Mafs, Prox1, and Pax6 Can Regulate Chicken βB1-Crystallin Gene Expression*

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Wenwu Cui§, Stanislav I. Tomarev¶, Joram Piatigorsky¶, Ana B. Chepelnksy¶, and Melinda K. Duncan¶

From the §Department of Biological Sciences, University of Delaware, Newark, Delaware 19716 and the ¶Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda, Maryland 20892-2730

During lens fiber cell differentiation, the regulation of crystallin gene expression is coupled with dramatic morphological changes. Here we report that Mafs, Prox1, and Pax6, which are essential transcription factors for normal lens development, bind to three functionally important cis elements, PL1, PL2, and OL2, in the chicken βB1-crystallin promoter and may cooperatively direct the transcription of this lens fiber cell preferred gene. Gel shift assays demonstrated that Mafs bind to the MARE-like sequences in the PL1 and PL2 elements, whereas Prox1, a sequence-specific DNA-binding protein like its homolog Prospero, interacts with the OL2 element. Furthermore, Pax6, a known repressor of the chicken βB1-crystallin promoter, binds to all three of these cis elements. In transfection assays, Mafs and Prox1 activated the chicken βB1-crystallin promoter; however, their transactivation ability was repressed when co-transfected with Pax6. Taken together with the known spatiotemporal expression patterns of Mafs, Prox1, and Pax6 in the developing lens, we propose that Pax6 occupies and represses the chicken βB1-crystallin promoter in lens epithelial cells, and is displaced by Prox1 and Mafs, which activate the promoter, in differentiating cortical fiber cells.

The lens is a transparent tissue composed of cuboidal epithelial cells on its anterior surface and fiber cells that comprise the remainder of the lens. Vertebrate lens development initiates when a portion of lens-competent cephalic ectoderm responds to an inductive signal from the optic neuroepithelium. The induced ectoderm, termed the lens placode, thickens, invaginates, and pinches off to form the lens vesicle. Cells in the anterior portion of the lens vesicle develop into lens epithelium, whereas cells in the posterior portion leave the cell cycle, elongate and differentiate into primary lens fibers. After the lens forms, its growth is mediated by lens epithelial proliferation and the differentiation of lens epithelial cells at the lens equator into lens fiber cells (1, 2). This differentiation event is marked by dramatic changes in cell shape, the down-regulation of epithelial markers such as vimentin, the initiation of aquaporin0/MIP and beaded filament expression, and the accumulation of crystallin proteins (3).

A number of transcription factors are essential for either the initiation of lens development or the differentiation of lens fiber cells from epithelial cells (4, 5). Mafs are a group of basic leucine zipper transcription factors that bind to a common recognition element (TPA-MARE or CRE-MARE) and regulate target gene expression (6). c-Mafs expression is first detected in the lens placode and is highly up-regulated during fiber cell differentiation (7–9). In c-Mafs knockout mice, the lens fiber cells fail to elongate and crystallin gene expression is severely repressed (8–10). L-Maf is expressed in the embryonic chicken lens and is sufficient to reprogram retinal-pigmented epithelial cells into lens cells. In gel shift assays, L-Maf binds to MARE-like elements in a number of crystallin promoters and can activate the expression of constructs consisting of these MAREs coupled with heterologous promoters (11). MafB is transcribed in the lens epithelium (8) and can transactivate the chicken αA-crystallin promoter in transfection assays (12).

Prox1, a divergent homeodomain protein, is expressed initially in the early lens placode and is up-regulated during fiber cell differentiation (13–15). Prox1 null lenses do not undergo lens fiber cell elongation, although the expression of several fiber cell-specific genes can be detected by RT-PCR (14). In co-transfection assays, Prox1 activates several γ-crystallin promoters (16). However, it has not been established whether Prox1 is a DNA binding transcription factor like its Drosophila homolog, Prospero (17, 18).

