Relationship of Pax6 activity levels to the extent of eye development in the mouse, Mus musculus


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ABSTRACT

In the present study we extend the mouse Pax6 mutant allelic series to include a homozygous and hemizygous viable hypomorph allele. The $\text{Pax6}^{132-14\text{Neu}}$ allele is a Phe272Ile missense mutation within the third helix of the homeodomain. The mutant Pax6 homeodomain shows greatly reduced binding activity to the P3 DNA binding target. Glucagon promoter activation by the entire mutant Pax6 product of a reporter gene driven by the G1 paired and homeodomain DNA binding target was slightly increased. We constructed mutant Pax6 genotypes such that Pax6 activity ranged between 100% and 0%, and show that the extent of eye development is progressively reduced as Pax6 activity decreased. Two apparent thresholds identify three groups in which the extent of eye development abruptly shifted from complete eye at the highest levels of Pax6, to a rudimentary eye at intermediate levels of Pax6, to very early termination of eye development at the lowest levels of Pax6. Of the two Pax6-positive regions which participate in eye development, the surface ectoderm, which develops into the lens vesicle and the cornea, is more sensitive to reduced levels of Pax6 activity than the optic vesicle, which develops into the inner and outer retinal layers.
INTRODUCTION


*Pax6* gene products control the transcriptional activity of target genes by directly or indirectly (via oligomerization with additional co-factors) binding to enhancer DNA target sequences (CHI and EPSTEIN 2002). The “level” of *Pax6* gene activity cannot be considered the sum of activities of the individual domains since missense mutations confined to a single domain can affect the binding activity of the full length gene product by the second non-mutated domain and can alter the spectrum of gene target sequences to which the mutated gene product binds (MISHRA *et al.* 2002; SINGH *et al.* 2000; TANG *et al.* 1997). Similarly, missense mutations in the C-terminal proline-, serine- and threonine-rich transcription activation domain can affect the DNA binding activity of the gene product by the paired or homeodomains (SINGH *et al.*
2001). The situation is further complicated since Pax6 is expressed as a number of isoforms (CARRIÈRE et al. 1993, 1995; GORLOV and SAUNDERS 2002; WAWERSIK et al. 2000). Thus, although DNA binding activity of isolated Pax6 domains to specific DNA target sequences or reporter gene activation by specific Pax6 isoforms can be measured, such results do not reflect the in vivo situation of a mixture of multiple isoforms simultaneously binding to a range of alternate target sites. Previous experimental approaches to address the question of the consequences of altering the levels of Pax6 on developmental outcome have included the use of mouse null mutations (VAN RAAMSDONK and TILGHMAN 2000), $\text{Pax6}^{-/-} \leftrightarrow \text{Pax6}^{+/+}$ chimera (COLLINSON et al. 2000, 2001, 2003; QUINN et al. 1996; TALAMILLO et al. 2003), conditional inactivation of Pax6 in a tissue- and stage-specific manner (ASHERY-PADAN et al. 2000; DAVIS-SILBERMAN et al. 2005), over expression of Pax6 via transgenic mutant constructs (DUNCAN et al. 2000; KIM and LAUDERDALE 2006, 2008; MANUEL et al. 2007; SCHEDL et al. 1996) and retrovirally-mediated Pax6 expression (HACK et al. 2005; HEINS et al. 2002). We have taken a genetic approach utilizing members of the mouse Pax6 allelic series. We identified and characterized the first homozygous viable Pax6 hypomorph allele. With this extension of the Pax6 allelic series we constructed Pax6 mutant genotypes such that the predicted Pax6 activity ranged from 100% normal to 0% and assessed the extent of eye development.

MATERIALS AND METHODS

Mice, mapping and slit lamp examination: The original mutant, designated 132, was recovered as a heterozygote expressing anterior pyramidal opacity with corneal adhesions in the offspring of a (102/El x C3H/El)F1 male exposed to 4.55 + 4.55 Gy gamma irradiation and mated to an Oak Ridge Tester-stock female. Confirmation crosses indicated the mutation to be autosomal dominant (KRATOSCHIVOLOVA and EHLING 1979). Subsequent analyses showed
the mutation to be homozygous viable and fertile, with homozygotes expressing microphthalmia and closed eyes, and that the 132 mutation was allelic with three additional eye mutations. The allelism group was designated Cat4 (KRATOCHVILOVA and FAVOR 1992) and the 132 mutation was previously assigned the mutant symbol Apyc or Apcat1. The 132 mutation was incorrectly (see results below) assigned linkage to Chr 8 (FAVOR et al. 1997).

Ophthalmological examinations were done as previously described (FAVOR 1983). Prior to mapping, a congenic C3H/HeJ 132 mutant line was established by more than 20 consecutive backcross generations of 132 heterozygotes to strain C3H/HeJ. Genome-wide linkage analysis of the mutation relative to 42 MIT-microsatellite markers which are distributed over the 19 autosomes was carried out as previously described (FAVOR et al. 1997). Since as will be shown below the 132 mutation is a hypomorph mutant allele of Pax6 with a high frequency of phenotypically misclassifying heterozygous carriers as wildtype, only animals classified as mutants were used in the linkage analysis. After localization of the mutation to Chr 2 the backcross mice were genotyped for additional MIT-microsatellite markers within the region. Segregation data were analysed with Map Manager version 2.6.5 (MANLY 1993) and the gene order was determined by minimizing the number of multiple crossovers. Animals were bred and maintained in our facilities according to the German law for the protection of animals. All inbred strains employed in this study (C3H/HeJ, C57BL/6Ei) were obtained from breeding colonies maintained by the Department of Animal Resources at Neuherberg.

**Morphology and histology:** Wildtype, heterozygous 132/+ and homozygous 132/132 mutants were weighed, and both eyes were classified for the degree of eye opacity and eye weight at P35 as previously described (FAVOR et al. 2001), with the addition of two eye classes to accommodate the minor eye phenotype expressed by heterozygous 132/+ mice (minor iris irregularities with no lens opacity) and the extreme phenotype expressed by homozygous 132/132 mice (extreme microphthalmia with lens/corneal opacity and iris
abnormality). Homozygous wildtype, heterozygous 132/+ and homozygous 132/132 embryos were produced from intercrosses of 132/+ heterozygotes. Compound heterozygotes were produced in crosses of homozygous 132/132 mutants with heterozygous carriers of various Pax6 mutations. The recovered compound heterozygotes were fertility tested by outcrossing to homozygous wildtype and homozygous 132/132 mutant partners. Compound heterozygote embryos and animals at weaning were identified as expressing extreme microphthalmia. Embryos were collected and processed for histology, and histological sectioning, staining and photography were all conducted as previously described (FAVOR et al. 2001, 2007).

