APPENDIX

METHODS

Construction of Transgenic Vectors

An novel $Bgl\text{II}$ site was inserted into the CPV2 vector at -86 relative to the murine $\alpha$A-crystallin transcription start site by overlapping PCR, creating the intermediate CPV7 vector. The precise sequence modification in CPV7 was CCCGagatctAGCTGA where the lowercase letters represent the insertion that created the $Bgl\text{II}$ site (underlined) and the endogenous $\alpha$A-crystallin promoter sequences are shown by uppercase letters. To create a consensus Pax6 binding site, two oligonucleotides: 22772 (5’-GATCCATCTTCACGCATGAGTGACTG-3’) and 22773 (5’-GATCCAGTCACTCATGCGTGAAGATG-3’) were made. These partially complementary oligonucleotides were annealed and phosphorylated to generate a copy of the Pax6 consensus binding site with 5’ overhangs compatible with the $Bgl\text{II}$ restriction site. Then the Pax6 consensus binding sequence was inserted into the $Bgl\text{II}$ site of CPV7 to generate CPV14 and CPV15 vectors, differing only by the orientation of the single Pax6 binding site as confirmed by sequence analysis.

A 2.2 kb $Bam\text{HI-}Eco\text{RI}$ genomic fragment containing the hGH gene from pOGH (obtained from Francesco DeMayo, Baylor College of Medicine) was subcloned into $Bam\text{HI}/Eco\text{RI}$ cut CPV2, CPV14 and CPV15, creating CPV2/hGH, CPV14/hGH and CPV15/hGH respectively.

For $\alpha B1/hGH$ construct, genomic sequence from -214 to +38 of the mouse $\alpha B$-crystallin gene (relative to the major lens transcription start site, Genbank Accession No. M73741) was amplified by PCR using two primers: (primer 25763, linker sequence containing $Bgl\text{II}$ and $Sac\text{I}$ sites italicized) 5’-GAAGATCTGAGCTCGTGAACAAAGACCATG-3’ and (primer 25762, linker sequence containing $Bam\text{HI}$, $Bgl\text{II}$ and $Xho\text{I}$ sites italicized) 5’-CGGGATCCAAAGATCTC
TCGAGATGAATGCAGAGTCGG-3’. The resultant 285-bp product was digested with SacI and BamHI and inserted into CPV2 that had been partially digested with SacI and completely digested with BamHI to replace the murine αA-crystallin promoter, creating the vector αB1.

The promoter of αB1 was isolated by KpnI and BamHI digestion and ligated into the vector backbone of KpnI/BamHI digested CPV2/hGH, replacing the αA-crystallin promoter to create αB1/hGH.

The αA/αB4/hGH construct was made through several steps. First, the 3’ untranslated region and polyadenylation signal of the murine αB-crystallin gene was amplified by PCR using primers: (primer 25508, linker sequence completing a SmaI site italicized, endogenous αB-crystallin stop codon underlined) 5’-TCCCCCGGGTAGATCCCCCTTTCCTCATTG-3’ and (primer 25764, linker sequence containing XbaI site italicized) 5’-GGTCTAGACCCCTGAATCATAGTTTG-3’. The resultant 248-bp PCR product was digested with SmaI and XbaI and ligated to the large fragment of SmaI/SpeI cut αB1, replacing the SV40-derived intron and polyadenylation sequence of αB1. This intermediate plasmid was called αB2. The first intron of the murine αB-crystallin gene was amplified by PCR with primers: (primer 24535, linker containing BamHI site italicized, and splice donor junction indicated by the slash) 5’-CAGGATCCGGACTCTCAGAG/GTGAGTCTGC-3’ and (primer 24536, linker completing a BamHI site italicized, and splice acceptor junction indicated by a slash. The underlined T represents an intentional base change to destroy a potential initiator ATG located downstream of the splice junction in the wild type αB-crystallin gene.) 5’-CCGGATCCAAACGTAT/CTAGAATAAC-3’. The resultant 1084-bp fragment was digested with BamHI and inserted between the BglII and BamHI sites of αB1, creating the intermediate vector αB3. A three-way ligation consisting of the KpnI/BamHI fragment of αB3 containing the αB-crystallin promoter and its
first intron, the BamHI/EcoRI fragment of pOGH 43 containing the hGH gene and the 
EcoRI/KpnI fragment of αB2 containing the αB-crystallin 3’ untranslated region /polyadenylation sequences and the plasmid backbone was performed to create αB4/hGH. To 
generate the composite αA/αB promoter, CPV7 (described above) was digested with BglII and 
BamHI to remove the −86/+43 region of the αA-crystallin gene. The PCR-amplified murine αB-
crystallin promoter (described above) was digested with BglII and ligated into the BglII/BamHI 
cut CPV7, creating the intermediate αA/αB composite promoter vector. The αA/αB composite 
promoter was released by digestion with KpnI and XhoI and ligated into KpnI/XhoI cut 
αB4/hGH to replace the αB-crystallin promoter, completing the αA/αB4/hGH construct.

