THE REGULATORY MECHANISMS AND FUNCTIONS OF PAX6 ISOFORMS
IN EYE DEVELOPMENT

by

JIHA KIM
(Under the Direction of James D. Lauderdale)

ABSTRACT

Pax6, a member of the paired-family transcription factors, exhibits restricted expression and essential functions to control the development and maintenance of the mammalian eye. In mammals, three different isoforms are known to be involved in diverse functions of Pax6. The goal of this study was to understand global regulatory mechanisms that control expression of different Pax6 isoforms during oculogenesis. Although previous studies have provided a wealth of information about regulatory elements located 3’, within, and 5’ of Pax6 transcription unit, genetic evidences from mice and human indicate that these elements act coordinately. The mechanism by which this happens to achieve tightly controlled Pax6 expression is not clear.

To study Pax6 regulation and isoform expression, I developed a set of BAC transgene reporters that serve as a useful tool to study mechanism(s) by which the 3’ Downstream Regulatory Region(DRR) exerts its master regulatory function to coordinate a global regulatory network. Using this transgenic mouse, I showed that the 3’ region between HV break point and Elp4 exon7 is sufficient to drive reporter expression that is comparable to endogenous Pax6 in the eye. Phylogenetic foot printing and an in vitro reporter assay revealed that several evolutionarily conserved regions within DRR are capable of inducing reporter expression in a
tissue specific manner. Subsequent in vivo assays using transgenic mouse showed that removal of CR2, one of the highly conserved region within the DRR, can abolish transgene expression in the entire embryo. Together, this evidence strongly argues that the Pax6 DRR is required for correct Pax6 expression in the eye.

In addition to a complex regulatory mechanism, diverse functions of Pax6 isoforms add more complexity in understanding the activity of Pax6 during oculogenesis. Three isoforms including Pax6, Pax6 (5a), and Pax6ΔPD have been identified in mammals. Here I demonstrate that Pax6ΔPD is predominantly expressed in the mammalian eye and can cause microphthalmia when it is over-expressed. These results suggest that Pax6ΔPD has a role during eye development. Genetic analysis suggests that Pax6ΔPD is acting possibly as dominant negative factor against full length Pax6. Although our detailed analysis suggests that Pax6ΔPD is acting on signaling pathway to control lens development, what type of signaling pathway is involved in this cascade is yet to be studied.

INDEX WORDS: Pax6, eye development, Downstream Regulatory Region (DRR), regulatory elements, BAC transgene, isoforms, reporter assay, evolutionarily conserved sequences, Locus Control Region (LCR)
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by

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THE REGULATORY MECHANISMS AND FUNCTIONS OF PAX6 ISOFORMS

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DEDICATION

This dissertation is dedicated to my parents. Without their encouragement and support, it would be impossible for me to make achievements. My deep gratitude goes to my father specially. Without his endless support and love, I would not be able to become a scientist and to realize what I am capable of.
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Chapter 1

General Introduction

Overview of Vertebrate Eye development

The eye is essentially a highly specialized extension of the brain (Dowling 1987). Vertebrate eyes are generated by a series of induction and regional specification events. Development of the eye can be subdivided into three phases; formation of the major structures of the eye, maturation of these structures to form the functional eye, and formation of neural connection between the retina and optic tectum. These processes are tightly regulated by signaling cascades and some members of these signaling cascades have been identified including secreted factors, receptors, and transcription factors (Oliver and Gruss 1997; Jean, Ewan et al. 1998). Early structures of the eye including the lens vesicle, double layered optic cup, optic stalk and precursor tissue of the cornea are generated by a series of induction and regional specification events from an outpocketing of the forebrain neuroectoderm and the overlying head ectoderm (Cvekl and Tamm 2004). The eye is one of the first regions to be defined in the anterior neural plate, which then evaginates from the forebrain to become first the optic pit (E8.5) and then the optic vesicle (E9.5). The optic vesicles come into contact with the overlying surface ectoderm, which responds with a local thickening, that becomes the lens placod. Coordinated invagination of the lens placode and the optic vesicle results in the formation of the lens vesicle and bilayered optic cup (E10.5) (Schwarz, Cecconi et al. 2000). The outer layer of the optic cup develops into the retinal pigmented epithelium (RPE) and the inner layer develops
into the neural retina with its different cell types. Finally, the lens vesicle detaches from the surface ectoderm and invaginates into the optic cup (E11) and becomes the lens. Shortly after the lens vesicle detaches from the surface ectoderm, mesenchymal cells start to migrate into the space between the lens vesicle and the surface ectoderm. At E14.5, cornea is beginning to form from posterior mesenchymal cells and surface ectoderm. Beginning around E15.5, the peripheral edge of the optic cup grows into this cavity along the anterior lens surface to give rise to the iris and ciliary body (Cvekl and Tamm 2004).

Figure 1.1. Schematic representation of the eye development. Representation of the main embryonic stage E8.5 to E11.5 of mouse eye development. ne, neural ectoderm; op, optic pit; ov, optic vesicle; lp, lens placode; RPE, retinal pigmented epithelium; os, optic stalk; lv, lens vesicle; se, surface ectoderm.

**Conserved pathways control eye development**

Although much of our understanding of how early developmental genes direct eye formation has come from work on *Drosophila* eye development, it is now evident that highly conserved networks and pathways govern oculogenesis in both vertebrates and invertebrates. In
vertebrates, the first events of eye development occur within the anterior neural plate. Several of the homeodomain transcription factors such as Pax6, Rx, and Six3 have been shown to be required to pattern the anterior neural plate during early embryonic development (Hill, Favor et al. 1991; Oliver, Mailhos et al. 1995; Mathers, Grinberg et al. 1997; Chow and Lang 2001). In *Drosophila*, the eye specification genes (*toy, ey/Pax6, eye, so, and eya*) form a regulatory network in which the direct control of any one of these genes may affect the others during eye development (Halder, Callaerts et al. 1995; Bonini, Bui et al. 1997; Pignoni, Hu et al. 1997; Czerny, Halder et al. 1999). It is likely that these networks are under the control of evolutionarily conserved signaling pathways. For example, the Notch signaling pathway has been shown to be involved in Pax6 activation during early eye development in both vertebrates and invertebrates. The Notch signaling pathway defines an evolutionarily conserved cell–cell interaction mechanism that controls the ability of precursor cells to respond to developmental signals (Artavanis-Tsakonas, Rand et al. 1999). In *Drosophila*, Notch signaling regulates the expression of *eyeless* and *twin of eyeless; twin of eyeless*(toy) is known to be an upstream regulator for *eyeless* (Kurata, Go et al. 2000). Also, in vertebrates, activation of Notch signaling activates *Pax6* and other eye-related genes and induces eye duplications as well as proximal eye defects in embryos. These results also suggest that the regulation of Pax6 expression and the function of Pax6 in eye development are conserved in vertebrates and invertebrates (Onuma, Takahashi et al. 2002). Although theses signaling pathways are expected to control both eye specification and morphogenesis by governing the eye specific genes, the mechanisms by which this is accomplished is unknown.
**Pax6: Regulator of eye morphogenesis**

Pax6 is highly conserved among metazoans and controls key steps during eye morphogenesis (Gehring and Ikeo 1999). First identified in mice (Hill, Favor et al. 1991; Walther, Gruss et al. 1991) and humans (Ton, Hirvonen et al. 1991; Glaser, Walton et al. 1992), homologs have been identified in a wide variety of species, including birds, *Drosophila*, (Plaza, Dozier et al. 1993), and nematodes (Chisholm and Horvitz 1995; Zhang and Emmons 1995). Pax6 is a key regulator of eye development that is both necessary for eye formation in different organisms as well as capable of inducing ectopic eyes in *Drosophila* (Halder, Callaerts et al. 1995) and *Xenopus* (Chow, Altmann et al. 1999) upon mis-expression.

In human, *PAX6* was identified by positional cloning as the causal gene for the developmental eye anomaly Aniridia (Ton, Hirvonen et al. 1991; Hanson, Seawright et al. 1993; Quiring, Walldorf et al. 1994). Aniridia in human and *Small eye* (*Sey*) in mouse are semidominant panocular disorders, resulting from heterozygosity of null alleles, which include severe iris hypoplasia, defects in the neural retina, and risk for acquired corneal opacification, cataracts, and glaucoma. Homozygous mouse mutants are anophthalmic and die at birth (Hogan, Horsburgh et al. 1986; Matsuo, Osumi-Yamashita et al. 1993; Grindley, Davidson et al. 1995). *Pax6* gene dosage is known to be important for normal eye development in mammals. As described above, Pax6 function is haploinsufficient so that heterozygosity of null alleles results in eye phenotypes. Also, mice that had more than two normal copies of the *Pax6* locus showed eye defects, while a single copy of the *Pax6* locus could rescue the *Sey* (heterozygous) phenotype (Schedl, Ross et al. 1996). This evidence implies that proper dosage is important for expression level of Pax6 protein.
From the earliest stage of vertebrate eye morphogenesis, Pax6 is expressed in the optic vesicle, giving rise to the retina and pigmented epithelium, and in the overlying ectoderm that forms the lens and cornea (Grindley, Davidson et al. 1995). This wide expression of Pax6 in the developing eye is consistent with the panocular features of aniridia mentioned above. Detailed analysis of its cellular functions suggests an involvement in processes like cell proliferation, differentiation, and adhesion/migration (Warren, Caric et al. 1999; van Heyningen, Williamson et al. 2002).

Most of the known functions of Pax6 in eye morphogenesis have been implicated from analysis of mutant model systems. Although, Sey mutant mice initiate the formation of the optic vesicle, they fail to constrict proximally and show abnormalities at early stages. In addition, differentiation of the developing optic cup into distinct layers, pigmented retinal epithelium and neural retina, also failed in Sey (Grindley, Davidson et al. 1995). In the developing retina, Pax6 expression is maintained in all proliferating retinal progenitor cells, but is down regulated following the differentiation of most neuronal cell types, except amacrine and bipolar cells. Also, another study has shown a requirement for Pax6 in the vertebrate retina during neurogenesis (Walther, Gruss et al. 1991). Furthermore, tissue recombination experiments, using the rat small eye, found that lens formation was dependent on the genotype of the surface ectoderm but not the genotype of the optic vesicle, suggesting that normal Pax6 expression in the surface ectoderm is responsible for lens formation (Fujiwara, Uchida et al. 1994). Reduced proliferative capability in the developing lens has been demonstrated for heterozygous Pax6 mutant cells (Xu, Saunders et al. 1997). Also, non-lens tissues that express Pax6 such as embryonic retina, iris and pigmented retinal epithelium are known to have the ability to transdifferentiate into lens (Okada 1991). The Pax6 protein also plays an important role in the induction of certain crystallines in the forming
lens at the stage of placode and lens vesicle. It was demonstrated that Pax6 had the capability of binding to the promoter region of the alpha-α-crystallin gene (Schedl, Ross et al. 1996) and alpha-β-crystallin gene (Ton, Miwa et al. 1992) in mouse, and several other types of crystallin genes in chicken and guinea pig. Loss of Pax6 function in lens development after the early role in placode formation had been completed, led to disruption not only of lens but also retinal development (Ashery-Padan, Marquardt et al. 2000), emphasizing the developmental interdependency of these two structures in the vertebrate eye.

**Transcription factor Pax6**

Pax6 belongs to the family of the Pax homeodomain proteins which were isolated by homology to the DNA binding domain of the *Drosophila* paired gene (Walther, Guenet et al. 1991). Human *PAX6*, on chromosome 11p13, encodes a 422 amino acid transcriptional regulator, spanning 16 exons distributed over a 30 kb region; in addition to exons 0 through 13, there are also exons alpha and 5a (Glaser et al., 1992; Kammandel et al., 1999; Plaza et al., 1999; St-Onge et al., 1997; Williams et al., 1998; Xu et al., 1999). The Pax6 protein contains three distinct domains. It has two DNA binding domains, a paired domain (PD) at the N-terminus and a paired-like homeodomain (HD) in the middle, and a transactivation domain at the C-terminus (Walther and Gruss 1991; Epstein, Glaser et al. 1994). Throughout the whole coding region, Pax6 showed strong similarity between human and rodent (100%), chick (96%) and zebra fish (93%) and especially the PD and the HD maintain 80-90% identity from mammals to *Drosophila* (Quiring, Walldorf et al. 1994) and *C. elegans* (Gehring and Ikeo 1999).

The PD of Pax6 can bind to a broad range of DNA sequences with high specificity. The PD consists of independent amino-terminal and carboxyl-terminal subdomains, and each
subdomain can identify and bind to distinct DNA sequences (Czerny, Schaffner et al. 1993; Epstein, Glaser et al. 1994). However, it is not clear how the Pax6 PD recognizes so many different DNA sequences. PDs can also bind DNA by interacting with other DNA binding domains such as the HD (Underhill, Vogan et al. 1995; Jun and Desplan 1996). In the PAX6 protein, the HD is about 80 amino acids downstream of the PD. Studies have shown that highly diverged HDs have indistinguishable DNA binding preferences (Wilson, Sheng et al. 1993). Several mechanisms have been suggested for the specificity of HD function. These include protein oligomerization, protein-protein interactions of homeoproteins with other factors, association of the HD with other DNA binding domains, and cooperative dimerization of paired class HDs (Wilson, Sheng et al. 1993). However, which of these mechanisms are utilized by PAX6 is not known. Although HD itself is able to activate target gene such as rhodopsin (Sheng, Thouvenot et al. 1997), other study suggests that the structural changes in the subdomains of PD influence the DNA binding of the HD of PAX6 differentially (Singh, Stellrecht et al. 2000). Mutation in C-terminal transactivation domain resulted in the loss of DNA binding ability of the PAX6 homeodomain and lower DNA binding ability through the paired domain (Singh, Chao et al. 2001). This evidence suggests that three functional domains of Pax6 are interacting with each other cooperatively.

**Expression pattern of Pax6**

Pax6 is expressed in the developing central nervous system, in the neuroretina and lens of the eye, in the nasal placode, and in the pancreas (Callaerts, Halder et al. 1997). Pax6 expression is turned on in neural ectoderm of the presumptive forebrain, optic pit, presumptive hindbrain, and presumptive spinal cord between day 8 to 8.5 when the first somites are formed.
and the neural folds begin to close in the cervical region. Later in development, Pax6 transcripts are present in the telencephalon and diencephalon and in the part of the hindbrain (Walther and Gruss 1991; Puschel, Gruss et al. 1992; Stoykova and Gruss 1994; Grindley, Davidson et al. 1995).

**Pax6 expression in the eye.**

Pax6 expression is observed early in mouse development (E8.0-E8.5) in a broad region of head surface ectoderm and in head neural ectoderm, including the optic pit which is the first indication of the eye. Around E9.5, Pax6 is expressed in the epithelial layer of the optic vesicle and in the developing optic stalk. As optic vesicles developed into optic cup, Pax6 expression is the highest around the rim of the developing optic cup but is slightly weaker in the proximal optic stalk (Baumer, Marquardt et al. 2003). While Pax6 expression in the optic cup is equally high in both presumptive neuroretina and retina pigmented epithelium (RPE) during early development of optic cup, in later stage expression is down regulated in the RPE but still maintained at high level in the presumptive neuroretina. In the developing optic cup, Pax6 is initially expressed throughout (Grindley, Davidson et al. 1995), but in the differentiated retina it is expressed strongly only in ganglion and amacrine cells (Belecky-Adams, Tomarev et al. 1997). The rim of the retina that will become the iris and ciliary body express Pax6 continuously throughout the development of anterior ocular structures.

Although Pax6 is expressed in a broad region of surface ectoderm in early stage, it becomes progressively restricted to the developing lens placode. The expression of Pax6 in the head surface ectoderm and subsequently in the lens placode is correlated with the lens-forming competence of these tissues (Grainger 1992; Grindley, Davidson et al. 1995). Pax6 expression
continues throughout the surface ectoderm, but as the lens begins to differentiates, it becomes restricted to the proliferating lens epithelial cells (Grindley, Davidson et al. 1995). After lens vesicle is detached, the overlaying surface ectoderm, which corresponds to the developing cornea, also continues to express Pax6 until late stage.

**Regulation of Pax6 expression**

A wide array of upstream enhancers, promoters and intronic regulatory elements has been defined for Pax6 over the years. Three different transcripts, initiated from P_0, P_1, and P_\alpha promoters, were shown to be found in different region during eye and CNS development (Xu, Zhang et al. 1999). Cis regulatory elements have been defined 5', within, and 3' of mammalian Pax6 using transgene reporter assay. Specific enhancers for the endocrine pancreas, retina, lens and cornea have been identified 5' to P_0 and within the intron alpha located between exons 4 and 5 (Kammandel, Chowdhury et al. 1999). Enhancers for the telencephalon, diencephalon, rhombencephalon, and spinal cord are located within the region between P_0 and P_1 (Kammandel, Chowdhury et al. 1999; Xu, Zhang et al. 1999). Enhancers that control reporter expression in the maturing eye, diencephalon, and hindbrain are located within intron7, and enhancers for the olfactory region, pretectum, and neural retina are located 3' to Pax6 in a region designated as C1170/Box 123 (Griffin, Kleinjan et al. 2002; Kleinjan, Seawright et al. 2004). Identified regulatory elements seem to be evolutionarily conserved between distant species (Plaza, Dozier et al. 1995; Xu, Saunders et al. 1997; Williams, Altmann et al. 1998; Plaza, Saule et al. 1999). Recent studies have identified a putative Pax6 regulatory region (Downstream regulatory region) located >150kb downstream of the major Pax6 promoters (Lauderdale, Wilensky et al. 2000; Kleinjan, Seawright et al. 2001). The presence of this region was first suggested by analysis of a set of aniridic patients in which chromosomal rearrangement disrupted the region 3' to Pax6 but
spared the *Pax6* transcription unit (Ton, Hirvonen et al. 1991; Fantes, Redeker et al. 1995; Lauderdale, Wilensky et al. 2000). Work over the past several years has provided several lines of evidence that this region regulates Pax6 expression. First, Pax6 was not expressed from the allele containing deletion within the region 3' to the *Pax6* in the human × mouse somatic cell hybrid experiment (Lauderdale, Wilensky et al. 2000). Second, human YAC transgenes spanning *PAX6* were able to rescue the *Sey* phenotype and homozygous *Sey* lethality only if the YAC contained this 3' region (Fantes, Redeker et al. 1995; Kleinjan, Seawright et al. 2001). Third, additional tissue specific regulatory elements were identified including lens specific enhancer, retina specific regulatory region (Kleinjan, Seawright et al. 2001), and most recently C1170/Box 123 which was active in the developing pretectum, neural retina, and olfactory region (Griffin, Kleinjan et al. 2002). Together, these results argue strongly that normal Pax6 expression in the eye requires critical control elements located 3' to the *Pax6* transcription unit.

Figure 1.2. Genomic organization of the *Pax6* gene with its control elements. Pax6 expression is shown by in situ hybridization on E10.5 mouse embryo. All known regulatory sequences are indicated by color boxes and described detail in the table.
**Pax6 isoforms**

Two different isoforms, canonical Pax6 and Pax6 (5a), which contains a 14 amino acid insertion in the N-terminal subunit of the PD, have been implicated as major Pax6 proteins. Recently, a third isoform, a paired-less isoform of Pax6 has been identified in birds (Carriere, Plaza et al. 1993), nematodes (Chisholm and Horvitz 1995; Zhang and Emmons 1995) and mouse (Kim and Lauderdale 2006). In Pax6 (5a) isoform, additional 14 amino acid insertion in PD caused disruption of paired domain and alter its DNA binding properties. Therefore, different two isoforms possibly activate different sets of downstream target genes (Epstein, Glaser et al. 1994). It has been shown that Pax6(5a) is expressed in the eye at about one-tenth the level of Pax6 during embryonic development (Epstein, Glaser et al. 1994; Richardson, Cvekl et al. 1995; Jaworski, Sperbeck et al. 1997; Kozmik, Czerny et al. 1997). However, recent studies showed that expression level of Pax6 (5a) isoform is up-regulated to the same level with Pax6 after birth (Zhang, Cveklova et al. 2001). Consistent with this result, eyes of newborn Pax6 (5a) null mice showed grossly similar phenotype with wild-type while adult Pax6 (5a) null mice showed iris hypoplasia, and distinct defects in the cornea, lens, and retina. This finding suggest that Pax6 (5a) is required later for the differentiation and/or maintenance of adult eye structures (Singh, Mishra et al. 2002). In addition, the ratio of the two Pax6 isoforms is appeared to be critical for the normal development and function of vertebrate eyes. Changes in the ratio of the two isoforms correlate with a distinct human ocular syndrome (Epstein, Glaser et al. 1994). Although functional interaction between Pax6 and Pax6 (5a) is not clear yet, Pax6 (5a) appears to act in a spatiotemporally restricted manner that is different than Pax6. Third isoform, paired-less Pax6 (Pax6ΔPD) has been identified first in *Quail* (Carriere, Plaza et al. 1993). Pax6ΔPD is a 32/33kda isoform that lacks the PD due to the use of an alternative start codon for translation in
the sequence between the PD and the HD. Also in *C. elegans* (Chisholm and Horvitz 1995; Zhang and Emmons 1995), two transcripts of the *Pax6* gene were identified, one that encodes the full-length protein and one initiated at an internal promoter producing a transcript that does not encode the PD. Transcripts predicted to encode for this isoform have been identified in mammal and can be generated from alternative internal promoters, $P_{\alpha}$ and $P_4$ (Kammandel et al., 1999; Kleinjan et al., 2004; Mishra et al., 2002), or by alternative splicing (Gorlov and Saunders, 2002; Mishra et al., 2002). Recently, in mouse 32/33kDa Pax6ΔPD protein has been detected specifically in the developing eye (Kim and Lauderdale 2006).

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Figure 1.3. Schematic diagram of Pax6 isoforms.

However, little is known about the biological function and expression pattern of paired-less Pax6 isoform. Although Pax6ΔPD has been identified first in quail, no biological function has been known in quail. In *C. elegans* this isoform is required for specification of a peripheral sense-organ (Zhang and Emmons 1995) and known to interact with paired-containing Pax6
additively, synergistically, or antagonistically, depending on the cellular context (Cinar and Chisholm 2004). In mouse, over-expression of Pax6ΔPD in cells that normally express this isoform results in severe microphthalmia in both Pax6+/+ and Pax6+/− animals. Although, over-expression of human PAX6 also caused microphthalmia in wild-type mice (Schedl, Ross et al. 1996), their phenotypes were different and more importantly, the human PAX6 locus rescued the Small eye mutant phenotype (Schedl et al., 1996) whereas Pax6ΔPD enhanced small eye phenotype (Kim and Lauderdale 2006). Taken together, these results indicate that Pax6ΔPD has functions that are different than Pax6, possibly acting as a dominant-negative isoform in eye development. However, detailed biological function during eye development and molecular mechanisms that control the balanced expression of different isoforms are largely unknown. Interestingly, studies from both Quail and C. elegans suggest that PAX6ΔPD isoform can be located both in cytoplasm and nucleus whereas Pax6 is exclusively located in nucleus (Carriere, Plaza et al. 1995; Zhang, Ferreira et al. 1998). Although, function of Pax6ΔPD in the cytoplasm is unclear, this distinct function of Pax6ΔPD is conserved between different species.

References


Chapter 2

Analysis of Pax6 expression using a BAC transgene reveals the presence of a paired-less isoform of Pax6 in the eye and olfactory bulb\(^1\)

Abstract

Pax6, a member of the paired-family of transcription factors, exhibits restricted expression and essential functions in the developing eye, olfactory system, central nervous system, and pancreas. To understand Pax6 function, which critically depends on induction of proper expression levels during development, it is necessary to elucidate the molecular mechanisms governing Pax6 transcription. Although previous studies using classic transgenic approaches have provided a wealth of information about the distribution and types of regulatory elements involved in Pax6 regulation, genetic studies in both humans and mice indicate that these enhancers alone are not sufficient for fully regulated Pax6 expression. We report here our analysis of mice transgenic for a 160 kb mouse Pax6 BAC transgene, which was generated as a necessary first step towards testing the long-range control of Pax6 expression in vivo. We show that this BAC transgene replicates Pax6 expression in the eye. This is the first time that a reporter transgene has been expressed in a normal Pax6-like pattern in all of the tissues of the eye and defines an eye regulatory region within the Pax6 downstream regulatory region (DRR). Second, we show that this BAC transgene contains all of the cis-regulatory elements required for normal Pax6 expression within the developing embryo, except for within the diencephalon and olfactory bulb. Third, we show that this transgene is subject to Pax6 autoregulation. Lastly, we identify, for the first time in mammals, an isoform of the Pax6 protein lacking the paired domain. This isoform is expressed in the developing olfactory bulb and eye. Over-expression of Pax6ΔPD causes a microphthalmic phenotype in both Pax6+/+ mice and Pax6+/- mice. These results demonstrate a role for Pax6ΔPD in eye development, which appears to be different than that ascribed to either canonical Pax6 or Pax6 (5a).
Introduction

The Pax6 transcription factor is highly conserved among metazoans and controls key steps in eye development (Gehring 2005). In the mammalian and avian eye, Pax6 is expressed in the developing optic vesicle, lens, cornea, iris, and neural retina (Walther and Gruss 1991; Martin, Carriere et al. 1992; Grindley, Davidson et al. 1995; Davis and Reed 1996; Koroma, Yang et al. 1997). These sites of expression correlate with Pax6 ocular function. In mammals, Pax6 mutations are associated with aniridia in humans (Ton, Hirvonen et al. 1991; Glaser, Walton et al. 1992; Hanson, Seawright et al. 1993) and the Small eye (Sey) trait in rodents (Hill, Favor et al. 1991; Matsuo, Clay et al. 1993). Aniridia and Sey are semidominant panocular disorders, resulting from heterozygosity of null alleles, which include severe iris hypoplasia, defects in the neural retina, and risk for acquired corneal opacification, cataracts, and glaucoma. Although the mechanisms underlying these haploinsufficient phenotypes are poorly understood, it is clear that Pax6 has multiple functions in both the developing and adult eye and affects cell fate, cell proliferation, and patterning. Recent evidence suggests that these diverse functions are mediated by different isoforms of the Pax6 protein (Azuma, Yamaguchi et al. 1999; Singh, Mishra et al. 2002; Haubst, Berger et al. 2004).

