field before and after sets of 10 to 20 gas measurements. Minimum detectable flux was ~0.05 ng cm^{-2} $hour^{-1}$.

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Induction of Lens Differentiation by Activation of a bZIP Transcription Factor, L-Maf

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After the vertebrate lens is induced from head ectoderm, lens-specific genes are expressed. Transcriptional regulation of the lens-specific αA-crystallin gene is controlled by an enhancer element, αCE2. A gene encoding an αCE2-binding protein, *L-maf* (lens-specific *maf*), was isolated. *L-maf* expression is initiated in the lens placode and is restricted to lens cells. The gene product L-Maf regulates the expression of multiple genes expressed in the lens, and ectopic expression of this transcription factor converts chick embryonic ectodermal cells and cultured cells into lens fibers. Thus, vertebrate lens induction and differentiation can be triggered by the activation of L-Maf.

During development, the vertebrate lens is induced upon contact between the presumptive retina and head ectoderm (*1*). Differentiation of the ectoderm into lens cells is accompanied by the specific upregulation of crystallin gene transcription (*2*). We previously identified a lens-specific enhancer element, termed αCE2, in the chicken αA-crystallin promoter (*3*, *4*). The αCE2 sequence, located 100 base pairs upstream of the transcription start site, represents a lens-specific enhancer element that is conserved in the regulatory regions of many crystallin genes (*5*). The integrity of this αCE2 sequence to direct lens-specific transcription has been demonstrated in both cell culture and transgenic mouse experiments.

To identify a factor or factors that bind to the αCE2 sequence and are expressed during the formation of the lens, we screened an expression library prepared from chick embryonic lens with oligonucleotide probes encoding the αCE2 sequence (*6*). Positive cDNA clones were classified by their patterns of tissue distribution. Northern (RNA) blot analysis of 8-day-old chick embryonic tissues revealed a 3.6-kb mRNA that was expressed almost exclusively in the lens, with very weak expression in the brain (Fig. 1A). The spatial and temporal patterns of expression were examined by whole-mount in situ hybridization analyses (*7*). The transcripts were first detected in the lens placode at stage 11, when the head ectoderm makes contact with the optic vesicle (Fig. 1B). The expression remains restricted to the invaginating lens placode (stage 13, Fig. 1, C and D), and subsequently to the developing lens vesicle (stage 15, Fig. 1, E and F), where localized transcription of the αA-crystallin gene later occurs (stage 18, Fig. 1G). Early expression of this factor in the lens placode preceded the induction of the δ1-crystallin gene, one of the earliest lens markers.

Full-length cDNA was obtained and the sequence was determined. A single open reading frame encoding 286 amino acids predicted a putative transcription factor with a bZIP motif and additional sequences characteristic of the *maf* proto-oncogene family (Fig. 2A). Thus, we named this protein L-Maf (lens-specific Maf). The gene product, which represents a previously undescribed member of the family, can be classified with the large Maf subfamily including MafB, c-Maf, and NRL (Fig. 2B) rather than with the small Mafs such as MafK, MafF, and MafG (*8*). L-Maf most closely resembles MafB/Kreisler, which has been shown to be involved in segmentation of the hindbrain (*9*). Members of the large Maf family are expressed in the lens of the rat, mouse, chick, *Xenopus*, and zebrafish (*10*). Interestingly, *mafB* and *c-maf* or *L-maf* are detected in the lens epithelial and fiber cells, respectively, of the rat and *Xenopus*.

We next tested the ability of L-Maf to control transcription in transfection assays that used chicken primary culture cells and a reporter construct encoding the chick αA-crystallin promoter (−244 to +89) linked to a luciferase gene (Fig. 3A). Cotransfection of an L-Maf expression plasmid (pEFX–L-Maf) and the reporter into chick embryonic lens cell cultures (*4*, *11*) resulted in luciferase activity 10 times that caused by transfection with a control plasmid (pEFX) (*12*). Replacement of the αCE2 sequence, located in the promoter region between base pairs −119 and −99, with a Bam HI linker abolished this response, indicating that L-Maf activation occurs through the αCE2 sequence. Efficient transactivation of the αA-crystallin promoter by L-Maf was also observed in chick neural retina cell cultures. In addition, activation was observed when L-Maf was overexpressed in lung cultures; otherwise, activity of the αA-crystallin promot-