Pax6 is a paired domain/homeodomain transcription factor hypothesized to be a master control gene of visual system development because it can induce ectopic eyes in both vertebrates and Drosophila (19–23). In vertebrates, Pax6 expression initiates in the head ectoderm and is essential for the development of the lens placode. Once the lens forms, Pax6 becomes restricted to the epithelium, which continues to express this transcription factor throughout life (24, 25). Tissue recombination experiments demonstrated that lens epithelial cells lacking one functional copy of the Pax6 gene preferentially undergo fiber cell differentiation, suggesting that Pax6 maintains the epithelial cell phenotype in the mature lens (26). Pax6 is a positive transcriptional regulator of the mouse ab (27, 28), chicken and mouse αA (29, 30), and chicken α1-crystallin (31) genes. Furthermore, Pax6 cooperates with Mafs to activate γ-crystallin (32, 33) and glucagon expression (34). However,

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¶ To whom all correspondence should be addressed: Dept. of Biological Sciences, University of Delaware, Newark, DE 19716. Tel.: 302-831-0533; Fax: 302-831-2281; E-mail: duncanm@udel.edu.
Pax6 directly represses the transcription of the fiber cell pre-
ferred crystallin genes, chicken βB (25) and mouse ye, yF (35).
During lens development, the morphological transition be-
tween epithelial cells and fiber cells coincides with specific,
regulated changes in crystallin gene expression. For example,
βB1-crystallin, which accounts for 8.5% of the total protein of
the newborn mouse lens (36), is not transcribed in lens epitel-
ial cells, but its expression begins concurrently with the initial
elongation of fiber cells (37, 38). Thus, βB1-crystallin is a spe-
cific lens fiber cell marker whose transcription is likely to be
controlled by molecular mechanisms that overlap those of fiber
cell differentiation. Furthermore, these mechanisms are evolu-
tionarily conserved because the chicken βB1-crystallin pro-
mot is fully functional in the transgenic mouse (38).

The minimal promoter required for chicken βB1-crystallin
transcription in transfected cells (128/30) contains at least
three functionally important cis acting elements, PL1 (116 to
–102), PL2 (−90 to −76), and OL2 (−75 to −68) (Fig. 1A) (39).
Site-directed mutagenesis of PL1 (M6A) and PL2 (M7) decrease
chicken βB1-crystallin promoter activity (39). PL1 and PL2 are
both similar to the TPA-MARE consensus sequence (11) and rec-
ognizes a distinct factor in lens cell nuclear extracts (39). In
the present study, we demonstrate that Mafs bind the PL1 and
PL2 elements, whereas Prox1 binds the OL2 element. Further-
in the, we propose that Mafs, Prox1, and Pax6 collaborate to
direct spatiotemporal expression of the chicken βB1-crystallin
gene during lens development.

EXPERIMENTAL PROCEDURES

RT-PCR Analysis of c-Maf Isoform Expression in Lens—Total lens
RNA was harvested from 5-day-old mice using the SV Total RNA
Isolation Kit (Promega, Madison, WI). RT-PCR was performed
using the SuperScript RT-PCR kit (Invitrogen, Carlsbad, CA). The common 5
primer for both c-Maf isoforms is GCAGCTGACACCATCACCAC,
3′ for the c-Maf long form is GCACACTGCAGAATGCTTGCCT,
and the 3′ primer for the c-Maf short form is AAAAAAGCCATCACAC-
CACCAC. The expected sizes of RT-PCR products are 249 bp for the
c-Maf long form and 300 bp for the c-Maf short form. After reverse
transcription, the products were PCR amplified for 35 cycles of 94
°C for 30 s, and 72°C for 30 s. The RT-PCR products were electro-
phoresed in 8% PAGE, isolated from the gel using Ultradee-DA DNA puri-
fication columns (Millipore, Billerica, MA), cloned into pcR 2.1-TOPO
(Invitrogen), and verified by DNA sequencing.

