fig. 7A–H). At st 18, *MyoIII* mRNA expression first appears in rudimentary lateral and median photoreceptor cells (Fig. 7A,B), but no expression was observed in the lateral sense organ. However, the anti-MyoIII antibody weakly labels the rudimentary lateral and median eyes, as well as the lateral sense organ beginning at st 18 (Fig. 7D and Harzsch et al., 2006). Because the MyoIII antisera, but not our *MyoIII* riboprobe, labeled the lateral sense organ, it suggests that another MyoIII-like protein is present in this sensory structure.

At st 20-1, *MyoIII* mRNA is expressed within the first lateral eye ommatidial cells, situated in the center of a crescent of rudimentary photoreceptors (Fig. 7C′ and C″ high magnifications of the lateral eyes in C). *MyoIII* is also maintained in the rudimentary median eye at this older age (Fig. 7C‴). During st 20, a small number of ventral photoreceptor cells express *MyoIII* mRNA (Fig. 7F) and protein (Fig. 7G). MyoIII protein is localized similarly to visual arrestin (VAR, a photoreceptor-specific

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A. *Limulus polyphemus* Atonal class bHLH domains PCR amplified

```
RRLAANARERSRMHSLNIAFDRLREVVPSIGNDRKLSKETLQMA     60% of PCR products
RRLAANARERRRMHSLNEAFDRLRENVPSIGEDRKLSKETLQMA     30% of PCR products
RRLAANARERRRMHSLNEAFDRLRENVHSIGEDRNLSKETLQMA     10% of PCR products
```

B. Partial amino acid sequence of a *Limulus Atonal* homologue

```
RERSRMHSLNIAFDRLREVVPSIGNDRKLSKYETLQMAQSYITALSELLLK
```

