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Gene expression pattern

A highly conserved lens transcriptional control element from the Pax-6 gene

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Abstract

We have identified a short segment of the mouse Pax-6 gene 5′ flanking region that is necessary and sufficient for reporter construct expression in components of the eye derived from non-neural ectoderm. This transcriptional control element has a highly conserved nucleotide sequence over 341 bp and is located approximately 3.5 kb upstream of the start-point for transcription from the most proximal promoter (PO) of the Pax-6 gene. The level of identity between the human and mouse Pax-6 genes in this region is 93%. When combined either with its natural promoter or a heterologous minimal promoter, this element directs reporter construct expression to a region of surface ectoderm overlying the optic cup beginning at E8.5–9.0 (12–14 somites). Subsequently, expression is restricted to the lens (primarily the lens epithelium) and the corneal epithelium. This element will provide an important tool in future transgenic analyses of lens formation and will allow identification of transcription factors with a central function in lens development.

Keywords: Pax-6 gene; Proximal promoter; Transcription factors

1. Introduction

The transcription factor Pax-6 is known to have a critical role in development of the eye in a number of species. In *Drosophila*, homozygous mutation of the Pax-6 orthologue *eyeless* results in missing eye structures (Quiring et al., 1994) while in humans, heterozygous Pax-6 mutations give the ocular defects aniridia (Jordan et al., 1992) and Peters' anomaly (Hanson et al., 1994). Analogous defects are seen in the *Small* eye (*Sey*) mice, with the homozygous condition resulting in severe cranio-facial abnormalities including absence of eyes (Hill et al., 1991).

Gain-of-function experiments suggest that in invertebrates, Pax-6 is sufficient for development of the eye. Overexpression of *Drosophila* Pax-6 in imaginal discs results in the development of ectopic compound eyes (Gehring, 1996) and remarkably, this activity can be mimicked by Pax-6 from other species (Glardon et al., 1997; Tomarev et al., 1997). More recently, it was shown that overexpressed Pax-6 can lead to the cell-autonomous formation of well-formed ectopic lenses in *Xenopus laevis* (Altmann et al., 1997) suggesting that in a vertebrate as well, Pax-6 is sufficient to direct the formation of large components of the eye.

Two sets of experiments argue that Pax-6 is necessary for development of the vertebrate lens. First, tissue recombinations have shown that when the *Small eye (Sey)* mutation is present in presumptive lens ectoderm, formation of a lens is prevented (Fujiwara et al., 1994). Second, chimeric mice generated by aggregating cells from wild-type and *Sey* 8-cell embryos show no *Sey* cells in the lens (Quinn et al., 1996). These experiments both argue that in mammals, Pax-6 is required for lens development from the stage of close contact between the optic vesicle and presumptive lens ectoderm.