Plasmids and Constructs—The chicken βB1-crystallin promoter plasmid 432/
+30/CAT (p432) was described previously (41). QuikChangeTM site-
directed mutagenesis (Stratagene, La Jolla, CA) was performed to make
PL1-deleted (p3PL1), PL2-deleted (p2PL2), PL1/PL2-deleted (p3PL2/
PL2), and OL2-mutated reporter (pOL2mut1) constructs from p432.
For p3PL1, the sense strand primer was 5′-GAGCTTTGCAGGATGCNGGC-
CACAGACTCTG-3′; for p2PL2, the sense strand primer was 5′-CGCG-
CCAGACAGACACTCTTGGCTG-3′; for p3PL1/PL2, the sense strand primer was 5′-
GAGCTTTGCAGGATGCTTCTCAGATGC-3′; for pOL2mut1, the sense strand primer was 5′-
GACAGCTGAGATGTGACGCTGGCC-3′. The mutated con-
structs were prepared as suggested by the manufacturer (Stratagene)
and confirmed by sequencing.

Chicken MafB, an intronless gene, was cloned from genomic DNA by
PCR using the 5′ primer AAGGAAGGCAAGGGCGCCGCAGCA-
GCAGACTCTG-3′ for p3PL1, the sense strand primer was 5′-ATTAAC-
GACATCAGCTCAGACT-3′. The PCR product was sub-
cloned into PCR-Script Amp. SK+ (+) (Stratagene) and subjected to
sequencing. The eukaryotic MafB expression vector was prepared by
subcloning the BamHI-Not1 fragment of PCR-Script-MafB, which con-
tains the full coding region of MafB into pcDNA3.1 Zeo (+) (Invitrogen).
Two EST clones, respectively comprising the 5′ (GenBankTM accession
number 2257419) and 3′ (GenBankTM accession number 2988788) ends of the mouse c-Maf long form cDNA separated by a Not1 site,
were purchased from Invitrogen. The Not1–EcoRI fragment of the 3′ portion
of mouse c-Maf EST was inserted into pVL1392 (BD Pharmingen, San Diego, CA)
to create an Xbal site at its 3′ end. The eukaryotic expression vector for
the c-Maf long form was then constructed by sequentially subcloning
the EcoRI–Not1 fragment of the 5′ EST and Not1–Xbal fragment of
pVL1392-c-Maf into pcDNA 3.1 Zeo (+) (Invitrogen). The c-Maf short
form expression vector was generated by inserting the Xbal fragment
from pCR-2.1 TOPO-c-Maf short form (see the RT-PCR step) into the
XhoI site of the long form expression vector. All of the c-Maf expression
vectors were confirmed by sequencing.

Pax6 (25) and Prox1 (16) expression vectors were previously
described. pcMVβGal was purchased from Clontech ( Palo Alto, CA);
pCMVβgal was purchased from Promega (Madison, WI).

Cell Culture and Transfection—N/N 1003A cells (a rabbit lens epi-
thelium derived cell line) (42) were transfected following the Lipo-
fectAMINE Plus reagent protocol provided by Invitrogen. Generally 6 ×
10⁵ cells were plated for each 60-mm dish at least 8 h before transac-
tion, each dish received 2.5 μg of promoter/chloramphenicol acetyl-
transferase (CAT) plasmid, 0.5 μg of pcMVβGal, and 0.5 μg of various
expression plasmids. Cells were harvested 48 h after transfection
and cellular extracts were prepared by multiple cycles of freeze/thaw.
The extracts were assayed for CAT and β-galactosidase activity as previ-
ously described (39). All co-transfection experiments were performed
at least twice in triplicate and analyzed statistically by Student’s t test.

Electrophoretic Mobility Shift Assay (EMSA)—The chicken
B1-crystallin gene expression (25), inhibits Prox1- and Maf-medi-
ated transcription by competitively binding all three ele-
ments. Thus, we propose that Mafs, Prox1, and Pax6 collaborate to
direct spatiotemporal expression of the chicken βB1-crystallin
gene during lens development. For example, Pax6, a known negative regulator of
βB1-crystallin gene expression (25), inhibits Prox1- and Maf-medi-
ated transcription by competitively binding all three ele-
ments. Thus, we propose that Mafs, Prox1, and Pax6 collaborate to
direct spatiotemporal expression of the chicken βB1-crystallin
gene during lens development.