Three isoforms of the Pax6 protein have been reported in vertebrates. The canonical form of Pax6 contains two DNA-binding domains, the paired domain (PD) and a paired-like homeodomain (HD) linked by a glycine-rich region, and a proline–serine–threonin (PST)-rich transactivation domain (Hill, Favor et al. 1991; Ton, Hirvonen et al. 1991; Walther and Gruss 1991; Glaser, Walton et al. 1992; Ton, Miwa et al. 1992). This isoform is expressed in most cells that express Pax6 (Xu, Woo et al. 1997; Kammandel, Chowdhury et al. 1999; Plaza, Aumercier et al. 1999; Xu, Zhang et al. 1999; Anderson, Hedlund et al. 2002). The second Pax6 isoform,
which is generated by alternative splicing, 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). This isoform has been detected in the brain, spinal cord, olfactory epithelium, and eye (Epstein, Glaser et al. 1994; Zhang, Cveklova et al. 2001; Azuma, Tadokoro et al. 2005; Pinson, Mason et al. 2005). The third isoform, which was identified in quail neuroretina extracts (Carriere, Plaza et al. 1993), lacks the paired domain (Pax6ΔPD). Transcripts predicted to encode for this isoform have been identified in mammals and can be generated from alternative internal promoters (Kammandel, Chowdhury et al. 1999; Mishra, Gorlov et al. 2002; Kleinjan, Seawright et al. 2004) or by alternative splicing (Gorlov, Saunders et al. 2002; Mishra, Gorlov et al. 2002). Little is known about where this isoform is expressed or its normal function in vivo.

Insights into different aspects of Pax6 function can be facilitated by elucidation of the mechanisms governing Pax6 expression. Modular cis regulatory elements controlling different aspects of Pax6 expression have been defined 5′, internal, and 3′ to the Pax6 transcript unit using transgene reporter assays (Williams, Altmann et al. 1998; Kammandel, Chowdhury et al. 1999; Plaza, Aumercier et al. 1999; Xu, Zhang et al. 1999; Kleinjan, Seawright et al. 2001; Griffin, Kleinjan et al. 2002; Kleinjan, Seawright et al. 2004). While some of these elements are widely spaced in the Pax6 locus, they exhibit overlapping tissue specificity, particularly in the eye and diencephalon. Although collectively these elements account for the majority of the Pax6 expression domain observed in developing mice, genetic evidence from humans and mice suggests that these enhancers are insufficient for normal Pax6 expression. Whereas aniridia is typically caused by heterozygous null mutations within PAX6 or cytogenetic deletions of chromosome 11p13 that encompass PAX6, chromosomal rearrangements also have been
described that disrupt 11p13 but spare the *PAX6* transcription unit (Simola, Knuutila et al. 1983; Ton, Hirvonen et al. 1991; Fukushima, Hoovers et al. 1993; Fantes, Redeker et al. 1995; Lauderdale, Wilensky et al. 2000; Kleinjan, Seawright et al. 2001). The breakpoints of these aniridia-associated rearrangements are all located 3' to *PAX6*, with the most distal, designated as “SIMO,” located ~124 kb 3' the *PAX6* polyadenylation sites (Simola, Knuutila et al. 1983; Fantes, Redeker et al. 1995; Lauderdale, Wilensky et al. 2000; Kleinjan, Seawright et al. 2001). These rearrangements presumably cause a loss of gene expression by removing a distant positively acting cis regulatory element required for *PAX6* eye expression. Consistent with this, in mice, human YAC transgenes spanning *PAX6* rescue the *Sey* phenotype and homozygous *Sey* lethality when the YAC contained DNA 3' to the SIMO breakpoint (Schedl, Ross et al. 1996; Kleinjan, Seawright et al. 2001). Similarly, we showed, using human×mouse somatic cell hybrids that chromosomal rearrangements 3' to human *PAX6* blocked gene expression even though the transcript unit was intact and included known eye elements (Lauderdale, Wilensky et al. 2000). These results argue strongly that Pax6 eye expression requires additional 3' long-range enhancers located within a region designated as the *Pax6* downstream regulatory region (DRR), which extends over a ~75 kb region 3' to the SIMO breakpoint (Kleinjan, Seawright et al. 2001). Although cis regulatory elements have been identified within the DRR (Kleinjan, Seawright et al. 2001), their mechanisms of action remain unknown.

We are studying the long-range regulatory mechanisms governing *Pax6* transcription in the mouse using bacterial artificial chromosomes (BACs). Because *Pax6* transcriptional control is complex, the use of large genomic constructs will avoid potential problems associated with “classical” reporter transgene approaches, which are subject to position effects and do not take into account the cooperative interactions between regulatory elements that would normally exist
within the genome. We report here that mice containing a *Pax6* BAC transgene, which included the *Pax6* transcript unit and a cluster of evolutionarily conserved sequences located ~100 kb 3' to the *Pax6* coding region, are sufficient to recapitulate the native Pax6 expression pattern in all regions of the developing mouse, except the diencephalon and olfactory bulb. Our data strongly suggest the presence of additional *Pax6* enhancers for the diencephalon and olfactory bulb that reside outside the genomic DNA contained in the *Pax6* BAC transgene. Additionally, we identify, for the first time in mammals, an isoform of the Pax6 protein lacking the paired domain, which is expressed in both the developing eye and olfactory bulb and has a role in eye development.

**Material and Methods**

**Comparative genomics**

The genomic sequence data used to generate the scale drawings and also for comparative genomics were obtained from the National Center for Biotechnology Information (NCBI) or the Sanger Institute. Sequences with the following GenBank accessions nos. were used in this study: human, Z95332, Z83307, Z83001, Z83006, Z83009, Z83008, Z83308, AC131571, Z86001, AL136384; mouse, AL512589, AL590380; chicken, AC14046, AC141860; zebrafish AL929172.

**Identification and molecular characterization of Pax6 BACs**

BACs containing sequences homologous to the SIMO region were identified by screening a mouse ES-129/SvJ BAC library (Release II; Genome Systems, Inc) with a $^{32}$P-labeled single copy probe derived from the SIMO breakpoint (Gessler and Bruns 1989). The four BACs that gave the strongest signal, 250b19, 293d08, 427g18, and 432m12, were then screened
for Pax6 exon 0 using a $^{32}$P labeled mouse Pax6 exon 0 probe, derived from a cDNA clone, at high stringency (65°C, 0.2× SSC final wash). This probe detected BACs 293d08 and 432m12. BAC ends were sequenced by automated DNA sequencing, and sequence analysis was performed using MacVector (Accelrys, Inc) and BLAST (NCBI). The internal structures of these two BACs were determined by fingerprint analysis using, separately, BamHI and EcoRI. Southern analysis using single copy $^{32}$P-labeled probes was used to directly compare BamHI restriction fragments from BAC DNA to those obtained from genomic DNA prepared from 129/SvJ mice.

**BAC transgene construction**

Pax6 BAC 293d08 was modified by targeted insertion of an EGFP pA reporter cassette into Pax6 exon 4 using the prophage BAC modification system (Yu, Ellis et al. 2000; Lee, Yu et al. 2001). The EGFPFRT- kan-FRT targeting cassette was PCR-amplified from pCS2+ MTeGFPFRT-kan-FRT using the Pax6 forward targeting primer, E4RECF, 5'-AGCCCCGTATTCGAGCCCCGTGGGATCCGGAGGCTGCCAACCAGCTCCAGCATGGTGAGCAAGGGCGAGGAG3'- and the Pax6 reverse targeting primer, E4RECR, 5'-TAACCCACGGCCCGCACGCCTCCAGCCCAACAGTCCAGAGAAAGACCTGAGTATTCCAGAAGTAGTGAG-3". Nucleotides in italics are homologous to Pax6 sequences and those in roman are homologous to amplification cassette. The pCS2+MTeGFP-FRT-kan-FRT plasmid (gift of X. Fan and S. Dougan, UGA) was constructed by inserting the FRT-kan-FRT SacII fragment of pIGCN21 (gift of N. Copeland, NCI'; (Lee, Yu et al. 2001) into the SacII site of pCS2+MTeGFP (gift of D. Turner, Univ. Mich.). There is a SV40 polyA addition sequence just downstream of the EGFP open reading frame. The resulting 2.4 kb PCR product was gel-purified using a Qiaquick gel
extraction kit (Qiagen) and DpnI-treated to remove template plasmid before use for homologous recombination. BAC recombination was performed following the protocol of Lee et al. (2001). Double-resistant colonies (Cm\textsuperscript{R} Kan\textsuperscript{R}) were assayed for homologous recombination by PCR using the following primers: F1 (5\textquotesingle-GCTGTCTCTCCCTCCCTCTCTAAAG-3\textquotesingle), F2 (5\textquotesingle-GCACCATCTTCTTACGACGAC-3\textquotesingle), R1 (5\textquotesingle-CCTTGAAGAGATGGTGCG-3\textquotesingle), and R2 (5\textquotesingle-GGGGATGTAGGATGGTGCG-3\textquotesingle). The kanamycin cassette was flipped out by induction of flipase, and the cells were screened for kanamycin sensitivity (Kan\textsuperscript{S}). In these colonies, removal of the kanamycin cassette was verified by PCR using the F1, F2, R1, and R2 primers. The overall structure of the modified BACs was examined by fingerprint analysis using BamHI and compared to that obtained for the unmodified BAC. The sequence of the targeted region was verified by automated sequencing of both DNA strands using primer F1 for the 5\textquotesingle junction and primer F2 for the 3\textquotesingle junction.

**Generation of BAC-transgenic mice**

Transgenic mice were generated from pronuclear injection of closed circular BAC DNA into mouse oocytes (Lee, Yu et al. 2001; Gong, Yang et al. 2002; 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 Pax6 intron 3, 5\textquotesingle-GCAAGTTTATGTTTGTGC TGTTTTGG-3\textquotesingle; reverse primer, located in EGFP, 5\textquotesingle-CCTTGAAGAGATGGTGCG-3\textquotesingle; the PCR product is 714 bp). Three out of twenty-one pups derived from pronuclear injection harbored the BAC transgene. F1 founders were generated by crossing founder males (lines 3 and 14) or a founder female (line 15) to CD-1 mice (Charles River Laboratories). These lines are being maintained in the CD-1 background. Transgene copy number was determined by
densitometric analysis of Southern blots performed on a G4 Power Macintosh computer running OSX using ImageJ (v1.33, developed at the U.S. National Institutes of Health; available on the Internet at http://rsb.info.nih.gov/ij/index.html). Small eye mice harboring the Pax6 BAC 293d08-E4-EGFP pA transgene were generated by crossing an F1 founder male (line 15) with Sey Neu females. The founder Sey Neu mice for our colony were generously provided by Dr. Nadean Brown and are maintained in the albino FVB/N 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 CO2, 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 35 mm 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. Embryos that were to be used for mRNA in situ hybridization had incisions made in their hindbrain and cerebral vesicles prior to fixation; these embryos were fixed overnight, dehydrated stepwise through a graded PBS/methanol series, and stored in methanol at −20°C.

Transgene expression was analyzed in wild-type embryos obtained by timed matings of transgenic males with non-transgenic CD-1 females (Charles River Laboratories). Non-transgenic littermates were used as controls. Transgene expression was analyzed in Sey mutant embryos obtained by intercrosses between mice compound heterozygous for the transgene and
the Pax6<sup>SeyNeu</sup> allele; non-transgenic littermates were used as controls. The genotype of each embryo was determined by PCR (Xu, Saunders et al. 1997), using tail or extra-embryonic membrane DNA (Laird, Zijderveld et al. 1991).

**Immunocytochemistry**

Immunostaining was performed on frozen sections using antibodies against Pax6 and secondary antibodies conjugated to Cy3 (Jackson ImmunoResearch). The Pax6 protein was detected using a Pax6 rabbit antisera against a 17-residue C-terminal peptide from the mouse Pax6 sequence (Mastick, Davis et al. 1997). For sectioning, fixed embryos were embedded in gelatin and cryosectioned (Mastick, Davis et al. 1997); this method did not affect GFP fluorescence. The tissue was blocked for 1 h using 4% milk, TST (4% nonfat powdered milk dissolved in 10mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) and then incubated overnight with α-Pax6 antibody diluted 1:1000 in 4% milk, TST. After several washes in TST, the slides were incubated for 30 min with biotinylated secondary antibody (Jackson) at 1:100 dilution followed by Cy3-conjugated Streptavidin (Jackson) at 1:200 dilution for 15 min. Specific signal was detected by either standard fluorescence microscopy or laser scanning confocal microscopy.

**In situ hybridization**

Whole-mount mRNA in situ hybridization was performed as described (Hargrave and Koopman 2000). Sense and antisense digoxigenin-labeled RNA probes were prepared from a Bg/II digest of the pMPX2-1 Pax6 cDNA clone (gift of T. Glaser, Univ. Michigan) using a DIG RNA labeling kit (Roche). Hybridization and stringent posthybridization wash steps were
performed at 70°C. After color development, embryos were destained for up to 4 h in 1% Triton X-100/PBS and then postfixed in 4% paraformaldehyde/PBS at 4°C.

**Western analysis**

Western analysis was performed following standard protocols (Harlow and Lane 1988; Sambrook 2001). Protein extracts were prepared from the heads or dissected eyes of both transgenic and non-transgenic E10.5 mouse embryos. Blots were probed using $\alpha$-GFP (A-11122, Molecular Probes) at a 1:2500 dilution, $\alpha$-Pax6 (against the C-terminus) at a 1:1000 dilution, or serum 11 (against the paired domain) (Carriere et al., 1993) at a 1:200 dilution. Bands were visualized by chemiluminescence (ECL, Pierce Co.).

**RNA analysis**

Total RNA from mouse embryonic tissue was prepared from tissue in TRIzol reagent (GibcoBRL). RNA was reverse-transcribed for 1 h at 42°C (SuperscriptII; GibcoBRL). To assess transcript initiation in the eyes of wild-type embryos, reverse transcription was performed using a primer complementary to the mouse Pax6 sequence around the stop codon. 5' RACE (Frohman 1990) was performed using primers directed to the homeodomain within exon 8. PCR products were cloned, sequenced, and compared with genomic DNA. We recovered 11, 20, and 16 RACE clones corresponding to transcripts that initiated from $P_0$, $P_1$, and $P_{\alpha}$, respectively. No clones were recovered that corresponded to transcripts that initiated from $P_4$. 
Results

The *Pax6–Elp4* locus is directly comparable between humans and mice

To construct a BAC transgene that contained all known cis-regulatory elements implicated in controlling Pax6 expression in the eye as well as remote 3' elements located in the DRR, it was necessary to first compare the Pax6 locus between mice and humans. Whereas several eye-specific regulatory elements 5' and internal to *Pax6* have been identified in mouse (Williams, Altmann et al. 1998; Kammandel, Chowdhury et al. 1999; Xu, Zhang et al. 1999), the locations of 3' regulatory elements, including the minimal extent of the DRR, have been defined using the human *PAX6* locus (Kleinjan, Seawright et al. 2001; Griffin, Kleinjan et al. 2002). Therefore, to provide a framework for our BAC studies, we determined the organization of the *Pax6* locus in mouse and compared it to human. Scale diagrams of human and mouse *Pax6* gene are presented in Figs. 2.1A and B.

In both mice and humans, the *Pax6* gene has 16 exons distributed over the ~30 kb region; in addition to exons 0 through 13, there are also exons *alpha* and 5a (Glaser, Walton et al. 1992; St-Onge, Sosa-Pineda et al. 1997; Williams, Altmann et al. 1998; Kammandel, Chowdhury et al. 1999; Plaza, Saule et al. 1999; Xu, Zhang et al. 1999) (see also, Fig. 2.10A). Transcription of *Pax6* initiates from four promoters, here designated as P₀, P₁, Pₘₐₜₐₓ, and P₄ (Figs. 2.1A, B) (Kammandel et al., 1999; Kleinjan et al., 2004; Plaza et al., 1999; Xu and Saunders, 1997; Xu et al., 1999). P₀ and P₁ are located 5' to exons 0 and 1, respectively. Pₘₐₜₐₓ is located 5' to the *alpha* exon, which is located between exon 4 and 5. P₄ is located in the intron between exon 7 and 8. P₀ and P₁ constitute the major promoters and initiate expression in most cell types that express Pax6 (Anderson et al., 2002; Kammandel et al., 1999; Plaza et al., 1999; Xu and Saunders, 1997; Xu et al., 1999). Modular *cis* regulatory elements controlling *Pax6* transcription in the eye have been
defined within a ~25 kb 5' region of mouse Pax6. Specific enhancers for the retina, lens, and cornea have been identified 5' to P₀, Pα, and P₄ (Kammandel et al., 1999; Kleinjan et al., 2004; Williams et al., 1998; Xu et al., 1999). The location of the surface ectodermal (“se”) enhancer, which is the 5' most eye enhancer and regulates lens and cornea expression (Kammandel et al., 1999; Williams et al., 1998; Xu et al., 1999), is indicated for both human and mouse (Figs. 2.1A and B). In humans, additional eye enhancers have been defined in two 3' distal regions, the C1170/Box 123 and DRR, which are located ~76 kb and >124 kb downstream from Pax6 exon 13, respectively (Fig. 2.1A) (Griffin et al., 2002; Kleinjan et al., 2001). A conserved, differentially methylated CpG island is located 5' to P₀ in both human and mouse (Figs. 2.1A, B). Because the methylation state of this island correlates with Pax6 transcriptional activity (Lauderdale and Glaser, unpublished) and is located upstream of all known Pax6 regulatory elements, we have tentatively designated this CpG island as the 5'-most extent of the Pax6 locus.

Another gene, ELP4, is located 3' to Pax6 and is arranged in antisense orientation (Figs. 1A, B) (Kleinjan et al., 2001, 2002). ELP4 encodes a subunit of the RNA polymerase II elongator Holoenzyme (Winkler, Petrakis et al. 2001). ELP4 has 10 exons with exon 10 located adjacent to Pax6 exon 13 (Fig. 2.1). Whereas in humans, ELP4 exons are distributed over a ~277 kb region, in mice, they are distributed over an ~202 kb region (compare Figs. 2.1A to B). Although ELP4 is disrupted by aniridia-associated rearrangements 3' to PAX6 (e.g. “SGL” and “SIMO” in Fig. 2.1A) (Kleinjan et al., 2001, 2002; Lauderdale et al., 2000), it is not thought to be causal for aniridia (Kleinjan et al., 2002). Because 3' Pax6 regulatory elements are located within ELP4 introns, we include it here to provide reference points between the mouse and human genomes.
Although the Pax6–Elp4 genes are more compact in mice (compare Fig. 2.1A with B), the structures of the Pax6 and Elp4 genes are highly conserved between mice and humans.

**Defining the location the Pax6 DRR**

The DRR is genetically defined for human PAX6 as the ~75 kb genomic region between the SIMO breakpoint and the telomeric end of human YAC Y593, which is near ELP4 exon 4 (Fig. 2.1A). Although elements controlling expression in the lens and retina (“l” and “r” in Fig. 2.1A) have been defined by classic transgenic assay at the PAX6-proximal end of the DRR (Kleinjan et al., 2001), it is not known if these elements are sufficient for DRR eye activity. To narrow the region likely to functionally constitute the DRR, we took a comparative genomics approach to identify evolutionarily conserved non-coding sequences within the genomic region extending from SIMO to the telomeric end-point of Y593 (Fig. 2.1A). Fig. 2.1C shows the results of a comparison between humans, mouse and chicken Pax6 genomic DNA sequences using the multi-LAGAN computer program (Brudno, Do et al. 2003) and displayed using the VISTA computer program (Dubchak, Brudno et al. 2000; Frazer, Pachter et al. 2004). This analysis revealed 14 regions of single-copy, noncoding genomic DNA sequence with >75% nucleotide identity shared between human, mouse, and chicken. The first of these regions corresponded with the position of the SIMO breakpoint (“SIMO” in Fig. 2.1C), which had been previously shown by cross-species Southern analysis to be conserved between mammals and birds (Gessler and Bruns 1989). The next eleven regions were located between the position of the SIMO breakpoint and ELP4 exon 7 (peaks 2–12 in Fig. 2.1C). The last two regions were located between ELP4 exon 4 and 7 (data not shown). We extended these analyses using genomic sequences from pig (Sus scrofa), opossum (Didelphis virginiana and Monodelphis domestica),
platypus (Ornithorhynchus anatinus), and zebrafish (Danio rerio). We found that regions 1–12 were similarly conserved in these mammals and regions 2,4,5, and 7 had >75% sequence conservation with the zebrafish Pax6a locus on linkage group 25 (data not shown). This high-density cluster of evolutionarily conserved, single-copy, non-coding sequences suggested that clusters of functionally important regulatory elements are located between SIMO and ELP4 exon 7.

Identification of BACs likely to recapitulate Pax6 expression in the eye

To test if the mouse genomic region extending from the differentially methylated CpG island 5' of P0 through Elp4 exon 7 was sufficient to drive transgene expression in the Pax6 expression pattern, we isolated two mouse genomic DNA BACs, BAC 432m12 and BAC 293d08, that each contains the Pax6 transcript unit and sequences homologous to SIMO. The end points of each BAC were determined by BAC end sequencing and alignment to mouse genomic DNA (Fig. 2.1B). BAC 432m12 is 189 kb in length and extends from 51.4 kb 5' of Pax6 exon 0 to just distal to Elp4 exon 8. BAC 293d08 is 160 kb in length and extends from 12 kb 5' of Pax6 exon 0 to within 0.6 kb of Elp4 exon 7 and contains the twelve conserved, non-coding sequences identified above. To verify that the BACs contain contiguous genomic DNA that spans the Pax6 locus, internal structures were determined by restriction or “fingerprint” analysis (Gong et al., 2003) using BamHI or EcoRI digests. The patterns and sizes of the restriction fragments for each BAC were compared in ethidium-stained gels. Selected fragments were compared to the endogenous Pax6 locus using digests of genomic DNA prepared from 129/SvJ mice in Southern blots. With the exception of BAC end fragments, restriction fragment sizes were comparable both between BACs and also with the endogenous locus (data not shown). Thus, BACs 432m12 and 293d08 are intact Pax6 genomic clones. Because BAC 293d08 includes the 5' differentially
methylated CpG island plus the DRR region between SIMO and *Elp4* exon 7, we chose this BAC to use as a transgene in mice.

**Generation and molecular analysis of transgenic mice**

To facilitate expression analysis and remove Pax6 function from the BAC transgene, we inserted an enhanced green fluorescent protein reporter cassette (EGFP pA) in-frame with the initiator ATG of *Pax6* exon 4 (Figs. 2.2A, B) using the prophage BAC modification system (Lee et al., 2001; Yu et al., 2000). Because *P₀* and *P₁* constitute the major promoters and initiate expression in most, if not all, cell types that express Pax6 (Anderson et al., 2002; Kammandel et al., 1999; Plaza et al., 1999; Xu and Saunders, 1997; Xu et al., 1999), placement of the EGFP reporter cassette in exon 4 was expected to prevent translation of Pax6 from the majority of transcripts initiating from the BAC. This was necessary because *Pax6* transcripts initiating from the unmodified BAC would increase the amount of Pax6 protein in the mouse, which would be expected to affect the development of the eye as was observed for mice harboring the human Y593 transgene (Schedl, Ross et al. 1996). We targeted exon 4 such that the remaining 7 bp of exon 4 and the first 8 bp of the following intron (CAGAACA/gtaagtgt) were deleted. No other intronic sequences were affected. Restriction fingerprint and sequence analyses revealed that the EGFP pA reporter cassette was correctly inserted into *Pax6* exon 4 of BAC 293d08 and that no other changes to the BAC were made (Figs. 2.2B, C).

Three independent lines of transgenic mice (lines 3, 14, and 15) were generated by pronuclear injection of closed circular BAC DNA into mouse oocytes (Copeland et al., 2001; Gong et al., 2002, 2003). Hybridization with an intron 3 probe (Fig. 2.3A) as well as PCR analysis with BAC vector arm primers (data not shown) confirmed that the BACs were intact in
the three lines of transgenic mice. Transgene copy number was determined by densitometric analysis of EcoRI fragments detected in a Southern blot (Fig. 2.3A). Because the probe detected both an endogenous Pax6 genomic fragment and a fragment from the BAC transgene, the endogenous fragment was used as an internal normalization standard for two gene copies. Mouse line 3 harbors 10 ± 1 copies, line 14 harbors 1 copy, and line 15 harbors 8 ± 1 copies of the transgene (n = 3 independent blots).

Although the placement of the EGFP reporter cassette in exon 4 was expected to prevent translation of Pax6 from the majority of transcripts initiating from the BAC, Pax6 isoforms could potentially be translated from transcripts initiating from either the P<sub>alpha</sub> or P<sub>4</sub> promoters, or from alternatively spliced P<sub>0</sub>-and P<sub>1</sub>-initiated transcripts that lack exon 4, which have been recently described (Gorlov, Saunders et al. 2002). Because eye development is a very sensitive change in Pax6 dosage, we tested by Western analysis if the BAC transgene acted as a significant source of Pax6 protein. First, we tested if the BAC produced an EGFP-Pax6 fusion protein by probing protein extracts prepared from the heads of either wild-type embryos or BAC-transgenic embryos from line 3 or 15. Both Pax6 protein and transgene EGFP were detected by specific antisera. No EGFP-Pax6 fusion protein was detected in embryos harboring the BAC transgene by either the α-GFP or the α-Pax6 (Mastick et al., 1997) antibodies (Fig. 2.3B), indicating that there was no read-through of P0- or P1-initiated transcripts. In fact, comparable results between wild-type and transgenic embryos were obtained using the α-Pax6 antisera, indicating that multiple copies of the BAC transgene did not significantly affect Pax6 protein levels in wild-type embryos harboring the transgene. To directly test if the BAC was a source of Pax6 protein, we probed extracts prepared from the heads of Sey<sup>Neu</sup> mutant embryos harboring 8 copies of the BAC transgene. The Sey<sup>Neu</sup> allele encodes a nonfunctional protein that lacks a key C-terminal
domain (Hill et al., 1991) that is normally recognized by the α-Pax6 antisera used (Fig. 2.3B). Therefore, any Pax6 protein detectable in extracts prepared from BAC-transgenic $^{\text{Neu}}_{\text{Sey}}$ homozygous mutant embryos would have originated from the BAC. Since no Pax6 protein was detected, we conclude that the $Pax6$ BAC 293d08-E4-EGFPpA transgene is not a significant source of Pax6 protein in the developing embryo. However, there may be subdomains of expression not detectable in whole-head extracts.