C. bHLH domain amino acid alignment

```
                  |  Basic  |   Helix1   |          Loop          |      Helix2     |          % Identity

Limulus Atonal          RERSRMHSLNIAFDRLREVVP--------------SIGNDRKLSKYETLQMAQSYITALSELL

Drosophila Ato    RRLAANARERRRMQNLNQAFDRLRQYLP--------------CLGNDRQLSKHETLQMAQTYISALGDLL          69%
Drosophila Amos   RRLAANARERRRMNSLNDAFDKLRDVVP--------------SLGHDRRLSKYETLQMAQAYIGDLVTLL          73%
Drosophila Cato   RRQAANARERKRMNGLNAAFERLREVVP--------------APSIDQKLSKFETLQMAQSYILALCDLL          71%
Platynereis Ato   RRMAANARERRRMNSLNGAFDRLRSVIP--------------GMKGQRQLSKYETFQ                       67%
Anopheles Ato     RRLAANARERRRMNSLNDAFDRLRDVVP--------------SLGNDRKLSKFETLQMAQTYIAALNELL          81%
Caenorhabditis    RRSAANERERRRMNTLNVAYDELREVLP--------------EIDSGKKLSKFETLQMAQKYIECLSQIL          63%

Homo Ath1         RRLAANARERRRMHGLNHAFDQLRNVIP--------------SFNNDKKLSKYETLQMAQIYINALSELL          78%
Mus Ath1          RRLAANARERRRMHGLNHAFDQLRNVIP--------------SFNNDKKLSKYETLQMAQIYINALSELL          78%
Gallus Ath1       RRLAANARERRRMHGLNHAFDQLRNVIP--------------SFNNDKKLSKYETLQMAQIYISALAELL          76%
Xenopus Ath1      RRLAANARERRRMHGLNHAFDQLRNVIP--------------SFNNDKKLSKYETLQMAQIYINALSDLL          78%
Danio Ath1        RRMAANARERRRMHGLNHAFDELRSVIP--------------AFDNDKKLSKYETLQMAQIYINALSDLL          73%

Homo Ath5         RRLAANARERRRMQGLNTAFDRLRRVVP--------------QWGQDKKLSKYETLQMALSYIMALTRIL          71%
Mus Ath5          RRLAANARERRRMQGLNTAFDRLRRVVP--------------QWGQDKKLSKYETLQMALSYIIALTRIL          71%
Gallus Ath5       RRLAANARERRRMQGLNTAFDRLRKVVP--------------QWGQDKKLSKYETLQMALSYIMALTRIL          71%
Xenopus Ath5a     RRLAANARERRRMQGLNTAFDSLRKVVP--------------QWGEDKQLSKYETLQMALSYIMALSRIL          69%
Danio Ath5        RRMAANARERKRMQGLNTAFDRLRKVVP--------------QWGQDKKLSKYETLQMALSYIMALNRIL          71%

NeuroD1           RRMKANARERNRMHGLNAALDNLRKVVP--------------CYSKTQKLSKIETLRLAKNYIWALSEIL          61%
NeuroD2           RRQKANARERNRMHDLNAALDNLRKVVP--------------CYSKTQKLSKIETLRLAKNYIWALSEIL          61%
Mus Ath2          RRQEANARERNRMHGLNDALDNLRKVVP--------------CYSKTQKLSKIETLRLAKNYIWALSEIL          61%
Mus Ath3          RRVKANARERTRMHGLNDALDNLRRVMP--------------CYSKTQKLSKIETLRLARNYIWALSEVL          59%
Gallus NeuroM     RRVKANARERTRMHGLNDALDNLRRVMP--------------CYSKTQKLSKIETLRLARNYIWALSEVL          59%
Xenopus Ath3      RRVKANARERSRMHGLNDALENLRRVMP--------------CYSKTQKLSKIETLRLARNYIWALSDIL          57%
Ngn1              RRVKANDRERNRMHNLNAALDALRSVLP--------------SFPDDTKLTKIETLRFAYNYIWALAETL          59%
Ngn2              RRLKANNRERNRMHNLNAALDALREVLP--------------TFPEDAKLTKIETLRFAHNYIWALTETL          59%
Ngn3              RRKKANDRERNRMHNLNSALDALRGVLP--------------TFPDDAKLTKIETLRFAHNYIWALTQTL          55%
Xenopus Ngnr1     RRVKANNRERNRMHNLNSALDSLREVLP--------------SLPEDAKLTKIETLRFAYNYIWALSETL          63%

Mus Mash1         ---RRNERERNRVKLVNLGFATLREHVP--------NG------AANKKMSKVETLRSAVEYIRALQQLL          49%
Mus Mash2         ---RRNERERNRVKLVNLGFQALRQHVP--------HG------GANKKLSKVETLRSAVEYIRALQRLL          51%
Drosophila Ac     SVIRRNARERNRVKQVNNGFSQLRQHIPAAVIADLSNGRRGIGPGANKKLSKVSTLKMAVEYIRRLQKVL          45%
Drosophila Sc     SVQRRNARERNRVKQVNNSFARLRQHIPQSIITDLTKGG---GRGPHKKISKVDTLRIAVEYIRSLQDLV          43%
```

Fig. 4. Molecular characterization of *Lp atonal*. **A:** Amino acid sequences of three types of atonal-related basic helix–loop–helix (bHLH) domains PCR amplified from *Limulus* genomic DNA. Degenerate PCR primers were designed for the underlined amino acids, based on *Drosophila atonal* and *Xenopus Ath5* bHLH domains (Brown et al., 1998). **B:** Partial amino acid sequence of a *Limulus atonal* homologue partially cloned. **C:** Comparison of bHLH domain amino acid sequence for *Lp* atonal with those of various invertebrate and vertebrate Atonal/Ath/ATOH proteins in the NIH/NCBI database. Dark shading indicates identical amino acids, light shading similar amino acids to *Lp* Atonal. Percent identities (uncorrected pairwise sequence divergences) are listed in the right column. The *Limulus* bHLH domain shares the highest percent identity with the *Anopheles* (mosquito) *atonal* gene.

protein) in the rudimentary ventral eyes, lateral and median optic nerves (Fig. 7G,H). In animals st 18 and older, *Lp atonal* and *Pax6* mRNA expression was observed in the lateral sense organ that lies ventral to the lateral rudimentary eye (black arrows in Fig. 7I–L). These two sense organs are readily distinguished by their dorsal–ventral positions and distinct morphologies (compare insets in Figs. 7A and 7I). Because we found no evidence of *Lp Pax6* or *atonal* expression analogous to that of *MyoIII*, we conclude that neither gene is expressed during the formation of the three different rudimentary eyes or early lateral compound eye and median ocellar development.

