

Secondary structure analysis of mutant PAX6 from patients provides insight to PAX6 associated variable phenotypes

Shukla, Sachin and Mishra, Rajnikant

Department of Zoology, Banaras Hindu University

The PAX6 (Paired Box 6) is a transcriptional regulator. It is expressed in brain, eyes, pituitary and pancreatic islets. The mutation in PAX6 leads to ocular (e.g., aniridia, anophthalmia, microphthalmia) and neural diseases (polymicrogyria, dementia). The phenotype is variable in penetrance and expressivity. The PAX6 contains two DNA binding domains (paired box domain (PD) and a homeodomain (HD)). The transactivation domain at C-terminus is rich in proline, serine and threonine. The PD consists of two sub-domains (N-terminal sub-domain (NTS) or PAI and C-terminal sub-domain (CTS) or RED). The missense mutation in any of its functional domains influences sequence recognition, DNA binding and transactivation properties. The mechanism of PAX6 function is not clearly understood because of presence of two DNA-binding domains and their independent and/or cooperative functions. It is also not feasible to study all aminoacid residues through mutagenesis. It is intended to study properties of the unique transcriptional regulator (PAX6) through InSilico. The secondary structure of PAX6 mutants reported from patients are selected for the present study. The missense mutations of PAX6 (G18W, R26G, A33P, I42S, S43P, L46R, C52R, V53L, G64V, G36A, G51A, V126D, R128C, R242T, V256E, S259P, P375Q and Q422R) spanning its functional domains were analyzed. We used various online servers like Jpred, PredictProtein, GOR IV, APSSP2, SOPMA, PSIPRED and SABLE for the analysis. The Relative solvent accessibilities (RSA) of the mutant and the wild type residues were also compared. The change in the contact residues of the mutants and calculations of energy level were done through SVMcon and MUpPro, respectively. The results show alteration in local conformation or misfolding of mutant PAX6 protein. The alteration in the contact residues of the PAX6 may influence modulation in DNA binding property and other cellular interactions. We correlate our *in silico* findings with the earlier reports obtained from the functional analysis of these mutants, *in vitro*. It is presumed to be the cause or effect of variable penetrance and expressivity of phenotypes. This report also shows one of the approaches to understand the molecular mechanisms and effects of mutations on the functional status of a protein like PAX6 whose structural properties are unknown.

Acknowledgments: Financial support from the CSIR, New Delhi (37(1256)/06/EMR-II) is gratefully acknowledged

Introduction:

The PAX6 (Paired box6), a member of *Pax* gene family is a transcriptional regulator that is involved in development of eyes, brain, spinal cord, pituitary and islets of pancreas. The PAX6 gene is located on chromosome 11p13 that encodes a highly conserved major protein of 422 amino acid residues [1-3]. It is a “master control gene” for eye development and also regulates the expression of large number of genes during embryonic development. The loss of its functions leads to anophthalmia, nasal hypoplasia and defects in central nervous system [4]. The PAX6 contains two DNA-binding domains, paired domain (PD) and homeodomain (HD). The PD function is contributed from fourth amino acid residue through 128 amino acids at N-terminus. The glycine (16.7%) and glutamine (12.8%) -rich region of 78 amino acids links PD to paired-like HD (61 amino acid residues). The PAX6 bears transactivation domain at C-terminus that is proline-serine and threonine (PST) rich. The PD is further differentiated into two [5] independent DNA-binding sub-domains (N-terminal sub-domain (NTS) or PAI, and C-terminus sub-domain (CTS) or RED). The PAI sub-domain is highly conserved whereas RED sub-domain is relatively variable. The NTS is proved to be more important for specific DNA-binding [6, 7]. The sequence which is recognized by NTS is called P6CON (*TTTTACGCTTGAGTTCAC*) whereas CTS is involved in binding with 5aCON site (*ATGCTCAGTGAATGTTTCATTG*) [8, 9]. The analysis of crystal structure of human PAX6 paired domain-DNA complex revealed that NTS contains two extended β -sheets at the N-terminal and three alpha helical regions between 20 to 60 amino acid residues. Some β -turns are also found in this region. The CTS mainly contains three alpha helical regions. The NTS uses a helix-turn-helix motif to dock against the major groove of the DNA binding region. The first few residues of the NTS form a β -hairpin that spans the minor groove of DNA and contacts sugar phosphate backbone of both DNA strands. This beta hairpin is followed by a β -turn that makes important base contacts in the minor groove [10, 11].