The BAC transgene expresses EGFP in a Pax6 expression pattern in most tissues

We examined transgenic animals for EGFP fluorescence at different stages of development and also in the adult brain (Fig. 2.4 and data not shown). Comparisons of EGFP fluorescence between live and fixed embryos revealed identical patterns of expression; however, the background fluorescence was higher in the fixed tissues. Although lines 3, 14, and 15 all exhibited EGFP expression patterns identical to Pax6 (Figs. 2.4D, E, G), lines 3 and 15 have the brightest fluorescence that exactly correlates with transgene copy number. In lines 3 and 15, EGFP fluorescence was first detected in the presumptive forebrain at E8.0, and by E8.5, robust EGFP expression was observed in the presumptive forebrain, hindbrain, and anterior spinal cord adjacent to somites (Fig. 2.4B). Within the forebrain, EGFP was strongly expressed in the optic pit (Fig. 4B”). No EGFP fluorescence was observed in E7.5 embryos (Fig. 2.4A). This expression pattern is directly comparable to Pax6 mRNA in situ hybridization (Fig. 2.4C) (Grindley et al., 1995; Walther and Gruss, 1991).

However, some differences between EGFP and Pax6 expression were observed at E9.5. Whereas a Pax6 pattern of EGFP expression was observed in the cerebral vesicles, eye, nasal pit, hindbrain, spinal cord, and endocrine pancreas (Figs. 2.4D–G), only a partial pattern was
observed in the diencephalon. Although Pax6 mRNA is strongly expressed in the dorsal diencephalon at E10.5 (Fig. 2.8A), EGFP was only expressed in two patches of cells: one located in the ventral thalamus (vt) and the other in the ventrocaudal pretectum (Figs. 2.4D', G). With the exception of the diencephalon, a Pax6 pattern of EGFP expression was observed in the embryo at both E12.5 (Fig. 2.4H) and E15 (Fig. 2.4I). At E15, strong EGFP expression was observed in the cerebral vesicles, olfactory bulb, and eye (Fig. 2.4I). EGFP continued to be expressed in the eye and cortex of postnatal and adult mice (Fig. 2.4J, data not shown). These results suggest that BAC 293d08 is sufficient to recapitulate the Pax6 expression pattern in all regions of the developing mouse, except the diencephalon.

**BAC 293d08 faithfully replicates Pax6 expression in the eye**

Our analysis of transgene expression in whole embryos revealed that the BAC transgene was expressed in the developing eye during oculogenesis. We further examined EGFP expression in cryosections cut through the eye at different stages of development to verify at the cellular level its faithfulness to the endogenous Pax6 pattern. Sections through E10.5 optic vesicles revealed EGFP expression in the neuroepithelial cells of the optic vesicle and the overlying surface ectoderm. EGFP is expressed in the neural retina, RPE, lens, and presumptive cornea identically to Pax6 protein (Figs. 2.5A, D). Within the developing retina, both retinal ganglion cells (RGCs) and amacrine cells express EGFP (Fig. 2.5D, data not shown). EGFP expression in the RGC axons is evident in the optic nerve (Fig. 2.5D).

To directly compare cells expressing EGFP with those expressing Pax6, sections were immunolabeled in red for Pax6 using the α-Pax6 antisera described above. Pax6 protein is nuclear, but EGFP is predominantly cytoplasmic. Consequently, at high magnification, double-
labeled cells contained red nuclei surrounded by green cytoplasm. Comparison of Pax6 expression (Figs. 2.5B, E) with EGFP (Figs. 2.5A, D) revealed coincident expression in all eye tissues at both E10.5 (Fig. 2.5C) and at E13.5 (Fig. 2.5F).

We next directly compared cells expressing EGFP with those expressing Pax6 in sections cut through the adult eye (Fig. 2.6). Just as in the developing eye, comparison of Pax6 expression with EGFP revealed coincident expression in the retina, including the RGCs, amacrine cells, and Müller glia (Fig. 2.6B). EGFP expression in the Müller glia is evident in the radial processes spanning the outer nuclear layer (ONL). Coincident expression of Pax6 and EGFP was also observed in the corneal epithelium, iris, and ciliary body (Fig. 2.6A). The green fluorescence of the lens at this time is due to EGFP protein that has been incorporated into this structure. Thus, transgene expression in both the developing and adult eye replicates Pax6 expression with high fidelity.

**Transgene expression in the developing olfactory epithelium and bulb**

Pax6 also plays a key role in the development of the sense of smell (Martha, Strong et al. 1995; Sisodiya, Free et al. 2001). To determine if the transgene was expressed in a Pax6 pattern within the developing olfactory system, we examined EGFP expression in cryosections cut through the developing olfactory epithelium and bulb. Only cells coexpressing EGFP and Pax6 were observed in the olfactory placode at E9.5 and in the olfactory epithelium at E10.5 and E13.5 (Fig. 2.7A, data not shown).

The olfactory bulb (OB) develops at the rostral end of the telencephalon and functions as the primary processing center for odor information. Over 80% of the cells produced in the olfactory bulb primordium between E11 and E13 migrate radially out of the ventricular zone and
differentiate as mitral cells (Hinds 1968). These cells, along with the tufted cells, which are born between E13 and E18 (Hinds, 1968), establish the lateral olfactory tract, which is located at the lateral edge of the telencephalon (Cajal 1890; Valverde-García 1965; Hinds 1972; Lopez-Mascaraque and de Castro 2002). At E13.5, three groups of Pax6-expressing cells were observed in the olfactory bulb region (Fig. 2.7E). One population was located in the ventricular zone of the OB, and two populations were located in the mantle region. The cells in the mantle were in two stripes, one located medially and the other located laterally (MS and LS, respectively; Fig. 2.7E). Cells in the lateral stripe were in the region of the lateral olfactory tract (LOT).

EGFP was also expressed in these regions and in axons in the LOT (Fig. 2.7D). The LOT was visualized by double-labeling with βIII tubulin (data not shown), which labels axons in the CNS (Moody, Quigg et al. 1989; Lee, Rebhun et al. 1990; Lee, Tuttle et al. 1990; Moody, Miller et al. 1996). Direct comparison of Pax6 and EGFP expression revealed that most, if not all, Pax6-expressing cells within the olfactory bulb and medial stripe coexpressed EGFP (Fig. 2.7F). However, both Pax6+/EGFP+ and Pax6+/EGFP− cells were located in the lateral stripe. Whereas most, if not all, cells near the olfactory bulb primordium coexpressed Pax6 and EGFP, cells located in the region denoted by the arrowhead in Fig. 7E expressed Pax6 but not EGFP. These results suggest that different mechanisms control Pax6 expression in the cells in the ventricular zone of the olfactory bulb relative to the population of cells in the mantle region. Alternatively, because cells are known to migrate into the embryonic olfactory bulb from other brain regions (Wichterle et al., 2001), it is possible that the Pax6+/EGFP− cells in mantle originated in a different part of the forebrain.
Transgene expression in the diencephalon exhibits a partial Pax6 pattern

In all three lines of transgenic mice, expression of the BAC 293d08 transgene did not match the pattern expected for Pax6 in the diencephalon. By E10.5, the diencephalon can be divided into three regions: the presumptive dorsal thalamus (dt), the presumptive ventral thalamus (vt), and the pretectum (pt) (Puelles and Rubenstein 1993; Stoykova, Fritsch et al. 1996). At this time, Pax6 mRNA is expressed by a broad swath of cells located in the dorsal diencephalon and ventrally in a large patch of cells in the ventral thalamus (Fig. 2.8A). Pax6 is also expressed in a ventrocaudal group of cells located at the pretectum/mesencephalon (pt/mes) boundary (Mastick et al., 1997). In contrast with Pax6 mRNA, EGFP is only expressed by a patch of cells located in the ventral thalamus and by a ventrocaudal cluster of cells in the pretectum (Fig. 2.8B). No EGFP expression was detected in the dorsal diencephalon (Fig. 2.8B). This finding was unexpected because this BAC contains all previously identified diencephalic regulatory elements, which have been shown by transgenic assays to drive reporter gene expression in the dorsal diencephalon (Griffin et al., 2002; Kleinjan et al., 2001, 2004).

To examine if the transgene was expressed in a partial Pax6 pattern within the diencephalon, we directly compared EGFP and Pax6 expression in cryosections cut through the diencephalons of transgenic embryos at E10.5 (Figs. 8D–F) or E13.5(Figs. 8G–I; J–L). Cells expressing Pax6 were visualized using anti-Pax6 antibody (Figs. 2.8E, H, K). At E10.5, Pax6 is broadly expressed in the diencephalon and sharply terminates at the pt/mes boundary (arrow, Fig. 2.8E). In contrast, EGFP expression in the caudal diencephalon is limited to a stripe at the pt/mes boundary (arrow, Fig. 2.8D). Direct comparison of the two expression patterns revealed that the EGFP-expressing cells represent a subset of the Pax6-expressing cells in the pretectum (Fig. 2.8F). However, in the ventral thalamus, most if not all cells that expressed Pax6 also expressed EGFP (data not shown).
These results suggest that Pax6 expression in the diencephalon is minimally controlled by at least three different regulatory mechanisms: one that controls expression in the ventral thalamus and two that control expression in dorsal thalamus and pretectum. The mechanism controlling Pax6 expression at the pt/mes boundary is likely to be involved in establishing this boundary and is different than the one controlling Pax6 expression in the rest of the dorsal diencephalon.

At E13.5, Pax6 is broadly expressed within the thalamic region of the diencephalon (Fig. 2.8H). Cells expressing Pax6 are located in the ventricular zone and mantle of the thalamus (Fig. 2.8H), and scattered Pax6-expressing cells are located in the hypothalamus (arrowheads, Fig. 2.8H). Expression in the ventricular zone includes both the dorsal and ventral aspects of the thalamus and ends within the hypothalamic sulcus, which demarcates the thalamic/hypothalamic boundary (Fig. 2.8H). Within the ventral thalamus, Pax6-positive nuclei are located in a broad swath extending medially from the ventricular zone laterally to the superficial edge of the thalamus (Fig. 2.8H). A similar pattern of expression is observed for EGFP in the ventral thalamus (Fig. 2.8G); however, EGFP is not expressed by cells in the ventricular zone adjacent to the interventricular foramen (ivf) nor in the scattered Pax6+ cells in the hypothalamus (Fig. 2.8I). Although not all Pax6-expressing cells coexpressed EGFP, direct comparison revealed that most if not all EGFP-expressing cells in the thalamus expressed Pax6 (Figs. 2.8I, L). Axon tracts comprised of EGFP-expressing axons are visible in this section (Figs. 2.8G, I). These data demonstrate that the majority of Pax6-expressing cells in the thalamus also express the BAC transgene. However, the lack of transgene expression in some Pax6+ cells in the thalamus is consistent with our observations in earlier embryos and reinforces the idea that Pax6 expression in the diencephalon is controlled by different mechanisms in different cell populations.
The BAC transgene and Pax6 are coexpressed by most cells in the embryo

Our analysis of transgene expression in whole embryos revealed that, with the exception of the developing diencephalon, the transgene was expressed in a Pax6-like spatiotemporal expression pattern in the embryo. However, our analysis of the developing olfactory bulb revealed a population of Pax6+ cells that, like in the diencephalon, did not coexpress EGFP, and this discrepancy was not visible in whole mounts. To investigate if the transgene was only expressed in a subset of Pax6+ cells in other regions of the embryo, we directly compared transgene expression with Pax6 in cryosections through the cerebral vesicles (Fig. 2.5C), hindbrain (Fig. 2.9C), spinal cord (data not shown), and pancreas (Fig. 2.9F) of E10.5 embryos. In these regions, all cells expressing Pax6 also express EGFP. In the hindbrain, cells coexpressing EGFP and Pax6 were observed both in horizontal sections along the rostral–caudal axis (Figs. 9A–C) and as well as along the dorsoventral axis (data not shown). Similar results were obtained for the spinal cord (data not shown). Cells coexpressing Pax6 and EGFP were also observed in Rathke's pouch at E13.5 (data not shown). These results suggest that, with the exception of the diencephalon and olfactory bulb, this BAC transgene replicates Pax6 expression.

BAC transgene expression is subject to Pax6 autoregulation

Pax6 transcription in the lens and olfactory placodes and in a subset of domains within the diencephalon requires Pax6 (Grindley, Davidson et al. 1995; Aota, Nakajima et al. 2003; Kleinjan, Seawright et al. 2004). Does Pax6 similarly regulate BAC transgene expression? To test this idea, mice heterozygous for both the transgene and Pax6SeyNeu allele were produced and mated together to generate Pax6 mutant homozygotes carrying one allele of the transgene (Tg: Pax6−/−). In these embryos, EGFP expression was observed in those regions where Pax6
expression is not subject to autoregulation, including the cerebral vesicles, optic vesicles, ventral thalamus, hindbrain, spinal cord, and pancreas (Fig. 2.10D, data not shown). Conversely, no EGFP expression was observed in those regions that require Pax6 for expression, including the lens surface ectoderm (Fig. 2.10E), nasal placodes (data not shown), and the ventrocaudal cluster of cells at the pt/mes region (compare Figs. 2.10A with D). These results demonstrate that BAC transgene expression in these domains is crossregulated by Pax6 comparable to autoregulation at the endogenous gene locus.

**P\textsubscript{alpha}/P\textsubscript{4} transcripts are expressed in subsets of cells that also express P\textsubscript{0}/P\textsubscript{1} transcripts**

The presence within the diencephalon and olfactory bulb of Pax6\textsuperscript{+/−}/EGFP\textsuperscript{−} cells could be explained if the BAC transgene lacked critical regulatory elements required for expression within these cells. Alternatively, these cells might express Pax6 using only the P\textsubscript{alpha} and/or P\textsubscript{4} promoters. Because the EGFP reporter would not be contained in transcripts initiating from these promoters in the transgene, it was possible that Pax6 transcription from the BAC transgene was regulated correctly in these Pax6\textsuperscript{+/−}/EGFP\textsuperscript{−} cells but was not detectable using EGFP. To assess if specific cell types within the embryo expressed Pax6 using the P\textsubscript{alpha} and/or P\textsubscript{4} promoters, cryosections from Tg; Pax6\textsuperscript{−/−} embryos at E10.5 and E13.5 were stained with α-Pax6 antibody (Figs. 2.10F, L). Because this antibody recognizes the C-terminal amino acids of Pax6 (Fig. 2.3B), it cannot detect the truncated form of Pax6 protein expressed by the Sey\textsuperscript{Neu} allele (Fig. 2.10I). Therefore, any Pax6 protein detected by the antisera in these mutant embryos would have originated from P\textsubscript{alpha}- or P\textsubscript{4}-initiated transcripts from the BAC transgene. A survey of sections cut from E10.5 and E13.5 Tg;Pax6\textsuperscript{−/−} embryos revealed Pax6 immunoreactivity in both the optic vesicle and rostral telencephalon (Figs. 2.10F, L); however, these cells also expressed EGFP
No Pax6 immunoreactivity was observed in sections through the cerebral vesicles, hindbrain, spinal cord, pancreas, or diencephalon at either E10.5 or E13.5 (Fig. 2.10F, data not shown). These results suggest that $P_{\alpha}/P_4$-initiated transcripts are only expressed in subsets of cells within the embryo, and, in these cells, Pax6 is also expressed using the P0 and/or P1 promoters. Therefore, the BAC transgene appears to be missing a critical regulatory element required for Pax6 expression in a subset of cells in diencephalon and also in the olfactory bulb.

**Identification of a paired-less Pax6 isoform in the eye and olfactory bulb**

The finding of Pax6 immunoreactivity, using the C-terminal Pax6 antisera, in the optic vesicles of $Tg;Pax6^{-/-}$ embryos at E10.5 (Fig. 2.10F) suggested that $P_{\alpha}$-and/or $P_4$-initiated transcripts are expressed in the developing eye. Although the optic vesicles in Pax6 homozygous mutant embryos are arrested in their development, they are regionalized along their proximodistal extent as optic stalk, neural retina, and RPE (Baumer, Marquardt et al. 2003). In contrast with EGFP expression, which was more strongly expressed in distal and medial regions and less strongly expressed in the proximal region (Fig. 2.10E), Pax6 immunoreactivity was strongest in the medial region and exhibited a sharp border of expression proximally (arrow, Fig. 2.10F). These cells coexpress Chx10 (data not shown), a marker for presumptive neural retina (Baumer et al., 2003; Burmeister et al., 1996). Pax6 immunoreactivity was also observed in the optic vesicles of $Tg;Pax6^{-/-}$ embryos at E13.5 (data not shown). These data suggested that the $P_{\alpha}$ and/or $P_4$ promoters were active in the developing retina.

To assess if the $P_{\alpha}$ and/or $P_4$ promoters were utilized in wild-type non-transgenic embryos, a 5' RACE analysis was performed using pooled eyes dissected from wild-type embryos between E10.5 and E13.5. Forty-seven (47) independent transcripts were analyzed.
Although P<sub>4</sub>-initiated transcripts were not detected in this assay, transcripts initiating from P<sub>0</sub>, P<sub>1</sub>, and P<sub>alpha</sub> were identified (Fig. 2.11A). Of these, 31 transcripts initiated from either the P<sub>0</sub> or P<sub>1</sub> promoters and 16 transcripts initiated from the P<sub>alpha</sub> promoter. All of the transcripts that initiated from the P0 and P1 promoters included exon 4. These results demonstrate the P<sub>alpha</sub> promoter is active during eye development and suggest that P<sub>alpha</sub>-initiated transcripts represent a source of Pax6 protein in the developing eye.

Whereas P<sub>0</sub>- and P<sub>1</sub>-initiated transcripts encode for a Pax6 protein that includes the paired domain, homeodomain, and PST domain (Carriere et al., 1993; Glaser et al., 1992; Martin et al., 1992; Ton et al., 1991; Walther and Gruss, 1991), P<sub>alpha</sub>-initiated transcripts are predicted to encode for a Pax6 protein that includes the homeodomain and PST domain but lacks the paired domain (Carriere et al., 1993). To directly test if a paired-less isoform of Pax6 (Pax6<sub>ΔPD</sub>) was made in the developing eye, protein extracts were prepared from eyes dissected from wildtype embryos at E10.5 and then probed with two different Pax6 antisera in Western blots, one directed towards the C-terminal portion of Pax6 and the other directed towards the paired domain (Carriere et al., 1993). Consistent with the results from the RACE experiments, the antisera directed towards the C-terminal portion of Pax6 detected a 32/33 kDa isoform of Pax6 in eye extracts but not whole-head extracts (Fig. 2.11B). This isoform was not detected using the paired domain antisera (serum 11), indicating that the 32/33 kDa isoform lacks the paired domain (Fig. 2.11C). In contrast, the 46 and 48 kDa Pax6 isoforms, which are translated from P<sub>0</sub>- and P<sub>1</sub>-initiated transcripts, were detected by both antisera in extracts from both the head and eye. The amount of 32/33 kDa isoform in eye extracts was small compared to the 46/48 kDa isoforms (Fig. 2.11B). To test if the transgene exhibited similar expression, extracts were prepared from the eyes and heads of transgenic embryos (line 3 heterozygotes) and probed for Pax6. As for wild-
type embryos, the antisera directed towards the C-terminal portion of Pax6 detected a 32/33 kDa isoform that was greatly enriched in eye extracts (Fig. 2.11B), and this isoform was not detected using serum 11 (Fig. 2.11C). Because these embryos harbored 10 copies of the transgene, the amount of the 32/33 kDa isoform expressed in the eye was greater than for non-transgenic embryos (Fig. 2.11B). Together, these data demonstrate, for the first time, that a paired-less isoform of Pax6 is expressed in the developing mouse eye.

Pax6 immunoreactivity using the C-terminal antisera was also detected in the rostral telencephalon of \( Tg;Pax6^{-/-} \) embryos (Fig. 2.10L). Although Pax6 mutant mice and rats lack an olfactory bulb (Grindley, Hargett et al. 1997; Dellovade, Pfaff et al. 1998), they develop an olfactory-bulb-like structure (OBLS) in the rostrolateral telencephalon (Jimenez, Garcia et al. 2000). The OBLS forms as the result of abnormal migration of mitral cell progenitors (Nomura and Osumi 2004). In both wild-type and Pax6 mutant rodents, the mitral cell progenitors originate from the rostral part of the telencephalon; however, in \( Pax6^{-/-} \) rodents, the mitral cells then mismigrate caudally toward the lateral part of the telencephalon forming the OBLS (Nomura and Osumi, 2004). The OBLS can be morphologically identified in sections cut through the rostral telencephalon of E13.5 Pax6 mutant mice (Figs. 2.10K, L) (see also, Jimenez et al., 2000; Lopez-Mascaraque et al., 1998). In these sections, EGFP is expressed by cells in the rostral telencephalon and strongly expressed by cells in the OBLS (Fig. 2.10K). Additionally, scattered EGFP-expressing cells are observed in the mantle throughout the rostral telencephalon (arrowheads, Fig. 2.10K). Similar to the developing optic vesicle, Pax6 immunoreactivity using the C-terminal antisera is observed in a subset of the EGFP-expressing cells (Fig. 2.10L). At this time, no immunoreactivity is observed in the EGFP-expressing cells in the ventricular region of the rostral telencephalon. However, strong immunoreactivity is observed in the OBLS and in
scattered cells in the mantle region (Fig. 2.10L). These Pax6-expressing cells also express EGFP. These data indicate that cells in the olfactory bulb express Pax6ΔPD.

Over-expression of Pax6ΔPD causes eye defects

To test if Pax6ΔPD had a role in eye development, we examined the eye phenotype in adult wild-type animals harboring different copy numbers of the BAC transgene. Whereas all animals carrying 8 (Tg^{15}) or 10 (Tg^{3}) copies of the transgene had morphologically normal eyes (Fig. 12B), all animals carrying 16 (Tg^{15}/Tg^{15}) or 18 (Tg^{3}, Tg^{15}) copies of the transgene had severely microphthalmic eyes (Fig. 2.12C). Histological analysis revealed that these eyes were highly disorganized and lacked lenses (Fig. 2.12F). The retina, though abnormal in appearance throughout much of the eye, had a ganglion cell layer, inner nuclear layer, and an outer nuclear layer (Fig. 2.12F, data not shown). The vitreous body was filled with mesenchymal cells. Because an identical phenotype was obtained in animal compound for lines 3 and 15, the eye defects cannot be explained by homozygosity at the transgene loci. These results demonstrate that Pax6ΔPD acts on eye development in a dosage-dependent fashion.

Previous studies have shown that the human \textit{PAX6} locus rescues the \textit{Sey} phenotype (Schedl et al., 1996). To test if Pax6ΔPD could rescue the \textit{Sey} phenotype, we examined the eyes of \textit{Pax6}^{+/−} mice carrying 8 or 10 copies of the transgene. Rather than exhibiting a rescue of the \textit{Sey} phenotype, all of these animals had severely microphthalmic eyes (Fig. 2.12E). Although the eye phenotype in \textit{Pax6}^{+/−} animals can be variable, all transgenic \textit{Sey} animals exhibited comparable microphthalmic phenotypes, and these phenotypes were always distinguishable from the \textit{Sey} phenotype. Histological analysis revealed that the microphthalmic eyes from transgenic \textit{Sey} mice were comparable to those obtained from wild-type mice carrying 16–18 copies of the
transgene. Together, these results demonstrate that Pax6ΔPD has a role in eye development and indicate that this isoform functions differently than the paired-containing isoform.

**Discussion**

We report here our analysis of mice transgenic for a 160 kb mouse *Pax6* BAC transgene. Because transcriptional control of *Pax6* is complex, the use of large genomic constructs avoids potential problems associated with conventional transgenic reporter gene approaches. Although conventional transgenes are effective in determining if a given sequence is sufficient for expression in a particular expression pattern or subdomain of a more complex expression pattern, this approach fails to take into account cooperative interactions between elements that would normally exist within the genome. Consequently, potential discrepancies can arise between the activities ascribed to a regulatory element based on its behavior in a classical transgenic assay and its normal function within the genome. Such discrepancies have been observed for cis regulatory elements within the HoxD complex (Beckers and Duboule 1998) and were suggested for *Pax6*, especially with respect to the elements controlling *Pax6* transcription in the eye and diencephalon.