In the mouse, Pax-6 has a complex pattern of expression

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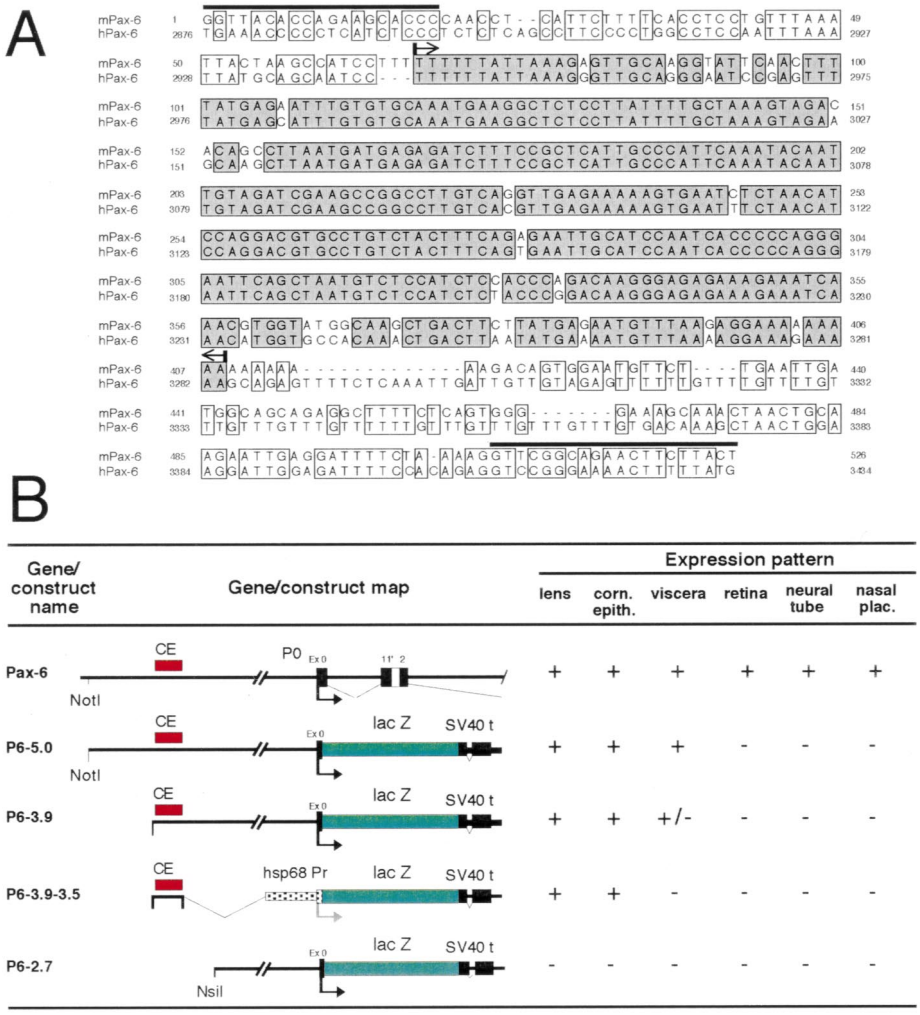
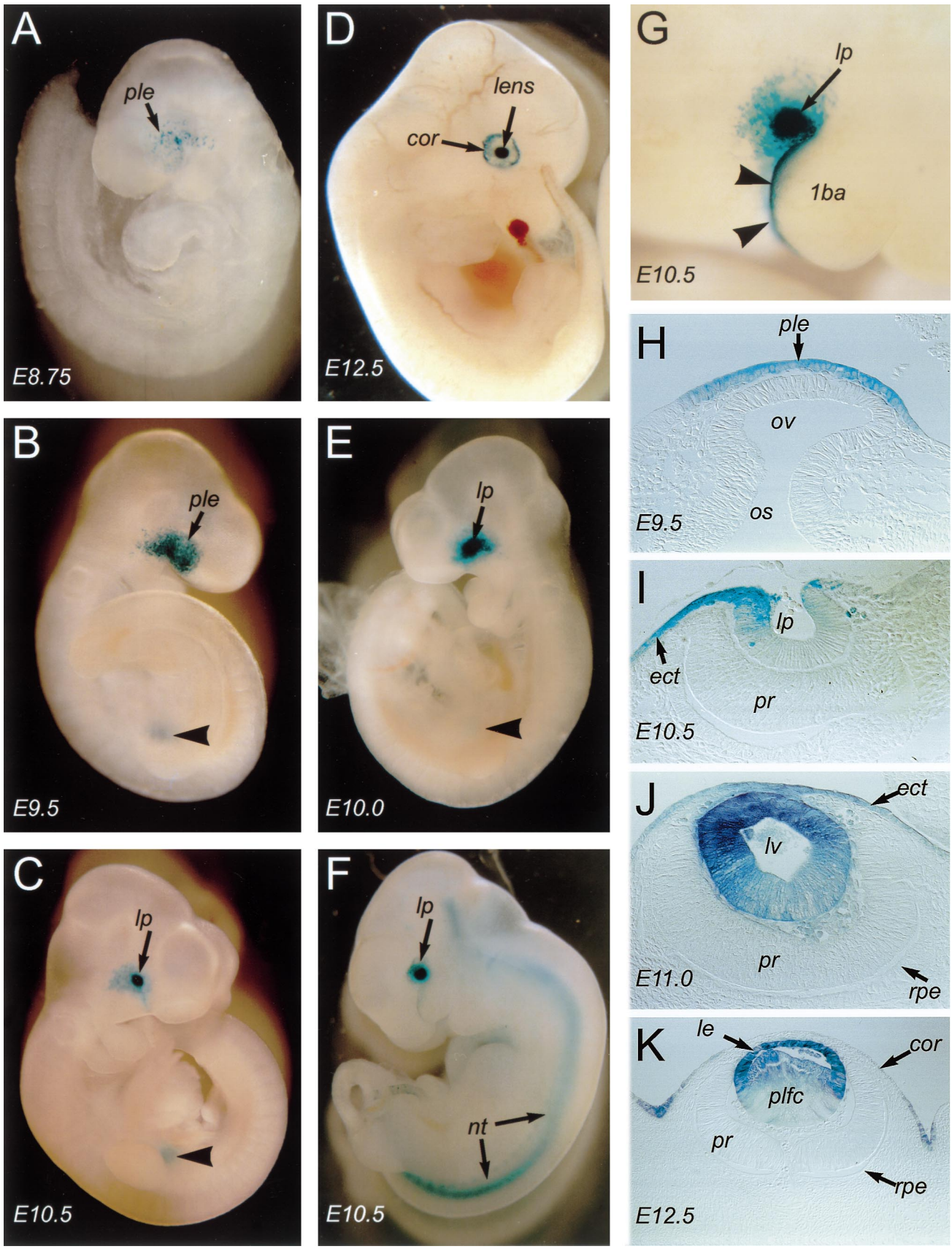


