Functional conservation of Pax6 regulatory elements in humans and mice demonstrated with a novel transgenic reporter mouse

Citation for published version:
Tyas, DA, Simpson, TI, Carr, CB, Kleinjan, DA, van Heyningen, V, Mason, JO & Price, D 2006, 'Functional conservation of Pax6 regulatory elements in humans and mice demonstrated with a novel transgenic reporter mouse', *BMC Developmental Biology*, vol. 6, 21. https://doi.org/10.1186/1471-213X-6-21

Digital Object Identifier (DOI):
10.1186/1471-213X-6-21

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published in:
*BMC Developmental Biology*

---

**General rights**
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

**Take down policy**
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Functional conservation of Pax6 regulatory elements in humans and mice demonstrated with a novel transgenic reporter mouse

David A Tyas1, T Ian Simpson1, Catherine B Carr1, Dirk A Kleinjan2, Veronica van Heyningen2, John O Mason1 and David J Price*1

Address: 1Genes and Development Group, Centres for Integrative Physiology and Neuroscience Research, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, UK and 2MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, UK

Email: David A Tyas - tyas_d@hotmail.com; T Ian Simpson - ian.simpson@ed.ac.uk; Catherine B Carr - c.b.carr@sms.ed.ac.uk; Dirk A Kleinjan - Dirk-Jan.Kleinjan@hgu.mrc.ac.uk; Veronica van Heyningen - veronica.vanheyningen@hgu.mrc.ac.uk; John O Mason - John.Mason@ed.ac.uk; David J Price* - David.Price@ed.ac.uk

* Corresponding author

Abstract

Background: The Pax6 transcription factor is expressed during development in the eyes and in specific CNS regions, where it is essential for normal cell proliferation and differentiation. Mice lacking one or both copies of the Pax6 gene model closely humans with loss-of-function mutations in the Pax6 locus. The sequence of the Pax6/PAX6 protein is identical in mice and humans and previous studies have shown structural conservation of the gene's regulatory regions.

Results: We generated a transgenic mouse expressing green fluorescent protein (GFP) and neomycin resistance under the control of the entire complement of human PAX6 regulatory elements using a modified yeast artificial chromosome (YAC). Expression of GFP was studied in embryos from 9.5 days on and was confined to cells known to express Pax6. GFP expression was sufficiently strong that expressing cells could be distinguished from non-expressing cells using flow cytometry.

Conclusion: This work demonstrates the functional conservation of the regulatory elements controlling Pax6/PAX6 expression in mice and humans. The transgene provides an excellent tool for studying the functions of different Pax6/PAX6 regulatory elements in controlling Pax6 expression in animals that are otherwise normal. It will allow the analysis and isolation of cells in which Pax6 is activated, irrespective of the status of the endogenous locus.

Background

Pax6 is a transcription factor containing an N-terminal DNA binding domain, a paired domain, separated by a glycine-rich linker sequence from a second DNA binding domain, a homeodomain, and a C-terminal proline-serine-threonine-rich transregulatory domain. It is highly conserved in very diverse species. In mammals, it is expressed during development in the eye, in specific regions of the CNS, in the nasal placodes and olfactory epithelium and in the pancreas [1-3]. Haploinsufficiency for Pax6 function (Pax6+/−) in the mouse results in the Small eye (Sey) phenotype [4]. Homozygotes (Pax6−/−) die perinatally with no eyes and many brain abnormalities [3-14]. PAX6 haploinsufficiency also causes eye and brain defects in humans [15,16].
Normal development requires not only that Pax6 be present in certain cells at certain times but also that it be present in the correct amounts. Schedl et al. [17] showed that severe eye abnormalities are caused not only by under-expression but also by over-expression of Pax6 in mice. Bishop et al. [18,19] and Muzio et al. [20] provided evidence that graded expression of Pax6 across the developing neocortex of mice is essential for the correct specification of its major areas. Such findings imply that Pax6 expression is tightly regulated and that different levels are maintained in different regions as they grow.

