ANALYSIS OF THE GENE REGULATORY MECHANISM CONTROLLING PAX6 EXPRESSION IN TELEOSTS AND MAMMALS

by

JORN LAKOWSKI

(Under the Direction of James D. Lauderdale)

ABSTRACT

The homeodomain transcription factor Pax6 is crucial to the proper development and maintenance of the brain, spinal cord, olfactory system, endocrine pancreas and the eye. A large body of evidence has demonstrated that Pax6 is of particular importance to the visual system and is part of an ancient genetic network of transcriptional regulators that controls various steps during oculogenesis in creatures as diverse as insects and mammals. Within the developing retina, the center piece of the visual system in vertebrates, the Pax6 gene plays several important roles, including control of cell proliferation, maintenance of the retinogenic potential of progenitor cells, and cell fate specification. All of those functions have been attributed to a Pax6 gene product containing a paired-domain, a homeodomain and a C-terminal transactivation domain. However, it was recently postulated that the different aspects of Pax6 function are mediated by different isoforms of the Pax6 protein; however, little is known about the spatiotemporal expression of Pax6 isoforms in the retina. Interestingly, a Pax6 isoform lacking the paired-DNA binding domain was recently identified but its exact expression domains and function remain elusive.

In this study, I have used modified bacterial artificial chromosomes (BAC) from zebrafish and mouse to distinguish the expression pattern of paired containing and paired-less Pax6 transcripts in retina. In both zebrafish and mice, the spatial and temporal onset of expression of these transcripts suggests that the paired-less isoform is involved in the cell fate decision leading to the generation of amacrine cells. Furthermore, I present evidence that the two Pax6 isoforms are differentially expressed within amacrine subpopulations, possibly controlling sub-cell type specification events therein. Furthermore, in an effort to determine the extent of conservation of mechanism controlling Pax6 expression, I have established a complement of transgenic mouse lines, carrying either the zebrafish Pax6a or Pax6b locus. Using this cross species comparison, I show that the gene regulatory control mechanism directing Pax6 activity has been highly conserved within the vertebrate lineage. In addition, my data suggests that the Pax6 loci in zebrafish have been retained due to degenerative and complementary mutations in essential tissue specific regulatory elements.

INDEX WORDS: Pax6, transgene, alternative promoter, retina, eye, zebrafish

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DEDICATION

To my family and friend, who have been so supportive throughout my graduate studies.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Of the five classical senses, vision may be considered the most valued one, as it enables us to obtain and integrate crucial information about our environment. Consequently, the loss thereof, either by disease or injury, has dire consequences for the individual affected and often results in a decreased quality of life. Debilitating degenerative diseases of the eye, such as retinitis pigmentosa, macular degeneration, glaucoma, cataracts, and retinoblastoma are quite common and currently afflict more than 3 million people within the United States alone. Therefore, in recent years, much research has been aimed at finding potential regenerative or replacement therapies for cases where eye sight is lost or severely impaired. In this context, it has become obvious that a thorough understanding of the fundamental principles and mechanisms leading to the proper formation of the visual system is a prerequisite for designing the appropriate treatment strategies for the various pathological conditions (Gong, Yang et al. 2002; Zaghloul, Yan et al. 2005).

In vertebrates, the eyes develop from an interaction of the neural tube with the overlying surface ectoderm (Graw 1996). During gastrulation, the involuting endoderm and mesoderm endow the adjacent anterior head ectoderm with a latent lens forming capability. However, the first morphological sign of oculogenesis, the optic vesicle, is visible as a bilateral evagination of the diencephalon during the early neurula stage. As development progresses, the optic vesicle extends towards the overlying non-neural surface ectoderm, displacing the mesenchyme tissue

separating both structures. Upon contact, the optic vesicle then induces the formation of the lens vesicle by a diffusible, but not yet identified signal. Interestingly, the signaling between lens placode and optic vesicle at this point in development is not unidirectional but reciprocal (Spemann 1938). This is obvious from recent experiments, in which ablation of the lens forming ectoderm prevented the proper formation of the optic cup from the optic vesicle (Hyer, Kuhlman et al. 2003). Analysis of chick eye morphogenesis has implicated fibroblast growth factors (FGFs), emanating from the surface ectoderm, in the process of optic cup formation (Hyer, Mima et al. 1998).

Following lens induction, the concerted morphogenetic movements of the lens vesicle and the double layer optic cup result in the establishment of the basic structure of the eye. Eventually, the lens vesicle pinches off from the surface ectoderm and assumes its characteristic polarity, in which a mitotically active anterior monolayer of lens epithelial cells overlies a posterior located terminally differentiated lens fiber cell mass. In addition, proliferating secondary lens fiber cells are positioned around the equatorial zone of the lens, directly juxtaposed to the ciliary body of the retina. The transparent cornea of the adult will be formed from the remaining surface ectodermal placode and neural crest derived mesenchyme, in a highly coordinated and multi-step process. First, the corneal epithelium, overlying the lens vesicle, secretes a collagen rich extracellular matrix, attracting a wave of mesenchyme cells. These cells then migrate into the space between the lens vesicle and the surface ectoderm, condensing and forming several layers of cells that constitute the corneal precursors. During their maturation process corneal precursors create tight junctions with each other and elevated levels of thyroxin then lead to dehydration and subsequent compaction of this posterior stroma.

The retina, essentially being an extension of the brain, is one of most highly conserved parts of the central nervous system in vertebrates and its relative simple structure and accessibility have made it a prime focus for the investigation of fundamental neurological processes and development (Masland 2001). The adult retina is structured as a laminar neural network, containing sensory neurons, interneurons as well as projection neurons, whose combined action makes possible sophisticated image detection, processing and signal transmission. In contrast, the retinal primordium, consists of a pseudostratified neural epithelium in which proliferating progenitor cells span the entire width between ventricular and vitreal surface (Gong, Yang et al. 2002).

Derived from the bilayered optic cup, the developing retina consists of an outer pigmented epithelium (pigmented retina) and an inner neural retina. While BMP signals from the surrounding mesenchyme induce the formation of pigmented epithelium, it has been shown that certain FGF signals from the surface ectoderm are required for the formation of the neural portion of the retina (Hyer, Mima et al. 1998; Hyer, Kuhlman et al. 2003; Dias da Silva, Tiffin et al. 2007). Whereas the pigmented retina has a supportive role for the later, the neural retina, on the other hand, is arguably the core of the vertebrate visual system. Within the inner layer of the optic cup, seven major cell types are generated from a common pool of precursor cells in certain order, which is relatively conserved within the vertebrate lineage (Turner and Cepko 1987; Wetts and Fraser 1988; Glaser, Jepeal et al. 1994; Kageyama, Ishibashi et al. 1997; Ashery-Padan, Zhou et al. 2004). Cellular differentiation is initiated in the center of the inner optic cup and proceeds concentrically, eventually reaching the tips of the optic cup. Preceding this differentiation process is a rapid amplification of retinal precursors, resulting in the creation of a large pool of undifferentiated cells that are used in subsequent developmental steps.

Retinal ganglion cells, the projection neurons of the eye, appear first, followed closely by horizontal cells. Cone-photoreceptors, amacrine and bipolar interneurons as well as rod-photoreceptors are subsequently generated in overlapping phases, establishing the characteristic multi layered structure to the mature retina. Mueller glia cells, ensuring structural integrity and possibly serving as a source of retinal stem cells, are generated last during retinogenesis. Therefore, the development of the retina is a three step process, encompassing, proliferation of the uncommitted progenitors, neurogenesis and eventually gliogenesis.

One of the main challenges for researchers in this field has been to unravel the molecular mechanisms, regulating the differentiation process of the various cell types from a multipotent precursor cell. The rationale being that once the system is fully understood, this knowledge could then be applied to various stem cell sources; e.g. leading undifferentiated cells down a particular pathway and eventually creating custom cells that can be used to treat pathological conditions.

A series of recent studies have revealed that the fate of the retinal precursors depends on the concerted action of both intrinsic and extrinsic signals. Furthermore, it has been shown that the competence of precursors to generate either of the possible retinal cell types, changes with progression of retinogenesis (Cepko, Austin et al. 1996; Cepko 1999; Livesey and Cepko 2001). Several secreted factors have been implicated in controlling certain steps during retinogenesis; among those are classical signaling molecules such as Sonic Hedgehog (Shh), TGF-β/BMP molecules, epidermal growth factors (EGF) and fibroblast growth factors (FGF). Within this group Shh and BMP-4, as well as related molecules, play a special role as they appear to act at different stages during retinogenesis and the preceding steps. The opposing activities of Shh, ventral, and BMP-4, dorsal, have been shown to define the dorsal-ventral polarity of the early optic vesicle, by controlling the expression of certain domain specific transcription factors such

as Tbx5 and Vax1/2, respectively (Gong, Yang et al. 2002). At later stages, Shh was shown to promote the progression of the proximo-distal wave of cellular differentiation as well as the retinal ganglion cell fate (Xu, Zhang et al. 1999). On the other hand, Activin A, a member of the TGF-β family, has an effect on photoreceptor differentiation (Davis, Matzuk et al. 2000). Furthermore, certain FGF, EGF growth factors as well as leukemia inhibiting factor (LIF) promote the production of particular retinal cell types from progenitors, while suppressing the generation of others (Pittack, Grunwald et al. 1997; Ashery-Padan, Zhou et al. 2004).

In addition to those extrinsic cues, a complex network of cell-autonomous factors is beginning to emerge, mediating the responsiveness of retinal precursor cells to extrinsic signals. Within this network, basic helix-loop-helix (bHLH) transcriptional activators and repressors have been shown to play a central role in the determination of the various retinal cell types (Kageyama, Ishibashi et al. 1997). While bHLH repressors, such as Hes-1 and Hes-5, maintain the progenitor pool and promote gliogenesis, bHLH transcriptional activators, like Math5, Mash1, Math3 or NeuroD, antagonize repressor function and initiate neural differentiation. However, it has become clear that other transcriptional regulators, namely homeodomain transcription factors, are required for the correct assembly of all retinal cell types. It has been postulated that homeodomain proteins such as Pax6, Prox1, Six3, Crx, Chx-10 and Otx-2 determine the layer specificity within the retina, while the neuronal identity of post mitotic cells depends on the combinatorial expression of certain bHLH proteins (Burmeister, Novak et al. 1996; Inoue, Hojo et al. 2002; Dyer 2003; Ashery-Padan, Zhou et al. 2004).

One of the most investigated players in this process is the homeodomain transcription factor Pax6. Pax6 is a member of the vertebrate paired box family of transcription factors, which were discovered through their similarity to the *Drosophila* segmentation gene paired *(Noll 1993)*.

Interestingly, Pax6 proteins from different organisms display a remarkable similarity even between distantly related species such as humans, mice or zebrafish, pointing to an evolutionarily conserved function (Onuma, Takahashi et al. 2002). Because of its involvement in eye morphogenesis it has often been described as the "master control gene" (Gehring 1996; Baker 2001; Tsonis and Fuentes 2006). In fact, it appears that Pax6 is located at the top of an evolutionarily conserved cascade of transcriptional regulators that control the development of ocular structures from insects to mammals (Wawersik and Maas 2000).

In vertebrates, the importance of Pax6 is demonstrated by a vast variety of known mutations, all having profoundly detrimental effects on eye and central nervous system development. Haploinsufficiency for Pax6 results in aniridia in humans and the *Small eye* phenotype in rodents, both heritable panocular disorders associated with foveal and iris hypoplasia, cataracts, corneal opacification as well as glaucoma (Glaser, Walton et al. 1992; Glaser, Jepeal et al. 1994; Grindley, Davidson et al. 1995). Homozygosity on the other hand, results in neonatal lethality in mammals (Glaser, Jepeal et al. 1994; Grindley, Davidson et al. 1995).

Similarly, the expression of Pax6 is highly conserved between different species. In mice, transcripts are first detected in the anterior neuroectoderm on day 8 of embryonic development (Callaerts, Leng et al. 2001). On day 8.5, expression is seen in the region that will eventually give rise to the optic cup. On day 10.5 Pax6 positive structures include the developing eyes, nose, diencephalon, rhombencephalon, neural tube, nasal placode and pancreas (Stoykova and Gruss 1994; Kammandel, Chowdhury et al. 1999). In contrast, expression in the adult is only maintained in the eye, cerebellum and the endocrine pancreas.

During early oculogenesis Pax6 is expressed in proliferating neural progenitor cells throughout the optic cup and in cells within both lens and the cornea (Chow and Lang 2001). As

development progresses Pax6 expression assumes a characteristic distal-high to proximal-low gradient within the optic cup. After retinal differentiation has been completed only amacrine, ganglion and horizontal cells maintain Pax6 expression. Pax6 expression in the lens and cornea is maintained throughout adulthood.

In both human and mouse, the Pax6 gene, encodes an identical 422 amino acid protein that contains four distinct domains (Glaser, Walton et al. 1992; Kammandel, Chowdhury et al. 1999). The characteristic paired domain (PD) is located at the N-terminus followed by a glycine-rich linker region, which connects to a central paired-type homeodomain (HD). Both PD and HD function in DNA binding, and can act independently as well as cooperatively, resulting in a large regulatory capacity (Epstein, Cai et al. 1994; Epstein, Glaser et al. 1994; Czerny and Busslinger 1995). In addition, Pax6 contains a C-terminal proline-serine-threonine-rich transactivation domain (PST). The genomic organization of the Pax6 locus in vertebrates, including human, mouse and quail, is highly conserved (Plaza, Dozier et al. 1993; Turque, Plaza et al. 1994; Plaza, Dozier et al. 1995). Among vertebrates certain teleost fish, such as zebrafish, take a special place as they have been shown to be the only lineage to possess two Pax6 genes. It is generally assumed that these paralogs arose through a gene duplication event, which happened at the split that gave rise to the teleost and mammalian lineages (Nornes, Clarkson et al. 1998). However, why both Pax6 genes have been preserved in these species is not well understood.

Studies from our lab and others have shown that the vertebrate Pax6 transcription unit contains between 15 and 17 exons, depending on the species under consideration. Transcription initiates from three promoters, which for historical reasons are designated as P0, P1, and P-*alpha*. Transcription is controlled by an array of modular tissue specific control elements located upstream, within and downstream of the Pax6 gene (Kammandel, Chowdhury et al. 1999;

Kleinjan, Seawright et al. 2001; Kleinjan, Seawright et al. 2004; Morgan 2004; Kleinjan, Seawright et al. 2006; Tyas, Simpson et al. 2006). Furthermore, it has been reported that the different Pax6 promoters are differentially active with respect to time and space during development (Anderson, Hedlund et al. 2002). Interestingly, the P-*alpha* promoter is located downstream of the canonical translation start site in exon 4 and has been shown to give rise to a paired-less isoform of the Pax6 protein (Kim and Lauderdale 2006). Isoforms lacking the paired DNA binding domain [Pax6(-PD)] have now been described in several species including *C.elegans*, quail and most recently in mouse (Turque, Plaza et al. 1994; Chisholm and Horvitz 1995; Xu, Zhang et al. 1999; Kim and Lauderdale 2006).

Initially, a study carried out in a quail primary cell culture system was able to show that a short Pax6 isoform lacking the paired DNA binding domain was present in the developing quail neural retina and pancreas, but not in the central nervous system (Turque, Plaza et al. 1994). It was suggested that translation of this truncated form of Pax6 was initiating at a start site in Pax6 exon 7, but the source of the transcripts was not determined. Subsequently, the first functional information concerning this truncated isoform was obtained through a study in *C. elegans*. In these nematodes, the Pax6 locus was shown to encode for two distinct Pax6 isoforms, either with (Vab3) or without the paired-domain (Mab-18), which, depending on the cellular context, could interact additively, antagonistically or synergistically. Furthermore, this study showed that the truncated, paired-less isoform of the *C. elegans* Pax6 ortholog was crucial for the proper development of the male specific sensory organ, with transcripts originating from an internal promoter (Xu, Zhang et al. 1999).

Recent work in mice suggested that non-paired containing transcripts can arise either by alternative splicing or through the utilization of the intragenic P-*alpha* promoter (Gorlov and

Saunders 2002; Kim and Lauderdale 2006; Kleinjan, Seawright et al. 2006). However, information on the function of the paired-less Pax6 isoform in vertebrates is very limited. So far the only functional data available in vertebrates was obtained through an overexpression approach using BAC or YAC modification technology. It was shown that when overexpressed from a mouse bacterial artificial chromosome (BAC) or a human yeast artificial chromosome (YAC), containing the complete Pax6 transcription unit, the pairedless isoform could cause a severe micropthalemic phenotype (Kim and Lauderdale 2006; Kleinjan, Seawright et al. 2006). Although, the mechanism of action is not yet clear, these results show that pairedless Pax6 can participate in the process of oculogenesis. Intriguingly, a recent study in a zebrafish *in vitro* system demonstrates that the pairedless isoform was capable of enhancing full-length Pax6 transcriptional activation (Mikkola, Bruun et al. 2001). However, it is currently unknown if this mechanism is also at work under *in vivo* conditions.

Most of our current knowledge of the role of Pax6 during oculogenesis comes from the study of both naturally occurring and induced Pax6 mutations, as well as targeted deletions of the Pax6 gene in mice (Grindley, Davidson et al. 1995; Li, Lu et al. 2006; Curto, Lara et al. 2007). Mutations of the Pax6 gene in mice are characterized by a small-eye (*Sey*) phenotype, mimicking the human panocular disease Aniridia. Although Pax6 null mutants in mice initiate the formation of the optic vesicle, retinal structures subsequently degenerate, due to the absence of the lens placode. It is believed that the optic vesicle induces the lens placode by secretion of a molecule(s) and that Pax6 confers the ability to respond to these signals. Furthermore, a reduced proliferative capability in the developing lens has been shown for heterozygous mutant cells (Xu, Zhang et al. 1999).

Unfortunately, the analysis of Pax6 functions during later stages of oculogenesis, e.g. the specification of the various retinal cell types, is precluded by the severity of the *Sey* pheno-type. However, the generation of a conditional knock-out mouse system, using the Cre-lox approach, has shed some light on aspects of Pax6 function in the process of retinogenesis (Ashery-Padan, Zhou et al. 2004). Elimination of the Pax6 gene from parts of the optic cup in this fashion clearly demonstrated that Pax6 function was required for the proper generation of the retinal cell types from a common precursor cell. Interestingly, of all retinal cell types available, only amacrine cells were generated by Pax6 deficient progenitors in this study. This result has been interpreted by some that Pax6 function is not required for the generation of amacrine cells. However, the same study clearly showed not all amacrine cell subtypes were produced, suggesting that Pax6 function is in fact required to generate certain amacrine subtypes.

A recent study by Philips et al. has investigated the effects of Pax6 loss of function during retinogenesis in homozygous *Sey* mice (Philips, Stair et al. 2005). The authors presented convincing evidence that Pax6 deficient progenitor cells in the mutant retina exited the cell cycle prematurely, and adopted generic, rather than specific neural fates during their short existence. These results indicated that Pax6 controls the timing of differentiation as well as cell type decisions made by progenitors.

How can we reconcile these seemingly contradictory results? One possibility is that different aspects of Pax6 function are carried out by different isoforms of the Pax6 protein. Supporting this notion is a finding that a specific Pax6 isoform, Pax6(5a), carrying an additional 14 amino acids in the paired DNA binding domain, is specifically required for proper iris development (Singh, Mishra et al. 2002). Intriguingly, based on studies in quail, it has been suggested that a pairedless isoform of the Pax6 protein [Pax6(-PD)] is specifically involved in the process of

retinogenesis (Turque, Plaza et al. 1994). Unfortunately, besides the absence of the paired DNA binding domain, both Pax6(PD) and Pax6(-PD) are practically identical and can therefore not be distinguished, using conventional methods such as immunohistochemistry or in situ hybridization.

In this study, I have focused on elucidating the role of Pax6(-PD) in the process of retinal cell type specification in vertebrates. To this end, I have employed a novel BAC modification strategy, which has allowed me to establish for the first time, the spatial and temporal expression patterns of this protein in both zebrafish and mice. Furthermore, by creating a complement of transgenic mice carrying either the zebrafish Pax6a or Pax6b locus, I have investigated the extent of evolutionary conservation of tissue specific Pax6 expression during development in a genome-like context.

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CHAPTER 2

MECHANISM CONTROLLING THE EXPRESSION OF PAX6 ISOFORMS IN THE EYE HAS BEEN CONSERVED BETWEEN TELEOSTS AND MAMMALS[1]

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Abstract

The Pax6 gene plays several roles in retinal development, including control of cell proliferation, maintenance of the retinogenic potential of progenitor cells, and cell fate specification. Emerging evidence suggests that these different aspects of Pax6 gene function are mediated by different isoforms of the Pax6 protein; however, relatively little is known about the spatiotemporal expression of Pax6 isoforms in the vertebrate retina. Using bacterial artificial chromosome (BAC) technology, we modified a zebrafish Pax6a BAC such that we could distinguish paired containing Pax6a transcripts from pairedless Pax6a transcripts. In the zebrafish, the spatial and temporal onset of expression of these transcripts suggests that the pairedless isoform is involved in the cell fate decision leading to the generation of amacrine cells; however, because of limitations associated with transient transgenic analysis, it was not feasible to establish whether this promoter was active in all amacrine cells or in a specific population of amacrine cells. By making mice transgenic for the zebrafish Pax6a BAC reporter transgene, we were able to show that paired containing and pairedless Pax6a transcripts were differentially expressed in amacrine subpopulations. Our study also directly demonstrates the functional conservation of the regulatory mechanisms governing Pax6 transcription in teleosts and mammals.

Introduction

The vertebrate retina contains six types of neurons and one type of glial cell, which are arranged in three distinct cellular layers. The outer nuclear layer (ONL) contains the rod and cone photoreceptors. The inner nuclear layer (INL) contains the cell bodies of the horizontal, bipolar, and amacrine interneurons and also Müller glial cells. The ganglion cell layer (GCL) contains ganglion and displaced amacrine cells. Birthdating studies have revealed that these distinct cell types are generated in a temporal order that appears to be (La Vail, Rapaport et al. 1991) generally conserved between fish, amphibians, birds, and mammals (Glaser, Jepeal et al. 1994; Belecky-Adams, Cook et al. 1996; Cepko, Austin et al. 1996; Hu and Easter 1999). The retinal ganglion cells (RGCs) differentiate first followed by horizontals, cones and amacrines; rods, bipolars and Müller glial cells differentiate last. Although there is a temporal order to the appearance of the different cell types, there is extensive overlap in the periods during which each cell type is produced. Lineage tracing studies have revealed that the different types of cells in the retina arise from a common multipotent progenitor (Turner and Cepko 1987; Holt, Bertsch et al. 1988; Wetts and Fraser 1988; Turner, Snyder et al. 1990; Glaser, Jepeal et al. 1994). These and other findings (Belliveau and Cepko 1999; Belliveau, Young et al. 2000) have given rise to the current model of retinogenesis in which cell fate decisions are governed by the interplay of extrinsic and intrinsic factors (Harris 1997; Cepko 1999; Livesey and Cepko 2001; Ashery-Padan, Zhou et al. 2004).

In the Competence model of retinal development (Cepko, Austin et al. 1996), retinal progenitors pass through successive and intrinsically distinct competence states, during each of which the progenitors are competent to produce a subset of retinal cell types (Livesey and Cepko 2001).Competence states appear to be largely controlled by intrinsic factors, especially genes

encoding for transcription factors from the basic helix-loop-helix (bHLH; Mash1, Math3, Math5, NeuroD1, Ngn2, Hes1, Hesr2, Bhlhb4), forkhead/winged helix (Foxn4), and homeodomain (Six3, Prox1, Crx, Otx2, Chx10, Rax, and Pax6) families ((Burmeister, Novak et al. 1996; Brown, Patel et al. 2001; Li, Gu et al. 2001; Yan, Ma et al. 2001; Inoue, Hojo et al. 2002; Dyer, Livesey et al. 2003; Ashery-Padan, Zhou et al. 2004; Bramblett, Pennesi et al. 2004; Brudno, Poliakov et al. 2004). Within a competence state, the generation of a particular cell type is controlled by environmental signals (Guillemot and Cepko 1992; Altshuler, Lo Turco et al. 1993; Kelley, Turner et al. 1994; Austin, Feldman et al. 1995; Kelley, Turner et al. 1995; Kelley, Turner et al. 1995; Lillien 1995; Ezzeddine, Yang et al. 1997; Xu, Zhang et al. 1999; Furukawa, Mukherjee et al. 2000).

Of the intrinsic factors, the Pax6 gene stands out because it plays several key roles in eye development. First, Pax6 is required for normal optic vesicle formation. Null mutations in Pax6 result in early arrest of eye morphogenesis at a primitive optic vesicle stage (Grindley, Davidson et al. 1995). Although arrested, molecular marker analyses reveal that these mutant optic vesicles are patterned into optic stalk, neural retina, and retinal pigmented epithelium (Baumer, Marquardt et al. 2003), however, these mutant optic vesicles exhibit reduced proliferation, coupled with precocious neurogenesis (Philips, Stair et al. 2005). Second, Pax6 is required in the optic cup to maintain both the proliferative state and retinogenic potential of retinal progenitor cells (RPCs). Conditional inactivation of Pax6 in the RPCs of the distal cup just prior to the onset of cell differentiation, resulted in both reduced RPC proliferation and a restriction in the Pax6 deficient RPCs to the GABAergic amacrine cell fate (Ashery-Padan, Zhou et al. 2004). In contrast misexpression of Pax6 in developing retinal explant cultures generated undifferentiated cells in the INL (Inoue, Hojo et al. 2002). Together, these studies indicate that the Pax6 gene has

at least three overlapping functions in the neural retina: promotion of progenitor proliferation, maintenance of progenitor potential, and regulation of the timing of cellular differentiation. However, it is not clear how the Pax6 gene performs these different tasks.

One possible explanation is that different isoforms of the Pax6 protein carry out different developmental functions. Three isoforms of the Pax6 protein have been reported in vertebrates: canonical Pax6, Pax6(5a), and pairedless Pax6 (Pax6-PD). The canonical form of Pax6 contains two DNA-binding domains, the paired domain (PD) and paired-type homeodomain (HD), which are linked by a glycine-rich region, followed by a proline-serine-threonine (PST) rich transactivation domain (Walther and Gruss 1991; Glaser, Walton et al. 1992). The second Pax6 isoform, Pax6(5a), is similar to canonical Pax6 with the exception of the PD. This isoform is generated by alternative splicing and contains an exon 5a-encoded 14 amino acid insertion in the N-terminal subunit of the PD (Walther and Gruss 1991; Glaser, Walton et al. 1992; Epstein, Glaser et al. 1994). In contrast with the other two isoforms, the third isoform, Pax6(-PD), lacks the paired-domain (Carriere 1993; Kim and Lauderdale 2006). Transcripts from an internal Pax6 promoter encode for Pax6ΔPD (Kammandel, Chowdhury et al. 1999; Kim and Lauderdale 2006).

Recent studies addressing the function of the paired domain and homeodomain have demonstrated that both the PD and HD are important for the regulation of proliferation and cell fate in the eye (Dominguez, Ferres-Marco et al. 2004; Haubst, Berger et al. 2004; Azuma, Tadokoro et al. 2005; Azuma, Tadokoro et al. 2005), however, little is known about the expression or function of the pairedless isoform (Pax6-PD). Although it was shown previously that Pax6(-PD) exhibits a restricted expression in cells of the developing quail neural retina

(Carriere 1993), that study was carried out using a primary cell culture approach and little could be said about the distribution of Pax6(-PD) in the embryo.

In this study, we tested the idea that Pax6(-PD) exhibits a restricted expression pattern within the developing eye. We provide the first direct evidence that Pax6(-PD) exists in teleosts. Using a dual reporter Pax6a BAC transgene, we examine the spatiotemporal expression of paired containing and paired-less Pax6a transcripts in the developing zebrafish embryo. We show that Pax6a paired transcripts are expressed by most, if not all, cells that express Pax6a, and that the Pax6a pairedless isoform is expressed within the retina in developing amacrine cells in zebrafish. We further demonstrate that the regulatory elements governing Pax6a transcription are functionally conserved between zebrafish and mice. Finally, we demonstrate that Pax6(-PD) transcripts are expressed in a population of GABAergic, but not glycinergic, amacrine cells in the adult mouse retina.

Materials and Methods

Zebrafish strains

Wild-type ("outbred", WIK or TL) embryos were obtained from zebrafish (Danio rerio) lines maintained following standard procedures (Westerfield, 2000). "Outbred" lines were originally obtained from a Florida fish farm and have been maintained for several years in our colony. WIK and Tuebingen long fin were obtained from the Zebrafish International Resource Center (ZIRC). Embryos were staged by hours postfertilization (hpf or h) and by standard staging criteria (Kimmel, Ballard et al. 1995). Gene symbols according to ZFIN (http://zfin.org). Organization of the Pax6a gene Each Pax6a exon was placed by comparison of Pax6a cDNA sequences with the genomic sequence from linkage group 25. The genomic sequence flanking each exon was then

used to define intron-exon boundaries using established criteria for donor and acceptor sites (Mount 1982; Jackson 1991; Burset, Seledtsov et al. 2000) (Shapiro and Senapathy 1987). To be consistent with the nomenclature used to describe the Pax6 loci of human (Ton, Miwa et al. 1992), mouse (Kammandel, Chowdhury et al. 1999; Xu, Zhang et al. 1999) and quail (Plaza, Dozier et al. 1993), the 5'-most exon of Pax6a was designated as "exon 0," and the third promoter was designated as "alpha." A detailed description will be provided in a separate publication.

BAC modification

Zebrafish BAC clone DKEYP-46C10 (Genebank accession: AL929172) was modified by targeted insertion of an enhanced green fluorescent protein (EGFP) pA reporter cassette into Pax6a exon 4 followed by targeted insertion of a Discosoma sp. red fluorescent protein (DsRed; Contech) pA reporter cassette into Pax6a exon 8, using the prophage BAC modification system (Yuan, Liang et al. 2000; Lee, Yu et al. 2001). The EGFP-FRT-kan-FRT targeting cassette was PCRamplified from pCS2+MTeGFP-FRT-kan-FRT (gift of X. Fan and S. Dougan, UGA) using the ax6a exon 4 forward targeting primer, Pax6a-E4-RecFor, 5′-AT-AGAATACTATAACCGG-GCCACGTGGGAGTCTGGTGTCGCGTCCATGATGGTGAGCAAGGGCGAGGAG-3′ and the exon 4 reverse targeting primer, Pax6a-E4-RecRev, 5′-AC-ATACGTCTTTACCAATATAC-CAAAACAAATGCCTTTATGGCTAGATTGTATTCCAGAAGTAGTGAG-3′. The DsRed-FRT-kan-FRT targeting cassette was PCR-amplified from pCS2+DsRed-FRT-kan-FRT using the Pax6a exon 8 forward targeting primer, Pax6a-E8-RecFor, 5'-GGTGAGAACACAAACT-CATAAGCTCCAATGGCGAGGACTCAGAT-GAGACCCAAATGGCCTCCTCCGAGGA-CGTCA-3' and the exon 8 reverse primer, Pax6a-E8-RecRev, 5'-TTTGTAACAGATGCTGTA-

ATATTCGCTATAATCTCCATGTTT-ACCTTTTTCAAGTA-TTCCAGAAGTAGTGAG-3'. Nucleotides in bold italics are homologous to Pax6a sequences and those in roman are homologous to the amplification cassettes. SV40 late polyadenylation (polyA or pA) addition sequences are located immediately after both the EGFP and DsRed open reading frames. The late polyA signal of SV40 is generally more efficient than the SV40 early polyA signal in mammalian cells (Carswell and Alwine 1989). The PCR products were gel purified using a Qiaquick gel extraction kit (Qiagen) and Dpn I treated to remove template plasmid before use for homologous recombination.

The pCS2+DsRed-FRT-kan-FRT plasmid was constructed in two steps. First, a 0.7 kb BamHI/EcoRI fragment encompassing the coding sequence of Discosoma red fluorescent protein from pDsRed-Express (Clontech) was subcloned into pCS2+ (gift of D. Turner, University of Michigan; (Turner and Weintraub 1994) to generate pCS2+DsRed. Second, the FRT-kan-FRT SacII fragment of pIGCN21 (gift of N. Copeland, NCI; (Lee, Yu et al. 2001)) was inserted into the SacII site of pCS2+DsRed. Recombinogenic targeting was performed following the protocol of Lee et al. (Lee, Yu et al. 2001; Gong, Zheng et al. 2003). The EGFP pA reporter cassette was inserted into the BAC first. Double-resistant colonies (CmR KanR) were assayed for homologous recombination by PCR using the following primers: E4F1 (5′-CTGGACATAAGT-GTATTGTGGAG-3′), E4R1 (5′-CCTTGAAGAAGATGGTGCG-3′), E4F2 (5′-TGCTGCCCG-ACAACCACTAC-3′), and E4R2 (5′-CTCTCTCTAACACACACA-GACGC-3′). The kanamycin cassette was flipped out by induction of flipase, and the cells were screened for kanamycin sensitivity (KanS). In these colonies, removal of the kanamycin cassette was verified by PCR using the E4F1, E4F2, E4R1, and E4R2 primers. The DsRed pA reporter cassette was then inserted into the EGFP-modified BAC. Double-resistant colonies (CmR KanR) were

assayed for homologous recombination by PCR using the following primers: E8F1 (5′-CGAGA-GAATCCTTTTGTC-GC-3′), E8R1 (5′-AGGGGAAGTCACGCCGATG-3′), E8F2 (5′-CGAGA-AAAGTTGCCAT-CCG-3′), and E8R2, (5′-GACAT-TCTCATTCTACAGAGCGTC-3′). The kanamycin cassette was flipped out by limited induction of flipase, and the cells were screened for kanamycin sensitivity (KanS). In these colonies, removal of the kanamycin cassette was verified by PCR using the E4F1, E4F2, E4R1, E4R2, E8F1, E8F2, E4R1, and E4R2 primers. The overall structure of the doubly modified BAC was examined by fingerprint analysis (Gong, Zheng et al. 2003; Kim and Lauderdale 2006) using XbaI and compared to that obtained for the unmodified BAC. The sequences of the targeted regions were verified by automated sequencing of both DNA strands (Supplemental Data Fig. S1). BAC injections into zebrafish BAC DNA was purified using the Sigma Phase Kit and diluted to between 80-162 ng/μl in either KCl injection buffer (0.1 M KCl, 0.05 M Tris-HCl, pH6.8, 1mM EGTA) or BAC injection buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 30 μM spermine, 70 μM spermidine, 100 mM NaCl). Both buffers contained 0.1% phenol red as a tracer. Although equivalent results were obtained using the KCl and BAC injection buffers and fresh BAC preparations, we prefer the BAC injection buffer because the salts and polyamines stabilize large DNA constructs (Schedl, Larin et al. 1993; Montoliu, Bock et al. 1995) and we have been able to store intact BAC DNA at 4°C for a year in polyamine buffer. Microinjections were performed using thin wall borosilicate capillary tubing (OD = 1.0 mm,ID = 0.75 mm) with an inner fiber for rapid fill (FHC, Brunswick), pulled to a fine tip using a micropipette puller. The micropipettes were backfilled with the injection solution and connected to a pressure injector (Picospritzer II; General Valve, Fairfield, NJ) driven by compressed nitrogen.

