

# Cooperative action between L-Maf and Sox2 on $\delta$ -crystallin gene expression during chick lens development

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## Abstract

Lens development is regulated by a variety of transcription factors with distinct properties. The lens-specific transcription factor, L-Maf, is essential for lens formation and induces lens-specific markers, such as the *crystallin* genes. In this study, we analyzed the mechanism by which L-Maf regulates  $\delta$ -crystallin expression. Misexpression of L-Maf in the head ectoderm of lens placode-forming embryos by in ovo electroporation induced  $\delta$ -crystallin only in the region surrounding the lens. To define this restricted expression, we misexpressed L-Maf together with other transcription factors implicated in  $\delta$ -crystallin expression. Sox2 plus L-Maf expanded the  $\delta$ -crystallin-inducible domain to the entire head ectoderm and simultaneously increased the quantity of  $\delta$ -crystallin mRNA expressed. In contrast, co-expression of L-Maf with other factors such as Pax6, Six3 and Prox1 had little or no effect on  $\delta$ -crystallin. We also observed that L-Maf and Sox2 cooperatively enhanced the transactivation of a reporter gene bearing the  $\delta$ -crystallin enhancer in ovo, implying that L-Maf and Sox2 can induce  $\delta$ -crystallin through the same enhancer. In conclusion, we report here that L-Maf and Sox2 cooperatively regulate the expression of  $\delta$ -crystallin during chick lens development.

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**Keywords:** L-Maf; Sox2; Lens development; Lens induction; In ovo reporter assay; Electroporation

## 1. Introduction

Lens formation during development has been studied for the last century because of its simple system with distinctive structures, tissue induction and differentiation. Lens induction in the head ectoderm is triggered by inductive signals from the optic vesicle. The lens is formed from the head ectoderm overlying the optic vesicle, initially via thickening of the presumptive lens ectoderm to form a lens placode. Subsequently, the lens placode forms a lens vesicle by invagination, fusion and separation from the head ectoderm. Cells in the anterior half of the lens vesicle continue to proliferate and contribute to the formation of lens fiber cells, which differentiate into lens tissues by elongation. Lens differentiation is accompanied by expression of several lens-specific genes, such as *crystallin* genes that encode structural proteins. In chick,  $\delta$ -crystallin is known as an early molecular marker of lens differentiation and is expressed at the lens placode stage (Piatigorsky, 1981). Analysis of the gene expression cascade from lens induction

to  $\delta$ -crystallin gene expression reveals the molecular mechanism underlying lens development. Some transcription factors implicated in lens development are known to regulate  $\delta$ -crystallin expression.

Pax6 is the paired-domain and homeodomain-containing transcription factor considered as a master regulator of eye development (Quiring et al., 1994; Halder et al., 1995). In *Drosophila* and *Xenopus* embryos, misexpression of Pax6 induces ectopic eye formation (Halder et al., 1995; Chow et al., 1999; Czerny et al., 1999). Pax6 expression is essential for the lens-forming competence of head ectoderm to respond to inductive signals from the optic vesicle (Fujiwara et al., 1994; Quinn et al., 1996; Collinson et al., 2000; Reza et al., 2002). During lens differentiation, Pax6 participates in the transactivation of lens-specific genes such as *L-maf*, *Prox1* and *crystallins* (Cvekl and Piatigorsky, 1996; Reza et al., 2002).

Several members of the Sox family of transcription factors, which contain both a transactivation domain and a highly conserved DNA-binding HMG domain, are involved in lens formation (Laudet et al., 1993; Kamachi et al., 1995). Sox proteins require a partner factor to transactivate their target genes (Kamachi et al., 2000). Among the members,

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the Sox1, 2, and 3 subgroups are implicated in lens development (Kamachi et al., 1998; Nishiguchi et al., 1998). In the chick, misexpression of Sox2 and Pax6 induces ectopic lens placode, characterized by the expression of L-Maf and  $\delta$ -crystallin (Kamachi et al., 2001; Reza et al., 2002). In addition, the cooperative effect on  $\delta$ -crystallin expression is elicited through a lens specific enhancer element DC5, located within the third intron of the  $\delta$ -crystallin gene (Kamachi et al., 2001). These data imply that Sox2 functions both in lens induction and differentiation.

Six3 is a homeobox-containing transcription factor homologous to *Drosophila sine oculis* (so), which is crucial for early eye development (Cheyette et al., 1994; Oliver et al., 1995). Overexpression of murine Six3 in the optic vesicle of medaka fish induces ectopic lens (Oliver et al., 1996). Six3 was recently shown to function as a repressor of eye formation and  $\delta$ -crystallin expression, in concert with Groucho co-repressors (Kobayashi et al., 2001; Zhu et al., 2002).

Maf family members containing a basic leucine zipper domain are also involved in lens formation (Sakai et al., 1997; Moens et al., 1998; Ogino and Yasuda, 1998; Kawauchi et al., 1999; Kim et al., 1999; Ishibashi and Yasuda, 2001; Kajihara et al., 2001; Muta et al., 2002; Reza et al., 2002; Yoshida and Yasuda, 2002). L-Maf is a transcription factor, which binds to lens-specific enhancer element  $\alpha$ CE2 in the chick  $\alpha$ A-crystallin promoter (Ogino and Yasuda, 1998). L-Maf is first expressed at the lens placode and is maintained specifically in lens cells. L-Maf induces the expression of several lens-specific markers including *crystallins* and *filensin* and can convert cultured chick retinal cells into lens cells. In ovo gain- and loss-of-function studies reveal that L-Maf functions downstream of Pax6 in the genetic cascade during lens development (Reza et al., 2002). Misexpression of L-Maf in ventral head ectoderm induces expression of  $\delta$ -crystallin and Prox1 (Ogino and Yasuda, 1998; Reza et al., 2002), a homeobox gene implicated in terminal differentiation of lens fiber cells (Wigle et al., 1999). Dominant-negative L-Maf inhibits the induction of both  $\delta$ -crystallin and Prox1, resulting in a lack of lens formation (Reza et al., 2002). It is clear therefore that L-Maf plays a key role in lens development and  $\delta$ -crystallin induction. However, the molecular mechanisms of L-Maf function on the regulation of  $\delta$ -crystallin expression remain undefined.

In this study, we performed gain-of-function experiments by in ovo microelectroporation of chick embryos. We electroporated *L-maf* and several other genes of interest into head ectoderm of embryos to determine their roles in  $\delta$ -crystallin expression. We observed that misexpression of L-Maf in head ectoderm induced expression of  $\delta$ -crystallin within the region surrounding the lens, while misexpression of L-Maf with Sox2 expanded the  $\delta$ -crystallin expression domain to include the entire head ectoderm. L-Maf and Sox2 cooperatively enhanced the transactivation of the  $\delta$ -crystallin gene and a reporter gene bearing the  $\delta$ -crystallin

enhancer in ovo. We also found that endogenous Sox2 expression was required when L-Maf induced  $\delta$ -crystallin ectopically. These results demonstrate that cooperative function of Sox2 is significant for the role of L-Maf in transactivating  $\delta$ -crystallin gene during chick lens development.