Fig. 1. A conserved sequence element in the Pax-6 gene and its incorporation into reporter constructs. (A) Nucleotide identities between mouse (m) and human (h) Pax-6 gene sequences for the conserved region are indicated by the boxing. The 341-bp region of high percentage identity is indicated by the arrows and gray shading. Sequence numbering is indicated for the human sequence on the lower line (accession number Z95332) and for the mouse sequence on the upper line (accession number pending) (note that the lower line in column 2, panel 4 should read 3028 and not 151 as shown). The regions of complementarity for oligonucleotides used in the construction of reporter plasmids are overlined (see Section 3). (B) The basic structure of the Pax-6 gene is indicated in the first line of the table. The approximate location of the conserved element (CE) is indicated by the red box. Reporter constructs carry a transcriptional start-point either from the Pax-6 gene (black arrow) or from the hsp68 gene (gray arrow). In each construct, the start-point of transcription is immediately upstream of the open reading frame for β-galactosidase (*lacZ*, blue box) which in turn is upstream of the splicing and polyadenylation signals from the Simian virus 40 small t gene (SV40 t) (Song et al., 1996). For the P6-3.9-3.5 construct, a minimal promoter from the mouse *hsp68* gene is employed. All other constructs use the Pax-6 gene PO promoter. The expression pattern resulting from the generation of transgenic mice is summarized in the columns at right, a + indicating strong expression, a ± indicating weak expression and a − indicating no detectable expression.

Fig. 2. The conserved element supports reporter construct expression in lens and corneal epithelium. (A–D) Whole mount X-gal staining for transgenic mice generated with construct P6-5.0. Staining is observed in the presumptive lens ectoderm (ple) at E8.75 (A) and E9.5 (B) in the lens pit (lp) at E10.5 (C) and in the lens and future corneal epithelium (cor) at E12.5 (D). At each developmental stage shown, X-gal labeling is also observed in the viscera (arrowheads). (E) Transgenic mice generated with the P6-3.9 construct showed strong X-gal staining in the lens pit (lp) of the developing eye and weak staining in a discrete component of the viscera (arrowhead). (F) The P6-3.9-3.5 construct showed X-gal staining in the lens pit (lp) and neural tube (nt). Staining in the neural tube is typical of the activity of the minimal promoter for the mouse hsp68 gene (Song et al., 1996). (G) The pattern of X-gal staining at E10.5 for the P6-3.9-3.5 construct in the region of the lens pit (lp) and first branchial arch (1ba). Staining is observed transiently at the rostral edge of the first branchial arch (G, arrowheads). (H–K) Histological sections through the eye at various stages of development. In all cases, rostral is to the left, caudal to the right. (H) At E9.5, X-gal staining is restricted to the presumptive lens ectoderm (ple) overlying the optic vesicle (ov). (I) At E10.5, staining is seen in the invaginating lens pit (lp) and in the surface ectoderm (ect) but not presumptive retina (pr). (J) At E11.0, *lacZ* expression is detected in the surface ectoderm (ect) and throughout the lens vesicle (lv). (K) At E12.5 when the primary lens fiber cells (plfc) are elongating and filling the volume of the lens vesicle, *lacZ* expression is seen primarily in the lens epithelium (le) but is also observed in the future corneal epithelium (cor). In all sections, labeling is not observed in the neurallyderived optic vesicle (ov), optic stalk (os), presumptive retina (pr) and presumptive retinal pigmented epithelium (rpe).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(Walther and Gruss, 1991; Grindley et al., 1995) that in the eye includes the presumptive and mature lens, retina and corneal epithelium (Walther and Gruss, 1991; Grindley et al., 1995). This expression pattern is well conserved in different vertebrate species including the frog (Li et al., 1997) and chick (Li et al., 1994). In the current study, we have addressed the transcriptional control of the Pax-6 gene as a means of identifying elements of lens development pathways. Thus far, we have identified a short region of the Pax-6 gene 5′ flanking sequence that is unusually conserved between mouse and man and is necessary and sufficient for directing expression of a reporter construct to the presumptive lens ectoderm and corneal epithelium.