Work on humans has indicated that the PAX6 regulatory elements extend over more than two hundred kilobases [21-24]. The locus is highly conserved, both in the coding regions (human and mouse proteins are identical) and also in the non-coding regions, where similar long-range control elements have been identified in mouse and Fugu by genomic sequence comparisons and DNasel hypersensitivity analysis [24-26]. A YAC (named Y593) containing 420 kb of the human coding sequence and flanking regions, extending beyond its putative regulatory elements, rescues the mouse Pax6-/- phenotype, whereas a YAC containing 110 kb less flanking sequence does not [17,24]. In the work described here, we modified Y593 by introducing tau-GFP and neomycin resistance cassettes so that they would be controlled by the gene's regulatory elements and would prevent the production of PAX6 protein from the translational start site in exon 4 (Fig. 1). This new YAC (called Y1123) was then used to generate transgenic mice.

**Results**

**Initial characterization of transgenic mice**

Y593 was successfully modified as illustrated in Fig. 1 and as described in Methods to generate Y1123. This YAC was used to generate transgenic mice. Three fertile transgenic founders, named DTy22, DTy42 and DTy54, were identified. They all appeared phenotypically normal and successfully transmitted a tau-GFP-expressing Y1123 transgene to their offspring. PCR with primers marked in Fig. 1 (sequences in Methods) was used to examine the minimum extent of incorporation of Y1123: the results indicated that only DTy54 had incorporated at least the majority of Y1123, whereas DTy22 and DTy42 had incorporated truncated versions. Neither DTy22 nor DTy42 recapitulated the full expression pattern of Pax6. It was possible to identify DTy54 and DTy42 transgenic mice using a hand-held torch emitting blue light, of the wavelength required to excite GFP, and an appropriate filter, as described in [27]. This revealed GFP expression in the eyes of living DTy54 and DTy42 mice; the eyes of DTy22 mice did not express GFP. DTy42 mice expressed GFP only in the eyes. Using DTy54 mice, Y1123 was crossed into embryos that were either Pax6-/- or Pax6+/. Unlike the unmodified YAC Y593 [17], Y1123 produced no rescue of either the eye or brain defects in these mutants, confirming its predicted lack of function.

Quantitative PCR (qPCR) was used to compare relative fluorescence intensities following amplification with primers specific for human PAX6 and mouse Pax6 or for human PAX6 and mouse Pax3. Intensities following amplification for mouse Pax6 and Pax3 were halved (since there are two copies of each in the mouse genome). The ratios between the intensities from PAX6 and half the intensities from Pax6 and Pax3 are shown in Table 1. We conclude that one copy of Y1123 (or a part of Y1123 in DTy22 and DTy42) had integrated into the genome of each founder. Fluorescent in situ hybridization (FISH) using the entire FAT5 cosmid (Fig. 1) as a probe [17] on blood smears from DTy54 showed a single signal per cell, confirming that there were not multiple sites of integration. DTy22 and DTy42 were not studied further here.
Expression of tau-GFP in DTy54 mice

To test whether the tau-GFP expression in DTy54 mice was consistent with the established Pax6 expression pattern, embryos were collected at embryonic day (E) 9.5, 10.5, 12.5, 14.5 and 16.5 and eyes were taken from adults. Expression of tau-GFP was seen in the eyes at all ages; examples are shown at E9.5 (Fig. 2A,B), E10.5 (Fig. 2D) and E14.5 (Fig. 3A,B). Expression was present in the retina and lens, as expected, and allowed cellular processes to be visualised due to cytoplasmic labelling by tau-GFP. Particularly striking was label in the axonal projections of retinal ganglion cells, which could be seen in the optic nerve (Fig. 3B–D) and followed through the optic chiasm into the optic tract (Fig. 3E). Elsewhere, tau-GFP expression was confined to regions known to express Pax6 [1,6,7,9,10]. As expected, expression at E9.5–10.5 was in the forebrain (in the telencephalic and diencephalic vesicles), with a sharp posterior boundary of expression at the diencephalic/mesencephalic boundary [7,10], and also in the hindbrain and spinal cord (Fig. 2). Figure 3F shows a parasagittal section through the brain at E14.5: expression was in the cerebral cortex, prethalamus (also known as the ventral thalamus), pretectum, the basal plate in the region of the pons [28] and in the cerebellar primordium [11]. The intensity of label in the cerebral cortex was graded from high rostrally to low caudally (Fig. 3F), in line with the known gradient of expression of Pax6, which is shown using an anti-Pax6 antibody in Fig. 3M [18,20]. Similar to earlier ages, there was a sharp posterior boundary of expression at the border between pretectum and midbrain (arrow in Fig. 3F) [7,10]. In coronal sections, expression of tau-GFP was seen in the pineal gland (Fig. 3G) and nearby in the posterior commissure (not shown), which are also sites of Pax6 expression [14]. There was expression in the prethalamus (Fig. 3H) in a pattern similar to that shown with an anti-Pax6 antibody (Fig. 3L). In the pallium (Fig. 3H), there was a boundary of expression at the border between the pallium and subpallium, in agreement with the known boundary of Pax6 expression (Fig. 3K) [7,10]. Label was seen running ventrally to the amygdaloid region (Fig. 3H), mirroring the known expression of Pax6 in this area [9,29]. In the cerebral cortex, tau-GFP was seen in the radial processes of cells located on the ventricular side of the cortical wall (Fig. 3I); again, this was anticipated since Pax6 is known to be expressed in radial glial cells [30]. There was expression in the olfactory epithelium (Fig. 3J) [1,2].