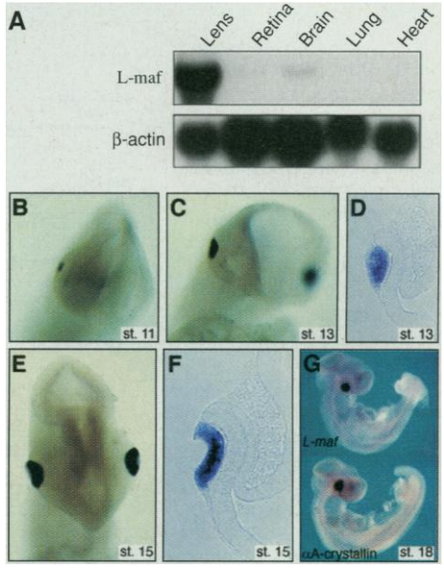

Fig. 1. Restricted expression of *L-maf* mRNA in the lens. (**A**) Northern blot analysis of mRNAs from 8-day-old chick embryonic tissues, including lens, neural retina, brain, lung, and heart tissues. In each lane, 10 μg of total RNA was blotted and hybridized with a randomly primed probe for *L-maf* (3.6 kb) or β-actin cDNAs. (**B** to **G**) Expression of *L-maf* during chick lens development. *L-maf* expression was analyzed by whole mount in situ hybridization from stages 11 to 18. Frontal views of embryos hybridized with antisense *L-maf* probes are shown for stages 11 (B), 13 (C), and 15 (E); coronal sections through the lens placodes are shown for stages 13 (D) and 15 (F). A lateral view of embryos at stage 18 (G) shows expression of *L-maf* and αA-crystallin.

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er is extremely low. Thus, L-Maf acts as a principal factor controlling transcriptional switching of the αA-crystallin promoter.

To analyze the binding specificity of L-Maf, we prepared purified recombinant proteins fused to maltose-binding protein (MBP) and tested them in gel mobility shift DNA binding assays (*13*) against regulatory elements conserved in avian and mamma-