## 2. Results

### 2.1. Restricted expression of $\delta$ -crystallin by L-Maf misexpression

We previously demonstrated that L-Maf can induce  $\delta$ -crystallin expression in head ectoderm of chick embryos. We also observed regions where L-Maf could not induce the  $\delta$ -crystallin expression (Ogino and Yasuda, 2000). These data led to the assumption that there was a distinct boundary between the  $\delta$ -crystallin-inducing and non-inducing regions and identification of that boundary might reveal molecular mechanisms underlying  $\delta$ -crystallin expression by L-Maf. To address this, we misexpressed L-Maf in various regions of head ectoderm in stage 9–10 embryos by microelectroporation. Following electroporation, these embryos were incubated for 24 h then stained with anti- $\delta$ -crystallin antibody. Control embryos, electroporated with pCAGGS-GFP expression plasmid (Fig. 1A), showed no  $\delta$ -crystallin expression in GFP-expressing regions (Fig. 1A'). Electroporation of the GFP expression plasmid containing L-Maf cDNA induced the expression of  $\delta$ -crystallin in head ectoderm regions around the lens (Fig. 1B,B'). In contrast, when L-Maf was misexpressed in other regions,  $\delta$ -crystallin expression was not detected (Fig. 1C,C'). The  $\delta$ -crystallin expression was restricted to the head ectoderm surrounding the lens (Fig. 1D), which raised a question as to what was lacking in the regions where L-Maf failed to cause  $\delta$ -crystallin expression.

### 2.2. In ovo assay to assess the transactivation ability of L-Maf

One possibility for the restricted induction of  $\delta$ -crystallin expression by L-Maf is that exogenous L-Maf has lost its transactivation potential in the region where L-Maf cannot induce  $\delta$ -crystallin expression, perhaps via phosphorylation by MAPK, which causes loss of the transactivation activity of L-Maf (Benkhelifa et al., 2001; Ochi et al., 2003). To test this possibility, we performed in ovo reporter assays with a  $\beta$ -gal reporter plasmid containing an L-Maf binding element ( $\alpha$ CE2) (Matsuo and Yasuda, 1992). We transfected the reporter construct ( $\alpha$ CE2-LacZ) alone and with a GFP expression plasmid into the embryonic head ectoderm. Expression of the reporter gene was detected specifically in lens cells, suggesting that endogenous L-Maf transactivates the reporter gene through the binding element  $\alpha$ CE2 (Fig. 2A–B'). We next co-transfected the  $\alpha$ CE2-LacZ reporter

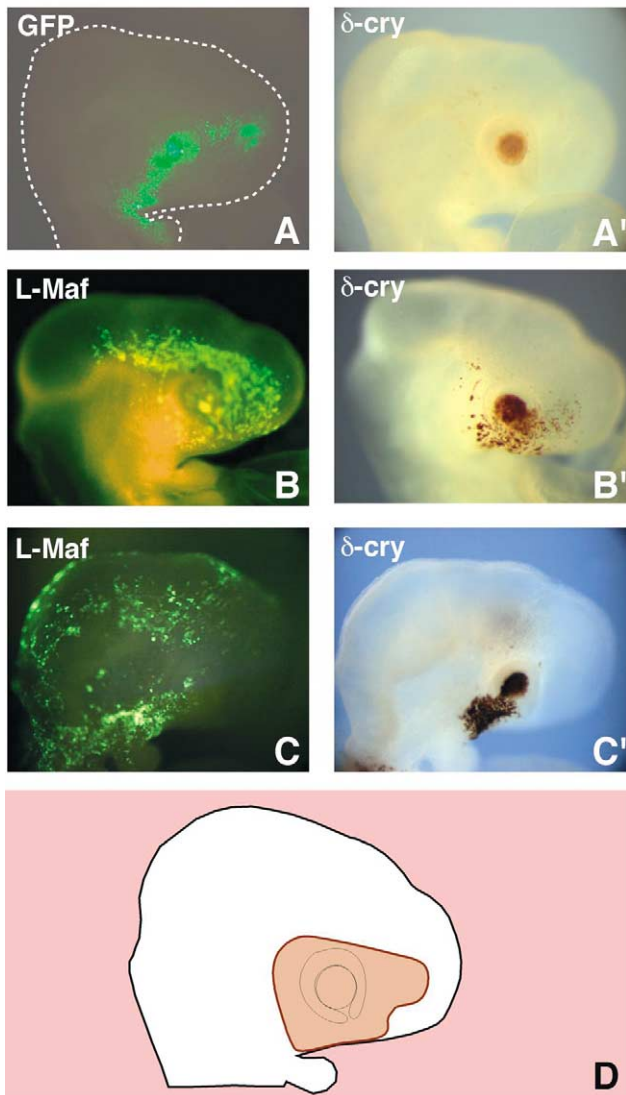


Fig. 1. Misexpression of L-Maf induces restricted expression of  $\delta$ -crystallin. Stage 9–10 embryos were electroporated with expression plasmids for L-Maf and GFP and incubated for 24 h. Embryos were then stained with anti- $\delta$ -crystallin antibody (A'–C'). To target different regions, embryos were electroporated by changing the position of two electrodes. GFP signals show the region where exogenous L-Maf is expressed (A–C). (D) Schematic drawing of the ectopic  $\delta$ -crystallin expression domain induced by L-Maf. 51 embryos out of 54 L-Maf expressing embryos showed the restricted  $\delta$ -crystallin expression.

and the L-Maf expression vector in the head ectoderm.  $\beta$ -Gal positive signals were detected even in the region where ectopic  $\delta$ -crystallin was not detected in the L-Maf misexpression experiments (Fig. 2C,C'). These results indicate that exogenous L-Maf is competent for transactivation in all parts of head ectoderm. Therefore, the restricted spatial ability of L-Maf to induce  $\delta$ -crystallin expression is not due to loss of its transactivation potential. These data also indicate that the limited expression of  $\delta$ -crystallin is probably due to the lack of other factors in head ectoderm.