Fertilized eggs were collected immediately after mating of adult pairs, and placed into an injection holder. Approximately 1 nl of BAC DNA was injected into the blastodisc within 10 minutes of fertilization. Injected embryos were removed from the injection holder, placed into embryo medium (Westerfield, 2000), and grown to the appropriate stage of development at 28.5°C. In pilot experiments, >90% of injected embryos retained the BAC after 1 dpf (Supplemental Data Fig. S2); however, for any given injection set, between 50% and 90% of the injected embryos exhibited reporter expression (n= 27 injection sets). Although the basis for this variability was unclear, it was dependent on individual BAC preparations and storage time. Fresh BAC preparations typically gave the best results. Only embryos with normal morphology were used for these analyses.

Analysis of transgene expression in zebrafish embryos

Injected embryos were screened for transient EGFP and DsRed expression starting at 1 dpf using a Zeiss Stemi SV11 Apo (Carl Zeiss, Thornwood, NY) dissecting microscope fitted for epifluorescence. All injected embryos were treated with 0.003% 1-phenyl-2-thiourea (PTU, -Sigma) to inhibit pigmentation (Westerfield, 2000). For live imaging, embryos were anesthetized with 0.02% ethyl 3-aminobenzoate methanesulfonate (tricaine, Sigma, A-5040) and mounted in 3% methylcellulose in embryo medium containing tricaine (Westerfield, 2000). To visualize reporter gene expression in sections, embryos were fixed overnight by immersion in 4% paraformaldehyde (PFA) in PBS and kept at 4°C. The next day, fixed embryos were embedded in gelatin (Mastick et al., 1997) and cryosectioned at 12 μm or 14 μm thickness; this method did not affect either EGFP or DsRed fluorescence.

Whole-mount mRNA in situ hybridization and immunocytochemistry. Whole-mount mRNA in situ hybridization was performed as described in Hargrave and Koopman (Hargrave and Koopman 2000) except that the blocking solution contained 1% BSA instead of 2% and the post-antibody washes contained 0.5% BSA instead of 0.1%. Sense and antisense digoxigenin-labeled RNA probes were synthesized as described in Jowett and Lettice (Jowett and Lettice, 1994) from the pPAX[zf-a] Pax6a cDNA clone (clone zf27; gift of A. Fjose, University of Tromso, Norway;(Krauss, Johansen et al. 1991)). Hybridization and post hybridization wash steps were performed at 65°C. Dig-labeled probes were detected using α-digoxigenin-AP (Roche) and NBT/BCIP substrate. After color development, embryos were postfixed in 4% paraformaldehyde/PBS at 4°C and cleared in 70% glycerol/PBS. Immunostaining was performed on frozen sections prepared from embryos embedded and sectioned in gelatin (Mastick, Davis et al. 1997). Sections were blocked for 2 hours in 4% milk/TST (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tween-20) and then incubated overnight at room temperature with the primary antibody (see below).

After removal of the primary antibody, the sections were washed several times in TST and then incubated for 30 min with the appropriate biotinylated secondary antibody (Jackson ImmunoResearch; 111-065-144, 715-065-150) at a 1:50 dilution followed by either Cy2, Cy3- or Cy5-conjugated to streptavidin (Jackson ImmunoResearch; 016-220-084, 016-170-084) at a 1:100 dilution for 30 min. After a final wash, VECTASHIELD (Vector) mounting medium was placed on the slides, and coverslips applied. Nuclei were labeled using Hoechst 33342 (Sigma, B-2261). Specific signals were visualized using either standard fluorescence microscopy or laser scanning confocal microscopy.

The following primary antibodies were used for immunohistochemistry: anti-Pax6 (Serum 11 and Serum 14, gifts of Dr. S. Saule; 1:1000); anti-Pax6 (C-terminal amino acids, gift of Dr. G. Mastick; 1:1000); anti-Prox1 (1:1000, Chemicon, AB5475), anti-Chx-10 (1:1000, Exalpha Biologicals, x1180P), anti-DSR (1:1000, Clontech, 8374-1), anti-glutamic acid decarboxylase (1:75; Developmental Studies Hybridoma Bank, GAD-6), anti-GFP-Alexa Fluor488 (1:200, Molecular probes, A-21311); anti-glycine transporter 1 (1:1000; Chemicon, AB1770), anti-Syntaxin (1:2000; Sigma, SO664). In double or triple stains in the mouse retina, DsRed protein was visualized using anti-rabbit Alexa Fluor594 (1:200, Molecular Probes, A11072).

Transcript Analysis

Alternative Pax6a transcripts were analyzed by cDNA library screen. Full-length Pax6a transcripts were identified by screening a zebrafish cDNA eye library, which was prepared from 4-5 day old larvae, at high-stringency (Sambrook and Russell, 2001) using a 32_P-labeled EcoRI fragment of the pPAX[zf-a] Pax6a cDNA, which encompasses exons 5-13. Twenty-three fulllength clones were recovered and sequenced. Transcripts from the BAC transgene were analyzed by RT-PCR. Total RNA was prepared from transiently transgenic zebrafish embryos at 3 dpf using TRIzol reagent (GibcoBRL). RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) following the manufacture's recommended conditions. To assess transcript structure, RT-PCR was performed using the primer pairs listed in Supplemental Data Table S1. To test for long transcripts, reactions were performed at 72°C with extension times of 4 minutes. We have previously shown that these conditions are sufficient to detect transcripts of ~ 4 kb in length (Lauderdale, Wilensky et al. 2000).

Generation of BAC transgenic mice.

Transgenic mice were generated from pronuclear injection of closed circular BAC DNA into mouse oocytes (Gong, Zheng et al. 2003). Mice carrying the BAC transgene were genotyped by PCR using tail DNA and primers to detect the EGFP reporter cassette (forward primer, located in the Pax6a intron 5' of exon 4, 5′-CTGGACATAAGTGTATTTGTGGAG-3′; reverse primer, located in EGFP, 5′-CGCACCATCTTCTTCAAGG-3′; the PCR product is 559 bp) or DsRed reporter cassette (forward primer, 5′-TCATCGGCGTGAACTTCC-3′; reverse primer, 5′-GTTTCA-GGTTCAGGGGGAG-3′; the PCR product is 551 bp). Two out of twelve pups derived from pronuclear injection harbored the BAC transgene. F1 founders were generated by crossing founder males to CD-1 mice (Charles River Laboratories). These lines are being maintained in the CD-1 background.

Analysis of transgene expression in whole mouse embryos

Mouse embryos were obtained from our breeding colony, with noon on the day of plug discovery designated as day 0.5 (E0.5). The pregnant females were killed using CO_2, and the uteri were washed in ice-cold phosphate-buffered saline (PBS). The embryos were dissected free and placed in ice-cold PBS. GFP expression was assessed in live embryos by fluorescence microscopy using a Zeiss Stemi SV11 Apo dissecting microscope fitted for epifluorescence and documented using a Zeiss AxioCam digital camera or 35mm SLR camera. All embryos were then fixed by immersion in 4% paraformaldehyde/PBS at 4°C. Embryos that were to be used for immunohistochemistry were kept in fixative for up to 2 weeks at 4°C. The genotype of each embryo was determined by PCR (Xu, Zhang et al. 1999), using tail or extra-embryonic membrane DNA.

Results

Organization and transcription of the zebrafish Pax6a gene

The zebrafish Pax6a gene has 17 exons distributed over ~ 21 kb region of chromosome 25 (Fig. 1A). In addition to exons 0 through 13, there are also exons alpha, 4a and 5a. Transcript analysis, using mixed stage embryos, revealed that Pax6a transcription initiates from three promoters, designated as P0, P1, and P-*alpha* (Figs. 1B, C). P0 and P1 are located 5' to exons 0 and 1, respectively. P-*alpha* is located 5' to the alpha exon, which is located between exons 4a and 5. P0 and P1 constitute the major promoters, and initiate expression in most cell-types that express Pax6a (data not shown). Whereas P0- and P1-initiated transcripts encode a Pax6 protein that includes the paired domain, homeodomain, and PST domain (Fig. 1B) (Nornes, Clarkson et al. 1998; Mikkola, Bruun et al. 2001) P-*alpha*-initiated transcripts are predicted to encode a Pax6 protein that includes the homeodomain and PST domain, but lacks the paired domain (Fig. 1C) (Kammandel, Chowdhury et al. 1999; Mishra, Gorlov et al. 2002).

BAC targeting strategy to visualize different Pax6a transcripts

Because paired-containing and pairedless isoforms of Pax6 may have different roles in the developing vertebrate (Mikkola, Bruun et al. 2001; Haubst, Berger et al. 2004; Kim and Lauderdale 2006), we wanted to examine the spatiotemporal pattern of P0- and P1-initiated transcription relative to P-*alpha*-initiated transcription during zebrafish development. In order to visualize sites of (P0 + P1)-initiated transcription compared to Palpha-initiated transcription, we introduced two reporter genes into a bacterial artificial chromosome (BAC) that encompassed the Pax6a transcript unit (Fig. 2A). To visualize P0- and P1-initiated expression, we inserted an

enhanced green fluorescent protein reporter cassette (EGFP-pA) in-frame with the second ATG of Pax6a exon 4 (Fig. 2B, Supplemental Fig. S1).

Unlike mammals or birds, where the initiator ATG of paired containing Pax6 is in exon 4, ray-finned fish can initiate translation of paired-containing Pax6 in either exon 2 or exon 4 (Krauss, Johansen et al. 1991; Behrens, Langecker et al. 1997, Puschel, Gruss et al. 1992; Miles, Elgar et al. 1998; Nornes, Clarkson et al. 1998) . Placement of EGFP in exon 4 was expected to report most, if not all, transcripts that initiated from either P0 or P1 (Fig. 1C). To visualize Palpha-initiated transcripts, we inserted a Discosoma red fluorescent protein reporter cassette (DsRed-pA) in-frame with the ATG of Pax6a exon 8 (Fig. 2B, Supplemental Fig. S1). Because the EGFP reporter cassette was expected to terminate P0 and P1-initiated transcripts, the placement of DsRed was expected to report most, if not all, transcripts that initiated from Palpha (Fig. 2C). We confirmed these predictions by RT-PCR (Fig. 2D). Total RNA was prepared from embryos transiently transgenic for the dual-reporter BAC at 3 dpf, and PCR analyses performed using primers directed towards Pax6a exons 1 (e1), 2 (e2), alpha, 5 (e5), 6 (e6) and 7 (e7), and also towards EGFP and DsRed. As expected transcripts from the BAC that contained exons 1 and/or 2 also contained EGFP, but not DsRed (Fig. 2D). Similarly transcripts that contained exons alpha, 5, 6 or 7 also contained DsRed but not EGFP (Fig. 2D and data not shown). No read-through transcripts containing EGFP and downstream exons were detected (Fig. 2D and data not shown). These data demonstrate that in the BAC transgene P0- and P1-initiated transcripts contain EGFP (but not DsRed), and P-*alpha*-initiated transcripts contain DsRed.

EGFP expression replicates the Pax6a pattern in developing zebrafish embryos

To assess if the BAC reporter transgene replicated Pax6a expression, we generated transiently transgenic zebrafish by microinjection of the transgene into early 1-cell embryos. We examined

these animals for EGFP and DsRed fluorescence for the first five days of development (Table 1; Figs. 3, 4). Although expression was mosaic, the over-all pattern was comparable to that observed for the endogenous Pax6a gene (compare Fig. 3B to A). EGFP was expressed by cells in Pax6a-expression domains in the developing forebrain, hindbrain, spinal cord, and eye at both 1 dpf (compare Figs. 3B to A) and 2 dpf (compare Figs. 3D to C). By 4 dpf, EGFP expression was mostly restricted to the developing eye and groups of neurons in the telencephalon and diencephalon (Figs. 3F, 4B), comparable to that of Pax6a (Fig. 3E). These results suggested that the BAC transgene contained most, if not all, of the regulatory elements required to replicate Pax6a transcription. Additionally, these results are consistent with our transcript analysis, which indicated that the P0- and P1-promoters initiate expression in most cell-types that express Pax6a. EGFP is expressed by multiple cell types in the developing retina in zebrafish.

To determine which cells in the developing zebrafish eye expressed EGFP, we examined EGFP expression in cryosections cut through the eye at different stages of development (Table 3; Fig. 5). Multiple cell types in the developing retina expressed EGFP, including retinal precursor cells (Fig. 5A, B, and data not shown), retinal ganglion cells (Fig. 5C, D), amacrine cells (Fig. 5B-D), and putative horizontal cells ("hc" in Fig. 5C). EGFP expression in the axons of retinal ganglion cells was visible within the optic nerve (Fig. 5C). To directly compare cells expressing EGFP with those expressing Pax6, sections cut through the eyes of transiently transgenic embryos at 2 or 3 dpf were immunolabeled for Pax6 using an antiserum directed towards the C-terminal 17 amino acids of Pax6. This antibody detects most, if not all, Pax6 isoforms in the zebrafish (data not shown). Direct comparison of EGFP-expression with Pax6 revealed coincident expression in retinal precursor cells (RPCs), retinal ganglion cells (RGCs), amacrine cells and horizontal cells. These results indicate that the zebrafish Pax6a BAC transgene was

sufficient to replicate P0- and/or P1-initiated Pax6a transcription in these cells in the developing zebrafish retina. However, because these analyses were performed in transiently transgenic embryos, it was not possible to determine if the transgene could be expressed by all of the cells that normally express Pax6. EGFP expression was also occasionally observed in putative Müller glia cells ("m" in Fig.5D) and putative bipolar cells (data not shown) at 3-4 dpf; however, it was unclear if these cells coexpressed Pax6.

Although Pax6 is not generally thought to be expressed in these cells, irregular weak Pax6 expression has been reported to occur in the Müller-bipolar region of the inner nuclear layer in the developing chick retina (Adler 1997). Thus, it was possible that transgene expression in these cells did in fact reflect continued (weak) Pax6 expression. Alternatively, given the speed at which the zebrafish retina develops, the EGFP in these cells could result from EGFP perdurance as a consequence of transgene expression in the RPCs that gave rise to these Müller glia or bipolar cells. Lastly, this expression could represent ectopic activity of the transgene; however, this BAC does not exhibit appreciable ectopic expression elsewhere in the embryo (Fig. 3, Supplemental Data Fig. S2).

DsRed is expressed by amacrine cells in transiently transgenic zebrafish

In contrast with EGFP, DsRed fluorescence was only detected in the developing eye (Tables 1, 2; Fig. 4B). Embryos that exhibited EGFP expression in the eye, telencephalon, diencephalon, hindbrain and spinal cord at 1 dpf were followed through 5 dpf (Table 1). In these animals, DsRed-expressing cells were typically first detected between 2.0 and 2.5 dpf and only in the eye (Tables 1, 2; Fig. 3C). No DsRed-expressing cells were observed in other parts of the embryo (Tables 1, 2; Fig. 3C). In order to detect even very low levels of the reporter gene products we

confirmed sites of expression by indirect immunofluorescence using antibodies to EGFP and DsRed, respectively. There was complete concordance between EGFP or DsRed fluorescence and protein expression (data not shown).

To determine which cells expressed DsRed, we examined both the onset and location of DsRed fluorescence in sections cut through the eyes at different stages of development (Fig. 5). At 1 dpf, just prior to the appearance of the first postmitotic cells (Hu and Easter 1999), EGFP but not DsRed, was expressed in the RPCs (data not shown). The first DsRed expressing cells were observed between 2 and 2.5 dpf (Fig. 5B, Table 1), and were most often located in the inner nuclear layer (Fig. 5C', D', G, H, Table 3). However, DsRed+ cells were occasionally located in the GCL (Fig. 5E, F, Table 3). In both cases, these cells sent projections into the inner plexiform layer (Fig. 5C', D', E, F, G, H), suggesting that they were amacrine cells. Consistent with this, the onset of DsRed expression was coincident with the appearance of amacrine cell population, which starts forming at 50 hpf (Schmitt and Dowling 1999). Direct comparison of EGFP and DsRed fluorescence revealed that DsRed expressing cells always represented a subset of the EGFP-expressing cell population (Figs. 5C, D). DsRed-expression was never observed in EGFP-negative cells (Fig. 5C, D and data not shown).

To confirm that the DsRed-expressing cells were in fact amacrine cells, we colabeled the retina with syntaxin, which is expressed in the dendritic processes and perikarya of all amacrine cells (Barnstable, Hofstein et al. 1985; Alexiades and Cepko 1997)). All DsRed+ cells coexpressed syntaxin (Fig. 5H). This data demonstrated that the DsRed+ cells in the INL were amacrines. To determine the identity of the DsRed+ cells in the GCL, we examined the ganglion cell axons in the optic nerve in both intact embryos and in sectioned material for spectral overlap of GFP and DsRed. Although the GCL contains the soma of both RGCs and displaced

amacrines, only the RGCs project axons to the brain. Whereas EGFP expression in the RGC axons was evident in the optic nerve (e.g. Fig. 5C), these axons did not express DsRed (data not shown), which suggested that the DsRed+ cells in the GCL were displaced amacrines. We confirmed by immuno-histochemistry that RGCs did not express DsRed (data not shown). Together these data indicated that amacrine cells in the developing zebrafish retina expressed DsRed. However, because these analyses were performed in transiently transgenic embryos, it was possible to miss small populations of other cells that normally express pairedless Pax6a transcripts in the developing zebrafish retina.

Putative eye regulatory regions are conserved between Zebrafish Pax6a and mouse Pax6

We next wanted to test whether Pax6(-PD) was expressed by all amacrine cells or by a specific population of amacrine cells. However, this type of analysis is best done in a transgenic line rather than in transiently transgenic animals. Because it is relatively easier to generate BAC transgenic mice as opposed to BAC-transgenic fish, we chose to do this experiment in mice. Prior to generating transgenic mice, we first evaluated the degree of conservation between the genomic sequence of the zebrafish Pax6a BAC with that of the Pax6 gene in mice (Fig. 6).

In mammals, the regulatory regions controlling transcription of the Pax6 gene are dispersed over an ~200 kb region, and are located 5', internal, and 3' to the Pax6 gene. The 3' Pax6 regulatory elements are located in the introns of a second gene, Elp4, which is located 3' to Pax6 and is arranged in antisense orientation (Fig. 6) (Kleinjan, Seawright et al. 2002). Although Elp4 has a similar arrangement in Fugu (Miles, Elgar et al. 1998) and for zebrafish Pax6b (Fig. 6B), this gene is not associated with zebrafish Pax6a (Fig. 6B). To identify putative regulatory regions conserved in the zebrafish Pax6a BAC transgene, we compared the sequence of this BAC to the

genomic sequences of human, mouse, Fugu, and zebrafish Pax6b (Fig. 6). We included Pax6b in this analysis because it was possible that Pax6 regulatory elements had been partitioned between Pax6a and Pax6b in zebrafish. Conserved sequences were identified using the multi-LAGAN computer program (Brudno, Do et al. 2003) and displayed using the VISTA computer program (Mayor, Brudno et al. 2000).

Although the Pax6a BAC extends >80kb 5' of the Pax6 gene (Fig. 6A), no appreciable sequence similarity was observed in the region 5' to a conserved CpG island that is differentially methylated in mammals (Kim and Lauderdale 2006). However starting with this CpG island, evolutionarily conserved noncoding sequences were detected within and 3' to the Pax6a transcript unit (Fig. 6B). Several of these sequences mapped to regions implicated in regulating mammalian Pax6 expression in the eye (Fig. 6B), and included the 3' human Pax6 regulatory regions designated as C1170, EI, and HS234 (Kleinjan, Seawright et al. 2001; Kleinjan, Seawright et al. 2006; Tyas, Simpson et al. 2006). Together these results strongly suggested that the zebrafish Pax6a BAC transgene would exhibit regulated expression in the mouse eye.

Zebrafish Pax6a P0/P1 promoters are active in the mouse retina

To directly test if the mechanisms controlling zebrafish Pax6a expression in the eye were conserved between zebrafish and mouse, we generated mice transgenic for the zebrafish Pax6a BAC reporter transgene. For these experiments, we assessed transgene expression in cryosections cut from the mouse retina at different developmental times (Fig. 7) and also from adults (Figs. 8-11). Like zebrafish, EGFP was expressed in the early neural retina (Fig. 7A). To directly compare cells expressing EGFP with those expressing mouse Pax6, sections were immunolabeled in red for Pax6 using an α-Pax6 antiserum (Fig. 7B). Pax6 protein is nuclear but

EGFP is predominantly cytoplasmic. Consequently, at high magnification double-labeled cells contained red nuclei surrounded by green cytoplasm. In contrast with the zebrafish, we were unable to visualize DsRed fluorescence in mice (data not shown); therefore, this allowed us to label epitopes, such as Pax6, using red fluorescence. However, we are able to detect DsRed by indirect immunofluorescence (see below and also Methods). Comparison with endogenous Pax6 protein (Fig. 7B) revealed that most, if not all, Pax6+ cells in the E10.5 retina also expressed EGFP (Fig. 7C). At both E13.5 (Fig. 7D-F) and E17.5 (Fig. 7G-I), EGFP appeared to be expressed at two different levels in the retina. Lower levels of expression were observed in the neuroepithelial cells in the retinal ventricular zone, and higher levels of expression were observed in putative differentiating cells.

At E13.5 EGFP expression in RGC axons was visible within the optic nerve (Fig. 7D, F). At E17.5 EGFP was strongly expressed by cells in the developing inner nuclear and RGC layers (Fig. 7G, I). Comparison with endogenous Pax6 revealed concordant expression at both E13.5 (Fig. 7D-F) and E17.5 (Fig. 7G-I). Together, these data indicate that the mechanisms controlling Pax6 expression in retinal neuroepithelial cells have been conserved between the zebrafish Pax6a gene and mouse Pax6, and that a second conserved mechanism acts to increase Pax6 transcription in at least some differentiating neurons. In the adult retina, EGFP was strongly expressed by cells in the inner nuclear and ganglion cell layers, and also within neurites in the OPL and IPL (Fig. 8A-B). The INL is populated by amacrine, horizontal and bipolar cells, and contains the nuclei of Müller glia. Both amacrine and ganglion cells populate the GCL (Drager and Olsen 1981; Perry 1981; Jeon, Strettoi et al. 1998). Based on the morphologies and locations of the EGFP-expressing cells, it was clear that the P0/P1 promoters from the zebrafish Pax6a transgene were active in at least some amacrine cells, horizontal cells, and RGCs. In the case of

RGCs, EGFP expression in RGC axons was visible in the optic nerve (data not shown; see Fig. 7D for optic nerve expression in developing retina). Additionally, a number of EGFP-expressing cells morphologically resembled Müller glia (Fig. 8A, and data not shown). These cells, whose nuclei were located in the middle of the INL, contained processes that spanned the radial dimension of the retina and ended at the outer limiting membrane (arrow, Fig. 8A). Because we could not unambiguously identify endfeet at the vitreal surface for all of these cells, it is also possible that EGFP was expressed in bipolar cells that contained ascending processes. We next directly compared cells expressing EGFP with those expressing Pax6 in the adult retina (Fig. 8B-D). In adults, Pax6 protein is strongly expressed in ganglion, amacrine, and horizontal cells (Fig. 8C) (Belecky-Adams, Cook et al. 1996; Hitchcock, Macdonald et al. 1996). Because EGFP expression represented transcripts encoding paired-containing Pax6, sections were immunolabeled in red for paired-containing Pax6 using an antiserum that recognized the paired-domain (serum 11; (Carriere 1993)). Most, if not all cells, that expressed paired-containing Pax6 coexpressed EGFP (Fig. 8B-D). Although it is possible that there were cells in the INL that expressed Pax6 but not EGFP, we did not find any unambiguous examples in these coimmunolabeling experiments. However, we did find examples of cells in the INL that expressed EGFP but did not appear to immunolabel for paired-containing Pax6 (arrowheads, Fig.8B-D).

Because it was possible that these cells preferentially expressed Pax6ΔPD, which would not be detected by serum 11, we repeated the experiment using Pax6 antibodies that recognize all Pax6 isoforms, with similar results. Thus, although these results confirm our initial impression that EGFP from the zebrafish Pax6a BAC transgene is expressed in ganglion, amacrine, and horizontal cells in mouse, they also indicate that the zebrafish Pax6a BAC transgene is

ectopically expressed in a discrete population of cells in the mouse retina. The ectopic expression in these cells is most likely due to either species differences in expression or misregulation of the zebrafish Pax6a BAC transgene in mice. Alternatively, transgene expression in these cells may reflect low levels of Pax6 expression, which cannot be easily detected using our Pax6 antibodies. Nevertheless, the fact that EGFP expression accurately replicated Pax6 expression in most cells in the adult mouse retina suggested that at least some of the mechanisms controlling P0/P1-initiated Pax6 transcription in the adult retina have been conserved between teleost and mammals. A subset of amacrine cells in the adult mouse retina expressed Pax6a P-*alpha*-initiated transcripts.

We next examined if the zebrafish Pax6a P-*alpha* promoter was active in the adult mouse retina. Because we were unable to visualize red fluorescent protein fluorescence in mice, we visualized DsRed using an antibody to this protein. Like zebrafish, cells in both the INL and GCL expressed DsRed (Fig. 9B, Supplemental Data Fig. S3). Comparison with EGFP expression revealed that most, if not all, DsRed-expressing cells also expressed EGFP (Fig. 9D, Supplemental Data Fig. S3). Conversely not all EGFP-expressing cells coexpressed DsRed (Fig. 9D, arrowheads in Supplemental Data Fig. S3). Notably, both horizontal and ganglion cells expressed EGFP but not DsRed. These results indicate that the zebrafish P-*alpha* promoter is regulated differently than the P0/P1 promoters in mice as in zebrafish.

We next tested if amacrine cells expressed DsRed. Amacrine cells are located in both the INL and also in the GCL, where they are termed displaced amacrines. In normal mice, displaced amacrines constitute 59% of the cells in the RGC layer. Amacrines in both the INL and GCL express syntaxin (Fig. 9C) (Barnstable, Hofstein et al. 1985; Alexiades and Cepko 1997). As expected the majority of DsRed+ cells in the retina coexpressed syntaxin (Fig. 9B-D) and were,

therefore, amacrine cells. Interestingly, only a subset of syntaxin+ cells coexpressed DsRed, and the neurites from these cells tended to be localized to the middle of the IPL (Fig. 9D, arrow in Supplemental Data S3). When EGFP expression was added to the comparison, we identified three distinct populations of syntaxin expressing cells: those that expressed EGFP (arrowheads in Fig. 9), those that coexpressed EGFP and DsRed (arrows in Fig. 9), and those that did not express either EGFP or DsRed (blue cells in Fig. 9D). Although this latter result was unexpected because, as described above, EGFP and Pax6 appeared to be coexpressed in the adult mouse retina, it showed that there was a population of amacrine cells that did not express the zebrafish Pax6a BAC transgene. Importantly, this data also indicated that the zebrafish Pax6a Palpha promoter was active in a specific group of amacrine cells.

Amacrine cells can be divided into two large generic cell groups, those containing GABA (γ-aminobutyric acid) or glycine, which together comprise about three fourths of the total population (Strettoi and Masland 1996; Haverkamp and Wassle 2000). To determine the identity of the DsRed expressing amacrine cells, we performed immunolabeling for Gad-6 (glutamic acid decarboxylase) and GlyT1 (glycine transporter 1), which mainly stain GABAergic and glycinergic amacrines, respectively, in the inner nuclear layer. Interestingly, while EGFP was expressed in both Gad-6 and Glyt1 positive amacrine cells (arrowheads in Fig. 10), DsRed expression was restricted to Gad-6 positive cells in the amacrine population (arrows in Fig. 10). These results suggest that Pax6a isoforms are differentially expressed between the two main amacrine groups.

A subset of bipolar cells in the adult mouse retina express Pax6a P-*alpha* initiated transcripts. Although the majority of DsRed-expressing cells in the retina were GABAergic amacrines, we also observed morphologically distinct cells in the INL that expressed DsRed and EGFP, but not

syntaxin (data not shown). These cells often occurred as pairs, were located in the bipolar region of the INL (arrows in Fig. 11A-H), and extended processes to both the OPL and IPL (see left-most cell denoted in Fig. 11F). Molecularly, these cells coexpressed Chx10 (Fig. 11C-D) and Prox1 (Fig. 11G-H), both of which are expressed by bipolar cells in the adult retina (Belecky-Adams, Cook et al. 1996; Chen and Cepko 2000). The majority of Chx10 or Prox1 expressing cells do not express the transgene (Fig. 11D and H, respectively). Although Prox1 is also expressed by horizontal cells and AII amacrine cells (Dyer 2003), these DsRed-expressing cells do not appear to be either of these cell types. Horizontal cells are easily identified using morphological and molecular criteria. These cells have relatively large soma and extend projections into the OPL. Molecularly, they express EGFP, Pax6 and Prox1, but not Chx10 or DsRed (Fig. 11H and data not shown). Similarly, the AII amacrine cells can be clearly identified in our transgenic animals. These neurons express EGFP, syntaxin, Prox1, and GlyT1, but not DsRed; their cell bodies are located immediately adjacent to the IPL (Fig. 11I-L, and data not shown). Together, these results suggest that the zebrafish Pax6a transgene is expressed in a specific group of bipolar neurons in the adult mouse retina. Although we have occasionally observed EGFP expression in bipolar cells in the developing zebrafish retina, these cells did not appear to express DsRed. This apparent discrepancy between zebrafish and mouse could reflect errors in transgene expression in the mouse. Alternatively, it could reflect differences between the developing and adult retina. Because of limitations with transient transgenics in zebrafish, we were not able to assess transgene expression in the adult zebrafish eye.

Conservation of putative regulatory elements in the Pax6a alpha-promoter region

Our results indicated that the regulatory mechanisms controlling Pax6a alpha promoter

activity had been largely conserved between mice and zebrafish. To identify potential regulatory elements conserved between mice and zebrafish, we compared the genomic sequence encompassed by zebrafish Pax6a exons 4 and 5 with the corresponding genomic sequences from human, mouse, and Fugu (Fig. 12). This region includes the alpha promoter and exon. Our analysis revealed two highly conserved noncoding regions, designated "αCR1" and "αCR2" respectively, 5' of the alpha exon (Fig. 12A). The first region, which has not been previously described, contains a highly conserved E-box (Fig. 12B) that could serve as a binding site for bHLH transcription factors involved in cell fate specification in the retina.

The αCR2 sequence (Fig. 12C) corresponds to the Pax6 retinal-specific enhancer region previously described in mice and quail(Plaza, Dozier et al. 1995; Kammandel, Chowdhury et al. 1999; Xu, Zhang et al. 1999). Sequences corresponding to the four neuroretina-specific protein-binding sites (designated DF1-DF4) identified in quail (Plaza, Dozier et al. 1995) are conserved in zebrafish Pax6a, as is a known Pax2 binding site (Kammandel, Chowdhury et al. 1999). The putative Msx1 binding site (CAATTAG; (Catron, Iler et al. 1993)) in DF3 and all of the sequence within DF4 are conserved. Multiple different neuroretina factors, including Brn3b (Pou4f2), have been shown to bind to DF4 (Plaza, Hennemann et al. 1999).

Discussion

Analysis of alternative Pax6 promoter activity in vivo using a dual-reporter BAC

The Pax6 gene plays a central role in metazoan eye development. Null mutations in Pax6 result in a failure in eye development in both insects and mammals (Ton, Miwa et al. 1992; Matsuo, Osumi-Yamashita et al. 1993; Quiring, Walldorf et al. 1994), and Pax6 misexpression is sufficient to induce ectopic eyes in both insects and amphibians (Chow, Altmann et al. 1999,

Halder, Callaerts et al. 1995; mOnuma, Takahashi et al. 2002). Gene inactivation studies in mice revealed that Pax6 is required for different aspects of retinal development, including control of cell proliferation (Ashery-Padan, Zhou et al. 2004; Philips, Stair et al. 2005), maintenance of the retinogenic potential of RPCs (Ashery-Padan, Zhou et al. 2004), regulation of the initiation of cell-specific differentiation programs (Ashery-Padan, Zhou et al. 2004; Philips, Stair et al. 2005), and possible arealization of the retina (Inoue, Hojo et al. 2002). Emerging evidence suggests that these different aspects of Pax6 gene function are likely to be mediated by different isoforms of the Pax6 protein (Dominguez, Ferres-Marco et al. 2004; Haubst, Berger et al. 2004; Azuma, Tadokoro et al. 2005; Azuma, Tadokoro et al. 2005); however, relatively little is known about the spatiotemporal expression of Pax6 isoforms during retinogenesis.

We report here our analyses of zebrafish and mice transgenic for a dual-reporter zebrafish Pax6a BAC transgene that separately reports on Pax6a (P0 + P1)-initiated transcripts and Palpha initiated transcripts. In both mice (Kammandel, Chowdhury et al. 1999; Xu, Zhang et al. 1999; Kim and Lauderdale 2006)) and zebrafish (this study; Lauderdale, unpublished), transcript analysis has revealed that expression of the different Pax6 isoforms is governed by both alternative promoter usage and splicing. However, determining the location and timing of Pax6 isoform expression in the context of the developing and adult retina is problematic. Transcripts encoding for Pax6, Pax6(5a), and Pax6ΔPD are largely identical, which makes it challenging to distinguish between the different transcripts by mRNA in situ hybridization. Although it is possible to develop an antibody that will specifically recognize Pax6(5a), the Pax6ΔPD isoform is identical to the full length Pax6 protein in all respects except the paired domain, and thus it is not possible to distinguish between pairedless and paired-containing Pax6 proteins in wild-type embryos using immunohistochemistry. Thus, our dual-reporter approach using a BAC transgene

is currently the most direct way to examine alternative promoter usage in vivo. Because transcriptional control of Pax6 is complex, the use of large genomic constructs avoids potential problems associated with conventional transgenic reporter gene approaches and permits examination of reporter expression in a genomic-like context.