Immunohistochemistry with anti-Pax6 and anti-GFP antibodies confirmed the presence of GFP in Pax6-expressing regions. An example of co-localization at E12.5 is shown in Fig. 4: Pax6 is expressed on the pallial side of the pallial/subpallial border (Fig. 4A), as is tau-GFP (Fig. 4B,C). Overall, we concluded that the patterns of label with tau-GFP are exactly as anticipated on the basis of the known expression of Pax6 and that regional differences in the intensity of label in the cerebral cortex reflect known differences in the level of expression of Pax6.

Analysis of DTy54 brains with flow cytometry

One of the potential uses of this transgenic mouse is to allow the isolation of Pax6-expressing cells. We demonstrated that this is possible using flow cytometry on dissociated cells from the brains of E14.5 embryos. The telencephalic vesicles were removed and each was cut into dorsal, lateral and ventral components. Data are shown in Fig. 5. Analysis of non-transgenic embryos provided frequency distributions of background fluorescence intensity (Fig. 5A). A gate was set to cover intensities above the upper limit of the fluorescence seen in these controls, on the basis that cells falling within this gate in transgenic embryos (Fig. 5B–D) were certain to be expressing tau-GFP. In samples from dorsal telencephalon of DTy54 embryos (Fig. 5B), a large proportion of cells had fluorescence levels within the gate. There were also large numbers of cells whose fluorescence intensities were not within the gate but were higher than the average intensity in non-transgenic controls. It is likely that these are cells expressing GFP at lower levels; for example, many may be in the process of down-regulating the transgene as they differentiate, which is the pattern of expression of Pax6 [1,6,12]. A similar picture was seen in samples from the lateral and ventral telencephalon (Fig. 5C,D). The average fluorescence intensity of cells within the gate was higher in the lateral telencephalon than in the dorsal telencephalon (peak shifted to the right in Fig. 5C compared to...
Fig. 5B), which agrees with the brighter fluorescence of cells in this region in sections (Fig. 3H). The proportion of cells within the gate was smallest in samples from the ventral telencephalon (Fig. 5D), which agrees with the fact that fewer cells in this region express Pax6 (Fig. 3H).

Discussion
In DTy54, the modified YAC that had integrated into the mouse genome did not affect the endogenous Pax6 locus, unlike an alternative strategy involving the insertion of a reporter gene into the endogenous locus [3]. The YAC1123 transgene can be crossed onto mice with any Pax6 status (e.g. Pax6\(^{+/+}\), Pax6\(^{+/-}\), Pax6\(^{-/-}\), Pax6\(^{loxP/loxP}\)) to identify and isolate those cells in which Pax6 is being activated by upstream factors. In addition to generating a useful new tool for understanding the role of Pax6, our results demonstrate that the elements regulating the human PAX6 gene present in Y1123 and Y593 [17] are necessary and sufficient to recapitulate accurately the expression of Pax6 in mice. This indicates that these elements are not only structurally [24,25] but also functionally highly conserved. In their original study of mice containing human PAX6-expressing YACs, Schedl et al. [17] suggested functional conservation of the regulatory elements controlling the human and mouse genes on the basis that the human locus is able to complement the Sey mutation in mouse. The introduction of PAX6-producing transgenes corrected the eye defects in heterozygotes and rescued homozygotes from perinatal death. It remained unclear, however, how accurately the human regulatory elements reproduce the pattern of endogenous mouse Pax6 expression. Although Y593 must have caused re-expression of the missing factor in those cells that normally express it, thereby rescuing their abnormal phenotypes, additional ectopic expression from Y593 might have gone undetected. Our current work complements that of Schedl et al. [17] by demonstrating a remarkable conservation of function of the Pax6/PAX6 regulatory elements in the two species.