The *Pax6* gene also encodes an alternatively spliced form (Pax6+5a) with 14-amino-acid insertion in the paired domain. It is expressed only in vertebrates and exhibits unique DNA-binding properties. The paired domain(s) usually bind DNA predominantly by their amino termini but the Pax6+5a interacts with DNA through its carboxyl terminus. It doesn't bind to the P6CON rather it binds with 5aCON sequences through CTS of PD. It can bind to either half of the 22-nucleotide 5aCON site and even form higher order complexes, whereas no such properties of PD of PAX6 have been reported so far. The functional analysis of PAX6 and PAX6+5a mutations from human suggest that activation by PAX6 and PAX6+5a is modulated by specific cellular interactions [12].

The human and rodent PAX6 proteins are 100% identical through the coding region. The chick (96%) and Zebrafish (93%) also show strong similarity. The PD and HD maintain 80-90% identity from mammals to *Drosophila* and *C.elegans* [13]. The HD of PAX6 is also supposed to have three alpha-helices as compared to the high resolution crystal structure of a Paired (Pax) class cooperative homeodomain dimer on DNA [14]. The third helix contacts the major groove of the DNA and is responsible for sequence recognition. The helix 1 is preceded by a flexible N-terminal arm and separated by a loop from helix 2, which forms a HTH motif with helix 3. The Helix 3 (the recognition helix) and the N-terminal arm make base-specific contacts in the major groove and in the minor groove, respectively. The homeodomain (s) preferentially bind cooperatively as dimers to palindromic sequences composed of two TAAT-half sites. The reports also suggest that protein-protein interactions with the DNA-binding domains of PAX6 and paired-type-homeodomain may be instrumental in regulating the activity of PAX6 [15]. The last 40 amino acids of the PST domain constitute a highly conserved C-terminal peptide that has been implicated in modulation of DNA-binding by the HD [16]. The mutations like PAX6-G18W [17], PAX6-R26G [18], PAX6-A33P, PAX6-S43P, PAX6-G64V, and PAX6-V126D [19] are reported to cause congenital cataracts, Peter's anomaly, secondary glaucoma, aniridia, nystagmus, peripheral corneal vascularization, foveal hypoplasia, partial aniridia, atypical aniridia, optic nerve hypoplasia, macular hypoplasia and mild limbal corneal dystrophy. The PAX6-G36A and G51A [20] mutations cause optic-nerve hypoplasia and optic-disc coloboma, respectively. The PAX6-L46R and V53L mutants [21] cause nystagmus, coloboma, juvenile cataracts and glaucoma. The PAX6-I87R [22] results into aniridia. The PAX6-R128C [23] causes foveal hypoplasia. The mutant from homeodomain binding region of PAX6, R242T [24] results into pseudo-coloboma, whereas PAX6-V256E [25] and S259P [26] are the reported missense mutations from mouse. The mutations from the TD of PAX6 (PAX6-P375Q and Q422R) result into aniridia [27]. The overall analysis of reports through human PAX6 Allelic Variant Database [28-29] suggests that more than two third of mutations are associated with congenital eye malformations. It is likely that phenotypes associated with PAX6 missense mutations originate from abnormal protein function in a restricted number

of ocular cell types. The mutation spectrum of PAX6 reveals two major ocular phenotypes (aniridia and non-aniridia). The most common of these is aniridia, which is mainly characterized by the congenital absence of the iris, but also affects the cornea, lens and retina. The PAX6 mutations also cause a series of non-aniridia phenotypes, such as optic nerve defects, keratitis, microphthalmia, and foveal hypoplasia [30-31]. It is also observed that the aniridia phenotype is predominantly associated with mutation in premature termination codon (PTC), while non-aniridia phenotypes are found to be primarily associated with missense mutations [32]. The missense mutations probably lead to the generation of hypomorphic proteins that are able to perform some but not all of the normal functions of PAX6. The functional studies on missense mutations of the HD and transactivation region are highly limited [33, 34]. It is suggested that partially impaired DNA-binding may be a major cause of variable phenotypes.