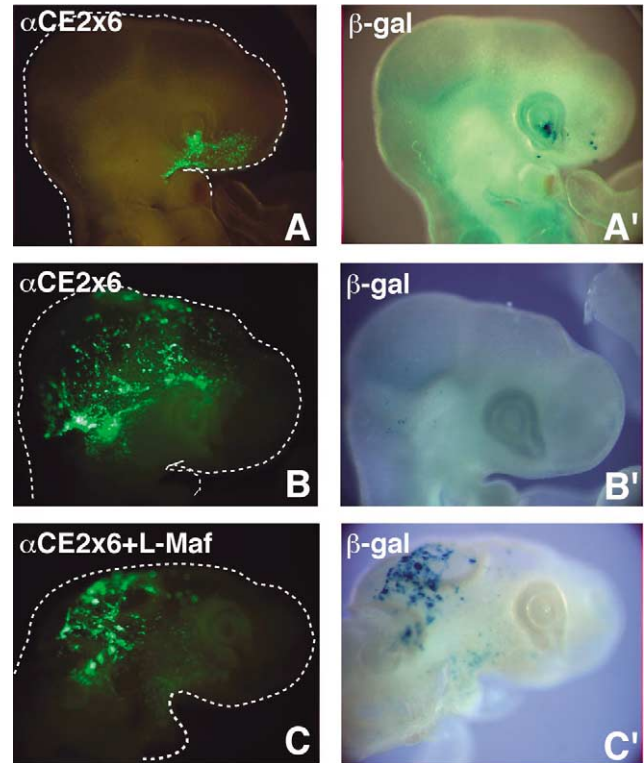


Fig. 2. Exogenous L-Maf possesses equal transactivation potential in all parts of the head ectoderm. X-gal staining of the embryos co-electroporated with the control GFP expression plasmid and  $\beta$ -gal reporter plasmid containing the hexameric fragment of L-Maf binding element,  $\alpha$ CE2 ( $\alpha$ CE2x6-LacZ) (A–C').  $\beta$ -gal activity was detected only in the lens (A,A') and not in other regions of the head ectoderm (B,B'), suggesting that endogenous L-Maf activates the reporter gene. (C,C') X-gal staining of the embryos electroporated with GFP, L-Maf and  $\alpha$ CE2-LacZ. When ectopically expressed, L-Maf can activate the reporter gene even in the region where  $\delta$ -crystallin expression is not induced by L-Maf in Fig. 1D.

### 2.3. Expansion of $\delta$ -crystallin expression domain by Sox2

According to the assumption that the region where L-Maf could not induce  $\delta$ -crystallin lacks other factors required for its expression, we designed the following experiments. Among transcription factors that function in lens development, Pax6, Six3, Prox1 and Sox2 are thought to be involved in  $\delta$ -crystallin expression (Kamachi et al., 2001; Reza et al., 2002; Zhu et al., 2002). We therefore misexpressed each of these together with L-Maf in the head ectoderm by electroporation. When L-Maf was misexpressed with either Pax6 or Six3, no  $\delta$ -crystallin expression was induced in a total of 29 embryos (15 and 14 embryos, respectively, Fig. 3A–B'). When L-Maf and Prox1 were misexpressed,  $\delta$ -crystallin-positive cells were detected intermittently in the head ectoderm of eight embryos out of 12 examined (Fig. 3C,C'). On the other hand, misexpression of L-Maf with Sox2 expanded the  $\delta$ -crystallin-expressing region to the entire head ectoderm in 23 embryos out of 25 examined (Fig. 3D,D'), although Sox2 alone induced  $\delta$ -crystallin only in the region contiguous to the lens (Kamachi et al., 2001). These findings support the idea that

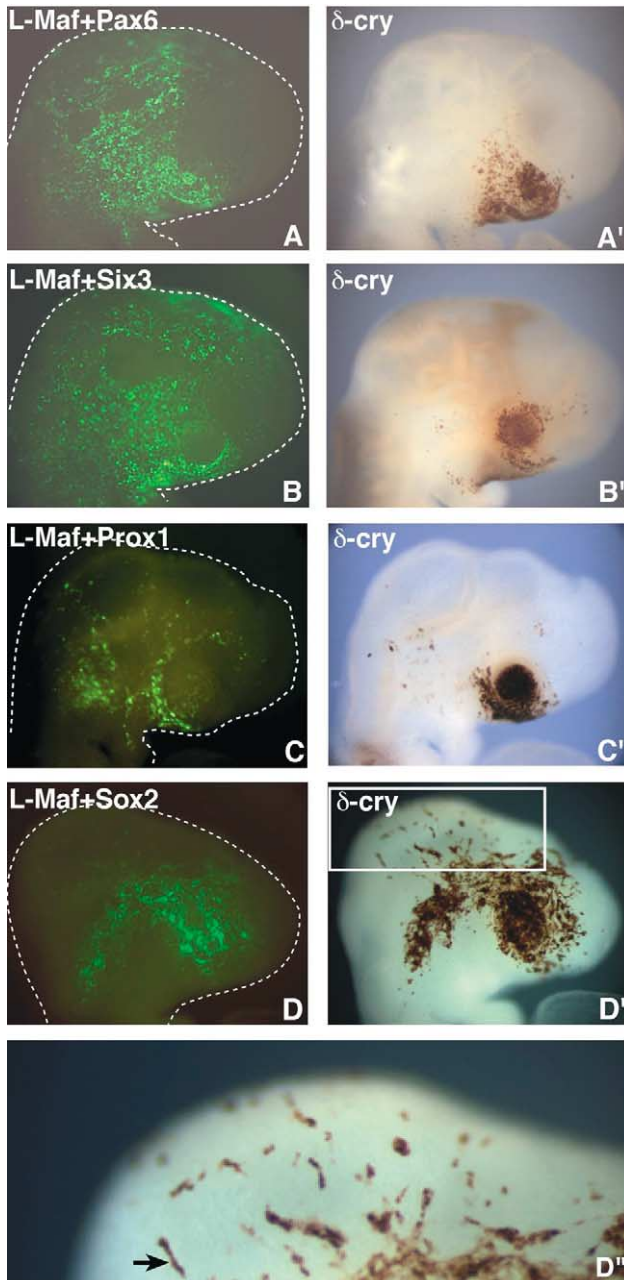


Fig. 3. L-Maf and Sox2 cooperatively induce  $\delta$ -crystallin expression. Lens placode-forming embryos were co-electroporated with L-Maf and (A) Pax6, (B) Six3, (C) Prox1, and (D) Sox2, then stained with anti- $\delta$ -crystallin antibody (A'–D'). Co-electroporation of L-Maf together with either Pax6 or Six3 induced  $\delta$ -crystallin in the same region as L-Maf alone (A', B'). Co-electroporation of L-Maf and Prox1 induced scattered expression of  $\delta$ -crystallin in wider regions of the head ectoderm (C'). In contrast, co-electroporation of L-Maf and Sox2 strongly induced  $\delta$ -crystallin in the entire head ectoderm (D'). (D'') Higher magnification of the white box in (D') shows elongated  $\delta$ -crystallin-positive cells (arrow).

L-Maf and Sox2 act cooperatively to enhance  $\delta$ -crystallin expression. Interestingly, the  $\delta$ -crystallin-positive cells were elongated (Fig. 3D''), similar to lens fiber cells, implying that L-Maf and Sox2 also act cooperatively on lens differentiation.