Recently, Kim and Lauderdale [31] described the generation of a bacterial artificial chromosome (BAC) transgenic reporter mouse containing 160 kb of mouse genomic DNA from around the mouse Pax6 gene. Unlike the YAC transgene described here, the BAC transgene did not generate expression in diencephalic and olfactory cells that are known to express Pax6. A likely explanation for this difference is that the shorter BAC transgene is missing some important regulatory elements. Figure 6 compares YAC Y593 (which spans the same genomic interval as its derivative, YAC Y1123) with BAC mBAC293d08 [31]. BAC mBAC293d08 lacks the genomic region between ELP4 exons 4 and 7 that comprises part of the downstream regulatory region (DRR) in the human. LAGAN/ VISTA pairwise alignment identifies five highly conserved regions that could contain regulatory elements responsible for differences in expression between the transgenic reporter mice carrying mBAC293d08 and Y1123 (Fig. 6).

Conclusion
This work provides further evidence that the Pax6/PAX6 regulatory elements are highly conserved not only structurally but also functionally in mice and humans. Y1123 provides an excellent tool for studying the functions of different Pax6/PAX6 regulatory elements and will allow the analysis and isolation of cells in which Pax6 is activated, irrespective of the status of the endogenous locus.
Expression of tau-GFP in DTy54 transgenic mice at E14.5. (A-E) Sections showing tau-GFP in the eye and optic tract. The optic nerve (on) contains tau-GFP (B-D). Axons containing tau-GFP are seen emerging from the retina at the optic nerve head (C) and forming the optic chiasm (E). (F) Parasagittal section through the brain at E14.5 showing tau-GFP in the cerebral cortex (cc), prethalamus (pth), pretectum (pt), optic chiasm (oc), basal plate of the pons (po) and primordial cerebellum (c). Arrow points to the posterior boundary of the pretectum. (G-I) Coronal sections at E14.5 showing tau-GFP in the brain: (G) the pineal gland, (H) the pallium (p), subpallium (sp) and prethalamus and (I) the cerebral cortex. (J) Section through the olfactory epithelium (oe). (K-M) Expression of Pax6 shown with immunohistochemistry on coronal sections. Scale bars: A,B 200 µm; C, 20 µm; D,E,G,H,J-L, 300 µm; F,M, 900 µm; I, 50 µm.
Figure 4

Expression of tau-GFP in DTy54 transgenic mice at E12.5. Coronal sections through the pallial/subpallial border of E12.5 embryos stained with antibodies against Pax6 (A) and GFP (B); images are merged in C. Scale bar: 200 µm.
Methods

Generation of the DTy54 transgenic mouse

All work on mice followed current Home Office (UK) regulations stipulated in the Animals (Scientific Procedures) Act 1986. An overview of the strategy is illustrated in Fig. 1. We inserted a tau-GFP reporter cassette and a neomycin resistance cassette, linked by an internal ribosomal entry site (IRES), in frame into the translation start site in exon 4 of the \textit{PAX6} gene in YAC Y593 [17] by homologous recombination using a yeast URA3 selectable marker. The manipulated YAC (named Y1123) was then used to generate transgenic mice.