**Long-range interacting elements control of *Pax6* transcription in the eye**

Normal *Pax6* transcription in the eye appears to require the coordinated actions of multiple cis regulatory elements, which are widely spaced within the *Pax6* locus. Although specific enhancers for the lens and cornea are located 5' to *P_0* and enhancers for the retina are located 5' to *P_*alpha* within intron 7, and 3' to *Pax6* in the C1170/Box 123 region (Griffin et al., 2002; Kammandel et al., 1999; Kleinjan et al., 2004; Williams et al., 1998; Xu et al., 1999),
activation of these elements requires the activity of elements located within the DRR. This idea first came from aniridia cases in which chromosomal rearrangements disrupted the region 3' to \textit{PAX6} but spared the \textit{PAX6} transcription unit (Fantes et al., 1995; Fukushima et al., 1993; Kleinjan et al., 2001; Lauderdale et al., 2000; Simola et al., 1983; Ton et al., 1991). Because the clinical manifestations of these patients are indistinguishable from aniridia patients with \textit{PAX6} coding deletions or truncations, it was suggested that cis regulatory elements located 3' to \textit{PAX6} are required for normal expression (Lauderdale et al., 2000). To better define distal 3' \textit{Pax6} enhancer elements, \textit{Pax6} BAC-transgenic mice were generated. Because the BAC 293d08 transgene faithfully replicated Pax6 expression in the eye, it is likely that this BAC contains all of the regulatory elements required for normal Pax6 eye expression. When compared to the results from mice harboring YACs Y589 and Y593 (Kleinjan et al., 2001; Schedl et al., 1996), this indicates that regulatory elements located between SIMO and \textit{Elp4} exon 7 are necessary for normal Pax6 transcription in the eye and implies that these elements interact with those elsewhere within the \textit{Pax6} transcript unit, including those 5' to \textit{P0}, \textit{Palpha} within intron 7, and in C1170/Box 123. Thus, Pax6 transcription in the eye appears to require the coordinated interactions of widely spaced regulatory elements, perhaps through combinatorial binding to the same regulatory proteins or complexes, as might be expected to occur via a looping model of long-range gene control (Bulger and Groudine 1999; Engel and Tanimoto 2000; Tolhuis, Palstra et al. 2002; de Laat and Grosveld 2003).

**Interacting elements control Pax6 transcription in the diencephalon**

In contrast with the eye, our BAC transgene only partially recapitulates Pax6 expression
in the diencephalon. Whereas Pax6 is normally expressed in the dorsal diencephalon, ventral thalamus, and at the pt/mes boundary, transgene expression was observed in the ventral thalamus and in a cluster of cells at the pt/mes boundary. This result was unexpected because this BAC contains all previously described diencephalic regulatory elements (Griffin et al., 2002; Kleinjan et al., 2001, 2004). In fact, this BAC contains diencephalic enhancers located between P₀ and P₁, (Kammandel et al., 1999; Xu et al., 1999), within intron 7 (Griffin et al., 2002; Kleinjan et al., 2004), C1170/Box 123 (Griffin et al., 2002; Kleinjan et al., 2004), and in the DRR (Kleinjan et al., 2001, 2004). This result suggests that the diencephalic enhancers controlling expression in the dorsal diencephalon are interacting with an as yet unidentified regulatory element that is missing from BAC 293d08.

Although it is possible that this missing diencephalic element is located 5' to Pax6, we propose that it is located remotely 3' to Pax6 and functionally divides the DRR into two regions. This proposal stems from comparison of the results of our BAC study to those reported for mice harboring the human PAX6 YACs (Kleinjan et al., 2001; Schedl et al., 1996). Whereas the YAC Y593 transgene was expressed in a Pax6 expression pattern in the diencephalon of transgenic mice, the Y589 transgene was not (Kleinjan et al., 2001). Because these two YACs are comparable 5' to Pax6 but differ significantly at their 3' ends, this difference in expression suggests that a diencephalic regulatory element, missing from both BAC 293d08 and YAC Y589, is located within the DRR. Comparison of the location of the 3' ends of YAC Y593 and BAC 293d08 (Fig. 2.1B) suggests that this element is located between Elp4 exon 4 and 7. Thus, it is likely that the DRR can be functionally divided into an eye regulatory region (between SIMO and Elp4 exon 7) and a diencephalic regulatory region (between Elp4 exon 7 and 4). We are currently testing this hypothesis. Alternatively, cells in the dorsal diencephalon could have
expressed transcripts that initiated only from the $P_{alpha}$ or $P_4$ promoters, which would not be reported by our BAC transgene. Although $P_{alpha}$-initiated transcripts were identified, cells transcribing Pax6 from this promoter also utilized the $P_0$ and $P_1$ promoters. Thus, Pax6 transcription in diencephalon, like the eye, appears to require the coordinated interactions of multiple cis regulatory elements widely distributed across the Pax6 locus.

**Paired-less Pax6 in the developing eye and olfactory bulb**

We provide evidence that a paired-less Pax6 isoform is expressed in the developing eye and olfactory bulb in mammals. Pax6 transcripts that initiate from $P_0$ and $P_1$ typically encode a Pax6 protein that contains a paired domain, paired-type homeodomain, and a PST-rich C-terminal region. However, a paired-less isoform of Pax6 has been identified in both birds (Carriere et al., 1993) and nematodes (Chisholm and Horvitz 1995; Zhang and Emmons 1995). The possibility that a truncated Pax6 isoform was also expressed in mammals was raised by the identification of the $P_{alpha}$ (Kammandel et al., 1999) and $P_4$ (Kleinjan et al., 2004) Pax6 promoters. Because this isoform is expected to be identical to the full-length Pax6 protein in all respects except the paired domain, it is not possible to distinguish between paired-less and paired-containing Pax6 proteins in wild-type embryos using immunohistochemistry. However, by taking advantage of the attributes of our BAC transgene and the SeyNeu allele, we were able to show that Pax6ΔPD is expressed in the optic vesicle and rostral telencephalon in Pax6 mutant embryos (Figs. 2.10F, L). Although both the eye and olfactory bulb develop abnormally in Pax6 mutant mice, these structures share some similarities with wild-type embryos (Jimenez, Garcia et al. 2000; Baumer, Marquardt et al. 2003). Therefore, the presence in $Tg:Pax6^{−/−}$ embryos of Pax6 immunoreactivity in the retinal portion of the optic vesicle and also in the OBLS suggests
that a truncated form of Pax6 is expressed in the developing eye and olfactory bulb of wild-type embryos. Consistent with this, we showed by Western blot that a paired-less isoform of Pax6 is present in protein extracts prepared from the developing eyes of wild-type embryos. Although transcripts initiating from either $P_{\alpha}$ or $P_4$ are predicted to encode for a Pax6$\Delta$PD protein, transcript analysis indicates that the $P_{\alpha}$ promoter is likely to be the primary source of this isoform in the developing eye (Fig. 2.11A). Both the transcript analysis and Western blot indicate that Pax6$\Delta$PD is expressed at lower levels than full-length Pax6. Although this low-level expression may indicate that the truncated protein is functionally unimportant and its expression an evolutionary vestige, alternatively, the paired-less isoform may have an important functional role, which necessitates tight control over its expression. This latter possibility raises some interesting questions about Pax6 function during oculogenesis and olfactory development.

Pax6 is involved in multiple aspects of eye development and affects cell proliferation, cell fate, and morphogenesis. 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 and indicate that different aspects of Pax6 gene function are mediated by the different isoforms of the Pax6 protein (Favor, Peters et al. 2001; Dominguez, Ferres-Marco et al. 2004; Haubst, Berger et al. 2004; Azuma, Tadokoro et al. 2005). Whereas Pax6 is necessary (Hogan, Horsburgh et al. 1986; Hill, Favor et al. 1991; Glaser, Jepeal et al. 1994) and sufficient (Chow, Altmann et al. 1999; Onuma, Takahashi et al. 2002) for eye development in vertebrates, Pax6(5a) appears to have a more restricted role. Mutations affecting the Pax6(5a) protein are found in patients with foveal hypoplasia (Azuma, Nishina et al. 1996; Azuma, Yamaguchi et al. 1999), and mice that lack exon 5a exhibit iris hypoplasia (Singh, Mishra et al. 2002). In patients, a splice acceptor mutation that increases the relative abundance
of the Pax6(5a) isoform causes distinct abnormalities in the iris (Epstein, Glaser et al. 1994). However, Pax6 and Pax6 (5a) both promote proliferation in the retina (Azuma et al., 2005; Marquardt et al., 2001). Thus, Pax6(5a) appears to act in a spatially restricted manner to control the development of specific structures within the eye. Could the same be true of Pax6∆PD?

We show that Pax6∆PD also has a role in eye development. Over-expression of Pax6∆PD in cells that normally express this isoform results in severe microphthalmia in both Pax6+/+ and Pax6+/− animals. Although the cellular defects underlying this phenotype remain to be determined, histological analysis revealed an abnormally shaped retina within the eye, mesenchymal cells in the vitreous body, and agenesis of the lens. The retina itself was layered and contained RGCs and photoreceptors. The lack of a lens was striking and suggests that Pax6∆PD has a role in lens development. Consistent with this idea, the HD of Pax6 appears to mediate the early role of Pax6 in the surface ectoderm for lens formation. Mice homozygous for a missense mutation in the homeodomain develop a rudimentary retinal neuroepithelium and the surface ectoderm begins to invaginate, however, no lens develops (Favor, Peters et al. 2001). Comparison of the eye phenotypes that result from over-expression of Pax6∆PD with those from over-expression of Pax6 suggests that these two isoforms function differently. Although mice carrying 5–10 copies of the human PAX6 locus on a wild-type background also exhibit a microphthalmic phenotype, their eyes had a distinct retina, iris, cornea, and lens (Schedl et al., 1996). More dramatically, the human PAX6 locus rescues the Small eye mutant phenotype (Schedl et al., 1996). Taken together, these results indicate that Pax6∆PD interacts antagonistically with Pax6 during eye development. Consistent with this interpretation, genetic analysis of the Pax6 locus in C. elegans indicates that Pax6 isoforms can interact additively, synergistically, or antagonistically, depending on the cellular context(Cinar and Chisholm 2004).
Together, these results suggest that Pax6ΔPD is likely to act as a dominant-negative isoform in eye development.

Acknowledgement

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References


Fig. 2.1. Identification of mouse BACs likely to recapitulate Pax6 expression in the eye.

(A) Physical map of human chromosome 11p13 showing the locations of *PAX6* and *ELP4*, which are in an antisense orientation relative to each other. Bent arrows denote the locations of transcript initiation and direction of transcription; black boxes denote exons. The breakpoints of the two distal-most aniridia-associated rearrangements, which disrupt 11p13 but spare the *PAX6* transcription unit, are indicated by “SGL” and “SIMO” (Fantes et al., 1995; Fukushima et al., 1993; Simola et al., 1983). The locations of eye-specific regulatory elements defined by transgenic reporter assays are indicated for the surface ectoderm (se), neural retina (nr), lens (l), and a retina (r) enhancer that drives expression in both the neural retina and RPE (Griffin et al., 2002; Kammandel et al., 1999; Kleinjan et al., 2001, 2004; Williams et al., 1998; Xu et al., 1999). “Box 123” denotes the location of the C1170/Box 123 regulatory region, which includes a regulatory element for the neural retina (Griffin et al., 2002). The 3" ends of YACs Y593 and Y589 are indicated. In transgenic mice, Y593, but not Y589, rescues the mouse *Sey* phenotype and homozygous *Sey* lethality (Kleinjan et al., 2001; Schedl et al., 1996). Together, these data define the DRR for the human *PAX6* locus. (B) Physical map of the *Pax6/Elp4* locus on mouse chromosome 2. A differentially methylated CpG island ( Nurs) is located upstream of the conserved surface ectoderm regulatory element in humans and mice. Comparison of homologous sequences between humans and mice revealed that the murine *Pax6/Elp4* locus is more compact. Scale bar in panel A applies to panel B. Mouse BACs 432m12 and 293d08 were identified by library screen. BAC 432m12 is 189 kb in length and extends from 51.4 kb 5" of *Pax6* exon 0 to just distal to *Elp4* exon 8. BAC 293d08 is 160 kb in length and extends from 12 kb 5" of *Pax6* exon 0 to within 0.6 kb of *Elp4* exon 7. (C) MLAGAN alignments of genomic sequence spanning SIMO to *ELP4* exon 7 for human (Z83308, AC131571, Z86001, AL136384), mouse (Al512589, AL590380), and chicken (AC14046, AC141860). Colored peaks represent regions of sequence conservation above 50% over at least 40 bp. Noncoding sequences are shaded in pink; *ELP4* exons are shaded in blue. Repetitive sequences are marked as follows: LINE (red), SINE (green), LTRs (pink), RNA (purple), DNA (orange), Other (yellow). Twelve single-copy, noncoding regions (arrows) exhibited N75% sequence identity between human, mouse, and chicken. Of these, four (*) were also conserved with the zebrafish *Pax6a* locus (AL929172). This high density of conserved sequences suggests that this region has regulatory activity.
Fig. 2.2. Construction of the *Pax6* BAC 293d08-E4-EGFP pA reporter transgene

(A) Mouse BAC 293d08 was modified by insertion of a green fluorescent protein reporter cassette (EGFP pA) in-frame with the initiator ATG of *Pax6* in exon 4 using a prophage BAC modification system (Lee et al., 2001; Yu et al., 2000). The EGFP pA reporter cassette was inserted such that the remaining 7 bp of exon 4 and the first 8 bp of the intron were deleted (CAGAACA/gtaagtgt). The targeting construct contained the EGFP-SV40 pA reporter cassette and the kanamycin resistance (Kan\(^R\)) gene flanked by FRT sequences. After homologous recombination, the kanamycin cassette was subsequently flipped out by induction of flipase. Bacteria harboring the modified BAC were identified by resistance to chloramphenicol (Cm\(^R\)) and sensitivity to kanamycin (Kan\(^S\)).

(B) Sequence of the targeted *Pax6* exon 4 in BAC 293d08-E4-EGFP pA. The EGFP coding sequence is in-frame with the initiator ATG of *Pax6*; the sequence 5' of EGFP is that of *Pax6*. (C) *BamHI* restriction analyses of the targeted BAC. The EGFP pA cassette introduced a novel *BamHI* site (B*) 3' to the endogenous *BamHI* site of *Pax6* exon 4, which resulted in a novel 970 bp fragment in the targeted BAC (arrowhead) and a 100 bp increase in the adjacent 3' 10.2 kb *BamHI* fragment (location denoted by arrow). No other changes were observed. B, *BamHI* site.
Fig. 2.3. Molecular analysis of the BAC 293d08-E4-EGFP pA transgene in mice

(A) Southern analysis of EcoRI-digested genomic DNA using a 32P-labeled probe. A 4kb EcoRI fragment is detected in mice harboring the transgene (lines 3, 14 and 15), but not in wild-type (WT) animals. Densitometric analysis, using the 3 kb EcoRI fragment from the endogenous gene as a reference, indicates that mouse line 3 harbors 10 ± 1 copies, line 14 harbors 1 copy, and line 15 harbors 8 ± 1 copies of the transgene. (B) Western analysis using protein extracted from the heads of E10.5 embryos. Antibodies to EGFP detected a single band of 27 kDa in animals harboring the transgene, suggesting that the EGFP reporter was not expressed as a fusion protein. Although the band in the WT;Tg lane appears slightly retarded relative to the control, this slight shift was not observed in other blots. Blots probed with α-Pax6 revealed comparable Pax6 expression between wild-type and wild-type transgenic embryos; no Pax6 protein was detected in extracts prepared from homozygous Sey mutant embryos harboring the transgene. Together, these data suggest that the Pax6 BAC 293d08-E4-EGFP pA transgene does not significantly alter the levels of Pax6 protein in the developing embryo. Both the α-GFP and α-Pax6 panels are from the same blot. The location of the SeyNea truncation and the epitope recognized by α-Pax6 are indicated on the schematic of the Pax6 protein.
A. Southern analysis

B. Western analysis
**Fig. 2.4.** The BAC transgene exhibits Pax6-like expression in most tissues. Unless indicated, data are from line 15. (A, A') Bright field and fluorescent images, respectively, of an E7.5 embryo (side-view). No EGFP expression was observed at E7.5. EGFP fluorescence is first detected at E8.0. (B, B') E8.5. EGFP fluorescence is observed in the neural ectoderm of the presumptive forebrain, optic pit (op), presumptive hindbrain (hb), and presumptive spinal cord adjacent to somites (s). (B") Front view, 11 somites, embryo different from the one shown in panel B. EGFP is expressed in both the neural ectoderm (ne) and optic pit. (C) At E8.5, Pax6 is expressed in the neural ectoderm of the presumptive forebrain, optic pit, presumptive hindbrain, and presumptive spinal cord as visualized by whole-mount mRNA in situ hybridization. Arrowheads denote expression in hindbrain and spinal cord. (D–H) A Pax6-like pattern of EGFP fluorescence is observed in the cerebral vesicles (cv), developing eye (ey) and nose (np), hindbrain (hb), and spinal cord (sc). In contrast, only a partial Pax6 pattern is observed in the diencephalon. EGFP expression is observed in the ventral thalamus (vt) and in a ventral cluster of cells (vc) at the pretectal/mesencephalic (pt/mes) boundary. No EGFP expression is observed in dorsal thalamus (dt). (F) EGFP expressed in the pancreas (p). (G) Although EGFP expression is less robust in line 14, which has one copy of the transgene, the pattern of expression is similar to lines 3 and 15, which harbor 10 and 8 copies of the transgene, respectively. (I) E15. Strong EGFP expression is observed in the cerebral vesicles, olfactory bulb (ob), and eye. (J) EGFP expression is observed in the cortex (ctx), olfactory bulb, and eyes of a 3-day-old pup. The expression in the brain is visible through the skull.
**Fig. 2.5.** BAC 293d08 faithfully replicates Pax6 expression in the developing eye
Sections cut through the eye of transgenic mouse embryos at E10.5 (A–C) or E13.5 (D–F). (A, D) EGFP fluorescence. (B, E) Endogenous Pax6 visualized by indirect immunofluorescence (red). (C, F) Merged images. EGFP and Pax6 are coexpressed in the cells in the cerebral vesicles, neural retina (nr), retinal pigmented epithelium (rpe), developing lens, and presumptive cornea. At E13.5, EGFP expression in retinal ganglion cells axons is visible within the optic nerve (on).
E10.5

A  di  rpe  lp  nr  cv

E13.5

D  c  rpe  nr

EGFP

Pax6

B  di  lp  cv

E  lens  on

Merge

C  di  lp  cv

F  lens  on
**Fig. 2.6.** BAC 293d08 replicates Pax6 expression in the adult eye. Sections cut through the eye of a transgenic mouse. (A) Anterior eye. EGFP and Pax6 are both expressed by cells in the corneal epithelium, iris, ciliary body, and retina. The EGFP fluorescence in the lens is the result of this protein being incorporated into this structure. (B) In the retina, EGFP and Pax6 are both expressed by cells in the ganglion cell layer (GCL) and a subset of cells in the inner nuclear layer (INL), including amacrine cells and Müller glia. EGFP expression in Müller glia is evident in the radial processes spanning the outer nuclear layer (ONL).
**Fig. 2.7.** Transgene expression in the developing olfactory system

Sections cut through the olfactory epithelium (A–C) or olfactory bulb primordium (D–F) of transgenic mouse embryos at E13.5. (A, D) EGFP fluorescence. (B, E) Endogenous Pax6 visualized by indirect immunofluorescence (red). (C, F) Merged images. (A–C) Cells in the olfactory epithelium coexpress EGFP and Pax6. (E) Within the rostral telencephalon, cells expressing Pax6 are located in the ventricular zone of the olfactory bulb, in a medial stripe (ms) near the midline, and in a lateral stripe (ls) in the region of the developing lateral olfactory tract (LOT). (D) EGFP-expressing cells are also in the ventricular zone of the olfactory bulb and in both the medial and lateral stripes. EGFP-positive axons are observed within the LOT. Arrows denote the lateral extent of the LOT. (F) Whereas cells coexpressing EGFP and Pax6 are located in the ventricular zone of the olfactory bulb, more Pax6-expressing cells than EGFP-expressing cells are observed in the lateral stripe. This suggests that cells within the olfactory bulb primordium differentially regulate Pax6 expression.
Fig. 2.8. Transgene expression in the diencephalon exhibits a partial Pax6 pattern

(A) At E10.5, Pax6 is broadly expressed in the diencephalon as visualized by wholemount mRNA in situ hybridization. The diencephalon can be divided into three regions, the pretectum (pt), dorsal thalamus (dt), and ventral thalamus (vt). Pax6 mRNA is expressed in all three of these regions and in a cluster of cells (vc) in the ventral pretectum at the pt/mes boundary. (B) In contrast, EGFP expression in the diencephalon is restricted to the ventral thalamus and the ventral cluster in the pretectum, suggesting that the transgene is expressed in only a subset of Pax6 expression domains. (D–F) Section through the diencephalon at E10.5; the plane of section shown in panel B. (E) Pax6, visualized by immunofluorescence, is expressed throughout the diencephalon and terminates in a sharp border at the pt/mes boundary. (D) EGFP is expressed by a narrow stripe of cells at the pt/mes boundary. (F) Direct comparison reveals that the cells at the pt/mes boundary coexpress EGFP and Pax6. (G–L) Section through the diencephalon at E13.5; plane of section shown in panel C. These panels are photomosaics, which show the thalamic region. (H) Pax6, visualized by immunofluorescence, is expressed by cells in the ventricular zone (vz) and mantle region of the thalamus (th). Scattered Pax6-expressing cells are also located in the hypothalamus (arrowheads). (G) EGFP is expressed by cells in the mantle region and the ventricular zone of the ventral thalamus, adjacent to the hypothalamic sulcus (sul). No EGFP-expressing cells were observed in the ventricular zone adjacent to the interventricular foramen (ivf). EGFP-positive axons were observed along the lateral edge of the thalamus and in bundles adjacent to the ivf. (I) Direct comparison revealed that all cells that expressed EGFP also expressed Pax6. In contrast, no EGFP was detected in the Pax6-expressing cells located in the ventricular zone adjacent to the ivf or in the scattered cells in the hypothalamus. (J–L) Cells in the mantle region of the ventral thalamus coexpress EGFP and Pax6 (arrowheads indicate a few such cells). These high-magnification views were taken from a section adjacent to the one shown in panels G–I. Rostral is down in all sections; mge, medial ganglionic eminence.
**Fig. 2.9.** The BAC transgene and Pax6 are coexpressed by cells in hindbrain and pancreas. Horizontal sections cut through the hindbrain (A–C) or pancreas (D–F) of transgenic mouse embryos at E10.5. (A, D) EGFP fluorescence. (B, E) Endogenous Pax6 visualized by indirect immunofluorescence (red). (C, F) Merged images. Cells in the hindbrain and pancreas coexpress EGFP and Pax6. otv, otic vesicles.
Fig. 2.10. BAC transgene expression in Pax6 mutant embryos. To test if the BAC transgene was subject to regulation by Pax6 and also to identify cells potentially utilizing the $P_{\alpha}$ and/or $P_4$ promoters, mice compound heterozygous for the transgene and the $Pax6^{\text{SeyNeu}}$ allele ($Tg;Pax6^{+/-}$) were produced and mated together to generate Pax6 homozygotes carrying the transgene ($Tg;Pax6^{-/-}$). (A–C) E10.5 wild-type littermate carrying the transgene. (D–F) E10.5 Pax6 homozygote carrying the transgene. (G–I) E10.5 non-transgenic Pax6 homozygote littermate. (J–L) E13.5 Pax6 homozygote carrying the transgene. (A, D, G, J) EGFP expression visualized in whole embryos; these panels were generated by an overlay of EGFP fluorescence on the corresponding bright field images. (C, F, I, L) Pax6 expression visualized by immunofluorescence using an $\alpha$-Pax6 antisera directed towards the C-terminal 17 amino acids of mouse Pax6 (Mastick et al., 1997). (D, E) As in wildtype embryos, EGFP is expressed in the optic vesicle, cerebral vesicle, presumptive ventral thalamus (vt), hindbrain, spinal cord, and pancreas in $Pax6^{-/-}$ embryos at E10.5. In contrast with wild-type littermates, transgene expression is not observed in the nasal pits, lens surface ectoderm (se), or in the ventral cluster (vc) of cells in pt (compare D with A, E with B), consistent with Pax6 autoregulation in these tissues. (F, I) Interestingly, a Pax6 protein was detected in the optic vesicle of $Pax6^{\text{SeyNeu}}$ mutant embryos harboring the transgene at E10.5 but not in non-transgenic $Pax6^{\text{SeyNeu}}$ mutant littermates (compare F with I). This protein is likely due to transcripts initiating from the $P_{\alpha}$, and/or $P_4$ promoter, located in the BAC transgene. These transcripts are predicted to encode a Pax6 isoform comprised of the homeodomain and C-terminal domain, but lacking the paired domain. (K, L) Both EGFP and Pax6 immunoreactivities were detected in the olfactory-bulb-like structure (OBLS) and in scattered cells (arrowheads) in the rostral telencephalon of E13.5 $Pax6^{\text{SeyNeu}}$ mutant embryos harboring the transgene.
Fig. 2.11. Paired-less Pax6 is expressed in the developing eye. (A) 5' RACE analysis using pooled eyes dissected from wild-type embryos between E10.5 and E13.5. Forty-seven independent clones were analyzed. Only transcripts initiating from P₀, P₁, and P_\text{alpha} were detected. All P₀- or P₁-initiated transcripts spliced into exon 4. Two P₁-initiated transcripts used an alternative splice site for exon 2. About half P_\text{alpha}-initiated transcripts included exon 5a, and four had an alternative splice site for the alpha exon (*). 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. (B–C) Western analysis of extracts prepared from eyes (ey) or heads (hd) dissected from wild-type (WT) or line 3 embryos (Tg) at E10.5. (B) The arrow denotes a 32/33 kDa protein detected using the α-Pax6 antisera (Mastick et al., 1997), which is directed towards the C-terminal 17 amino acids of Pax6. This protein was enriched in the eye but not head extracts of both wild-type and transgenic embryos. (C) The 32/33 kDa protein was not detected using serum 11 (Carriere et al., 1993), which is directed towards the paired domain of Pax6. Both α-Pax6 and serum 11 detected the 46/48 kDa bands, which are full-length Pax6. Because in wildtype embryos the amount of the 32/33 kDa isoform was low relative to the 46 and 48 kDa isoforms, it was necessary to develop the Western to the point where the separation between the 46 and 48 kDa isoforms in all lanes and the 32 and 33 kDa isoform in the eye extracts prepared from transgenic embryos became obscured. Together, these data demonstrate that a paired-less isoform of Pax6 is expressed in the developing mouse eye.
A. Pax6 promoter usage in early mouse optic cup