Zebrafish was chosen for this study because it has two Pax6 genes, designated Pax6a and Pax6b (Nornes, Clarkson et al. 1998). These two genes map to different linkage groups (Pax6a, LG25; Pax6b, LG7) and likely arose from a genome duplication that occurred after the split between the lineages that gave rise to the tetrapods and teleost (Christoffels, Koh et al. 2004) (Postlethwait, Woods et al. 2000) (Vandepoele, De Vos et al. 2004). The Pax6 proteins encoded by Pax6a and Pax6b share 95% amino-acid identity (98.8% similarity) over their entire length and both generate ectopic eyes in *Drosophila* (Nornes, Clarkson et al. 1998) suggesting that the two proteins have retained similar biochemical functions. Thus, the duplicated Pax6 loci in zebrafish may have been retained through partitioning of cis-regulatory elements (Lynch and Conery 2000; Lynch, O'Hely et al. 2001). Consistent with this idea, the expression patterns of Pax6a and Pax6b together in the zebrafish embryo are similar to the expression pattern of the single Pax6 gene in a comparably staged mouse embryo (Lakowski, Majumder and Lauderdale, unpublished). Thus, zebrafish Pax6a and Pax6b BAC transgenes could be exceptionally useful tools for identifying and studying the functions of different regulatory elements that control the activities of the different Pax6 promoters in the developing and adult eye.

Mechanisms controlling Pax6 transcription are highly conserved. The long-range genomic organization of eukaryotic genes is often important for their normal expression, and this appears to be particularly true for the Pax6 gene in mammals. The mammalian Pax6 transcript unit is complex. It has 16 exons distributed over ~ 30 kb region, and initiates transcription from three

different promoters (Glaser, Walton et al. 1992; Kammandel, Chowdhury et al. 1999; Kim and Lauderdale 2006) (Kleinjan, Seawright et al. 2004) (St-Onge, Sosa-Pineda et al. 1997; Williams, Altmann et al. 1998; Xu, Zhang et al. 1999) . Transcription appears to be controlled through the coordinate activity of multiple, widely spaced regulatory elements, some of which are located >100 kb 3' to the promoters (Griffin, Kleinjan et al. 2002), (Kammandel, Chowdhury et al. 1999; Kim and Lauderdale 2006), (Williams, Altmann et al. 1998; Xu, Zhang et al. 1999; Kleinjan, Seawright et al. 2001; Kleinjan, Seawright et al. 2004). We determined that organization and transcription of the Pax6a gene in zebrafish was comparable to that of mammals (Figs. 1, 6A). Concordantly, comparative genomics revealed active conservation of several noncoding sequences in both mammals and fish (Figs. 6B and 12). The majority of these mapped to regions previously identified by transgenic analysis as Pax6 regulatory regions in mammals, and included 3' regions critical for Pax6 expression in the eye (Williams, Altmann et al. 1998; Kammandel, Chowdhury et al. 1999; Xu, Zhang et al. 1999; Kleinjan, Seawright et al. 2001; Griffin, Kleinjan et al. 2002; Kleinjan, Seawright et al. 2004; Kim and Lauderdale 2006; Kleinjan, Seawright et al. 2006). These results indicate that, like mammalian Pax6, the long-range genomic organization of the Pax6a transcript unit in zebrafish is important for normal gene function. Normal Pax6 transcription appears to require the coordinated actions of multiple cis regulatory elements, which are widely spaced in the Pax6 locus. Although this idea first came from human aniridia cases in which chromosomal rearrangements disrupted the region 3' to PAX6 but spared the PAX6 transcription unit (Ton, Miwa et al. 1992; Fantes, Redeker et al. 1995; Lauderdale, Wilensky et al. 2000; Kleinjan, Seawright et al. 2001), several recent studies have demonstrated that it is necessary to use large genomic constructs in order to replicate mammalian Pax6 expression in mice (Schedl, Ross et al. 1996; Kleinjan, Seawright et al. 2004;

Kim and Lauderdale 2006; Kleinjan, Seawright et al. 2006; Tyas, Simpson et al. 2006). In this study, we provide evidence that the zebrafish Pax6a BAC DKEYP 46C10 is sufficient to recapitulate Pax6a expression in the zebrafish. Although small differences between endogenous Pax6a and transgene expression would be missed in our transient transgenic approach, our results suggest that this BAC contains most, if not all, of the elements necessary to govern Pax6a expression in the zebrafish. One of the main findings of our study is that the mechanisms controlling Pax6 transcription in the retina are mostly conserved between teleost and mammals. Although small classic transgenes containing different Pax6 regulatory elements from fish have been shown to exhibit activity in mice (Kammandel, Chowdhury et al. 1999; Griffin, Kleinjan et al. 2002), it was unclear if the Pax6 transcript unit from fish would exhibit regulated expression in the mammal. Remarkably, the zebrafish Pax6a BAC transgene exhibited largely comparable patterns of expression in both the zebrafish and mouse retina, even though mammals and teleosts diverged ~430 Myr ago (Charnov 1991), and the zebrafish has two Pax6 genes that have been subjected to evolutionary pressure. Although the zebrafish Pax6a BAC transgene did not perfectly replicate Pax6 expression in the adult mouse retina, the fact that it accurately reported Pax6 expression in the vast majority of cells in both developing and adult retina is significant and demonstrates that regulatory elements controlling Pax6a transcription in zebrafish, including the mechanisms controlling alternative promoter usage, are largely sufficient to recapitulate Pax6 expression in the mouse retina.

Pax6a P-*alpha*-initiated transcripts are expressed in a subset of cells in the retina.

Our second main finding is that zebrafish Pax6a P-*alpha*-initiated transcripts are expressed in amacrine cells in both zebrafish and mice. We demonstrated that P-*alpha* initiated transcripts are

expressed in amacrines in the developing zebrafish retina; however, because of limitations associated with transient transgenic analysis, it was not feasible to establish whether this promoter was active in all amacrine cells or in a specific population of amacrine cells. By making mice transgenic for the zebrafish Pax6a BAC reporter transgene, we were able to demonstrate directly that the zebrafish Pax6a alpha-promoter drove expression in a population of GABAergic amacrine cells (Fig. 10). These results suggest that at least some of the mechanisms governing Palpha-initiated transcription in amacrine cells have been conserved between teleost and mammals. Consistent with this, the zebrafish Pax6a alpha promoter region contains highly conserved consensus binding sites for several transcription factors, including those that are known to bind proteins in mice or quail (Plaza, Dozier et al. 1995; Kammandel, Chowdhury et al. 1999). Of these Pax2 and Msx1 stand out; Pax2 is known to regulate Pax6 expression during eye morphogenesis (Schwarz, Cecconi et al. 2000; Baumer, Marquardt et al. 2003) , and a regulatory link between Msx1 and Pax6 has been suggested based on genetic analysis (Grindley, Davidson et al. 1995).

Our results indicate that these regulatory interactions have been conserved between zebrafish Pax6a and mouse. Additionally, we identified a putative bHLH binding site that is conserved between zebrafish Pax6a, humans, mice, and birds (Fig. 12B, and data not shown). Members of the bHLH family of transcription factors bind specifically to a hexanucleotide sequence (CANNTG), known as an E-box (Ephrussi, Church et al. 1985). The central two nucleotides of the E-box and the flanking sequences influence the binding preference of individual bHLH dimer pairs (Blackwell, Kretzner et al. 1990; Blackwell and Weintraub 1990). The conservation of the central two nucleotides and the flanking sequence of the Ebox in αCR1 suggest that it may act as a binding site for bHLH transcription factors involved in cell fate specification in the retina. Pax6

isoforms are differentially regulated in amacrine subpopulations Our results suggest that paired-containing and pairedless Pax6 may have distinct roles in glycinergic and GABAergic amacrine cells, respectively.

Amacrines, originally named because they have no axons, are inter-neurons that modulate and integrate visual signals from photoreceptors via bipolar cells to ganglion cells in the retinal circuitry. Although amacrine cells are a heterogeneous group comprised of at least 30 different morphologically and molecularly distinct types, they can be divided into three generic populations based on neurotransmitter phenotypes (Strettoi and Masland 1996; MacNeil and Masland 1998; MacNeil, Heussy et al. 1999). The two largest populations contain GABA or glycine (Strettoi and Masland 1996; Marc and Cameron 2001). In the mouse, glycinergic amacrine cells comprise ~35% and GABAergic amacrine cells comprise ~40% of the amacrine cell population (Pourcho and Goebel 1987; Glaser, Jepeal et al. 1994; Ashery-Padan, Zhou et al. 2004). The GABAergic amacrine cells can be further subdivided based on the presence of other neurotransmitters such as acetylcholine and dopamine, and the glycinergic amacrine cells include AII amacrines (Glaser, Jepeal et al. 1994; Strettoi and Masland 1996). Although amacrine cells in both the developing and adult retina were known to express the Pax6 gene (Belecky-Adams, Cook et al. 1996; Hitchcock, Macdonald et al. 1996), we provide evidence that glycinergic amacrine cells express paired-containing Pax6 (Fig. 10A-E; Fig. 11I-L), and a subpopulation of GABAergic amacrine cells express both paired-containing and paired-less Pax6 (Fig. 10F-J). These results are intriguing because conditional deletion of Pax6 in the mouse retina led to the loss of all retinal cell types except GABAergic amacrine cells (Ashery-Padan, Zhou et al. 2004). Thus, the Pax6 gene appears to be required for specification of glycinergic amacrine cells, but dispensable for the generation of GABAergic and unidentified amacrines. Our finding that

glycinergic amacrine cells express transcripts encoding for paired-containing Pax6, in conjunction with the Pax6 conditional deletion data, indicates that paired-containing Pax6 is required for specification of glycinergic amacrine cells. Our data also suggests that Pax6ΔPD may have a distinct function in GABAergic neurons. One possible function could be to control the differentiation of specific types of GABAergic amacrine cells. There are at least two ways that Pax6ΔPD could function. Because Pax6ΔPD can only bind DNA through the HD, it is possible that it may bind to downstream targets different than those of paired-containing Pax6. Alternatively, Pax6ΔPD may alter the range of Pax6 targets by directly interacting with paired-containing Pax6 or other factors. Biochemical and genetic studies have shown that the PD and HD bind to specific DNA target sites and can influence each other's binding to DNA (Duncan, Haynes et al. 1998; Mikkola, Bruun et al. 2001; Mishra, Gorlov et al. 2002; Haubst, Berger et al. 2004). Analysis of the in vivo function of Pax6ΔPD is expected to clarify its role in GABAergic amacrines.

Transgene expression in bipolar and Müller glial cells. We observed transgene expression in populations of bipolar and putative Müller glial cells in the retinas of both developing zebrafish and adult mouse. Although these cells are not normally thought to express Pax6, irregular weak Pax6 expression has been reported in the Müller-bipolar region in the developing chick retina (Adler 1997), and we have detected weak endogenous Pax6 expression in a small number of cells in the Müller-bipolar region of the adult zebrafish retina (Lakowski and Lauderdale, unpublished). Although we do not know the identity of these cells, they do not express syntaxin. Thus it is possible that a group of bipolar cells in birds and fish express the Pax6 gene at low levels, and the transgene detects this. Alternatively, the transgene may be ectopically expressed in these cells for some reason. In the adult mouse retina, ectopic expression of the zebrafish

Pax6a BAC transgene could result from either species differences in gene regulation or misregulation due to sequence differences between zebrafish and mice in some cis-regulatory elements. It is worth noting, however, that the majority of bipolar cells in the adult mouse retina did not express the transgene (Fig. 11), and so the mechanism responsible for this ectopic expression was operational in only a subset of bipolar cells. The Pax6 BAC transgene expression in Müller glial cells is intriguing, and is present in all of our transgenic lines. In addition to the zebrafish Pax6a BAC transgene reported here, we have several lines of mice harboring one of three different mouse Pax6 BACs (Kim and Lauderdale 2006), Kim and Lauderdale, submitted; Kim and Lauderdale, unpublished]. All lines exhibit EGFP expression in Müller glial cells. While it is possible that all these BAC transgenes lack negative regulatory elements that keep Pax6 from being expressed in these cells, an alternative explanation is that Pax6 is normally transcribed in Müller glial cells, although at very low levels. Alternatively, the apparent lack of Pax6 immuno-reactivity in these cells may result from posttranscriptional control mechanisms. There is precedence for a BAC transgene unveiling regions of low expression of a gene; in this case a Chx10 reporter BAC transgene revealed Chx10 expression in Müller glial cells (Rowan et al., 2004). It will be interesting to determine if Pax6 is in fact expressed in Müller glial cells in the adult retina, as is suggested by our Pax6 BAC transgenes. Pax6 functions to maintain the neurogenic potential of radial glial cells elsewhere in the brain (Heins, Malatesta et al. 2002). Perhaps it plays a similar function in Müller glial cells in the retina.

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Fig. 2.1. Genomic organization and transcript analysis of the zebrafish Pax6a gene.

(A) Physical map of the zebrafish Pax6a transcript unit. Bent arrows denote the locations of transcript initiation and direction of transcription. Boxes denote exons. Shaded exons are coding; unshaded exons are non-coding. Although exon 4a is reported to be coding in Astyanax fasciatus (Behrens, Langecker et al. 1997), the use of an alternative splice site renders this exon noncoding in all of the zebrafish Pax6a transcripts identified to date (Lauderdale, unpublished). However, it is possible that Pax6a transcripts exist in which this exon is coding. (B-C) Transcript analysis. (B) All P0- or P1-initiated transcripts spliced into exon 4. These transcripts encode paired-containing Pax6. Translation can initiate in either exon 2 or 4. The ATG in exon 4 denotes two ATGs, which are adjacent. Exon 1' contains exon 1, the region between exons 1 and 2, and exon 2. (C) P-*alpha* initiated transcripts encode for pairedless Pax6; translation is expected to begin in exon 7. The ATG in exon 7 denotes three evolutionarily conserved ATGs that could be used for initiation of translation. There are no other ATGs located between exon 4 and 7. Five of six P-*alpha*-initiated transcripts contained exon 5a; one of these contained alternative splice sites for both exons alpha and 5a, which is denoted using asterisks (*).

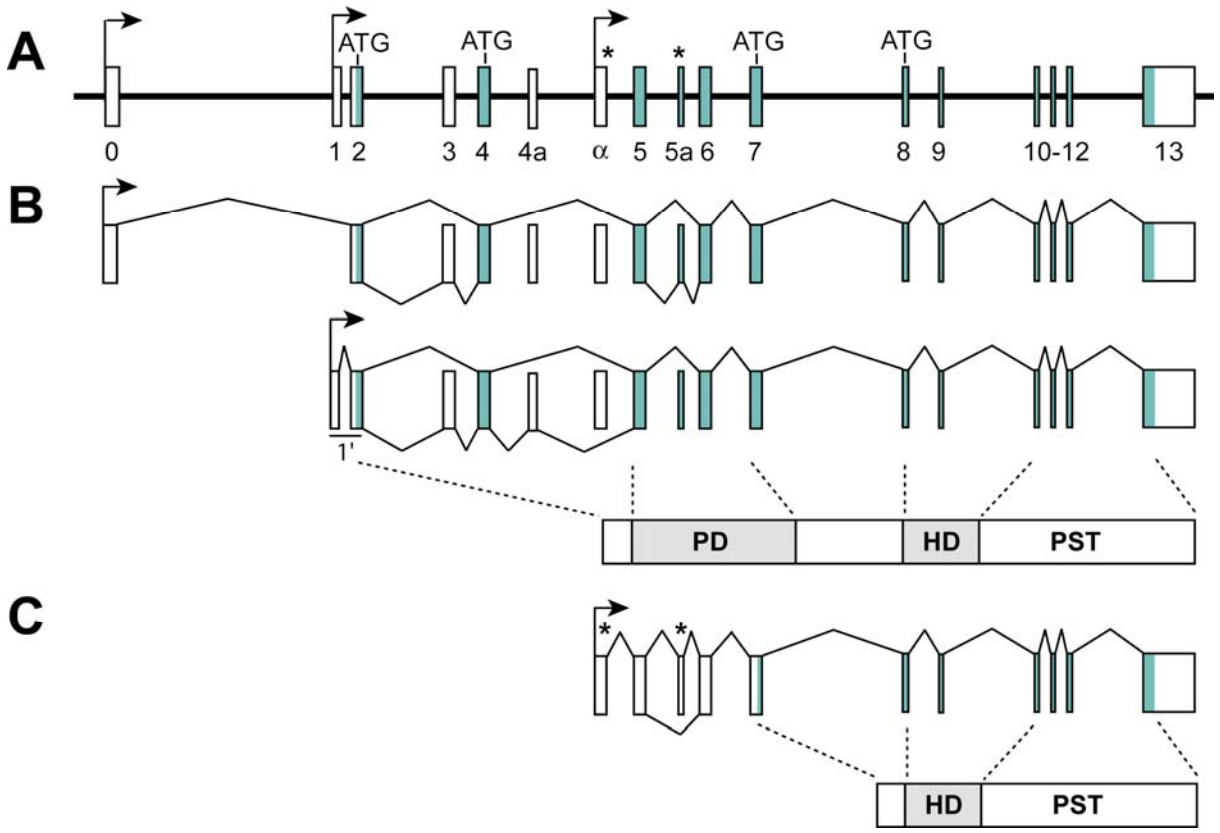
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Fig. 2.2. Structure and transcript analysis of the zebrafish Pax6a dual-reporter BAC transgene.

(A) Schematic representation of zebrafish BAC DKEY-46C10 showing the location of the Pax6a transcript unit within the BAC. Black boxes denote exons, and bent arrows denote promoters P0, P1, and P-*alpha*. (B) The zebrafish Pax6a BAC was modified by insertion of an enhanced green fluorescent protein reporter cassette (EGFP pA) in-frame with the evolutionarily conserved ATG in exon 4. A Discosoma red fluorescent protein reporter cassette (DsRed-pA) was inserted inframe into the conserved ATG in exon 8. (C) Whereas transcripts that initiate from the P0 and P1 promoters encode for EGFP, P-*alpha*-initiated transcripts encode for DsRed. (D) Analysis of transcripts from the BAC transgene. RNA was prepared from 3-day old embryos transiently transgenic for the dual-reporter BAC and analyzed by RT-PCR. Transcripts with exon 1 (e1) or exon 2 (e2) also included EGFP, but not DsRed. Transcripts with exons alpha, 5 (e5), 6 (e6), or 7 (e7) also included DsRed. No P1- or P0-initiated transcripts contained DsRed.

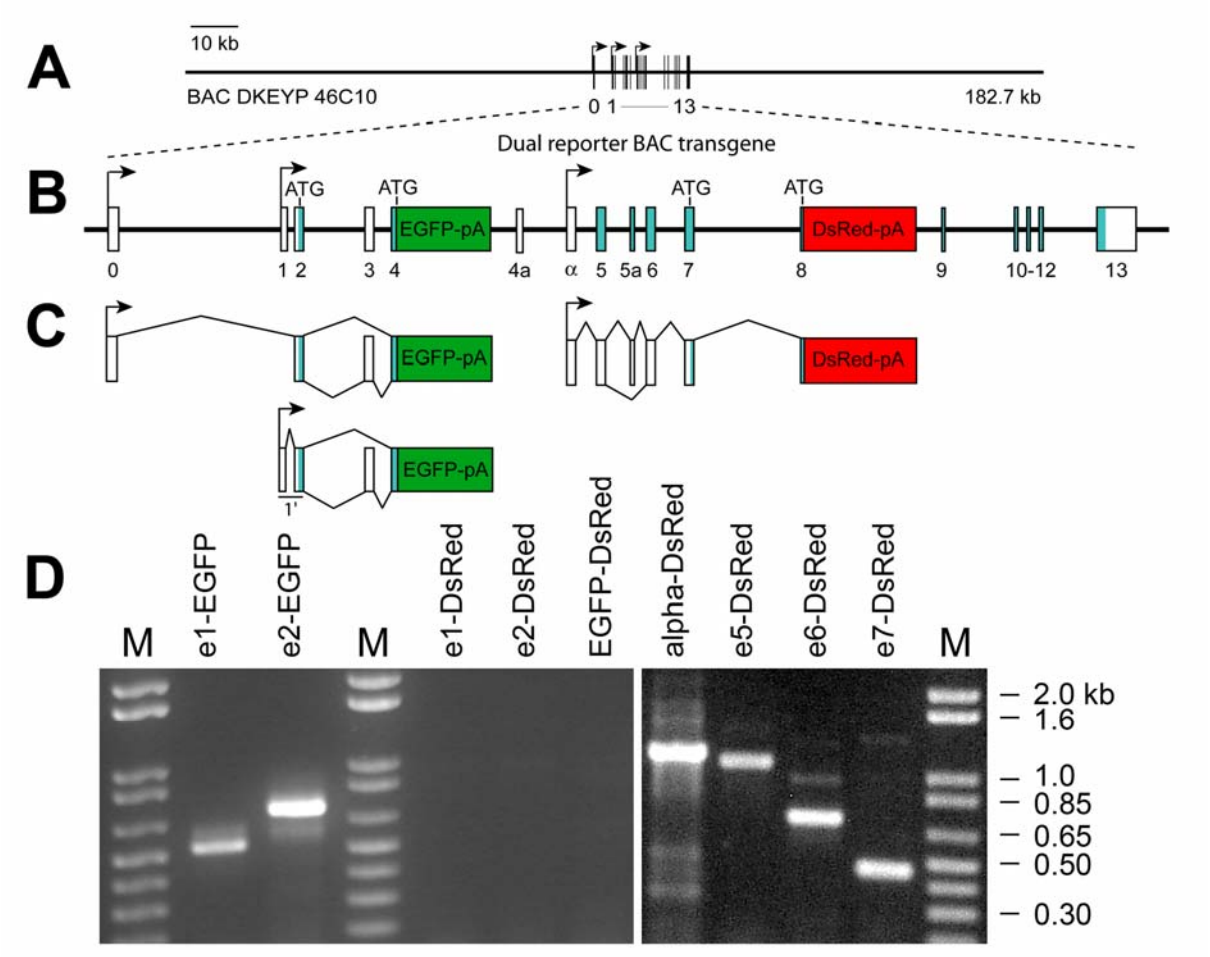

A
10 kb
BAC DKEYP 46C10
182.7 kb
0 1
13
Dual reporter BAC transgene
B
ATG
ATG
EGFP-pA
ATG
ATG
DsRed-pA
0
1 2
3 4
4a
α
5
5a 6
7
8
9
10-12
13
C
EGFP-pA
DsRed-pA
EGFP-pA
1'
D
M
e1-EGFP
e2-EGFP
M
e1-DsRed
e2-DsRed
EGFP-DsRed
alpha-DsRed
e5-DsRed
e6-DsRed
e7-DsRed
M
2.0 kb
1.6
1.0
0.85
0.65
0.50
0.30

Fig. 2.3. EGFP is expressed in a Pax6a-like pattern in developing zebrafish.

(A, C, E) Pax6a expression visualized by whole-mount mRNA in situ hybridization. (B, D, F) Transgene expression visualized by EGFP fluorescence. EGFP was expressed by cells within domains of Pax6a expression. These results suggest that BAC DKEYP-46C10 is sufficient to recapitulate the Pax6a expression pattern in all regions of the developing zebrafish.

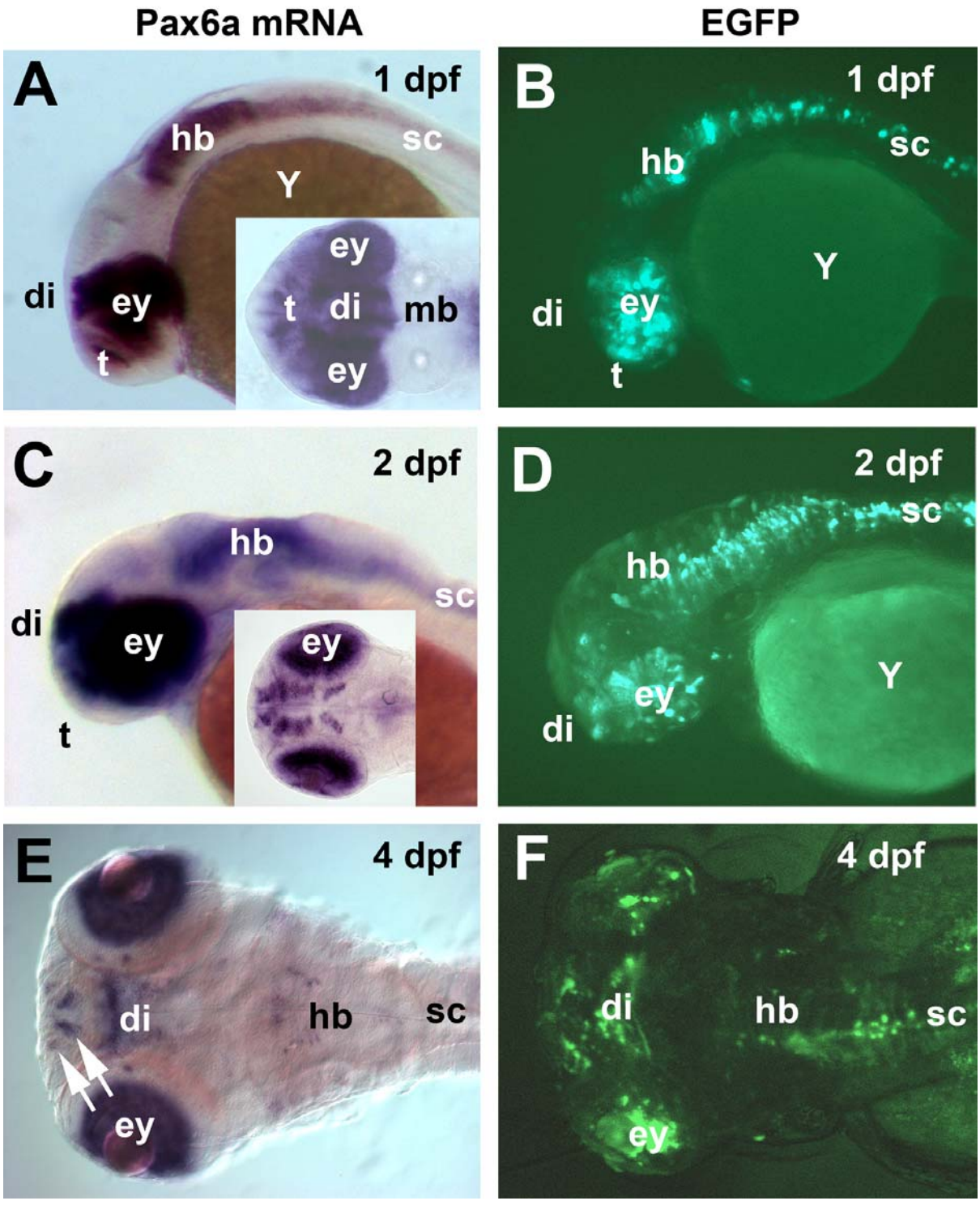

Pax6a mRNA
EGFP
A
1 dpf
hb
sc
Y
di
ey
t
ey
t
di
mb
ey
B
1 dpf
hb
sc
Y
di
ey
t
C
2 dpf
hb
sc
di
ey
t
ey
D
2 dpf
sc
hb
Y
ey
di
E
4 dpf
di
hb
sc
ey
F
4 dpf
di
hb
sc
ey

Fig. 2.4. DsRed expression is restricted to the developing zebrafish eye.

(A) Live zebrafish embryo at 3-days post fertilization (dpf) transiently transgenic for the dual-reporter Pax6a BAC. In this embryo, cells in the eye, diencephalon, hindbrain and spinal cord express EGFP (B), but only cells in the eye express DsRed (C). DsRed expression in the eye was typically first detected between 2.0 and 2.5 dpf.

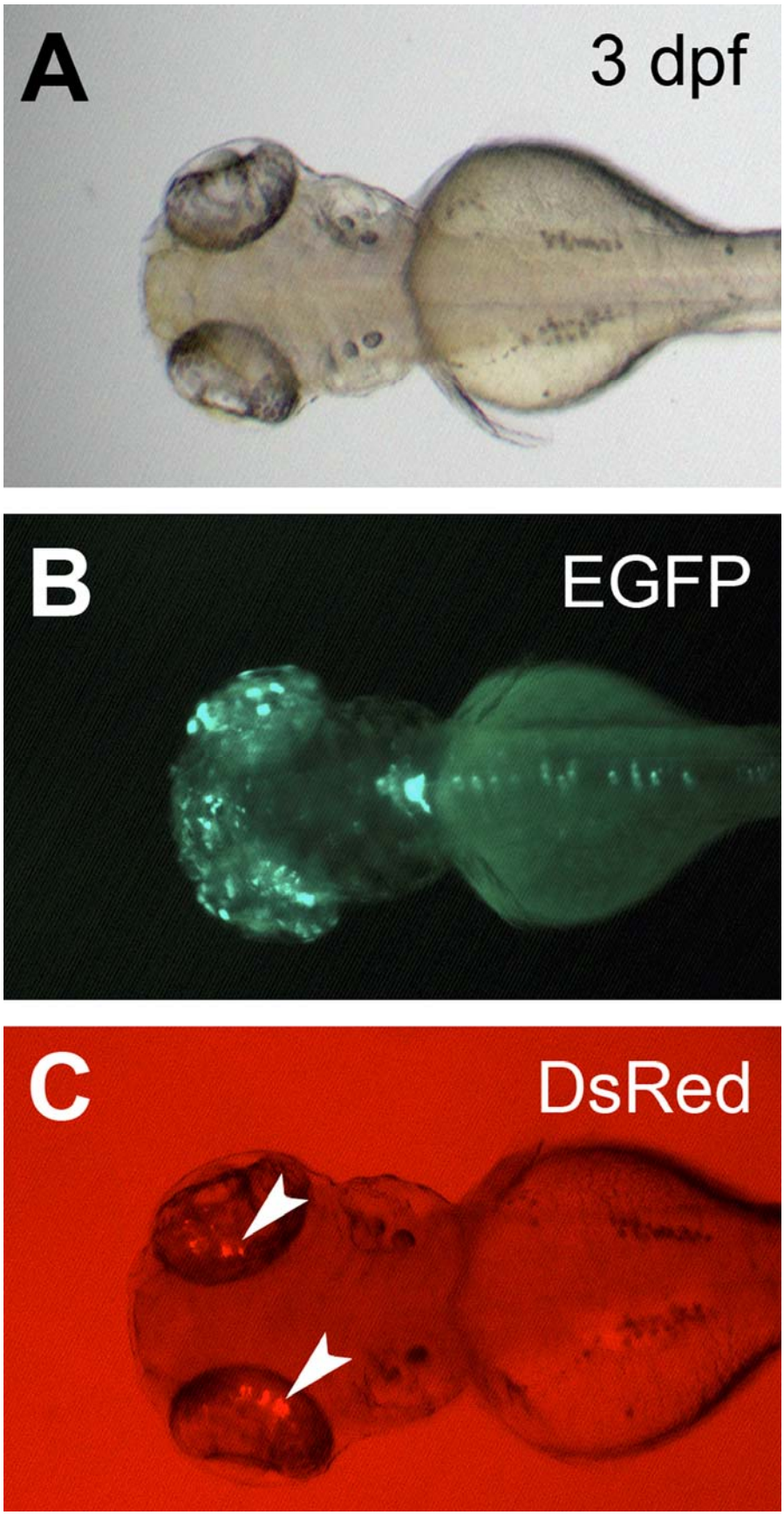

A
3 dpf
B
EGFP
C
DsRed

Fig. 2.5. Amacrine cells in the developing zebrafish retina express DsRed.

Sections cut through the eye of transiently transgenic embryos at different stages of development. (A) At 28 hpf EGFP, but not DsRed, is expressed by retinal progenitor cells (arrowheads). EGFP-expressing cells are also visible in the diencephalon (di) and hindbrain (*). Inset: lateral view of a 28 hpf embryo showing the plane of section. Rostral is left and dorsal is up. (B) Starting at the time of amacrine cell differentiation (50 hpf), a subset of EGFP-expressing cells begin expressing DsRed (arrows). (C, D) Whereas differentiating cells in the ONL, INL and GCL expressed EGFP, only amacrine cells appeared to express DsRed. EGFP expression in retinal ganglion cell axons was visible within the optic nerve by 72 hpf (on). Cell nuclei in D are labeled (blue) with Hoechst. (E-H) Sections cut through the eye from four different transiently transgenic embryos that were allowed to develop to 72 hpf. (E, F) DsRed is expressed by displaced amacrines (arrows), which have cell bodies in the GCL. (G) DsRed expressing cells (red) coexpress Pax6 (immunostained in green). (H) DsRed expressing cells (red) coexpress syntaxin (immunostained in blue), which is expressed in the dendritic processes and perikarya of all amacrine cells. hb, hindbrain; ln, lens; nr, neural retina.

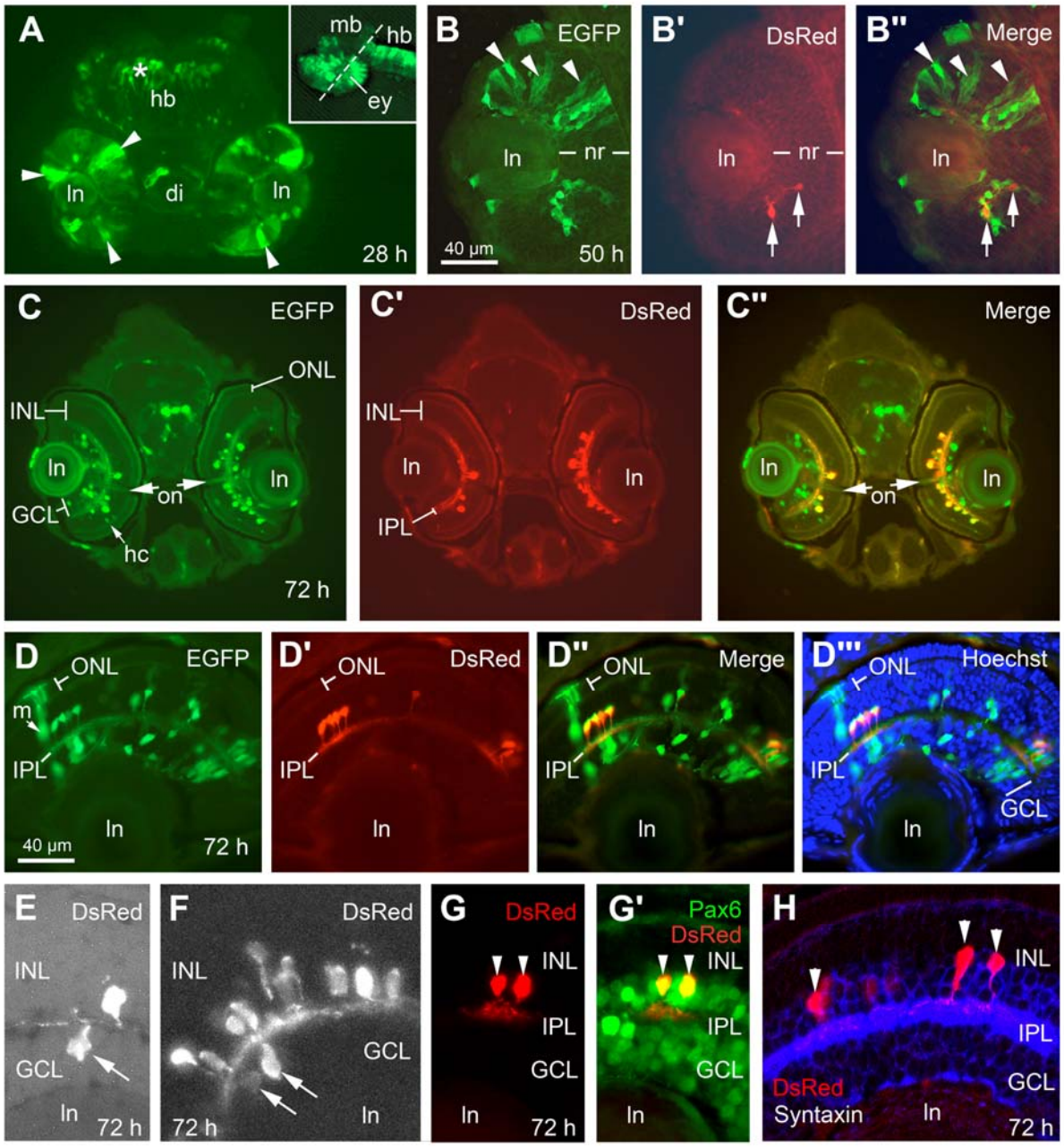

A
mb
hb
ey
hb
ln
di
ln
28 h
B
EGFP
ln
nr
40 µm
50 h
B'
DsRed
ln
nr
B''
Merge
ln
C
EGFP
ONL
INL
ln
ln
on
GCL
hc
72 h
C'
DsRed
INL
ln
ln
IPL
C''
Merge
ln
ln
on
D
ONL
EGFP
m
IPL
ln
40 µm
72 h
D'
ONL
DsRed
IPL
ln
D''
ONL
Merge
IPL
ln
D'''
ONL
Hoechst
IPL
GCL
ln
E
DsRed
INL
GCL
ln
72 h
F
DsRed
INL
GCL
72 h
ln
G
DsRed
INL
IPL
GCL
ln
72 h
G'
Pax6
DsRed
INL
IPL
GCL
ln
H
INL
IPL
GCL
DsRed
Syntaxin
ln
72 h

Fig. 2.6. Comparison of mouse and zebrafish Pax6 transcript units.

