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Review Article

Molecular Regulation of Eye Development: Role of the PAX Gene

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Abstract

The development of *eye* has long been studied using classical embryological methods in birds, amphibians and primates. Early research has described interactions between different elements that take part in formation of the *eye* at the tissue level. But molecular regulation of *eye* development is a field that seems to invite further exploration. A number of ocular mutations and syndromes have been studied to elucidate regulatory molecules in *eye* development. Positional cloning of genes at mutated loci form the basis of functional and chromosomal location data in responsible molecules. Particular attention has been devoted in recent researches to the molecular biology of the *PAX6(paired box)* gene because of its high degree of conservation and its ability to regulate development of compound and simple *eye* structures in both vertebrates and invertebrates through loss and gain of function mechanisms. This paper comprehensively explores the molecular structure of PAX 6 gene, its role in *eye* development, its analogues and the full potential of its therapeutic and developmental applications.

Introduction

The *eye* develops from an out-pouching of the diencephalon. A series of induction and regionalization events in the neuro-ectoderm generate and shape the optic cup, vesicle, lens etc. The origin of Pax(paired box protein) genes predates the origin of *eye*s and even the nervous system. The ancestral Pax gene (PaxB) was related to Pax2,5 and 8. Interestingly, it was identified in the sponge that has neither *eye*s nor the nervous system. Cnidaria are the most basal animal models that have simple or complex (lens containing) *eye*s as well as Pax genes. Cnidarian (*Tripedalia*) PaxB gene is expressed in lens and retina and activates both lens *crystallin* and *opsin* reporter genes. This data indicates that modern the *Pax2* and *Pax6* genes evolved from a cnidarian PaxB ancestor by the mechanism of duplication and diversification in Bilateria with Pax2 loosing its HD and *PAX6* loosing its octapeptide (yellow box) along the way. Aniridia, a human genetic disease manifested by alterations in structure and function of the *eye*, includes reduced iris size, absence of fovea, and lens deformities. [1] It was documented as a genetic disease more than a hundred years ago. It is an ideal model for demonstrating autosomal dominant genetic disorders because of the high penetrance of its mutant alleles, the ease of diagnosis at birth, and a relatively similar incidence in various populations. [2] Recently, the aniridia gene (AN) was mapped (to chromosome band 11p13).[3] Pax-6, also called the aniridia type II protein (AN2) or oculorhombin is a protein that is encoded by the *PAX6* gene in humans. The gene *PAX6* was determined to be the k*ey* regulator of *eye* development as well as that of the central nervous system, with researches proving that *PAX2* expresses more in the optic stalk and the *PAX6* expressing predominantly in the optic cup [1]. Many studies that led to the identification of *PAX6* as the human aniridia gene were conducted in lower mammals. These studies demonstrated *PAX6* as a very sophisticated gene, that is highly conserved among vertebrates and lower animals. The *PAX6* 'dosage effect' in cases of aniridia ranges from mild loss of visual acuity and cataracts to severe nervous system defects and synopthalmia (conjoint *eye*s) or anopthalmia (complete absence of the *eye*s) [4] *PAX6* homologues have been located in *Drosophila* fly (*eye*less), with similar functions and similar mutant phenotypes. The amino acid sequence identities of approximately 90% are found [5]. *PAX6* is 96% identical in its amino acid sequence to pax(zf-a) in zebrafish; but the two species diverged over 400 million years [6]. This degree of conservation parallels that of histones, one of the most highly conserved proteins known. The knowledge of the mechanism of *PAX6* explained

unique evolution trends that Darwin had not been able to. In his notable work, 'Origin of the Species', Darwin found it difficult to explain the evolution of structures as dissimilar as simple vertebrae *eye*s and compound insect *eye*s. He speculated that these structures might have developed separately through the mechanism of 'convergent evolution' [7]. However, in both insects and vertebrates, *PAX6* is expressed in the embryo just prior to the formation of the optic vesicle. It has been found that mis-expression of a *PAX6* homologue in flies and chicks can induce ectopic *eye*s. Based on this evidence, it has been speculated that *PAX6* is a master regulatory gene that induces *eye* development in a broad range of animals [8].