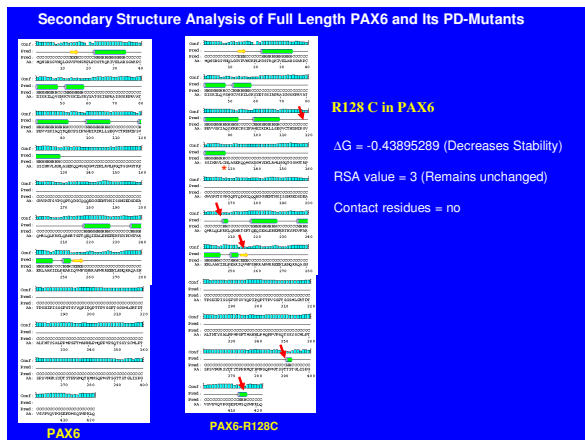
The role of PAX6 in development of eye, brain, spinal cord and pancreatic islets is known but the mechanism of its function is not clear. The reports emphasizing structure function relationship are also lacking. Secondary structure prediction is often regarded as a first step in predicting the structure of a protein. When suitable related 3-D template structures do not exist for a particular protein, secondary structure prediction is a strong viable alternative. It is also an important analytical tool, when total protein has not been crystallized, as is the case with PAX6. Unlike comparative modeling, it does not provide a full atom model of the tertiary structure, but provides a prediction of the secondary structure state of each residue, either helical, strand or extended sheet, or coil. Practically, the functional analysis of all amino acid residues of a protein like PAX6 is not always feasible through wet-laboratory experiments following site-directed mutagenesis. The *in silico* approach seems to be one of the best alternatives to study the effect of mutations on the structure and function of a protein like PAX6. The mutants from PAX6-PD (G18W, R26G, A33P, G36A, I42S, S43P, L46R, G51A, C52R, V53L, G64V, I87R, V126D, and R128C), PAX6-HD (R242T, V256E, and S259P) and the PST (P375Q and Q422R) were selected from PAX6 Allelic Variant Database (<http://pax6.hgu.mrc.ac.uk/>). In this report, the analysis (*in silico*) of secondary structures of selected missense mutations of PAX6 is described. It provides an approach to understand the molecular mechanisms and effects of mutations on the functional status of a protein like PAX6 whose structural properties are unknown.

Methodology: The PAX6 missense mutations, spanning all the three functional domains and showing variable phenotypes were selected from PAX6 Allelic Variant Database (<http://pax6.hgu.mrc.ac.uk/>). The mutations with known functional properties were preferred over those whose functional analyses are still under investigation. This was done for the purpose of establishing a correlation between the *In silico* and the experimental findings. Protein sequence database was explored for Pax protein family using BLAST [35] and analyzed through ClustalW [36] for MSA of Pax6 protein. Human PAX6 protein sequence was used as query template. Various online secondary structure-predicting servers, involving latest neural network based like Jpred [37], PredictProtein [38], NNpredict [39], APSSP [40], SAM-T06 [41-42], SOPMA [43] and PSIPred [44] have been used. Some traditional servers like Chou-Fasman [45] and GOR-IV were also explored in validation of the data. In absence of the 3-D modelling data, results have been supported with RSA and free energy change values. The change in the relative solvent accessibility of the residues has been counted through SABLE (<http://sable.cchmc.org/>) server [46]. The contact residues chart has been generated through SVMcon (<http://www.igb.uci.edu/>) server [47], whereas the effect of mutation on the stability of protein has been calculated through [MUpro](http://www.ics.uci.edu/%7Ebaldig/mutation.html) (<http://www.ics.uci.edu/%7Ebaldig/mutation.html>) [48].

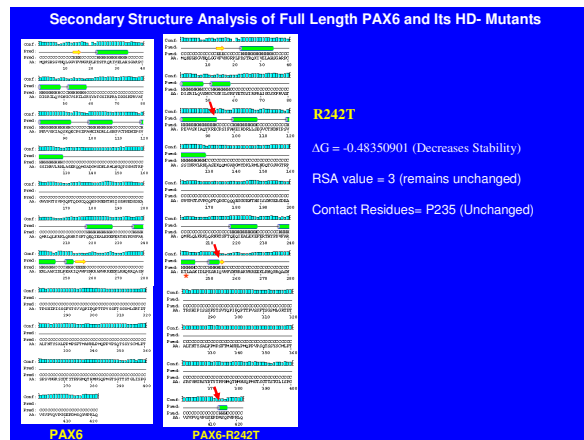
Significant results: Several missense mutations spanning all the three domains were analysed for their secondary structures through PSIPRED server. The predicted results were mapped to full length PAX6. It reveals that PAX6 contains a β -stranded sheet formed by 14, 15 and 16 residues, and three alpha helices from 23rd to 35th residue, 40th to 46th residues and from 50th to 58th residues. Significant changes were observed in PAX6 mutants as compared to wild-type PAX6. Most of the selected missense mutations were found to cause structural alterations with respect to the size and position of the helices and sheets in the secondary structure of PAX6. In most of the cases, these alterations were found to occur in the regions which are directly involved in the target recognition and DNA-binding, thus providing a strong evidence for their variable binding and transactivation leading to variable expressivities. Most of these mutations were found to alter the RSA values of the mutant residues and also increasing their free energy change values, leading to decreased stability of the mutant protein. From the contact residue chart, it is also clear that in most cases old contacts are destroyed whereas several new are formed.