2. Results and discussion

The conserved element we describe was recognized through an alignment of the 5′ flanking sequence of the human and mouse Pax-6 genes. The mouse Pax-6 gene was isolated and sequenced over a distance of 7 kb upstream of the PO promoter (previously identified in the quail gene (Plaza et al., 1995)). Alignment of the mouse and human (accession number Z95332) sequences revealed a region of high identity located between 3.5 and 3.9 kb upstream of the PO promoter in the mouse Pax-6 gene (Fig. 1A, gray shading; note that the lower line in column 2, panel 4 should read 3028 and not 151 as shown). Remarkably, this element showed 93% conservation but was demarcated by regions where identity was more limited. Based on the assumption that conserved regions of sequence located outside a gene might be involved in control of transcription, we generated reporter constructs to test the activity of this element

Construct P6-5.0 (Fig. 1B) contained sequences from the P0 promoter transcription start to a *Not*I site located approximately 5 kb upstream and contained the 341-bp region of high identity. When transgenic mice were generated with this construct, X-gal staining revealed expression of *lacZ* in the surface ectoderm overlying the optic cup from E8.5 (12 somites) onwards (Fig. 2A–D). Staining in the presumptive eye region appeared initially as scattered X-gal positive cells (Fig. 2A). At E9.5, a tear-drop shaped area of ectodermal staining arose that encompassed the optic eminence and had a sharply-defined rostral boundary corresponding to the edge of the underlying optic vesicle (Fig. 2B). This region extended caudally towards the maxillary component of the first branchial arch (Fig. 2B). At E10.5, X-gal labeling became more intense in the lens pit at the center of the area of ectodermal staining but had diminished peripherally (Fig. 2C). At E12.5, intense X-gal labeling was observed in the lens together with lighter staining of an annular region of future corneal epithelium (Fig. 2D). Labeling in a discrete region of the viscera at the level of the fore-limb buds was observed through the body wall (Fig. 2B and C).

To determine if any sequences 5′ to the conserved element had any role in the expression pattern observed with P6-5.0, we generated construct P6-3.9. This contained DNA in the Pax-6 gene from the 5′ boundary of the conserved element to the start-point of transcription for the PO promoter (Fig. 1B). In transgenic mice, this gave an identical pattern of expression to construct P6-5.0 in components of the eye (Fig. 2E) at all stages of development. Expression in the viscera was still present though very much reduced in intensity (Fig. 2E, arrowhead).

The conserved element was shown to have an eye-specific expression activity in isolation with construct P6-3.9-3.5 where the conserved region was placed adjacent to a minimal promoter from the mouse *hsp68* gene (Fig. 1B). In transgenic mice, this construct gave expression in the eye region with an identical pattern to that of both P6-5.0 and P6-3.9 but excluded expression from the viscera (Fig. 2F). Background expression in the neural tube was observed and is typical of the activity of the *hsp68* gene minimal promoter (Song et al., 1996). With all constructs containing the conserved element, *lacZ* expression was transiently observed at the rostral edge (Fig. 2G) and in the medial ectoderm (not shown) of the first branchial arch. A construct excluding the conserved element (P6-2.7, Fig. 1B) gave either no expression of *lacZ* or ectopic expression unrelated to the pattern expected for Pax-6 (data not shown).