Discussion

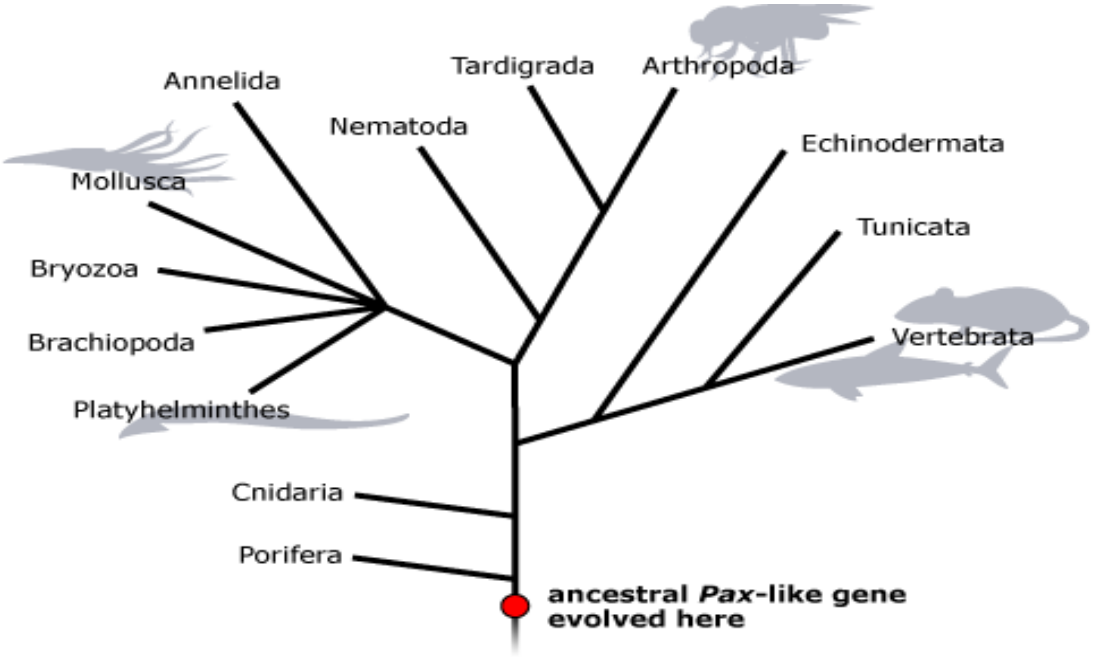
The multi-gene family of PAX

PAX6 belongs to the *PAX* multigene family of transcription factors that help regulate embryonic differentiation. These genes encode proteins that include a 128-amino acid sequence-specific DNA-binding domain, the Paired box, which can regulate the expression of other genes. Recent research on *Pax6* paired domain binding has revealed a possible structure of three A-helixes [9], a consensus DNA-binding sequence, and evidence for conformation changes in protein binding [10]. Nine unlinked PAX genes have been identified in *Drosophila* larvae. The *PAX6* protein is one among the many transcription factors that induce embryonic differentiation along the major body axis. These transcription factors bind to specific DNA sequences of other genes in response to concentration gradients of regulatory proteins, and adjust their expression; thus translating positional information into developmental patterns for distinct structures [11]. Like *PAX6*, many other *PAX* genes are expressed in the developing nervous system. These are believed to regulate neurogenesis. In mice, Pax1 is expressed in the developing vertebral column and thymus; *Pax3* in the early neural tube; *Pax6*, in the developing hindbrain and forebrain upon closure of the neural tube, *Pax8* in the neural tube, hindbrain, and thyroid. *PAX3* mutants result in deafness, depigmentation, and spina bifida (failure of neural tube closure) [8]. *PAX* genes number 3,4,6,7 also contain another common DNA-binding element, the homeobox(*HOX*). The homeobox was first discovered in *Drosophila*. It encodes a 60-amino acid homeo-domain that is known to be part of more than 0.2% of the total vertebrate genes. The homeo-domain also contains three A-helices, one of which is responsible for target sequence recognition [5]. Recent research has shown that the Pax 6 paired domain and homeo-domain may interact cooperatively to recognize multiple DNA binding sites [6]. Unlike the *HoX* family of homeobox-containing genes, which regulate many aspects of embryonic morphogenesis, PAX genes are not clustered but are dispersed randomly throughout the genome [12].