It was interesting to observe the cross-talk between the functional domains of PAX6 (PD, HD, and TD). In some cases (R128C, R242T and P375Q), it was observed that a missense mutation in a particular domain causes structural alterations in the nearby domain. This suggests that various functional domains of PAX6 act together to carry out a particular function and thus supposed to work in a co-operative manner.

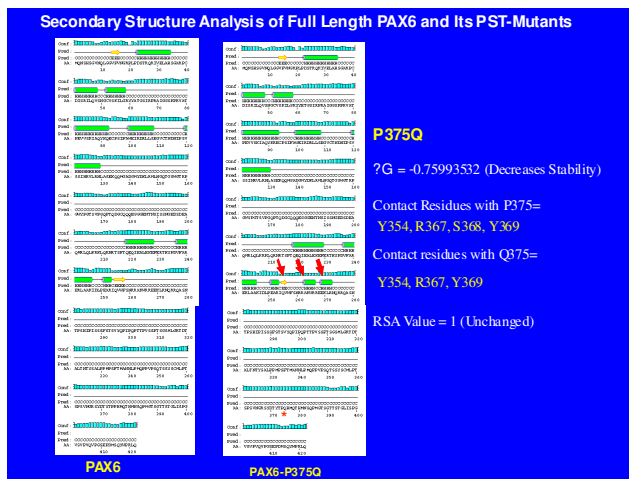
Conclusion:The report presents structural properties of PAX6. The local alterations in the secondary structure of the protein were observed which is referred as "distortion" as compared to the native structure of PAX6 in the PAI sub-domain of the PD of PAX6. It is explained that a point mutation of PAX6 abolishes a beta sheet in some cases or make it shorter. Such alterations are possible due to conformational changes induced by missense mutations in PAX6. It is supported through contact residue chart. It is also interesting to note that due to a single mutation several contacts are destroyed and many new contacts are introduced. Although the point mutation is in different region, but it causes conformational changes in the protein due to which β -strand is lost. It is likely due to space constraints or by some force constraints. The missense mutations in this report do not have same binding properties, which is also supported by our results that alteration in their secondary structure is also not similar. It is likely that missense mutations induce conformational changes in the entire PAX6 protein (Figure 1) along with structural sub-domains (Figure 2). The characteristic of missense mutations in PAX6 is consistent with the *in vitro* observations of the mutant proteins. The results show alteration in local conformation or misfolding of mutant PAX6 protein. The alteration in the contact residues of the PAX6 may influence modulation in DNA binding property and other cellular interactions. A direct indication of this possibility is the variation in the state of the residues involved in the formation of secondary structure (Helix, sheet, turns and coils) of the PAX6. Consequently, PAX6-mutated proteins may differentially interact with tissue-restricted proteins of the transcriptional machineries. It is presumed to be the cause or effect of variable penetrance and expressivity of phenotypes. This report also shows one of the approaches to understand the molecular mechanisms and effects of mutations on the functional status of a protein like PAX6 whose structural properties are unknown.



a) Secondary structure analysis of full length PAX6 and its PD-mutant (R128C)