DISCUSSION

Limulus polyphemus (*Lp*) embryology has been studied since the late 1800s. Much more recently, investigations of chelicerate embryonic body patterning and neural development were reported by multiple groups (Telford and Thomas, 1998; Abzhanov et al., 1999; Stollewerk et al., 2001; Dearden et al., 2002, 2203; Mittmann, 2002; Mittmann and Scholtz, 2003; Maxmen et al., 2005; Pioro and Stollewerk, 2006). However, only a handful of developmentally expressed *Limulus* genes have been characterized (Cartwright et al., 1993; Cook et al., 2001; Mittmann and Scholtz, 2001; Davis et al., 2005). Likewise, the *Lp* visual system has been investigated for decades (reviewed in Barlow, 2003; Battelle, 2006) but, the molecular mechanisms of eye development in *Limulus* and other chelicerates, is still unknown. The goal of this study was to link *Lp Pax6* and *atonal* genes to the formation of one or more types of *Limulus* eyes. Much to our surprise, we found neither gene is expressed during the formation of different eyes. Instead, we observed *Lp Pax6* expression in the brain and ventral nerve cord and the expression of both genes in the lateral sense organ.

There are multiple explanations for these findings that are not mutually

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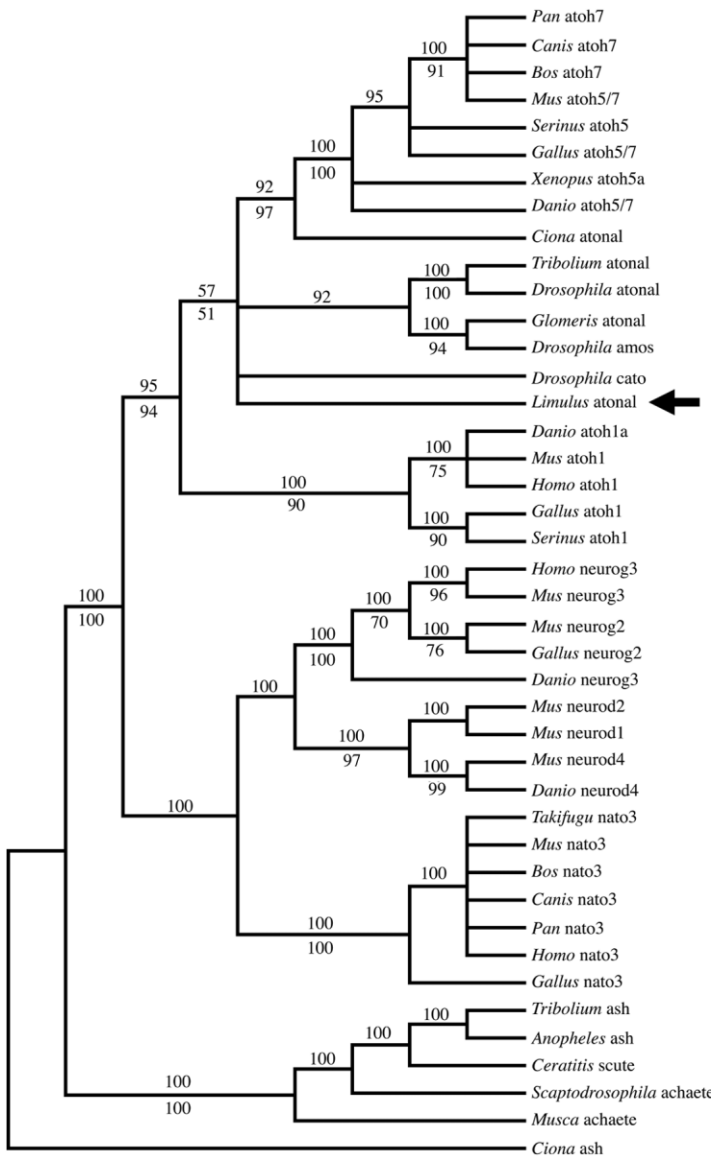