Structure of the PAX6 Gene

Human *PAX6* is transcribed as 2.7kb mRNA and encodes a 422-amino-acid protein. This protein includes the paired box, the homeo box, and a DNA-binding motif called the PST domain-Proline, Serine, and Threonine-rich sequence [3]. (Figure1). Interestingly, *PAX6* contains an alternative mRNA splice-site in the paired domain which can result in a 42-nucleotide insertion. This insertion allows the carboxy terminal subregion of the paired domain to recognize a novel DNA sequence, therefore allowing *PAX6* to regulate an expanded or restricted set of genes depending upon how the mRNA is spliced (Epstein, et al. 1994). *PAX6* extends over 22kb and contains 14 exons and intron sequences in the homeobox. Additionally, there is a CCAGCATGC translation start site in exon 4, a TAA stop codon in exon 13, a transcription start site and promoter region with TATA, CAAT, and GC regulatory elements, and atleast three possible polyadenylation signals in several converging lines of research. Mutations at various locations within the *PAX6* gene give rise to "dosage effects" that support the hypothesis that *PAX6* regulates gene expression during *eye* development through concentration gradients with other transcription factors. In a popular study by Glaser on a family with congenital cataracts, aniridia, anophthalmia and central nervous system defects, it was found that truncation of *PAX6* in the PST domain by a point mutation in exon 12 led to cataracts and decreased visual acuity in the father; truncation of *PAX6* in the paired domain by a point mutation in exon 6 led to iris absence, cataracts, severely decreased visual acuity, and other ocular malformations in the mother; and a compound heterozygote daughter with a copy of each of the parent's mutated *PAX6* genes died eight days after birth with severe central nervous system and craniofacial anomalies. Research on *PAX6* was facilitated by the discovery of a *PAX6* homologue in *Drosophila*, called the *eyeless (ey)* gene [4]. Mutations in *ey* produced *eye* defects similar to those produced by S*ey* in mouse and *PAX6* mutations in humans. *Drosophila* provides a convenient model for *PAX6* because its growth is rapid, its genetic and embryonic mechanisms have been well-characterized, and it is simple and inexpensive to maintain. Like *PAX6*, *ey* is expressed in nerve cord, specific regions of the brain, and in embryonic *eye* precursors. Most researches on the *PAX6* focus on developmental pathways that lead to *eye* formation. Upstream and downstream regulatory genes have been investigated for *ey* in *Drosophila*. A number of regulatory elements in the *PAX6* and *ey* genes have been identified through gel-shift and foot-printing assays and transgeneic in vivo studies in mice expressing a lacZ reporter under the control of various *PAX6* regulatory elements. The popular hypothesized downstream targets of *ey* include *eyes absent, sine oculis*, and *dac*hshund [10]. Relatively few upstream regulatory protein products of *PAX6* or *ey* have been identified. Possible upstream regulators of *PAX6* include activin A [9] and *sonic hedgehog* (SHH), another developmental control gene that produces separation of the *eye* primordial into two separate *eye* fields [12]. Defects in SHH signalling lead

to conditions like synopthalmia.