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**Fig. 2.12.** Over-expression of Pax6ΔPD causes a microphthalmic phenotype in wild-type mice and enhances the *Sey* phenotype. (A–C, F) *Pax6<sup>+/+</sup>* animals. (D–E) *Pax6<sup>+</sup>/−* animals. (B–C) Whereas wild-type mice (*Pax6<sup>+/+</sup>*) carrying 8 (Tg<sup>15</sup>) or 10 (Tg<sup>3</sup>) copies of the BAC transgene have morphologically normal eyes (B), all animals carrying 16 (Tg<sup>15</sup>/Tg<sup>15</sup>) or 18 (Tg<sup>3</sup>; Tg<sup>15</sup>) copies exhibit severely microphthalmic eyes (C). (E) A similar phenotype is observed in *Pax6<sup>+</sup>/−* mice carrying either 8 (Tg<sup>15</sup>) or 10 (Tg<sup>3</sup>) copies of the BAC transgene. (F) Section cut through the eye of a mouse carrying 18 copies of the BAC transgene on a wildtype background. Nuclei are labeled in blue with Hoechst. Cells expressing Pax6 are labeled in red, and those expressing EGFP are green. The microphthalmic eye is disorganized and lacks a lens. Although abnormal in appearance, the retina has a ganglion cell layer, inner nuclear layer, and outer nuclear layer. The vitreous body is filled with mesenchymal cells. Because the BAC transgene is a source of Pax6ΔPD, these data demonstrate that Pax6ΔPD acts on eye development in a dosage-dependent fashion and suggests that this isoform interacts antagonistically with paired-containing Pax6 isoforms.
Chapter 3

Paired-less Pax6 has a role in eye development\(^1\)

\(^1\) Jiha Kim, James D. Lauderdale. To be submitted to *Development.*
Abstract

Pax6 is required for development of the eye. Recent studies have shown that Pax6 affects cell proliferation, cell fate decisions, and patterning. These diverse functions appear to be mediated by different isoforms of the Pax6 protein. Three isoforms of the Pax6 protein have been reported in mammals. The canonical form contains the paired domain (PD), paired-type homeodomain (HD), and a transactivation domain (PST). This isoform is expressed in most cells that express Pax6. The second isoform designated as Pax6 (5a) contains a 14 amino acid insertion in the N-terminal subunit of the PD. The third isoform lacks the paired-domain (Pax6∆PD); however, little is known about where this isoform is expressed or its normal function in vivo. To investigate the role of Pax6∆PD, I used a Pax6 BAC transgene that I developed to over-express this isoform in those cells that normally express it. Over-expression of Pax6∆PD causes a severe microphthalmic phenotype in wild-type mice carrying 18 copies of the transgene. A similar phenotype was observed in Small eye mice carrying 8-10 copies of the transgene. Microphthalmic eyes appear to lack lenses and exhibit several morphological defects. Analysis of lens development in these mice revealed that the lens degenerated via apoptotic cell death between E12 and E12.75. These results suggest a role for Pax6∆PD in eye development, which may be different than that ascribed to canonical Pax6. In addition to biological function of paired-less isoform in eye development, I present the evidence that Pax6∆PD is dominantly expressed within the distal tip of neuroretina during early eye development and amacrine cell population in the adult eye.
Introduction

The transcription factor Pax6, a member of the paired-family of transcription factors, is necessary for the correct development of the eyes, olfactory system, brain, spinal cord, pituitary and pancreas (Walther and Gruss 1991; Callaerts, Halder et al. 1997; St-Onge, Sosa-Pineda et al. 1997; Kioussi, O'Connell et al. 1999). Heterozygous loss-of-function mutations in Pax6 result in aniridia in humans and Small eye phenotype in rodents (Hogan, Horsburgh et al. 1986; Hill, Favor et al. 1991; Glaser, Walton et al. 1992; Jordan, Hanson et al. 1992; Fujiwara, Uchida et al. 1994). Aniridia is a semidominant disorder in which development of the iris, lens, cornea and retina is disturbed (Hanson, Seawright et al. 1993; Grindley, Davidson et al. 1995). A complex spatio-temporal expression pattern has been reported for this important developmental regulator in various animal phyla (Callaerts, Halder et al. 1997; Kammermeier, Leemans et al. 2001). Consistent with a role in eye development, Pax6 is expressed in the developing optic vesicle, lens, cornea, iris, and neural retina in vertebrates (Walther, Gruss et al. 1991; Grindley, Davidson et al. 1995; Koroma, Yang et al. 1997). Pax6 has been known to play several key roles including cell proliferation, differentiation, and cell fate decision in different tissues at different time point. However, the mechanism by which Pax6 performs these multitasks are not clear. One possible explanation is that different Pax6 isoforms are functionally interacting with each other to achieve diverse functions or performing different tasks independently. The Pax6 protein contains three distinct domains. It has two DNA binding domains, a paired domain (PD) at the N-terminus and a paired-like homeodomain (HD) in the middle, and a transactivation domain at the C-terminus (Walther and Gruss 1991; Epstein, Glaser et al. 1994). Two different isoforms, canonical Pax6 and Pax6 (5a) that contains a 14 amino acid insertion in the N-terminal subunit of the PD, have been implicated as major Pax6 proteins. Recently, a third isoform, a paired-less isoform of Pax6
has been identified in birds (Carriere, Plaza et al. 1993), nematodes (Chisholm and Horvitz 1995; Zhang and Emmons 1995) and mouse (Kim and Lauderdale 2006). In Pax6 (5a) isoform, additional 14 amino acid insertion in PD caused disruption of paired domain and alter its DNA binding properties. Therefore, different two isoforms possibly activate different sets of downstream target genes (Epstein, Glaser et al. 1994). However, little is known about the biological function and expression pattern of paired-less Pax6 isoform. Although Pax6\(\Delta PD\) has been identified first in quail, no biological function is known in quail. In C. elegans this isoform is required for specification of a peripheral sense-organ (Zhang and Emmons 1995) and known to interact with paired-containing Pax6 additively, synergistically, or antagonistically, depending on the cellular context (Cinar and Chisholm 2004). Even though biological function of Pax6\(\Delta PD\) is largely unknown, previous studies indicate that this isoform in bird (Carriere, Plaza et al. 1995), nematode (Zhang, Ferreira et al. 1998), and mouse (Kim and Lauderdale, unpublished) have similar biochemical characteristics.

In this study, I used a transgenic approach using Pax6 BAC that over-expresses Pax6\(\Delta PD\) but not the full length Pax6. Unlike conventional transgenic technique, Pax6 BAC expresses Pax6\(\Delta PD\) and also EGFP as a reporter for full length Pax6 at same time, under the native regulatory condition. Also, because Pax6 BAC contains the entire Pax6 transcription unit including the DRR, I can modify more than one locus within the BAC and use single transgenic BAC for multiple purpose. Double heterozygote transgenic mice that over-express Pax6\(\Delta PD\) exhibited severe microphthalmic phenotype as a result of over-expressing Pax6\(\Delta PD\) in the eye. Although the primary defects in microphthalmic eyes were degenerating lenses, Pax6\(\Delta PD\) expression was restricted to the distal neuroretina and amacrine cells. Lens degeneration was first evident around E12.5 when the lens vesicle is already formed and primary lens fibers are
initiated to elongate. Taken together, our data suggests that Pax6ΔPD is predominantly expressed at the distal tip of neuroretina where it is involved in a cell non-autonomous signaling pathway to help proper lens development.

**Material and Methods**

**BAC transgene construction**

Mouse *Pax6* BAC clone RPCI24 304E12 was modified by targeted insertion of an Enhanced Green Fluorescent Protein (EGFP) pA reporter cassette into *Pax6* exon 4 followed by targeted insertion of a Red Fluorescent Protein (DsRFP) pA reporter cassette into *Pax6* exon 8, using the prophage BAC modification system (Yu, Ellis et al. 2000; Lee, Yu et al. 2001). The EGFP FRT- kan-FRT targeting cassette was PCR-amplified from pCS2+MTeGFPFRT- kan-FRT using the *Pax6* forward targeting primer, E4RECF, 5'-AGCCCCGTATTGAGGCCCGGTG GGATCCGGAGGGCTGCCAACCAGCTCCAGCATGTTGAGCAAGGGCGAGGAG-3' and the *Pax6* reverse targeting primer, E4RECR, 5'-TAACCCACGGCCCGACGCCTCCAGCATGTTGAGCAAGGGCGAGGAG-3'. The DsRFP-FRT-kan-FRT targeting cassette was PCR-amplified from pCS2+DsRED-FRT-kan-FRT using the *Pax6* exon 8 forward targeting primer, E8RECF, 5'-AGAACACCCAATCCATCAGTTCTAACGGAGAAGACT CGGATGAAGCTCAGATGGCCTCCCTCCGGAGGACGTCA-3' and the *Pax6* reverse targeting primer, E8RECR, 5'-TATGCAAAAGGCCCTAGCTAAATTTAGGCCCTTTGTGTGCTTGTGG CAGATCAGTTATCTAGATCCGG-3'. Nucleotides in italics are homologous to *Pax6* sequences and those in roman are homologous to amplification cassette. SV40 late polyadenylation (polyA or pA) addition sequences are located immediately after both the EGFP and DsRFP 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 *Bam*HI/*Eco*RI 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`; Rupp, Snider et al. 1994; Turner and Weintraub 1994) to generate pCS2+DsRFP. Second, the FRT-kan-FRT *Sac*II fragment of pIGCN21 (gift of N. Copeland, NCI`; Lee, Yu et al. 2001) was inserted into the *Sac*II site of pCS2+DsRFP. BAC recombination was performed in two steps using modified protocol of Lee et al. (2001). First, the EGFP pA reporter cassette was inserted into *Pax6* exon4 and double-resistant colonies (Cm R Kan R) were assayed for homologous recombination as previously described. The kanamycin cassette was flipped out by induction of flipase, and the cells were screened for kanamycin sensitivity (Kan S). The DsRFP pA reporter cassette was then inserted into *Pax6* exon8 in the EGFP-modified BAC. Double-resistant colonies (Cm R Kan R) were assayed for homologous recombination by PCR using the following primers: mE8F1 (5'-GTTTCTAACTGTGCCGAGTCC-3'), mE9R1 (5'-CAAACACATCTGGATAATGGGTCC-3'). The kanamycin cassette was flipped out by limited induction of *flipase*, and the cells were screened for kanamycin sensitivity (Kan S). In these colonies, removal of the kanamycin cassette was verified by PCR using the E4F1, E4F2, E4R1, E4R2, mE8F1, mE9R1, RedF, and RedR primers. The overall structure of the doubly-modified BAC was examined by fingerprint analysis (Gong, Zheng et al. 2003) using *Bam*HI and southern blot analysis. Fingerprint for the modified BAC was 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 mice**

Transgenic mice carrying Pax6 BAC 293d08-EGFP pA transgene were generated and maintained as described (Kim and Lauderdale 2006). Small eye mice harboring the Pax6 BAC 293d08-EGFP pA (Tg^3;Pax6^+/−) transgene were generated by crossing an F1 founder male (line 3) with either Pax6^Sey-Neu or Pax6^Sey-Dey females. The founder Pax6^Sey-Neu mice for our colony were generously provided by Dr. Nadean Brown and are maintained in the albino FVB/N background. The founder Pax6^Sey-Dey mice were obtained from Jackson laboratory. Double heterozygote mice for line3 and line15 (Tg^3;Tg^15;Pax6^+/+) were generated by crossing F1 founder male (line 3) with F1 founder female (line15). The offspring phenotypically scored on the basis of their eye size and subsequently DNA analysis was used to determine the genotype and copy number of individual mice.

Doubly modified Pax6 BAC 304E12-EGFP/RFP was purified using NucleoBond® BAC Maxi Kit (Clontech) and linearized by PI-SceI (Spitz, Gonzalez et al. 2001). 160kb linearized BAC DNA was purified by Phenol/chloroform extraction followed by ethanol precipitation. Purified linear BAC DNA was dissolved in microinjection buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 30µM spermine, 70µM spermidine, 100 mM NaCl) and injected into mouse oocytes. Mice carrying the BAC transgene were genotyped using tail DNA, by southern blot analysis using either EGFP or DsRFP probe, and by PCR using primers, E4F1, E4F2, E4R1, E4R2, mE8F1, mE9R1, RedF, and RedR to detect the EGFP and/or DsRFP reporter cassette. Four transgenic founder mice (line 897,898,939, and 949) were generated from pronuclear injection.
F1 founders were generated by crossing a founder males (line 939) or founder females (lines 897, 898, and 942) to CD-1 mice (Charles River Laboratories). These lines are being maintained in the CD-1 background. Transgene copy number was determined by densitometric analysis of Southern blots performed on a G4 PowerMacintosh computer running OSX using ImageJ (v1.33, developed at the U.S. National Institutes of Health; available on the Internet at http://rsb.info.nih.gov/ij/index.html).

**Immunofluorescence labeling**

For cryosectioning, staged embryos were fixed with 4% Paraformaldehyde (1hr to overnight), cryoprotected in 5% and then 15% sucrose and embedded in gelatin. Sections for Brn3b antibody staining were fixed with 4% PFA for only 1hr and embedded in OCT (Optical Cutting Temperature, Tissue-Tek 4583). 12µm frozen sections were prepared. Sections were blocked for 1 hour in 4% milk/TST (10 mM Tris–HCl, pH 7.4; 150 mM NaCl; 0.1% Tween20). Overnight primary antibody incubation was performed at room temperature followed by secondary antibody incubation for 30 min. Primary antibody identities and dilutions are as follows: polyclonal rabbit anti-Pax6 (1:1000, Mastick et al., 1997), polyclonal rabbit anti-Prox1 (1:5000, Chemicon AB5475), polyclonal rabbit anti-histone H3(1:100, Abcam ab-5176), polyclonal goat anti-Brn3b(1:100, Santa Cruz sc-6026), rabbit anti-β–crystallin (1:400, Santa Cruz sc-22745), rabbit anti-αA crystallin(1:200, Santa Cruz sc-22389) and rabbit anti-cleaved PARP(1:100, Cell Signaling #9544). To amplify DsRFP signal, polyclonal rabbit anti-DsRed antibody (1:2000, Clontech) was used. After several washes in TST, the slides were incubated for 30 min with biotinylated secondary antibody (Jackson) at 1:100 dilution followed by Cy3-
conjugated Streptavidin (Jackson) at 1:200 dilution for 15 min. Specific signal was detected by either standard fluorescence microscopy or laser scanning confocal microscopy.

**Analysis of transgene expression in whole mouse embryos and adult eye ball**

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 CO₂, and the uteri were washed in ice-cold phosphate-buffered saline (PBS). The embryos were dissected free and placed in ice-cold PBS. Adult eye balls were dissected from 2-3 months old transgenic and wild type littermate and washed 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. All embryos and eye balls that were to be used for immunohistochemistry or histology were kept in fixative (4% paraformaldehyde/PBS) for up to 2 weeks at 4°C. Transgene expression was analyzed in wild-type embryos obtained by timed mating of transgenic males with non-transgenic CD-1 females (Charles River Laboratories). Non-transgenic littermates were used as controls.

**Histology**

For paraffin sections, dissected eye balls were fixed with 4% Paraformaldehyde at 4°C for overnight and perfused overnight in 5% sucrose in phosphate buffer (pH7.3) accompanied by several changes of solution. Following this, the embryos were then dehydrated in a graded series of 50, 75, 90, 96 and 100% ethanol, equilibrated with xylene, embedded in paraffin and sectioned at a thickness of 10 μm. Sections were stained with Mayer's hematoxylin (Sigma) and eosin solution after dewaxing with xylene, and mounted on slides.
Cell culture and Pax6 expression constructs

Pax6 cDNA clones corresponding to P₀ (I.M.A.G.E. Consortium Clone ID#4504106) and P_alpha-initiated transcript (I.M.A.G.E. Consortium Clone ID#4527976) were obtained from the I.M.A.G.E. Consortium [LLNL] EST project (Lennon et al., 1996). The cDNA derived from the P₀-transcripts did not contain exon5a. Both clones were in the pCMV-SPORT6 expression vector. pCS2⁺-EGFP construct was used to monitor transfected cells. NIH/3T3 mouse fibroblasts (gift from Dr. Tom Glazer) were used to express Pax6 isoforms and cultured in Dulbecco modified Eagle medium (DMEM)–supplemented with 10% calf serum, 20mM glutamine, 100units/ml penicillin and 100ug/ml streptomycin. Cells were plated at a density of 1-1.5×10⁶ cells per 100-mm-diameter dish and 1×10⁵ cells per well in 6 well plate 24h prior to transfection. Transfections were performed using Fugene 6 reagent (Roche) according to manufacturer recommendations. Cells were transfected with mixture of 1ug Pax6 expression construct and 1ug of pCS2⁺-EGFP per each well in 6 well plate and mixture of 10ug Pax6 expression construct and 1ug of pCS2⁺-EGFP per 100-mm-diameter dish. Transfected cells in 100-mm-diameter dish were used for protein extraction and cells in 6 well plates were used for immunofluorescent staining.

Protein extraction and Western blot

72hrs post transfection, cells were lysed to prepare protein extract for western blot. Cells were washed with PBS and lysed with lysis buffer (1% triton-X 100, 0.1% SDS, 0.5% deoxycholic acid, 20mM Tris-Cl pH 7.5, 10% glycerol, 0.5M EDTA, 100mM PMSF, and protease inhibitor cocktail) for 5min on ice. Lysed cells were collected into 1.5ml tube and incubated for 15min in ice with occasional vortexing. Cell debris was separated from total
protein solution by centrifugation at 13,000g for 20min at 4°C. Supernatant was stored at -20°C until used. Western analysis was performed following standard protocols (Harlow and Lane 1988; Sambrook 2001). Blots were probed using anti-Pax6 (against the C-terminus) at a 1:1000 dilution, or serum 11 (against the paired domain) (Carriere, Plaza et al. 1993) at a 1:400 dilution. Bands were visualized by chemiluminescence (ECL, Pierce Co.).

**Immunocytochemistry**

72hrs post transfection, cells in 6 well plates were washed three times with PBS and fixed with 3.7% Formaldehyde for 10min at room temperature. Fixed cells were washed three times with PBS and permeabilized with 0.2% TritonX-100 for 5min at room temperature. Cells were washed again and incubated with blocking solution (5% BSA) for at least 1hour at room temperature. Primary antibodies, a-Pax6 (C-terminal) at 1:1000 and serum11 at 1:400, were applied onto cells and incubated for over night at 4°C. After remove primary antibody, cells were washed and incubated with secondary antibody, biotin-conjugated anti-rabbit IgG(1:100), for 30 min followed by Cy3-conjugated streptavidin at a 1:200 dilution for 15min. Nuclei were labeled using Hoechst 33342(Sigma, B-2261). Specific signals were visualized using fluorescence microscopy.

**Results**

**Overexpression of Pax6ΔPD causes microphthalmic phenotype**

Recently, paired-less isoform of Pax6 (Pax6ΔPD) protein has been identified to be expressed within developing mouse eye and to have a role in eye development. As previously described (Kim and Lauderdale 2006), over-expression of Pax6ΔPD using \textit{Pax6BAC} 293d08
transgene (Fig. 3.1A-C) caused severe microphthalmic phenotype. In this study, I analyzed this microphthalmic phenotype in detail and also defined spatiotemporal expression pattern of Pax6ΔPD using dually modified Pax6BAC 304E12 transgenic mice.

To examine the phenotype and cellular structures of the microphthalmic eyes, adult eyes were dissected out and compared to that of wild-type mice. Size of microphthalmic eye was approximately one fourth of that of wild-type eyes (Fig. 3.3A, B). Histological analysis of the microphthalmic eye revealed that these eyes were highly disorganized and lacking lenses. H and E staining on the sections showed that only very small, lens-like structure remained (Fig. 3.3F). Although overall retina structures appeared to be highly disorganized as they folded toward inside, most of cell layers within the retina include ganglion cell layer, inner nuclear layer, and an outer nuclear layer were evident (Fig. 3.3F, I). Also development of other anterior structures including cornea, iris, and ciliary body were abnormal. The space that would normally have been the anterior chamber was filled with pigmented cells and entire retina seemed to be attached to cornea. Although there was very short and small iris structure that is Pax6 positive, its morphology was abnormal (Fig. 3.3F).

Previous studies have shown that the human PAX6 locus rescues the Sey phenotype (Schedl, Ross et al. 1996). To test if Pax6ΔPD could rescue the Sey phenotype, I examined the eyes of Pax6+/− mice carrying 8 or 10 copies of the transgene. Rather than exhibiting a rescue of the Sey phenotype, all of these animals had severely microphthalmic eyes (Fig. 3.2D, and 3.3D). Although the eye phenotype in Sey animals can be variable, all transgenic Sey animals exhibited comparable microphthalmic phenotypes, and these phenotypes were always distinguishable from the Sey phenotype (Fig. 3.2C). Histological analysis revealed that the microphthalmic eyes from
transgenic Sey mice (Fig. 3.3G, J) were comparable to those obtained from wild-type mice carrying 16–18 copies of the transgene (Fig. 3.3F,I).

However, Sey mice that I used in this study were Pax6 mutant mice that express C-terminally truncated Pax6 protein. Although this truncated protein is believed to be non-functional due to absence of transactivation domain (Hill, Favor et al. 1991), there is a possibility that truncated Pax6 protein may interact with over-expressed Pax6\(^{\Delta PD}\) and caused microphthalmic phenotype. Therefore I decided to confirm this experiment by using Pax6\(^{\text{Sey-Dey}}\) strain (Fig. 3.2E) that contains a large deletion on chromosome 2 including Pax6 locus (Theiler, Varnum et al. 1978; MT 1986; Glaser, Lane et al. 1990). As expected, Pax6\(^{\text{Sey-Dey}}\) containing transgene (Tg\(^3\); Pax6\(^{+/}\)) exhibited comparable microphthalmic phenotype (Fig. 3.2F) suggesting that this eye phenotype is caused by over-expression of Pax6\(^{\Delta PD}\) protein in animal. Together, these results demonstrate that Pax6\(^{\Delta PD}\) has a role in eye development and indicate that this isoform functions differently than the paired-containing isoform.

**Lenses degenerate during embryogenesis**

To understand the underlying molecular mechanism that causes these severe microphthalmic phenotypes, I examined series of double heterozygote (Tg\(^3\)Tg\(^{15}\); Pax6\(^{+/}\)) embryos for BAC transgene at different developmental time points (Fig. 3.4). Surprisingly, despite severe abnormality in lens development, lens vesicles appeared to form normally and no visible difference in retina development was observed until E12 (Compare Fig. 3.4E, F with Fig. 3.4M, N). However, dramatic change in size of lens vesicles was evident from E12.5 and lenses were rapidly degenerated from then. By E15, most of lens structures were degenerated and only small tissue connected to cornea was remained (Fig. 3.4O, P). In spite of rapid lens degeneration,
no obvious defect in the retina was detected at this stage. The fact that lens vesicles were initiated to form normally at early stage suggests that early inductive signals required for lens development such as Bmp4 and Bmp7 (Furuta and Hogan 1998; Wawersik, Purcell et al. 1999) were properly induced.

**Lens Differentiation in the Pax6ΔPD over-expressing animal**

To determine whether lens fate has been changed in degenerating lens, I assessed expression of lens fate markers, Prox1, β-crystallin and α-crystallin. The transcription factor Prox1 is required for lens fiber differentiation (Wigle, Chowdhury et al. 1999) and is expressed in the lens placode starting at E9.5 mouse embryo. At E12.5, Prox1 is observed in anterior dividing epithelium and lens fibers in normal lens (Fig. 3.5A, C). Despite their abnormal size and morphology, degenerating lenses still expressed Prox1 (Fig. 3.5B, D). Fiber cell differentiation is characterized by the onset of fiber cell-specific crystallin expression (e.g., β and γ–crystallins in the mouse), elongation, and the loss of organelles including the nucleus, mitochondria and endoplasmic reticulum. Crystallins are the predominant soluble proteins in the mammalian lens and conferring the property of transparency on the lens (McAvoy 1978). At E12.5 mouse embryos, α and β-crystallin is up-regulated in differentiating lens fibers (Fig. 3.5E, G, K). Similar to Prox1, degenerating lens cells also expressed both α (Fig. 3.5L) and β-crystallin (Fig. 3.5F,H,L) protein suggesting that lens fate has not been altered by over-expression of Pax6ΔPD.

**Cell proliferation and Death in degenerating lenses**

To test whether the degeneration of the lens was due to reduced cell proliferation, I examined phospho-Histone H3 level in the eye of $T_{g}^{3}T_{g}^{15}$; $Pax6^{+/+}$ embryos. Histone H3 phosphorylation has long been implicated in chromosome condensation during mitosis and
meiosis (Hendzel, Wei et al. 1997). Therefore phospho histone H3 is a good marker for actively proliferating cells. In E12.5 wild-type mouse embryos, anti-phospho histone H3 antibody labels proliferating cells at the lumen side of neuro-retina and anterior epithelial cells of the lenses (Fig. 3.6E, G). Similarly, equivalent numbers of histone H3 positive cells were detected in the eyes of \( Tg^3Tg^{15}; Pax6^{+/+} \) embryos (Fig. 3.6F,H) suggesting proliferation has not been affected by over-expression of Pax6ΔPD.

Because \( Tg^3Tg^{15}; Pax6^{+/+} \) embryos have severe degeneration of the lenses, but showed no significant differences in cell proliferation during early embryogenesis, I examined degenerating lenses for apoptotic cell death. PARP, nuclear poly (ADP-ribose) polymerase, is one of the main cleavage targets of active caspase-3 which is expressed abundantly during apoptotic cell death. Therefore, I stained sections of E12.5 eyes from both wild-type and \( Tg^3Tg^{15}; Pax6^{+/+} \) embryos with anti-PARP antibody. Compare to wild-type lenses at E13.5 (Fig. 3.6A, C), \( Tg^3Tg^{15}; Pax6^{+/+} \) embryos showed increased number of apoptotic cells within the lenses including anterior epithelial cells and fibers (Fig. 3.6B, D). These results suggest that sudden degeneration of the lenses during early embryogenesis is most likely caused by increased number of apoptotic cells.