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The PL1 and PL2 elements are essential for chicken βB1-crystallin promoter activity. A, schematic diagram of the chicken βB1-crystallin promoter. Pros-like, sequence similar to the binding site for Drosophila Prospero; PL1, PL2 (sequences are underlined), and OL2 (sequence is shown in bold) are cis elements known to be important for βB1-crystallin promoter function (38, 39). B, transfection analysis of the chicken βB1-crystallin promoter in lens-derived N/N 1003A cells. Loss of either or both the PL1 and PL2 element significantly decreases promoter activity ($p = 0.003$ for pPL1, $p = 0.003$ for pPL2, $p = 0.002$ for pPL1/PL2). CAT activity is expressed relative to the activity of pCAT-Base, which was arbitrarily set at 1. $p432$, the wild type chicken βB1-crystallin promoter ($−432/+30$) linked to the cat gene; $pPL1$, $p432$ without the PL1 element; $pPL2$, $p432$ without the PL2 element; $pPL1/PL2$, $p432$ without both the PL1 and the PL2 elements.

**RESULTS**

PL1 and PL2 Elements Are Essential for Chicken βB1-Crystallin Promoter Activity—The full-length chicken βB1-crystallin promoter ($−432/+30$) can be separated into two portions: $−126/+30$ is the minimal promoter in transfections (39) and contains all of the signals necessary for lens fiber cell-specific gene expression (38); whereas $−432/−126$ directs crystallin level gene expression (25, 45) (Fig. 1A). Three distinct functional cis elements have been identified in the minimal promoter by DNase I footprinting and transfection analyses (39). The PL1 and PL2 elements are similar to each other and to the TPA-MARE sequence for Maf transcription factors (11). In contrast, the OL2 element is similar to the consensus DNA recognition sequence for the Drosophila protein, Prospero (18) (Figs. 1A and 6A).

The contribution of PL1 and PL2 to the activity of the chicken βB1-crystallin promoter was tested by generating $−432/+30$ reporter constructs with either PL1, PL2 or both deleted. Transfection assays performed in lens-derived N/N 1003A cells demonstrated that deletion of either one or both of the PL1 and PL2 elements reduced βB1-crystallin promoter activity by at least 70% (Fig. 1B). Similar results were also obtained in the non-lens cell line CHO (data not shown).

MafB and c-Maf Isoforms Are Expressed in Lens—Because TPA-MARE consensus sites can be recognized by multiple Maf family members, we investigated Maf expression in the adult lens. Previous in situ hybridization studies showed that MafB mRNA was present in the embryonic lens (8, 46). Expression of MafB in the adult lens was determined by Western immunoblotting of extracts obtained from microdissected adult lens fibers, lens epithelial cells, and extracts of two lens-derived cell lines, $\alpha$TN4-1 (43) and N/N 1003A (42). MafB protein was detected in microdissected lens epithelial cells as well as in the established lens epithelial cell lines, but not in the fiber cells (Fig. 2A).

c-Maf protein is expressed at moderate levels in the lens epithelium, and its expression increases sharply with fiber cell differentiation (9). However, the mouse c-Maf gene is alternatively spliced, producing a long form with 10 extra amino acids at the carboxyl terminus compared with the short form (47). RT-PCR analysis detected the transcripts of both isoforms in 5-day-old mouse lenses (Fig. 2B), although this qualitative RT-PCR analysis suggests that the short form predominates over the long form.