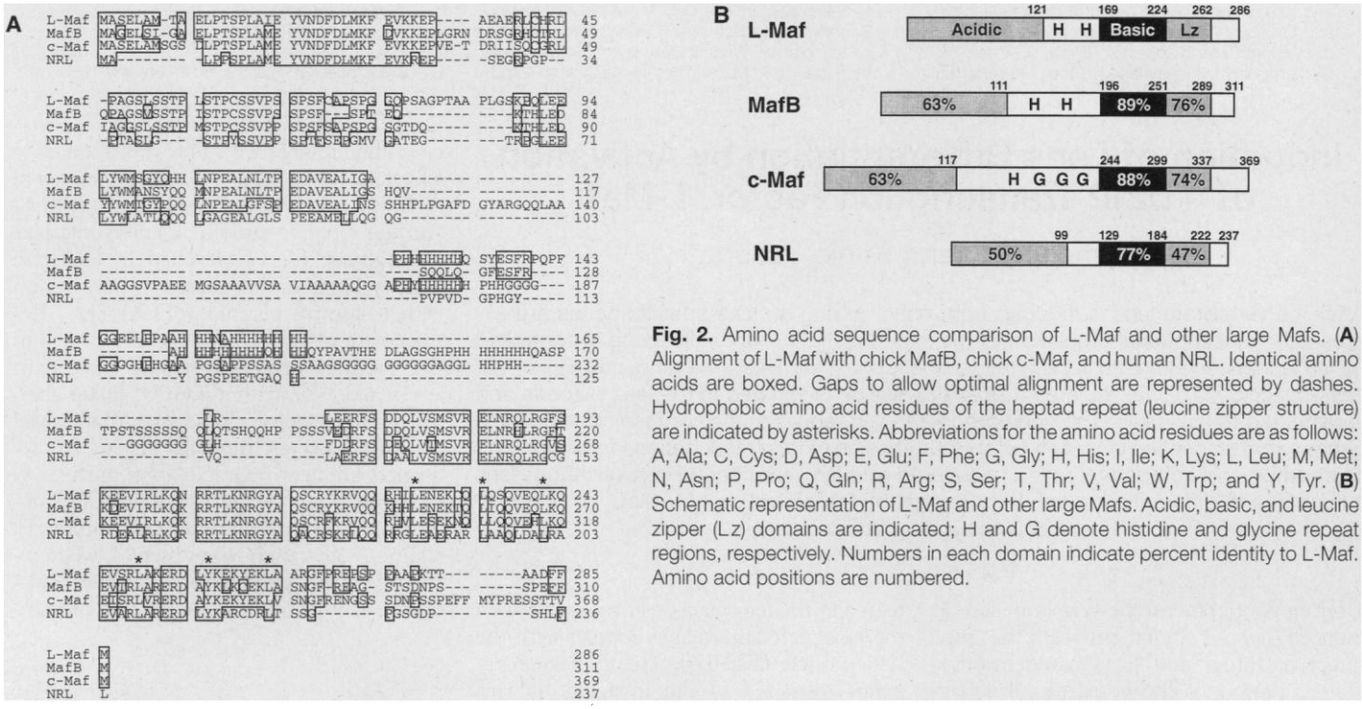

Fig. 2. Amino acid sequence comparison of L-Maf and other large Mafs. (**A**) Alignment of L-Maf with chick MafB, chick c-Maf, and human NRL. Identical amino acids are boxed. Gaps to allow optimal alignment are represented by dashes. Hydrophobic amino acid residues of the heptad repeat (leucine zipper structure) are indicated by asterisks. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B**) Schematic representation of L-Maf and other large Mafs. Acidic, basic, and leucine zipper (Lz) domains are indicated; H and G denote histidine and glycine repeat regions, respectively. Numbers in each domain indicate percent identity to L-Maf. Amino acid positions are numbered.