**Sequencing, DNA binding assay and glucagon promoter assay:** RNA and genomic DNA were extracted from the heads and bodies, respectively, of homozygous wildtype and homozygous 132/132 mutant E15 embryos. Preparation and sequencing procedures were as previously described (FAVOR et al. 2001). Two primer pairs were used to generate overlapping amplification products across the Pax6 cDNA. These amplification products were used as substrates to sequence the Pax6 transcript. The sequencing results using cDNA as substrate were confirmed by sequencing the mutation site with genomic DNA as substrate in the initial embryos analysed as well as from additional heterozygous and homozygous mutants. The primer pair used to amplify and to sequence the mutation site from genomic DNA was; 5’ ACCCATTATCCAGATGTGTTTGCC and 5’ GGAATGTGACTAGGAGTGTTGCTG. Numbering of the transcript and the translation products corresponds to EMSMUSG00000027168/ENUMUST00000111087 (Ensembl, release 48).

The electrophoretic mobility shift assay to ascertain homeodomain binding to the P3 DNA target was conducted as previously described (FAVOR et al. 2001). Briefly, sub-clones of the wildtype, Pax64Neu and the 132 mutant Pax6 cDNAs, coding for the entire homeodomain with six additional amino acids upstream and four amino acids downstream (Pax6 amino acids
218-288), were inserted between the *PstI* and the *HindIII* restriction sites of the pQE-41 vector (QIAGEN). The pQE expression constructs were transformed into *E. coli* strain M15 [pREP4]. Expression of the homeodomains in exponentially growing bacterial cultures was induced with 0.1mM isopropyl thiogalactoside for 2 h at 30°. Bacterial pellets were lysed, crude extracts were electrophoresed in 10% SDS-PAGE and proteins visualized by staining with Coomassie brilliant blue. Crude extracts from the transformed bacteria were incubated with 15 fmol of the target oligonucleotide, which was 3’ end-labeled with digoxygenin-11-ddUTP as recommended by the supplier (Roche Diagnostics, Mannheim, Germany). The single strand oligonucleotide target sequence (with the P3 homeodomain binding site underlined) was, 5’-TCGAGGGCATCAGGATGCTAATTGAATTA
GCATCCGATCGGG-3’, to which the *Pax6* homeodomain binds via cooperative dimerization (CZERNY and BUSSLINGER 1995; WILSON et al. 1993). The rabbit anti-Pax6-homeodomain antiserum used to control for the specificity of the homeodomain-DNA complex was serum 13 (CARRIERE et al. 1993).

To assess transcriptional activation by the *Pax6* wildtype, *Pax6*<sup>4Neu</sup> and 132 mutant gene products we carried out glucagon promoter assays (PLANQUE et al. 2001; RITZ-LASER et al. 1999). *Pax6* is expressed in the pancreas and is required for α-cell development and expression of glucagon (ST-ONGE et al. 1997). Transcriptional activation of glucagon by *Pax6* is mediated by the interaction of *Pax6* with two AT-rich sequences, designated G1 and G3, of the glucagon gene (RITZ-LASER et al. 1999). The glucagon promoter assay was carried out according to the previously described procedures (PLANQUE et al. 2001; RITZ-LASER et al. 1999). The full length wildtype, *Pax6*<sup>4Neu</sup> mutant and 132 mutant *Pax6* canonical transcripts (isoforms not containing the exon 5a) were amplified with the primer set 5’
AGCTCCAGCATGCAGAACAGTCAC and 5’ ACTGCTGTGTCACATAGTCATCGGC using the Titan RT-PCR system (Roche Diagnostics, Mannheim, Germany). The amplification products were isolated by electrophoretic separation in 1% agarose gels and
extracted with the MiniElute kit (QIAGEN, Chatsworth, CA). The blunt-end PCR products were modified to sticky-end 3’ A overhangs with the QIAGEN PCR Cloning<sup>plus</sup> kit (QIAGEN) and ligated into the QIAGEN pDrive Cloning Vector orientated to the T7 promoter (QIAGEN). Transformed <i>E. coli</i> strain M15[pREP4] colonies were isolated, cultured and plasmid DNA extracted for sequencing to identify clones containing the full length transcript sequences correctly orientated to the T7 promoter. The <i>KpnI-HindIII</i> restriction fragment of each clone containing the entire <i>Pax6</i> coding sequence was inserted into the pVNC vector. <i>Pax6</i> was therefore expressed under the control of the CMV promoter. BHK-21 cells were co-transfected with DNA from a CAT reporter gene construct driven by the -138 glucagon promoter bearing the G1 <i>Pax6</i>-binding element, a <i>Pax6</i> expression vector containing the full length open reading frame of the mouse wildtype, <i>Pax6<sup>4Neu</sup></i> mutant or the <i>Pax6<sup>132-14Neu</sup></i> mutant cDNA sequence, and the pcDNA3-LacZ vector (for normalization of the CAT assay). The -138 glucagon promoter is a 196 bp sequence from the proximal region of the glucagon gene. The G1 sequence containing two 7 bp AT-rich sequences (underlined) within the -138 promoter was: 5’ CCCCATATTATTTACAGGAAATTTATTTATTTG (RITZ-LASER et al. 1999). The CAT assays were performed as previously described (PLAZA et al. 1999).

RESULTS

**Breeding, eye morphology and mapping:** In an attempt to increase the accuracy of our initial linkage studies (FAVOR et al. 1997) we noted that the mutation was not linked to Chr 8. We undertook another genome-wide mapping study and localised the 132 mutation to Chr 2 with the following locus order (frequencies of crossovers between adjacent loci are given in parentheses): <i>D2Mit249</i>-(1/71)-132-(1/71)-<i>D2Mit102</i>-(2/71)-<i>D2Mit258</i>-(13/71)-<i>Agouti</i>. Based on the chromosomal region and the eye phenotype we considered <i>Pax6</i> to be a
candidate gene for mutation analysis. Sequencing analyses confirmed the 132 mutation to be a nucleotide substitution (c.T1099A) within the coding region of the Pax6 gene. The base-pair substitution results in an amino acid substitution (Phe272Ile) within the third helix of the homeodomain. We have assigned the mutation the allele symbol $\text{Pax}6^{132-14Neu}$ which has been approved by the Mouse Genetic Nomenclature Committee (accession number MGI: 1856585). Heterozygous $\text{Pax}6^{132-14Neu}$ mutant embryos expressed microphthalmia, anterior pyramidal opacity, adhesion of the lens to the cornea, and a reduced anterior chamber (Figure 3, D). Homozygous $\text{Pax}6^{132-14Neu}$ mutant embryos expressed extreme microphthalmia with more extreme lens and corneal defects than observed in heterozygotes (Figure 3, H).