#### 2.4. Sox2 enhances the transactivation of the $\delta$ -crystallin gene

We showed that  $\delta$ -crystallin expression was enhanced by the misexpression of L-Maf with Sox2. This effect probably occurs at a transcriptional level, since Sox2 requires cell-specific partner factors to elicit its transactivation ability and acts with these factors to synergistically transactivate target genes (Kamachi et al., 2000). Therefore, co-electroporation of L-Maf and Sox2 was expected to cooperatively increase the quantity of induced  $\delta$ -crystallin mRNA and the expression induced by both proteins might be detected earlier than that induced by L-Maf alone. This experiment allows us to assess the enhancement ability of Sox2 besides cooperative action in  $\delta$ -crystallin expression. To measure this proposed timing difference, we misexpressed L-Maf alone or with Sox2 in head ectoderm and timed the onset of  $\delta$ -crystallin mRNA expression by in situ hybridization. With L-Maf alone,  $\delta$ -crystallin mRNA positive-cells were not detected in any of eight embryos at 4 h of incubation following electroporation (Fig. 4A, A'), but were observed after 4.5 h in seven embryos out of 12 examined (Fig. 4B, B'). In the presence of L-Maf and Sox2, however,  $\delta$ -crystallin mRNA expression was first observed after 2.5 h of incubation in eight embryos out of 11 examined (Fig. 4C–D'). Thus, there was approximately 2 h difference in the onset of the  $\delta$ -crystallin mRNA expression between misexpression of L-Maf alone and that of L-Maf and Sox2 together. These results suggest that Sox2 enhances the transactivation of the  $\delta$ -crystallin gene via a cooperative action with L-Maf.

#### 2.5. L-Maf induces $\delta$ -crystallin in the Sox2-positive domain of head ectoderm

Since Sox2 expands the region of L-Maf-induced  $\delta$ -crystallin expression, we speculate that L-Maf requires Sox2 to induce  $\delta$ -crystallin expression. Namely, L-Maf misexpression can cause  $\delta$ -crystallin expression only where endogenous Sox2 is present. To test this hypothesis, we first checked Sox2 expression patterns by immunostaining on frozen sections of stage 18 embryos using an anti-Sox2 antibody. Sox2 protein was expressed with the same pattern (Fig 5A, A') as Sox2 mRNA (Kamachi et al., 1998). The Sox2 expression in the head ectoderm was restricted to the ventral region including the area overlying the optic vesicle. As proposed, the ventrally restricted expression of Sox2 was overlapped by the  $\delta$ -crystallin expression induced by L-Maf (Fig. 5A–E). Other sections expressing exogenous L-Maf also exhibited  $\delta$ -crystallin where Sox2 was normally expressed (Fig. 5F). These data suggest that endogenous Sox2 is essential for induction of  $\delta$ -crystallin expression by L-Maf.

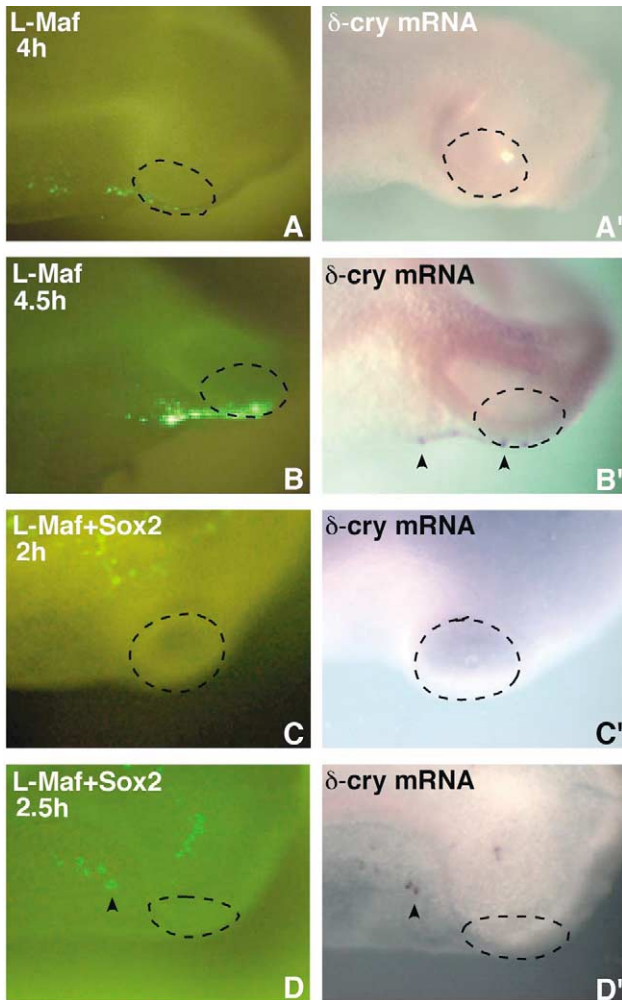


Fig. 4. L-Maf and Sox2 cooperatively enhance transcription of the  $\delta$ -crystallin gene. Lens placode-forming embryos were electroporated with L-Maf alone (A,B) or L-Maf and Sox2 (C,D). After electroporation, the embryos were incubated for 4 or 4.5 h (A or B), and 2 or 2.5 h (C or D), then  $\delta$ -crystallin mRNA was detected by whole-mount in situ hybridization (A'–D'). The  $\delta$ -crystallin mRNA was detectable at the earliest time point, 4.5 h (B') and 2.5 h (D'), respectively. Co-expression of L-Maf and Sox2 shortened the time at which  $\delta$ -crystallin expression was first detected by 2 h compared with L-Maf alone. Presumptive lens placodes and representative  $\delta$ -crystallin-positive cells are indicated by black dotted lines and black arrowheads (A–D'), respectively.

### 2.6. L-Maf and Sox2 cooperatively activate the $\delta$ -crystallin enhancer

The results presented thus far show that co-electroporation of L-Maf and Sox2 can induce an accelerated and increased level of  $\delta$ -crystallin expression compared with that induced by L-Maf alone. This cooperative effect on  $\delta$ -crystallin expression is thought to be elicited through the same promoter region on the  $\delta$ -crystallin gene. Two Maf binding sites (MafD and a second predicted site) and three Sox2 binding sites (SoxD, SoxM and SoxU) are present in the enhancer region of the gene (Fig. 6; Ogino and Yasuda, 1998; Kamachi et al., 2001; Muta et al., 2002). The MafD site and two of the Sox2 sites (SoxD and SoxU) exhibit

enhancer activity in mouse lens (Muta et al., 2002). To examine the possible site(s) on the  $\delta$ -crystallin gene involved in the cooperative induction, we performed in ovo reporter assays in chicks using a  $\beta$ -gal reporter gene bearing these possible Maf and Sox2 sites (BHd-LacZ). We electroporated the reporter plasmid and GFP expression vector into embryonic head ectoderm and, after incubation for 24 h, embryos were stained with X-gal. Transactivation of the reporter gene was detected only in the lens cells (Fig. 6A,A'). Section staining also revealed that  $\beta$ -gal expression was positive both in the presumptive lens epithelium (Fig. 6B) and lens fibers (Fig. 6C), suggesting that the enhancer recapitulates  $\delta$ -crystallin expression during early lens development. When the reporter gene was co-electroporated with either L-Maf or Sox2, only weak  $\beta$ -gal signals were detected (Fig. 6D–E'). In contrast, when the reporter gene was electroporated with L-Maf and Sox2 together, among 18 embryos examined, 16 embryos showed significant enhancement of the reporter activity (Fig. 6F,F'), which is similar to that seen with Sox2 and Pax6 in 18 out of 21 embryos (Fig. 6G,G'). These results suggest that the cooperative effect of Sox2 on L-Maf action is exerted through the  $\delta$ -crystallin enhancer as observed in the case of Sox2 and Pax6.