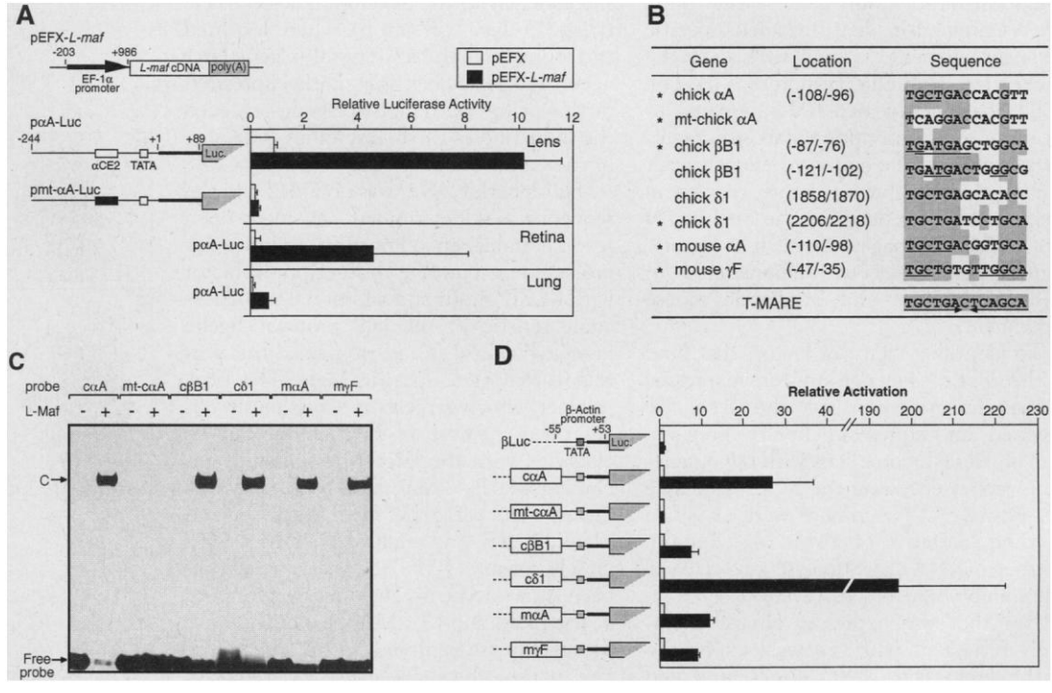

Fig. 3. L-Maf binds and transactivates through lens-specific enhancer elements of crystallin genes. (**A**) Comparison of transactivation by L-Maf from an αA-crystallin promoter in different tissues. Plasmid pαA-Luc contains the chick αA-crystallin promoter (base pairs −244 to +89) upstream of the luciferase gene; plasmid pmt-αA-Luc was constructed from pαA-Luc by replacing the region containing the αCE2 core sequence (base pairs −126 to −96) with an 8–base pair Bam HI linker. pαA-Luc and pmt-αA-Luc were transfected with pEFX or pEFX–L-Maf into lens, retina, or lung cultures. Luciferase activities are shown relative to activities of tkLuc in each culture (assigned as 10); tkLuc is the luciferase reporter plasmid carrying the HSV tk promoter (base pairs −197 to +56). (**B**) Alignment of enhancer sequences of crystallin genes and the consensus binding sequence of the proto-oncogene *maf* (*v-maf*) (T-MARE, TPA-responsive type Maf recognition element). The nucleotides that have been shown by mutational analyses to be essential for enhancer activity are underlined, and those that match the T-MARE consensus are shaded. Arrows indicate a palindrome sequence in T-MARE. (**C**) Gel retardation experiments using a bacterially expressed fusion protein of maltose-binding protein and L-Maf (MBP–L-Maf). Radiolabeled oligonucleotides containing the sequences marked by asterisks in (B) were incubated with MBP (−) or MBP–L-Maf (+) and electrophoretically separated. C represents the position of the DNA-protein complex. For abbreviations for probes and their complete nucleotide sequences, see (*13*). (**D**) Luciferase activity assay. Lens cultures were transfected with reporter plasmids carrying enhancer elements of crystallin genes and pEFX or pEFX–L-Maf. In the reporter plasmids, six copies of oligonucleotides that were used as the probe in gel retardation assays (B) were inserted upstream of a chick β-actin basal promoter (base pairs −55 to +53) linked to a luciferase reporter gene.

lian crystallin genes (Fig. 3B). MBP–L-Maf bound specifically to αCE2 and other crystallin sequences tested (Fig. 3C). Substitution of three nucleotides in the αCE2 sequence (mt-cαA) resulted in complete loss of binding. L-Maf was also expressed in lens cells along with luciferase reporter plasmids containing six copies of each crystallin element in front of a heterologous β-actin basal promoter (Fig. 3D). Regardless of the crystallin element, overexpression of L-Maf resulted in efficient transcriptional activation by a factor of up to 200. In contrast, no transcriptional stimulation was observed through the reporters without any crystallin element or with the mutant αCE2 element, mt-cαA.

Next, we tested whether L-Maf could initiate the genetic cascade toward lens cell differentiation. pEFX–L-Maf was introduced into neural retina cell cultures prepared from 8-day-old chick embryos by transient transfection. Cultures were double-stained with L-Maf and αA- or δ1-crystallin antiserum (*14*). We were able to visualize L-Maf–positive cells, and these cells were also found to be positive for αA- and δ1-crystallin antisera (Fig. 4A). Morphologically, these crystallin-positive cells also exhibited the large, elongated shape characteristic of terminally differentiated lens fiber cells. When the retina cells were transfected with a control expression vector encoding β-galactosidase (β-gal), no cells were stained with L-Maf antiserum or crystallin monoclonal antibodies (mAbs) even among β-gal–positive cells, indicating the absence of lens differentiation. Lens differentiation was also induced in other primary cultures, including the retinal pigmented epithelium, forebrain, and midbrain cells (*15*). We then transfected eukaryotic expression vectors encoding L-Maf (pCAGGS–L-Maf) and a green fluorescent protein (pCAGGS-GFP) into chick embryonic head ectoderms at stage 9 by in ovo electroporation (*16*) to examine whether ectopic expression of L-Maf can induce lens differentiation in chick embryos. Whole-mount immunostaining with δ1-crystallin antiserum showed that δ1-crystallin–positive cells were clearly detected in ectodermal cells (Fig. 4B). In a control embryo, no crystallin-positive cells were detected (Fig. 4B). Thus, consistent with the ability of L-Maf to convert retinal cells into lens cells in vitro, L-Maf expression is capable of ectopically inducing crystallin genes in ovo.