Mouse *Pax6* gene:

GTATCCAACGGTTGTGTGAGTAAAATTCTGGGCAGGTATTACGAGACTGGCTCCATCAGA

Fly *eyeless* gene:

Genetic similarity to mouse: 76.66%
Protein similarity to mouse: 100%

GTATCAAATGGATGTGTGAGCAAAATTCTCGGGAGGTATTATGAAACAGGAAGCATACGA

Shark eye control gene:

Genetic similarity to mouse: 85%
Protein similarity to mouse: 100%

GTGTCCAACGGTTGTGTCAGTAAAATCCTGGGCAGATACTATGAAACAGGATCCATCAGA

Squid eye control gene:

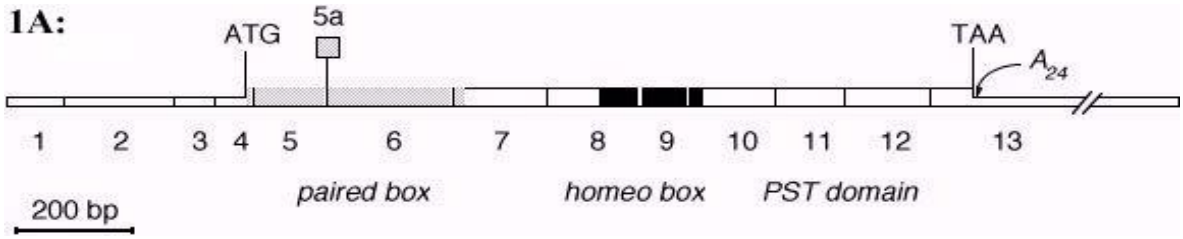
Genetic similarity to mouse: 78.33%
Protein similarity to mouse: 100%

GTCTCCAACGGCTGCGTTAGCAAGATTCTCGGACGGTACTATGAGACGGGCTCCATAAGA

Flatworm eye control gene:

Genetic similarity to mouse: 71.66%
Protein similarity to mouse: 100%

GTGTCTAATGGTTGTGTTAGTAAAATACTTTGCCGATATTATGGAACAGGTTCTATTAAA



Human PAX6 gene exon/intron structure

	PAX6 variant 1		PAX6 variant 2		PAX6 variant 3	
#	Exon	Intron	Exon	Intron	Exon	Intron
1	217	100	119	194	153	6794
2	188	3903	188	3903	188	3903
3	77	387	77	387	77	387
4	61	3568	61	3568	61	3568
5	131	928	131	792	131	940
6	216	705	42	95	216	705
7	166	5903	216	705	166	5903
8	159	516	166	5903	159	516
9	83	230	159	516	83	230
10	151	99	83	230	151	99
11	116	2578	151	99	116	2578
12	151	691	116	2578	151	691
13	5228		151	691	5228	
14			5228			

Exons for CDS in red. Sequence length in base pair

Figure 2 - Multi gene family and molecular structure of PAX. Image courtesy- Understanding evolution- available at:
http://evolution.berkeley.edu/evolibrary/article/1_0_0/eyes_10

PAX Isoforms

The vertebrate *PAX6* locus encodes three different protein isoforms-the canonical *PAX6*, the *PAX6* (5a), and the *PAX6* (ΔPD). The canonical *PAX6* protein includes an N-terminal paired domain, connected by linker regions to paired-type homeodomain, and to a proline/ serine/ threonine (P/S/T)-rich C-terminal domain. Each of the paired domain and paired-type homeodomain have DNA binding activities, whereas the P/S/T-rich domain possesses a transactivation function [1]. *PAX6* (5a), being a product of the alternatively spliced exon 5a, results in a 14 residue insertion in the paired domain that alters the specificity of this DNA binding activity. The nucleotide sequence corresponding to the linker region encodes a set of three alternative translation start codons from which the third *PAX6* isoform originates. Collectively, these are known as the *PAX6* (ΔPD) or pairedless isoforms. These three gene products lack a paired domain. The paired less proteins have molecular weights of 43, 33, or 32kDa, depending upon the particular start codon used. *PAX6* transactivation function is attributed to the variable length C-terminal P/S/T-rich domain that stretches 153 residues long in human and mouse proteins.