MafB and c-Maf Can Activate the Chicken βB1-Crystallin Promoter through the MARE-like Elements—Because the PL1 and PL2 elements, each containing a putative MARE, are critical for βB1-crystallin promoter activity, it is likely that these elements are binding sites for Mafs. To determine whether MafB and c-Maf are capable of activating the chicken βB1-crystallin promoter, the expression vector for MafB or c-Maf (either long form or short form) was co-transfected with the p432 reporter into N/N 1003A cells. MafB appears to be a stronger transactivator because it activates the promoter 15.5 ± 3.1-fold, whereas c-Maf short form and long form only activated the promoter 4.0 ± 0.2- and 1.7 ± 0.1-fold, respec-
lacking either the PL1 or PL2 elements (p/H11005 by MafB (C form)).

The relative CAT activity of the MafB expression vector and p/H9004 PL1/PL2 co-transfected cells was even lower than that of p432 (pPL2). The relative CAT activity of the MafB expression vector and p/H9004 was arbitrarily set at 1.

pH9004 preservation (Prox1). B (p432, PL1, p/H9004 p432, pPL1, PL1, and plasmids encoding MafB, c-Maf, and Pax6. PL1, p/H9004 p432, pPL1/PL2, and plasmids encoding MafB, c-Maf long/short form, Prox1 or Pax6. A, MafB activated the p432 promoter (lane 3) (p = 0.001); Pax6 repressed βB1-crystallin promoter activity when overexpressed (lane 5) (p = 0.02); MafB- and Prox1-mediated transactivation was repressed when co-transfected with a Pax6 expression vector (lanes 7 and 8) (p = 0.005 for MafB and p = 0.000002 for Prox1). B, both c-Maf isoforms activated the p432 promoter (lanes 3 and 4) (p = 0.00001 for c-Maf short form, p = 0.0004 for c-Maf long form). B, both c-Maf isoforms can additively function with Prox1 to activate the promoter (lanes 7 and 9) (p = 0.03 for c-Maf short form, p = 0.02 for c-Maf long form). Moreover, Pax6 repressed c-Maf-mediated transactivation (p = 0.0001 for c-Maf short form, p = 0.000006 for c-Maf long form). C, the wild type reporter, p432 was significantly transactivated by MafB (p = 0.00007). However, MafB could not activate the reporters lacking either the PL1 or PL2 elements (p = 0.36 for pPL1, p = 0.78 for pPL2). The relative CAT activity of the MafB expression vector and pPL1/PL2 co-transfected cells was even lower than that of p432 (p = 0.002). CAT activity is expressed relative to the activity of p432, which was arbitrarily set at 1.

deletion of either PL1 or PL2 diminished MafB-mediated transactivation in N/N 1003A cells (Fig. 3C). Similar results were also obtained in the non-lens cell line CHO (data not shown).

Maf Specifically Binds to the PL1 and PL2 Elements of the Chicken βB1-Crystallin Promoter—Because Mafs can transactivate the chicken βB1-crystallin promoter and this transactivation is dependent on the MARE-like PL1 and PL2 elements, we tested whether these elements can bind Maf proteins in gel shift assays (Fig. 4). When there was no non-radioabeled PL2/OL2 competitor present, MafB/PL2/OL2 complexes can be visualized by autoradiography. The formation of a labeled complex was significantly reduced by competition with nonradioactive self-oligonucleotide PL2/OL2, PL1, −126/−46, or −126/−46 (mut PL1), but not with PL1 mut1 and PL2 mut1, suggesting that MafB can specifically bind to PL1 and PL2 elements of the chicken βB1-crystallin promoter. The PL2 element appears to be more important for MafB binding because −126/−46 (mut PL2), which has an intact copy of PL1, competed less efficiently than −126/−46 (mut PL1), which has an intact copy of PL2.

Prox1 Activates the Chicken βB1-Crystallin Promoter—The vertebrate homolog of Drosophila Prospero, Prox1, is crucial for lens fiber cell elongation (14) and is able to activate γ-crystallin promoters in transfection assays (16). To test whether Prox1 is also capable of activating the chicken βB1-crystallin promoter, co-transfection assays were performed with a Prox1 expression vector in N/N 1003A cells. The relative cat activity was increased 7.0 ± 0.3-fold when the Prox1 expression vector was co-transfected (Fig. 3A, lane 4).