(A) Physical maps of mouse chromosome 2 showing the locations of Pax6 and Elp4, which are in an antisense orientation relative to each other, and zebrafish chromosome 25 showing the locations of Pax6a and zebrafish BAC DKEYP 46C10. In mammals, 3' Pax6 regulatory elements are located within Elp4 introns; therefore, the Elp4 gene is often used to provide reference points between genomes (e.g. Griffin et al., 2002; Kim and Lauderdale, 2006; Kleinjan et al., 2006; Kleinjan et al., 2001). Because Elp4 has not been conserved on zebrafish chromosome 25, the positions of zebrafish sequences with a high-degree of conservation with selected regulatory regions from mice are indicated. Comparison of homologous sequences between mice and zebrafish revealed that the zebrafish Pax6a locus is more compact. In addition to regulatory regions 5' and internal to the Pax6 transcript unit, zebrafish BAC 46C10 includes sequences conserved with the C1170, EI, and HS234 3' Pax6 regulatory regions identified in humans and mice (see below). (B) To identify putative regulatory sequences within zebrafish Pax6a BAC46C10, the genomic sequence from this BAC (AL929172) was compared to that from human (Z83308, AC131571, Z86001, AL136384), mouse (Al512589, AL590380), Fugu (AL021531), and zebrafish Pax6b (AC127461, BX957347) using MLAGAN and displayed using VISTA (Brudno et al., 2003). Human was used as the base sequence. Colored peaks represent regions of sequence conservation above 50% over at least 40 bp. Noncoding sequences are shaded in pink; exons are shaded in blue. No appreciable sequence similarity was observed 5' to a conserved CpG island, which is differentially methylated in mammals (¥) (Kim and Lauderdale, 2006). Enhancer regions identified by transgenic analysis in mammals are shown as red ovals. The regions in which each enhancer directs expression of a reporter construct are as follows: p, pancreas; se, lens, cornea, lacrimal gland, and conjunctiva; tel-hb-sc, telencephalon,

hindbrain and spinal cord; NTDP, post-mitotic, non-terminally differentiated neurons; α-en, amacrine cells, iris, ciliary body and RPE; intron-7, diencephalon, hindbrain and in late eye development; C1170, pretectum, neural retina and olfactory region; EI, lens, diencephalon and hindbrain; and HS234, neural retina, RPE and nasal pits (Griffin et al., 2002; Kammandel et al., 1999; Kleinjan et al., 2004; Kleinjan et al., 2001; Williams et al., 1998; Xu et al., 1999). The asterisk in the tel-hb-sc enhancer region denotes the location of a conserved E-box (Morgan, 2004).

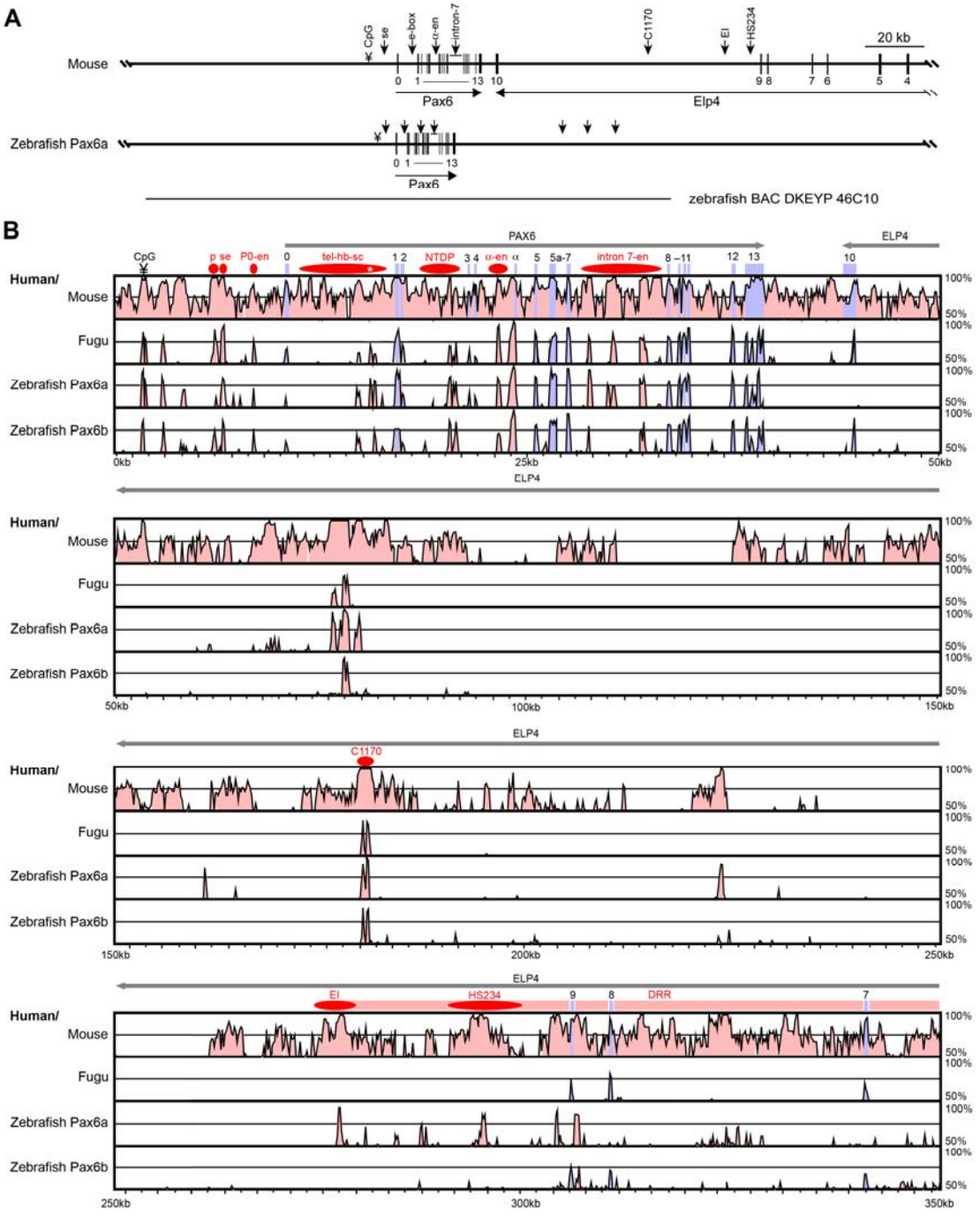

A
Mouse
CpG
se
e-box
α-en
intron-7
C1170
EI
HS234
20 kb
Pax6
Elp4
Zebrafish Pax6a
zebrafish BAC DKEYP 46C10
B
PAX6
ELP4
p se
P0-en
tel-hb-sc
NTDP
α-en
5a-7
intron 7-en
8 –11
12
13
10
Human/
Mouse
Fugu
Zebrafish Pax6a
Zebrafish Pax6b
100%
50%
0kb
25kb
50kb
50kb
100kb
150kb
150kb
200kb
250kb
250kb
300kb
350kb
C1170
EI
HS234
9
8
DRR
7

Fig. 2.7. Zebrafish Pax6a P0/P1 promoters are active in Pax6-expressing cells in the developing mouse retina.

Sections cut through the eyes of transgenic mouse embryos at E10.5 (A-C), E13.5 (D-F), or E17.5 (G-I). Both EGFP (A, D, G) and endogenous Pax6 (B, E, H) were visualized by indirect immunofluorescence (EGFP, green; Pax6, red). (C, F, I) Merged images. EGFP and Pax6 are coexpressed in cells in the neural retina (nr). At E10.5, EGFP expression replicated mouse Pax6. At both E13.5 (D-F) and E17.5 (G-I), EGFP was more strongly expressed in putative differentiating neurons (arrowheads) than in neuroepithelial cells. At E13.5 EGFP expression in retinal ganglion cell axons is visible within the optic nerve (on). Arrowheads in DF denote ganglion cells with axonal projections into the developing optic nerve. Scale bars = 25μm; bar in A applies to B-C; bar in D applies to E-I.

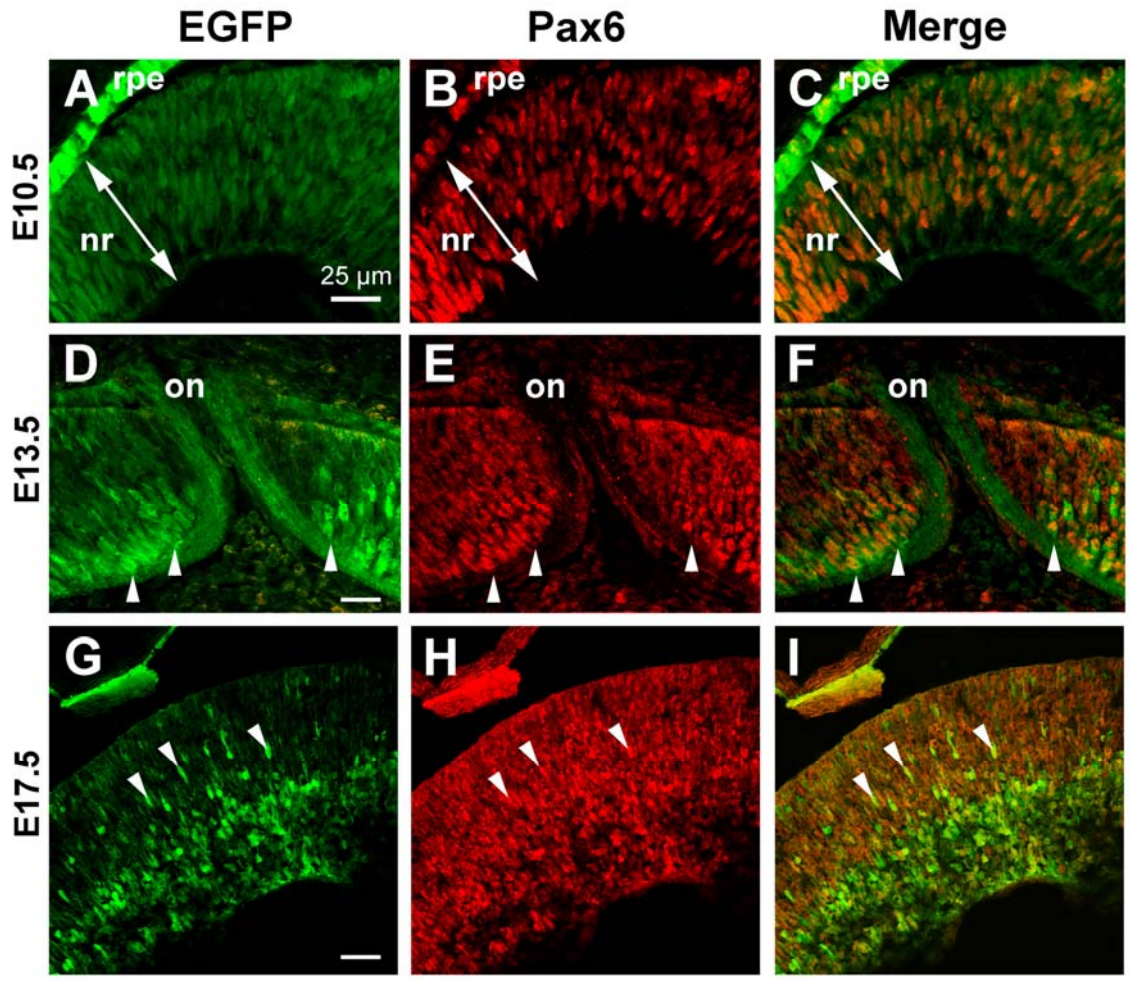

EGFP
Pax6
Merge
E10.5
E13.5
E17.5
A
B
C
D
E
F
G
H
I
rpe
nr
on
25 µm

Fig. 2.8. Zebrafish Pax6a P0/P1 promoters are active in Pax6-expressing cells in the adult mouse retina.

(A-B) EGFP from the zebrafish Pax6a BAC transgene is expressed by cells in the inner nuclear layer (INL) and ganglion cell layer (GCL). (A) EGFP+ radial processes also span the outer nuclear layer (ONL) and end at the outer limiting membrane (arrow). This suggests that Müller glia express the zebrafish Pax6a BAC transgene. (B-D) Direct comparison of EGFP and Pax6 expression in the adult mouse retina revealed that most, if not all, Pax6+ cells coexpressed EGFP; however, a few EGFP+ cells did not express Pax6 (arrowheads). The ectopic expression in these cells is most likely due to either species differences in expression or misregulation of the zebrafish Pax6a BAC transgene in mice. Scale bar in B applies to C-D.

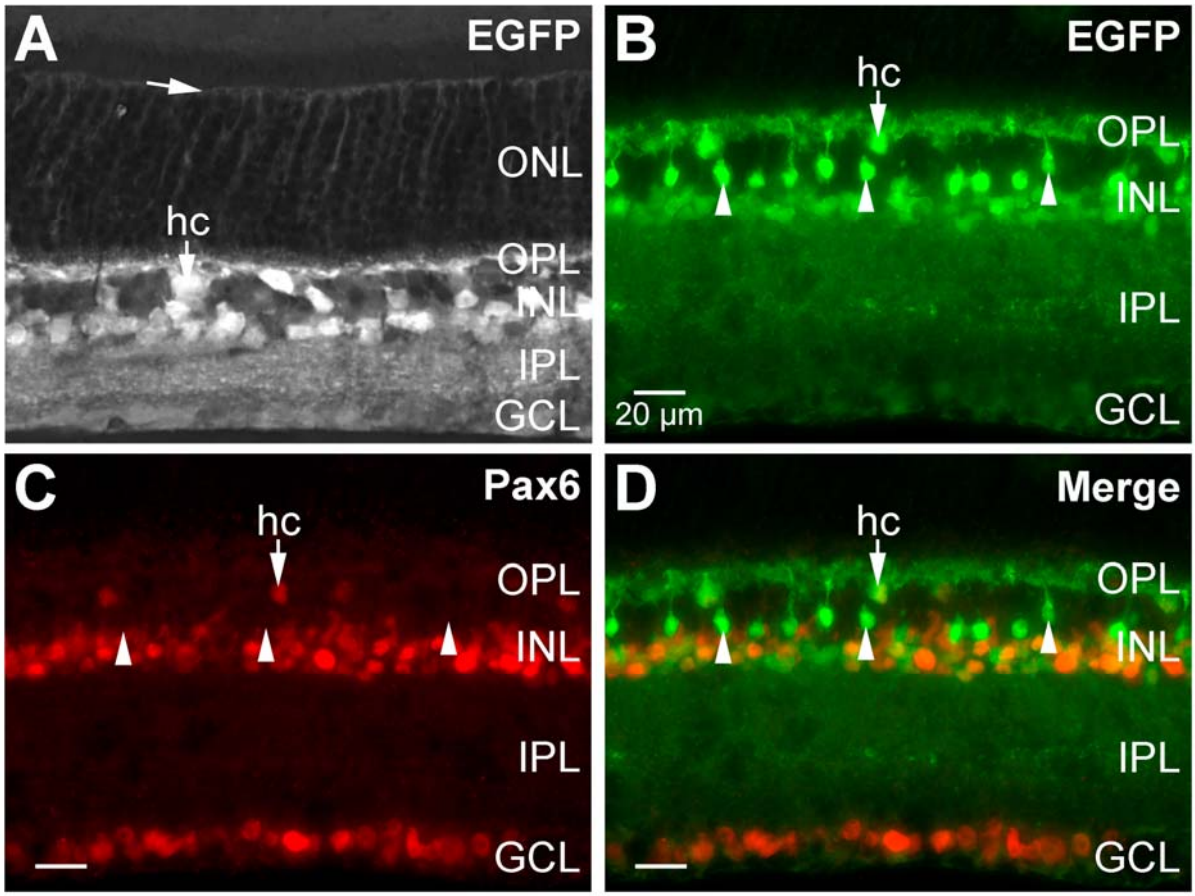

A
EGFP
ONL
hc
OPL
INL
IPL
GCL
B
EGFP
hc
OPL
INL
IPL
20 µm
GCL
C
Pax6
hc
OPL
INL
IPL
GCL
D
Merge
hc
OPL
INL
IPL
GCL

Fig. 2.9. Paired-containing and pairedless transcripts from the zebrafish Pax6a BAC transgene are differentially expressed by amacrine cells in the adult mouse retina.

(A-B, D) A subset of EGFP expressing cells in the adult mouse retina coexpress DsRed. Colabeling with syntaxin (C, D) reveals three populations of amacrine cells: syntaxin+/EGFP-/DsRed- (blue cells in D), syntaxin+/EGFP+/DsRed- (arrowheads) and syntaxin+/EGFP+/DsRed+ (arrows). DsRed expressing amacrine cells tend to project neurites to the middle strata of the IPL. Strata divisions were determined based on work by Masland and co-workers (Masland, 2001; Masland, 2004). Asterisks denote non-specific staining from a secondary antibody needed for these triple-label experiments. Scale bar in A applies to all panels.

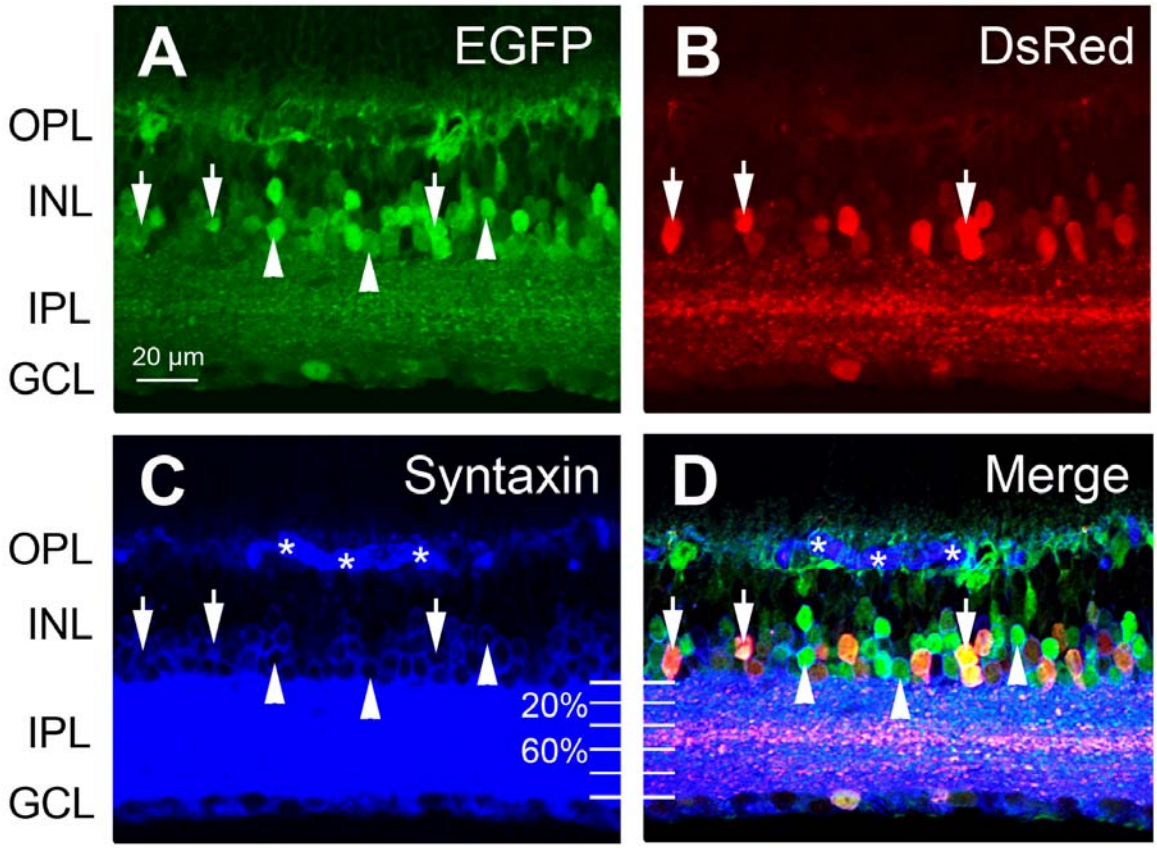

A
EGFP
B
DsRed
OPL
INL
IPL
GCL
20 µm
C
Syntaxin
D
Merge
OPL
INL
IPL
GCL
20%
60%

Fig. 2.10. Zebrafish Pax6a pairedless transcripts are expressed in GABAergic, but not glycinergic, amacrine cells in the adult mouse retina.

(A-E) Comparison of glycine transporter 1 (GlyT1), DsRed, and EGFP expression by coimmunostaining reveals that GlyT1+ cells express EGFP but not DsRed (arrowheads). (F-J) Comparison of glutamic acid decarboxylase (GAD), DsRed, and EGFP expression by coimmunostaining reveals that a population of GAD+ cells express EGFP and DsRed (arrows). The cell denoted by an arrow with an asterisk is weakly EGFP+.

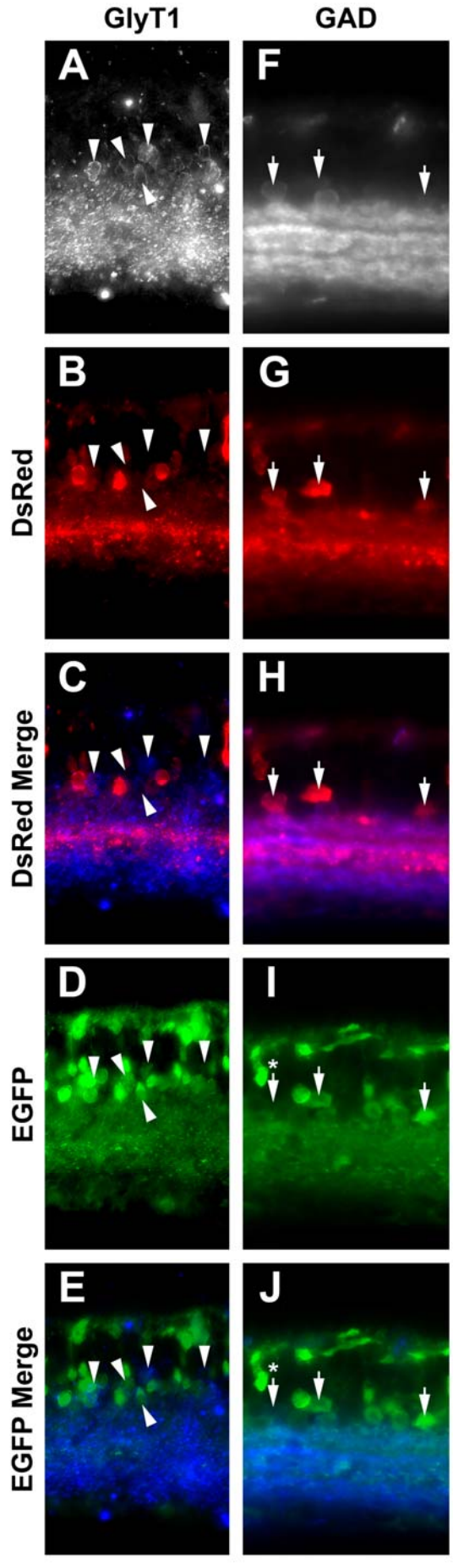

GlyT1
GAD
A
F
B
G
DsRed
C
H
DsRed Merge
D
I
EGFP
E
J
EGFP Merge

Fig. 2.11. A subset of bipolar cells in the adult mouse retina express the zebrafish Pax6a BAC transgene.

(A-D) In addition to being expressed in amacrine cells, DsRed is also expressed in a small number of morphologically distinct cells in the bipolar region of the INL, which are specifically imaged in this figure (arrows Fig. 11). These cells also express EGFP (B and D, F and H), Chx10 (C, D), and Prox1 (G, H); Chx10 and Prox1 are both expressed by bipolar cells in the adult retina. However, the majority of Chx10 or Prox1 expressing cells (blue-labeled cells in D and H, respectively) do not express the transgene. (E-H) Prox1-expressing horizontal cells (hc) coexpress EGFP, but not DsRed. (I-L) AII amacrine cells coexpress Prox1, GlyT1, and EGFP (arrowheads). Asterisks denote non-specific staining from the secondary used in this triple-label experiment.

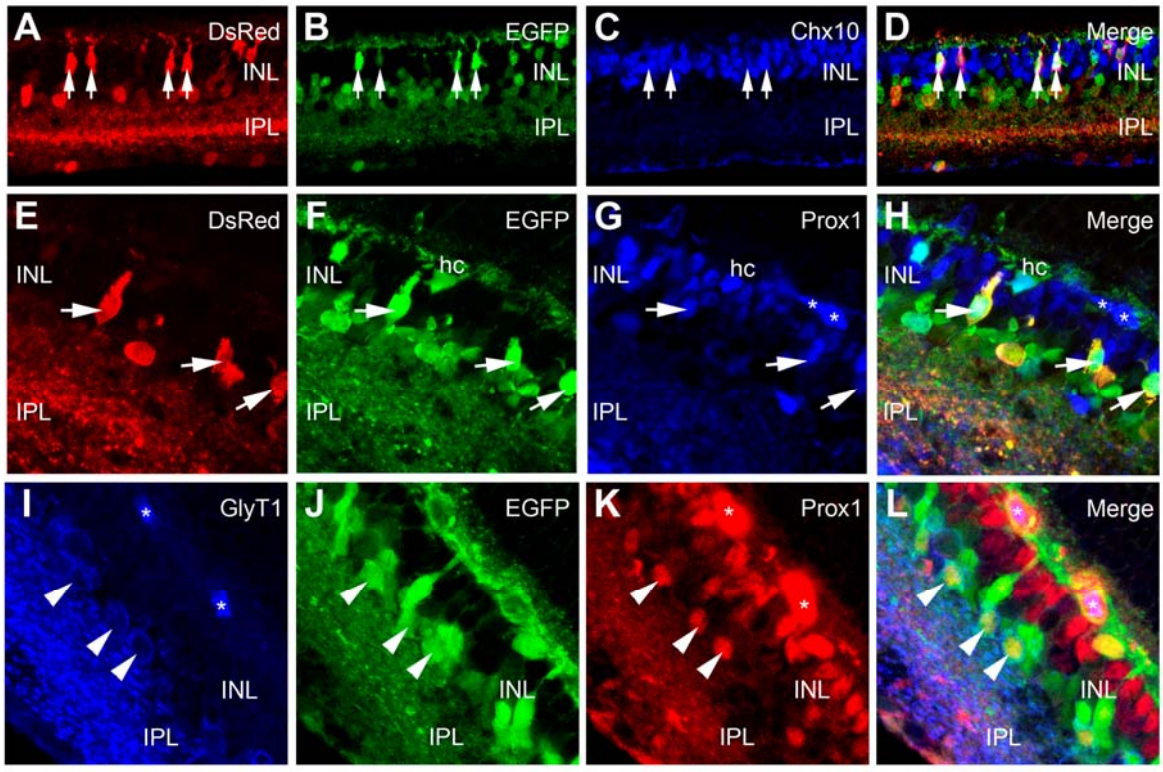
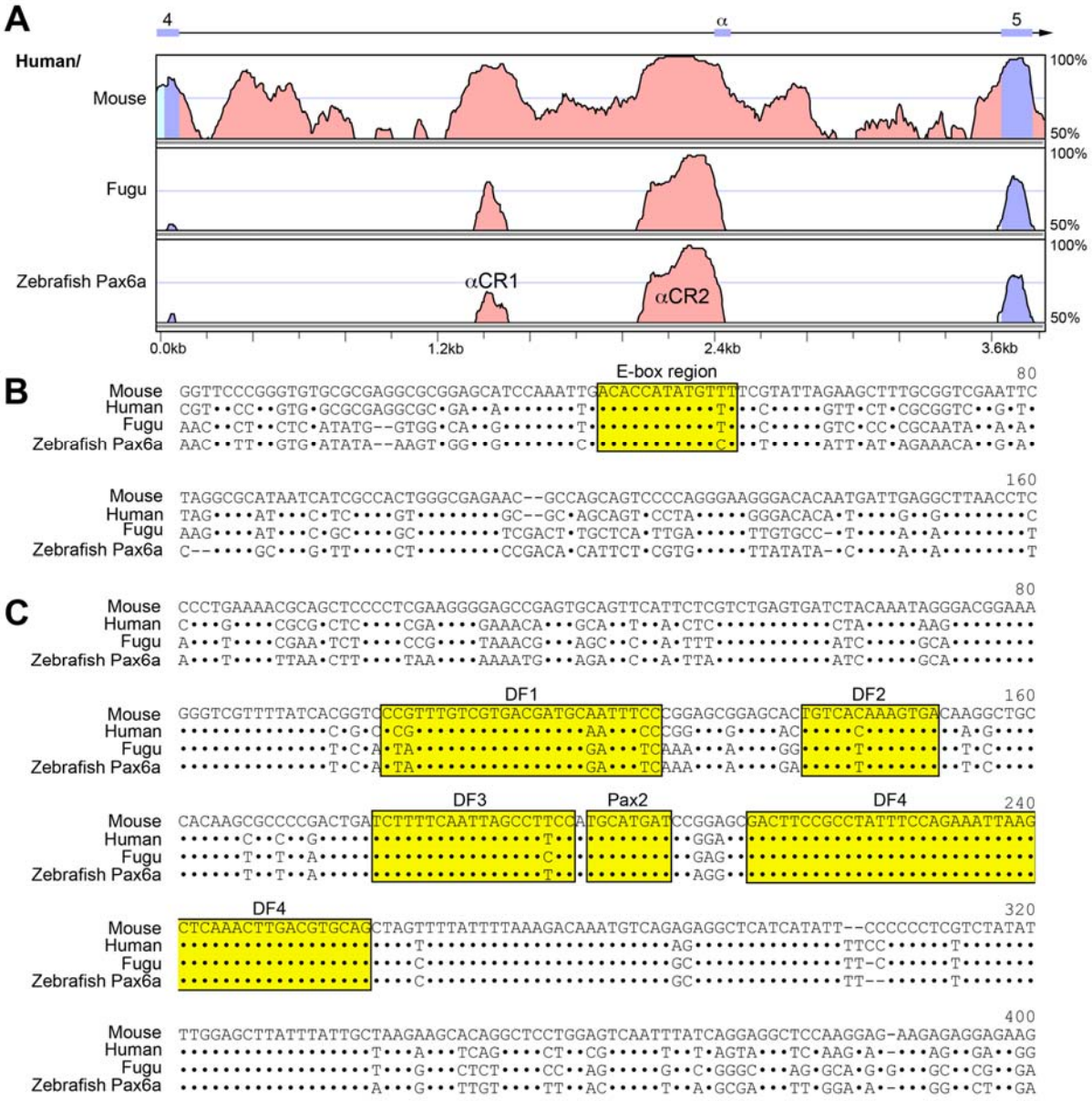

A
DsRed
INL
IPL
B
EGFP
INL
IPL
C
Chx10
INL
IPL
D
Merge
INL
IPL
E
DsRed
INL
IPL
F
EGFP
hc
INL
IPL
G
Prox1
hc
INL
IPL
H
Merge
hc
INL
IPL
I
GlyT1
INL
IPL
J
EGFP
INL
IPL
K
Prox1
INL
IPL
L
Merge
INL
IPL

Fig. 2.12. The zebrafish Pax6a *alpha* enhancer region is highly conserved.

(A) MLAGAN analysis of the alpha enhancer region between human, mouse, Fugu, and zebrafish Pax6a reveals two regions, denoted αCR1 and αCR2 respectively, that exhibit significant sequence conservation. (B-C) Sequence comparison of the two highly conserved regions in mouse, human, Fugu, and zebrafish Pax6a. (B) Conserved region 1. The boxed sequence (E-box region) contains potential binding sites for bHLH transcription factors. (C) Conserved region 2. Sequences corresponding to the four neuroretina-specific protein-binding sites (DF) identified in quail (Plaza et al., 1995) and a Pax2 binding site (Kammandel et al., 1999) are boxed. DF3 encompasses an Msx1 binding site (Kammandel et al., 1999). Multiple different neuroretina factors, including Brn3b (Pou4f2), have been shown to bind to DF4 (Plaza et al., 1999a).