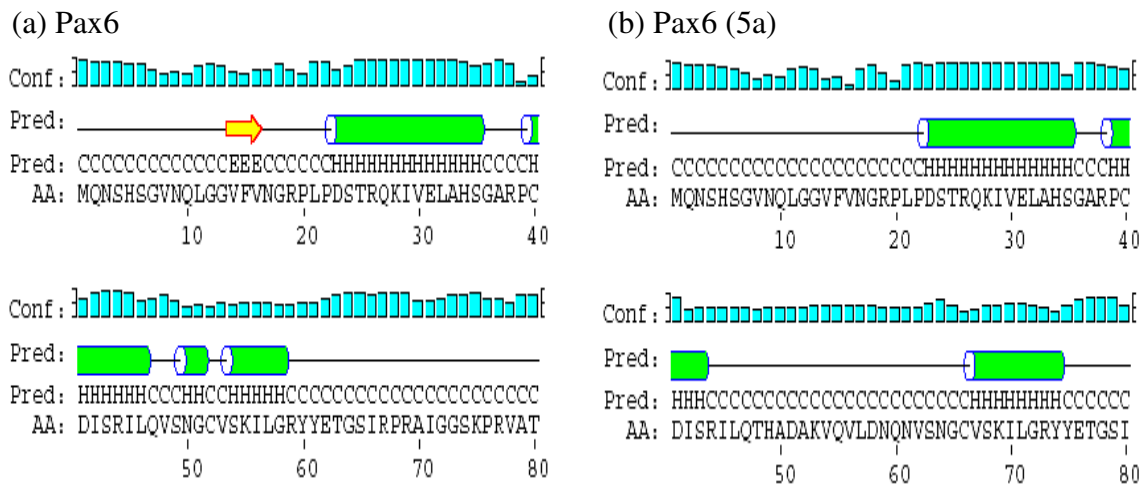


b) Secondary structure analysis of full length PAX6 and its HD-mutant (R242T)

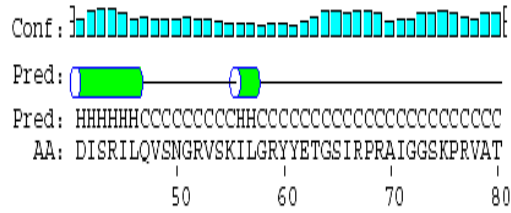
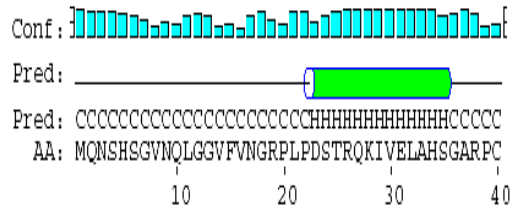


c) Secondary structure analysis of full length PAX6 and its PST-mutant (P375Q)

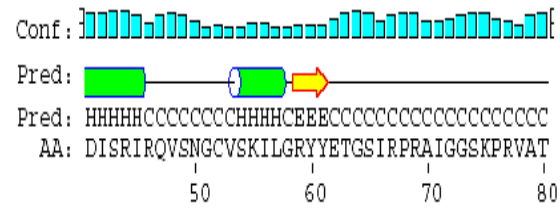
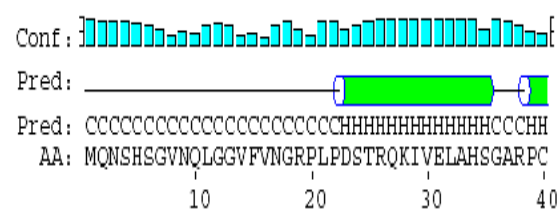
Figure 1: Secondary structure model of Full length PAX6 and its mutants, generated through PSIPRED server. The ‘E’ indicates β -strand region, ‘H’ indicates α -helix region and ‘C’ indicates coiled region. a) PAX6 and PAX6-R128C, b) PAX6 and PAX6-R242T, and c) PAX6 and PAX6-P375Q. The red arrows indicate significant alterations in the mutants compared to the PAX6.



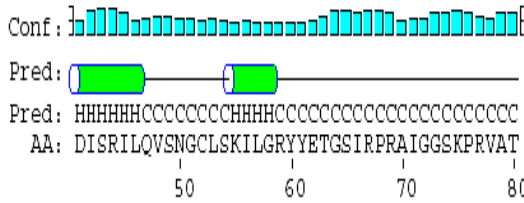
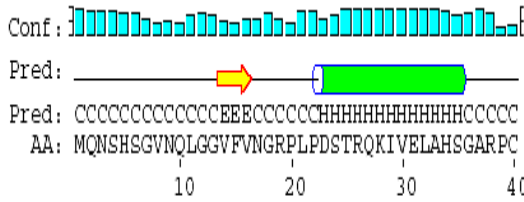
(c) C52R



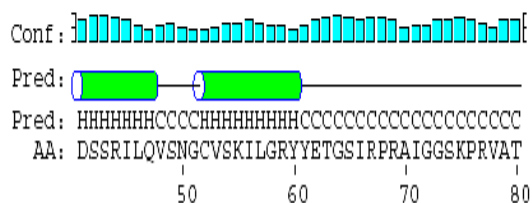
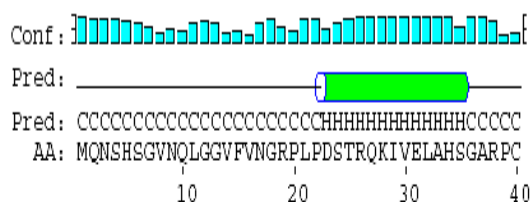
(d) L46R