Prox1 Is a DNA-binding Protein—Whereas Prox1 can activate crystallin gene expression, its ability to function as a DNA binding transcription factor has not been demonstrated. However, the COOH terminus (amino acids 572 to 736) of chicken Prox1 consisting of its homeo- and Prospero domains is 78% similar to Drosophila Prospero (amino acid 1241 to 1403) (13), a sequence-specific DNA-binding protein and transcription fac-
The OL2 Element Is Important for Chicken \( \beta B1 \)-crystallin Promoter—Because the OL2 element of the chicken \( \beta B1 \)-crystallin promoter is similar to the Prospero consensus binding sequence (Fig. 6A), we tested the ability of Prox1 to bind to the PL2/OL2 region. EMSA revealed that Prox1 can interact with the radiolabeled PL2/OL2 element in the absence of nonradioactive competitor (Fig. 6B). We designed a series of PL2/OL2 mutations, PL2 mut1 (the M7 mutation of the PL2/MARE that significantly reduces chicken \( \beta B1 \)-crystallin promoter activity (38, 39), PL2 mut2 (mutation of the 5' end of the PL2/MARE), OL2 mut1 (mutation of the 5' end of OL2), OL2 mut2 (mutation of the 3' end of OL2) (Fig. 6A) to test the binding specificity of Prox1 in gel shift assays. The formation of the Prox1-PL2/OL2 complex was completely eliminated by competition with the nonradioactive self-oligonucleotide PL2/OL2. The mutated oligonucleotides, PL2 mut1 (M7), PL2 mut2, and OL2 mut2, competed for HD + Pros binding, suggesting that these sites were not important for Prox1 binding. However, OL2 mut1 and PL1 (which lacks a Prospero consensus site) competed less efficiently for HD + Pros binding (Fig. 6B). These data suggest that the 5' end of the Prospero consensus element is critical for Prox1 binding.

The OL2 Element Is Important for Chicken \( \beta B1 \)-crystallin Promoter Activity and Prox1-mediated Transactivation—Because recombinant Prox1 binds the chicken \( \beta B1 \)-crystallin promoter via the 5' end of the OL2 element, we tested whether these nucleotides are important for \( \beta B1 \)-crystallin promoter activity and Prox1-mediated transactivation. In N/N 1003A cells, the three-nucleotide mutation (OL2 mut1) reduced promoter activity by half over wild type. Prox1 activated the p432 promoter 5.8 ± 0.9-fold. In contrast, Prox1 could only activate the pOL2mut1 reporter 0.4-fold, which was significantly lower than its ability to transactivate the p432 promoter (p = 0.02).
Transcriptional Regulation of \( \beta B1 \)-Crystallin

**Fig. 7. Pax6 specifically binds to the chicken \( \beta B1 \)-crystallin promoter.** The PL2/OL2 oligonucleotide was radiolabeled and used for EMSAs with the Pax6/GST fusion protein.

than wild type \((p = 0.02)\) (Fig. 6C). These data suggest that the Prospero consensus site found in the OL2 element contributes to Prox1-mediated transactivation of p432.

Prox1 and Mafs Cooperate to Activate the Chicken \( \beta B1 \)-Crystallin Promoter—It has been shown that Prox1 and the c-Maf short form synergistically activate the mouse \( \beta B2 \)-crystallin promoter in transfaction assays (48). Here we investigate whether Prox1 and Mafs can cooperatively activate the \( \beta B1 \)-crystallin promoter in transfaction assays. Co-transfection of Prox1 with MafB did not significantly increase MafB-mediated transactivation in either N/N 1003A (Fig. 3A, lane 6) or CHO cells (data not shown). In contrast, Prox1 and the c-Maf short form alone activated the p432 promoter 4.0 ± 0.2- and 2.4 ± 0.3-fold, respectively, co-transfection of both Prox1 and the c-Maf short form expression vectors increased promoter activity by 5.3 ± 0.7-fold (Fig. 3B, lane 7). Similar results were also obtained when Prox1 and c-Maf long form expression vectors were co-transfected (Fig. 3B, lane 9).