A
4
α
5
Human/
Mouse
Fugu
Zebrafish Pax6a
100%
50%
αCR1
αCR2
0.0kb
1.2kb
2.4kb
3.6kb
B
E-box region
Mouse GGTTCCCGGGTGTGCGCGAGGCGCGGAGCATCCAAATTGACACCATATGTTTTCGTATTAGAAGCTTTGCGGTCGAATTC
Human CGT••CC••GTG•GCGCGAGGCGC•GA••A•••••••T•••••••••••T•••C•••••GTT•CT•CGCGGTC••G•T•
Fugu AAC••CT••CTC•ATATG--GTGG•CA••G•••••••T•••••••••••T•••C•••••GTC•CC•CGCAATA••A•A•
Zebrafish Pax6a AAC••TT••GTG•ATATA--AAGT•GG••G•••••••C•••••••••••C•••T•••••ATT•AT•AGAAACA••G•A•
Mouse TAGGCGCATAATCATCGCCACTGGGCGAGAAC--GCCAGCAGTCCCCAGGGAAGGGACACAATGATTGAGGCTTAACCTC
Human TAG••••AT•••C•TC••••GT••••••••GC--GC•AGCAGT•CCTA•••••GGGACACA•T••••G••G••••••••C
Fugu AAG••••AT•••C•GC••••GC••••••••TCGACT•TGCTCA•TTGA•••••TTGTGCC-•T••••A••A••••••••T
Zebrafish Pax6a C--••••GC•••G•TT••••CT••••••••CCGACA•CATTCT•CGTG•••••TTATATA-•C••••A••A••••••••T
C
Mouse CCCTGAAAACGCAGCTCCCCTCGAAGGGGAGCCGAGTGCAGTTCATTCTCGTCTGAGTGATCTACAAATAGGGACGGAAA
Human C•••G••••CGCG•CTC••••CGA••••GAAACA•••GCA••T••A•CTC••••••••••CTA•••••AAG••••••••
Fugu A•••T••••CGAA•TCT••••CCG••••TAAACG•••AGC••C••A•TTT••••••••••ATC•••••GCA••••••••
Zebrafish Pax6a A•••T••••TTAA•CTT••••TAA••••AAAATG•••AGA••C••A•TTA••••••••••ATC•••••GCA••••••••
DF1
DF2
Mouse GGGTCGTTTTATCACGGTCCCGTTTGTCGTGACGATGCAATTTCCCGGAGCGGAGCACTGTCACAAAGTGACAAGGCTGC
Human ••••••••••••••C•G•C•CG•••••••••••••••AA•••CCCGG•••G••••AC•••••C•••••••••A•G••••
Fugu ••••••••••••••T•C•A•TA•••••••••••••••GA•••TCAAA•••A••••GG•••••T•••••••••T•C••••
Zebrafish Pax6a ••••••••••••••T•C•A•TA•••••••••••••••GA•••TCAAA•••A••••GA•••••T•••••••••T•C••••
DF3
Pax2
DF4
Mouse CACAAGCGCCCCGACTGATCTTTTCAATTAGCCTTCCATGCATGATCCGGAGCGACTTCCGCCTATTTCCAGAAATTAAG
Human ••••••C••C••G•••••••••••••••••••T•••••••••••GGA••••••••••••••••••••••••••••••
Fugu ••••••T••T••A•••••••••••••••••••C•••••••••••GAG••••••••••••••••••••••••••••••
Zebrafish Pax6a ••••••T••T••A•••••••••••••••••••T•••••••••••AGG••••••••••••••••••••••••••••••
DF4
Mouse CTCAAACTTGACGTGCAGCTAGTTTTATTTTAAAGACAAATGTCAGAGAGGCTCATCATATT--CCCCCCTCGTCTATAT
Human ••••••••••••••••••••••T•••••••••••••••••••••••AG•••••••••••••TTCC••••••T•••••••
Fugu ••••••••••••••••••••••C•••••••••••••••••••••••GC•••••••••••••TT-C••••••T•••••••
Zebrafish Pax6a ••••••••••••••••••••••C•••••••••••••••••••••••GC•••••••••••••TT--•••••••T•••••••
Mouse TTGGAGCTTATTTATTGCTAAGAAGCACAGGCTCCTGGAGTCAATTTATCAGGAGGCTCCAAGGAG-AAGAGAGGAGAAG
Human ••••••••••••••••••T•••A•••TCAG••••CT••CG•••••T••T•AGTA•••TC•AAG•A•-•••AG••GA••GG
Fugu ••••••••••••••••••T•••G•••CTCT••••CC••AG•••••G••C•GGGC•••AG•GCA•G•G•••GC••CG••GA
Zebrafish Pax6a ••••••••••••••••••A•••G•••TTGT••••TT••AC•••••T••A•GCGA•••TT•GGA•A•-•••GG••CT••GA

CHAPTER 3

PAX6 ISOFORMS ARE DIFFERENTIALLY EXPRESSED IN AMACRINE CELL SUBPOPULATIONS OF THE MURINE RETINA

[1] Jorn Lakowski, Jiha Kim, Amy Riesenberg, James D. Lauderdale. To be submitted to Developmental Biology

Abstract

During retinogenesis in vertebrates, the seven major cell types are generated from a common pool of precursor cells, in a sequence that is conserved within the vertebrate lineage. Controlling this process is an evolutionarily conserved network of transcription factors, including basic helix-loop-helix transcriptional activators and repressors as well as homeodomain proteins. After the various main cell types have been established, a multitude of sub-cell types are generated, most of which remain largely unknown and the mechanism leading to their production are poorly understood. A central component of the network, directing oculogenesis in vertebrates, is the homeodomain transcription factor Pax6, which has been shown to mediate certain aspects of progenitor proliferation as well as cell type specification in the retina. While, its exact role during retinogenesis is not well defined, there is emerging evidence that its various functions during embryogenesis are carried out by distinct isoforms. Recent studies in several species, including zebrafish, mouse and quail have implicated a pairedless Pax6 isoform in the process of oculogenesis. In this study, we have examined the distribution of the paired-containing and pairedless Pax6 isoforms in the developing and mature murine retina. Using a bacterial artificial chromosome, containing the mouse Pax6 locus, we demonstrate that the various Pax6 isoforms exhibit a differential expression pattern within the amacrine cell population in the mouse retina. While paired-containing Pax6 is localized to both glycinergic and GABAergic amacrines, pairedless transcripts are restricted to GABAergic amacrine interneurons. While paired-containing Pax6 exhibits an exclusively nuclear localization, the pairedless isoform is also found in the cytoplasm. Furthermore, we demonstrate that the phenotype observed in Pax6 $^{flox/flox}$; α-Cre mice, is due to a differential inactivation of the two Pax6 isoforms.

INDEX WORDS: Pax6, isoform, retinogenesis, amacrine cells, specification

Introduction

The vertebrate retina contains seven major cell types, all of which are derived from a common multi-potent precursor cell during retinogenesis (Marquardt and Gruss 2002). They are generated in an evolutionarily conserved chronological order and their correct assembly depends on the concerted action of both intrinsic and extrinsic factors in the developing eye (Hatakeyama, Tomita et al. 2001; Hatakeyama and Kageyama 2004). The first cells to differentiate from the pool of precursors are ganglion and horizontal cells, followed by cones, rods, amacrines and bipolar cells. Mueller glia cells are generated last (Cepko 1999). The genetic network underlying this process is remarkably conserved within the vertebrate lineage and several important players have been identified in recent years. However, despite the progress made in elucidating the steps that lead to the correct formation of the retina, many open questions still remain.

Amacrines are one of the most abundant cells in the vertebrate retina, constituting approximately 30% of the total number of cells within the inner nuclear layer in the murine retina (Strettoi and Masland 1996; Jeon, Strettoi et al. 1998). With about 26 morphologically distinct types in mice, they present a highly diverse group of interneurons, integrating and relaying information from bipolar cells to ganglion cell, which then project axons into the optic nerve and eventually towards the appropriate processing center in the brain. Despite their diversity, amacrine cells can be separated into three main categories based on their neurotransmitter usage. In the murine retina, approximately 40% of all amacrines utilize GABA as their primary neurotransmitter, followed by 35% amacrines using glycine (Strettoi and Masland 1996; Jeon, Strettoi et al. 1998; Marquardt, Ashery-Padan et al. 2001). Within these two groups, cells can be further subdivided according to the use of secondary neurotransmitters, such

as dopamine or acetylcholine. The remaining amacrine cells are usually categorized as "unidentified", due to the lack of information regarding their transmitter usage.

Although several factors have been identified that appear to be involved in the generation of amacrine interneurons, none has been shown to mediate this process alone, probably reflecting the vast variety of subtypes within this cell class. The expression of the transcription factors NeuroD, Math3, Foxn4, Barhl2, Bhlhb-5 and Pax6 correlate with amacrine differentiation. Mutations in any one alone, however, do not completely abolish amacrine cell formation (Marquardt, Ashery-Padan et al. 2001; Inoue, Hojo et al. 2002; Li, Mo et al. 2004; Mo, Li et al. 2004; Feng, Xie et al. 2006). While factors such as NeuroD, Math3 and Foxn4 affect amacrine cells as a whole, other factors including Pax6, Bhlhb-5 (Feng, Xie et al. 2006) as well as Barhl-2 (Mo, Li et al. 2004) appear to be involved in generating distinct amacrine subpopulations. Mutations in NeuroD and Math3 alone do not perturb amacrine cell genesis, however, mice lacking both genes completely lack this cell class (Morrow, Furukawa et al. 1999; Inoue, Hojo et al. 2002). In these double mutant mice, retinal progenitors, destined to become amacrines transdifferentiate and adopt a ganglion cell or Mueller glia fate instead. On the other hand, missexpression of both factors alone, surprisingly, results in the formation of extra rod photoreceptors instead of additional amacrines. Furthermore, recent studies suggest that NeuroD and Math3 are downstream of Foxn4, a member of the winged helix/forkhead family of transcription factors (Li, Mo et al. 2004). Loss of function of this protein results in a reduced expression of both factors in amacrines. Another protein implicated in amacrine cell genesis is the homeodomain transcription factor Pax6.

In vertebrates, the importance of Pax6 is demonstrated by a vast variety of known mutations, all having profoundly detrimental effects on eye development or maintenance (Hanson, Churchill

et al. 1999). Haploinsufficiency for Pax6 results in aniridia in humans and the small eye phenotype (Sey) in mice, both heritable panocular disorders associated with foveal and iris hypoplasia, cataracts, corneal opacification as well as glaucoma (OMIM 106210). Homozygosity on the other hand, results in neonatal lethality in both species (Glaser, Jepeal et al. 1994; Grindley, Davidson et al. 1995; Grindley, Hargett et al. 1997). However, the exact role of Pax6 during retinogenesis is poorly understood and somewhat controversial. Using a *Cre*/lox approach, Marquardt and colleagues showed that conditional inactivation of Pax6 in the distal tips of the optic cup results in the exclusive generation of amacrine cells in this area, at the expense of all other cell types (Marquardt, Ashery-Padan et al. 2001; Marquardt and Gruss 2002). This result has been interpreted by some that Pax6 function is not required for the generation of the amacrine cell class and that this cell type may be the default fate of all retinal precursors. However, it is noteworthy that, although only cells of amacrine identity were observed, not all amacrine cell subtypes were produced by Pax6 deficient progenitor cells, suggesting a role in the differentiation of at least part of the amacrine population.

Inactivation of Pax6 eliminated almost completely glycinergic amacrines, which comprise about 34% of the entire amacrine cell population. Interestingly, the loss of glycinergic amacrine cells did not result in a corresponding increase in the number of GABAergic amacrines but in a shift of Pax6 deficient cells towards a mature yet "unidentified" amacrine cell fate. Interestingly, in the developing retina of homozygous *Sey* mice, it was recently shown that neurons differentiate prematurely, do not persist and adopt a generic, rather than specific fate during their short lives (Philips, Stair et al. 2005). These findings indicate that Pax6 controls the timing of retinal cell differentiation, as well as the cell fate decision made by the retinal progenitors. Taken together, Pax6 appears to be responsible for certain cell fate decisions made within the amacrine

cell subpopulation in addition to controlling the generation of all other retinal cell types in the retina.

It has been postulated that the different functional aspects of Pax6 are mediated by various isoforms of the same protein. One example is the Pax6(5a) isoform, which arises through alternative splicing, resulting in the insertion of 14 additional amino acids into the Paired DNA binding domain. This variant of Pax6 has been shown to possess different DNA binding properties than the conventional protein, and when deleted results in an iris specific phenotype in mice (Duncan, Kozmik et al. 2000; Singh, Mishra et al. 2002).

Our laboratory (Kim and Lauderdale 2006) and others (Kleinjan, Seawright et al. 2006) recently provided evidence for the existence of a Pax6 isoform [Pax6(-PD)] in mammals, containing the homeodomain but lacking the paired DNA binding domain and that is expressed in the developing eyes of mice and fish. While transcription of conventional Pax6 in vertebrates is initiated from two upstream promoters, P0 and P1, we have previously shown that pairedless Pax6 expression starts at the internally located *alpha* promoter, situated in intron 4 of this gene. Transcripts initiating at the P-*alpha* promoter therefore do not contain the evolutionarily conserved translation start site in exon 4, and are believed to utilize a translation start site in exon 7, just upstream of the sequence coding for the homeo-DNA binding domain. Furthermore, we and others have shown that over-expression of this pairedless isoform of Pax6 results in severe lens defects and subsequent disorganization of the neural retina, demonstrating a role of this isoform in oculogenesis (Kim and Lauderdale 2006; Kleinjan, Seawright et al. 2006). In the present study, using a dually modified mouse bacterial artificial chromosome (BAC), we show that the various Pax6 isoforms are differentially expressed in sub-populations of amacrine cells within the mouse retina. While paired-containing Pax6 [Pax6(PD)] is expressed in glycinergic

and GABAergic amacrines, pairedless Pax6 expression is restricted to GABAergic amacrines, suggesting that Pax6(-PD) may play a role in specifying this subtype.

Furthermore, using antibodies against the C-terminal and N-terminal parts of the Pax6 protein, we demonstrate that the paired containing but not pairedless Pax6 protein is eliminated in the distal tips of the optic cup of *Pax6*$^{flox/flox}$*;alpha-Cre* mice. Our results indicate that the phenotype observed in *Pax6*$^{flox/flox}$*;alpha-Cre* mice is due to a differential inactivation of Pax6 isoforms, controlling different cell fates within the amacrine cell population.

Results

Pax6(-PD) is expressed in the developing and adult retina

We have previously shown that zebrafish Pax6(-PD) is expressed in differentiating and mature amacrine cells in the retina (Lakowski, Majumder et al. 2007) and therefore wished to determine the distribution of the various Pax6 isoforms in the mouse retina. In the mouse, differentiation of this cell type commences at day 11 of embryonic development and continues until postnatal day 4, while certain markers for specific amacrine cell subtypes can be detected as early as e11.5 (Feng, Xie et al. 2006).

To visualize the expression domains of conventional Pax6 and Pax6(-PD) we decided to employ the dually modified murine BAC construct (Kim et al. unpublished). In this reporter construct the mouse Pax6 locus was modified by targeted placement of two different reporter cassettes. An EGFP gene was inserted in frame with the translation start site in exon 4, and a second reporter gene, DsRed, was inserted in the same fashion into exon 8. This strategy allowed us to distinguish between these two Pax6 isoforms, which is not possible by other conventional means such as immunohistochemistry or in situ hybridization.

We first wished to establish the expression domains of paired-containing Pax6 and pairedless Pax6 transcripts in the developing murine retina at e17.5, a time when retinal differentiation is well under way. EGFP, the reporter for paired containing Pax6, was expressed in a broad pattern throughout the neural retina, the ciliary margin, lens, cornea and the retinal pigmented epithelium (Fig.1 A, C, D, F). EGFP expression was also observed in the optic nerve, indicating that retinoganglial cells expressed the paired containing Pax6, isoform, although EGFP signal was relatively weak in the ganglion cell layer compared to the proliferating neuroblastic layer (Fig. 1 and data not shown). While there was some overlay in the expression patterns of both reporter genes, there were striking differences. In contrast to EGFP, DsRed, the reporter for Pax6(-PD) was completely absent from derivatives of the head surface ectoderm (e.g. lens and cornea) as well as the pigmented epithelium, consistent with previous observations of zebrafish Pax6(-PD). On the other hand, similar to EGFP, the DsRed reporter was most strongly expressed in the distal tips of the optic cup (Fig.1B, C), which gives rise to the ciliary margin, the future stem cell niche of the retina. Interestingly, the reporter gene expression seen for both isoforms in the ciliary margin was separated from the rest of the neural retina by a steep gradient, implying strict transcriptional control (Fig.1B).

Within the neural retina, we observed the main portion of the DsRed positive cells at the interface between ganglion cell and inner neuroblastic layer (Fig.1E, F), most if not all of them also being positive for EGFP. A few additional DsRed positive cells were scattered throughout both inner and outer neuroblastic layers (E, F) indicating that Pax6 (-PD) may have a function in either cycling retinal progenitor cells or cells that have just exited the cell cycle. Interestingly, while DsRed expressing cells were relatively restricted in the neural retina, EGFP signal of varying levels was visible throughout the neural retina (Fig. D, E, F), indicating distinct

functions for the two Pax6 isoforms. The observation that DsRed was expressed only in a subset of cells in the neural retina at this time of development was consistent with the notion that Pax6 (-PD) was involved in the generation of a particular retinal cell type or cell-subtype.
The position at the interface between ganglion cell layer and inner neuroblast layer is normally occupied but amacrine interneurons, projecting their processes into the forming inner plexiform layer. As most DsRed expressing cells were aligned along the periphery of the inner neuroblast layer (Fig.1E, F), it is very likely that those cells in fact represent differentiated amacrine interneurons. Furthermore, DsRed expressing cells located within the proliferating neuroblastic layer may be recently differentiated amacrine cells that have not yet migrated to their laminar layer within the retina.

In the adult retina, both reporter genes continued to be expressed in derivatives of structures that had expressed them during the embryonic development. DsRed exhibited a restricted expression in cells within the inner nuclear layer as well as ciliary body (H and data not shown), while being absent from lens and cornea. Similar to the embryonic stage, all cells that expressed the DsRed reporter gene were also positive for EGFP. Immunostaining showed that all DsRed positive cells in the retina were also positive for syntaxin, a pan-amacrine cell marker. Consistent with this conclusion, strong DsRed staining was also visible within three discrete strata of the inner plexiform layer that correspond to projections of amacrine cells, residing in both inner nuclear layer and ganglion cell layer. On the other hand, EGFP expression in the adult mouse eye was localized to the lens, cornea, ciliary body and inner nuclear layer. Position and morphology of those cells within the inner nuclear layer suggested that the majority of them belonged to the amacrine and horizontal cell types (G, J). In addition, we consistently observed EGFP expression

in the radial processes of Mueller glia cells, indicating that also this cell type expresses paired containing Pax6 (data not shown).

Pax6 isoforms are differentially expressed in amacrine cell subpopulations

Factors controlling the generation of specific subtypes of amacrine cells remain largely elusive, due to the vast diversity within this cell class. Because the distribution of the different Pax6 isoforms in the mouse retina is not clear and conditional inactivation of Pax6 in the retina results in a fate shift within the amacrine cell population, we set out to determine the distribution of paired containing and pairedless Pax6 in this cell class.

To investigate the expression domain of conventional paired containing Pax6 we performed a double immunostaining using an antibody directed against the paired DNA binding domain of Pax6 in conjunction with antibodies specific to either of the two main amacrine subtypes. Immunolabelling for glycine transporter 1 (Glyt-1), which marks glycinergic amacrine cells, was strongly visible in the inner nuclear layer and to a lesser degree in the ganglion cell layer (Fig 2A, C). Diffuse labeling, corresponding to projections of amacrine cells was observed in the inner plexiform layer (Fig 2A, C). We found that paired containing Pax6 was localized to the nuclei of those cells, consistent with its role as transcription factor (Fig2 B, C). Glutamic acid decarboxylase-6 is involved in γ-amino butyric acid (GABA) metabolism and can therefore be used to label GABAergic amacrine cells. Pax6(PD) was present in all GAD-6 positive amacrines (Fig.2 D,E, F). In addition, Pax6(PD) staining was also readily visible in cells residing in the ganglion cell layer (Fig.2 D) as well as putative horizontal cell abutting the outer plexiform layer (data not shown).

Previously, we showed that transcripts, giving rise to zebrafish Pax6(-PD), exhibit a restricted expression within the amacrine population. Analysis of our dually modified mouse BAC revealed that DsRed, corresponding to Pax6(-PD), was present in cells also positive for GAD-6 (Fig.2 G, H, I). Furthermore, strong DsRed staining was observed in three different strata within in the inner plexiform layer (Fig 2 H, I), matching the projections of GABAergic amacrine cells. Interestingly, DsRed did not co-localize with Glyt-1, a result consistent with our previous observations (Fig.2 J, K, L). Furthermore, using markers for cholinergic (ChAT) and dopaminergic (TH) amacrine cells, we found that Pax6(-PD) did not co-localize with either of the two subpopulations (data not shown). In contrast, Pax6(+PD) was strongly expressed in those cells (data not shown). We thus concluded that the different Pax6 isoforms are subject to a differential regulatory mechanism within the mouse amacrine cell population.

Conditional inactivation of Pax6 affects paired containing but not pairedless isoform

Work by Marquardt and colleagues showed that Pax6 function is required for the generation of all photoreceptors, horizontal,-ganglion,-bipolar,-Mueller glia as well as glycinergic amacrine cells in the mouse retina (Marquardt, Ashery-Padan et al. 2001). GABAergic amacrines, however, remained almost unaffected in this study (Marquardt, Ashery-Padan et al. 2001; Marquardt and Gruss 2002). These results imply that another factor(s) is responsible for the generation of GABAergic amacrines, the identity of which remains elusive.

Given the restricted expression of Pax6(-PD) in GABAergic amacrine cells in the murine retina, we wished to reexamine the effects of Pax6 inactivation in the distal tips of the optic cup following *Cre*-loxP mediated deletion. *α-Cre-GFP* and $Pax6^{flox/flox}$ transgenic mice have been described previously. Briefly; In $Pax6^{flox/flox}$, the endogenous Pax6 gene was replaced by

homologous recombination with an altered Pax6 allele, in which exon 4-6 are flanked by two loxP recombination sites. This genomic region contains both the translation start site and the paired box motif. To achieve neural retina specific inactivation of Pax6, the *Cre* recombinase gene was placed under the control of the Pax6 α-enhancer, a retinal specific regulatory element, driving in the distal tips of the optic cup, as well as putative amacrine cells in the central retina. In this construct, the use of an internal ribosome entry site, results in the simultaneous production of *Cre* protein and GFP, from one bicistronic mRNA, marking the cells effected by the recombination event.

In order to investigate the efficiency of Pax6 inactivation in this experimental system, we performed immunostaining for Pax6 using antibodies directed against different parts of the Pax6 protein. As previously reported, at E13.5, activity of the Pax6(+PD) α-enhancer driving transcription of *Cre* recombinase and EGFP, was visible in the distal tips of the developing neural retina that normally give rise to the ciliary body (Fig.3 A). Using an antibody specific to the N-terminal portion of the Pax6 protein, we confirmed that no paired domain containing Pax6 was present in the part of the retina (Fig.3 B), affected by *Cre* mediated modification of the Pax6 locus. However, Pax6 positive cells were observed, as expected, in the central neural retina, retinal pigmented epithelium, lens and the developing cornea (Gig.3 B, C). Surprisingly, however, an antibody specific for the C-terminal portion of the Pax6 protein showed strong reactivity in the domain of *Cre* recombinase expression (Fig.3, F), indicating that Pax6 protein was still being produced by cell having undergone *Cre* mediated recombination at the Pax6 locus. While Pax6 signal outside the range of *Cre* activity was localized exclusively to the nucleus of cells, we observed both nuclear and cytoplasmic Pax6 stain in those cells (Fig.3, E, F). This was surprising to us, as Pax6 is a transcription factor, usually restricted to the nucleus.

However, these results demonstrate that *Cre* mediated deletion of Pax6 as reported previously effects the paired containing but not the pairedless isoform of Pax6.

Pax6(-PD) is located in both cytoplasm and nucleus

In accordance with its role as a transcription factor the Pax6 protein is normally localized to the nucleus. Because our immunohistochemical analysis of *Pax6*$^{flox/flox}$*;α-Cre*; mice suggested that Pax6(-PD) was mostly cytoplasmic (Fig.4E), we wished to verify the cellular localization of both Pax6 isoforms in a cell culture assay. Expression plasmids containing the coding sequences for the Pax6(+PD), Pax6(-PD) and EGFP gene respectively, were prepared and transfected into mouse fibroblast cells (NIH3T3), which normally do not express Pax6 (Fig.4C). Expression of those genes was directed by the cytomegalovirus (CMV) promoter/enhancer, shown to function efficiently in most mammalian cell types. Transfection of the control pCMV-EGFP construct resulted in cytoplasmic and nuclear EGFP expression, while no Pax6 staining was observed (Fig. 4 B, D). We then co-transfected of the pCMV-Pax6 together with the pCMV-EGFP control vector and performed immunostaining using antibodies directed at either the paired-domain (PD) or the carboxy-terminal transactivation domain (C-term). As expected, strong nuclear Pax6 was detected with either of the two antibodies, indicating that paired containing Pax6, is entirely restricted to the nucleus of cells. EGFP signal was readily visible in both the cytoplasmic and nuclear domains.

In contrast, pairedless Pax6(-PD) protein was found to be localized mostly in the cytoplasm but could also be observed, yet to a lesser extent, in the nucleus (Fig.4H). Although somewhat surprising, a similar observation has been reported in quail. The differential cellular distribution of the two Pax6 isoforms is intriguing and suggests distinct physiological roles.

Discussion

The mechanisms controlling the correct special and temporal generation all cell types in the developing vertebrate retina from a common pool of precursor cells are beginning to be unraveled. However, many aspects of cellular differentiation, especially the specification of certain neuronal subtypes are still only poorly understood. To find regenerative or cell replacement therapies for the visual system, it is imperative to have a comprehensive understanding of the factors and pathways directing the fate of all cell in the retina, including those responsible for creating cell subtype diversity. In this study we have investigated the expression of two Pax6 isoforms in the developing and mature murine retina. We show that both isoforms are differentially regulated, suggesting distinct functions during development and in the adult.

Differential expression of Pax6 isoforms in amacrine cell subtypes

Differentiation of amacrine interneurons in the murine retina commences at E11.5, peaks around E15 and continues until postnatal day 4 (Feng, Xie et al. 2006). Like the other retinal neurons, amacrine cells are born in the neuroblast layer and then migrate to their specific laminar positions, where they form inner plexiform connections with other cells such as ganglions cells or bipolar cells. Our analysis reveals that the temporal and spatial expression patterns of both Pax6 variants coincides with the generation of amacrine cells during embryogenesis; although, the broader expression of Pax6(PD) implies a function of this isoform in the generation of additional cell types in the eye at this time. Furthermore, our expression analysis demonstrates that paired containing and pairedless Pax6 isoforms are expressed in not only newly generated but also mature amacrines in the mouse retina. The fact that Pax6(-PD) is only present in a subset

of retinal progenitors and putative amacrine cells, suggests that it's role may lie with the generation of specific cellular subtypes after a cell has been determined to be an amacrine interneuron. Consistent with this conclusion, Pax6(-PD) is restricted to select GABAergic cells within the adult retina, while Pax6(PD) is localized to both glycinergic and GABAergic cells within the amacrine cell population. Therefore, the Pax6(-PD) isoform could serve as early subtype specific cellular marker for this cell sub-type.

It is noteworthy that while Pax6(-PD) is restricted to GABAergic amacrines, its paired containing counterpart is expressed in additional cell types within the adult retina. Based on position, morphology and specific marker expression analysis, Pax6(+PD) is strongly expressed in both horizontal and Mueller glia cells (data not shown). Furthermore, EGFP expression in the optic nerve suggests that this isoform is also present in retinoganglial cells (data not shown).

Effect of *Cre*-mediated inactivation of Pax6 on its isoforms in the retina

Together with other factors, such as Six3, Six6 and Rx1, Pax6 appears to be acting in an evolutionarily conserved genetic network, controlling the initiation of oculogenesis (Cepko 1999; Hatakeyama, Tomita et al. 2001; Hatakeyama and Kageyama 2004). The Pax6 gene is expressed in retinal precursor cells prior to the onset of retinal differentiation and during subsequent steps of retinogenesis in vertebrates. While overexpression of Pax6 results in the generation of ectopic retinal tissue in frogs, loss of function leads to an early arrest of optic vesicle formation, preventing a further analysis of Pax6 function in processes following this event. Previously, Marquardt et al. employed a conditional deletion approach, using the *Cre*-loxP system to investigate to function of Pax6 during retinal differentiation, thereby sidestepping the problems associated with null mutations of this gene (Marquardt, Ashery-Padan et al. 2001;

Marquardt and Gruss 2002). While this approach eliminates the conserved translation start site located in exon 4, it does not affect translation initiation from a site downstream of exon 6.

In this study we demonstrate, using antibodies directed against the paired domain and C-terminal transactivation domain that conditional inactivation of Pax6 using the *Cre*-loxP approach results in a differential deletion of the two Pax6 isoforms. While the Pax6 protein containing the paired domain is deleted in the region of *Cre* mediated recombination, pairedless Pax6 does not appear to be affected by this event. Therefore it is tempting to speculate that after deletion of Pax6, the action of Pax6(-PD) variant contributes to fate decisions made by retinal precursor cells.

Potential translation start sites of the pairedless Pax6 isoform

Our analysis of human, mouse and zebrafish sequences has revealed that four potential in frame translation start sites exist downstream of Pax6 exon 6 that could give rise to a pairedless Pax6 isoform still retaining the homeo-DNA binding domain (Fig.5). Three closely spaced potential start sites (PSS), located in exon 7, each display an "adequate" *Kozak* consensus sequence, while a fourth in frame ATG in exon 8 only constitutes a "weak" consensus sequence (Kozak 1984). It is therefore likely, that translation of a pairedless Pax6 isoform initiates at one of the evolutionarily conserved putative translation start sites in exon 7.

Model for action of Pax6 isoforms in the process of amacrine subtype genesis

It has been shown that the redundant function of the transcription factors Math3 and NeuroD, probably controlled by Foxn4, is required to specify amacrine cells from multipotent progenitors in the retina (Inoue, Hojo et al. 2002; Li, Mo et al. 2004). The cell fate decision towards one of

the three different amacrine subtypes is then determined by the presence or absence of additional transcription factors. For example, the homeodomain factor Barhl2 (Mo, Li et al. 2004) is expressed by all amacrines but promotes the generation of glycinergic amacrines, while not affecting cells adopting a GABAergic character. In addition, inactivation of paired containing Pax6 clearly demonstrates the necessity of this protein for the generation of this cell subtype, however, it is currently unclear if this is achieved synergistically or through parallel action. It is conceivable that both proteins direct the differentiation of selective glycinergic cells within this subpopulation of amacrines. Bhlhb5, a bHLH transcription factor of the Olig family, has been implicated in the specification of GABAergic amacrines as well as cone bipolar cells (Feng, Xie et al. 2006). Homozygous Bhlhb5 mutants, show a loss of approximately 44% of all GABAergic amacrines, and a significant reduction in TH positive amacrines, while leaving the number of Chat positive amacrines unaffected. These findings imply that another factor(s) is responsible for the genesis of the remaining GABAergic amacrine cells. The restricted expression of Pax6(-PD) in this cell subtype, starting during early retinogenesis and continuing into adulthood, suggests that it may be involved in the specification and maintenance of those neurons.

As glycinergic and GABAergic amacrines only account for about three fourth of the total population of amacrine cells in the murine retina, additional factors may be required for the generation of other “unidentified” amacrines.

Materials and Methods

Construction of a dual reporter Pax6 BAC transgene

We modified a mouse Pax6 BAC clone RPCI-24 304E12 (BAC-PAC Resource Center at Children's HospitalOakland Research Institute in Oakland, California) by targeted insertion of an

Enhanced Green Fluorescent Protein (EGFP) pA reporter cassette into the conserved translation start site of Pax6 exon 4, followed by a second insertion of a Red Fluorescent Protein (DsRFP) pA reporter cassette into Pax6 exon 8, using the prophage BAC modification system (Lee et al., 2001; Yu et al., 2000). The EGFP reporter cassette and targeting were as previously described (Kim and Lauderdale, 2006). The DsRFP-FRT-kan-FRT targeting cassette was PCR-amplified from pCS2+DsRFP-FRT-kan-FRT plasmid (Lakowski et al., *in press*) using the Pax6 exon 8 forward targeting primer, E8RECF, 5'-*AGAACACCAACTCCATCAGTTCTAACGGAGAAGACT-CGGATGAAGCTCAG*ATGG-CCTCCTCCGAGGACGTCA-3' and the Pax6 reverse targeting primer, E8RECR, 5'-*TATGCAAAGAGCCCTAGCTAAATTTAGCCCTTTGTGCTTTTGCTGTGG-CA*GATCAGTTATCT-AGATCCGG-3'. Nucleotides in italics are homologous to Pax6 sequences and those in roman are homologous to amplification cassette. Both, the EGFP and DsRED open reading frames, are followed immediately by SV40 late polyadenylation (polyAor pA) sequences, which has been shown to be more efficient than the SV40 early polyA signal. Following PCR, the respective targeting construct was gel purified using a Qiaquick gel extraction kit (Qiagen) and treated with *DpnI* to remove remaining template plasmid before use for homologous recombination. BAC recombination was performed as described in Lakowski et al. (*in press*); details available upon request. The structural integrity of the doubly-modified BAC was examined by fingerprint analysis (Gong et al., 2003; Kim and Lauderdale, 2006) using *Bam*HI and by Southern blot analysis and sequences of the targeted regions were verified by automated sequencing of both DNA strands (Supplemental Data).

Generation of mice transgenic for the dual-reporter Pax6 BAC

Doubly modified Pax6 BAC 304E12-EGFP/RFP was purified using NucleoBond® BAC Maxi Kit (Clontech) and linearized by PI-SceI (Spitz et al., 2001). To remove remaining impurities the linearized BAC DNA was purified by phenol/chloroform extraction followed by ethanol precipitation, and finally dissolved in microinjection buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 30μM spermine, 70μM spermidine, 100 mM NaCl). The BAC was delivered by male pronuclear injection, using standard procedures. Mice carrying the BAC transgene were genotyped using tail DNA, by Southern blot analysis using either EGFP or DsRFP probes, and by PCR detect the EGFP and/or DsRFP reporter cassettes. Four transgenic founder mice (lines 897, 898, 939, and 949) were generated and F1 founders were generated by crossing a founder male (line 939) or founder females (lines 897, 898, and 942) to CD-1 mice (Charles River Laboratories). Transgene copy number was determined by densitometric analysis of Southern blots using ImageJ (v1.33) as previously described (Kim and Lauderdale, 2006). Comparison with the endogenous Pax6 gene as a reference indicates that line 942 harbors 2 copies, lines 897 and 939 both harbor 5±1 copies, and line 898 harbors 7±1 copies of the transgene.