Integration of YAC Y593 into yeast window strain W3

Before modifying the parental YAC Y593 with the reporter construct, it was introduced into a yeast window strain. This was necessary because Y593 co-migrates with similar sized endogenous yeast chromosomes in pulse field gel electrophoresis, making it difficult to isolate from the endogenous chromosomes. Each window strain contains defined alterations in its karyotype, which provide a large size interval, or window, devoid of endogenous chromosomes [32,33]. Window strain W3 was mated with Y593 using the \textit{kar}-cross method [34,35]. Y593, in addition to the \textit{PAX6} gene locus, contains the genes allowing yeast cells to produce adenine and tryptophan. By removing adenine hemisulfate salt and tryptophan from the growth medium, it was possible to select for yeast colonies expressing these genes and, therefore, containing Y593.

Integration of reporter cassette into Y593 by homologous recombination

Y593 was modified to generate a new YAC (named Y1123) using the plasmid pDT-1 (Fig. 7), which contained the following elements (Figs. 1, 7). (i) Coding sequence for tau-GFP fusion protein; the microtubule binding protein tau would allow the visualisation of the processes of expressing cells [13]. (ii) An IRES followed by an optimised Kozak translation consensus start site (IRESKozak) to allow the translation of two cis genes from a single transcript [36]. (iii) A neomycin resistance (neo) cassette to allow G418-based selection of expressing cells. (iv) A polyadenylation (pA) site to allow polyadenylation of the tau-GFP-IRES-neo mRNA. (v) A C2MAZ site to slow RNA polymerase II [37] and promote transcription termination; the aim was to further reduce the chances of transcription of the entire targeted locus, which might have reduced marker expression through splicing around exon 4.

Once Y593 had been moved into the window strain it was transformed with the bacterial construct pDT-1 using modified lithium acetate yeast transformation. W3 has a defective URA3 gene and is unable to survive pyrimidine starvation. Since pDT-1 contained the yeast gene URA3 (Fig. 7), any cell harbouring Y593 into which pDT-1 had recombined survived pyrimidine starvation and was selected by the omission of uracil from the growth medium.

Several colonies were picked and screened for the likely presence of a complete pDT-1 using PCR for parts of pDT-1 distant from the URA3 gene and with Southern blots. Of 18 clones screened, one showed correct first round recombination. This clone was grown in the presence of 5-fluoroorotic acid (5-FOA), which prevents yeast cells containing the URA3 gene from growing and provides selection for removal of the URA3 gene by internal homologous recombination [38,39]. Nine clones were picked from the 5-FOA plate, designated 1121 to 1129, and Southern blots were done to identify correct clones. One clone (1123) was identified as correct, giving a suc-
cess rate of about 11%. PCR combined with restriction digests on some of the PCR products was used to confirm that the individual parts of the reporter cassette were present in the clone. The junction between PAX6 and tauGFP was checked by sequencing in both directions, confirming that the PAX6 ATG in exon 4 was followed immediately by tauGFP.

Microinjection of Y1123 and initial assessment of transgenic mice

Y1123 DNA was isolated for microinjection using alternating contour-clamped homogeneous electric field pulse field gel electrophoresis [40]. Injected one-cell embryos (from crosses of C57Bl/6 and CBA mice) were either replaced immediately into pseudopregnant female mice or first cultured overnight until two-cell. About 5% of injected one-cell embryos were born. Subsequent breeding was such that all mice carrying Y1123 studied here were hemizygous for the transgene. Southern blotting with a full-length cDNA probe was used to confirm that the modified PAX6 coding region had integrated. PCR with primers shown in Fig. 1 was used to confirm the extent of incorporation of Y1123. The primer sequences were:

- 3163F AAGCCATTTTGTTGGTGAGC
- 3163R TTCCAGTTATACAGGGGCTGA
- 3140F AAGGTGCCCAGCCTAATTCT
- 3140R TCGTCTCGATCTCCTGACCT
- 5003F CAGAGGGAGGACCTCTCAGG
- 5003R TTTGCCTTTAGGGCTCACTG
- 3004F CTTCCCTGGCTACCATGTCT
- 3004R CGGCCCAGTGAATTAGAAAA
- 3080F TGAAAATGCAAACAGGTTCC
- 3080R AAGCCGTCAGACCACTTTTG
- 5083F TGAGAGCTGTGCAGAGCAGA
- 5083R GAAAGCAAAACCCTGGACAA
- 5405F GCCATCTGAAAGCTGAGGAG
- 5405R CCAGCCTACCTTGACATGCT
- 5395F GACACGCTGGTCACCAAGTA
- 5395R TTACAGCGGACCCCTCTTC