Pax6 Represses MafB-, c-Maf-, and Prox1-mediated Transactivation—Pax6 and Mafs cooperate to activate \( \gamma \)-crystallin (33) and glucagon (34) promoter activity. To examine whether the chicken \( \beta B1 \)-crystallin promoter is regulated by the same way, a Pax6 expression vector was co-transfected with MafB, Prox1, or c-Maf expression vectors into N/N 1003A cells. As previously shown (25), Pax6 represses the chicken \( \beta B1 \)-crystallin promoter (Fig. 3A, lane 5). Moreover, when the Pax6 expression vector is co-transfected with MafB or c-Maf, transactivation was lower than in transfection tests with MafB or c-Maf alone (compare Fig. 3, A, lanes 3 and 7; B, lanes 3 and 8; and lanes 4 and 10). Furthermore, Prox1-mediated transactivation is decreased at least by half when co-transfected with Pax6 expression vector (compare Fig. 3A, lanes 4 and 8). Similar results were also obtained in the non-lens cell line CHO (data not shown).

Pax6 Can Bind to cis-Elements of the Chicken \( \beta B1 \)-Crystallin Promoter—In previous studies, Pax6 was able to bind to an oligonucleotide consisting of a PL2 trimer (25). Here we demonstrate that Pax6 can bind a single copy of the PL2/OL2 element and this complex was reduced by competition with nonradioactive self-oligonucleotide (PL2/OL2), PL1, -126/-46 or -126/-46 (mut PL1) (which has an intact copy of PL2), but not with PL2 mut1 (Fig. 7), suggesting that Pax6 can specifically bind to PL1 and PL2/OL2 elements of the chicken \( \beta B1 \)-crystallin promoter. The PL2 element appears to be more important for Pax6 binding because -126/-46 (mut PL2), which has an intact copy of PL1, competed less efficiently than -126/-46 (mut PL1), which has an intact copy of PL2.

**DISCUSSION**

Crystallin genes are among the most transcriptionally active single copy genes and produce from 1 to 50% of total lens mRNA depending on gene, species, and developmental stage (49). Some crystallin promoters are active in all lens cells as well as some non-lens tissues (50, 51). In contrast, \( \beta B1 \)-crystallin transcription is highly lens fiber cell preferred (38, 41). In this study, we demonstrate that Pax6 can repress Maf-mediated transactivation by binding to the same composite element of the chicken \( \beta B1 \)-crystallin promoter. We also show that Prox1 activates the chicken \( \beta B1 \)-crystallin promoter by specifically binding to sequences adjacent to the MARE-like PL2 composite element. Furthermore, this Prox1-mediated transactivation is inhibited by Pax6. Thus, the promoter activity depends on occupancy of these transcription factors on the promoter; if Pax6 concentrations are high (as seen in the lens epithelium) (19), \( \beta B1 \)-crystallin gene expression is off; if Prox1 and Maf concentrations are high and Pax6 concentrations are low (as in lens fiber cells) (8, 14, 15), Pax6 is displaced from the promoter by c-Maf and Prox1, which stimulate \( \beta B1 \)-crystallin gene expression (Fig. 8).