### 3. Discussion

Previously, we reported that overexpression of L-Maf could induce  $\delta$ -crystallin expression in specific tissues: cultured lens cells, neural retina cells and ventral head ectoderm of chick embryo (Ogino and Yasuda, 1998; Ogino and Yasuda, 2000). The molecular mechanisms underlying this tissue specific induction of  $\delta$ -crystallin by L-Maf, however, remained unclear. In this study, we have identified the  $\delta$ -crystallin expression domain inducible by L-Maf misexpression in the head ectoderm of lens placode-forming chick embryos. We show that the  $\delta$ -crystallin expression is restricted to a head ectoderm region surrounding the lens. We also found that the  $\delta$ -crystallin-inducing ability of L-Maf is not solely determined by its transactivation activity, and that a cooperative effect with Sox2 is also crucial. We demonstrate that Sox2 is in fact required for the transactivation function of L-Maf as well as the transcriptional enhancement of  $\delta$ -crystallin expression together with L-Maf. Taken together, our results indicate that L-Maf and Sox2 cooperatively regulate  $\delta$ -crystallin expression during lens differentiation.

#### 3.1. Function of L-Maf and Sox2 during lens formation

Misexpression of L-Maf and Sox2 expanded the  $\delta$ -crystallin-inducible domain in the head ectoderm compared with that of L-Maf alone. Furthermore,  $\delta$ -crystallin expression induced by L-Maf was observed only where Sox2 was also expressed. These findings can be taken to

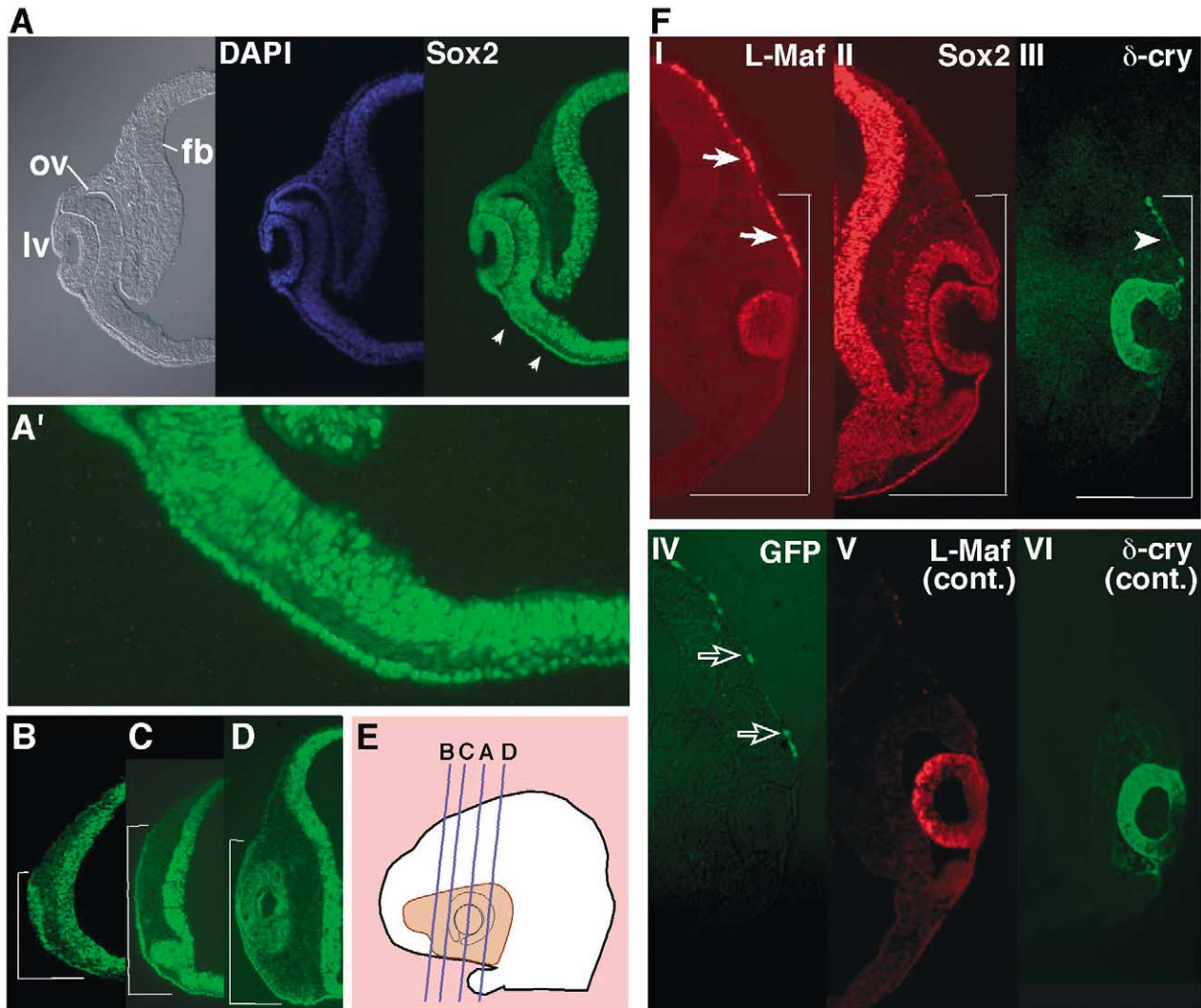


Fig. 5. L-Maf can induce the  $\delta$ -crystallin gene only in the Sox2 expression domain. Stage 18 embryos were transversely sectioned at different planes of the head ectoderm (E, line A–D) and stained with anti-Sox2 antibody (A–D, green) and DAPI (A, blue). (E) Schematic representation of a stage 18 embryo depicting the ectopic  $\delta$ -crystallin expression domain (brown). (A') A higher magnification of the ventral head ectoderm indicated by white arrowheads in (A). Ectopic  $\delta$ -crystallin expression was detected only in the Sox2 expression domain (compare E, brown with A–D, green). (F) Sections of a stage 18 embryo electroporated with L-Maf. GFP fluorescence indicates the L-Maf electroporated area (open arrows, FIV). Ectopic L-Maf expression was followed in both Sox2 expressing and non-expressing regions (white arrows, FI). Ectopic  $\delta$ -crystallin (green) was detected only in the Sox2 expression domain (arrowhead, FIII) The Sox2-positive domain is indicated by white lines (FI–III). Six embryos were analyzed for each experiment. lv, lens vesicle; ov, optic vesicle; fb, forebrain.

suggest that L-Maf exerts its transactivation activity on  $\delta$ -crystallin in a Sox2-dependent manner. Interestingly, elongated cells, which are characteristic of differentiated lens cells, were observed with misexpression of L-Maf and Sox2 together. Transfection of L-Maf can convert cultured neural retina cells into lens cells (Ogino and Yasuda, 1998). In addition, *Sox1* or *c-maf* knockout mice exhibit similar defects in elongation of lens fiber cells associated with expression of *crystallin* genes (Nishiguchi et al., 1998; Kawachi et al., 1999). These results support the notion that L-Maf and Sox2 function cooperatively during lens differentiation. In another recent report, Sox2 and Pax6 were shown to ectopically induce lens placode (Kamachi et al., 2001). Likewise, L-Maf is essential for lens placode formation (Reza et al., 2002). Sox2 up-regulation is also

observed in head ectoderm apposed to the optic vesicle (Furuta and Hogan, 1998; Kamachi et al., 1998) when L-Maf starts its expression in placodal cells (Ogino and Yasuda, 1998), implying that L-Maf and Sox2 can also act harmoniously during lens induction mechanisms.