Fig. 5. Phylogenetic analyses of *Lp Atonal* bHLH domain amino acids. Majority rule consensus tree of the 37 most parsimonious trees found in a heuristic search using maximum parsimony; numbers above branches are the percentage of the trees in which the topology appears. Numbers below branches are the posterior probabilities for that topology found through a Bayesian phylogenetic analysis, using a mixed amino acid model. *Ath5/ATOH7*, *Ath1/ATOH1*, and atonal bHLH domains form a clade that is strongly supported by both analyses. Among these bHLH domains, the relationship of the *Lp* atonal bHLH domain (black arrow) to those of other *atonal* genes is unresolved, although there is weak support that it may be more closely related to vertebrate *Ath5* and *atonal* genes.

exclusive. First, because these transcription factors are expressed at younger ages than our in situ experiments could assay, it is plausible they act in the eye primoridia(s) before stage 13. Second, there are other instances of bilaterian eyes that do not express *Pax6*, and this may also be the case for *Limulus*. For example, *Platynereis* larval eyes express *Pax6*, but their adult eyes form by means of a *Pax6*-independent mechanism that involves *So*/*Six* gene expression (Arendt et al., 2002). The myriapod *Glomeris* has two *Pax6* genes, yet neither is associated with eye formation, and amphioxus *Pax6* is present in its multiple eye types, but not the Organ of Hesse that contains photoreceptors (Glardon et al., 1998; Prpic, 2005). Analogously, deuterostome eye specification requires the *Rx* gene that acts upstream of *Pax6* (Furukawa et al., 1997; Mathers et al., 1997; D'Aniello et al., 2006). Finally, multiple *Pax6* and/or *atonal* genes may exist now or during the evolutionary history of *Limulus*, meaning a *Pax6* or *atonal* paralogue may regulate eye development. Indeed, our isolation of three different *atonal*-class bHLH domains, suggests there are multiple *Lp atonal*-like genes. The *Lp atonal* described here is only expressed in the lateral sense organ, a structure of unknown function. The high amino acid identity among *Lp* Atonal, *Drosophila* Amos, and *Mus* Math1 proteins, whose functions in auditory or olfactory development are known (Bermingham et al., 1999; zur Lage et al., 2003), may indicate that the lateral sense organ is involved in auditory or olfactory sensation.

Horseshoe crabs are often considered "living fossils" as they obtained their present morphological form at least by the mid Mesozoic (ca. 160 million years ago, Fisher, 1984), but possibly as early as the late Ordovican (ca. 445 million years ago, Rudkin et al., 2008). However, the last common ancestor of living taxa was more recent, probably within the last 80 million years (Avise et al., 1994). Here, we describe several distinct features of the *Limulus Pax6* gene, as compared to those of other arthropods. Along with the absence of *Pax6* mRNA expression in multiple developing eye types, another unanticipated characteristic of *Lp Pax6* is its predicted multiple splice products that would give rise to distinct isoforms. Intriguingly, distinct splice products and protein isoforms are features of vertebrate *Pax6* genes. Together our findings suggest that *Limulus* embryos use divergent gene networks in their developing visual systems and appendages. In the future, it will be exciting to understand whether these are also characteristics of other chelicerate species. While morphological stasis may characterize horseshoe crab evolution, further research on their embryology may provide important insight into the evolution of novel gene networks.