**Generation of Pax6BAC 304E12DT to examine Pax6ΔPD expression**

Although the most obvious effect of over-expression of Pax6ΔPD in the \( Tg^3Tg^{15}; Pax6^{+/+} \) mice is rapid lens degeneration during early eye development, whether this is cell autonomous or non-autonomous effect still remained to be answered. To answer to that question directly, studying the expression pattern of Pax6ΔPD is a necessary step. Unfortunately, because Pax6ΔPD is identical to full length Pax6 protein except that Pax6ΔPD lacks paired-domain (Fig. 3.1B, C), localizing only the Pax6ΔPD in animal is almost impossible. Therefore I decided to
take advantage of modified \textit{Pax6BAC} technology in which full length Pax6 protein expression is represented by GFP expression and Pax6\(\Delta\text{PD}\) protein expression is represented by Red fluorescent protein expression. To generate modified Pax6BAC, I first isolated mouse BAC RPC1 304E12 that contains the Pax6 transcription unit starting from differentially methylated CpG Island 5' of \(P_0\) and 3' downstream regulatory region ends at \textit{Elp4} exon4. I inserted EGFP-pA targeting construct into \textit{Pax6} exon4 and DsRed-pA targeting construct downstream of putative translation initiation site ATG within \textit{Pax6} exon8. There are two putative translation initiation sites downstream of \(P_{\alpha}\) promoter. One located within \textit{Pax6} exon7 and another one within exon 8. Because I decide that ATG in exon8 is a likely initiation codon for translation of Pax6\(\Delta\text{PD}\) protein from \(P_{\alpha}\) transcript, I inserted DsRed-pA targeting construct into exon8(Fig. 3.7I).

mBAC 304E12 was modified as described in methods and used to generate transgenic mice. Although I previously had successfully generated transgenic mice with supercoil mBAC293d08, this time I decided to used linear transgenic BAC in which I could easily determine whether the end of the BAC is intact or not. Linearized BAC transgene was injected into mouse oocytes and four transgenic founder mice (line 897, 898, 924, 939) were identified. One of which didn’t transmit a transgene to its offspring. PCR using primers described in the method and southern blot were used to examine the mBAC304E12 transgene within genomic locus. Although all 4 lines had multiple copies (1-7 copies) of transgene, I used line 898 that carried the highest copies of transgene. As expected, none of these lines showed any phenotype.

**Comparison of expression pattern between Pax6 and Pax6\(\Delta\text{PD}\)**

To examine transgene expression, embryos were collect at different embryonic days and visualized by fluorescent microscope. Expression of EGFP was detected at E10.5, E12.5 and E16.5 and the expression pattern was consistent with the established Pax6 expression. EGFP
expression was detected in developing lens pit, neuroretina, and RPE at E10.5 (Fig. 3.7A). As eyes are continued to develop, EGFP expression was also observed in optic nerve, retina ganglion cell layer, anterior epithelium cells and differentiating fiber cells within the lenses (Fig. 3.7D,G). No significant differences between \textit{Pax6}BAC293d08 and \textit{Pax6}BAC304E12 were detected in EGFP transgene expression.

To examine the DsRed-pA transgene expression in mBAC304E12 transgenic mice, embryos at different stages were analyzed for red fluorescence. Although transgenic line898 contains 7 copies of transgene, DsRed protein expression was not visualized in whole mouse embryo at any stage by standard dissecting fluorescent microscope. To test whether this is due to low expression level of DsRed protein, I examined sections of embryos expressing EGFP by anti-DsRed antibody staining. At E10.5, DsRed transgene was detected at the distal tip of neuro-retina but not in the central retina (Fig. 3.7B). Whereas EGFP transgene was expressed in the entire neuroretina, RPE, and lens vesicle, consistent with endogenous Pax6 expression, DsRed transgene expression was restricted to the distal tip of neuro-retina at E13.5. (Fig. 3.7C). At E16.5, although DsRed transgene expression is still highest at the distal tip of neuroretina, cells within inner nuclear layers, most likely amacrine cells, showed DsRed transgene expression (Fig. 3.7H,I).

\textbf{Pax6\DeltaPD is localized in both nucleus and cytoplasm}

As a first step of characterizing Pax6\DeltaPD protein, I decide to examine the nuclear localization properties of Pax6\DeltaPD protein using a cell culture system. Similar to most transcription factors, Pax6 is localized exclusively in the nucleus. However, previous studies in \textit{Quail} and \textit{C. elegans} showed that nuclear localization of paired-less Pax6 is regulated
spatiotemporally. (Carriere, Plaza et al. 1993; Carriere, Plaza et al. 1995; Zhang, Ferreira et al. 1998). Whereas, 46/48 kDa of Quail Pax6 (Pax6-QNR) isoforms were only localized in the nucleus (100%), 32/33kDa paired-less isoforms were localized in both the cytoplasm and the nucleus (100%) (Carriere, Plaza et al. 1995). In *C. elegans*, the non-PD isoform, MAB-18, is localized in cytoplasm in several neuronal sublineages in the male tail (Zhang, Ferreira et al. 1998). To test whether mammalian Pax6ΔPD is also localized in both the cytoplasm and the nucleus, I decide to use a cell culture system. I transfected NIH3T3 mouse fibroblast cells with Pax6 cDNAs that were synthesized from either P₀ transcripts or P_α transcripts. Cells were cotransfected with an EGFP expression construct to visualize transfected cells (Figs. 3.8D-F) and immunostained with anti-Pax6 antibody that binds to the C-terminus of Pax6 protein (Figs. 3.8A-C). Because NIH3T3 cells do not express Pax6 proteins normally, cells showing Pax6 positive signals should be transfected cells. Immunostaining showed that paired-containing Pax6 protein is localized mostly in the nucleus (88%) (Fig. 3.8B, G), whereas the Pax6ΔPD protein was in both the nucleus and the cytoplasm (84.5%) (Fig. 3.8C, G). This result suggests that mammalian Pax6ΔPD isoform also localizes in both the nucleus and the cytoplasm as in *C. elegans* and Quail. However, the mechanism that controls the localization of Pax6 is unclear. In Quail, there are two nuclear localizing signals, one in exon5 and the other one in exon7. Because the NLS in exon5 is stronger than the one in exon7, paired-less Pax6 that lacks the N-terminal can be localized in both nucleus and cytoplasm. Although, it is not clear how the nuclear localization of Pax6ΔPD in mouse is regulated, these similar results from three different species suggest that Pax6ΔPD may act by a similar mechanism.
Discussion

I here reported analysis of microphthalmic phenotype caused by over-expression of Pax6ΔPD using Pax6 BAC transgenes. Because the canonical form of Pax6 and the paired-less Pax6 isoform are identical except the paired-domain and $P_{\alpha}$ transcripts are expressed in subsets of cells that also express $P_0/P_1$ transcripts, it is almost impossible to study expression pattern and biological function of Pax6ΔPD in wild-type mice. Fortunately, the Pax6 BAC293d08-EGFP transgenic mice, that I previously generated, express only Pax6ΔPD isoform but not full length Pax6. In addition to that, because this transgenic mouse expresses EGFP instead of full length Pax6, this mouse served as a great tool to study the expression pattern and more importantly the biological function of Pax6ΔPD in vivo. Using double heterozygous embryos that carry 18-20 copies of transgene, I was able to uncover that Pax6ΔPD has a potential role during eye development. To investigate the precise expression pattern of Pax6ΔPD, I also generated dually-modified Pax6 BAC304E12-EGFP/RFP. This dually-modified BAC transgene expresses both EGFP and DsRFP that recapitulates full length Pax6 and Pax6ΔPD expression respectively.

Over-expression of Pax6ΔPD causes microphthalmic phenotype

A paired-less isoform of Pax6 has been identified in birds (Carriere, Plaza et al. 1993), nematodes (Chisholm and Horvitz 1995; Zhang and Emmons 1995), and mice (Kim and Lauderdale 2006). In mice, paired-less isoform was thought to arise by alternative splicing (Mishra, Gorlov et al. 2002) or by transcription from an internal promoter, as in C. elegans (Kleinjan, Seawright et al. 2004). In C. elegans, paired-less Pax6 is known to interact with paired-containing Pax6 additively, synergistically, or antagonistically, depending on the cellular
context (Cinar and Chisholm 2004). However, the function of this pax6 isoform is unknown in quail. As I previously described (Kim and Lauderdale 2006), in mice, paired-less Pax6 has a role in eye development. Over-expression of paired-less Pax6 in the mouse transgenic for the Pax6BAC 293d08 exhibit microphthalmic phenotype in both $Pax6^{+/+}$ and $Pax6^{+-}$ animals. In contrast to the full length Pax6 that rescued small eye phenotype when over-expressed in the mouse, Pax6ΔPD enhanced small eye phenotype in our experiment suggesting that Pax6ΔPD have potential dominant negative function against full length Pax6. Alternatively, microphthalmic phenotype caused by over-expression of Pax6ΔPD might be in part due to disruption of relative levels of Pax6 and Pax6ΔPD. Pax6 has multiple roles in eye development: promotion of progenitor proliferation, maintenance of progenitor potential, and regulation of the timing of neural differentiation (Marquardt, Ashery-Padan et al. 2001; Inoue, Hojo et al. 2002; Philips, Stair et al. 2005). One possible explanation of these multi tasks of Pax6 is that different isoforms of the Pax6 protein carry out different developmental functions. Also, recent studies suggest that differences between its function in different time point and different tissues might be due to a shift in the relative levels of Pax6 and Pax6(5a) isoform and stabilizing the relative levels of the isoforms is important to maintain diverse functions of Pax6 in normal development (Pinson, Mason et al. 2005; Pinson, Simpson et al. 2006). Although functions of different isoform are not clear yet, their spatiotemporal expression is overlapped with Pax6. Therefore, the precise ratio between Pax6 and Pax6ΔPD might be important factor to maintain functions of Pax6 during eye development. In this view, microphthalmic phenotype in our experiment may have been resulted by disrupting the balance between Pax6 and Pax6ΔPD by over-expressing only Pax6ΔPD. However, functional relationship between Pax6 and Pax6ΔPD still need to be studied.
Over-expression of Pax6ΔPD causes defects in lens development

I provide evidence that paired-less Pax6 has a role in eye development. Over-expression of Pax6ΔPD affected continued differentiation of lens cells at later stages accompanied with abnormalities in anterior eye structures. Lens development is a multi step process that involves a complex series of interactions between two different cell populations; ectodermally-derived presumptive lens ectoderm (PLE) and neurally-derived optic vesicle. Grainger et al. showed that early induction of the lens is controlled by four different events: competence, bias, specification, and differentiation (Grainger 1992). Lens forming competence is the ability of head ectoderm to respond to inductive signal. This lens forming competence may be specified autonomously within ectoderm on the basis of the age of the ectoderm (Servetnick and Grainger 1991). Bias for lens induction is acquired throughout the head ectoderm during the neural plate stage and thought to be mediated, in part, by signals from presumptive anterior neural plate tissue (Henry and Grainger 1990). Lens specification is the third event that occurred as the optic vesicle comes into contact with biased lens ectoderm. Usually, lens specification is characterized by the formation of a small, crystallin-expressing structure. Finally lens differentiation starts at the time of lens placode formation and lens morphology first becomes detectable at this time point (Grainger, Henry et al. 1992). To specify presumptive lens ectoderm prior to placode formation, numerous regulatory proteins are known to be activated in these tissues. Among these regulatory proteins, Pax6 has been implicated as one of the earliest and an essential regulatory protein along with others in both vertebrate and invertebrate eye development (Gehring and Ikeo 1999). To better understand the underlying mechanism that causes microphthalmic phenotype in double-heterozygote animal, I assessed a series of embryos at different time point to see which step of lens development has been affected. At E12.5 lens vesicle has been successfully formed
and primary lens fibers were elongated as in wild-type. Markers for anterior epithelium and fiber cell differentiation were expressed in correct time and location, although morphology of the lens was abnormal and small. These results indicate that initial inductive signals for lens development have taken place normally. This lens degeneration was largely due to increased apoptosis within the lens, whereas no apparent changes in cell proliferation were detected. Also over-expression of \textit{Pax6\textDelta PD} affected development of anterior structures, including iris, cornea, ciliary body, and anterior chamber. Defects in these tissues are most likely due to abnormalities in signaling from the lens. Previous studies have shown that lens produces growth factors and other signaling molecules (de Iongh and McAvoy 1993) and influences the development of the ciliary body, iris, and cornea (Genis-Galvez 1966; Beebe 1986; Beebe and Coats 2000).

\textbf{Pax6\textDelta PD is expressed in the neuro-retina but not in the lens}

Although \textit{P\textalpha} transcripts are thought to be the source of Pax6\textDelta PD, at present, there is no clear technique to localize only Pax6\textDelta PD protein in wild-type embryo because Pax6\textDelta PD is expected to be identical with Pax6 except the paired-domain. In this study, using Dual-reporter BAC transgene system, I showed precise expression pattern of Pax6\textDelta PD without causing any phenotype. Despite a severe lens defect in the microphthalmic phenotype, Pax6\textDelta PD was not detected in the lens implicating that lens degeneration is likely due to a cell non-autonomous effect. During embryogenesis, Pax6\textDelta PD is expressed highly in the distal optic cup and a distinct cell population within the inner nuclear layer at later stages. Although I can not rule out the possibility that Pax6\textDelta PD is generated by alternative splicing from \textit{P\textalpha/P\textbeta} transcripts, Pax6\textDelta PD protein is more likely expressed from \textit{P\textalpha} initiated transcripts based on previous studies (Mikkola, Bruun et al. 2001) and cell culture study where I was able to express Pax6\textDelta PD protein.
using cDNA synthesized from \( P_{\alpha} \) transcripts (I.M.A.G.E. Consortium Clone ID#4527976)(unpublished data, Kim and Lauderdale). \( P_{\alpha} \) promoter is located within intron4 just downstream of Pax6 distal retina enhancer (\( \alpha \) enhancer) that is well characterized by several studies (Kammandel, Chowdhury et al. 1999). Recent studies using \( \alpha \)-Cre transgenic line that express Cre under the control of \( \alpha \)-enhancer, showed that \( \alpha \)-enhancer is capable to derived Cre activity in the distal neuroretina including the most distal tips, destined for the iris and the CB, but at no stage in the lens or the retinal pigment epithelium or any other tissue(Marquardt, Ashery-Padan et al. 2001; Davis-Silberman, Kalich et al. 2005). This expression pattern is largely similar with Pax6\( \Delta \)PD expression pattern in this study and support the idea that Pax6\( \Delta \)PD is initiated from \( P_{\alpha} \) transcripts instead of alternative splicing. Also, when sequences between Pax6 exon 4 and exon7 were specifically deleted by \( \alpha \)-Cre activity, Pax6 expression in the distal neuroretina was decreased and \( Pax6^{\alpha/4} \), \( \alpha \)-Cre eyes showed reduced size of iris and CB indicating that maintaining certain level of Pax6 expression in the distal neuroretina is critical for anterior ocular development (Davis-Silberman, Kalich et al. 2005). The lens has been shown to play a central role in the development of the anterior segment, while lens growth depends on growth factors secreted from the CB, iris and retina into the aqueous and vitreous compartments (McAvoy, Chamberlain et al. 1999). Based on these observations, I propose that the lens defect in this microphthalmic eye is due to altered signals from distal neuroretina that over-express Pax6\( \Delta \)PD, and the lack of lens is caused abnormal anterior ocular segments. However, whether over-expression of Pax6\( \Delta \)PD in the distal neuroretina has affected development of anterior ocular structures directly or indirectly, as I proposed, is not clear.
Signaling molecules affect lens differentiation

If our hypothesis is correct, there must be specific signaling molecules secreted from the retina that is normally involved in lens cell differentiation but has been altered by over-expression of Pax6ΔPD. It is well known that signaling molecules that are secreted from the RPE and retina are required for proper lens development and maintenance (Chow and Lang 2001; Reza and Yasuda 2004). Because Pax6ΔPD is expressed in the retina but not in the lens, our best candidate molecules are the ones that are expressed in the retina. For example, FGF 3, 5, 8, 12, 15 are known to be expressed either in the neuroretina or RPE during eye development (Wilkinson, Bhatt et al. 1989; Kitaoka, Aotaki-Keen et al. 1994; Hartung, Feldman et al. 1997; McWhirter, Goulding et al. 1997; Vogel-Hopker, Momose et al. 2000) whereas FGF receptors including FGFR1, 2b, C, and 3 are expressed in the lens (Garcia, Yu et al. 2005). Although BMP4 is known to play important roles in the early stages of lens development, and expressed in the lens placode, BMP4 is also expressed in the tip of retina during later stage of eye development (Furuta and Hogan 1998; Faber, Robinson et al. 2002). Also jagged, ligand for Notch 2 is expressed in the distal retina (Bao and Cepko 1997). Among these molecules, so far, the best candidate signaling molecules fitting to our model are BMP4 and FGF8 considering their expression pattern and expression timing. As I described early, BMP4 is expressed in similar spatiotemporal pattern with Pax6ΔPD. FGF8 is expressed in the central part of the presumptive retina during early stages and middle of the developing inner nuclear layer (approximate level of the cell bodies of Muller glia and bipolar cells in the adult) but not in the lens or RPE at any stage of development. Although FGF8 is not detected in the distal part of retina, I cannot rule out that FGF8 expression is affected by over-expression of Pax6ΔPD in the inner nuclear layer of the retina. Therefore, studies to test whether these signaling pathways have
been altered by over-expression of Pax6ΔPD are necessary step to understand the function of
Pax6ΔPD in molecular level.

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**Fig. 3.1.** Transcript analysis of *Pax6* and structure of the Pax6 BAC 293d08-EGFP

(A) Physical map of the mouse Pax6 transcription unit. Bent arrows denote the locations of transcript initiation and direction of transcription. (B) All P₀- or P₁-initiated transcripts spliced into exon 4. These transcripts encode paired-containing Pax6. Translation initiate at the ATG win exon 4. (C) Pα-initiated transcripts encode for paired-less 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.

(D) Structure and transcript analysis of the Pax6 BAC 293d08-EGFP. BAC 293d08 has been generated as previously described. All P₀- or P₁-initiated transcripts encode EGFP. Because EGFP reporter cassette is inserted to 5' of *Pα*, Pax6BAC 293d08 is expected to express paired-less Pax6 in animal.
Fig. 3.2. Over-expression of Pax6ΔPD causes microphthalmic phenotype in wild-type mice carrying 18 copies of transgene (B). Same phenotype was observed in Pax6 heterozygote mutant mice, Pax6<sup>Sey-Neu</sup> or Pax6<sup>Sey-Dey</sup>, carrying 9-10 copies of transgene (C-F). Whereas 9 copies of transgene does not effects eye phenotype in wild-type mice (A), same copy number of transgene enhanced small eye phenotype (D, F) suggesting that this isoform interacts antagonistically with paired-containing Pax6 isoforms. Eye phenotype is consistent between different lines.
Fig.3.3. Microphthalmic eyes have lens defects. Microphthalmic eyes from both $Tg^3;Tg^{l5};Pax6^{+/+}$ and $Tg^3;Pax6^{Sey-Neu/+}$ showed greatly reduced size compared to wild-type eye and surrounded by abundant fibrotic tissues (A-D). (E-J) Histological analysis of adult eyes by H&E staining. $Tg^3;Pax6^{+/+}$ mice have normal eyes (E,F). Both $Tg^3;Tg^{l5};Pax6^{+/+}$ and $Tg^3;Pax6^{Sey-Neu/+}$ mice have extremely small or no lenses with disorganized retina. The anterior chamber in these microphthalmic eyes is appeared to be filled with pigmented cells and corneal endothelium is adhered to RPE (retina pigmented epithelium). Although overall retina structures are disorganized, distinct RPE, ONL (outer nuclear layer), INL (inner nuclear layer) and GCL (ganglion cell layer) are visible (G-J).
Fig. 3.4. Lenses degenerate during embryogenesis. To further investigate the eye phenotype in $Tg^3;Tg^{15};Pax6^{+/+}$ transgenic mice, series of embryos at different developmental time point (I-P) were compared with embryos from $Tg^3;Pax6^{+/+}$ transgenic mice that exhibit wild-type phenotype (A-H). GFP expression was assessed in either live embryos (A-D, and I-L) or frozen sections (E-H, and M-P) by fluorescence microscopy. Both gross eye phenotype (A and I) and transgene expression (E and M) at E10.5 were indistinguishable from each other except expression level of GFP. By E12, both lens vesicles were successfully formed and lens fiber cells initiated to elongate. However, at E13 lenses were largely degenerated in both size and morphology. Ganglion layer seemed slightly thicker and have more proliferating cells in $Tg^3;Tg^{15};Pax6^{+/+}$ embryos than their wild-type littermates. No obvious change in retina structure was observed at this stage. At E15, the anterior cells persist in the developing lens as a living single layer of cells along the anterior surface. These cells will continue to divide and the cells along the edges will begin to form secondary lens fibers. Pax6 is consistently expressed in the anterior epithelium and nucleus of equatorial. Unlikely, lenses in $Tg^3;Tg^{15};Pax6^{+/+}$ embryos were mostly degenerated and remained lens like structures (LLs) were attached to the cornea. lp, lens pit; rpe, retina pigmented epithelium; nr, neuro retina; on, optic nerve; c, cornea.
Fig. 3.5. Lenses of Tg3;Tg15;Pax6+/+ transgenic mice have normal expression of lens cell markers. Sections from Tg3;Pax6+/+ embryos and from Tg3;Tg15;Pax6+/+ littermates embryos at E13.5 were immunostained for Prox1(A-D), β-crystallin (E-H), α-crystallin (K,L), and Brn3b (I, J). Tg3;Tg15;Pax6+/+ embryos have smaller lenses than their Tg3;Pax6+/+ littermates. At E12.5, Prox1 protein is observed in the anterior proliferating epithelium and fibre cells (Wigle, Chowdhury et al. 1999) (A,C). Although lenses were largely degenerated and have abnormal morphology in Tg3;Tg15;Pax6+/+ embryos, Prox1 expression was observed within remaining lens structure (B,D). β-crystallin and α-crystallin are most abundant proteins expressed in the lens (Harding 1991). β-crystallin is present in the fiber cells of both Tg3;Tg15;Pax6+/+ and Tg3;Pax6+/+ embryos (E-H). α-crystallin is detected in epithelial and fiber cells in both genotypes (K, L). Sections from both genotype embryos were stained with antibody for Brn3b that is expressed in differentiating and retinal ganglion cells (de Melo, Qiu et al. 2003) . At E13, Brn3b expression is localized primarily to the inner retina, especially the central region in both Tg3;Tg15;Pax6+/+(I) and Tg3;Pax6+/+(J) embryos.
Fig. 3.6. Increased apoptotic cell death in the lens of \( Tg^3;Tg^{15};Pax6^{+/+} \) mice. To test whether rapid lenses degeneration is due to apoptotic cell death, OCT sections were immunostained for PARP that is one of the main cleavage targets of active caspase-3 in vivo during apoptosis (Nicholson, Ali et al. 1995; Tewari, Quan et al. 1995) (C, D). A and B show GFP expression in the eye of \( Tg^3;Pax6^{+/+} \) and \( Tg^3;Tg^{15};Pax6^{+/+} \) embryos respectively. Degenerating lens from \( Tg^3;Tg^{15};Pax6^{+/+} \) embryos had higher percentage of PARP positive cells that are undergoing apoptosis than wild-type lens. Also sections were assessed for histone H3 activity that is a good indicator of proliferating cells. Unlike, PARP staining, no noticeable differences were detected in histone H3 staining.
**Fig. 3.7.** Pax6ΔPD reporter is expressed in the peripheral neuroretina and amacrine cells.

J. Schematic drawing of the Pax6 BAC 304E12-EGFP/RFP construct used in this study. EGFP and RFP reporter cassettes were inserted at the translation initiation sites (ATG) in exon4 and exon8 respectively. Both reporter genes were followed by strong SV40 PolyA sequences to prevent Pax6 expression. To assess transgene expression in the eye, frozen sections of the eyes were prepared from E10.5, E13.5, and E16.5 embryos. Sections were stained with α-DsRed antibody to amplify RFP signal and visualized for GFP (A, D, and G) and RFP (B, E, and H) by fluorescence microscopy. GFP expression pattern of Pax6 BAC 304E12-EGFP/RFP transgenic mice was equivalent to that of Pax6 BAC 293d08-EGFP throughout development (A, D, and G) as previously described (Kim and Lauderdale 2006). (B, E, and H) At E10.5, DsRFP expression was evident at the peripheral neuroretina and excluded from central neuroretina and the lens pit (B). Similarly, DsRFP expression was restricted to the distal tip of neuroretina (E, F) whereas GFP expression was widely spread throughout entire retina, RPE, lens, cornea, and optic nerve (D, F). Although DsRFP expression was still highest at the ciliary margin of the retina at E16.5, distinct DsRFP expression was evident in the proliferating cells that are most likely amacrine cell population (H, I). inbl, inner neuroblast layer; onbl, outer neuroblast layer.
J. Schematic drawing of the Pax6 BAC 304E12-EGFP/RFP
**Fig. 3.8.** Subcellular localization of Pax6 isoforms. NIH3T3 cells were transfected with expression vector encoding either Pax6 (B,E) or Pax6ΔPD (C,F). Transfected cell were fixed 72h after the transfection and immunostained with α-Pax6 antibody(red) and counterstained with hoechst to visualize the nucleus. EGFP expression represents the transfected cells. (A, D) No 3T3 cells showed immunoreactivity without Pax6 construct. Whereas cells transfected with Pax6 expressing construct showed immunoreactivity only in the nucleus (B, E), cells transfected with Pax6ΔPD expressing construct showed immnoreactivity both in nucleus and cytoplasm (C,F).
<table>
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<th>Cellular localization</th>
<th>3T3/Pax6</th>
<th>3T3/Pax6ΔPD</th>
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<tr>
<td>(%)</td>
<td>21(12%)</td>
<td>1068(84.6%)</td>
</tr>
<tr>
<td>Cytoplasm + Nucleus</td>
<td>152(88%)</td>
<td>195(15.4%)</td>
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<tr>
<td>Nucleus only</td>
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<tr>
<td>Cytoplasm + Nucleus</td>
<td>1068(84.6%)</td>
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3T3/ GFP, 3T3/Pax6, 3T3/ΔPDPax6

a-Pax6(C-terminal) Antibody
Chapter 4

Identification of Potential Pax6 Locus Control Region
Abstract

Pax6, a member of the paired-family transcription factors, is required for development of the eye, central nervous system, and endocrine pancreas. Whereas heterozygous mutations in Pax6 cause defects in eye development, homozygous mutations result in loss of eyes, nose, forebrain structures and endocrine pancreas. This complex expression pattern and dosage sensitivity suggests that Pax6 transcription is tightly regulated. However, despite intensive study, the mechanisms controlling Pax6 expression are not well understood. In mammals, Pax6 gene transcription initiates from three different promoters and is mediated by several promoter proximal regions. However, recent work by our lab and others suggest that a 3' DOWNSTREAM REGULATORY REGION (DRR) is required for Pax6 expression and is expected to control the promoter proximal regulatory elements.