### 3.2. Cooperation of Sox2 with L-Maf is via the $\delta$ -crystallin enhancer region

Misexpression of L-Maf and Sox2 effectively increased the quantity of  $\delta$ -crystallin mRNA expressed in ovo compared to L-Maf misexpression alone, suggesting that L-Maf also acts in cooperation with Sox2 to regulate  $\delta$ -crystallin gene transcription. This cooperative effect was predicted to occur through an enhancer in the third intron of

the  $\delta$ -crystallin gene since this region contains putative binding sites for both Maf and Sox2 (Kamachi et al., 1995; Ogino and Yasuda, 2000; Muta et al., 2002). We performed in ovo reporter assays using a partial region of the enhancer

(BHd), containing two Maf binding sites (including a predicted site) and three Sox2 binding sites. We confirmed that BHd showed lens specific enhancer activity and that co-electroporation of L-Maf and Sox2 significantly enhanced the reporter activity compared to that measured for L-Maf or Sox2 alone.

In contrast, reporter assays using a plasmid encoding a smaller fragment of the  $\delta$ -crystallin enhancer (HN), which has lens-specific enhancer activity (Hayashi et al., 1987; Funahashi et al., 1991), showed considerably less reporter activity (data not shown). Furthermore, co-electroporation of L-Maf and Sox2 did not enhance the reporter activity (data not shown), although an appreciable increase was observed when Sox2 and Pax6 were co-electroporated (data not shown), as shown previously in cultured cells (Kamachi et al., 2001). These results demonstrate that an enhancer region longer than the HN region is required for transcriptional enhancement of  $\delta$ -crystallin expression by L-Maf and Sox2 in ovo. In fact, the v-Maf binding site (MafD) was shown to be downstream of the HN region (Muta et al., 2002), implying that Sox2 exerts its cooperative action with L-Maf through this region. Sox proteins themselves cannot transactivate their target genes because of their low DNA-binding affinity, and therefore require DNA-binding partners that act in a cell-specific fashion (Kamachi et al., 2000). On the other hand, L-Maf shows lens-specific expression and possesses high DNA-binding affinity (Ogino and Yasuda, 1998). Thus, it is possible that L-Maf functions as a DNA binding co-factor for Sox2. Sox2 and one of its partners, Oct-3/4, synergistically activate FGF-4 and UTF-1 enhancers, and this activation is dependent on protein-protein interactions between them (Ambrosetti et al., 1997, 2000; Nishimoto et al., 1999). In addition, Sox2 and Pax6 interact directly with each other on DC5, a subfragment of HN, and this ternary complex is required for the synergistic action of Sox2 and Pax6 (Kamachi et al., 2001). Though we have not studied the potential physical interaction between L-Maf and Sox2, the acidic and hinge region (AH) of L-Maf is essential for its transactivation ability in inducing  $\delta$ -crystallin expression (Yoshida and Yasuda, 2002). It is therefore possible that Sox2 directly binds to this AH domain to cooperatively elicit the induction of  $\delta$ -crystallin with L-Maf. Sox proteins also have the ability to bend DNA, which allows the assembly of other necessary transactivation machinery (Connor et al., 1994; Lefebvre et al., 1997; Kawachi et al., 1999; Wegner, 1999; Scaffidi and Bianchi, 2001; Weiss, 2001). In this system, Sox2 may play a similar

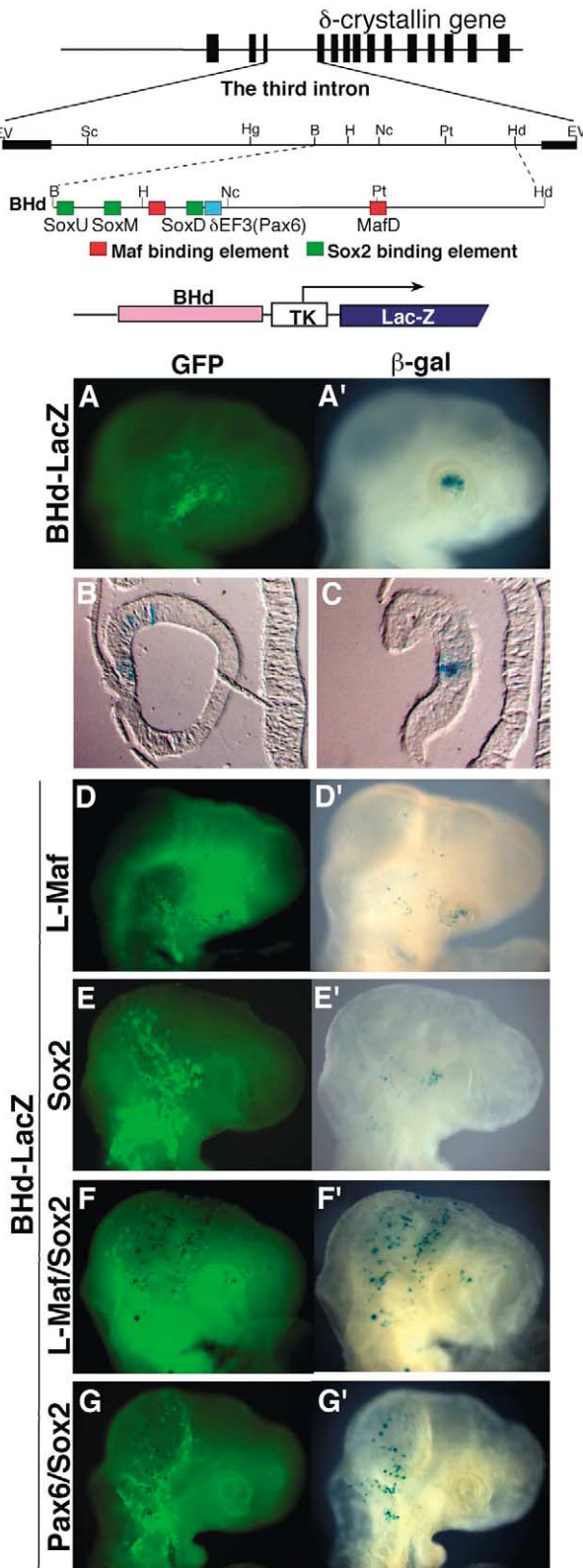


Fig. 6. X-gal staining of the embryos electroporated with GFP and the  $\beta$ -gal reporter gene bearing the partial fragment of the  $\delta$ -crystallin enhancer element, including both L-Maf and Sox2 binding sites (BHd) (A–G'). The reporter gene was activated only in the lens (A,A'). Embryos electroporated with the reporter gene were transversely sectioned through the lens.  $\beta$ -Gal signal was observed both in presumptive lens epithelium (B) and lens fibers (C). Electroporation of Sox2 with either L-Maf or Pax6 enhanced the reporter activity more effectively (F–G') than L-Maf or Sox2 alone (D–E').

role in promoting recruitment of L-Maf to its binding site via its DNA bending activity.