Immunofluorescence labeling

All immunostainings were carried out on frozen sections prepared from embryos or tissues. Specimen were fixed with 4% paraformaldehyde overnight and taken through a gradient pf sucrose solutions (5%, 15%) followed by an overnight incubation period in gelatin (Mastick et al.,1997). Incubation with the primary antibody was usually performed overnight at room temperature followed by secondary antibody incubation for 30 min. The following primary antibodies and dilutions were used: polyclonal rabbit anti-Pax6 (1:1000, Mastick et al., 1997),

polyclonal rabbit anti-Prox1 (1:5000, Chemicon AB5475), In order to amplify DsRFP signal, polyclonal rabbit anti-DsRed antibody (1:2000, Clontech) was used. Finally, after several washes in TST, the slides were incubated for 30 min with biotinylated secondary antibody (Jackson ImmunoResearch Laboratories; 111-065-144, 705-065-147) at 1:100 dilution followed by Cy3-conjugated Streptavidin (Jackson ImmunoResearch Laboratories, 016-160-0840) at 1:200 dilution for 30 min. Specific signal was detected by either standard fluorescence microscopy or laser scanning confocal microscopy.

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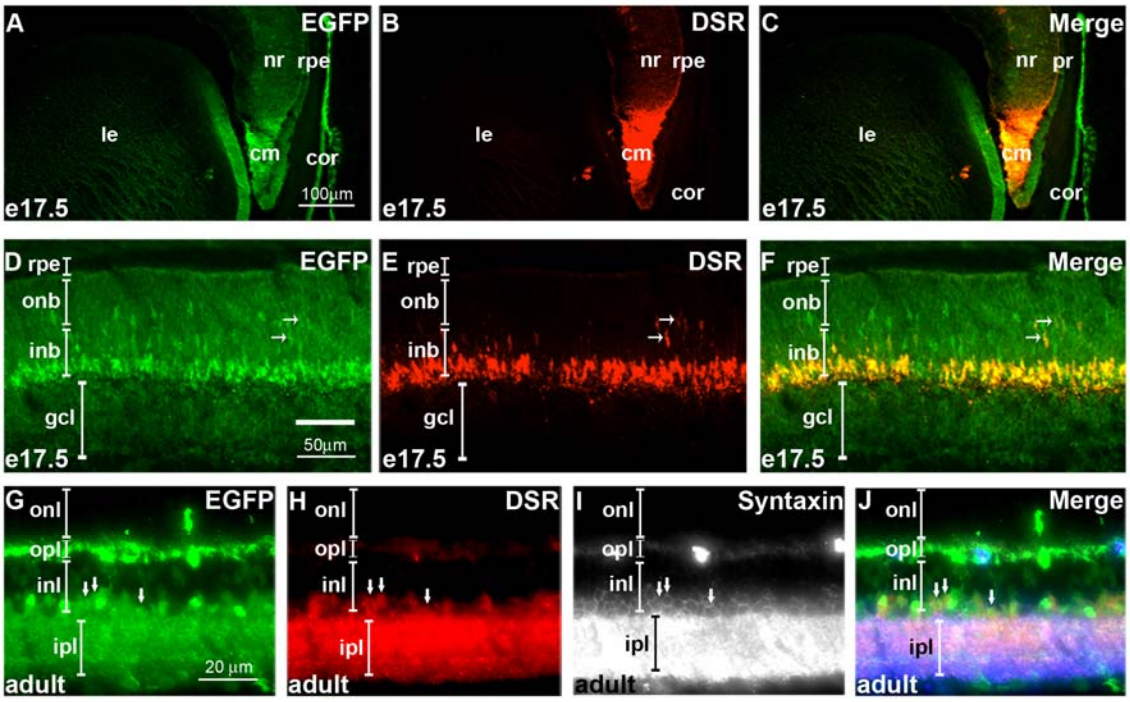
Fig 3.1. Expression of murine Pax6 isoforms in the embryonic and adult retina.

Analysis of BAC transgene expression at E17.5 of murine development shows that EGFP signal is present in the cornea, retinal pigmented epithelium, lens, ciliary margin and the neural retina (A, C). DsRed staining, however, is absent from the cornea and lens and is restricted to the ciliary margin as well as cells in the neural retina (B, C). While, weak EGFP expression is present throughout the entire neural retina, including the pool of progenitor cells in the neuroblastic layer, the strongest expressing cells were putative amacrine cells, aligned at the border between inner neuroblastic layer and ganglion cell layer. (D, F). DsRed was most highly expressed in the ciliary margin and a subset of retinal progenitor cells (arrows in B, D, E, F) as well as putative amacrine interneurons. Most, if not all, DsRed expressing cells were also EGFP positive (A-F).

In the mature mouse retina, immunostaining with syntaxin, a pan amacrine cell marker, revealed a restriction of DsRed expression to amacrine cells (arrows), which also co-expressed EGFP (G, H, I, J).

Abreviations: cm, ciliary margin; h, horizontal cell; cor cornea; gcl, ganglion cell layer; ipl, inner plexiform layer; inb, inner neuroblastic layer; le, lens; nr, neural retina; rpe, retinal pigmented epithelium

(data contributed by Jorn Lakowski)


A
EGFP
nr
rpe
le
cm
cor
100μm
e17.5
B
DSR
nr
rpe
le
cm
cor
e17.5
C
Merge
nr
pr
le
cm
cor
e17.5
D
rpe
EGFP
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inb
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50μm
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E
rpe
DSR
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inb
gcl
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F
rpe
Merge
onb
inb
gcl
e17.5
G
onl
EGFP
opl
inl
ipl
20 μm
adult
H
onl
DSR
opl
inl
ipl
adult
I
onl
Syntaxin
opl
inl
ipl
adult
J
onl
Merge
opl
inl
ipl
adult

Fig. 3.2. Differential expression of Pax6 isoforms in amacrine cell subpopulations.

Immunohistochemical analysis of Pax6 isoform expression in the adult retina of wild type and BAC transgenic mice. A-F, 12μm cryosections stained with an antibody directed at the paired DNA binding domain of Pax6, which specifically recognizes the paired containing Pax6 isoform. G-I, 12μm cryosections stained with GAD-6 (GABAergic neurons) and Glyt-1 (glycinergic neurons) respectively.

In wild type adult mice, paired containing Pax6 protein co-localizes with Glyt-1 and GAD-6 positive cells in the inner nuclear layer of the retina (A-F). Additional Pax6 expressing cells are visible in the ganglion cell layer and within the inner plexiform layer (interplexiform amacrines, arrows in E). Double labeling for DsRed and GAD-6 in BAC transgenic mice reveals that most, if not all, cells expressing the pairedless Pax6 isoform are GABAergic amacrine cells (G-I). Three DsRed positive strata are visible within the inner plexiform layer, corresponding to the characteristic cellular projections of GABAergic amacrines. In contrast, DsRed staining is absent from, most if not all, amacrine cells positive for Glyt-1, indicating that glycinergic amacrine interneurons usually do not express the pairedless Pax6 isoform.

Abbreviations: gcl, ganglion cell layer; inl, inner nuclear layer; ipl, inner plexiform layer; opl, outer plexiform layer

(data contributed by Jorn Lakowski, University of Georgia, Athens GA)

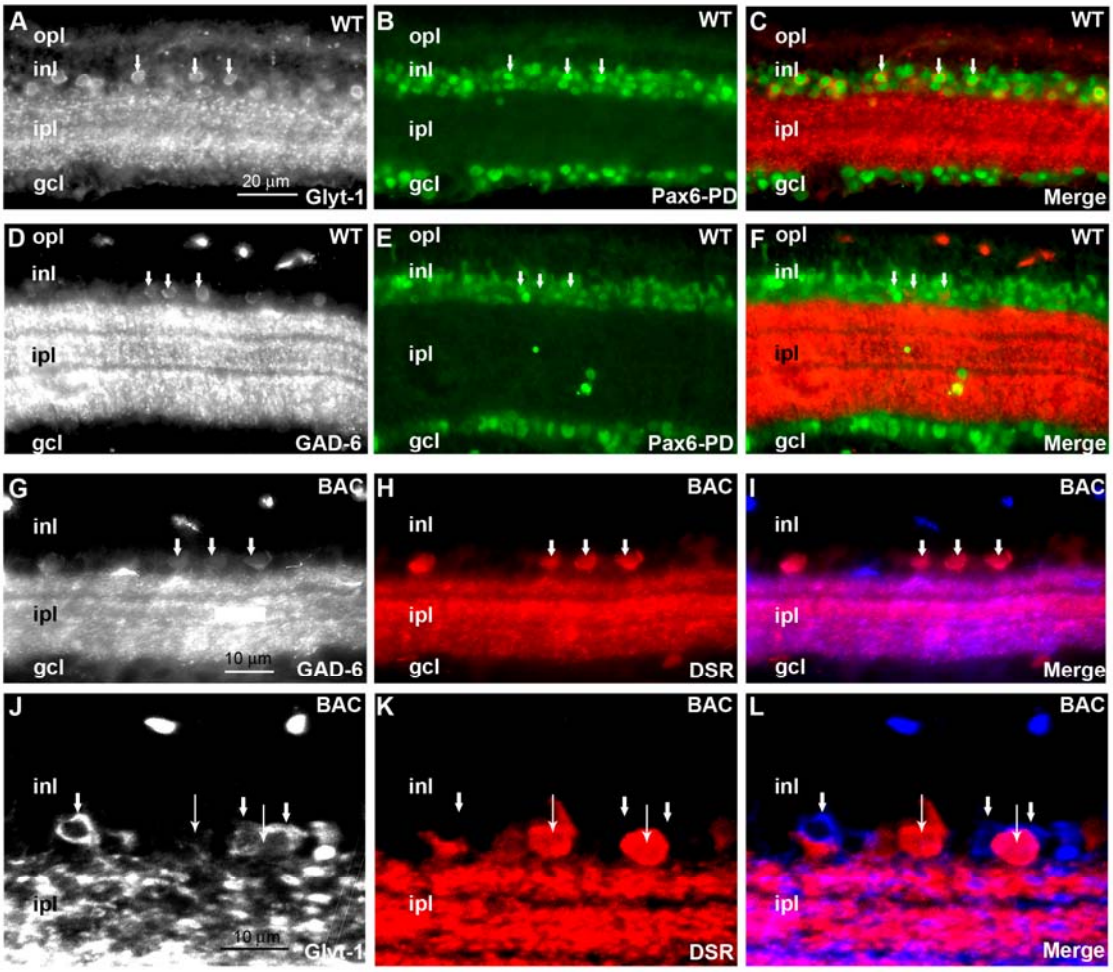

A
WT
opl
inl
ipl
gcl
20 µm
Glyt-1
B
WT
opl
inl
ipl
gcl
Pax6-PD
C
WT
opl
inl
ipl
gcl
Merge
D
opl
WT
inl
ipl
gcl
GAD-6
E
opl
WT
inl
ipl
gcl
Pax6-PD
F
opl
WT
inl
ipl
gcl
Merge
G
BAC
inl
ipl
gcl
10 µm
GAD-6
H
BAC
inl
ipl
gcl
DSR
I
BAC
inl
ipl
gcl
Merge
J
BAC
inl
ipl
10 µm
Glyt-1
K
BAC
inl
ipl
DSR
L
BAC
inl
ipl
Merge

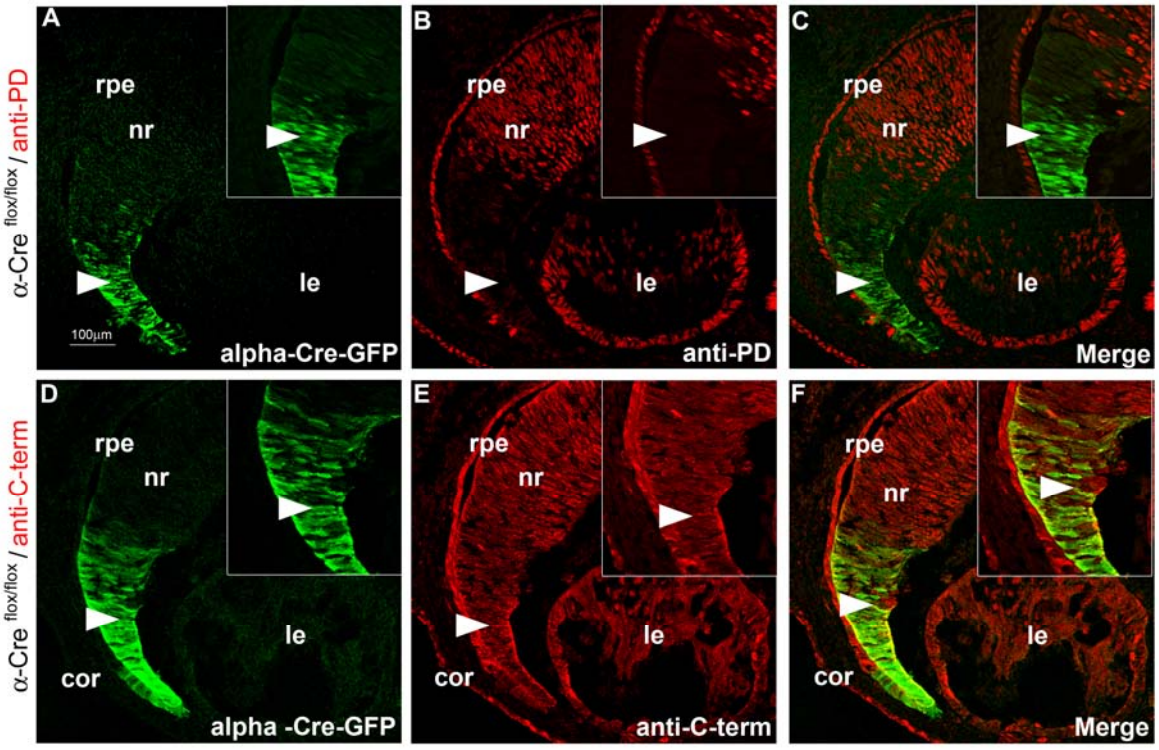
Fig. 3.3. Deletion of conserved translational start site does not affect Pax6(-PD).

Immunohistochemical localization of Pax6 on 12μm sections from α-Cre/Pax6$^{flox/flox}$ embryos, at E13.5 of murine development (A-F). Anti-PD, antibody specific to paired domain; anti-PST, antibody directed against the c-terminal transactivation domain of Pax6 (G). Expression of EGFP, marking the domain of *Cre*-recombinase activity, can be seen in the distal tips of the optic cup of α-*Cre*/Pax6$^{flox/flox}$ embryos (A). The expression of Cre recombinase occurs in a distal high to proximal low gradient within the developing neural retina (A, C). Arrowheads serve as reference points (A-F). An antibody, specific for the N-terminal portion of the Pax6 proteins shows that the paired containing isoform is eliminated in the region affected by *Cre* recombinase activity within the neural retina (arrow head in B, C and insets therein). Nuclear Pax6 staining is strong in cells of the retinal pigmented epithelium, lens, cornea and the proximal part of the neural retina (B, C).

In contrast, immunostaining, using an antibody directed towards the C-terminal trans-activation domain reveals that pairedless Pax6 is still present in cells affected by *Cre* mediated recombination (E, F and insets therein). However, this staining appears to be of cytoplasmic rather than nuclear nature.

Abbreviations: cor, cornea; le, lens; nr, neural retina; rpe, retinal pigmented epithelium; PD, paired domain; LNK, linker region; HD, homeodomain; PST, praline, serine, threonine rich transactivation domain

(data contributed by Amy Riesenber, Childrens's Hospital, Cincinnati OH)


A
B
C
D
E
F
rpe
nr
le
cor
100µm
α-Cre flox/flox / anti-PD
α-Cre flox/flox / anti-C-term
alpha-Cre-GFP
anti-PD
Merge
alpha -Cre-GFP
anti-C-term
Merge

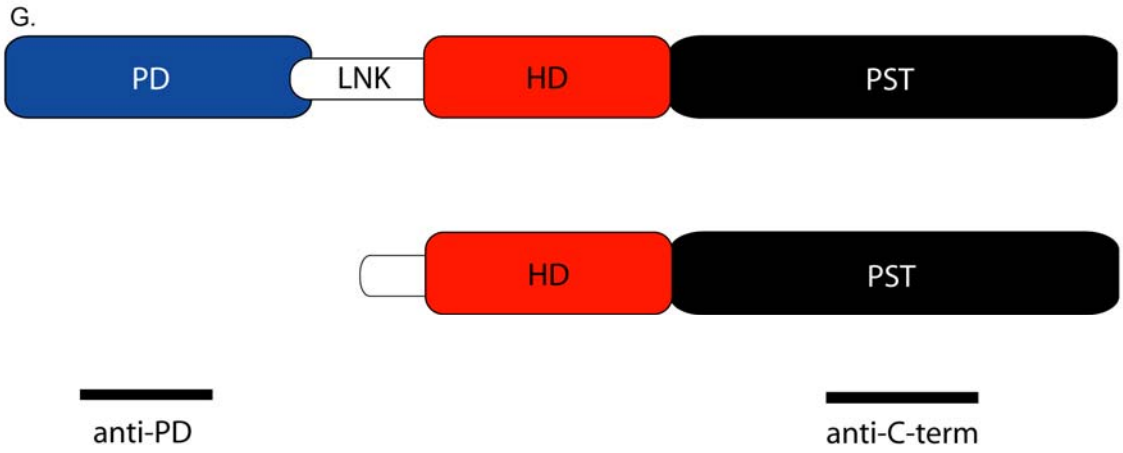

G.
PD
LNK
HD
PST
HD
PST
anti-PD
anti-C-term

Fig. 3.4. Cellular localization of Pax6 isoforms.

Characterization of Pax6 isoform localization in NHI-3T3 fibroblast cells, using antibodies directed against the paired DNA binding domain (PD) and the proline, serine, threonine rich c-terminal transactivation domain (PST). A-D, Pax6 antibody staining on fibroblast cells, transfected with pCMV-EGFP control vector. EGFP is present in both nucleus and cytoplasm of transfected cells (arrow heads in A, B, C, D); while no endogenous Pax6 staining is observed (A, C). Cotransfection of pCMV-EGFP and the pCMV-Pax6(PD) expression construct, which contains paired containing Pax6 cDNA, results in strong nuclear Pax6(PD) expression (D, E, F, G), detectable with both Pax6 antibodies (D, F). Interestingly, transfection of an expression construct, containing a cDNA encoding for pairedless Pax6 leads to strong staining in the cytoplasm (H). Furthermore, cells transfected with this expression construct sometimes also displayed an onset of nuclear paired containing Pax6 protein expression, detectable by using the anti-PD antibody. This observation suggests that Pax6(-PD) is capable of activating Pax6(PD) expression under certain circumstances.

(data contributed by Jiha Kim, University of Georgia, Athens GA)

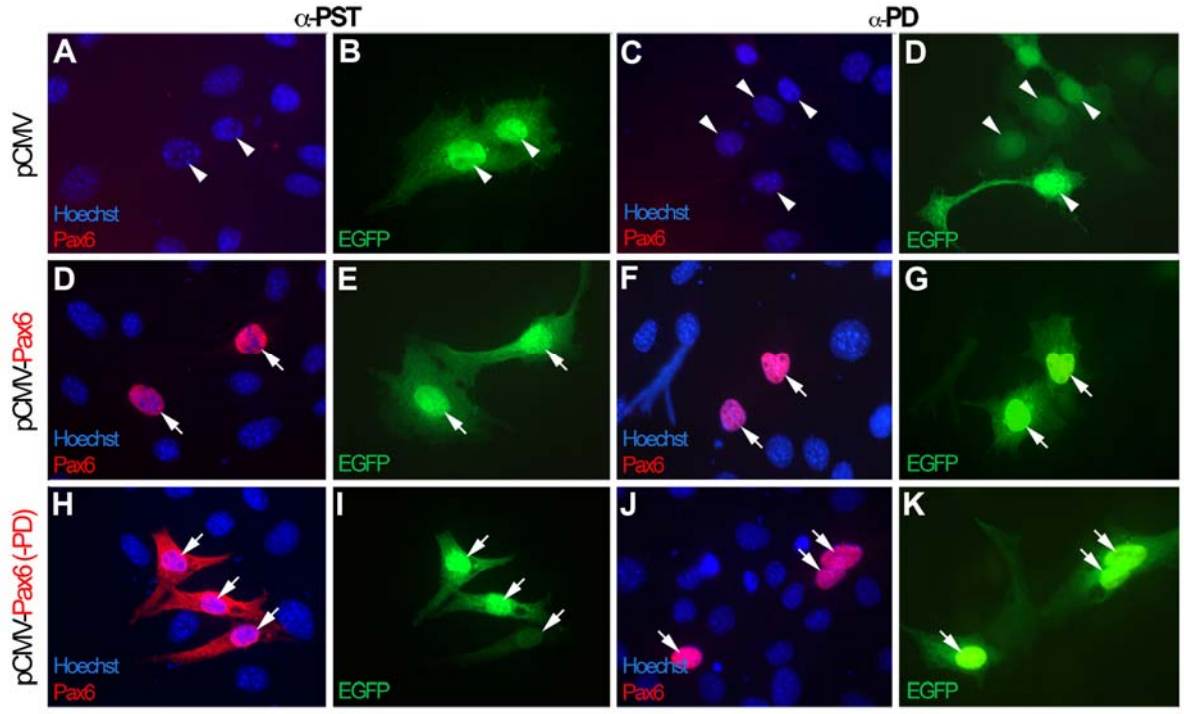

α-PST
α-PD
pCMV
pCMV-Pax6
pCMV-Pax6 (-PD)
A
B
C
D
D
E
F
G
H
I
J
K
Hoechst
Pax6
EGFP

Fig. 3.5. Potential translation start site of pairedless Pax6 are located in exon 7.

Sequence analysis of putative translation start site of pairedless Pax6 in exon 7 and exon 8. Alignment of human, mouse and zebrafish sequences reveals that four conserved in frame translation start sites exist that which could give rise to a pairedless Pax6 isoform. The core of the *Kozak* consensus sequence is shown as a red box. Sequence elements contributing to the overall strength of the translation start site are highlighted in red. Putative start sites (PSS) 1, 2 and 3, located in Pax6 exon 7, contain the crucial ATG as well as a purine base at the -3 position (A of ATG is +1) or a guanine at the +4 position. This arrangement is considered an "adequate" consensus sequence. In contrast, the conserved ATG in exon 8, lacking additional sequence elements, only presents a "weak" *Kozak* consensus sequence.
(data contributed by Jorn Lakowski, University of Georgia, Athens GA)

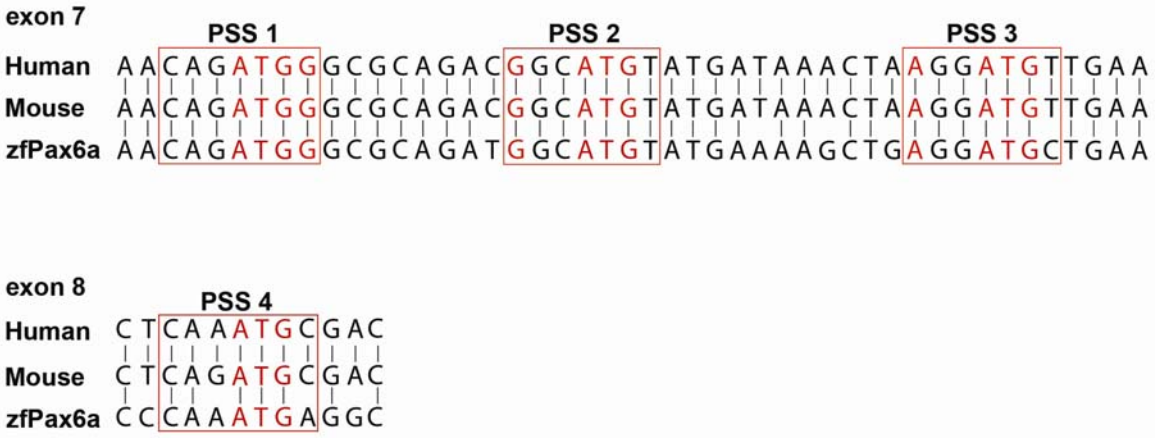
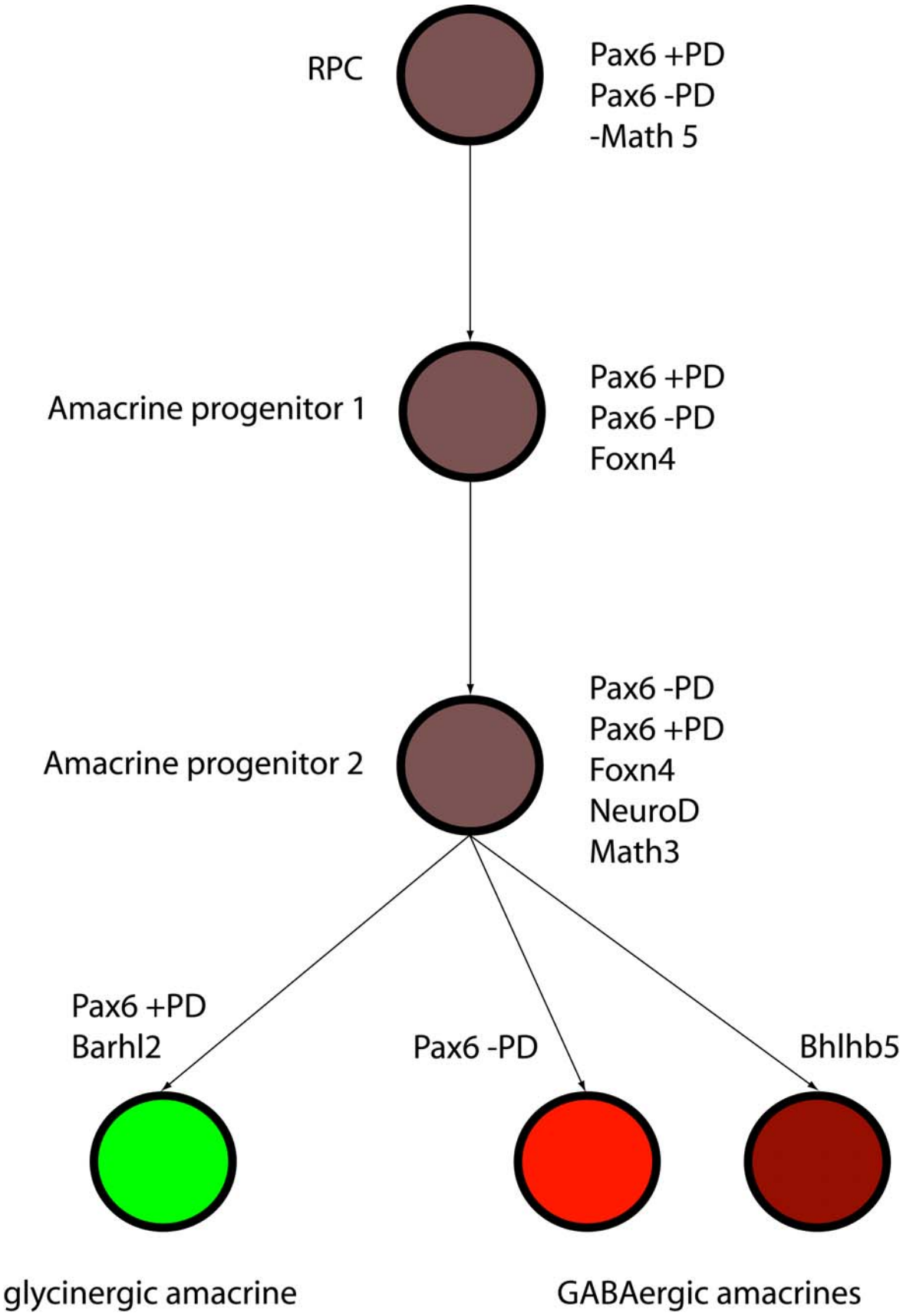

exon 7
PSS 1
PSS 2
PSS 3
Human AACAGATGGGCGCAGACGGCATGTATGATAAACTAAGGATGTTGAA
Mouse AACAGATGGGCGCAGACGGCATGTATGATAAACTAAGGATGTTGAA
zfPax6a AACAGATGGGCGCAGATGGCATGTATGAAAAGCTGAGGATGCTGAA
exon 8
PSS 4
Human CTCAAATGCGAC
Mouse CTCAGATGCGAC
zfPax6a CCCAAATGAGGC

Fig. 3.6. Model for the generation of amacrine cell subtypes in the mouse retina.

A retinal precursor cell or a cell that has recently exited the cell cycle expresses both paired containing and pairedless Pax6 isoforms but not Math5 and is destined to become an amacrine cell. Upregulation of the transcription factor Foxn4 then induces expression of Math3 and NeuroD. In the presence of Pax6(+PD) and Barhl2 glycinergic amacrine cells are produced. In contrast, the expression of Pax6 (-PD) and Bhlhb5 proteins results in the production of two distinct subtypes of GABAergic amacrine cells.


RPC
Pax6 +PD
Pax6 -PD
-Math 5
Amacrine progenitor 1
Pax6 +PD
Pax6 -PD
Foxn4
Amacrine progenitor 2
Pax6 -PD
Pax6 +PD
Foxn4
NeuroD
Math3
Pax6 +PD
Barhl2
Pax6 -PD
Bhlhb5
glycinergic amacrine
GABAergic amacrines

Chapter 4

EVIDENCE FOR THE FUNCTIONAL SEPARATION OF CRUCIAL REGULATORY ELEMENTS BETWEEN THE TWO PAX6 GENES IN ZEBRAFISH

[1] Jorn Lakowski, Anirban Majumder, James D. Lauderdale. To be submitted to Developmental Biology

Abstract

The evolutionarily conserved transcription factor Pax6 is expressed in the eye, parts of the central nervous system as well as endocrine pancreas, where it has been shown to control certain aspects of cell proliferation and differentiation. Pax6 is a member of the Pax family of transcriptional regulators, which were first identified through their similarity to the *Drosophila* segmentation gene paired. Reflecting its significance during embryogenesis, the Pax6 protein is under stringent transcriptional control, and a large number of tissue specific control elements have been identified in several species, including quail, mouse and human. While isolated *cis*-acting elements have been shown to recapitulate certain aspects of Pax6 expression in cross species studies, the extent of evolutionary conservation of Pax6 regulation, beyond the mammalian lineage, has not been tested. Interestingly, some teleost, such as zebrafish, possess two Pax6 paralogs, named Pax6a and Pax6b, which are though to have originated from a genome wide duplication that occurred at the beginning of the teleost lineage. However, it is unclear why both Pax6 genes have been retained since the duplication event. In this study, we have investigated the degree of evolutionary conservation of Pax6 expression between teleost and mammals, using transgenic mice that carry either the Pax6a or Pax6b locus. We show that certain aspects of Pax6 expression have been separated between the two loci, while others were retained in both. Furthermore, we present evidence that the two Pax6 genes in zebrafish have been retained due to the separation of crucial tissue specific control elements between ZfPax6a and ZfPax6b.

INDEX WORDS: Pax6, BAC, zebrafish, evolution

Introduction

The Pax6 protein is an important transcription factor, which is crucial to the normal development of the vertebrate eye, central nervous system and endocrine pancreas.
It is a member of the vertebrate paired box family of transcription factors, which were defined through their similarity to the *Drosophila* segmentation gene paired *(Hanson and Van Heyningen 1995; van Heyningen and Williamson 2002)*. Interestingly, Pax6 proteins from different organisms display a remarkable similarity even between distantly related species such as human and mouse, pointing to evolutionarily conserved function (Xu, Zhang et al. 1999). In vertebrates, the importance of Pax6 is demonstrated by a vast variety of known mutations, all having profoundly detrimental effects on eye development (Hogan, Horsburgh et al. 1986; Grindley, Davidson et al. 1995). Haploinsufficiency for Pax6 results in aniridia in humans and the small eye phenotype in mice, both heritable panocular disorders associated with foveal and iris hypoplasia, cataracts, corneal opacification as well as glaucoma. Homozygosity on the other hand, results in neonatal lethality in both species (Glaser, Jepeal et al. 1994). In addition, it was recently shown that mutations in Pax6 also lead to abnormal development of the brain, olfactory apparatus and the endocrine portion of the pancreas (Sander, Neubuser et al. 1997; St-Onge, Sosa-Pineda et al. 1997; Callaerts, Leng et al. 2001; Sisodiya, Free et al. 2001; Kuroda, Kaneto et al. 2004; Manuel and Price 2005; Hamasaki, Yamada et al. 2007). Reflecting its importance during development, the Pax6 transcript unit is highly regulated (Morgan 2004). Transcription initiates at three promoter regions, designated P0, P1 and P-alpha, while additional tissue specific regulatory elements are dispersed throughout the transcription unit, with some being localized far downstream of the last exon (Kammandel, Chowdhury et al. 1999; Lauderdale, Wilensky et al. 2000; Kim and Lauderdale 2006). P0 and P1 initiated transcripts encode a 46kDa(-5a) and

48kDa(+5a) Pax6 isoform, while transcripts initiated at the P-alpha promoter encodes a truncated isoform of the protein (Kammandel, Chowdhury et al. 1999). Although, mammals contain only one Pax6 gene, some teleost fish, including zebrafish, possess two paralogous Pax6 genes (Nornes, Clarkson et al. 1998), which are thought to have originated from a genome wide duplication event, which gave rise to the teleost lineage. Designated ZfPax6a and ZfPax6b, these two genes map to different linkage groups (ZfPax6a, LG25; ZfPax6b LG7) in the zebrafish genome. The Pax6 proteins encoded by ZfPax6a and ZfPax6b share 95% amino acid identity (98.8% similarity) over their entire length and both are capable of inducing ectopic eye in *Drosophila* (Nornes, Clarkson et al. 1998), suggesting that both proteins have retained a redundant biochemical function. While these two Pax6 genes are expressed in an overlapping and discrete pattern in zebrafish, the mechanisms that lead to the retention of these two functionally redundant genes is not well understood. Furthermore, as it is known that the Pax6 protein acts in a dosage sensitive fashion, it is currently not known what mechanisms are in place to ensure that the appropriate protein levels in the developing zebrafish embryo.

With the availability of fully sequenced genomes from an ever increasing number of species, it has become obvious that the number of duplicated genes therein is much larger than previously anticipated. For example, in the human genome, approximately 15% of all genes are thought to be a result of duplication events (Li, Gu et al. 2001). Moreover, the zebrafish genome has retained about 20% of all gene pairs that were generated during the latest duplication event in that lineage, which happened more than 110 Mya (million years ago)(Postlethwait, Woods et al. 2000). Duplications can occur on a local, regional or even whole genome level, resulting in initially identical pairs that over time diverge and become scattered throughout the ever rearranging genome (Lynch and Conery 2000).