EXPERIMENTAL PROCEDURES

Animals

The collection protocols of Harzsch et al. (2006) were followed, except that water was changed every other day

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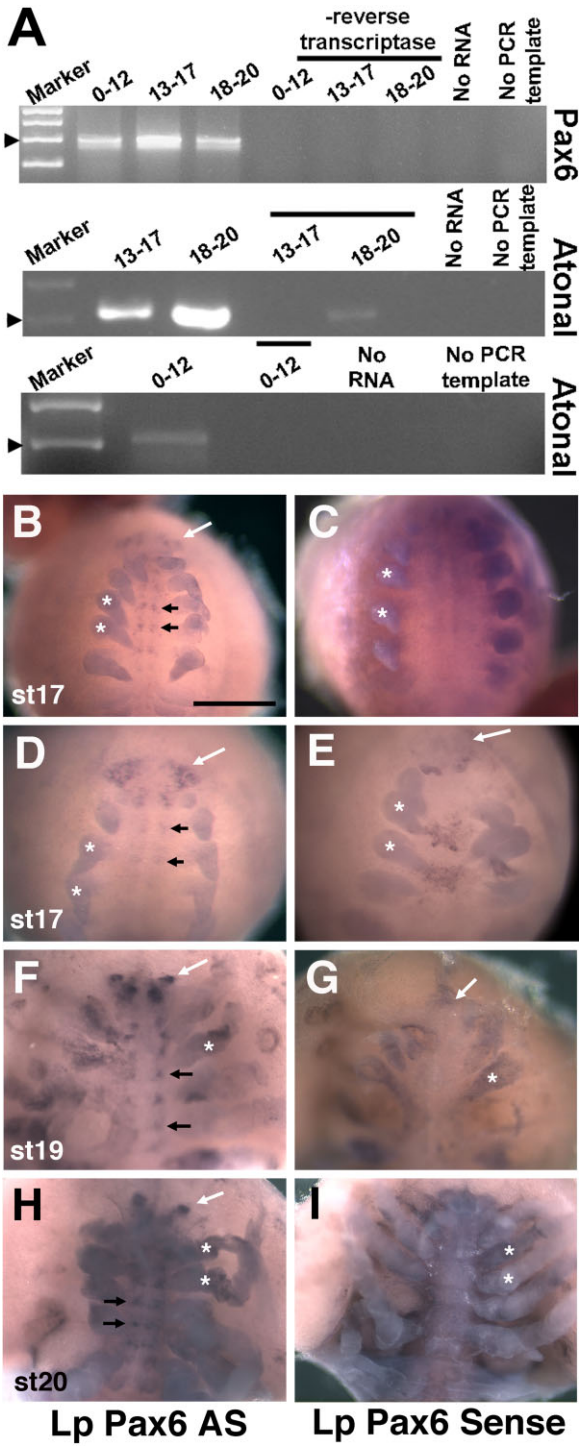

Fig. 6. Embryonic expression of *Limulus Pax6* and *Atonal* mRNA. **A:** Reverse transcriptase-polymerase chain reaction (RT-PCR) for *Lp Pax6* and *Lp Atonal* indicating both genes are expressed throughout embryogenesis to hatching at late st 20. All PCR and RT controls exhibited little to no background amplification. B–I: In situ hybridization for *Lp Pax6* antisense (AS) and sense probes, with all panels showing ventral views. **B–E:** St 17 embryos with *Lp Pax6* mRNA expression in the forming brain (white arrows) and ventral nerve cord (black arrows), compared with sense controls. **F,G:** St 19 embryo with discrete brain (white arrow) and clustered ventral nerve cord (black arrows) expression domains. **H,I:** St 20 embryo with brain lobe (white arrows) and clustered ventral nerve cord expression (black arrows). Asterisks in all panels label forming appendages that are nonspecifically stained in both antisense and sense probed embryos. Embryo staging followed Harzsch et al. (2006). Scale bar = 500 μm in B.