Histological sections revealed that for all constructs containing the conserved element, *lacZ* expression was restricted to eye components derived from non-neural ectoderm. Expression of the reporter constructs was detectable as early as E8.5 (12 somites) with intense X-gal staining arising at E9.5 (20 somites) in the ectoderm immediately overlying the optic vesicle (Fig. 2H). At E10.5, the invaginating ectoderm of the lens pit was labeled (Fig. 2I) and at E11.0, the lens vesicle (Fig. 2J). At E12.5, the maturing lens expressed *lacZ* with the strongest staining appearing in the lens epithelium (Fig. 2K). From E10.5 to E12.5, *lacZ* expression was detectable in the surface ectoderm destined to become the corneal epithelium (Fig. 2I–K). Importantly, with all reporter constructs used to make transgenic mice, *lacZ* expression was excluded from both the retinal and pigmented epithelial layers of the optic cup, as well as other sites where endogenous Pax-6 expression is normally observed (Walther and Gruss, 1991; Grindley et al., 1995). The identity of the tissue responsible for staining in the viscera with P6-5.0 and P6-3.9 is currently under investigation.

Combined, these experiments argue that the conserved region described has activity in directing transgene expression to the ectoderm that will form the lens and corneal epithelium. This region is currently delimited to 527 bp with a highly conserved core sequence of 341 bp. The identification of this transcriptional control element has two important consequences for future work on lens development. First, it will be a very powerful tool for analysis of lens development using transgenic mice and second, since Pax-6 expression in ectoderm appears to be necessary and sufficient for lens development (Fujiwara et al., 1994; Grindley et al., 1995; Altmann et al., 1997), this conserved

element probably represents an essential component of a lens development pathway.

3. Materials and methods

3.1. Cloning of the mouse Pax-6 gene

The Pax-6 gene was isolated from a mouse genomic library generated from the 129sv strain in the λFIXII vector (Stratagene) using a PCR-derived probe from the region just upstream of the PO promoter (Plaza et al., 1995). Three distinct λ clones were isolated, restriction mapped and found to span a 15-kb region that included at least 7 kb 5′ of the PO promoter. The 7-kb region upstream of the PO promoter was subcloned and sequenced using automated techniques. Sequence alignments were performed using the Lasergene software suite.

3.2. Generation of reporter constructs

lacZ reporter constructs (Fig. 1B) were generated using the plasmid p*hspPTlacZpA* (Song et al., 1996) kindly provided by Dr. D. Epstein. p*hspPTlacZpA* contains promoter sequences (−664 to +224 relative to the start-point of transcription) from the mouse *hsp68* gene and includes the translational start codon fused in-frame to a *lacZ* open reading frame. For the P6-3.9-3.5 construct, the conserved element was amplified by PCR using the oligonucleotides indicated below (see Fig. 1) and was subcloned upstream of the minimal *hsp68* promoter. For all other reporter constructs, the *hsp68* promoter was eliminated and a region of the Pax-6 gene that included the PO promoter was subcloned upstream of *lacZ* (Fig. 1B). The following oligonucleotides were used to amplify the conserved region for subcloning into the p*hspPTlacZpA* vector using NotI and BamHI restriction sites: 5′ primer; aag gaa aaa agc ggc cgc ggt tac acc aga agc acc c, 3′ primer; gcg gga tcc agt aag aag ttc tgc cga ac.

3.3. Generation of transgenic mice

Regions of the Pax-6 gene were tested for activity in transcriptional regulation using a transient transgenic mouse assay (Song et al., 1996). Reporter construct DNA was injected into mouse zygotes (Hogan et al., 1986) and the embryos allowed to develop to an appropriate stage. They were then dissected from the uterus, fixed and stained for *lacZ* activity according to established protocols (Song et al., 1996).

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