While we are now becoming aware that duplication events play an important role in shaping the organization of vertebrate genomes, the mechanisms, which cause the retention of duplicated genes, however, are only beginning to be understood. In the classical model, the duplicated gene can have one of two fates; either it is lost or it can acquire a novel function and persist (Prince and Pickett 2002). It is assumed that in the course of time, both alleles will accumulate mutations, which can be neutral, deleterious or beneficial (Force, Lynch et al. 1999). If a mutation results in the null allele, preventing proper transcription, translation or protein function, non-functionalization ensues and the affected allele becomes a pseudogene (Ohno, Wolf et al. 1968; Force, Lynch et al. 1999). This is possible as there is no selective pressure to retain two completely redundant alleles. Mutations rendering those alleles non-functional can occur in either regulatory or coding sequences and are predicted to be relatively frequent.

Alternatively, one allele can acquire a new advantageous role (neo-functionalization), preventing it from being lost, and leaving this previously redundant gene exposed to new selective conditions. As deleterious mutations are believed to occur more frequently a beneficial ones, the classical model predicts that most gene duplicates are likely to become non-functional, and are lost in a relatively short amount of time (Lynch and Conery 2000). Interestingly, several recent studies have now shown that the classical model can not account for the large number of duplicated genes retained in the various vertebrate lineages which have been under investigation (Force, Lynch et al. 1999).

Recently, Force and colleagues proposed an alternative model, attempting to explain the relative high prevalence of retained gene duplicates (Force, Lynch et al. 1999). Named the duplication-degeneration-complementation model, the concept is based on the assumption that any given gene is multifunctional in nature and that it contains certain independently mutable

structural elements, such as regulatory regions, protein domains or alternative splice sites (Force, Lynch et al. 1999; Prince and Pickett 2002). According to this model, both alleles initially retain all subfunctions of the ancestral gene, provided that no gene dosage sensitivity exists, which would prevent both alleles from being fixed in the population. Over time, however, random mutations are thought to occur in discrete sub-functions of both alleles that do not affect one another. It has been suggested that in vertebrates, *cis*-acting regulatory elements often represent subfunctions because of their modular nature. Because they are usually short, point mutations can quickly lead to a creation or disruption of a particular site. For example, degenerative mutations in select independent *cis*-regulatory elements may result in the parceling out of the various tissue/cell type specific sites between the duplicated genes, eventually leading to two less complex loci. As a result, both alleles must now be retained to complement each other and recapitulate the original function of the single ancestral gene.

In this study we have evaluated the degree of evolutionary conservation of the regulatory mechanism governing Pax6 expression between teleost and mammals, while testing aspects of the DDC model in the context of the zebrafish Pax6 paralogs. To this end, we have generated transgenic mice, carrying either a modified zebrafish ZfPax6a or ZfPax6b bacterial artificial chromosome (BAC). Although, small modular cis-acting elements from teleost have been shown to be functional in transgenic assays in mice (Kammandel, Chowdhury et al. 1999; Griffin, Kleinjan et al. 2002), they are unlikely to recapitulate the complexity of an actual genomic locus. The use of large modified BAC constructs enabled us to investigate more exactly the extent of evolutionary conservation in a genome-like environment. Using this approach, we demonstrate that the mechanism controlling Pax6 expression has been conserved to a high degree between teleost and mammals. While either ZfPax6a or ZfPax6b alone are expressed in the mouse in

similar domains as in zebrafish, the combined expression of both genes almost completely recapitulates the endogenous murine Pax6 expression pattern. Detailed sequence analysis of select known tissue specific regulatory elements reveals that their separation between ZfPax6a and ZfPax6b has led to the preservation of both zebrafish alleles in the course of evolution, suggesting the applicability of the DDC model to the evolving Pax6 loci in zebrafish.

Results

Organization and conservation of the Pax6 locus in mice and zebrafish

In mice, the Pax6 transcription unit encompasses 15 exons, spanning a region of 25kb (Kim and Lauderdale 2006). Mapping experiments in different species have shown that Pax6 transcripts can initiate from three alternative promoter regions P0, P1 and P-alpha, giving rise to different Pax6 isoforms (Kammandel, Chowdhury et al. 1999; Kim and Lauderdale 2006). In addition, a variety of tissue/cell type specific regulatory elements have been found 5', within and even far 3' to the Pax6 gene in mice (Kammandel, Chowdhury et al. 1999; Lauderdale, Wilensky et al. 2000; Griffin, Kleinjan et al. 2002; Kleinjan, Seawright et al. 2004; Kleinjan, Seawright et al. 2006). The comparison of homologous sequences between select mammals and teleost shows that in zebrafish the Pax6 loci are more compact, while maintaining a similar genomic organization as the single mouse locus (Fig.1A). In order to identify putative regulatory sequences within zebrafish ZfPax6a and ZfPax6b loci, we aligned them with the homologous human, mouse and pufferfish (fugu) genomic sequences, using MLAGAN and displayed the resulting alignment using VISTA (Mayor, Brudno et al. 2000; Brudno, Poliakov et al. 2004).While the coding sequences between the two zebrafish paralogs appear to have been comparably conserved, we observed an intriguing differential conservation of certain *cis*-acting

regulatory elements for zebrafish ZfPax6a and ZfPax6b. As reported previously, the zebrafish ZfPax6a transcript unit contains the 5' located elements for head surface ectoderm, the internal CNS, intron-7 elements and P-alpha regions as well as 3' regulatory sequences, including the C1170, EI, and HS234 regions, which were functionally characterized in humans and mice. In contrast, ZfPax6b contains the pancreas, lens/cornea, CNS and some aspects of the C1170 regulatory elements, while missing the intron-7, and the far downstream located EI and HS234 DRR elements (downstream regulatory region).

In mice and humans, the DRR was recently shown to be crucial to proper Pax6 expression, encompassing several tissue specific control elements for ocular and brain structures that are scattered over a large area far downstream of the Pax6 coding region. Interestingly, in mammals the Pax6 gene exists in a syntenic relationship with the Elp4 (elongator protein 4) (Kleinjan, Seawright et al. 2002), with both genes being transcribed in an antisense orientation relative to each other. In this genomic context, the DRR is placed within the intronic sequence of the neighboring housekeeping gene. Our comparison of the different vertebrate Pax6 loci shows that this conserved synteny is only partially preserved for either of the two zebrafish Pax6 paralogs. While most regulatory elements usually found in the DRR in mammals (C1170, EI, HS234) can be located in the appropriate regions of the ZfPax6a locus, they are not present in the ZfPax6b transcription unit. Reversely, the Elp4 gene has been maintained only in the ZfPax6b but not ZfPax6a locus.

The mechanism of Pax6 expression has been conserved between teleost and mammals

The proper special and temporal expression of a gene depends on the concerted activity of a variety of regulatory elements, such as promoters, enhancers/repressors and locus control

regions. To experimentally evaluate the extent to which the Pax6 transcriptional mechanism has been conserved throughout evolution, we decided to employ a BAC modification approach as this would be more likely to recapitulate the genomic environment. We obtained BAC constructs, containing either the zebrafish ZfPax6a or ZfPax6b genes, surrounded by vast flanking regions to ensure the presence of most, if not all, regulatory elements, required for the correct gene regulation. The manipulation strategy for both Pax6 loci were basically the same, although the zebrafish ZfPax6a BAC has been described previously and contains an additional modification (Fig.1A). In order to visualize the expression from the two upstream promoters P0 and P1, we inserted an enhanced green fluorescent protein reporter cassette into the evolutionarily conserved translation start site in Pax6 exon 4, using the prophage BAC modification system (Lee, Yu et al. 2001). It was shown previously that translation of Pax6 in certain ray finned fish can initiate in either exon 2 or exon 4 (Behrens, Langecker et al. 1997; Nornes, Clarkson et al. 1998). Insertion of the reporter gene in exon 4, however, was expected to report on the majority of P0 and P1 initiated transcripts. Efficient transcriptional termination and message stability was achieved by inclusion of a SV-40 polyadenylation signal. We generated transgenic mice by pronuclear injection and obtained several lines (4) carrying zebrafish ZfPax6b locus, which all expressed the transgene in a comparable pattern. As this modification strategy does not affect the transcripts giving rise to Pax6(-PD), it was possible that overexpression of the zebrafish ZfPax6b(-PD) protein in such a way, could result in ocular defects, as reported previously (Kim and Lauderdale 2006; Kleinjan, Seawright et al. 2006). However, we never observed any developmental abnormalities in our transgenic lines, possibly due to a lower copy number.

We examined the expression of the EGFP reporter genes at different stages of development. In order to assess and compare the transgene expression in the mouse to the endogenous gene expression pattern in zebrafish, we carried out *in situ* hybridization for either of the two zebrafish Pax6 genes at different developmental time points. It has previously been shown that the two paralogs are dynamically expressed in distinct and overlapping domains. Our observations are consistent with these previous results. Whole mount analysis in the zebrafish embryo at 1dpf revealed that ZfPax6a is strongly expressed in the developing eye, telencephalon, part of the diencephalon and rhombencephalon and more weakly in the spinal chord. Over the course of the next two days (2-4dpf), expression of ZfPax6a was down regulated in the hindbrain and spinal chord, while maintained and refined in parts of the diencephalon and eye (data not shown). Similarly, transcripts for ZfPax6b were visible in the eye, spinal chord and certain parts of the forebrain at 1dpf. While ZfPax6a staining was absent from the pancreas at all stages surveyed, strong ZfPax6b was visible in this organ. Interestingly, while both genes were expressed in the hindbrain and spinal chord, the expression levels were strikingly different. While ZfPax6a showed strong hindbrain and weak spinal chord expression, ZfPax6b displayed opposite levels of expression in these two structures. As our sequence analysis of known and potential regulatory sequences for both zebrafish Pax6 loci had revealed a high degree of evolutionary conservation, we anticipated our two reporter constructs to recapitulate most of the particularities of zebrafish Pax6 expression patterns in the mouse embryo. However, considering the vast evolutionary distance between teleost and mice, a certain degree of misregulation was expected.

At E10.5 of murine development, Pax6 is normally expressed in a variety of tissues, including the developing telencephalon, rhombencephalon, spinal chord, nasal placode, pancreas and eyes (Fig.2C). As expected, the ZfPax6a BAC recapitulated part of the endogenous mouse Pax6

pattern, while displaying several interesting differences. Strong reporter gene expression was observed in the developing eye, pretectum (prosomere 1) and prethalamus (prosomere 3) regions (Fig.2D and inset). In addition, expression could be seen in the telencephalic vesicles as well as the nasal epithelium, which was confirmed by cryosectioning (Fig.2D inset and data not shown). Interestingly, we also observed reporter gene expression in two unexpected locations. First, a band of intense EGFP expression was located in the dorsal spinal chord (Fig.2D, in which Pax6 is usually not expressed. A second domain of ectopic expression was visible in the proximal portion of the forelimb (Fig.2D). Although, Pax6 is normally expressed in the developing hindbrain and ventral spinal chord at this stage of development, the zebrafish ZfPax6a transgene, however, failed to report in these domains.

Consistent with the idea of a partial functional separation of the two paralogs in zebrafish, the ZfPax6b transgene, directed reporter gene activity in overlapping but also distinct domains within the embryo compared to ZfPax6a. The most striking difference between the expression patterns of the two transgenes in the mouse, was the apparent ZfPax6b signal in the ventro-medial portion of the spinal chord as well as the hindbrain, which was not present with the ZfPax6a reporter (Fig.2 F, G). In contrast to ZfPax6a, ZfPax6b did not appear to be expressed in the telencephalic vesicles (Fig.2F inset and G), consistent with observations in zebrafish were ZfPax6a carries the main transcriptional load for this part of the forebrain (Fig.2A). However, the ZfPax6b transgene exhibited strong activity in eye at e10.5 and prosomere 3 (Fig. 2F, inset) one day later at e11.5. Those are domains of the embryo where ZfPax6a expression had been observed previously (Fig.2D). Surprisingly, all four lines of transgenic mice carrying the ZfPax6b locus exhibited strong EGFP expression in the proximal forelimb (Fig.2F inset 2), similar to the one seen with the ZfPax6a reporter (Fig.2D). The fact that this ectopic expression

was obtained with both zebrafish Pax6 loci, and was reproducible among several transgenic lines, argues against a misregulation due to the site of transgene integration. Instead, it is likely that the factors normally preventing Pax6 expression in this location failed to recognize their specific binding sites, due to the vast evolutionary distance between teleost and mice.

To evaluate the combined domains of zebrafish Pax6 expression, we crossed mice heterozygous for either of the two transgenes and analyzed reporter gene activity of the double heterozygote embryos in whole mount. As expected, while ZfPax6a or ZfPax6b alone only partially recapitulated endogenous murine Pax6 pattern, the combined activity of both transgenes accounted for almost all expression domains. However, while we observed transgene activity in most tissues that normally express Pax6 at that time, we never observed EGFP expression in the prosomere 2 region. A tissue specific regulatory elements controlling Pax6 expression in this region was recently identified and is located far downstream of the last coding exon of Pax6, inside intron 4 of the neighboring gene ELP4. Phylogenetic sequence analysis of both our BAC constructs shows that neither one of them extends this far, explaining the apparent absence of reporter gene expression in this region in our assay.

Selective retention of tissue specific regulatory elements between ZfPax6a and ZfPax6b

Because our whole mount analysis of the ZfPax6a and ZfPax6b genes showed an apparent separation of transcriptional activity between the two zebrafish genes for certain expression domains, we wished to investigate the molecular underpinnings of such behavior. A large variety of regulatory elements have been identified for the Pax6 genes in several species. However, because of the complexity of the expression patterns and frequent overlap of activity, the exact role of individual elements and the interaction with one another remain poorly understood.

Nevertheless, a small number of regulatory elements have been characterized in great detail and their expression appears to be restricted to only one or few tissues. Among those are the ectodermal enhancer and the adjacent pancreatic enhancer, both of which are located upstream the Pax6 P0 promoter regions. An overall comparison of the two zebrafish loci to the single murine locus by sequence alignment had shown that many of the known regulatory elements were differentially preserved between the two zebrafish paralogs. This was also the case for the head surface ectodermal and pancreatic enhancer elements. Visual inspection of these known enhancer region showed that while the ectodermal enhancer appeared to be conserved in the transcription units of both zebrafish genes, the pancreatic element was only present for ZfPax6b gene. This conclusion is consistent with findings that both ZfPax6a and ZfPax6b are expressed in derivatives of the head surface ectoderm (e.g. lens placode, lens vesicle), while only ZfPax6b is expressed in the pancreas (Krauss, Johansen et al. 1991; Nornes, Clarkson et al. 1998; Biemar, Argenton et al. 2001). Within the pancreatic enhancer of Pax6, two putative transcription factor binding sites, Motif F and a Pbx-1 binding site, have been identified (Kammandel, Chowdhury et al. 1999). Sequence alignments of the pancreatic enhancer region from several vertebrates species showed that the nucleotide sequence of Motif F in ZfPax6a was completely absent, while highly conserved for ZfPax6b. A similar situation was found for the putative Pbx-1 binding site, which was degenerate in the ZfPax6a but conserved in ZfPax6b.

Recent experiments have suggested that Pax6 is under direct transcriptional control of both Meis-1/2 proteins in the prospective lens ectoderm. A Meis-1/2 binding site, located in the ectodermal enhancer, has been shown to be necessary for directing proper Pax6 expression in the lens. In agreement with our finding that both ZfPax6a and ZfPax6b are expressed in the lens, we find that this Meis-1/2 binding site is highly conserved across all vertebrates analyzed. As this

particular binding site was not sufficient to direct reporter gene expression in transgenic mice, it is likely that it interacts with other regulatory elements in its native genomic context. Consistent with this idea, several other conserved sequence motifs are located in close proximity. Their function, however, remains to be determined.

Furthermore, the auto-regulation capability of Pax6 in the surface ectoderm has been known for some time, yet until recently, the mechanism through which this was achieved was unknown. A recent study proposed that the Pax6 autoregulation is mediated through a Pax6 binding site, located within the surface ectodermal enhancer region. Although, binding of Pax6 to its consensus sequence in this region was shown to efficiently drive reporter gene expression, it was also demonstrated that transcription factors of the SOX family could positively modify its interactions with the control element (Aota, Nakajima et al. 2003). Consistent with previous reports, the Pax6 auto-regulation site is relatively long and not well conserved among different species. However, since functional activity has been confirmed for this site, and the fact that Pax family paired domain binding sites often times appear degenerate, we have to assume that this protein binding motif may in fact possess functional significance across different species. On the other hand, the core of the SOX-2/3 consensus sequence, located close to the Pax6 binding site is well conserved the various conserved among the various vertebrates we compared.

To investigate the functional conservation of mechanism governing the activity of these two important regulatory elements, we decided to explore the expression of zebrafish ZfPax6a and ZfPax6b in the developing mouse embryo. Cryosections of embryos from different time points were prepared and co-immunolabeled with an antibody specific for EGFP and endogenous Pax6 respectively. Immunohistochemical analysis at E10.5 in the murine eye shows that the endogenous Pax6 is expressed in the surface ectoderm, which gives rise to the adult cornea, and

the lens vesicle (Fig. 3A). Furthermore, strong Pax6 expression is visible in both pigmented retina and precursor cells within the neural retina (Fig.3A). The EGFP expression of both reporter constructs in the eye at this stage appeared to be largely coincident, and could be observed in pattern similar to the endogenous Pax6 (Fig.4 B, E, C and F). Consistent with this finding that the ectodermal Pax6 enhancer is conserved in both zebrafish paralogs, we observed EGFP expression in both lens and surface ectoderm (Fig.4 B, E). The expression in these two structures was maintained in similar pattern through out embryonic development (Fig.3 H, K) and was still present in the adult eye (data not shown). Interestingly, while ZfPax6a and ZfPax6b expression was similar in lens and cornea, the developing neural retina showed a much different situation. At e14.5, a time when retinal differentiation is well underway, the zebrafish genes were expressed in common and different subpopulation of putative neurons and precursor cells. While, ZfPax6a signal was mostly localized to cells in the forming ganglion cell layer and few cells in the outer neuroblast layer (Fig.4 H, I), ZfPax6b displayed an almost reverse pattern with most cells found throughout the outer neuroblast regions (Fig.4 K, L). However, it appeared that expression in the distal tips of the optic cup, which gives rise to the iris and is also a precursor of the ciliary body, the stem cell niche of the retina, is common to both ZfPax6a and ZfPax6b (Fig.4 H, I, K, L).

Next, we next wished examine the expression of both zebrafish Pax6 genes in the developing pancreas. As the regulatory region, directing pancreatic Pax6 expression in zebrafish, was highly degenerate in the ZfPax6a locus, we expected to see expression only with the ZfPax6b transgene. It has been reported that during mouse embryonic development, Pax6 protein can be detected as early as E9.0 in the pancreatic endoderm, and its expression is maintained in all endocrine cells throughout development and during adulthood (Sander, Neubuser et al. 1997; St-Onge, Sosa-

Pineda et al. 1997; Ashery-Padan, Zhou et al. 2004). Consistent with these previous findings, we were able to observe endogenous Pax6 expression in forming pancreatic islets at E10.5 (Fig.5A). As expected, the regulatory elements contained in the ZfPax6a BAC were not capable of directing EGFP expression in the mouse pancreas at any time during murine development or at the adult stage (Fig.5B, I, G). On the other hand, ZfPax6b was visible in cytoplasm of forming pancreatic islet cells at E10.5, E14.5 and in the adult pancreas, recapitulating the endogenous Pax6 expression (Fig. 5E, F, K, L).

Discussion

Evolutionary Conservation of Pax6 control elements

The Pax6 protein has been shown to mediate crucial steps of development in both vertebrates and invertebrates (Pichaud and Desplan 2002; Simpson and Price 2002; van Heyningen and Williamson 2002; Treisman 2004; Tsonis and Fuentes 2006). The high degree of conservation on the amino acid level between orthologs from different distantly related species suggests that Pax6 has maintained similar biochemical properties throughout the course of evolution. In this study we have investigated the conservation of the regulatory mechanism directing Pax6 activity between teleost and mammals, lineages that separated more than 400 Mya. We demonstrate that the various tissue specific *cis*-acting regulatory sequences of the Pax6 locus in both mice and zebrafish have been extraordinarily preserved throughout evolution. By extension, these findings indicate that the molecular cascades directing expression of Pax6 have also been preserved despite the vast evolutionary distance between these two species.

According to the DDC model, for paralogs to be retained after a duplication event, certain crucial subfunctions must be separated between the resulting genes. In the case of the Pax6 locus

several tissue specific control elements were differentially preserved on either of the two zebrafish loci. Following a duplication event, accumulating degenerative mutations lead to the elimination of the pancreatic enhancer from the ZfPax6a locus. However, the ectodermal enhancer, controlling Pax6 expression in the developing cornea as well as lens, was preserved in both loci.

As the proper function of the pancreas is essential for survival, and presents a selectable advantage, both zebrafish Pax6 genes were maintained. Although, the expression pattern of zebrafish ZfPax6a and ZfPax6b suggest that additional regulatory elements, such as; telencephalon, nasal placode, prosomere 1 region and spinal cord, have been divided between the two loci as well, they are not well defined. Therefore we were not able include them in our sequence analysis. However, based on our observations of pancreatic and ectodermal enhancer elements, we predict that the specific transcription factor binding sites, mediating Pax6 activity in those tissues are degenerate or absent, and therefore non-functional in the ZfPax6b locus.

Interestingly, the Pax6 protein has be reported to act in a dosage sensitive fashion, with the eye being especially susceptible to inappropriate levels of Pax6 during development. In this light, it is somewhat surprising that both zebrafish Pax6 are expressed at high levels in the eye throughout development as this would mean an overall increase in Pax6 in this organ. One explanation could be that in zebrafish the expression levels of both genes have been fine tuned in the ocular system, following duplication, to prevent elevated protein levels, which would cause developmental abnormalities. Already a two fold increase in copy number of the Pax6 gene has been shown to results in a small eye like phenotype in mice. Alternatively, it is possible that the functional load has been divided between the paralogs on a cell type level within the eye. In the vertebrate eye, 6 different types of neurons and 1 types of glia cell are created from one common

precursor cell during retinogenesis. Those cell types can then be further grouped into specific sub-cell types, all carrying out individual functions in the vision process. It is conceivable that zebrafish ZfPax6a and ZfPax6b are differentially expressed in these retinal cell types or sub-cell types, thereby keeping the overall Pax6 level within a physiological range. Taken together, the data presented here shows that the Pax6 loci in zebrafish have evolved according the DDC model, with the combined activity of both paralogs recapitulating the function of the single ancestral gene. Because different functional aspects of Pax6 function have been divided among the two zebrafish genes, our transgenic lines present useful tools for the further exploration Pax6's role during embryonic development as well as the mature organism.

Material and Methodes

BAC modification

Zebrafish BAC clone BX000452 was modified by targeted insertion of an enhanced green fluorescent protein (EGFP) pA reporter cassette into ZfPax6b exon 4, using the prophage BAC modification system (Lee, Yu et al. 2001) The EGFP-FRT-*kan*-FRT targeting cassette was PCRamplified from pCS2+MTeGFP-FRT-*kan*-FRT (gift of X. Fan and S. Dougan, UGA) using the ZfPax6b exon 4 forward targeting primer, ZfPax6a-E4-RecFor, 5′-*TAGAATACTATAACCG-GGCCACGTGGGAGTCTGGTGTCGCGTCCATGATG*GTGAGCA-AGGGCGAGG-AG-3′ and the exon 4 reverse targeting primer, ZfPax6a-E4-RecRev, 5′-*CA-TACGTCTTTACCAATATACC-AAAACAAATGCCTTTATGGCTAGATTG*TATTCCAGAAG-TAGTGAG-3′. Nucleotides in bold italics are homologous to ZfPax6a sequences and those in roman are homologous to the amplification cassettes. SV40 late polyadenylation (polyA or pA) addition sequences are located immediately after the EGFP open reading frames. The late polyA signal of SV40 is has been

reported to bemore efficient than the SV40 early polyA signal in mammalian cells. After PCR, amplicons were gel purified using a standard Qiaquick gel extraction kit (Qiagen) and *Dpn* I treated to remove (Carswell and Alwine 1989) template plasmid before use for homologous recombination. Targeted recombination was carried out according to Lee et al. (Lee, Yu et al. 2001). Double-resistant colonies (CmR KanR) were assayed for homologous recombination by PCR using the following primers: *E4F1 (5′-CT-GGACATAAG-TGTATTGTGGAG-3′), E4R1 (5′-CCTTGAAG-AAGATGGTGCG-3′), E4F2 (5′-TGCTGCCC-GACAACCACTAC-3′), and E4R2 (5′-CTC-TCTCTAACACACACAGACGC-3′)*. The kanamycin cassette was flipped out by induction of *flipase*, and the cells were screened for kanamycin sensitivity (KanS). Removal of the kanamycin cassette from those colonieswas verified by PCR using the E4F1, E4F2, E4R1, and E4R2 primers. To ensure the integrity of the modified BAC we examined the construct by fingerprint analysis (Gong, Zheng et al. 2003; Kim and Lauderdale 2006) using XbaI and compared to that obtained for the unmodified BAC. The sequences of the targeted regions were verified by automated sequencing of both DNA strands.

Generation of BAC transgenic ZfPax6b mice

All transgenic mice lines were generated from pronuclear injection of closed circular BAC DNA into mouse oocytes (Gong, Yang et al. 2002; Gong, Zheng et al. 2003). Mice were genotyped by PCR using tail DNA and primers to detect the EGFP reporter cassette (forward primer, located in the ZfPax6b intron 5′ of exon 4, 5′-TGGACATAAGTGTATTTGTGGAG-3′; reverse primer, located in EGFP, 5′-CGCA-CCATCTTCTTCAA GG-3′; the PCR product is 559 bp) or DsRed reporter cassette (forward primer, 5′-CATCGG- C G TGAACTTCC-3′; reverse primer, 5′-GTTTCAGGTTCAGGGG-GAG-3′; the PCR product is 551 bp). We obtained 4

independent transgenic mouse lines carrying theZfPax6b locus. F1 founders were generated by crossing founder males or females to CD-1 mice (Charles River Laboratories). These lines are being maintained in the CD-1 background.

Transgene analysis in mice

For mouse breeding, the day of plug discovery was designated E0.5. The pregnant mice were euthanized using CO at the desired time points, and the uterine horns were washed several times in ice-cold phosphate-buffered saline (PBS). The embryos were removed from extraembryonic tissue and placed in ice-cold PBS. EGFP reporter gene expression was assessed in live embryos by fluorescence microscopy using a Zeiss Stemi SV11 Apo dissecting microscope fitted for epifluorescence and documented using a Zeiss AxioCam digital camera. All embryos were then fixed overnight by immersion in 4% paraformaldehyde/PBS at 4°C.

Immunohistochemistry/*In situ* hybridization

We carried out whole-mount mRNA *in situ* hybridization as described previously (Hargrave and Koopman, 2000). Usually, all embryos that were to be used for immunohistochemistry were kept in fixative (4% PFA, PBS) for at least 2 weeks at 4°C. In order to prevent trapping of the RNA probe, mouse embryos that were to be used for mRNA *in situ* hybridization had incisions made in their hindbrain and/or cerebral vesicles prior to fixation. Those embryos were fixed overnight, followed by a stepwise dehydration in a graded PBS/methanol series, and stored in methanol at −20°C. Sense and antisense digoxigenin-labeled RNA probes were prepared from a BglII digest of the pMPX2-1 Pax6 cDNA clone (gift of T. Glaser, Univ. Michigan) using a DIG RNA labeling kit (Roche).The hybridization and stringent posthybridization wash steps were

carried out at 70°C. After color development, embryos were destained for several hours in 1% Triton X-100/PBS and subsequently postfixed in 4% paraformaldehyde/PBS at 4°C.

Immunohistochemistry, using antibodies against Pax6 and EGFP was carried out as described previously. Tissues and embryos from different stages of development were obtained and fixed in 4% paraformaldehyde (PFA), PBS overnight at 4°C. After extensive washes in PBS, embryos were incubated in sucrose PBS solutions (5%, 15%) for >6h each, followed by an overnight incubation in gelatine/sucrose solution (7% gelatine, 15% sucrose). Subsequently, embryos were embedded in cryomolds and quick frozen in methylbutane/dry ice slurry. Cryosections of 12µm thickness were prepared, using a Leica cryostat. After removal of gelatine, specimen were blocked in 4% milk/TST solution for 30 minutes and subsequently incubated with the primary antibody. The polyclonal Pax6 antibody (Serum 11) was a gift of Dr. Saule and was used at a 1:1000 dilution. The polyclonal EGFP antibody was obtained from Chemicon and used at 1:2000 dilution. Following the incubation with the primary antibody, sections were washed with TST washing buffer and incubated with biotinylated secondary antibody. Detection was carried out with a Cy-3-streptavidin conjugated.

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Fig. 4.1. Genomic organization of the mammalian and teleost Pax6 loci.

Map of the murine Pax6 and zebrafish ZfPax6a and ZfPax6b transcript unit. The mouse Pax6 gene is located on chromosome 2 and contains 15 exons. Transcription from three alternative promoters occurs in an antisense orientation to the neighboring Elp4 gene, which is located 3' to the last Pax6 exon. The red sphere marks the position of the conserved CpG island upstream of exon 0, serving as the 5' delineation of the Pax6 locus. The downstream regulatory region (DRR), located entirely in the intronic sequence of the Elp4 gene, is often used as a reference point (arrows), and encompasses the C1170 (olfactory region, retina, pretectum), EI (lens) and HS234 (retina, telencephalic surface ectoderm, olfactory region) regulatory Pax6 elements.

The zebrafish ZfPax6a transcription unit has 17 exon, distributed over a 20.7 kb region of LG25, and ZfPax6b encompassed 16 exons contained in a 25.6kb region.
Comparison of the genomic organization from ZfPax6a and ZfPax6b to the murine counterpart, using the markers such as the DRR and Elp4, shows that the teleost transcript units are more compact. While exons, coding for Elp4, can be mapped to the ZfPax6b gene, they are absent from ZfPax6a. Position of the reporter genes in the BAC constructs is indicated by colored boxes and red line underneath each zebrafish locus represents the sequences contained in the respective BAC.

B. MLAGAN/VISTA analysis of vertebrate Pax6 loci.

In order to identify regulatory elements, which were differentially preserved between zebrafish ZfPax6a and ZfPax6b we compared the respective genomic sequences to the homologous sequences from human, mouse and pufferfish, by MLAGAN/VISTA analysis.

The human locus was determined to serve as the base sequence. Colored peaks represent sequences with equal or more than 50% conservation over a 40 base pair region, while exons are colored in blue. The location of known regulatory sequences is indicated by red ovals; p, pancreas; se (surface ectoderm), lens, cornea, lacrimal gland, and conjunctiva; tel-hb-sc, telencephalon, hindbrain and spinal cord; NTDP, post-mitotic, non-terminally differentiated neurons; α-en, amacrine cells, iris, ciliary body and RPE; intron-7, C1170, EI , HS234, neural retina, RPE and nasal pits.

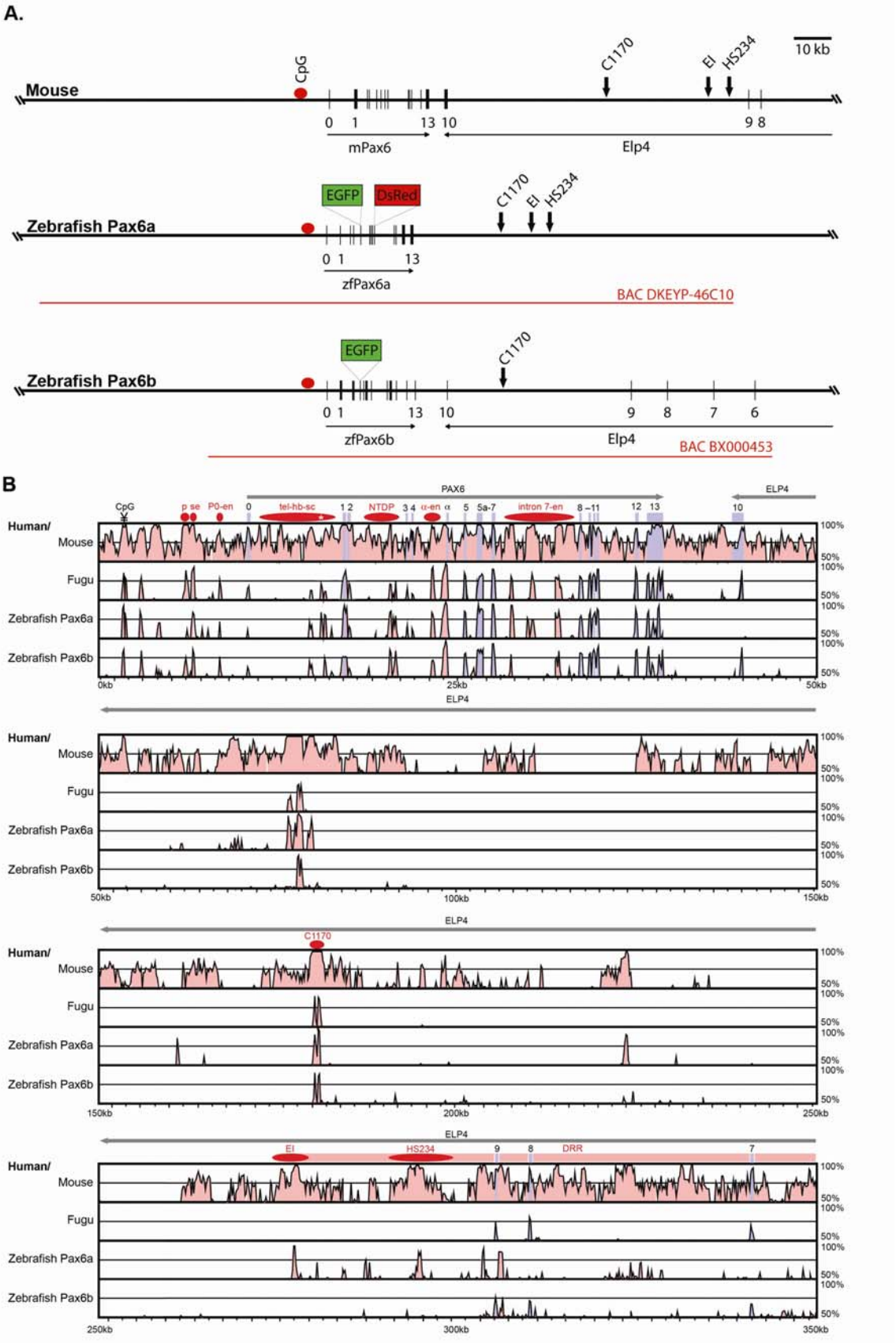

A.
Mouse
CpG
C1170
EI
HS234
10 kb
0 1 13 10 9 8
mPax6
Elp4
Zebrafish Pax6a
EGFP
DsRed
C1170
EI
HS234
0 1 13
zfPax6a
BAC DKEYP-46C10
Zebrafish Pax6b
EGFP
C1170
0 1 13 10 9 8 7 6
zfPax6b
Elp4
BAC BX000453
B
PAX6
ELP4
CpG
p se
P0-en
tel-hb-sc
NTDP
α-en
intron 7-en
Human/
Mouse
Fugu
Zebrafish Pax6a
Zebrafish Pax6b
100%
50%
0kb
25kb
50kb
50kb
100kb
150kb
150kb
200kb
250kb
C1170
EI
HS234
DRR
250kb
300kb
350kb

Fig. 4.2. Comparison of mouse and zebrafish Pax6 expression patterns.