We confirmed that the $\text{Pax}6^{132-14Neu}$ mutation is homozygous viable and that heterozygous and homozygous mutants are fully fertile with no significant differences ($F_{3, 145} = 1.33, P = 0.26$) in average litter size among the various crosses (+/- x +/-, $5.77 \pm 0.32$, $n = 57$; +/- x +/-, $4.76 \pm 0.52$, $n = 25$; -/- x +/-, $5.80 \pm 0.56$, $n = 20$; -/- x -/-, $5.83 \pm 0.30$, $n = 47$). The frequency of presumed heterozygous carriers was less than expected based on a phenotypic classification of offspring (data not shown). We carefully characterized the eye phenotypes in P35 animals of known genotype. In heterozygous $\text{Pax}6^{132-14Neu}$ mutants there was a high frequency of eyes with no observable defects or with only minor iris irregularities which fall outside the range of eye phenotypes previously associated (FAVOR et al. 2001) with heterozygous Pax6 mutants (Table 1), and eye size was slightly reduced (+/+, $18.90\text{mg} \pm 0.07$, $n = 116$; +/-, $16.68\text{mg} \pm 0.05$, $n = 259$). This observation explains the distortion in the ratio of presumed wildtype and heterozygous mutant carriers when classified phenotypically according to our previous classification criteria for Pax6 mutants (FAVOR et al. 2001). All homozygous $\text{Pax}6^{132-14Neu}$ mutants expressed extreme microphthalmia with lens/corneal opacity and iris abnormality (Table 1) and eye weight was extremely reduced (-/-, $2.88\text{mg} \pm 0.12$, $n = 32$). Body weight (+/+, $19.49\text{g} \pm 0.24$, $n = 58$; +/-, $17.94\text{g} \pm 0.18$, $n = 128$; -/-, $17.96\text{g} \pm 0.29$, $n = 16$) of heterozygous and homozygous $\text{Pax}6^{132-14Neu}$ mutants was less than the body weight of
homozygous wildtypes (+/+ vs +/-, t = 4.94, \( P_{\text{two-tailed}} = 1.73 \times 10^{-6}, \text{df} = 184 \); +/+ vs -/-, t = 3.20, \( P_{\text{two-tailed}} = 0.002, \text{df} = 72 \)). Since mutant \( \text{Pax}\delta^{132-14\text{Neu}} \) mice were associated with a significant reduction in body size, eye weights were normalized for body weight (Table 2). As compared to wildtype, the normalized eye weight of heterozygous \( \text{Pax}\delta^{132-14\text{Neu}} \) mutants was moderately but significantly reduced (t = 2.30, \( P_{\text{two-tailed}} = 0.02, \text{df} = 369 \)). The normalized eye weight of homozygous \( \text{Pax}\delta^{132-14\text{Neu}} \) mutants was extremely reduced (t = 58.37, \( P_{\text{two-tailed}} = 4.27 \times 10^{-103}, \text{df} = 146 \)).

**DNA-binding and glucagon promoter activation associated with the \( \text{Pax}\delta^{132-14\text{Neu}} \) gene product:** Given the functional significance of the third helix of the homeodomain (BRUUN et al. 2005; GEHRING et al. 1994; HANES and BRENT 1989; QIAN et al. 1994; TREISMAN et al. 1989; WILSON et al. 1993), we characterised the binding activity of the \( \text{Pax}\delta^{132-14\text{Neu}} \) mutant homeodomain to the P3 target sequence and the glucagon promoter activation of the \( \text{Pax}\delta^{132-14\text{Neu}} \) gene product. Results indicated loss of binding activity of the \( \text{Pax}\delta^{132-14\text{Neu}} \) mutant homeodomain to the P3 DNA binding target (Figure 1). The glucagon promoter activation by the mutant \( \text{Pax}\delta^{132-14\text{Neu}} \) was slightly higher than wildtype \( \text{Pax}\delta \) (Transformations with 100 ng DNA: wildtype, 7.19 ± 0.19, n = 2; \( \text{Pax}\delta^{132-14\text{Neu}} \), 10.87 ± 0.31, n = 2, t = 14.31, \( P_{\text{two-tailed}} < 0.05 \); Transformations with 300 ng DNA: wildtype, 8.49 ± 0.64, n = 2; \( \text{Pax}\delta^{132-14\text{Neu}} \), 11.74 ± 0.23, n = 2, t = 6.75, \( P_{\text{two-tailed}} < 0.05 \) (Figure 2). In these assays we included a previously described \( \text{Pax}\delta \) hypomorph mutation, \( \text{Pax}\delta^{4\text{Neu}} \), also due to an amino acid substitution within the third helix of the homeodomain (Ser273Pro). We observed that the binding activity of the \( \text{Pax}\delta^{4\text{Neu}} \) mutant homeodomain to the P3 target sequence was ablated (Figure 1) and there was greatly reduced glucagon promoter activation by the mutant \( \text{Pax}\delta^{4\text{Neu}} \) (Transformations with 100 ng DNA: wildtype, 7.19 ± 0.19, n = 2; \( \text{Pax}\delta^{4\text{Neu}} \), 2.15 ± 0.12, n = 2, t = 31.75, \( P_{\text{two-tailed}} < 0.05 \); Transformations with 300 ng DNA: wildtype, 8.49 ± 0.64, n = 2; \( \text{Pax}\delta^{4\text{Neu}} \), 3.29 ± 0.44, n = 2, t = 9.47, \( P_{\text{two-tailed}} < 0.05 \) (Figure 2). Co-transfection of cells with mutant \( \text{Pax}\delta^{4\text{Neu}} \) and
wildtype Pax6 indicated that the mutant Pax6\textsuperscript{4Neu} did not interfere with the normal promoter activation of the wildtype Pax6. Taken together, these results indicate that the Pax6\textsuperscript{132-14Neu} and Pax6\textsuperscript{4Neu} missense mutations in the third helix of the homeodomain both affect the binding activities of the mutant homeodomains. However, whereas the glucagon promoter activity of the Pax6\textsuperscript{132-14Neu} gene product was increased, the activity of the Pax6\textsuperscript{4Neu} gene product was greatly reduced. Since the reporter gene employed for this assay was driven by the Gl Pax6 paired and homeodomain binding site (PLANQUE et al. 2001) our results suggest that dysfunction due to the Pax6\textsuperscript{132-14Neu} missense mutation is confined to the homeodomain and allowed transcriptional activation by the intact paired domain, while the Pax6\textsuperscript{4Neu} homeodomain missense mutation also affects the binding activity of the gene product to paired domain targets.