To confirm quantitatively the ability of L-Maf to initiate lens cell differentiation, we used an L-Maf–expressing retrovirus (RCAS–L-maf) to infect retina cultures (*17*). The expression patterns of αA-crystallin and two additional terminally differentiated lens fiber–specific markers, βB1-crystallin and filensin, were examined. Many αA-, βB1-, and δ1-crystallin–positive colonies were present in cultures 5 days after infection with RCAS–L-maf, but not in those infected with a RCAS control virus (Fig. 4C). The reverse transcription–polymerase chain reaction (RT-PCR) (*18*) was used to confirm the presence of specific transcripts for these crystallins and filensin (Fig. 4D). Thus, L-Maf is able to induce the transcription of lens marker genes and promote differentiation into lens fiber.

L-Maf can induce lens differentiation through direct binding to lens-specific genes. Transcription factors such as *Pax-6* (*19–21*), *Sox-1/2* (*22*), *RAR/RXR* (*23*), *Six3* (*24*), *Rx* (*25*), and *Lhx2* (*26*) regulate the expression of crystallin genes through direct binding to their target sites or are involved in eye formation. In particular, homozygous mutations in the *Pax-6* gene result in a complete absence of eyes and nose (*19*, *27*), whereas ectopic expression in *Drosophila* induces supernumerary eyes (*20*). Ectopic expression of the murine homeobox gene *Six3* induces lens formation in the area of the otic vesicle in fish embryos (*24*). However, none of these genes are expressed as strictly as L-Maf in the developing lens. Most tissue-specific genes are known to be regulated by a combination of transcription factors that have overlapping yet distinct expression patterns. Indeed, in situ analyses show that the expression patterns of *Pax-6*, *Sox-1/2*, and *Six3* overlap in the eye primordium, the optic vesicle, and the lens placode, suggesting that they might be involved in the control of *L-maf* expression. Judging from the time of onset and lens specificity of *L-maf* expression and its ability to induce lens differentiation, L-Maf appears to be a prime candidate for a direct target of inductive signals from the optic vesicle.