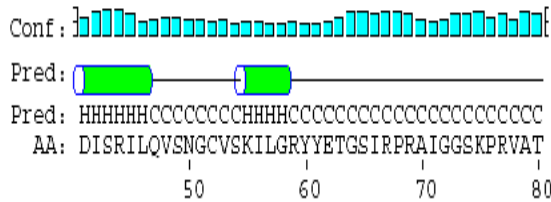
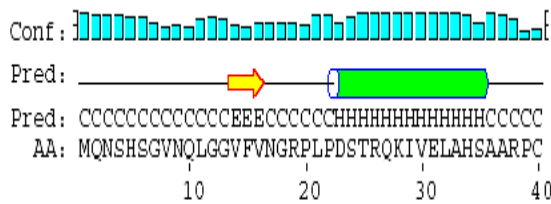
(e) V53L



(f) I42S



(g) G36A



(h) G51A

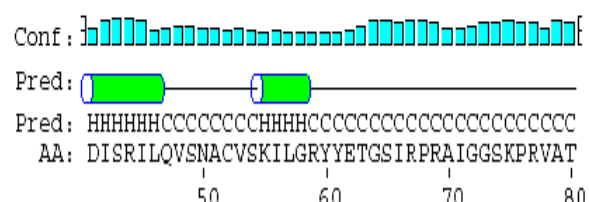


Figure 2

Secondary structure model of Pax6 and mutant proteins, generated through PSIPRED server. 'E' indicates β -strand region, 'H' indicates α -helix region and 'C' indicates coiled region. (a) Model of Pax6 indicating a β -strand at N-terminus and three α -helical regions. (b) Model structure of Pax6 (5a) isoform. Due to insertion of 14 amino acids at 47-70th position in PD, β -strand is absent, 2nd helix is curtailed and 3rd helix is shifted to a greater distance from the 2nd helix. (c) Model structure of Pax6 C52R mutant. Note the absence of β -strand and reduction in 3rd helix size. (d) Model structure of Pax6 L46R mutant. In this case, N-terminal β -strand is absent and a new strand develops after 3rd helix. (e) Model structure of Pax6 V53L mutant. β -strand is intact, but size of 3rd helix is reduced. (f) Model structure of Pax6 I42S mutant. All helices are intact but N-terminal β -strand is absent. (g) Model structure of G36A mutant. (i) Model structure of G51A mutant. In last two models, β -strand is present but 2nd helix is shifted and shortened and 3rd helix size is also reduced.

References:

- [1] Chi, N., Epstein, J.A., (2002). Getting your Pax Straight: Pax Proteins in development and disease. *Trends Genet.* **18**, 41-47.
- [2] Halder, G., Callaerts, P., Gehring, W.J., (1995). Induction of ectopic eyes by targeted expression of the eyeless gene in *Drosophila*. *Science* **267**, 1788-1792.
- [3] Tomarev, S.I., Chi, N., Epstein, J.A. (2002). Getting Your Pax Straight: Pax proteins in development and disease. *Int. J. Dev. Biol.* **41**, 835-842.
- [4] Glaser, T., Jepeal, L., Edwards, J.G., Young, S.R., Favor, J., Maas, R.L., (1994). PAX6 gene dosage effect in a family with congenital cataracts, aniridia, anophthalmia and central nervous system defects. *Nat. Genet* **7**, 463-471.
- [5] Jun, S., and Desplan, C., (1996). Cooperative interactions between paired domain and homeodomain. *Development* **122** , 2639-2650.
- [6] Chalepakis, G., Fritsch, R., Fickenscher, H., Deutsch, U., Goulding, M., Gruss, P., (1991). The molecular basis of the undulated/ Pax-1 mutation. *Cell* **66**, 873-884.
- [7] Treisman, J., Harris, E., Desplan, C., (1991). The paired box encodes a second DNA- binding domain in the paired homeodomain protein. *Cell* **67**, 1059-1074.
- [8] Epstein, J.A., Cai, J., Glaser, T., Jepeal, L., Maas, R.L., (1994a). Identification of a Pax paired domain recognition sequence & evidence for DNA dependent conformational changes. *J. Biol. Chem.* **269**, 8355-8361.
- [9] Epstein, J.A., Glaser, T., Cai, J., Jepeal, L., Walton, D.S., Maas, R.L., (1994b). Two independent and interactive DNA-binding subdomains of the Pax6 paired domain are regulated by alternative splicing. *Genes & Dev.* **8**, 2022-2034.
- [10] Xu, H.E., Rould, M.A., Xu, W., Epstein, J.A., Maas, R.L., Pabo, C.O., (1993). Crystal structure of the human Pax6 paired domain-DNA complex reveals specific roles for the linker region and carboxy-terminal subdomain in DNA binding. *Genes & Dev* **13**, 1263-1275.
- [11] Xu, W., Rould, M.A., Jun, S., Desplan, C., Pabo, C.O., (1995). Crystal structure of a paired domain-DNA complex at 2.5Å resolution reveals structural basis for pax developmental mutations. *Cell* **80**, 639-650.
- [12] Chauhan, B.K., Yang, Y., Cveklova, K., Cvekl, A., (2004). Functional properties of natural human PAX6 and PAX6(5a) mutants. *Inves. Ophthalmol Vis. Science* **45**, 385-392.
- [13] Bruun, J.A., Thomassen, E.I., Kristiansen, K., Tylden, G., Holm, T., Mikkola, I., Bjørkøy, G., Johansen, T., (2005). The third helix of the homeodomain of paired class homeodomain