### 3.3. Roles of L-Maf, Sox2 and Pax6 in regulation of $\delta$ -crystallin expression

L-Maf, Sox2 and Pax6 are all required for full expression of  $\delta$ -crystallin (Kamachi et al., 2001; Reza et al., 2002). Recently, transgenic mice carrying a  $\delta$ -crystallin enhancer region mutated in different Maf, Sox2 and Pax6 binding sites showed distinct enhancer activity during lens development. It was found that Sox2 is required for activation of the downstream  $\delta$ -crystallin enhancer in all situations, Pax6 functions as both activator and suppressor through different binding sites, and Maf regulates the enhancer only in lens fiber cells (Muta et al., 2002). However, the roles of Maf, Sox2 and Pax6 in  $\delta$ -crystallin regulation during lens induction are not clearly resolved. Our gain-of-function experiments show the cooperative action of L-Maf and Sox2 on  $\delta$ -crystallin expression. This is the first demonstration in relation with these factors during lens development. Co-electroporation of L-Maf and Sox2 causes higher  $\delta$ -crystallin induction than Sox2 and Pax6 (Kamachi et al., 2001). It has been suggested that Sox2 and Pax6 induce L-Maf or  $\delta$ -crystallin only upon the receipt of inductive signals from the optic vesicle (Reza et al., 2002). Therefore, the combination of L-Maf and Sox2 is thought to be the major  $\delta$ -crystallin inducer. This was proposed because, despite the early expression of Pax6 and Sox2 in presumptive lens ectoderm,  $\delta$ -crystallin expression takes place only after L-Maf expression in the placodal cells (Ogino and Yasuda, 1998; Reza et al., 2002). Furthermore, we observe here that Sox2 and Pax6 cooperatively activate the BHD enhancer in ovo. This result is supported by several other pieces of evidence. Sox2 and Pax6 synergistically activate the lens-specific enhancer DC5 in cultured cells (Kamachi et al., 2001). Transgenic mice bearing chick  $\delta$ -crystallin enhancer mutated in both the Sox2D and Pax6 ( $\delta$ EF3) binding sites show complete loss of enhancer activity, implying that these sites are essential for  $\delta$ -crystallin enhancer activity both in the epithelial and fiber state of lens cells (Muta et al., 2002).

When L-Maf was misexpressed together with Pax6, no significant change in  $\delta$ -crystallin expression was observed over that seen with L-Maf alone, although it has been reported that both Pax6 and Nrl-Maf regulate  $\zeta$ -crystallin gene expression in mouse (Sharon-Friling et al., 1998). In apparent contradiction of this, Pax6 has also been shown to inhibit the DNA binding ability of Maf (Kataoka et al., 2001) and it can both activate and repress the  $\delta$ -crystallin enhancer (Muta et al., 2002). Therefore, our finding with the Pax6 and L-Maf misexpression may simply reflect the complex nature of Pax6 function.

### 3.4. Gene cascade for lens development

Prior to lens induction, Pax6 and Sox2 are expressed more

broadly throughout the head ectoderm overlying the optic vesicle (Grindley et al., 1995; Kamachi et al., 1998) and act cooperatively to establish lens-forming competence. Misexpression of these factors can induce ectopic lens placode and  $\delta$ -crystallin expression in a restricted manner (Kamachi et al., 2001). We previously identified that both Pax6 and Sox2 induce L-Maf, which is expressed in lens placode prior to  $\delta$ -crystallin expression (Reza et al., 2002). Moreover, misexpression of the dominant-negative form of L-Maf in the lens primordium of chick embryo causes complete loss of lens tissue and  $\delta$ -crystallin expression (Reza et al., 2002), supporting L-Maf as the best candidate for controlling lens induction and specification. We show in this report that L-Maf regulates  $\delta$ -crystallin induction and probably lens formation in a Sox2-dependent manner. Apart from generating lens competence, Sox2 functions differently on induction of  $\delta$ -crystallin expression at the lens placode stage. Although misexpression of L-Maf and Prox1 could induce  $\delta$ -crystallin in a wider region of head ectoderm, the expression levels were lower than that caused by misexpression of L-Maf and Sox2 together, suggesting that L-Maf and Sox2 play the major role in  $\delta$ -crystallin induction interdependently. Nonetheless, Prox1 regulated by L-Maf must play a minor role in  $\delta$ -crystallin expression. L-Maf and Prox1 probably exert other distinct functions in later stages, as indicated by other studies (Wigle et al., 1999; Reza et al., 2002).

Our present finding of the cooperative function of Sox2 with L-Maf essentially upgrades the earlier model for lens induction, which is shown in Fig. 7. In summary, we report that despite the distinct functional roles of Pax6, Sox2 and L-Maf, L-Maf and Sox2 also function cooperatively to induce  $\delta$ -crystallin expression through the same enhancer during early lens development. Further genetic and molecular analysis of the interactions between these factors is now needed to further our picture of lens development.

## 4. Experimental procedures

### 4.1. Embryo maintenance

Fertilized eggs from Shiroyama and Takeuchi poultry farm in Japan were incubated at 38.5 °C with 100% humidity. Embryos were staged as described by Hamburger and Hamilton (1951).

### 4.2. Plasmid construction

A 982 bp cSix3-fragment was amplified by reverse transcription–polymerase chain reaction (RT–PCR) from total RNA isolated from the lens of 10-day-old chick embryos using upper primer 5'-TGTAAGCTTGTCATCC-CATGGTGTTCAGG-3' and lower primer 5'-GCTGAGCCT-TACACTACATACTCCTAGGTCA-3'. After *Hind*III and *Bam*HI digestion, these fragments were inserted into the *Hind*III and *Bgl*II sites of expression vector pCAGGS