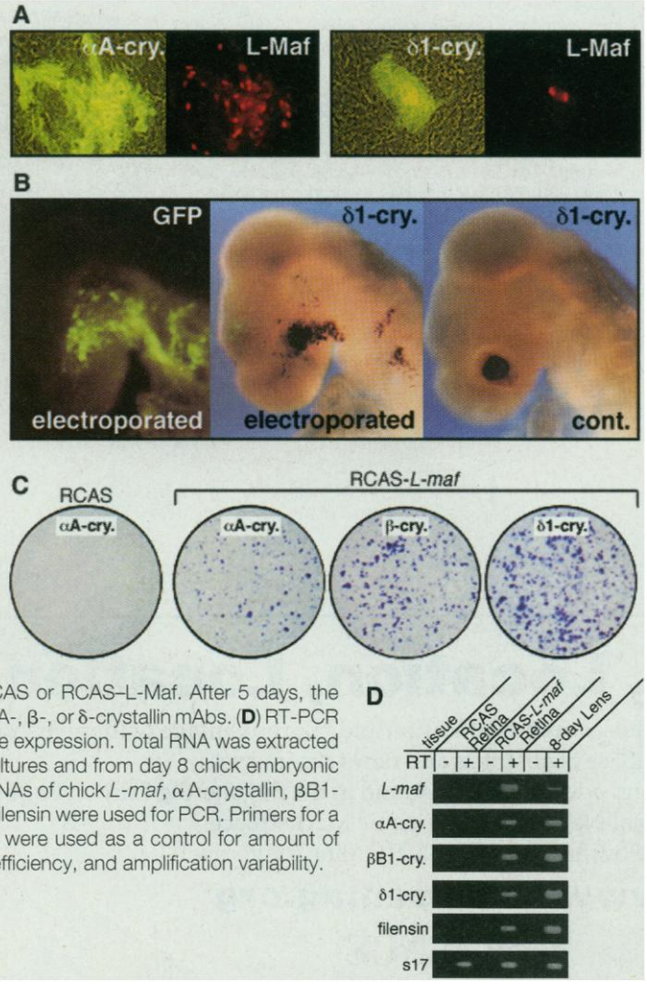

Fig. 4. Ectopic expression of L-Maf induces lens differentiation in neural retina cultures and chick embryos. (**A**) Immunostaining of neural retina cultures transfected with pEFX–L-Maf. Five days after transfection, cultures were double-stained with L-Maf antiserum and αA- or δ-crystallin mAbs. (**B**) Whole mount immunostaining of chick embryos electroporated with pCAGGS–L-Maf. After 36 hours of incubation, embryos were immunostained with δ-crystallin mAb. δ-Crystallin–positive cells are overlapped with GFP-positive ectodermal cells, but no cells stained in a control embryo. (**C**) Infection of L-Maf expression retrovirus. Neural retina cultures were infected with RCAS or RCAS–L-Maf. After 5 days, the cultures were stained with αA-, β-, or δ-crystallin mAbs. (**D**) RT-PCR analysis of lens-specific gene expression. Total RNA was extracted from virus-infected retina cultures and from day 8 chick embryonic lens. Primers specific for cDNAs of chick *L-maf*, αA-crystallin, βB1-crystallin, δ1-crystallin, and filensin were used for PCR. Primers for a ribosomal protein gene s17 were used as a control for amount of RNA, reverse transcription efficiency, and amplification variability.