proteins acts as a recognition helix both for DNA and protein interactions. *Nucleic Acids Res.* **33**, 2661-2675.

- [14] Wilson, D.S., Guenther, B., Desplan, C. and Kuriyan, J. (1995). High resolution crystal structure of a Paired (Pax) class cooperative homeodomain dimer on DNA. *Cell* **82**, 709-719.
- [15] Wilson, D., Sheng, G., Lecuit, T., Dostatni, N., Desplan, C., (1993). Cooperative dimerization of Paired class homeodomains on DNA. *Genes Dev.* **7**, 2120-2134.
- [16] Glaser, T., Walton, D.S., Mass, R.L., (1992). Genomic structure, evolutionary conservation and aniridia mutations in the human PAX6 genes, *Nat. Genet* **2**, 232-239.
- [17] Wolf, M.T., Lorenz, B., Winterpacht, A. et al., (1998). Ten novel mutations found in aniridia. *Hum. Mutat.* **12**, 304-313.
- [18] Hanson, I.M., Fletcher, J.M., Jordan, T. et al., (1994). Mutations at the *PAX6* locus are found in heterozygous anterior segment malformations including Peter's anomaly. *Nat. Genet.* **6**, 168-173.
- [19] Hanson, I., Churchill, A., Love, J., et al., (1999). Missense mutations in the most ancient residues of the PAX6 paired domain underlie a spectrum of human congenital diseases. *Hum. Mol. Genet.* **8**, 165-172.
- [20] Nallathambi, J., Neethirajan, G., Shashikant, S., Vijaylakshmi, P., Sundaresan, P., (2006). *PAX6* missense mutations associated in patients with optic nerve malformation, *Mol. Vis.* **12**, 236-242.
- [21] Chao, L.Y., Mishra, R., Strong, L.C., Saunders, G.F., (2003). Missense mutations in the DNA-binding region and termination codon in *PAX6*, *Hum. Mutat.* **21**, 138-145.
- [22] Tang, H.K., Chao, L.Y., Saunders, G.F., (1997). Functional analysis of paired box missense mutations in the *PAX6* gene. *Hum. Mol. Genet.* **6**, 381-386.
- [23] Yamaguchi, Y., Sawada, J., Yamada, M., Handa, H., Azuma, N., (1997). Autoregulation of Pax6 transcriptional activation by two distinct DNA-binding subdomains of the paired domain. *Genes Cells.* **2**, 255-261.
- [24] Morrison, D., FitzPatrick, D., Hanson, I. et al., (2002). National study of microphthalmia, anophthalmia, and coloboma (MAC) in Scotland: investigation of genetic aetiology. *J. Med. Genet.* **39**, 16-22.
- [25] Thaug, C., West, K., Clark, B.J. et al., (2002). Novel ENU-induced eye mutations in the mouse: models for human eye disease. *Hum. Mol. Genet.* **11**, 755-767.
- [26] Favor, J., Peters, H., Hermann, T., et al., (2001). Molecular characterization of Pax6(2Neu) through Pax6 (10 Neu): an extension of the Pax6 allelic series and the identification of two possible hypomorph alleles in the mouse *Mus musculus*. *Genetics.* **159**, 1689-1700.
- [27] Azuma, N. and Yamada, M., (1998). Missense mutations at the C-terminus of the PAX6 gene in ocular anterior segment anomalies. *Invest. Ophthalmol. Vis. Sci.* **39**, 828-830.
- [28] Brown, A., Mckie, M., van Heyningen, V., Prosser, J., (1998). The Human *PAX6* mutation database. *Nucleic Acids Res.* **26**, 259-264.
- [29] The PAX6 Allelic Variant Database (<http://pax6.hgu.mrc.ac.uk/>).
- [30] van Heyningen, V., Williamson, K.A., (2000). Pax6 in sensory development. *Hum. Mol. Genet.* **11**, 1161-1167.
- [31] Mirzayans, F., Pearce, W.G., MacDonald, I.M., Walter, M.A., (1995). Mutation of the *PAX6* gene in patients with autosomal dominant keratitis. *Am. J. Hum. Genet.* **57**, 539-548.