Analysis of mouse and zebrafish Pax6 genes by *in situ* hybridization. Whole mount mouse and zebrafish embryos were hybridized with Pax6 specific labeled ribo-probe. Lateral view in A-D, anterior is left. Zebrafish Pax6 paralogs are expressed in distinct and overlapping domains. At 24hpf zebrafish ZfPax6a is localized to the developing telencephalon, diencephalon, eye, rhombencephalon and the spinal chord, while staining in strikingly absent from the forming pancreas (A). For ZfPax6b, strong signal is observed in the eye, pancreas (inset in B) and spinal chord (B). Weaker expression is also seen in the rhombencephalon (B). Furthermore, ZfPax6b appears to be expressed in a subset of neurons located in the diencephalon (B and data not shown). The expression levels of ZfPax6a and ZfPax6b appear to be different for the various tissues (A, B). At E10.5 of murine development, Pax6 is expressed in the telencepahlic vesicles, eye, olfactory pit, diencephalon, rhombencephalon, spinal cord as well as pancreas primordium (C). Transgenic mice carrying the zebrafish ZfPax6a and ZfPax6b BACs drive EGFP reporter gene expression in patterns, consistent with their normal expression in zebrafish (D, E, F, G). The expression levels of the zebrafish ZfPax6a BAC were highest in the developing eyes, telencephalic vesicles and parts of the diencephalon (D, inset in E). In addition expression was also seen in the olfactory region, dorsal spinal cord and proximal forelimb buds, the later being ectopic expression domains (D, E). In contrast, ZfPax6b expression was expressed in the eye, rhombencephalon, ventro-medial spinal cord and at e11.5 also in P3 (F, inset 2). As for the ZfPax6a construct, ectopic gene expression was visible in the forelimb (inset 1 in F). The combined expression of both zebrafish reporter constructs recapitulates almost completely the endogenous murine Pax6 expression (I, inset in I). Abreviations: di, diencepahlon; dsc, dorsal spinal cord, fl, forelimb, eye; np, nasal placode; P1, P3, prosomere 1/3; pan, pancreas rhm,

rhombencephalon; sc, spinal cord; dsc, dorsal spinal cord; vsc, ventral spinal cord; tel, telencephalon, vsc, ventral spinal cord

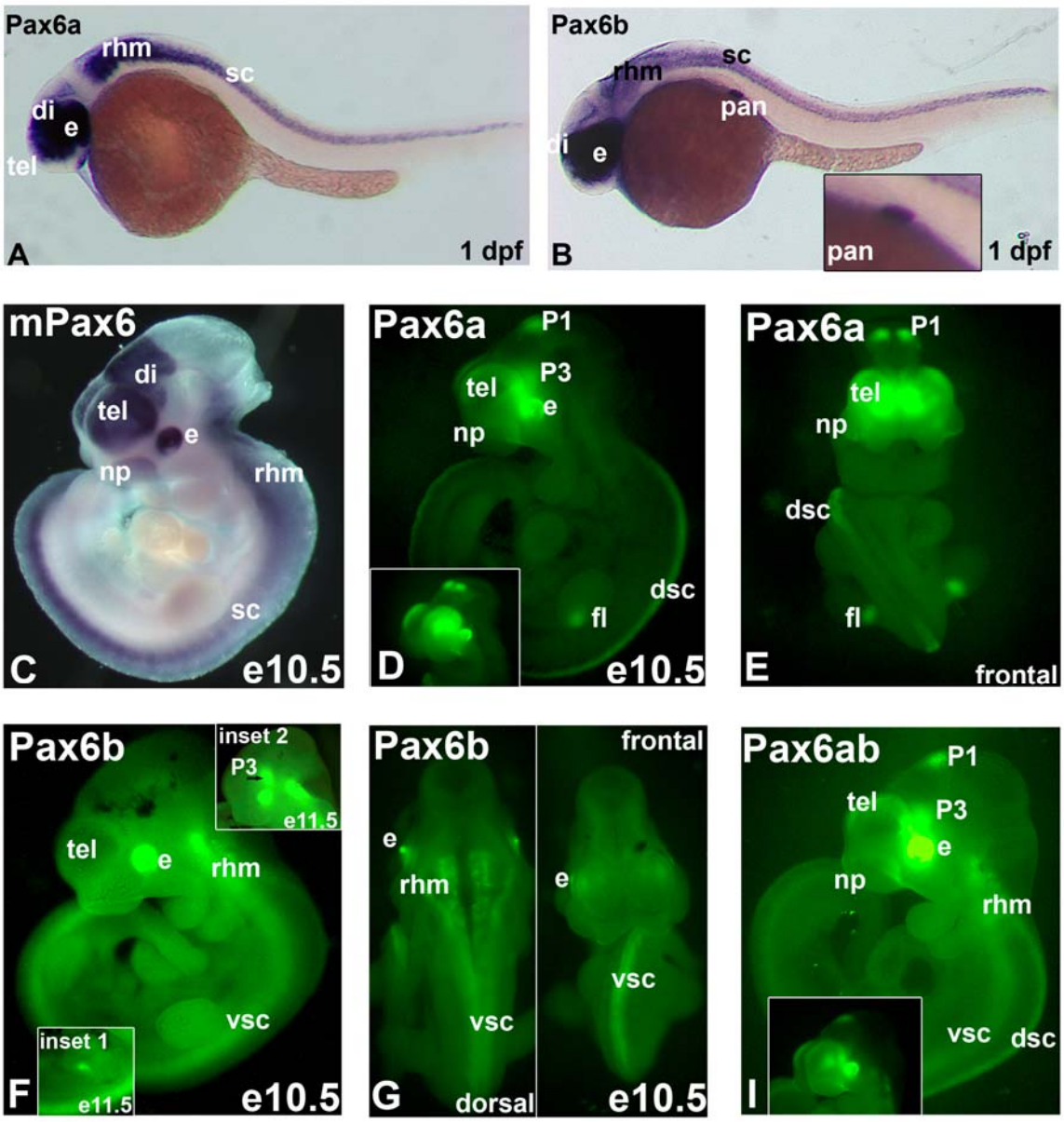

Pax6a
rhm
sc
di
e
tel
A
1 dpf
Pax6b
rhm
sc
pan
di
e
pan
B
1 dpf
mPax6
di
tel
e
np
rhm
sc
C
e10.5
Pax6a
P1
P3
tel
e
np
dsc
fl
D
e10.5
Pax6a
P1
tel
np
dsc
fl
E
frontal
Pax6b
inset 2
P3
e11.5
tel
e
rhm
vsc
inset 1
e11.5
F
e10.5
Pax6b
frontal
e
rhm
vsc
e
vsc
G
dorsal
e10.5
Pax6ab
P1
tel
P3
e
np
rhm
vsc
dsc
I

Fig. 4.3. Sequence analysis of the pancreatic and surface ectodermal Pax6 enhancers.

Regions of high homology, corresponding to the pancreas and head surface ectodermal enhancer can be identified using MLAGAN and VISTA, upstream of the Pax6 transcript unit in all vertebrate species (A). The different loci are displayed in order of their overall conservation with respect to the human base sequence. Pink peaks represent sequences of more than 50% conservation over a 40bp window. Red starts indicate conserved nucleotides. With 94% murine locus in this region is most similar the human reference sequence, followed by fugu (74%), zebrafish ZfPax6a (56%) and zebrafish ZfPax6b (48%). Nucleotides, conserved across all species, are marked by red stars. *Bona fide* transcription factor binding sites are delineated by red boxes. The pancreatic enhancer has been shown to contain two conserved transcription factor binding sites; Motif F and a Pbx-1 consensus sequence (B). While these two motifs are highly conserved between human, mouse, fugu and ZfPax6b, these sequences are degenerate in the zebrafish ZfPax6a locus, possibly rendering these binding sites non-functional. In the surface ectodermal enhancer, controlling Pax6 expression in lens and cornea, the crucial Meis-1/2 binding site is conserved between all vertebrate species compared here. The autoregulatory Pax6 binding site, is poorly conserved between the different vertebrate species. However, three previously identified Sox-2/3 consensus sequences, thought to influence Pax6 autoregulatory activity, are well conserved. In addition several other potential regulatory sequences of yet unknown function can be found in this region.

Figure 3.

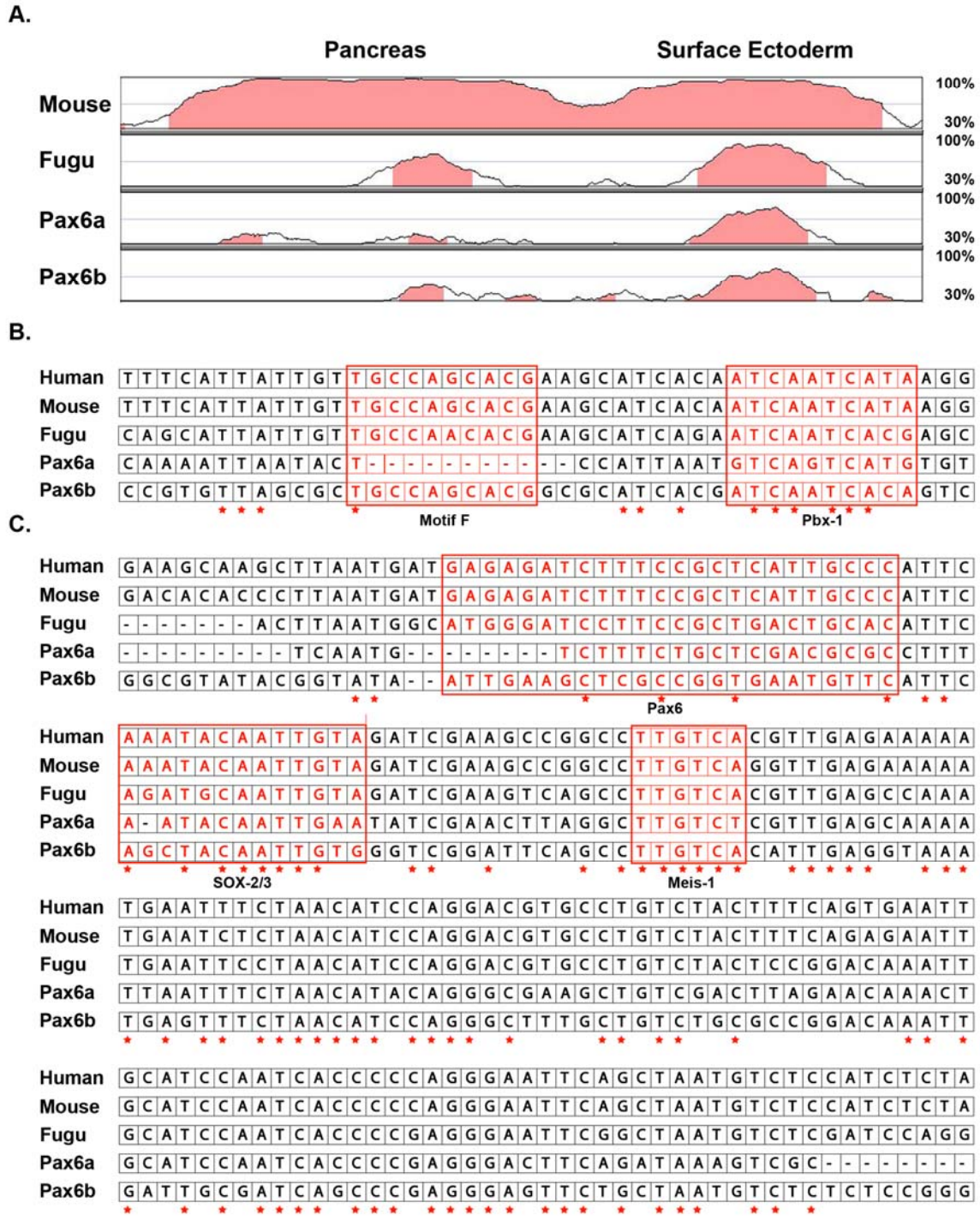

A.
Pancreas
Surface Ectoderm
Mouse
Fugu
Pax6a
Pax6b
100%
30%
B.
Human
Mouse
Fugu
Pax6a
Pax6b
Motif F
Pbx-1
C.
Pax6
SOX-2/3
Meis-1

Fig. 4.4. Expression of ZfPax6a and ZfPax6b in the developing eye.

Immunohistochemistry performed on 12μm transverse cryosections from e10.5 and e14.5 mouse transgenic mouse embryos. The zebrafish reporter constructs direct EGFP in patterns similar to the endogenous murine Pax6. At E10.5 expression of endogenous mouse Pax6 is readily visible in the nuclei of cells in the surface ectoderm, lens, pigmented and neural retina (A). While zebrafish ZfPax6a is most strongly expressed in the tips of the optic cups and in the pigmented retina, weak but consistent EGFP signal can also be seen throughout the neural retina (B). Additional staining is present in the lens and surface ectoderm (B). The zebrafish ZfPax6b transgene directs reporter expression in retinal precursor cells throughout the neural retina, with some cells spanning the entire width of this cell layer (D). The lens vesicle and surface ectoderm also express the transgene although at a weaker level.

At e14.5, strong endogenous Pax6 signal is observed in the cornea, lens, neural retina, and to a lesser extend in the pigmented retina (G). Interestingly, both zebrafish transgenes drive EGFP expression in the lens epithelial and fiber cells, cornea, pigmented retina as wells at developing ciliary body (G, J). However, the expression patterns in the neural retina are quite distinct. While ZfPax6a is found in cells of the forming ganglion cell layer and few cells abutting the pigmented retina, ZfPax6b is mostly expressed in a uniform pattern throughout the developing outer neuroblast layer.

Abbreviations: gcl, ganglion cell layer; le, lens; nr, neural retina; onb, outer neuroblast layer; rpe, retinal pigmented epithelium; se, surface ectoderm

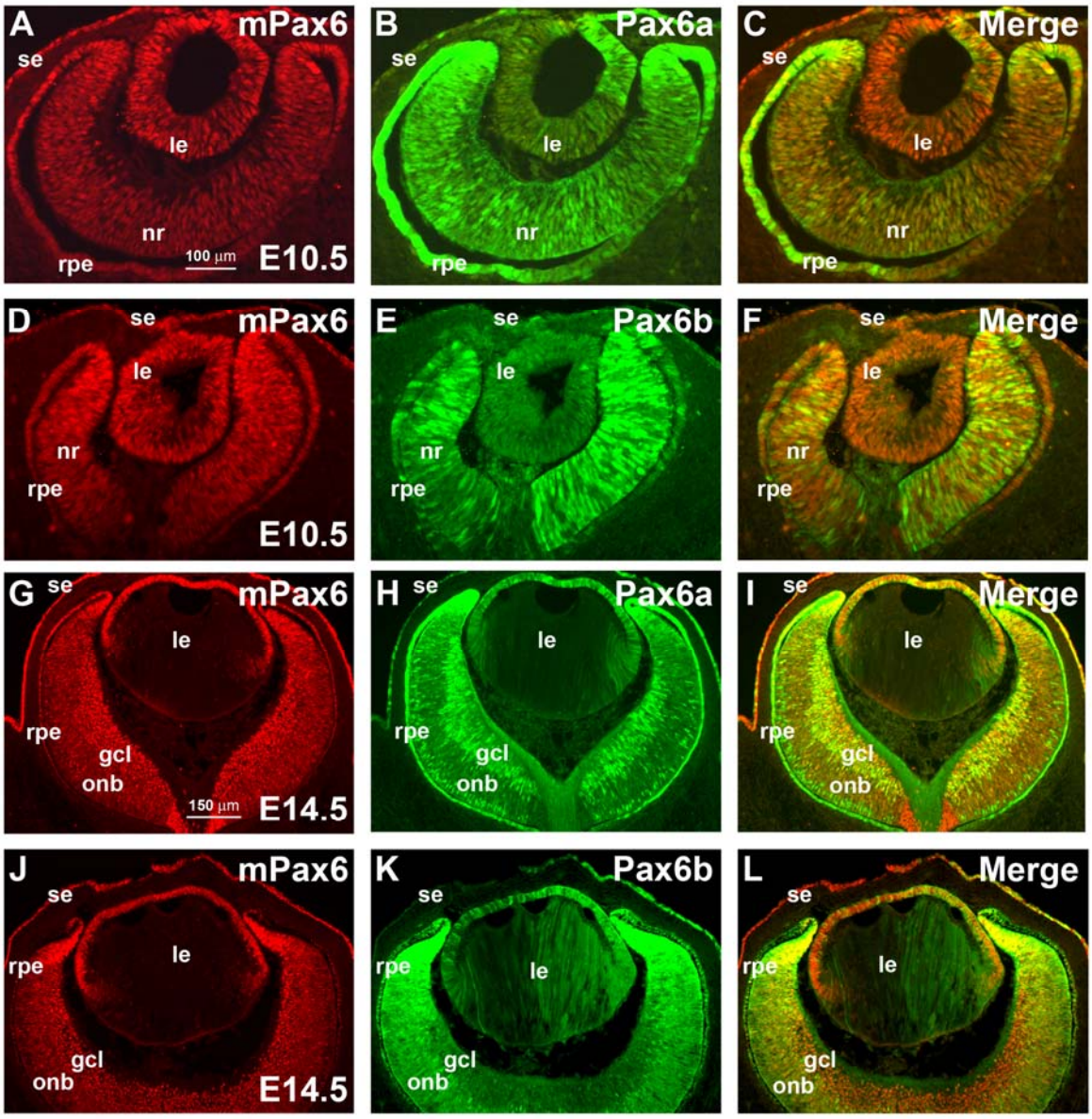

A
mPax6
se
le
nr
rpe
100 µm
E10.5
B
Pax6a
se
le
nr
rpe
C
Merge
se
le
nr
rpe
D
se
mPax6
le
nr
rpe
E10.5
E
se
Pax6b
le
nr
rpe
F
se
Merge
le
nr
rpe
G
se
mPax6
le
rpe
gcl
onb
150 µm
E14.5
H
se
Pax6a
le
rpe
gcl
onb
I
se
Merge
le
rpe
gcl
onb
J
mPax6
se
rpe
le
gcl
onb
E14.5
K
Pax6b
se
rpe
le
gcl
onb
L
Merge
se
rpe
le
gcl
onb

Fig. 4.5. Regulation of Pax6 in the pancreas has been divided in zebrafish.

Transgene EGFP expression driven by zebrafish ZfPax6a and ZfPax6b BAC during murine pancreatic development. Immunohistochemical analysis on 12 μm cryosections. Endogenous nuclear Pax6 expression is visible throughout development in the forming endocrine islet cells of the pancreas (A, D, G, J). The zebrafish ZfPax6a BAC is not capable of directing EGFP reporter gene expression in the pancreas at any time of murine development (B, C). Some autofluorescent blood cells are visible in panel B. In contrast, zebrafish ZfPax6b is co-expressed with endogenous murine Pax6 at E10.5 and E14.5 in clusters of pancreatic islet cells.

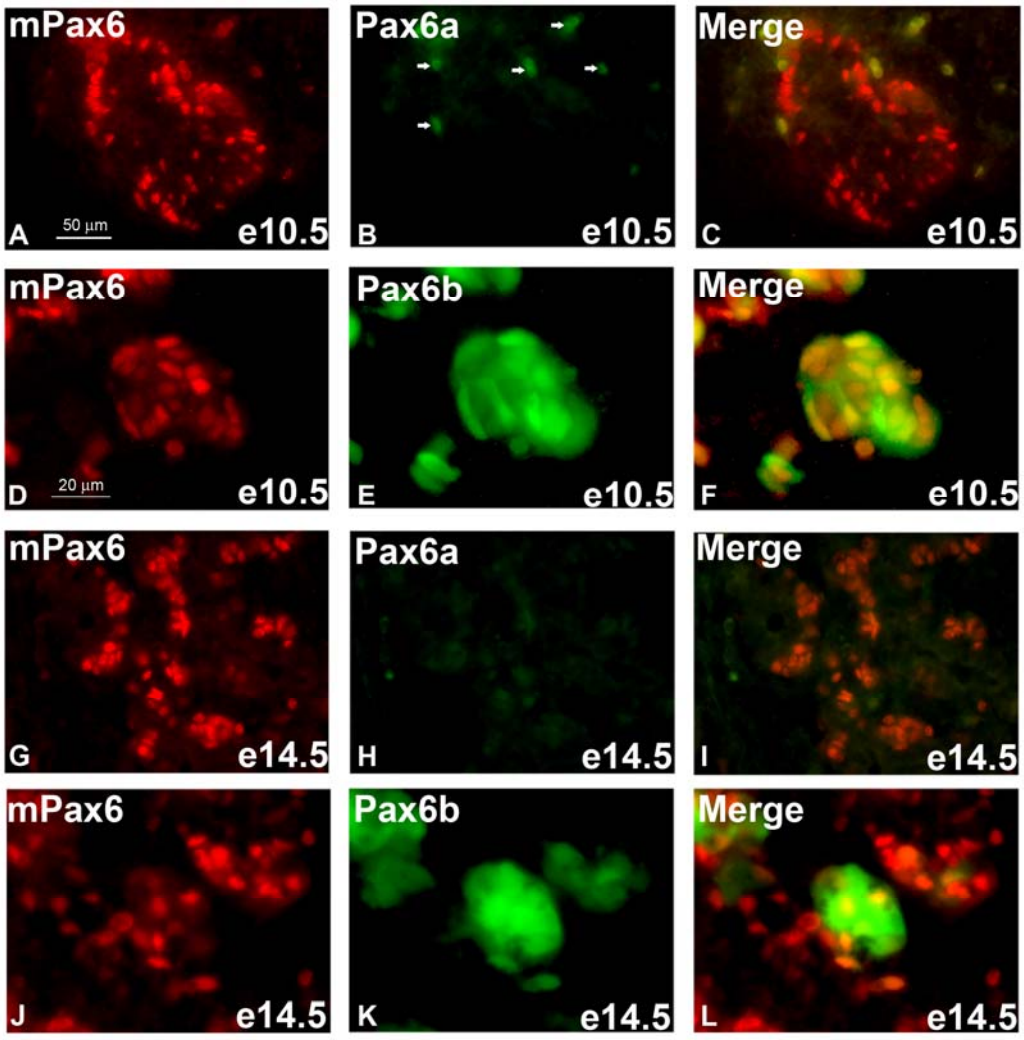
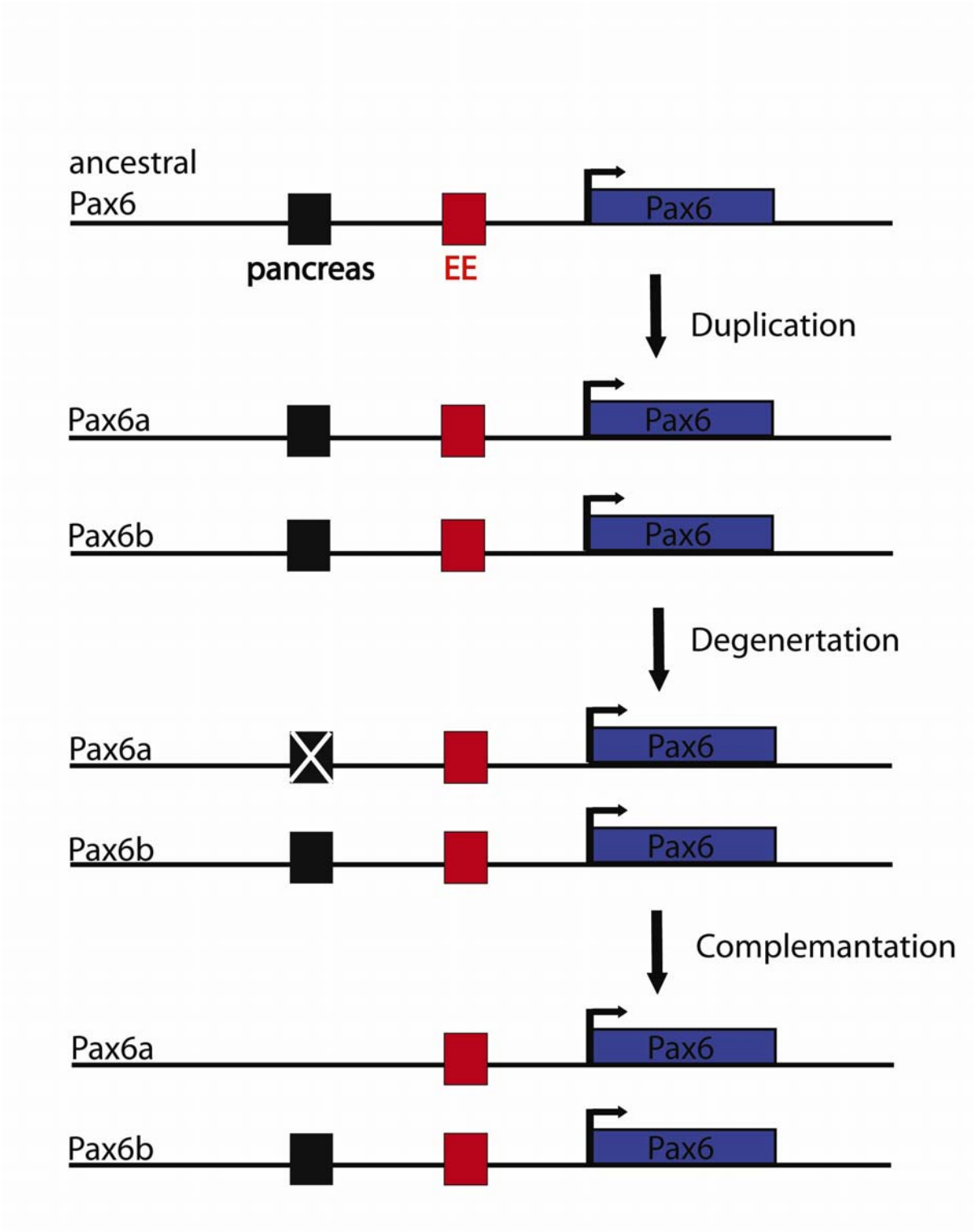

mPax6
Pax6a
Merge
A
50 µm
e10.5
B
e10.5
C
e10.5
mPax6
Pax6b
Merge
D
20 µm
e10.5
E
e10.5
F
e10.5
mPax6
Pax6a
Merge
G
e14.5
H
e14.5
I
e14.5
mPax6
Pax6b
Merge
J
e14.5
K
e14.5
L
e14.5

Fig. 4.6. Application of the DDC model to zebrafish Pax6 locus.

In the ancestral Pax6 gene, tissue specific enhancer elements, directing expression in pancreas and surface ectoderm, are located 5' to the first exon. (Duplication) Following a duplication event, of possibly genome wide scale, the two Pax6 loci co-exist for some time and accumulate degenerative mutations in the sequences of the regulatory elements as well as coding regions. (Degeneration) Due to these detrimental nucleotide changes, the pancreatic enhancer in the ZfPax6a locus is rendered non-functional, while the same element located in the ZfPax6b transcription unit persists. In contrast, the ectodermal enhancer, regulating Pax6 expression in some derivatives of the head surface ectoderm, is conserved in both zebrafish Pax6 genes. (Complementation) As the crucial pancreatic element, probably along with other cis-acting sequences, has been parceled out between the duplicates, both loci have to be maintained in order to recapitulate the functional ancestral Pax6 transcript unit.


ancestral
Pax6
pancreas
EE
Pax6
Duplication
Pax6a
Pax6
Pax6b
Pax6
Degenertation
Pax6a
Pax6
Pax6b
Pax6
Complemantation
Pax6a
Pax6
Pax6b
Pax6

CHAPTER 5

Conclusion

Blindness and other severe visual impairments affect a great number of people in our modern society, and can be caused by disease or injury to the visual system. The world health organization (WHO) has estimated that in the year 2002, approximately 161 million people world wide, suffered from visual impairments, 37 million of whom were legally blind. These numbers are expected to double by the year 2020, as the world's population increases. Statistically, cataracts (48%) and glaucoma (12%) are the leading causes of disease induced blindness, followed by uveitis, macula degeneration, trachoma, corneal opacity and diabetic retinopathy. However, besides the illnesses mentioned above, there is a multitude of other pathological conditions, which can lead to visual impairments by affecting one or more components of the visual system (Organization 2002).

Recent advances in the fields of embryonic and adult stem cell research, tissue engineering as well as gene transfer, have sparked the hope that in some instances vision can be restored or the loss thereof be prevented, using these novel approaches (Tropepe, Coles et al. 2000; Tropepe, Hitoshi et al. 2001; Dang and Tropepe 2006; Xu, Sunderland et al. 2007). However, a success in this area depends on our understanding of the molecular and morphogenetic mechanisms, underlying the formation of the visual system, and of the eye in particular. Interesting, with the identification and isolation of stem cells from the various sources in the adult nervous system, including the eye, it has become apparent that molecules acting during early development, are also at work in the adult organism (Tropepe, Coles et al. 2000). Therefore, it seems plausible that

an understanding of the functions of those factors during embryogenesis may facilitate a possible clinical application in pathological conditions.

An accumulating body of research suggests that eye development depends on a genetic network of transcriptional regulators that has been highly conserved from insects to mammals (Belecky-Adams, Cook et al. 1996; Gehring 1996; Oliver and Gruss 1997; Xu, Zhang et al. 1999; Hanson 2001; Kumar and Moses 2001; van Heyningen and Williamson 2002; Hanson 2003). The homeobox transcription factor Pax6 is a member of this network and has been shown to be located at the very top of its hierarchy in most species. Genetic studies have shown that Pax6 is involved in a wide variety of processes during oculogenesis, including the control of cell proliferation and cell type specification in the neural retina (Marquardt, Ashery-Padan et al. 2001; Philips, Stair et al. 2005). In addition, it was recently shown that Pax6 function is also required for retinal stem cell proliferation and maintenance in the adult retina of mammals (Xu, Sunderland et al. 2007). However, it is largely unknown, how exactly Pax6 carries out these important tasks. Because of the significance of Pax6 activity in embryogenesis and adulthood, it is imperative that we understand all aspects of Pax6 function as well as the regulatory mechanisms controlling its expression under physiological as well as pathological conditions. Recently, it has been suggested that the wide range of Pax6 functions is carried out by different isoforms of the protein, although their mechanism of action and their production are only beginning to be unveiled (Singh, Mishra et al. 2002; Kim and Lauderdale 2006). Interestingly, among those, a recently identified pairedless Pax6 isoform has been implicated in retinogenesis (Turque, Plaza et al. 1994). However, its exact role in this process has remained largely enigmatic.

In the first part of this study, I have used a transgenesis and cross species approach, to evaluate the potential role of Pax6(-PD) in the process of retinal cell type specification. This strategy has allowed me to established, for the first time, the spatial and temporal expression patterns of this protein in both zebrafish and mice. My findings suggest that the mechanism controlling the expression of the different Pax6 isoforms in the eye is highly conserved between teleosts and mammals, phyla, which are separated by more than 400 million years of evolution. Moreover, I present evidence that the different Pax6 isoforms differentially regulate the generation of the two main subtypes of amacrine interneurons during retinogenesis, a finding that reconciles the conflicting results of recent studies investigating Pax6 function in this process. For example, the analysis of a conditional Pax6 knock-out mouse, has suggested that Pax6 function is required for all retinal cell types, except amacrine cells (Marquardt, Ashery-Padan et al. 2001). This has lead to the general assumption that this particular cell type may be the default fate of all retinal precursors. However, it is often overlooked, that in this study Pax6 function was in fact needed to generate one particular subclass of amacrine interneurons, the glycinergic amacrines that make up approximately one third of the entire amacrine population. The cells remaining, after removal of Pax6 functions, were GABAergic and unidentified amacrines, both constituting about 30% of the total amacrine class. Interestingly, there was no significant decrease in the total number of amacrine cells, but a shift in cell fate within this cell class. In contrast, in homozygous Pax6 mice, retinal progenitors differentiate prematurely and assume a generic rather than specific fate, before being cleared from the retina (Philips, Stair et al. 2005). This result indicates that Pax6 is regulating the timing of differentiation as well as the fate of all retinal progenitors.

The data presented in this study, suggests that the results observed in the conditional knock-out system are due to the differential inactivation of the two Pax6 isoforms, Pax6(PD) and Pax6(-

PD) in the developing murine retina. While Pax6(PD) is required for the production of glycinergic amacrines, Pax6(-PD) appears to be involved in the generation of GABAergic amacrine cells, explaining the discrepancy observed in the two studies described above. Interestingly, these findings have implication beyond retinogenesis and this concept may also apply to the developing pancreas. Intriguingly, in Pax6 homozygous mutant mice, all of main endocrine cell types of the pancreas are affected, contributing to early postnatal mortality (Hill 1991; Sander, Neubuser et al. 1997). However, removal of Pax6 function by targeted deletion, on the other hand, leads to an absence of glucagon secreting α-cells only (St-Onge, Sosa-Pineda et al. 1997).

Interestingly, early studies in quail reported that the pairedless Pax6 isoform was in fact present in the developing retina as well as pancreas, but was absent from other parts of the embryo (Turque, Plaza et al. 1994). Therefore, it will be interesting to see if Pax6(-PD) also has a function in the differentiation of the different pancreatic cell types in addition to its role in retinogenesis. A thorough investigation of Pax6(-PD) expression in the pancreas in our experimental system will surely be informative.

In addition to investigating the role of Pax6(-PD) during retinogenesis, I have investigated the evolutionary conservation of the mechanism of Pax6 regulation. By creating a compliment of transgenic mice, carrying either the zebrafish Pax6a or Pax6b locus, I have been able to determine the extent of evolutionary conservation of tissue specific Pax6 expression during development. The evidence presented here demonstrates that most, if not all, aspects of the gene regulatory control mechanism, directing Pax6 expression in the various tissues have been conserved within the vertebrate lineage. In addition, I show that the Pax6 genes in zebrafish were maintained due to degenerative and complementary mutations within crucial regulatory

elements. The usefulness of the genetic tools generated in this dissertation goes beyond this study and present useful genetic markers for researchers, trying to address particular biological questions involving Pax6 function.

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