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6. Screening was performed essentially as described [C. R. Vinson, K. L. LaMarco, P. F. Johnson, W. H. Landschulz, S. L. McKnight, *Genes Dev.* **2**, 801 (1988)]. A cDNA expression library (λgt11) was prepared from primary cultures of lens tissues isolated from 8-day-old chick embryos. The enhancer of the chick αA-crystallin gene (base pairs −119 to −99) was tandemly ligated and labeled for use as a probe.
7. An *L-maf* antisense probe was generated from a construct containing 0.8 kb of the 5′ noncoding and the 0.6-kb coding sequence of its cDNA in reverse orientation to a T7 promoter. An αA-crystallin antisense probe corresponding to the 0.6-kb Pst I fragment of its cDNA was also prepared in the same way. Whole mount in situ hybridization using digoxigenin-labeled riboprobes was performed as described [D. G. Wilkinson, in *Essential Developmental Biology: A Practical Approach*, C. D. Stern and P. W. H. Holland, Eds. (IRL Press, Oxford, 1993), pp. 257–274]. Embryos were embedded in Tissue-TEK compound (Miles) and sectioned at 10 μm.
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11. Culturing of lens, neural retina, and lung tissues was performed as described (*4*). For virus experiments, specific pathogen-free white Leghorn eggs were used (line M, supplied by Nisseiken Ltd.).
12. *L-maf* cDNA or the Hind III–Bam HI fragment of pCHIIO (Pharmacia) encoding β-galactosidase were inserted into pEFX, resulting in pEFX–L-Maf and pEFX–β-gal, respectively. Cotransfection was carried out using a calcium phosphate method with 0.3 μg of luciferase reporter plasmid, 0.25 μg of pEFX–β-gal, 0.45 μg of pUC119 (carrier), and 20 ng of effector plasmid (pEFX or pEFX–L-Maf) in 22-mm dishes. Cell extracts were prepared 48 hours after transfection for assay of luciferase activities. β-Galactosidase was used to determine relative transfection efficiencies and to normalize luciferase activity. For forced expression experiments, 2 μg of pEFX–β-gal or pEFX–L-Maf was transfected into a 35-mm dish.
13. *L-maf* cDNA containing the open reading frame was inserted into the Eco RI site of pMAL-cRI vector (New England Biolabs). MBP–L-Maf fusion protein was purified by amylose resin affinity-column chromatography as recommended by the supplier (New England Biolabs); 250 ng of MBP–L-Maf or MBP (control) was incubated with the following end-labeled oligonucleotides and analyzed by polyacrylamide gel electrophoresis (*3*, *4*): cαA, chick αA-crystallin (base pairs −114 to −90, 5′-CATTTCTGCTGACCACGTTGCCTTC-3′); mt-cαA, a mutated version of cαA (5′-CATTTCTCAGGACCACGTTGCCTTC-3′); cβB1, chick βB1-crystallin (base pairs −93 to −69, 5′-AGACACTGATGAGCTGGCACTTCCA-3′); cδ1, chick δ1-crystallin (base pairs 2200 to 2224, 5′-CAGGACTGCAGGATCAGCATGATC-3′); mαA, mouse αA-crystallin (base pairs −116 to −92, 5′-TCCAGCTGCTGACGGTGCAGCCTCT-3′); and mγF, mouse γF-crystallin (base pairs −53 to −29, 5′-TGTTCCTGCCAACACAGCAGACCTC-3′).
14. L-Maf antiserum was generated by immunizing a rabbit with MBP–L-Maf. The crystallin mAbs were provided by K. Sawada and G. Eguchi [K. Sawada, K. Agata, A. Yoshiki, G. Eguchi, *Jpn. J. Ophthalmol.* **37**, 355 (1993)]. L-Maf and β-gal immune complexes were detected with antibodies to rabbit immunoglobulin G (IgG) conjugated to tetramethyl rhodamine isothiocyanate (DAKO); crystallin immune complexes were detected with antibody to mouse IgG conjugated to fluorescein isothiocyanate (DAKO).
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16. In ovo electroporation was basically performed as described [T. Muramatsu, Y. Mizutani, J. Okumura, *Anim. Sci. Technol.* **67**, 906 (1996)]. Plasmid pCAGGS–L-Maf, containing an *L-maf* cDNA fused to a plasmid pCAGGS bearing a CMV–β-actin promoter [M. Tokui *et al.*, *Biochem. Biophys. Res. Commun.* **233**, 527 (1997)], was electroporated into chick embryos at stage 9 to 10 together with plasmid pCAGGS-GFP to monitor incorporation of DNA into embryos. Whole mount immunostaining was performed as described [Y. M. Lee *et al.*, *Development* **121**, 825 (1995)]. We have found that not all tissues are equally sensitive to uptake of DNA delivered by this method. In particular, the branchial arch region appears less sensitive than the surrounding ectoderm for induction of L-Maf expression.
17. RCAS–L-Maf was constructed by inserting the *L-maf* cDNA into the Cla I site of a RCAS(A) vector [S. Hughes, J. J. Greenhouse, C. J. Petropoulos, P. Sutrave, *J. Virol.* **61**, 3004 (1987)]. Retrovirus preparation was performed as described [C. Cepko, in *Current Protocols in Molecular Biology*, F. M. Ausubel *et al.*, Eds. (Wiley, New York, 1992), Unit 9.11.
18. For a quantitative analysis, cycle numbers were determined for each primer pair to maintain exponential amplification. Primer pairs and numbers of cycles were as follows: *L-maf*, 5′-GAGGCCGAGAGGCTGTGCCAC-3′ and 5′-GCAGCTCCTCGCCCCCAAAGG-3′, 25 cycles; αA-crystallin, 5′-GCCTTTGTTCTCCTCCACTATCAG-3′ and 5′-GTGGAACTCACGAGAGATGTAGC-3′, 25 cycles; βB1-crystallin, 5′-AGCAGCTGCCCAGACCCGAG-3′ and 5′-GCTGACGATGACACTGCGCAC-3′, 28 cycles; δ1-crystallin, 5′-CTGAGCTGGAGAAGATCCTGAG-3′ and 5′-TCCACCAGGGTCTTGATGAGC-3′, 25 cycles; filensin, 5′-TCGCCAGCTACATCAAACCG-3′ and 5′-TGTGGTACTCATCAAGCATGC-3′, 28 cycles; s17, 5′-TACACCCGTCTGGGCAACGAC-3′ and 5′-CCGCTGGATGCGCTTCATCAG-3′, 25 cycles. All of these primers were designed for chick cDNA.
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28. We thank K. Umesono, R. Yu, K. Shimamoto, M. Nakafuku, J. Kato, and M. Takeichi for critical reading and comments on the manuscript; T. Momose and K. Ono for help with whole mount in situ hybridization; K. Umesono, H. Ogawa, and J. Miyazaki for plasmids; and K. Sawada and G. Eguchi for mAbs. Supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan and by a grant from the Human Frontier Science Program. The GenBank accession number for *L-maf* is AF034570.

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