The Cre coding sequence and intron/polyadenylation sequences contained in the murine 
metallothionein gene were excised from pBS216 (obtained from Brian Sauer, 2) by XhoI/HindIII 
digestion. The resulting 2613 bp DNA band was ligated into HindIII/SalI cut CPV14 and CPV2, 
creating CPV14/Cre and CPV2/Cre respectively.

**Generation of Transgenic Mice**

All animals were treated in adherence to the ARVO Statement for the Use of Animals in 
Ophthalmic and Vision Research. To generate microinjection fragments: CPV2/hGH, 
CPV14/hGH and CPV15/hGH were digested with SstII, EcoRI and ScaI , αB1/hGH and TYBS 
(a tyrosinase minigene) were digested with KpnI and EcoRI 45, αA/αB4/hGH was digested with 
SacII, CPV14/Cre and CPV2/Cre were digested with KpnI and HindIII. Microinjection 
fragments of ~2.6 kb for CPV2/hGH, CPV14/hGH, CPV15/hGH and αB1/hGH, ~3 kb for 
CPV14/Cre and CPV2/Cre and ~4 kb for αA/αB4/hGH and TYBS were gel-purified using the 
QiagenII gel extraction kit (Qiagen, Hilden, Germany). All micro-injection constructs were
injected into pronuclear stage FVB/N mouse embryos as described \(^\text{46}\). All hGH transgenic constructs were injected independently, but Cre constructs were either injected independently or co-injected with the TYBS cassette \(^\text{45}\).

All transgenic mice made with hGH transgenes with the exception of those made with the \(\alpha\)B1/hGH construct were screened by PCR with PR4 (5’-GCATTCCAGCTGCTGACGGT-3’), derived from the \(\alpha\)A-crystallin promoter and anti-hGH (5’-CTCATCTGCCTGCATTTTCGC-3’), derived from the hGH gene. \(\alpha\)B1/hGH transgenic mice were screened by PCR with primers 25763 and anti-hGH. Cre transgenic mice were screened by PCR with primers PR4 and CRE-AS (5’-CAGCCCGGACCGACGATGAAG-3’), derived from the Cre coding sequence.

**Histology, Immunohistochemistry and In-situ Hybridization**

Eyes or embryos were collected and fixed in 4% paraformaldehyde, processed and embedded in paraffin. Embedded samples were sectioned at 5 µm. Immunohistochemical staining for hGH was performed as described previously \(^\text{43}\). An hGH-specific riboprobe vector, CB4, was created by ligating a 630bp EcoRI-HindIII fragment containing the 3’untranslated region of the human growth hormone gene from the pSW2 \(^\text{47}\) vector (obtained from Lewis T. Williams) into pBluescript II KS(-)(Strategene, La Jolla, CA). Antisense \(^{35}\)S-UTP-labeled probes were synthesized using HindIII-digested CB4 template and T7 RNA polymerase (Promega, Madison, WI). In-situ hybridization of tissue sections was performed as described previously \(^\text{42}\).

**X-gal Staining for \(\beta\)-galactosidase Activity**

To evaluate the expression pattern of Cre transgene, Cre transgenic mouse lines were crossed to homozygous ROSA reporter line \(^\text{48}\). Mouse embryos, neonatal eyes and lens epithelium from
mice at weaning age were collected and analyzed for β-Galactosidase activity by x-gal staining as described 42.