with artificial seawater plus 100 U/ml of penicillin (Sigma) and 100 μg/ml of streptomyocin (Sigma), to prevent microorganism growth that reduced viability. Published embryo staging criteria were used (Sekiguchi et al., 1982; Harzsch et al., 2006). Stage 13–17 embryos were dechorionated in 50% bleach for 5 min, rinsed three times in dH_2O and fixed for 1 hr, rocking in a 50:50 mixture of 4% paraformaldehyde/phosphate buffered saline (PBS) and heptane. The aqueous phase was replaced with methanol and embryos vigorously shaken in heptane–methanol for 30 sec to rupture outer membranes. Both heptane and methanol were replaced with three changes of fresh methanol and embryos stored in methanol at −20°C. Older embryos and trilobite larvae were hand dissected from outer membranes and processed as younger embryos minus dechorionation.

Molecular Cloning

A previously published PCR strategy (Arendt et al., 2002) was used to amplify *Pax6* conserved domains from mixed stage *Limulus* (*Lp*) cDNA. The remainder of the *Pax6* coding region was isolated by means of 5′ and 3′ RACE (rapid amplification of cDNA ends). *Lp atonal* bHLH domains were PCR amplified from genomic DNA, with degenerate primers (Brown et al., 1998) and 40 cycles of 95°C × 30 sec, 60°C × 60 sec, 68°C × 2 min. Three highly related bHLH domains were found. Primers for RACE cloning were designed from the most frequently isolated bHLH domain. 5′ and 3′ RACE for *Lp Pax6* and *Lp atonal* used the SMART RACE cloning kit and protocol (BD/Invitrogen) and mixed embryo cDNA templates. *Pax6:* 5′RACE 5′-TAGAGCAACTGGCGATGATGTCGGAGG-3′; 3′RACE 5′-CAGGAGTGTCCTTCCGTATTTGCCT GG-3′; *atonal*: 3′RACE 5′-CCGTGAACGAAGCCGAAGCACAGTC-3′ gene-specific primers were used. Multiple products from each RACE experiment were DNA sequenced and analyzed with MacVector (v9.0) and AssemblyLIGN (v1.1, Accelrys) computer programs. GenBank accession number for *Lp atonal* is EU673469. The GenBank accession numbers for *Lp Pax6* are: EU673470 Pax6 mRNA predicted

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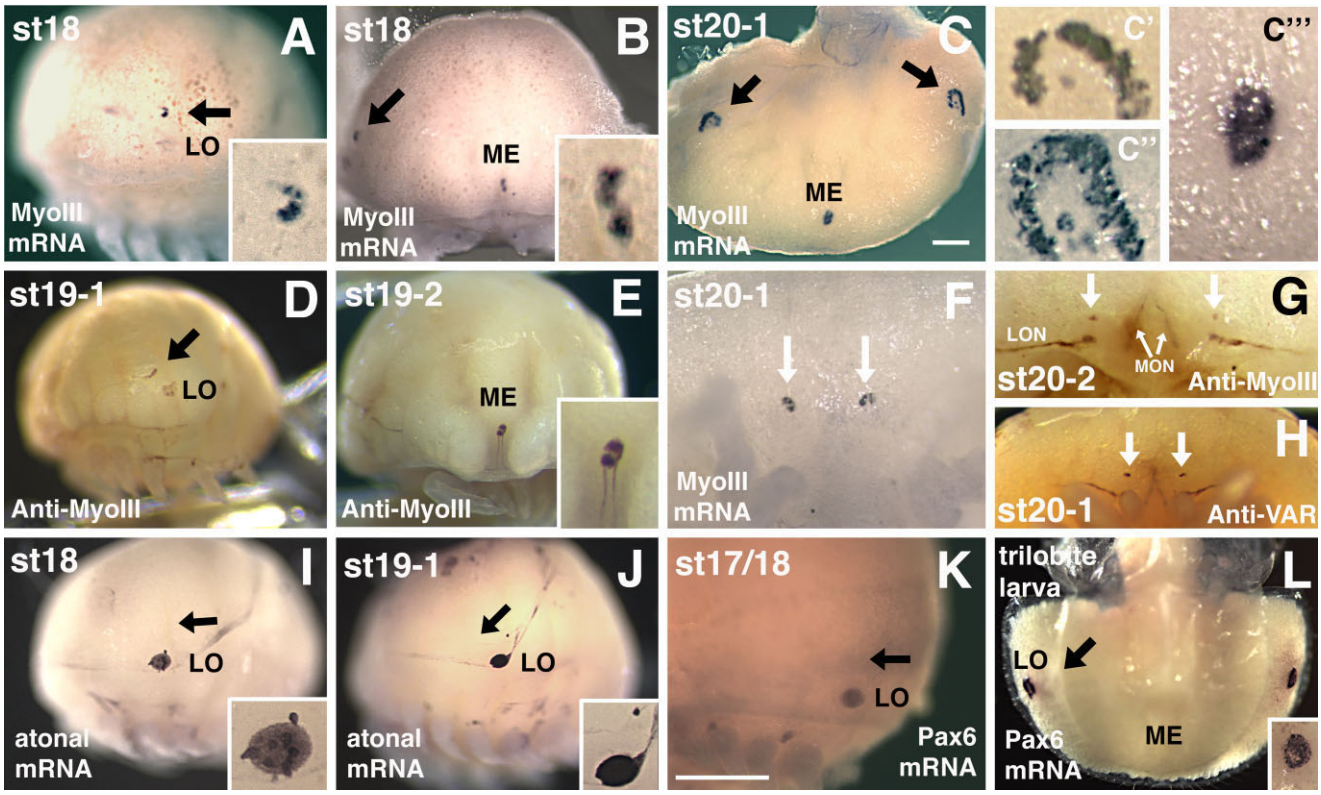