FISH on blood smears, with cosmid FAT5 as a probe (marked as CFAT5 in Fig. 1) and methods described in Schedl et al. [17], was used to search for possible multiple integration sites. Quantitative PCR (qPCR; Quantitect SYBR Green PCR Kit, Qiagen) was used to identify the number of copies of Y1123 present in the genome, using three sets of primers, one specific for human PAX6, one specific for mouse Pax6 and one specific for mouse Pax3. The latter two sets were standards (detecting genes with copy numbers of 2) against which to compare the intensity of the product from Y1123. The three sets of primers were as follows: (i) human PAX6 specific primers (PAX6HumF CGCGTGCCGCTACCCGTGTA, PAX6HumR CACGGTTAATGACGTTCTG); (ii) mouse Pax6 specific primers (Pax6MouF CGCAATACCTTTGCACTCA, Pax6MouR GAGGTGTTCTCGATGCTG); (iii) mouse Pax3 specific primers (Pax3MouF AACAGGCGAGGAGAGGACAA, Pax3MouR CTCGTTAAGCGTTCGCTC).

Assessing the transgene's expression

Pregnant females were killed at various ages by cervical dislocation and embryos were fixed overnight in ice cold 4% paraformaldehyde and embedded in 4% low melting point agarose (the day of the vaginal plug was designated E0.5). Vibratome sections were cut at 200 µm, counterstained with TOPRO3 (Molecular Probes, NL), mounted on glass slides and imaged using a Leica confocal microscope. For immunohistochemistry, tissue was fixed overnight in 4% paraformaldehyde, transferred to 15% sucrose, embedded in 7.5% gelatin/15% sucrose in phosphate buffered saline and placed in 30% sucrose overnight. Cryostat sections (15 µm) were cut and transferred to 20% goat serum in phosphate buffered saline containing 0.1% Triton-X for 30 min at room temperature. Sections were incubated with mouse anti-Pax6 ascites (1:5000; Developmental Studies Hybridoma Bank) and rabbit anti-GFP antibody (1:10000; Abcam) overnight at 4 °C and for 1 hr at room temperature the following day. Secondary antibodies were goat anti-mouse and goat anti-rabbit Alexa fluor 568 and 488 respectively (1:150; Molecular Probes), applied for 1 hr at room temperature.

Flow cytometry

Telencephalic tissue from E14.5 wild-type and DTy54 embryos were dissociated with papain (Papain Dissocia-
tion System, Worthington Biochemical). Cells in suspension were analysed on a Beckman-Coulter XL flow cytometer (10,000–20,000 cells were analysed per sample).

**Abbreviations**

BAC, bacterial artificial chromosome; c, cerebellum; cc, cerebral cortex; di, diencephalon; DRR, downstream regulatory region; E, embryonic day; 5-FOA, 5-fluoroorotic acid; FISH, fluorescent in situ hybridization; GFP, green fluorescent protein; hb, hindbrain; HR, homology region; IRES, internal ribosomal entry site; neo, neomycin resist-
Figure 7
Map of plasmid PDT-1. The components of the construct shown in Fig. 1A and the URA3 sequence are marked.