To better understand the function of DRR and mechanism by which DRR affect transcriptional activity of Pax6, I identified and analyzed several conserved regions within the DRR through evolutionary sequence comparison. Because highly conserved non-coding sequences often contain important transcription factor binding sites, I used reporter assay system to test these regions. As results of this study, I identified CR2 that shows the increased reporter activity in retina derived 661W cells line and also contains several potential factor binding sites. To further characterize in vivo function of this element, I tested CR2 coupled to mouse Pax6 P0 minimal promoter in zebrafish. When mP0min was coupled with CR2, reporter expression was spatiotemporally regulated compared to promoter itself. However, because of the complexity of regulatory mechanism of Pax6 in animal, it was necessary to test the function of individual cis-regulatory element in native context. Therefore, I deleted CR2 elements from the 150kb Pax6 BAC293d08-EGFP transgene that contains entire Pax6 transcription unit including DRR and
express EGFP, which is comparable to endogenous Pax6 expression pattern. Unexpectedly, this experiment results in absence of EGFP expression in entire embryos and led us to hypothesized that DRR may have Locus Control Region (LCR) like function in vivo to modulate global Pax6 transcription along with proximal components.

**Introduction**

A highly complex system of transcriptional regulatory elements exists for the Pax6 gene. Differential splicing and promoter usage, acting in combination with multiple cis-acting elements, direct the complex Pax6 gene expression pattern observed (Epstein et al., 1994; Plaza et al., 1995a; Jaworski et al., 1997; Okladnova et al., 1998). In mammals, the Pax6 gene has 16 exons distributed over ~30kb region (Fig. 4-1) (Glaser, Walton et al. 1992). Transcription initiates from three different promoters, designated P₀, P₁, and Palpha; P₁ and Palpha are internal promoters (Xu, Saunders et al. 1997; Kammandel, Chowdhury et al. 1999; Xu, Zhang et al. 1999; Anderson, Hedlund et al. 2002). Three different transcripts were shown to be found in different region during eye and CNS development possibly by presences of different upstream regulatory elements (Xu, Zhang et al. 1999). Recent analysis of the Pax6 promoters in transient transfection assay identified multiple cis-regulatory elements with distinct function in different cell lines (Kammandel, Chowdhury et al. 1999). In addition, approaches involving the generation of transgenic mice have been particularly useful to in localizing independent regulatory elements controlling the tissue specific expression of the Pax6. Identified regulatory elements seem to be evolutionarily conserved between distant species.
For example, the $P_0$ promoter upstream region was shown to contain two distinct enhancer elements, which are required for correct Pax6 expression in the lens, cornea, lacrimal gland and pancreas (Kammandel, Chowdhury et al. 1999). Results from in vitro experiments with the quail Pax6 gene revealed a 7.5kb downstream region of the quail $P_0$ promoter, acting as an enhancer in neuro retinal cells (Plaza, Dozier et al. 1995). Consistent with this finding, another mouse enhancer fragment, located downstream of the Pax6 translational start site, within intron 4, was identified and shown to be required for the Pax6 expression in the neural retina, the pigment layer of the retina, and the iris. This region displayed a number of potential homeobox- protein binding sites, including Pax2 and Msx1, which are the crucial factors involved in eye development. Finally, a 5kb fragment located between the $P_0$ and $P_1$ promoters has been known to mediate expression in the dorsal telencephalon, the hindbrain, and the spinal cord (Kammandel, Chowdhury et al. 1999).

The identified Pax6 cis-regulatory elements showed high sequence conservation in puffer fish, mouse, and human. In addition, a mouse retinal enhancer had the capability to drive reporter gene expression in a subset of eye structures in Drosophila (Xu, Zhang et al. 1999). This and other findings clearly demonstrate that the mechanisms regulating Pax6 expression are highly conserved. In addition to these well characterized promoters, 5' upstream enhancers, and intronic regulatory elements, a whole series of additional downstream regulators of Pax6 have been discovered and are now being functionally assessed. The existence of a Downstream Regulatory Region (DRR) was first deduced from a subset of aniridia patients (Lauderdale, Wilensky et al. 2000). Although aniridia is typically caused by heterozygous null mutation within the $PAX6$ gene (Glaser, Walton et al. 1992; Hanson, Seawright et al. 1993; Glaser, Jepeal


et al. 1994; Hanson, Brown et al. 1995), chromosomal rearrangements located 3' to the *PAX6* transcription unit have been shown to cause aniridia.

Work over the past several years has provided several pieces of evidence that this region (DRR) regulates *Pax6* expression. In the human × mouse somatic cell hybrid experiment, *PAX6* was only transcribed from the normal allele but not from the allele containing deletions within the region 3' to the *PAX6* (Lauderdale, Wilensky et al. 2000). Also, in transgenic experiments, it was demonstrated that a human YAC of 420kb, spanning the *PAX6* transcription unit, 5' and 3' flanking regions can rescue homozygous *Small eye* lethality and correct the heterozygous eye phenotype (Schedl, Ross et al. 1996). However, a shorter human YAC of 310kb, spanning the *PAX6* transcription unit and 5' flanking region, but lacking part of the 3' flanking region, failed to rescue the *small eye* lethality and only partially recapitulated normal *Pax6* expression (Kleinjan, Seawright et al. 2001). This finding indicates the existence of an essential regulatory region 150kb downstream from the *P1* promoter and redefined the functional *Pax6* gene domain extending far beyond the transcription unit. Using evolutionary sequence comparison, *DNase* I hypersensitivity analysis, and transgenic mice studies, additional tissues specific regulatory elements were identified including lens specific enhancer located near a HV breakpoint, retina specific regulatory module approximately 8kb downstream from the lens enhancer (Kleinjan, Seawright et al. 2001). Most recently a region was described, located 3kb downstream of exon13 of *Pax6*, which was shown to be active in the developing pretectum, neural retina, and olfactory region (Griffin, Kleinjan et al. 2002). The finding of these additional regulatory elements underlines the significance of the DRR in the context of a global regulatory mechanism governed by 5' upstream, intronic, and 3' downstream regulatory regions. For instance, in the YAC transgenic experiment, the 310Kb truncated YAC failed to drive *PAX6* expression in the retina,
although it contained a well known neuroretinal specific enhancer within intron4. This suggests
the requirement of the additional neuroretina specific enhancer elements in the DRR and a global
regulatory mechanism to control Pax6 expression. Taken together, this and other findings imply
that controlling the Pax6 expression in the eye requires 5’ upstream, intronic regulation and 3’
remote regulation. Thus, the DRR may act as a master regulator for correct Pax6 expression
during eye development.

In this study, I defined control elements within the DRR to better understand the molecular
mechanisms that governs the complex spatiotemporal expression of Pax6. Also I used a
transgenic approach with a BAC recombination system to test the biological function of
identified elements in their native context.

Material and Methods

Generation of reporter constructs

Genomic fragment A, C, and D that contains highly conserved region 1, 2, and 3
respectively, were PCR amplified from mouse genomic DNA using high-fidelity-DNA
polymerase and following primer sets.
Fragment A forward (5'-CGTGCTTGGGGTTATGGAG-3'), reverse (5'-CCACTGTTAACE-3'); Fragment B.2 forward (5'-TGGAACATCATATCCCCG-3'), reverse (5'-TGGATCCAGGAAATCTGACGAGG-3'); Fragment C forward (5'-GGCAAGAATCTGAGGGTG-3'), reverse (5'-CAGCAAAGAATAGAGGAG-3'); Fragment D forward (5'-CCAACTTTGACCTGCGAAGG-3'), reverse (5'-ATCCCCTTACCTGGACCCGTC-3'). Each PCR products were subcloned into the luciferase reporter vector pGL3-promoter containing SV40 promoter to drive luciferase gene expression at a basal level.

To perform the deletion analysis of fragment C containing CR2, series of small fragments were subcloned into luciferase reporter vector or pGL3-FC was serially modified. All constructs used in this study were generated by standard molecular cloning techniques (Sambrook 2001) and are shown in Fig 4. D1 was derived from pGL3-FC by deletion of the BglII fragment and religation of the vector. D2 was derived from pGL3-FC by removal of MluI/NdeI fragment and religation of blunt-ended vector. D3 was generated by subcloning the 695bp MluI-DraI fragment from pGL3-FC into MluI-SmaI sites in pGL3-promoter vector. D3Δp was generated by removing of 1.2kb of HindIII fragment from pGL3-FC to use as negative control lacking promoter. D4 was constructed by subcloning the 764bp NdeI-BglII fragment from pGL3-FC into pGL3-promoter vector. To generate D5 construct, pGL3-FC was digested with HindIII first and klenow treated. Blunt-ended HindIII fragment was digested with XhoI and subcloned into SmaI-XhoI sites in pGL3-promoter vector. D6 was generated by subcloning 500bp BglII fragment into BglII site in pGL3-promoter vector. To generate D7 construct, pGL3-FC was cut with HindIII and 355bp fragment was klenow treated. Blunt-ended 355bp fragment was subcloned into SmaI site in pGL3-promoter vector. D8 was generated by subcloning the 254bp DraI-NdeI fragment into pGL3-promoter vector.
Cell culture

NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum, 20 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. 661W cells, which were developed from the retinal tumor of a transgenic mouse expressing SV40 large T antigen under the control of the human interphotoreceptor retinoid-binding protein (IRBP) promoter (al-Ubaidi, Font et al. 1992), were grown in DMEM supplemented with 10% fetal bovine serum, 20 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin.

Cell transfection and luciferase reporter assays

Cells grown in 96-well plates to 50% confluence were transfected with 1µg reporter plasmid and 100 ng pRL-TK (Promega, Renilla luciferase) control vector using FuGENE 6 reagent (Roche Molecular Biochemicals). pGL3-basic (Promega), which lacks both a promoter and enhancer, was used as a negative control; pGL3-control (Promega), which contains SV40 promoter and enhancer sequences, was used as a positive control. At 48 h after transfection, when the cultures reached 70-80% confluence, cells were lysed, and luciferase activity was analyzed using the Dual Luciferase Reporter System (Promega) according to the manufacturer's protocol. Firefly and Renilla luciferase activities in cell lysates were sequentially measured using the Dual-Luciferase Reporter Assay System (Promega) and Lmax microplate reader (Molecular Devices). Because Renilla luciferase was simultaneously expressed and assayed from the same sample as the experimental construct, the activity of the experimental reporter for each sample was normalized by dividing the raw luminescence obtained from firefly luciferase by the raw luminescence obtained from Renilla luciferase. The mean values for individual constructs were
derived from three or more replicate experiments, each with three or more trials per construct. Statistical comparisons between constructs were performed using Student’s t-test.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared by standard procedure. Harvested 661W cells were washed with cold PBS and resuspended in 100ul NB1 (10mM Tris pH8.0, 10mM NaCl, 3mM MgCl₂, 0.5mM DTT, 0.1% Triton X-100, 0.1M sucrose) per 1×10⁷ cells. After gentle mix, same volume of NB2 (10mM Tris pH8.0, 10mM NaCl, 3mM MgCl₂, 0.5mM DTT, 0.1% Triton X-100, 0.25M sucrose) was added into tube and mixed gently. 50ul of NB3 (10mM Tris pH8.0, 5mM MgCl₂, 0.5mM DTT, 0.33M Sucrose) per 1×10⁷ cells were layered under the cell suspension and nuclei were collected by centrifugation at 2500rpm for 5min at 4°C. After remove supernatant (cytoplasmic fraction), nuclei were washed with 1ml of NB3 and resuspended in appropriate volumn (30µl per 1×10⁷ cells) of low salt buffer (20mM HEPES, pH7.9, 1.5mM MgCl₂, 20mM KCl, 0.2mM EDTA, 25% Glycerol, 0.5mM DTT, 0.5mM PMSF) by gentle tapping. Add equal volume of EB (high salt buffer) (25mM HEPES, 420mM NaCl, 1.5mM MgCl₂, 0.2mM EDTA, 0.5mM DTT, 25% Glycerol, 0.5mM PMSF, protease inhibitor cocktail) very slowly (possibly in small aliquots) while mixing with pipette tip. Incubate samples for 30-45 min at 4°C on a shaker or rotator and spin down at 14000g for 15min (4°C). Supernatant was dialyzed against buffer D (gel shift assay buffer; 20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP40, 2 mM DTT, and 0.1 mM PMSF) for 1hr at 4°C (Plaza, Dozier et al. 1995). Transfer supernatant to new tube and measure protein concentration. Nuclear extracts were stored at -70°C until needed.
To prepare the DNA probe for gel-shift analysis, an oligonucleotide for the site 1, potential activator binding site within CR2, (5'-ATTCCAATTGATGAAACTGCCAT-3’) and its complementary strand were synthesized, annealed, and labeled with \([\gamma^{32}P]\) ATP using polynucleotide kinase. Oligoprobe encompassing Meis1 binding site (5’-CGAAGCCGGCCTT GTCAGGTTGAGAA-3), which was identified and tested as a 5' lens enhancer from the other experiment (Chang, Jacobs et al. 1997; Zhang, Friedman et al. 2002), was radiolabeled and used as positive control. Binding reactions were performed as follows. Binding buffer containing 10 mM Na-HEPES (pH 7.4), 50 mM KCl, 5 Mm MgCl₂, 1mM DTT, 10% glycerol, 25ng/µL poly (dIdC), nuclear extracts, and 100X specific competitor were pre-incubated for 10min on ice. 7pmol of radiolabeled probe was added into pre-incubated mixture and incubated for 30min on ice. Samples were loaded on a 6% native polyacrylamide mini gel, run in 0.5X TBE buffer for 40min at 200V and examined by autoradiography after exposure of the dried gel to a Kodak XAR film at -70°C with an intensifying screen.

**Generation of CR2 deletion BAC**

*Pax6* BAC 293d08-E4-EGFP pA transgene was generated as previously described (Kim and Lauderdale 2006). FRT-kan-FRT targeting cassette was PCR-amplified from pCS2+ MTe GFP-FRT-kan-FRT using the *Pax6* forward targeting primer 5’-CAAGATCATGCTTTAAATTTCTG CAAAATATCATTATGAATAAATATAAGGCTCGGATCCACCGGATCTA-3’, and the *Pax6* reverse targeting primer 5’-CCAACTAGTGTAGCCAAAAAGAGTCTCTCCAAGTTCTTGAGA GACCCTATCTCGAAGTTCCATTCTCTTAGGAAAG-3’. Nucleotides in italics are homologous to *Pax6* sequences flanking CR2 region and those in roman are homologous to amplification cassette. BAC recombination was performed following the protocol of (Lee, Yu et al. 2001).
Double-resistant colonies (Cm\textsuperscript{R} Kan\textsuperscript{R}) were assayed for homologous recombination by PCR using the following primers: CR2flkF1 (5\textquotesingle-GCTAAACCACAAGCACTGGGG-3\textquotesingle), and CR2flkR1 (5\textquotesingle-ACACTGGGAGGGTAGAGACAGACG-3\textquotesingle). The kanamycin cassette was flipped out by limited induction of \textit{flipase}, and the cells were screened for kanamycin sensitivity (Kan\textsuperscript{S}). In these colonies, removal of the kanamycin cassette was verified by PCR using the CR2flkF1, and CR2flkR1 primers. The overall structure of the BAC293d08-\Delta CR2 was examined by fingerprint analysis (Gong, Zheng et al. 2003) using \textit{Bam}HI and southern blot analysis. Fingerprint for the BAC293d08\Delta CR2 was compared to that obtained for the BAC293d08+CR2. The sequences of the targeted regions were verified by automated sequencing of both DNA strands.

\textbf{Generation of Transgenic mice}

Transgenic mice were generated from pronuclear injection of closed circular BAC DNA into mouse oocytes (Lee, Yu et al. 2001; Gong, Yang et al. 2002; 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 \textit{Pax6} intron 3, 5\textquotesingle-GCAAGTTTTATGGTGTTTTGG-3\textquotesingle; reverse primer, located in \textit{EGFP}, 5\textquotesingle-CCTTGAAGAAGATGGTGCG-3\textquotesingle; the PCR product is 714 bp) and the deletion of CR2 (forward primer, CR2flkF1, 5\textquotesingle-GCTAAACCACAAGCACTGGGG-3\textquotesingle; reverse primer, CR2flkR1, 5\textquotesingle-ACACTGGGAGGGTAGAGACAGACG-3\textquotesingle). Because the endogenous CR2 locus are intact in mouse genomic DNA, these primer set will detect both endogenous CR2 region (1.9kb) and \Delta CR2 region from the BAC transgene (300bp).
Analysis of transgenic embryos for transgene expression

The pregnant females were killed using CO₂, 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 35 mm SLR camera. All embryos were then fixed by immersion in 4% paraformaldehyde/PBS at 4°C.

Results

Define the downstream regulatory region (DRR)

To identify evolutionarily conserved sequences 3’ to Pax6, I compared 80kb of the human genomic sequence extending from the HV breakpoint region to ELP4 exon4, which is near the telomeric end of YAC T593(Kleinjan, Seawright et al. 2001), with homologous region from pig, mouse, opossum, platypus, chicken, fugu, and the two Pax6 loci in zebrafish. Conserved sequences were identified by ranking gapped alignment scores based on length and identity; alignments with >70% nucleotide identity over 65bp were plotted. This alignment approach has been shown experimentally to be effective at detecting bona fide regulatory region(Flint, Tufarelli et al. 2001). Among several conserved sequences, the 5 distinct regions of non-coding sequence were highly conserved from mammals to birds and three of them also showed significant conservation with LG25 in zebrafish. I have designated these conserved regions CR1, CR2, CR3, CR4, and CR5 (Fig 4.1C). Consistent with this observation, several DNase1 hypersensitive(DH) sites, present only in PAX6-expression cell lines derived from the eye, were identified and mapped between the HV break point and ELP4 exon 7, indicating the
existence of putative regulatory factor binding sites (Gross and Garrard 1988). CR1 containing the HV breakpoint, is located in the lens element that has been tested and identified by transgenic mice experiment recently (Kleinjan, Seawright et al. 2001). CR2 is located in the 4.5Kb putative retina element that was also shown to drive reporter gene expression in neural retina, pigmented retina, and nasal pit and also contain three DH sites (Kleinjan, Seawright et al. 2001). These results suggest that the DRR is located between the HV breakpoint and $ELP4$ exon7 (Fig. 4.1A) and also provide several candidate genomic fragments that I will focus on to identify and define individual cis-regulatory elements.

The fragment C encompassing evolutionary conserved region (CR) 2 exhibit regulatory activity in the neural retina, RPE, and olfactory bulb.

**Development of a cell culture based transient genetic reporter assay.**

I have developed a cell-culture based transient genetic reporter assay for rapidly identifying and functionally characterizing candidate cis-regulatory elements within the $Pax6$ DRR. Because evolutionarily conserved sequence often contains important factor binding sites such as enhancer, silencer, or locus control region, cell-culture based reporter assay can provide fast and useful information to analyze and dissect the cis-acting genetic elements. In this approach, two different independent luciferase enzymes are simultaneously expressed and sequentially measured in a single cell system by Lmax™ Microplate Luminometer. Also I chose to use $Pax6$ expressing cell lines, 661W murine embryonic retinal cells (al-Ubaidi, Font et al. 1992) and $\alpha$TN4 murine lens cells (Yamada, Nakamura et al. 1990), for reporter assay. Because the tissue-specific factors that normally interact with regulatory elements within the DRR will be present in $Pax6$-expressing cell lines from the eye. To characterize theses cell lines prior to the
experiments, we performed RT-PCR for $P_0$, $P_1$, and $P_{\text{alpha}}$ transcripts. As previously reported, both 661W and $\alpha$TN4 cell lines expressed both $P_0$ and $P_1$ transcripts whereas NIH3T3 did not express $Pax6$ transcripts (Fig. 4.2A, B).

The fragment $C$ conferred tissue specific enhancer activity.

In our pilot screen, we tested 4 conserved fragment located in the DRR (fragments A, B.2, C, and D) (Fig. 4.2C). All of these fragments were subcloned into pGL3-promoter vector (Promega), which contained the SV40 promoter upstream of the firefly luciferase gene. Theses constructs were tested in parallel in 661W and NIH3T3 cells. In this assay, we identified two genomic fragments, fragment $C$ and $D$, showing clear enhancement over the promoter value in only $Pax6$ expressing 661W cells but not in $Pax6$ non-expressing NIH3T3 cells (Fig. 4.2D). In contrast, the genomic fragment containing non conserved sequences or flanking sequences of the DRR conferred little or no enhancer activity compare to promoter (Fig. 4.2D and data not shown). This finding demonstrates that genomic fragments encompassing conserved region contain regulatory elements that are functional in retina cells and that this assay can detect the existence of the regulatory elements. Of the fragments tested so far, fragment $C$ showed the highest level of activity in the 661W retina cells (Fig. 4.2D and data not shown). Therefore I have chosen fragment $C$ to further investigate for tissue specific regulatory activity in the context of a whole animal and also to functionally characterize the individual cis-regulatory elements within fragment $C$.

Although we used the SV40 promoter for reporter assay to normalize reporter activity against both pGL3-promoter and also pGL3-control vectors, it became necessary to use native $Pax6$ promoter to test the identified potential cis-regulatory fragment in vivo. Therefore, I
decided to use the mouse *Pax6* P₀ promoter (mP₀₀ₘᵟᵣₖ) to test conserved regions in the reporter system. The mouse minimal P₀ promoter (mP₀₀ₘᵟᵣᵣ) is 650bp long (Xu, Saunders et al. 1997), containing part of *Pax6* exon 0 (Fig. 4.3A) (Xu, Saunders et al. 1997; Xu, Zhang et al. 1999) and does not drive reporter gene expression by itself in the eye (Williams, Altmann et al. 1998; Kammandel, Chowdhury et al. 1999; Anderson, Hedlund et al. 2002). mP₀₀ₘᵟᵣᵣ has shown to drive reporter gene expression in various tissues including the lens, retina, pancreas and nasal placode with known *cis*-regulatory elements (Williams, Altmann et al. 1998; Kammandel, Chowdhury et al. 1999). Therefore, mP₀₀ₘᵟᵣᵣ is the ideal promoter to test potential retinal enhancer elements. I cloned fragment C with mP₀₀ₘᵟᵣᵣ into an EGFP reporter vector and tested for enhancer activity in *Pax6* expressing cell line (Fig. 4.3A). Cells were transiently transfected with mP₀₀ₘᵟᵣᵣ::eGFP or fragment C /mP₀₀ₘᵟᵣᵣ::eGFP constructs using Fugene transfection reagent (Promega) as described previously. Fragment C /mP₀₀ₘᵟᵣᵣ::eGFP construct showed enhancer activity in the density of expressed GFP and also the number of the cells expressing GFP (Fig. 4.3D, E). This observation demonstrates that mP₀₀ₘᵟᵣᵣ is functional in our cell culture system, and that the fragment C exhibit enhancer activity with the endogenous promoter mP₀₀ₘᵟᵣᵣ.

Because of the dynamic and complex control mechanism of *Pax6* expression, confirmation of the regulatory function of the identified regulatory element requires detailed analysis in the context of a whole animal system. We have chosen to use Zebrafish as an intermediate bioassay model system because transgenic analysis in mouse is both time consuming and expensive. Although we cannot rule out the possibility that there are species-specificity between fish and mammals, we have several lines of the evidences supporting the idea that the fragment C will exhibit normal activity in Zebrafish. First, phylogenetic footprinting demonstrate that CR2 is highly conserved from mammals to fish (Fig. 4.1), second, we have
shown that mBAC293d08 (Kim and Lauderdale 2006) can express mouse Pax6 protein in the retina, lens, forebrain, olfactory bulb, hindbrain, and spinal cord (data not shown). Therefore, I proposed to directly assay the potential regulatory element, fragment C, by analysis of mosaic expression of the reporter gene activity after microinjection into embryos at early cleavage stages. mP0\textsubscript{min}::eGFP construct alone drove very low level of EGFP expression in a variety of cell types including cells in the CNS, somite, blood and gut (Fig. 4.3B), whereas the C/mP0\textsubscript{min}::eGFP construct expressed relatively high levels of EGFP in the developing retina, RPE, and olfactory bulb (Fig. 4.3C). The 4.5 Kb fragment encompassing CR2 drove reporter gene expression in the retina, RPE, part of telencephalon, and nasal placode in other study (Kleinjan, Seawright et al. 2001), which is consistent with our observation. This data support the ideas that fragment C, likely within CR2, exhibits tissue specific regulatory activity in the transgenic animal and also that zebrafish can serve as a useful model system to functionally characterize potential regulatory elements.