**Compound heterozygotes:** We next attempted to produce compound Pax6 heterozygotes with Pax6\textsuperscript{132-14Neu} and 2 previously described Pax6 hypomorph alleles (Pax6\textsuperscript{4Neu} and Pax6\textsuperscript{7Neu}) which are more severely affected than carriers of the Pax6\textsuperscript{132-14Neu} mutation (FAVOR et al. 2001) or with the previously described null alleles Pax6\textsuperscript{Sey-Neu}, Pax6\textsuperscript{2Neu} and Pax6\textsuperscript{3Neu} (FAVOR et al. 2001; HILL et al. 1991) (Table 3). All compound heterozygote combinations expressed extreme microphthalmia and were viable, and the compound heterozygotes Pax6\textsuperscript{2Neu}/Pax6\textsuperscript{132-14Neu}, Pax6\textsuperscript{4Neu}/Pax6\textsuperscript{132-14Neu}, Pax6\textsuperscript{7Neu}/Pax6\textsuperscript{132-14Neu}, and Pax6\textsuperscript{Sey-Neu}/Pax6\textsuperscript{132-14Neu} were fertile. The compound heterozygote Pax6\textsuperscript{3Neu}/Pax6\textsuperscript{132-14Neu} was not fertility tested.

In previous characterizations of our extensive Pax6 allelic series we have concluded that most alleles (Pax6\textsuperscript{Sey-Neu}, Pax6\textsuperscript{2Neu}, Pax6\textsuperscript{3Neu}, Pax6\textsuperscript{5Neu}, Pax6\textsuperscript{6Neu}, Pax6\textsuperscript{8Neu}, Pax6\textsuperscript{9Neu}, Pax6\textsuperscript{10Neu} and Pax6\textsuperscript{Coop}) result in premature truncation of the Pax6 translation product (FAVOR et al. 2001; HILL et al. 1991; LYON et al. 2000). The hypomorph alleles Pax6\textsuperscript{4Neu} and Pax6\textsuperscript{7Neu} express delayed perinatal lethality when compared with homozygous Pax6 null mutations (FAVOR et al. 2001), and thus they must have less Pax6 activity than the Pax6\textsuperscript{132-14Neu} mutation which is
homozygous viable and fertile. The phenotype expressed by homozygous mutant \textit{Pax6}^{132-14Neu} mice was more severe than that of heterozygous \textit{Pax6} null mutants, and therefore \textit{Pax6}^{132-14Neu} homozygous mutants must express less than 50\% \textit{Pax6} activity. Since the compound heterozygotes of \textit{Pax6}^{132-14Neu} carried over \textit{Pax6} null alleles are viable and fertile, a single copy of the \textit{Pax6}^{132-14Neu} mutation carried over a null allele must have more activity than 2 copies of \textit{Pax6}^{4Neu} or \textit{Pax6}^{7Neu} carried by the respective homozygotes. Based on these results we could predict the order of \textit{Pax6} activity in wildtype and mutant \textit{Pax6} genotypes as follows: \textit{Pax6} +/- = 100\% > \textit{Pax6}^{132-14Neu}/+ > \textit{Pax6}^{3Neu}/+ = 50\% > \textit{Pax6}^{132-14Neu}/\textit{Pax6}^{132-14Neu} > \textit{Pax6}^{4Neu}/\textit{Pax6}^{132-14Neu} \approx \textit{Pax6}^{7Neu}/\textit{Pax6}^{132-14Neu} > \textit{Pax6}^{3Neu}/\textit{Pax6}^{132-14Neu} > \textit{Pax6}^{7Neu}/\textit{Pax6}^{7Neu} > \textit{Pax6}^{3Neu}/\textit{Pax6}^{3Neu} = 0\%. We constructed these genotypes, and assessed the degree of eye development in E15 embryos when the expected \textit{Pax6} activity in the embryos ranged from 100\% normal to 0\% (Figure 3). Results indicated that the degree of eye development is progressively affected as the expected \textit{Pax6} activity is reduced. Considering the degree of eye development, the various genotypes assessed can be grouped into three distinct classes. In the first class, embryos have a higher predicted \textit{Pax6} activity (\textit{Pax6} +/- (A, B), \textit{Pax6}^{132-14Neu}/+ (C, D), \textit{Pax6}^{3Neu}/+ (E, F) and \textit{Pax6}^{132-14Neu}/\textit{Pax6}^{132-14Neu} (G, H)) and the basic eye plan developed with a definite cornea, lens and retina. However, as the predicted level of \textit{Pax6} activity was reduced there was a progressive reduction in eye size and the degree of anterior segment abnormalities increased (thickened cornea, failure of the lens to detach from the cornea, degree of development of the anterior chamber between the cornea and lens). The second class (\textit{Pax6}^{4Neu}/\textit{Pax6}^{132-14Neu} (I, J) and \textit{Pax6}^{7Neu}/\textit{Pax6}^{132-14Neu} (K, L)) consists of embryos with less predicted \textit{Pax6} activity than that in the first class. A rudimentary eye developed consisting of a retina and a distinct optic pit. However, there was no apparent invagination of the surface ectoderm into the optic cup nor was there development of lenticular tissue. The third class consists of embryos with the least predicted \textit{Pax6} activity (\textit{Pax6}^{3Neu}/\textit{Pax6}^{132-14Neu} (M, N), \textit{Pax6}^{7Neu}/\textit{Pax6}^{7Neu} (O, P) and \textit{Pax6}^{3Neu}/\textit{Pax6}^{3Neu} (Q, R)). In all
three genotypes within this class the tissue observed had no apparent resemblance to a rudimentary eye. The progression of eye development in the genotype within this class with the higher predicted activity \((Pax6^{3Neu}/Pax6^{132-14Neu} \text{ (M, N)})\) was more extensive with more tissue derived from the optic vesicle in the vicinity of the surface ectoderm and an apparent rudimentary optic pit. By comparison, there was minimal progression of eye development in the genotype with zero \(Pax6\) activity \((Pax6^{3Neu}/Pax6^{3Neu} \text{ (Q, R)})\). The tissue derived from the optic vesicle (arrowheads) was observed to be distant from the surface ectoderm and there was no evidence of the formation of a rudimentary optic pit. The \(Pax6^{7Neu}/Pax6^{7Neu}\) genotype \((O, P)\) with predicted \(Pax6\) activity between the other two genotypes in this class expressed an intermediate progression in eye development.