'Gain of function mechanisms' showing PAX6 as a master regulator

PAX6 is hypothesized to be a "master regulator of *eye* development", because *ey* expression is not affected by mutations in other *eye*-determining genes, and also because *PAX6* is so high-

ly conserved [2]. A "knockout" model was created using mice during the point of time when the mouse did not express *PAX6*. This 'knockout" model is eyeless or has very underdeveloped *ey*es further indicating *PAX6* is required for proper *ey*e development. This hypothesis was recently supported by the finding that misexpression of *ey* could induce ectopic eye formation on appendages in *Drosophila* [1]. Interestingly, these ectopic eyes were fully formed and included the full complement of cell types and structures, including photoreceptors. Later research revealed that genes falling "downstream" of *ey* could also induce ectopic eyes. The products of Sine oculis (so), and eyes absent (*ey*a), form a complex that can induce ectopic eyes similar to those formed by ectopic *ey* expression [12]. More significantly, so and *ey*a could together induce *ey* expression, which is not consistent with the idea of *ey* being the master regulator of those genes. Additionally, *dac*hshund (*dac*); which is induced by *ey* expression and encodes a novel nuclear protein, and *ey*a mis-expression resulted in full ectopic *ey*e production as well, whereas *dac* and *ey*a alone could each weakly induce ectopic *ey*e formation [5]. These experiments suggest that the protein products of *ey* and its human homologue *PAX6* operate not in a hierarchical dogmatic pathway. Rather, they operate as a network with numerous feedback loops. Another hypothesis is that, because *ey*e regulatory genes are activated several times during development, th*ey* are turned on and off in sequence at each stage. The repeated use of the same regulatory genes during *ey*e development has been explained in conjunction with the high degree of conservation of the genes. During evolution, as eye formation progressed from simple photoreceptors to complex visual systems in insects and vertebrates, the same regulatory genes were co-opted for each new developmental pathway for the logic of convenience in familiarity [11]. It could well be likely, that *PAX6* and other *ey*e development genes also play a role in the development of other unrelated organs, such as pancreas and olfactory system [12]. *PAX6* may be involved in the larger process of organogenesis rather than only oculogenesis. The highly conserved *PAX6* and its *Drosophila* homologue *ey* are k*ey* regulators in the highly complex developmental pathway leading to the formation of both simple and compound eyes and possibly other organs as well in lower animals and humans. Currant researches are directed towards characterizing the complex network of regulatory genes involved. Several *PAX6* enhancer elements for upstream regulation of *PAX6*, and possibly even downstream products of *ey*a, so, or *dac* may play a regulatory role. In addition, there is limited evidence suggesting that *PAX6* may be expressed by the damaged *ey*e tissue to induce limited regeneration. Artificial upstream regulation of *PAX6* may eventually induce such regeneration. Finally, certain cancers, including alveolar rhabdo-myosarcoma, may be caused by PAX mutations.

Conclusion

Pax genes are developmental control genes that have a prominent role in encoding transcription factors. Their fundamental role in animal development has been well illustrated by genetic studies. Loss-of-function mutations cause specific developmental defects. Additionally, gain-of-function mutations have been implicated in cancer. For a fuller insight into the molecular mechanisms underlying Pax gene function, it is necessary to define Pax target genes, recognize proteins that modulate properties of Pax transcription factors by direct physical interaction and expose the basis of the haploinsufficiency phenomenon. With their multiple functions during development in various animal phyla, Pax genes can serve as a paradigm of how transcription factors control development. In addition, due to high Pax gene conservation throughout the metazoan kingdom it is possible to understand how the individual gene functions have been modified during the course of evolution. In fact, by focusing on one gene family in well-defined systems one can hope to contribute towards a better understanding of the sophisticated mechanisms underlying evolution, development and disease in general. In recent years, several medical applications have arisen based on researches on the *PAX6* gene family. Because of gene sequencing, prenatal diagnosis of aniridia is now possible. Further research is needed to explore the full potential of therapeutic applications of *PAX6* and a more comprehensive understanding of its role in *ey*e development.

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