- [32] Tzoulaki, I., White, I.M.S., Hanson, I.M., (2005). *PAX6* mutations: genotype-phenotype correlations. *BMC Genetics* **6**, 27.
- [33] Singh, S., Chao, L.Y., Mishra, R., Davies, J., Saunders, G.F., (2001). Missense mutations at the c-terminus of *PAX6* negatively modulates homeodomain function. *Hum. Mol. Genet.* **10**, 911-918.
- [34] D'Elia, A.V., Puppini, C., Pellizzari, L., Pianta, A. et al., (2006). Molecular analysis of a human *PAX6* homeobox mutant. *Eur. J. Hum. Genet.* **14**, 744-751.
- [35] Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410. BLAST. National Centre for Biotechnology Information [<http://www.ncbi.nlm.nih.gov/>].
- [36] *ClustalW*. [<http://www.ebi.ac.uk/Tools/clustalw/index.html>].
- [37] Cuff, J. A., Clamp, M. E., Siddiqui, A. S., Finlay, M. and Barton, G. J. (1998). Jpred: A Consensus Secondary Structure Prediction Server, *Bioinformatics* **14**, 892-893.
- [38] Rost, B., Yachdav, G., and Liu, J., (2004). The PredictProtein Server. *Nucleic Acids Research* **32**, (Web Server issue), W321-W326.
- [39] Kneller, D.G., Cohen, F.E., and Langridge, R., (1990). Improvements in Protein Secondary Structure Prediction by an Enhanced Neural Network. *J. Mol. Biol.* **214**, 171-182.
- [40] Raghava, G. P. S., (2002). APSSP2: A combination method for protein secondary structure prediction based on neural network and example based learning. *CASP5*, A-132.
- [41] Karplus, K., Katzman, S., Shackelford, G., Koeva, M., Draper, J., Barnes, B., Soriano, M., and Hughey, R., (2005) .SAM-T04: what's new in protein-structure prediction for CASP6. *Proteins: Structure, Function, and Bioinformatics*, **61**(S7),135-142.
- [42] Karplus, K., Karchin, R., Draper, J., Casper, J., Mandel-Gutfreund, Y., Diekhans, M. and Hughey, R. (2003) Combining local-structure, fold-recognition, and new-fold methods for protein structure prediction. *Proteins: Structure Function and Genetics*. **53**(S6),491-496.
- [43] *Geourjon and Deleage, (1995), SOPMA SECONDARY STRUCTURE PREDICTION METHOD.*
[http://npsa-pbil.ibcp.fr/cgi/bin/npsa_automat.pl?page=npsa_sopma.html]
- [44] Jones, D.T., McGuffin, L.J., Bryson, K., (1999). *The PSIPRED Protein Structure Prediction Server.* [<http://bioinf.cs.ucl.ac.uk/psipred/psiform.html>]
- [45] Chou, P.Y., Fasman, G.D., (1978). Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol. Relat. Areas Mol. Biol.* **47**,45-148.
- [46] *The SABLE Server: General Information.*[<http://sable.cchmc.org/>],
[<http://folding.cchmc.org/software/software.html>].
- [47] *SVMcon: Prediction of amino acid contact maps using Support Vector Machines. Institute for Genomics and Bioinformatics, University of California, Irvine.*
[<http://www.igb.uci.edu/?page=tools&subPage=psss>]
- [48] *MUpro: Prediction of Protein Stability Changes for Single-Site Mutations from Sequences. Institute for Genomics and Bioinformatics, University of California, Irvine.*
[<http://www.ics.uci.edu/%7Ebaldig/mutation.html>]