**DISCUSSION**

In the present study we have characterised a \(Pax6\) missense hypomorph mutant allele and, utilising our extensive \(Pax6\) mutant allelic series, constructed genotypes such that the predicted \(Pax6\) activity varied between 100\% and 0\%. We show that the extent of eye development was directly related to the levels of \(Pax6\) activity. The genotypes may be grouped into three distinct classes which define apparent thresholds in the relationship between \(Pax6\) activity and an abrupt shift in the extent of eye development. The first threshold at which \(Pax6\) activity was less than 50\% demarks a shift from development of a complete eye in genotypes above the threshold to a group of genotypes which only develop a rudimentary eye consisting of a retina and no lenticular tissue. The second threshold at a very low level of \(Pax6\) activity is associated with a shift from development of a rudimentary eye in genotypes with higher levels of \(Pax6\) to a group of genotypes in which eye development terminated very early and the tissues had no resemblances to a rudimentary eye.
**Hypomorph \(Pax6^{132-14Neu}\) allele:** We show that heterozygous carriers of the \(Pax6^{132-14Neu}\) mutation express minor eye abnormalities and homozygous mutants are viable and fertile. These results extend the mouse \(Pax6\) allelic series to include a hypomorph mutant allele with a level of \(Pax6\) residual activity sufficient for homozygous and hemizygous mutants to be viable and fertile. We have previously described two hypomorph alleles, \(Pax6^{4Neu}\) and \(Pax6^{7Neu}\), in which homozygous embryos express more optic tissues than that seen in homozygous \(Pax6\) null mutants and there was delayed time of perinatal death (FAVOR *et al.* 2001). The site of the \(Pax6^{132-14Neu}\) missense mutation, Phe272Ile, is in the third helix of the homeodomain and is highly conserved across all paired-like homeodomain sequences. The aromatic sidechain is buried within the protein tertiary structure in close proximity to a second Phe site from helix 1 of the homeodomain and may participate in stabilizing the homeodomain tertiary structure. The third helix of the homeodomain makes direct contact to the DNA and our observation that the binding activity of the mutant \(Pax6^{132-14Neu}\) homeodomain to the P3 target DNA sequence is lost is compatible with the conclusion that the Phe272Ile missense mutation alters the tertiary structure of the homeodomain and prevents proper binding to the DNA target.

The glucagon promoter activity assays of the full length \(Pax6\) wildtype and mutant gene products employed the CAT expression vector driven by the -138 glucagon promoter. This promoter contains the G1 sequence which is a target site for binding of the \(Pax6\) paired and homeodomains and the paired domain alone is sufficient for activation when the homeodomain is deleted from the \(Pax6\) gene product (PLANQUE *et al.* 2001; RITZ-LASER *et al.* 1999). We observed that the glucagon promoter activity of the mutant \(Pax6^{132-14Neu}\) gene product was slightly increased. Thus, the mutated Phe272Ile site in the homeodomain may result in an increase of the glucagon promoter activation via the paired domain in the mutant \(Pax6\) product. In contrast, the Ser273Pro \(Pax6^{4Neu}\) missense mutation, also within the third alpha helix of the homeodomain, has reduced glucagon promoter activation in the CAT.
reporter gene construct driven by the -138 glucagon promoter. This indicates that this mutation, which is predicted to interrupt the third alpha helix structure (FAVOR et al. 2001), also affects the glucagon promoter activation via the paired domain. It likely does not affect Pax6 dimerization since it did not affect the wildtype Pax6 glucagon promoter activation in the $Pax6$ wildtype + $Pax6^{4Neu}$ co-transfection assays. Rather, we interpret these results to indicate that, within the $Pax6^{4Neu}$ mutant gene product, the mutant Pax6 homeodomain interferes with the paired domain DNA binding activity to the paired domain target sites.

The third hypomorph mutant allele utilised in the present studies, $Pax6^{7Neu}$, has been shown to be a c.A-3T base pair substitution outside the Pax6 ORF in the Kozak sequence and results in greatly reduced translation product (FAVOR et al. 2001).

**Correlation of Pax6 activity and the extent of eye development:** Previous studies have shown that normal eye development requires a narrow range of Pax6 activity. Heterozygous null mutations (HILL et al. 1991) as well as transgenic mice which overexpress Pax6 (SCHEDL et al. 1996) were associated with abnormal eye development. The identification of the $Pax6^{132-14Neu}$ hypomorph mutation, which is homozygous viable and has a higher level of Pax6 activity than the $Pax6^{4Neu}$ or $Pax6^{7Neu}$ hypomorph alleles, has extended the range of the mouse Pax6 allelic series. With the availability of an extended Pax6 allelic series we were able to construct wildtype and mutant Pax6 genotypes, which provides information regarding the extent of eye development and animal viability for four new levels of predicted Pax6 activity; one genotype ($Pax6^{132-4Neu/+}$) with Pax6 activity between 100% and 50%, and three genotypes within the critical region between 50% and 0% Pax6 activity ($Pax6^{132-4Neu}/Pax6^{132-4Neu}$, $Pax6^{132-4Neu}/Pax6^{4Neu}$, $Pax6^{132-4Neu}/Pax6^{7Neu}$, $Pax6^{132-14Neu}/Pax6^{7Neu}$, $Pax6^{132-14Neu}/Pax6^{7Neu}$). We observed that as the level of predicted Pax6 activity was reduced there was a progressive reduction in the extent of eye development. In the early stages of eye development two regions in the head critical for eye development show Pax6 expression: the early optic vesicle and the surface
ectoderm. Studies in the chick have shown that the regional localization of the Pax6-positive cells in the surface ectoderm is independent of a neighboring Pax6-positive optic vesicle (Li et al. 1994). The early optic vesicle is an evagination of the prospective forebrain and makes contact with the Pax6-positive region of the surface ectoderm. Upon contact of the optic vesicle with the surface ectoderm, the optic vesicle invaginates to form the optic cup, consisting of the outer layer (presumptive pigmented retinal layer) and the inner layer (presumptive neural retina layer). The lens placode, which is the Pax6-positive surface ectoderm at the region of contact between the optic vesicle and the surface ectoderm, invaginates into the optic cup to form the lens vesicle. In the class of genotypes with the highest level of predicted Pax6 activity the basic eye plan developed, consisting of a cornea, lens and retina. Thus, within this group of genotypes the levels of Pax6 activity were sufficient for the optic vesicle to make proper contact with the Pax6-positive surface ectoderm and the lens placode was competent to invaginate and form a lens.

The second group of mutant genotypes had an intermediate level of predicted Pax6 activity. The optic vesicle evaginated towards and maintained contact with the surface ectoderm. A retina developed but the presumptive lens placode of the surface ectoderm did not invaginate and form the lens vesicle. Conditional inactivation of Pax6 in a stage- and cell-specific manner has shown that at later stages the developmental competency of eye tissues depends upon the endogenous Pax6 activity of the cells from which the tissues are derived.