Pax6 is essential for lens specification and early development (52). At later stages, loss of Pax6 expression in lens fibers is essential for normal fiber cell differentiation and crystallin gene expression. In transgenic mice overexpressing Pax6 in lens fiber cells, the lens is reduced in size and secondary fiber cell elongation is incomplete. Notably, \( \beta B1 \)-crystallin transcription is repressed by 78% relative to total lens RNA, confirming the importance of Pax6 down-regulation for \( \beta B1 \)-crystallin expression.2

Previous studies showed that c-Maf is critical for lens crystallin gene expression. In c-Maf null mice, the expression of \( \alpha \), \( \beta \), and \( \gamma \)-crystallins is severely repressed or completely abolished (8–10). In transfection assays, c-Maf was able to activate the \( \alpha \) (12), \( \beta B2 \) (48), \( \delta I \) (12), and \( \gamma \) (53) crystallin promoters. Other members of the large Maf family, L-Maf/MafA, MafB, and N-Ras are detected in the developing lenses of vertebrates (8, 46, 54, 55). All of them can activate crystallin promoters in co-transfection assays, even though there are quantitative, but not qualitative, differences in their transactivation capacities (12, 33). Thus, transcriptional activation of a crystallin gene may result from various Maf members at different developmental stages.

The spatiotemporal expression patterns of crystallin genes can be attributed to the placement of cis elements and assortment of a small set of developmentally essential transcription factors (56, 57). Here we show that, for the chicken \( \beta B1 \)-crystallin gene, the activators, Mafs bind to MARE-like sequences in both the PL1 and PL2 elements; the repressor, Pax6, binds to both of these elements. Notably, the distribution of Maf and Pax6 binding sites appears to be unique to the chicken \( \beta B1 \)-crystallin promoter and may be essential for its stringently regulated transcription (38). In crystallin promoters that function in both lens epithelial and fiber cells, such as mouse \( \alpha \) (9, 30), chicken \( \alpha \) (11, 29), and chicken \( \delta I \) (31, 58), Pax6 activates these genes by binding to cis elements that are separated, or at most, partially overlapping with the consensus MAREs. It is possible that Maf and Pax6 bind to the corresponding elements and cooperatively activate the transcription of these crystallin

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2 M. Duncan, L. Xie, L. David, M. Robinson, J. Taube, W. Cui, and L. Reneker, submitted for publication.
Concentrations are high, Pax6 binds to the PL1, PL2, and OL2 elements of the promoter, which inhibits access of Mafs and Prox1, and prevents β1-crystallin gene expression. In lens fiber cells, Pax6 levels diminish while Prox1 and c-Maf levels increase. c-Maf (dimers) can then bind to the PL1 and PL2 elements and Prox1 binds to OL2 to activate gene expression.

Prox1 is another transcription factor known to be critical for fiber cell differentiation (14). In this study, we determined that the COOH terminus of chicken Prox1 can specifically interact with both the Prospero consensus sequence (18) and a similar binding sequence located in the OL2 element that is adjacent to the COOH terminus of chicken Prox1 can specifically interact with the chicken 1-crystallin promoter in gel shift assays. Recently, it has become apparent that Prox1 probably interacts with both fiber cell preferred elements in vitro and negatively regulates the expression of fiber cell preferred elements.

A model describing the spatiotemporal regulation of chicken β1-crystallin expression. In lens epithelial cells where Pax6 concentrations are high, Pax6 binds to the PL1, PL2, and OL2 elements of the promoter, which inhibits access of Mafs and Prox1, and prevents β1-crystallin gene expression. In lens fiber cells, Pax6 levels diminish while Prox1 and c-Maf levels increase. c-Maf (dimers) can then bind to the PL1 and PL2 elements and Prox1 binds to OL2 to activate gene expression.

In conclusion, the regulated expression of chicken β1-crystallin appears to be achieved at two levels: at the promoter level, cis elements are recognized by positive (Maf and Prox1) and negative (Pax6) transcription factors simultaneously; at the transcription factor level, the repressor, Pax6, is expressed at high levels in the lens epithelium but not in lens fiber cells. In contrast, the activators, Prox1 and c-Maf are strongly up-regulated in lens fiber cells coincident with the initiation and up-regulation of chicken β1-crystallin expression. Thus, the spatiotemporal expression of chicken β1-crystallin could be harmoniously directed during lens differentiation.

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Wenwu Cui, Stanislav I. Tomarev, Joram Piatigorsky, Ana B. Chepelinsky and Melinda K. Duncan

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