References
1. Walther C, Gruss P: Pax-6, a murine paired box gene, is expressed in the developing CNS. Development 1991, 113:1435-1449.
2. Grindlay JC, Davidson DR, Hill RE: The role of Pax6 in eye and nasal development. Development 1995, 212:1433-1442.
3. St-Onge L, Sosa-Pineda B, Chowdhury K, Mansouri A, Gruss P: Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. Nature 1997, 387:406-409.
4. Hill RE, Favor J, Hogan BL, Ton CC, Saunders GF, Hanson IM, Prosser J, Jordan T, Hastie ND, van Heyningen V: Mouse small eye results from mutations in a paired-like homeobox-containing gene. Nature 1991, 354:522-525.
5. Hogan BL, Horsburgh G, Cohen J, Hetherington CM, Fisher G, Lyon MF: Small eyes (Sey): a homozygous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. J Embryol Exp Morphol 1986, 97:95-110.
6. Caric D, Gooday D, Hill RE, McConnell SK, Price DJ: Determination of the migratory capacity of embryonic cortical cells lacking the transcription factor Pax-6. Development 1997, 124:5087-5096.
7. Mastick GS, Davis NM, Andrew GL, Easter SS Jr: Pax-6 functions in boundary formation and axon guidance in the embryonic mouse forebrain. Development 1997, 124:1985-1997.
8. Stoykova A, Fritsch R, Walther C, Gruss P: Forebrain patterning defects in small eye mutant mice. Development 1996, 122:3453-3465.
9. Stoykova A, Gozi M, Gruss P, Price J: Pax6-dependent regulation of adhesive patterning, R-cadherin expression and boundary formation in developing forebrain. Development 1997, 124:3765-3777.
10. Warren N, Price DJ: Roles of Pax-6 in murine diencephalic development. Development 1997, 124:1573-1582.
11. Engelkamp D, Rashbass P, Seawright A, van Heyningen V: Role of Pax6 in development of the cerebellar system. Development 1999, 126:3585-3596.
12. Warren N, Caric D, Pratt T, Clausen JA, Asavariyakul P, Mason JO, Hill RE, Price DJ: The transcription factor, Pax6, is required for cell proliferation and differentiation in the developing cerebral cortex. Cereb Cortex 1999, 9:627-635.
13. Pratt T, Vitalis T, Warren N, Edgar JM, Mason JO, Price DJ: A role for Pax6 in the normal development of dorsal thalamus and its cortical connections. Development 2000, 127:5167-5178.
14. Estivill-Torrus G, Vitalis T, Fernandez-Llebrez P, Price DJ: The transcription factor Pax6 is required for development of the diencephalic dorsal midline secretory radial glia that form the subcommissural organ. Mech Dev 2001, 109:215-224.
15. Sudohiya SM, Frey SL, Williams KA, Mitchell TH, Willis C, Stevens JM, Kendall BE, Sharvon SD, Hansen I, Moore AT, Van Heyningen V: Pax6 haploinsufficiency causes cerebral malformation and olfactory dysfunction in humans. Nature Genetics 2001, 28:214-216.
16. Ton CC, Hirvonen H, Miwa H, Wei MM, Monaghan P, Jordan T, Van Heyningen V, Hastie ND, Meijers-Heijboer H, Drechsler M: Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. Cell 1991, 67:1059-1074.
17. Schedl A, Ross A, Lee M, Engelkamp D, Rashbass P, van Heyningen V, Hastie ND: Influence of Pax6 gene dosage on development: overexpression causes severe eye abnormalities. Cell 1996, 86:71-82.
18. Bishop KM, Goudreau G, O'Leary DD: Regulation of area identity in the mammalian neocortex by Emx2 and Pax6. Science 2000, 288:344-349.
19. Bishop KM, Rubenstein JL, O'Leary DD: Distinct actions of Emx1, Emx2, and Pax6 in regulating the specification of areas in the developing neocortex. J Neurosci 2002, 22:7627-7638.
20. Muzio L, DiBenedetto B, Stoykova A, Boncinielli E, Gruss P, Mallamaci A: Emx2 and Pax6 Control Regionalization of the Pre-neurogenic Cortical Primordium. Cerebral Cortex 2002, 12:129-139.
21. Fantes J, Redeker B, Breen M, Boyle S, Brown J, Fletcher J, Jones S, Bickmore W, Fukushima Y, Mannens M: Aniridia-associated cytogenetic rearrangements suggest that a position effect may cause the mutant phenotype. Hum Mol Genet 1995, 4:415-422.
22. Crolla JA, Cross I, Atkey N, Wright M, Oley CA: FISH studies in a patient with sporadic aniridia and t(7;11) (q31.2;p13). J Med Genet 1996, 33:66-68.

23. Lauderdale JD, Wilensky JS, Oliver ER, Walton DS, Glaser T: 3’ deletions cause aniridia by preventing PAX6 gene expression. Proc Natl Acad Sci USA 2000, 97:13755-13759.