Inactivation of a single copy of Pax6 in the distal optic cup allows proper development of the lens and cornea but developmental abnormalities of the iris were observed. Similarly, inactivation of a single copy of Pax6 in the lens placode resulted in abnormal lens and cornea development. Retina development was normal and there were only mild non-cell-autonomous iris abnormalities (Davis-Silberman et al. 2005). Microsurgical removal of the Pax6-positive neural plate and thus ablation of optic vesicle development in the chick resulted in Pax6-positive surface ectoderm cells but lens development was blocked (Li et al. 1994).
Inactivation of *Pax6* in the early optic vesicle of the chick resulted in death of the optic vesicle cells and blocked lens development from the *Pax6*-positive surface epithelium (CANTO-SOLER and ADLER 2006). Thus, the maintenance of *Pax6* expression in the surface ectoderm is not dependent upon optic vesicle contact, but when optic vesicle contact to the surface ectoderm is prevented lens development is blocked. Complete inactivation of *Pax6* in the lens placode blocked lens development. Multiple, fully differentiated retinae were observed within the optic cup indicating that the presence of a lens was not required for the development of the retina (ASHERY-PADAN et al. 2000). Our observations for the second class of genotypes with intermediate levels of *Pax6* activity are compatible with the hypothesis that the levels of *Pax6* activity in the optic vesicle and the surface ectoderm were sufficient for retina development but were insufficient for development of the lens vesicle.

In the class of mutant genotypes with the lowest levels of predicted *Pax6* activity, the levels of *Pax6* activity were insufficient for proper eye development beyond a very early stage. Although there was an initiation of optic vesicle development, it terminated very prematurely and the resultant tissue had no resemblance to a rudimentary eye.

Taken together, these results indicate that the level of *Pax6* activity is less critical for the progression of optic vesicle evagination and retina development than it is for the invagination of the lens placode to develop into the lens vesicle. Characterization of the *Pax6*Sey null mutation (HOGAN et al. 1986) demonstrated that in homozygous mutant embryos evagination of the optic vesicle was initiated but contact with the surface ectoderm was lost at a very early stage. Studies utilizing *Pax6*+/− ↔ *Pax6*+/+ chimera have shown that upon contact of the optic vesicle to the surface ectoderm *Pax6* activity deduced from the fraction of *Pax6*+/+ and *Pax6*+/− cells in both the optic vesicle and the surface ectoderm is critical for the maintenance of this contact and for the induction of a lens placode (COLLINSO et al. 2000). Thus, lens development requires maintenance of the contact of the optic vesicle with the surface ectoderm and proper lens development is dependent upon *Pax6* activity in both structures.
Although we have assigned abstract relative Pax6 activity levels to the Pax6 mutant alleles based on viability of mutant genotypes, the actual situation is more complex due to the spectrum of genes targeted for activation by the different isoforms of the mutant alleles. Despite our simplistic notion of predicted Pax6 activity, it was very convincing to observe that the extent of eye development completely followed our a priori prediction of Pax6 activity.

**Mouse and human Pax6 allele databases:** PAX6/Pax6 functions as a transcription factor via DNA binding of its binding domains to different target genes and mutations affecting different specific regions of the Pax6 gene product may result in different aberrant phenotypes (Haubst et al. 2004; Singh et al. 2000; Tang et al. 1997). There is an extensive human PAX6 allelic variant database (http://pax6.hgu.mrc.ac.uk) last updated 27 September 2007, with 408 entries. Most PAX6 mutations result in premature termination of the translation product and there were 61 missense mutations: 43 in the paired domain, one in the linker region, five in the homeodomain, 12 in the P/S/T region, four at the Met start codon and 15 at the stop codon. Genotype-phenotype correlations have shown that mutations resulting in premature termination of the translation of the PAX6 gene product are mostly associated with aniridia, while missense mutations are often associated with non-aniridia phenotypes (Azuma and Yamada 1998; Azuma et al. 1996, 1998, 1999; Tzoulaki et al. 2005). The mouse mutant allele database (http://www.informatics.jax.org) contains 32 entries. Twenty-four mutant alleles were recovered in phenotype screens and eight entries represent mutant knock-out or conditional knock-out constructs. Of the 24 mutant alleles recovered in phenotype-based screens, most result in premature truncation of the translation product or deletions of the Pax6 gene. Two Pax6 alleles are missense mutations in the paired domain, Pax6<sup>Leca2</sup> and Pax6<sup>Leca4</sup> (Thaung et al. 2002) and four missense mutations have been identified in the homeodomain, Pax6<sup>4Neu</sup> (Favor et al. 2001), Pax6<sup>Leca1</sup> (Thaung et al. 2002), Pax6<sup>13rt</sup>
(ROSSANT 2003), and \( \text{Pax6}^{132-14Neu} \) (present study). It is interesting to note that all missense mutations in the mouse in the homeodomain are at amino acid sites within the third helix of the homeodomain.

**General conclusions:** An extensive allelic series is useful for genetic, molecular and development analyses of gene function. Detailed *in vivo* analyses are usually only possible in model laboratory organisms. The present study has extended the mouse allelic series of the \( \text{Pax6} \) gene to include a homozygous and hemizygous viable and fertile hypomorph allele. In our genotype-phenotype studies to correlate levels of \( \text{Pax6} \) activity with the degree of aberrant development, we have focused on the eye. Obviously, similar studies with other organs in which \( \text{Pax6} \) plays an important role in development would be interesting. For example, the mechanisms leading to lethality of \( \text{Pax6} \) mutant neonates is still not known. The best candidates are neurologic or metabolic dysfunctions due to the brain and the pancreas developmental defects. Here, analyses of brain or pancreas development in the \( \text{Pax6} \) mutant genotype constructs could be informative.

**ACKNOWLEDGMENTS**

Research was partially supported by National Institutes of Health Grant R01EY10321. We thank Dr. Laure Bally-Cuif and Dr. Magdalena Götz for critically reading our manuscript, and we greatly appreciate the expert technical assistance of Oceane Anezo, Sibylle Frischholz, Bianca Hildebrand, Brigitta May and Sylvia Wolf.


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dimerization of paired class homeo domains on DNA. Genes Dev 7: 2120-2134.
Table 1: Characterization of eye phenotypes in P35 $Pax6^{132-14Neu}$ mutant mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Eye Class$^b$</th>
<th>0%</th>
<th>minor iris</th>
<th>25%</th>
<th>50%</th>
<th>75%</th>
<th>100%</th>
<th>extreme microphthalmia</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td></td>
<td>118</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/-</td>
<td></td>
<td>38</td>
<td>190</td>
<td>28</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32</td>
</tr>
</tbody>
</table>

$^a$ +/- were wildtype strain C3H mice; +/- mice were produced in the cross -/- x +/-; -/- mice were from the cross -/- x -/-.

$^b$ Class 0%, 25%, 50%, 75% and 100% denote lens/corneal opacities affecting 0, 25, 50, 75 or 100% of the eye, respectively.
Table 2: Normalized eye weight in P35 Pαx6<sup>132-14Neu</sup> mutant mice.

<table>
<thead>
<tr>
<th>Genotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Normalized Eye Weight&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>+/+</td>
<td>116</td>
</tr>
<tr>
<td>+/-</td>
<td>255</td>
</tr>
<tr>
<td>-/-</td>
<td>32</td>
</tr>
</tbody>
</table>

<sup>a</sup> Genotype origins as in Table 1.