Fig. 7. *Limulus Pax6* and *atonal* are not expressed in developing eyes. A–C,F: *Myosin III* mRNA expression in the rudimentary lateral eye (black arrows in A–C), early lateral ommatidial eyes (C′, C″ show first ommatidial photoreceptors surrounded by crescent of rudimentary photoreceptors), rudimentary median eye (ME; B,C,C‴), and rudimentary ventral photoreceptors (arrows in F). The lateral sense organ (LO) is not involved in vision and does not express MyoIII mRNA (A and data not shown). **D,E:** Anti-Myo III antibody labels the embryonic rudimentary lateral eye (arrow in D), median eye (E), and more weakly the LO (D). **F:** High magnification of ventral photoreceptors (arrows) expressing MyoIII mRNA. **G,H:** Anti-MyoIII (G) and Anti-Visual Arrestin (VAR) staining of ventral photoreceptors (arrows) at approximately the same age as in F. These antibodies also label forming optic nerves innervating the brain from the rudimentary lateral (LON) and median (MON) eyes. **I,J:** *Lp Atonal* expression in the LO but not rudimentary lateral eye (black arrows point to eye). Insets show higher magnification of LO. **K,L:** Throughout ages when MyoIII mRNA and protein are expressed in rudimentary eyes or forming lateral ommatidial eyes, *Pax6* mRNA is observed in the LO but is absent from these eyes (arrow points to rudimentary lateral eye). Panels A,D,I,J and K are lateral; B and E frontal; C and L are top-down dorsal; and F–H are ventral views. Scale bars = 500 μm in A,C; inset magnification is an additional 400×.

to encode the predominant isoform; EU673471 Pax6 mRNA predicted to encode alternate isoform; EU673472 Pax6 5′ RACE clone 2-6,12; Pax6 EU673473 5′ RACE clone 7; EU673474 Pax6 5′ RACE clone 1,8,9; EU673475 Pax6 5′ RACE clone 10; and EU673476 Pax6 5′RACE clone 11.