24. Kleinjan DA, Seawright A, Schedl A, Quinlan RA, Danes S, van Heyningen V: Aniridia-associated translocations, DNase hypersensitivity, sequence comparison and transgenic analysis redefine the functional domain of PAX6. Hum Mol Genet 2001, 10:2049-2059.

25. Griffin C, Kleinjan DA, Doe B, van Heyningen V: New 3’ elements control Pax6 expression in the developing pretectum, neural retina and olfactory region. Mech Dev 2002, 112:89-100.

26. Kleinjan DA, Seawright A, Childs AJ, van Heyningen V: Conserved elements in Pax6 intron 7 involved in (auto)regulation and alternative transcription. Dev Biol 2004, 265:462-477.

27. Tong D, Pratt T, Simpson TJ, Mason JG, Price DJ: Identifying GFP transgenic mice by flashlight. Biotechniques 2003, 34:474-476.

28. Vitalis T, Cases O, Engelkamp D, Verney C, Price DJ: Defect of tyrosine hydroxylase-immunoreactive neurons in the brains of mice lacking the transcription factor Pax6. J Neurosci 2000, 20:6501-6516.

29. Fernandez AS, Pieau C, Reperant J, Boncinelli E, Wassef M: Expression of the Ema-x and Dlx-1 homeobox genes define three molecularly distinct domains in the telencephalon of mouse, chick, turtle and frog embryos: implications for the evolution of telencephalic subdivisions in amniotes. Development 1998, 125:2099-2111.

30. Heins N, Malatesta P, Cecconi F, Nakafuku M, Tucker KL, Hack MA, Chapouton P, Barde YA, Gotz M: Glial cells generate neurons: the role of the transcription factor Pax6. Nat Neurosci 2000, 3:308-315.

31. Kim J, Lauderdale JD: Analysis of Pax6 expression using a BAC transgene reveals the presence of a paired-less isoform of Pax6 in the eye and olfactory bulb. Dev Biol 2006 in press.

32. Hamer L, Johnston M, Green ED: Isolation of yeast artificial chromosomes free of endogenous yeast chromosomes: construction of alternate hosts with defined karyotypic alterations. Proc Natl Acad Sci USA 1995, 92:11706-10.

33. Vollrath D, Davis RW, Connelly C, Hieter P: Physical mapping of large DNA by chromosome fragmentation. Proc Natl Acad Sci USA 1988, 85:6027-31.

34. Spencer F, Hugarat Y, Simchen G, Hurko O, Connelly C, Hieter P: Yeast kar1 mutants provide an effective method for YAC transfer to new hosts. Genomics 1994, 22:118-26.

35. Hugarat Y, Spencer F, Zenwirth D, Simchen G: A versatile method for efficient YAC transfer between any two strains. Genomics 1994, 22:108-17.

36. Vagner S, Galy B, Pyronnet S: Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. EMBO Rep 2001, 2:893-898.

37. Yonaha M, Proudfoot NJ: Transcriptional termination and coupled polyadenylation in vitro. EMBO J 2000, 19:3770-3777.

38. Soderholm J, Bevis BJ, Glick BS: Vector for pop-in/pop-out gene replacement in Pichia pastoris. Biotechniques 2001, 31:306-10.

39. Boeke JD, Trueheart J, Natoulsiu G, Fink GP: 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol 1987, 154:164-75.

40. Chu G, Vollrath D, Davis RW: Separation of large DNA molecules by contour-clamped homogenized electric fields. Science 1996, 274:1582-5.

41. Brudno M, Do C, Cooper G, Kim MF, Davydov E, Green ED, Sidow A, Batzoglou S: LAGAN and Multi-LAGAN: efficient tools for large-scale multiple alignment of genomic DNA. Genome Research 2003, 13:721-31.

42. Mayor C, Brudno M, Schwarz JR, Poliakov A, Rubin EM, Frazer KA, Pachter LS, Dubchak I: VISTA: Visualizing Global DNA Sequence Alignments of Arbitrary Length. Bioinformatics 2000, 16:1046.

43. Loos G, Ovcharenko I, Pachter L, Dubchak I, Rubin E: rVISTA for comparative sequence-based discovery of functional transcription factor binding sites. Genome Res 2002, 12:832-839.