**Functional characterization of individual cis-regulatory element within CR2**

To functionally characterize and define cis-regulatory elements within CR2, fragment C encompassing CR2 was subjected to deletion analysis using standard techniques. Figure 4.4A shows detailed restriction map and structures of the construct used in deletion analysis and their activity in 661W cells was determined using the dual luciferase assay. In this analysis, D3 construct containing 5' 363bp conserved sequence of CR2 showed a 2 fold higher luciferase activity than pGL3-FC containing full length CR2 (Fig. 4.4A). This data indicates the presence of transcriptional activator binding sites within the 363bp fragment and that this 363bp fragment itself is sufficient to drive reporter gene expression in the retina cell line. In addition, activity
from the construct D8 and D2 suggests the presence of some kind of negative regulatory factor binding sites within the *HindIII-NdeI* fragment.

**Determine and define the factor binding sites within regulatory element**

Screening for potential transcription factor binding sites using MatInspector software (Quandt, Frech et al. 1995)(Fig. 4.5), and also phylogenetic footprinting of the individual potential regulatory sequences (Fig. 4.4B and 5), revealed an array of putative factor binding sites that are conserved between mammals and birds. In this analysis, program (MatInspector software) matched known transcription factor binding sites from the library with subjected sequences with fixed core similarity (=1) and optimized matrix similarity. Using these techniques, I was able to identify a list of the putative transcription factor binding sites within the 363bp fragment and narrow down the protein profiles that are possibly responsible for enhancer activity of the regulatory elements (Fig. 4.5).

Interestingly, Meis1-Pbx1 heterodimeric complex binding site (site1) was one of the highly conserved sites within the 363bp fragment (Fig. 4.5B). Previously, Meis1 has been shown to directly bind to 5' lens enhancer to regulate *Pax6* expression during early eye development. Also Meis1 is known to be expressed in both lens ectoderm, and optic cup during early embryogenesis (Chang, Jacobs et al. 1997). Therefore, this site1 can be a potential activator binding site that is responsible for enhancer activity of 363bp fragment. Similarly, potential negative regulatory sequence within CR2 was also conserved between mammals and birds (Fig. 4.4B). However, in contrast with positive regulatory elements, I could not find any known factor binding sites within the negative regulatory element, suggesting that there are additional novel factors whose role in the *Pax6* regulation has yet to be determined (Fig. 4.5A).
To test factor binding ability of site1, 24bp oligoprobe (5'-ATTCCAAATGATTGAAAC TGCCAT-3') was subjected to gel mobility sift assay. 10ug of nuclear extracts from 661W or HeLa cells were used (Fig. 4.5C). As a positive control, previously known 26bp oligoprobe, which contains Meis1 binding sequences, was synthesized and used. Both oligoproses didn’t shift when nuclear extracts were absent. Specific DNA complex formation on both probes was observed with nuclear extracts. Complex formation (arrow head) is competed by unlabeled specific oligonucleotide at 100× molar ratios. This result indicates that site 1 harbors factor binding sequences that can be acting as a cis-regulatory element. Although I have here provided evidence that the 363bp fragment is sufficient to drive reporter activity in the retina cell line and that site1 within this fragment is capable of forming DNA-protein complex, further molecular characterization is necessary to prove that site1 is truly a Meis1 binding sequence and that this element is necessary for enhancer activity of CR2.

**Determine biological function of CR2 in native context.**

Previous studies from others and our lab, such as YAC rescue experiment (Kleinjan, Seawright et al. 2001), and human aniridic patients analysis(Lauderdale, Wilensky et al. 2000), suggested that Pax6 transcription in the eye is controlled by a hierarchy of regulatory elements, with the “master” elements located in the DRR. As a first step towards testing the function of specific Pax6 regulatory elements within the normal regulatory environment, I decide to remove DRR fragment C from BAC 293d08-EGFP, which uses EGFP as a reporter for Pax6 expression (Kim and Lauderdale 2006) (Fig. 4.6). In the BAC transgene 293d08-EGFPΔCR2, the CR2 region was replaced by a FRT-kan-FRT targeting cassette by homologous recombination and then the targeting cassette was flipped out by limited induction of flipase (Fig. 4.6A). This
doubly modified BAC was characterized by finger print analysis, southern blot, and sequencing (Fig. 4.6B, C). If our hypothesis was correct, transgene expression would be down-regulated in the developing eye, but remained normal level in the telencephalon, hindbrain, spinal cord, and pancreas. Alternatively, if separate elements within the DRR function to maintain an open chromatin configuration in ocular tissues, then we would observe only a fractional change in the pattern of transgene expression in the eye. To test transgene expression pattern, E13.5 embryos that were transiently transgenic for the BAC293d08ΔCR2 (Fig. 4.7E, F) were compared to the same stage of embryos from wild-type (Fig. 4.7A, B) or Tg293d08 line3 (Fig. 4.7C, D) mice. Tg293d08 line 3 mice express EGFP transgene that is comparable to endogenous Pax6 expression (Kim and Lauderdale 2006). When I compared these embryos, surprisingly, deletion of CR2 resulted in complete absence of transgene expression (Fig. 4.7F and data not shown). This result was unexpected because there are other known regulatory elements that have been shown to be involved in *Pax6* transcription in the eye, such as neuroretina enhancer (NRE) located in intron4 (Kammandel, Chowdhury et al. 1999), and C1170 Box 123 located 77kb downstream of *Pax6* transcription unit (Griffin, Kleinjan et al. 2002). Because there was no EGFP transgene expression, I did several molecular analyses to screen the transgenic embryos, including PCR, southern blot, and finger print analysis. First, I used PCR primers for EGFP transgene to see whether reporter region is intact in correct location. 4 out of 21 embryos showed positive signal for EGFP reporter region (Fig. 4.7G and data not shown). These 4 embryos also showed deleted CR2 region as well as intact CR2 region from endogenous locus with specific primer sets (Fig. 4.7H and data not shown). The fact that I have 4 independent lines showing identical results and that all of them have the same molecular characteristics suggests the possibility that CR2 may have a function that is different than a classical enhancer. One possible
explanation is that CR2 might act as part of Locus Control Region (LCR) to modulate global Pax6 transcription by changing chromatin structure. Although there is emerging evidence suggesting that LCRs are critical components to regulate gene transcription level, the function of CR2 in our experiment is still an open question.

Discussion

I here reported the identification of cis-regulatory element CR2 that is evolutionarily conserved from mammal to fish. Because highly conserved non-coding sequences often contain important factor binding sites, I used phylogenetic foot printing approach to identify such elements and characterized them by reporter assay using cell-culture system and also transient transgenic zebrafish as an intermediate step. Then to test the biological function of the identified elements in their native context, I generated transgenic mouse harboring BAC293d08-EGFP lacking the CR2 region. As result of this experiment, I discovered that CR2 might act as part of a LCR to regulate global Pax6 transcription.

Evolutionarily conserved non-coding sequences within DRR confer transcriptional activity in retinal cell line.

80kb long DRR has been implicated as a “master” regulatory region to control global Pax6 expression. However, little is known about individual elements within the DRR and the mechanism by which Pax6 transcription is affected. Although BOX 1, 2, 3(Griffin, Kleinjan et al. 2002) and 4.5kb non-coding region(Kleinjan, Seawright et al. 2001) within DRR have been identified and shown to have regulatory activity, we are far from understanding the entire structure or mechanism of the DRR. Therefore we performed pilot-screening of DRR using a
phylogenetic approach, and identified several potential regulatory elements. Of these conserved sequences, CR2 showed the highest transcriptional activity in 661W retina cell line and appears to have several interesting factor binding sites, including both positive and negative factor binding sites. Based on deletion analysis of the CR2 fragment, I concluded that 5’ 363bp fragment of CR2 contains activator binding sites that are sufficient to drive reporter expression in 661W cell lines and also transiently transgenic zebrafish (Fig. 4.3, and 4.4). In contrast, a 254bp HindIII/Ndel fragment showed repressive activity in the reporter assay. Further biochemical studies suggest (Fig. 4.5) that Meis 1 is a potential factor that binds to site1 within 363bp elements. Meis 1 is not only known as a factor that regulates Pax6 expression in the lens ectoderm (Zhang, Friedman et al. 2002), but also is known to be expressed within the retina.

Interestingly, in our deletion analysis of CR2, the D3 construct showed relatively high enhancer activity in Pax6 non-expressing 3T3 cells while full length CR2 showed no reporter activity in 3T3 cells (data not shown). This finding demonstrates that this ectopic enhancer activity from 363bp fragment in 3T3 cells is driven by transcriptional activators, which are expressed in both Pax6-expressing and non-expressing cells. In fact, when I tested the expression of Meis1 protein, by RT-PCR, I observed that Meis1 was expressed in both cell lines. In addition, this observation supports the idea that there are negative regulatory sequences adjacent to the 363bp positive regulatory sequence that modulate overall enhancer activity of the fragment C in a cell type specific manner. Although I have described molecular characteristics of CR2 and the possible mechanism by which CR2 affects gene transcription, studying the factors that are actually responsible for the CR2 transcriptional activity is still a necessary step to understand the complex Pax6 regulatory mechanism.
DRR might acting as Locus Control Region (LCR)

As a first attempt to understand the biological function of CR2 in the native context, I specifically deleted CR2 from the BAC293d08-EGFP transgene. Unexpectedly, removal of 1.67kb of CR2 caused the absence of transgene expression in the entire embryo. Although there are many open questions, based on our analysis, we proposed that CR2 is part of an LCR like structure within the DRR.

Locus control regions are emerging as important regulatory elements required for tissue-specific gene transcription in eukaryotes (Li, Peterson et al. 2002). LCRs are operationally defined by their ability to direct high-level, copy-number dependent expression of linked genes in transgenic mice irrespective of the chromosomal site of transgenic integration (Li, Peterson et al. 2002). Structurally, LCRs are composed of varying numbers of tissue-specific DNaseI hypersensitive (HS) sites in the chromatin of expressing cells. HSs typically encompass tissue-specific transcription factor binding sites and function as classical enhancer elements. LCR and LCR-like elements have been characterized for diverse genes in mammals and, where studies, are
evolutionarily conserved (Li, Peterson et al. 2002). LCR concept was first described from studies of the $\beta$-globin (Li, Zhou et al. 1990; Slightom, Bock et al. 1997). Studies in both human and mice have demonstrated that the $\beta$-globin LCR is required for high-level transcription of the globin genes (Forrester, Epner et al. 1990; Schubeler, Groudine et al. 2001). Although there have been several models to understand the mechanism of LCRs by which transcriptional activity is modulated, recent studies suggest that LCR is involved in forming an “active chromatin hub (ACH)”. The ACH is a special structure in which individual HS sites interact with each other and also with distant elements through looping (Tolhuis, Palstra et al. 2002; de Laat and Grosveld 2003; Patrinos, de Krom et al. 2004). In the $\beta$-globin locus, the LCR appeared to be required to maintain an open chromatin configuration (Patrinos, de Krom et al. 2004).

In our study, disrupting CR2 from the Pax6 native context results in loss of transgene expression. Also, as previously described, CR2 is evolutionarily conserved and contains factor binding sites and HS sites. Therefore, one possible model to explain the function of CR2 in its native context is that the CR2 elements located within the DRR enables the function of the regulatory elements located proximal to the transcript unit, perhaps by forming an ACH analogous to the $\beta$-globin locus. Although I only tested a small part of the DRR and there are many more conserved elements yet to be characterized, this finding led us to hypothesize that the DRR functions as a LCR or LCR-like element for the Pax6 locus and we speculate that it may control transcript initiation from the three Pax6 promoters by physically interacting with them.

References


**Fig. 4.1.** Defining the Pax6 Downstream Regulatory Region. (a) Physical map of human chromosome 11p13. (b) In transgenic mice, YAC construct used in rescue experiment in transgenic mice. YAC Y593, but not YAC Y589 rescues the mouse small eye (Sey) phenotype and homozygous lethality (Kleinjan, Seawright et al. 2001). (c) The proximal end of DRR is defined by the HV breakpoint. The human genomic sequences that drove expression in the lens, and retina, respectively, of transgenic mice are indicated by arrow. Phylogenetic analysis revealed five conserved region (*CR1-5*) between mammal and birds. CR1-3 are partially conserved on LG25 of zebrafish. DNaseI hypersensitive sites are indicated by star’(*)

Fig. 4.2. Conserved sequences exhibit the regulatory activity. (A, B) *Pax6* transcripts are initiated from both the P₀ and P₁ promoters in αTN4 and 661W cells. (A) Location of PCR primers used for RT-PCR. (B) RT-PCR analysis for *Pax6* transcripts. P₁-initiated transcripts containing (upper band) or lacking (lower band) the intron between exon1 and 2 are observed for both cell types; no amplicons were observed in the "no RT" control. Transcripts level was normalized by G3-PD. (C, D) Identification of *cis*-regulatory elements within the DRR. (C) Schematic diagram showing genomic fragments that are tested in this assay. Each fragment contains evolutionarily conserved sequences that are denoted as shade boxes below line. Genomic fragments were cloned into pGL3-promoter construct to assay regulatory activity. (D) Relative luciferase activity (±SEM) after transient transfection in 661W (n=7 experiment) or NIH3T3 (n=7 experiments) cells. Fragment C and D showed significant increase of luciferase activity while fragment A and B.2 did not show any activity in 661W cells.
**Fig. 4.3.** Fragment C is capable of driving reporter gene expression with tissue-specific manner *in vivo*. (A) Schematic diagram of reporter construct used in this assay. 650bp mouse minimal P₀ promoter was cloned into pEGFP-N1 vector to test promoter activity. Fragment C was cloned upstream of mP₀min to test tissue-specific activity of fragment C. (B) Zebrafish embryos transiently transgenic for mP₀min::eGFP; in this embryos EGFP is very weekly expressed in a few skin cells. Most embryos exhibit little to no EGFP expression. (C) Embryos transiently transgenic for mouse fragment C coupled to mP₀min::eGFP. 16/22 embryos expressed GFP; all expressed in the retina and lens (arrowheads), and 7/16 also expressed in the olfactory placode (arrow). (D, E) Same constructs were tested in 661W cell lines. Whereas mP₀min::eGFP construct drove very low EGFP expression (D), fragment C::mP₀min::eGFP expressed high level of EGFP(E).
Fig. 4.4. (A) Deletion analysis of fragment C revealed the presence of an enhancer and repressor element. Schematic showing the restriction map and deletion constructs tested. All constructs were cloned upstream of the SV40 promoter and tested for luciferase activity in 661W cells. The activity of each construct was compared to that of the promoter only construct (dotted line). Fragment C, construct D1, and D2 all showed comparable levels of activity, whereas D3 exhibited increased activity, which was promoter dependent (compared with d3Δp). No significant enhancement was observed for constructs D4, D5 and D6. However, activity was repressed from D7, and D8. (± SD; n= 12-15 trails/construct from 3-4 experiments). (B) Phylogenetic foot printing of fragment C showing highly conserved regions; boxes denote regions with nucleotide identities > 87%.
Fig. 4.5. Putative factor binding sites within cis-element CR2. (A) Schematic diagram showing putative factor binding sites within different fragments that showed transcriptional activity either positively or negatively in reporter assay. MatInspector software matched known transcription factor binding sites from the library for all vertebrates with subjected sequences with fixed core similarity (=1) and optimized matrix similarity. (B) Potential transcriptional activator Meis1/Pbx-1 heterodimer binding sequences is evolutionarily conserved except few differences in zebrafish LG3. Red indicates nucleotides that are not conserved. (C) Putative activator binding site 1 (24bp) from fragment d3 was subjected to EMSA. Oligoprobe encompassing Meis1 binding site (26bp), which was identified and tested as a 5' lens enhancer (LE) (Chang, Jacobs et al. 1997; Zhang, Friedman et al. 2002), was used as a positive control. 10µg of 661 nuclear extract was used for assay. No shifted band was detected when nuclear extract was absent for both LE and site1 oligoprobe (Lane 1 and 4). Both LE and site1 probes formed complex when 661W nuclear extracts were added (Lane 2, 3, 5, 6).
A

Min fragment C (363 bp)

Meis1/Pbx-1

OCT1

POUL-factor/Oct6

Hox1,3

NKX2.5

Mss1/2

Pax2/5/8

B

Meis1/Pbx-1 heterodimer binding site

<table>
<thead>
<tr>
<th>Species</th>
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**Fig. 4.6.** CR2 Deletion Strategy. We have deleted 1.67kb fragment C, which encompass CR2, from 160Kb BAC293d08-EGFP to test the biological function of CR2 in native context. Kanamycin gene flanked by FRT sequences were PCR amplified using primers that are homologous to sequences surrounding CR2 fragment. Homologous arms for targeting are denoted as pink boxes. (B) Sequence of the deleted CR2 region in BAC 293d08-EGFP. 1.67Kb of CR2 (red characters) is replaced by 70bp of transgene (blue characters) including FRT sequence. (C) BamHI restriction analyses of the BAC293d08-EGFPΔCR2. The deletion of CR2 region introduced a novel 9.1kb fragment in the ΔCR2 BAC (arrowhead). No other changes were observed. B, BamHI site.
Fig. 4.7. Transgene expression in CR2KO transgenic mice at E13.5. Embryos from wild-type, $Transgene^{+CR2}$, and $transgene^{ΔCR2}$ were compared to each other. (A, B) Bright field and fluorescence images, respectively, of an E13.5 wild-type embryos (A, C, D). No EGFP expression was detected. (C, D) At E13.5 EGFP transgene expression was observed in the cerebral vesicles (cv), developing eye (eye), spinal cord (sc), and hindbrain (hb) of $transgene^{+CR2}$ embryo. However, No EGFP expression was detected in $transgene^{ΔCR2}$ embryos (E, F). This pattern was observed in 4 independent lines. (G, H) PCR analysis for transgene in genomic DNA from each embryo. Genomic DNAs were isolated from chorion and used for PCR with primer sets described in material and methods.
Chapter 5

Conclusion

Millions of people suffer from a variety of visual impairments and ocular diseases. Aniridia is one of the rare panocular disorders in that many different parts of the eye may be affected. Aniridia is produced by a failure in eye development during embryogenesis due to a genetic mutation in the PAX6 locus. Pax6 expression is crucial to several steps in eye development. Mutations in *Pax6* cause panocular and progressive developmental disease that is characterized by severe iris hypoplasia, foveal/macular hypoplasia, cataracts, glaucoma, and corneal limbal insufficiency. Therefore understanding the activity and regulation of the *Pax6* gene would greatly facilitate the development of therapies that could restore vision or correct vision-related problems. Work over the past several years have shown that Pax6 expression is regulated in complex spatiotemporal patterns by a large number of regulatory elements spread over the entire *Pax6* transcription locus including 5', intronic, and 3' regions. However, the mechanism by which these regulatory elements work in coordination is far from understood. Because the regulatory regions are spread out over approximately 200 Kb genomic DNA, there are no tools that could facilitate the study of global regulatory mechanism(s) of Pax6 expression in its native environment. In addition, there are different isoforms of Pax6 including Pax6 (5a) and Pax6ΔPD which have potentially different unknown complicated regulatory mechanisms. Although Pax6 isoforms are believed to be involved in diverse functions of Pax6 during eye development, how and when these isoforms function is largely unknown. There were two goals of this dissertation. The first goal was to generate the tools for testing the long-range control of
Pax6 expression in vivo using transgenic mice and understand the function of downstream regulatory region (DRR) by isolating and characterizing new regulatory elements within the Pax6 DRR. The second goal was to understand the specific roles of Pax6ΔPD isoform during eye development using transgenic mice.

**Functionally define the Pax6 Downstream Regulatory Region**

The human PAX6 locus has been intensively studied (http://pax6.hgu.mrc.ac.uk/). In contrast, there are few studies of the mouse locus although most of the work on the function and regulation of Pax6 are performed in the mouse model system. In order to study and understand the regulatory mechanism of Pax6 expression, it was necessary to define the mouse Pax6 locus, the DRR within, and locate the known regulatory elements. I generated the BAC reporter system, which contains the upstream region, the entire Pax6 transcription unit, and the 30 kb DRR that we defined, thus transgene expression was under the control of endogenous Pax6 regulatory mechanism. As we expected, the transgene faithfully replicated Pax6 expression on cell by cell basis in all tissues within the eye through entire oculogenesis. Transgene expression was also maintained in the adult eyes. The BAC reporter system could be a useful tool in two ways. First, this BAC transgene will be used to understand the long-range regulatory mechanism that is controlled by the DRR. Pax6 expression is complex, studying the individual elements will not answer the questions regarding the coordinated regulation and the necessity of individual cis-elements in its native context. Second, the BAC transgene could be used to study the function of Pax6 in adult eye. There are stem cell colonies in the limbus region of adult cornea and the limbal stem cells (LSC) that are known to be involved in renewal of corneal epithelium (Collinson, Morris et al. 2002). LSC produce progeny cells called transiently amplifying (TA)
cells that express Pax6. However, it is not clear whether LSC itself express Pax6 or not. Recent studies suggest that Pax6 is involved in either function of LSC or maintenance (Wolosin, Budak et al. 2004). Cornea transplantation is one of the treatments for the patients of genetic ocular disorder or severe injuries in the eye. However, cornea transplantation is not an effective treatment for aniridic patients and it is believed that heterozygous mutation in Pax6 results in deficiency in limbal stem cells (Wolosin, Budak et al. 2004). Despite intensive studies, there are still no useful molecular markers that can be used to isolate stem cell populations. Potentially, the BAC transgene can be used to isolate LSC and also study the behavior of LSC or TA cells after transplantation. This research will greatly facilitate the understanding the role of Pax6 in adult cornea and the treatment of genetic ocular disorders.

**Identification of Pax6ΔPD isoform in mouse**

Three different Pax6 isoforms have been identified including Pax6, Pax6(5a), and paired-less Pax6 (Pax6ΔPD). Pax6ΔPD was first identified in quail (Carriere, Plaza et al. 1993) and subsequently in *C.elegans* (Chisholm and Horvitz 1995). In this study, I identified Pax6ΔPD in mammals for the first time using transgenic mice. As previously described, over-expression of Pax6ΔPD caused microphthalmic phenotype and the most obvious phenotype in the microphthalmic eye was severe lens degeneration. Although lens vesicle initiated to form and develop normally until E12, increased apoptotic cell death caused rapid lens degeneration starting around E12.5 and almost absence of lens by E15. Interestingly, this time point coincides with the developmental process in which the signaling pathways for lens differentiation are activated. It is known that many of the developmental pathways critical for lens induction are also critical for later stages of lens development (Lang 2004). The Fgf signaling pathway is
involved both in inductive signaling and in the regulation of lens fiber cell differentiation (McAvoy, Chamberlain et al. 1999). In the mouse, Pax6 is required for lens induction, but also activates β-crystallin later in lens development (Piatigorsky 1998). Therefore, if Pax6ΔPD has a role in signaling pathways for lens differentiation, we would expect to see some changes in expression of one of the downstream genes within the lens. However, the fact that we didn’t see any changes in lens fate marker expression such as Prox1 and β-crystallin suggest that degeneration of the lens is possibly caused by indirect effect of Pax6ΔPD on lens differentiation. Considering the restricted expression pattern of Pax6ΔPD in the retina but not in the lens, we concluded that over-expression of Pax6ΔPD in the retina affects lens development. These results suggest that Pax6ΔPD has a role during eye development in a cell non-autonomous manner to mediate signaling pathways for lens differentiation. In our genetic analysis, only double heterozygote (18-20 copies of transgene) but not the heterozygote (8-10 copies) showed microphthalmic phenotype. This finding suggests that Pax6ΔPD functions in dosage dependent fashion. Although over-expression of full length Pax6 results in microphthalmia (Schedl, Ross et al. 1996), the phenotype was varied and slightly different compare to the phenotype that I described here. Unlike Pax6 that rescued small eye phenotype when expressed in Sey/+ and Sey/Sey backgrounds, Pax6ΔPD enhanced the Sey phenotype suggesting that Pax6ΔPD is acting as dominant negative factor against Pax6. Alternatively, Pax6ΔPD might activate different sets of downstream genes and over-activation of certain downstream gene(s) caused the microphthalmic phenotype. Although Pax6ΔPD lacks the paired-domain, the homeodomain itself is also capable to bind to certain DNA sequences such as a palindromic HD binding site P3/RCS1 (Sheng, Thouvenot et al. 1997). Therefore, we can not rule out the possibility that Pax6ΔPD up-regulated different sets of downstream genes that are related to signaling pathways
for lens differentiation. Understanding the distinct function of Pax6ΔPD will greatly facilitate analyzing a complex regulatory mechanism(s) and functions of Pax6 isoforms during eye development. Unfortunately, there is little information available for paired-less Pax6 in Quail and C. elegans. Most studies of paired-less Pax6 in quail were performed in cell culture system, therefore, it is not clear what is the normal function of this isoform in vivo (Carriere, Plaza et al. 1993; Dozier, Carriere et al. 1993). In C. elegans, Pax6 has a slightly different function compared to mammalian Pax6. Therefore, if our analysis can provide the clue(s) to understand the different functions of Pax6 isoforms, we can begin to make the connections between each DNA binding domain (PD or HD) and their downstream genes during eye development. Also, unlike Pax6, Pax6ΔPD was localized in both the cytoplasm and the nucleus. This finding was consistent with study of Pax6 paired-less isoform in Quail (Carriere, Plaza et al. 1995), and C. elegans (Zhang, Ferreira et al. 1998). Although it is known that a positive feedback loop of paired-less Pax6 isoform (MAB-1) is activated by regulated nuclear entry of this isoform in C. elegans, why paired-less Pax6 in Quail, and mouse is also localized in both the nucleus and cytoplasm is not clear. In addition to Pax6, Prox1 and Tbox transcription factors were shown to be localized in both the cytoplasm and the nucleus. Although the mechanism that controls the nuclear entry of Pax6ΔPD is not clear yet, it is a very interesting observation that such an important transcription factors are sharing similar features.
References