Genomic Southern Blots

Lp genomic DNA was isolated from six juvenile crabs. Nylon blots (Pall Corp.) containing 5 μg of digested, transferred and immobilized genomic DNA were prehybridized in 50% formamide, 6× standard saline citrate (SSC), 5× Denhardt's, 0.5% sodium dodecyl sulfate (SDS), 100 μg/ml herring sperm DNA (Sigma) at 42°C, and then hybridized at either 37°C or 42°C (Glaser et al., 1992). A total of 1–2 × 10^7 CPM of each gel purified and 32P-labeled cDNA probe was added to 10 ml of hybridization buffer (40% formamide, 4× SSC, 0.8× Denhardt's, 10% dextran sulphate [American Bioanalytical], 7 mM Tris (pH7.4), 100 μg/ml herring sperm DNA). Blots were washed at high (3 times in 2×SSC/0.2%SDS at 55°C, twice in 0.2×SSC/0.2%SDS at 65°C) or low (2 times in 2×SSC/0.2%SDS at 50°C, twice in the same buffer at 55°C) stringency and exposed to X-ray film.

Phylogenetic Analysis

A heuristic search using maximum parsimony in PAUP (v 4.0b10, Altivec) and a Bayesian analysis using MrBayes (v 3.0b4) were performed for both a matrix of Pax6 and related paired domains and a matrix of atonal and related bHLH domains. Amino acid sequences were downloaded from GenBank in FASTA format and aligned using CLUSTALX. For paired domain analyses, the dataset was defined as 139 amino acids comprising the paired domain and homeodomain in Pax6, *Drosophila* eyeless (ey) and twin of eyeless (toy), with human and rat Pax4 included as outgroups. For atonal bHLH analyses, the data set was limited to 70 amino acids that comprise the bHLH domain in atonal homologues and six achaete-scute homologues included as an outgroup. In the Bayesian analyses, the prior probability model for the amino acid rate matrix was set to mixed, allowing the analysis to sample a range of different models for molecular evolution. Bayesian analyses were conducted for one million generations, with trees sampled every 100 generations; sta-

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tionarity was obtained after approximately 10,000 generations, thus the first 100 trees were discarded as burnin.

RT-PCR

Total RNA was isolated from > 100 embryos stage 0–12, and at least 50 embryos for the older age groups, These RNAs were pretreated with DNase before reverse transcription into cDNA. PCR parameters were 40 cycles of 95°C × 30 sec, 60°C × 60 sec, 68°C × 2 min with primers PAX6FOR 5′-TAGCACCACCTAACGGACGACT-TC-3′, PAX6REV 5′-CACCTGGAGAA-ATGACACCTGC-3′; or ATOFOR 5′-CGTGAACGAAGCCGAATGCAC-AGTCT-3′, ATOREV 5′-TGTAAAT-GAGGCTTTCCCACATGTATT-3′.

In Situ Hybridization and Immunohistochemistry

Lp Pax6 and *atonal* antisense and sense cRNA digoxigenin-labeled riboprobes contained coding plus 3′UTR nucleotide sequences. A *MyoIII* antisense riboprobe was synthesized from a full-length cDNA clone (Accession no. AF062069). Several modifications were made to a whole mount in situ protocol (Hargrave and Koopman, 2000). After rehydration into PBS/0.1% Triton X-100, (PBTX) embryos ≥ stage 18 were incubated overnight at room temperature in a 1:50 dilution of 5 U/μl chitinase (Sigma) in DEPC-dH_2O, to remove a nearly invisible chitin cuticle. Then embryos were washed in PBTX and proteinase K digested. After refixation, but before prehybridization, embryos and larvae were heated to 75°C for 30 min to quench endogenous alkaline phosphatase. Nonspecific background was lowered by preincubating a 1:500 dilution of sheep anti-digoxigenin antibody (Roche) in 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% Sheep serum (Sigma), 2% BSA, 0.2% sodium azide (Sigma) plus 1 mg of horseshoe crab protein powder, by means of rocking at 4°C for ≥4 hr. Powder was removed by brief centrifugation, and the antibody used at 1:2,000. Crab powder was made by cold acetone extraction of homogenized juvenile *Lp* soft body parts. Anti MyoIII (1:1,000) or Anti-Arrestin (VAR, 1:50) antibody labeling of *Lp* embryos used published protocols (Davis et al., 2001; Harzsch et al., 2006) and HRP color development.

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