<sup>b</sup> Normalized eye weight = [(Eye weight(mg)/Body weight(g)) x 10]
Table 3: Recovery and fertility testing of *Pax6* compound heterozygotes.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Phenotype class&lt;sup&gt;a&lt;/sup&gt;</th>
<th>0%</th>
<th>minor iris anomaly, 25%, 50%, 75%, or 100% ext. microphthalmia</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Pax6&lt;sup&gt;4Neu&lt;/sup&gt;-/+ x *Pax6&lt;sup&gt;132-14Neu -/-&lt;/sup&gt;</td>
<td>2</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>*Pax6&lt;sup&gt;4Neu&lt;/sup&gt;/Pax6&lt;sup&gt;132-14Neu&lt;/sup&gt; x +/+</td>
<td>3</td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td>*Pax6&lt;sup&gt;4Neu&lt;/sup&gt;/Pax6&lt;sup&gt;132-14Neu&lt;/sup&gt; x *Pax6&lt;sup&gt;132-14Neu -/-&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>36</td>
</tr>
<tr>
<td>*Pax6&lt;sup&gt;2Neu&lt;/sup&gt;-/+ x *Pax6&lt;sup&gt;132-14Neu -/-&lt;/sup&gt;</td>
<td>1</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>*Pax6&lt;sup&gt;2Neu&lt;/sup&gt;/Pax6&lt;sup&gt;132-14Neu&lt;/sup&gt; x +/+</td>
<td>-</td>
<td>29</td>
<td>-</td>
</tr>
<tr>
<td>*Pax6&lt;sup&gt;2Neu&lt;/sup&gt;/Pax6&lt;sup&gt;132-14Neu&lt;/sup&gt; x *Pax6&lt;sup&gt;132-14Neu -/-&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>*Pax6&lt;sup&gt;Sey-Neu&lt;/sup&gt;-/+ x *Pax6&lt;sup&gt;132-14Neu -/-&lt;/sup&gt;</td>
<td>2</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>*Pax6&lt;sup&gt;Sey-Neu&lt;/sup&gt;/Pax6&lt;sup&gt;132-14Neu&lt;/sup&gt; x +/+</td>
<td>1</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>*Pax6&lt;sup&gt;Sey-Neu&lt;/sup&gt;/Pax6&lt;sup&gt;132-14Neu&lt;/sup&gt; x *Pax6&lt;sup&gt;132-14Neu -/-&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>*Pax6&lt;sup&gt;2Neu&lt;/sup&gt;-/+ x *Pax6&lt;sup&gt;132-14Neu -/-&lt;/sup&gt;</td>
<td>-</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>*Pax6&lt;sup&gt;2Neu&lt;/sup&gt;/Pax6&lt;sup&gt;132-14Neu&lt;/sup&gt; x +/+</td>
<td>-</td>
<td>26</td>
<td>-</td>
</tr>
<tr>
<td>*Pax6&lt;sup&gt;2Neu&lt;/sup&gt;/Pax6&lt;sup&gt;132-14Neu&lt;/sup&gt; x *Pax6&lt;sup&gt;132-14Neu -/-&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>9</td>
</tr>
</tbody>
</table>
$P_{\text{axb}}^{3\text{Neu}^{+}} \times P_{\text{axb}}^{132-14\text{Neu}^{+}}$  

- 9 7

*Phenotype classes as in Table 1.*
Legends to the Figures:

Figure 1. Binding activity of the wildtype, $\text{Pax6}^{4\text{Neu}}$ and $\text{Pax6}^{132-14\text{Neu}}$ $\text{Pax6}$-homeodomains to the P3 DNA target sequence. Whole cell extracts from the transformed $\text{E. coli}$ strain M15[pREP4] were incubated with 15 fmol of the digoxygenin-labeled P3 target oligonucleotide. Binding specificity was demonstrated by competition of the labelled P3 target oligonucleotide with an excess of unlabeled P3 target oligonucleotide and a supershift assay with the $\text{Pax6}$ homeodomain-specific antibody. (Lane 1) P3 oligonucleotide alone; (Lane 2) P3 oligonucleotide with 50 ng wildtype $\text{Pax6}$ homeodomain; (Lane 3) P3 oligonucleotide with 50 ng wildtype $\text{Pax6}$ homeodomain and 3.3 pmol unmarked P3 oligonucleotide; (Lane 4) P3 oligonucleotide with 50 ng wildtype $\text{Pax6}$ homeodomain and 2µl of the $\text{Pax6}$ homeodomain-specific antiserum; (Lane 5) P3 oligonucleotide with 50 ng mutant $\text{Pax6}^{4\text{Neu}}$ homeodomain; (Lane 6) P3 oligonucleotide with 50 ng mutant $\text{Pax6}^{132-14\text{Neu}}$ homeodomain. The positions of the free oligonucleotide (O), the monomeric (M) and dimeric (D) DNA-protein complexes, and the shifted antibody DNA-protein complex (S) are marked.

Figure 2. Glucagon promoter activation by wildtype, $\text{Pax6}^{4\text{Neu}}$ and $\text{Pax6}^{132-14\text{Neu}}$ gene products. A CAT assay was employed to measure the glucagon promoter activity of the wildtype or mutant Pax6 in BHK-21 co-transfected cells. CAT activities were normalized to $\beta$-galactosidase activity obtained from co-transfection with the LacZ expression vector. Fold activation represents the observed normalized CAT activities relative to the normalized CAT activity in cells co-transfected with the empty expression plasmid DNA. (Lane 1) cells transfected with 100 ng empty plasmid DNA; (Lanes 2 and 3) cells transfected with 100 ng (Lane 2) or 300 ng (Lane 3) wildtype $\text{Pax6}$ plasmid DNA constructs; (Lanes 4 and 5) cells transfected with 100 ng (Lane 4) or 300 ng (Lane 5) mutant $\text{Pax6}^{4\text{Neu}}$ plasmid DNA constructs; (Lanes 6 – 9) cells co-transfected with plasmid DNA from wildtype $\text{Pax6}$ and
mutant Pax6<sup>4Neu</sup> constructs: (Lane 6) 100 ng DNA from wildtype Pax6 and 100 ng from mutant Pax6<sup>4Neu</sup>; (Lane 7) 100 ng DNA from wildtype Pax6 and 300 ng from mutant Pax6<sup>4Neu</sup>; (Lane 8) 300 ng DNA from wildtype Pax6 and 100 ng from mutant Pax6<sup>4Neu</sup>; (Lane 9) 300 ng DNA from wildtype Pax6 and 300 ng from mutant Pax6<sup>4Neu</sup>; (Lanes 10 and 11) cells transfected with 100 ng (Lane 10) or 300 ng (Lane 11) DNA from mutant Pax6<sup>132-14Neu</sup> plasmid constructs; (Lanes 12 – 15) cells co-transfected with plasmid DNA from wildtype Pax6 and mutant Pax6<sup>132-14Neu</sup> constructs: (Lane 12) 100 ng DNA from wildtype Pax6 and 100 ng from mutant Pax6<sup>132-14Neu</sup>; (Lane 13) 100 ng DNA from wildtype Pax6 and 300 ng from mutant Pax6<sup>132-14Neu</sup>; (Lane 14) 300 ng DNA from wildtype Pax6 and 100 ng from mutant Pax6<sup>132-14Neu</sup>; (Lane 15) 300 ng DNA from wildtype Pax6 and 300 ng from mutant Pax6<sup>132-14Neu</sup>. Columns represent mean ± S.D. fold activation from two determinations.

Figure 3. The extent of eye development in mutant Pax6 genotype constructs with varying levels of predicted Pax6 activity. From the Pax6 allelic series we have utilized Pax6 wildtype, the Pax6<sup>3Neu</sup> null (a frame shift mutation which ablates the linker, homeodomain and transactivation domain (FAVOR et al. 2001), the Pax6<sup>4Neu</sup> hypomorph (a homeodomain missense mutation which ablates homeodomain binding and reduces translation activation at paired domain target sites (FAVOR et al. 2001; this study), the Pax6<sup>7Neu</sup> hypomorph (a Kozak sequence mutation which greatly reduces translation levels of Pax6 (FAVOR et al. 2001) and the Pax6<sup>132-14Neu</sup> hypomorph (this study) alleles. The genotypes are arranged in the figure from top to bottom in a descending order of predicted Pax6 activity. The genotypes in A, B (wildtype) up to and including M, N (Pax6<sup>3Neu</sup>/Pax6<sup>132-14Neu</sup>) are viable and fertile. The genotypes in O, P (Pax6<sup>7Neu</sup>/Pax6<sup>7Neu</sup>) and Q, R (Pax6<sup>3Neu</sup>/Pax6<sup>7Neu</sup>) are lethal. Regions at which there was an abrupt shift in the extent of eye development are indicated by horizontal lines and identify three groups: Class 1 = highest levels of Pax6 activity and eye development was complete; Class 2 = intermediate levels of Pax6 activity and a rudimentary eye
developed; Class 3 = lowest levels of *Pax6* activity and eye development terminated very early. Head overview (A, C, E, G, I, K, M, O, Q) and eye (B, D, F, H, J, L, N, P, R) histology of E15 wildtype and *Pax6* mutant constructs. Eye development is normal in homozygous wildtype (A, B) embryos with 100% *Pax6* activity, showing a well developed cornea (co), lens (le), retina (ret) and a distinct anterior chamber between the cornea and the anterior surface of the lens. In heterozygous *Pax6*^{132-14Neu} embryos (C, D) with *Pax6* activity greater than 50% but less than 100% eye size is slightly reduced and the separation of the anterior surface of the lens from the cornea is incomplete resulting in a reduced anterior chamber. Heterozygotes of the *Pax6*^{3Neu} null mutation (E, F) with 50% *Pax6* activity show a further reduction in eye size as compared to heterozygous *Pax6*^{132-14Neu} mutants, the lens remains attached to the cornea, there is no anterior chamber and a plug of persistent epithelial cells remains in the cornea (arrow). Homozygous *Pax6*^{132-14Neu} mutants (G, H) have less than 50% *Pax6* activity. All major eye tissues (cornea, lens and retina) develop. However eye size is reduced and there is a large plug of persistent epithelial cells which remains attached between the cornea and the lens (arrow). Compound heterozygotes between *Pax6*^{132-14Neu} and the hypomorph *Pax6*^{6Neu} (I, J) or *Pax6*^{7Neu} (K, L) mutant alleles have *Pax6* activity less than that in homozygous *Pax6*^{132-14Neu} mutants. Eye development is incomplete. The optic vesicle makes contact with the surface ectoderm and a retina is formed with characteristic displacements in the dorsal tip region (arrowheads). A distinct optic pit is present (arrows). However the lens placode does not invaginate and there are no lenticular tissues. Compound heterozygote *Pax6*^{3Neu}/*Pax6*^{132-14Neu} embryos (M, N) have less *Pax6* activity than that in the *Pax6*^{4Neu}/*Pax6*^{132-14Neu} or *Pax6*^{7Neu}/*Pax6*^{132-14Neu} compound heterozygotes and eye development terminates at an earlier stage. The optic vesicle appears to have made contact with the surface ectoderm but contact has not been maintained, there is no apparent invagination of the optic vesicle to initiate formation of an optic cup, there is only a rudimentary response of the surface ectoderm to form an optic pit (arrow), and apparently no
lenticular tissue develops from the presumptive lens placode. *Pax6* activity in *Pax6*<sup>2Neu</sup> homozygous mutants (O, P) is less than that in *Pax3Neu/Pax6*<sup>132-14Neu</sup> and eye development terminates at a very early stage. There is no invagination of the optic vesicle, a very rudimentary response of the surface ectoderm (arrows) and no apparent invagination of the presumptive lens placode to form lenticular tissue. Homozygous *Pax6*<sup>3Neu</sup> mutants (Q, R) have 0% *Pax6* activity and although optic vesicle development is initiated it terminates extremely early and the resultant tissue (arrowheads) was displaced and found extremely distant from the surface ectoderm. There was no observed initial invagination of the surface ectoderm. Bar in A represents 400µm and bar in B represents 100µm.
Figure 2

Glucagon promoter activation

Fold activation

Transfection DNA
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Pax6 activity</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Pax6^{+/+}$</td>
<td>100%</td>
<td>1 = eye plan complete</td>
</tr>
<tr>
<td>$Pax6^{+14Neu+}$</td>
<td>&lt;100%, &gt;50%</td>
<td>1</td>
</tr>
<tr>
<td>$Pax6^{3Neu+}$</td>
<td>50%</td>
<td>1</td>
</tr>
<tr>
<td>$Pax6^{+14Neu/14Neu}$</td>
<td>&lt;50%</td>
<td>1</td>
</tr>
<tr>
<td>$Pax6^{4Neu/14Neu}$</td>
<td>&lt;50%</td>
<td>2 = retina present, lens vesicle did not develop</td>
</tr>
<tr>
<td>$Pax6^{7Neu/14Neu}$</td>
<td>&lt;50%</td>
<td>2</td>
</tr>
<tr>
<td>$Pax6^{3Neu/14Neu}$</td>
<td>&lt;50%, &gt;0%</td>
<td>3 = very early termination of eye development</td>
</tr>
<tr>
<td>$Pax6^{7Neu/7Neu}$</td>
<td>&gt;0%</td>
<td>3</td>
</tr>
<tr>
<td>$Pax6^{3Neu/3Neu}$</td>
<td>0%</td>
<td>3</td>
</tr>
</tbody>
</table>