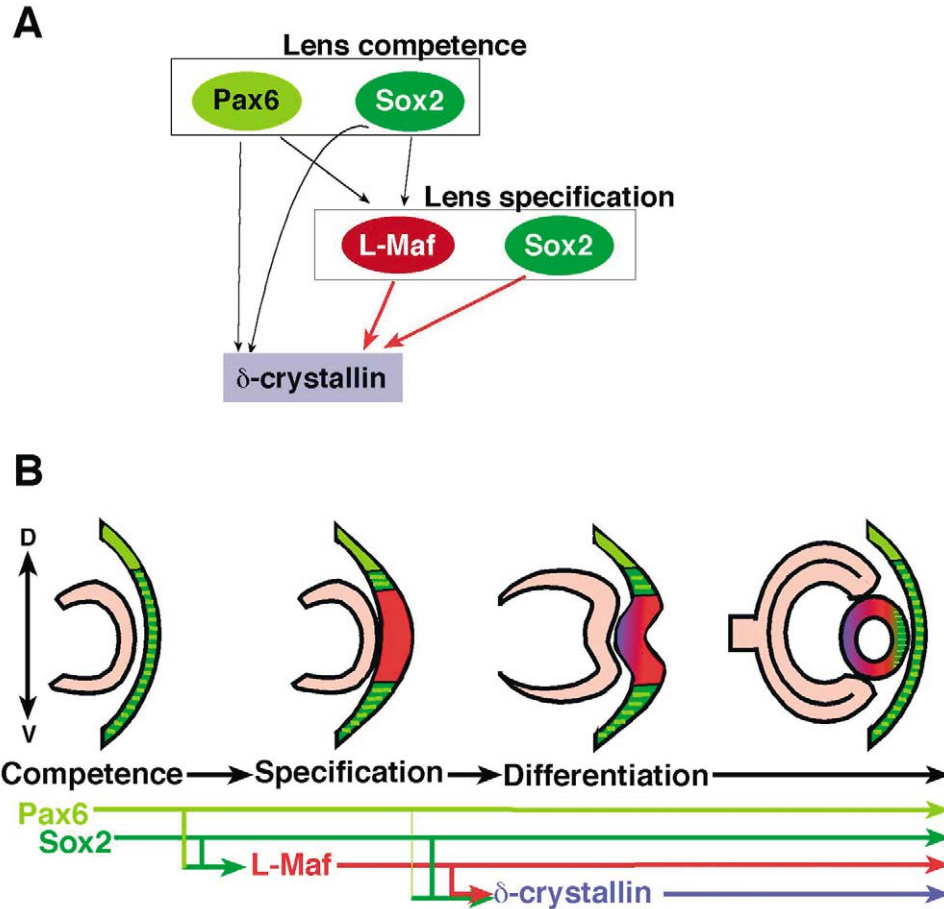


Fig. 7. (A) A proposed model of the gene expression cascade in lens development. Prior to the lens placode formation, progressive and cumulative functions of Pax6 and Sox2 generate and maintain competence for lens formation. Subsequently, Pax6 and Sox2 cooperatively regulate the lens-specific expression of L-Maf. Following L-Maf expression,  $\delta$ -crystallin is expressed in lens placode. Expression of  $\delta$ -crystallin is likely to occur by three pathways: direct regulation by L-Maf and Sox2; direct regulation by Sox2 and Pax6; and probably via weak regulation by Prox1 as a mediator of L-Maf. (B) Schematic representation of different stages of lens development.

containing the cytomegalovirus (CMV-IE) enhancer and chick  $\beta$ -actin promoter to yield pCAGGS-cSix3. Chick Prox1 cDNA (accession no. U46563), kindly provided by Dr S. Tomarev (Tomarev et al., 1996), was amplified as a *Not*I PCR fragment and inserted into the expression vector, pcDNAIII (Reza et al., 2002). To construct the reporter plasmid, a  $\beta$ -gal fragment digested with *Hind*III and *Bam*HI from pCH110 (Pharmacia) was inserted into the *Hind*III and *Bam*HI sites of expression vector pGV3 (Promega) (pGV3/*tk* $\beta$ -gal).  $\delta$ -crystallin enhancer (Bhd), the third intron of the  $\delta$ -crystallin gene was amplified by RT-PCR from a day 3 embryonic chick genomic library using upper primer 5'-GATCGGATCCATCATGGAGATTCTCAGCTC-3' and lower primer 5'-CAGTTGTCACACCTCGTCATGTTCCGAAGTAG-3', and digested with *Bam*HI and *Hind*III. The DNA fragments were filled in using *Klenow* fragment and cloned into pGV3/*tk* $\beta$ -gal digested with *Sma*I.

The above vectors and pCAGGS-GFP (Ogawa et al., 1995), pCAGGS-L-Maf (Ogino and Yasuda, 1998), pEFX3FLAG-Pax6, pCAGGS-cSox2 (Reza et al., 2002)

and p $\alpha$ CE2X6 $\beta$ -gal (Matsuo and Yasuda, 1992) were diluted in TE to 5 mg/ml for electroporation.

#### 4.3. Electroporation

The electroporations were performed according to the procedure described previously (Momose et al., 1999). Each expression vector and pCAGGS-GFP were mixed in a ratio of 9:1 and electroporated into the head ectoderm of stage 9–10 chick embryos. Fluorescent images were captured by a MZFLIII fluorescent microscope (Leica).

#### 4.4. X-gal staining

$\beta$ -Galactosidase staining was carried out as described previously (Momose et al., 1999).

#### 4.5. Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as

described (Henrique et al., 1995) with some minor modification. Embryos were refixed without any protease treatment. A digoxigenin-labeled (Boehringer Mannheim)  $\delta$ -crystallin riboprobe (Ogino and Yasuda, 1998) was prepared and hybridization was performed overnight at 68 °C. For blocking, hybridized embryos were incubated for 2 h in 20% sheep serum in 1.16% maleic acid, 0.87% NaCl, 1.1% Tween-20 in H<sub>2</sub>O (MABT). The  $\delta$ -crystallin mRNA expression was visualized using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim).

#### 4.6. Preparation of polyclonal antibody against Sox2

A His fusion protein of cSox2 (His-Sox2) was expressed in *Escherichia coli* strain BL21 codon + using the pET System (Novagen), and purified using a glutathione Sepharose 4B affinity column, according to the manufacturer's instructions (Pharmacia Biotech). Polyclonal antibodies were raised in rabbits (New Zealand White, Kitayama Labs Co. Ltd.). To confirm the antibody specificity, Sox1, Sox2 and Sox3 proteins were translated from pCMX-cSox1, -Sox2 and -Sox3 plasmids (kindly provided by Dr. H. Kondoh; Kamachi et al., 1999) using TNT Coupled Reticulocyte Lysate System (Promega) and analyzed by Western blot.

#### 4.7. Whole-mount immunostaining

Whole-mount immunostaining was carried out as described previously (Lee et al., 1995; Radice et al., 1997) with the following minor changes. A  $\delta$ -crystallin monoclonal antibody (a gift from Dr. G. Eguchi; Sawada et al., 1993) was added at a dilution of 1:10 as the primary antibody. Anti-mouse IgG-HRP (Amersham) was used as the secondary antibody at a 1:200 dilution.

#### 4.8. Section immunostaining

Dissected embryos were fixed overnight in 4% paraformaldehyde and embedded in Optimal Cutting Temperature compound (Sakura) then snap frozen. Cryostat sections were cut at 10  $\mu$ m, washed in 0.03% Tween-20 in Hanks' (Hanks'-Tw) then incubated for 1 h in 10% goat serum in Hanks'-Tw. Rabbit polyclonal anti-L-Maf (Ogino and Yasuda, 1998), anti-Sox2 and the  $\delta$ -crystallin monoclonal antibody were then added at dilutions of 1:1000, 1:2000 and 1:50, respectively. After washing with Hanks'-Tw, antibody binding was visualized using a 1:1000 dilution of Alexa Fluor 594, 488 or 350-conjugated secondary anti-mouse or -rabbit antibody (Molecular Probes) in Hanks'-Tw. All antibody incubations were carried out overnight at 4 °C. Fluorescent images were captured by AxioCam (Zeiss Co., Germany).

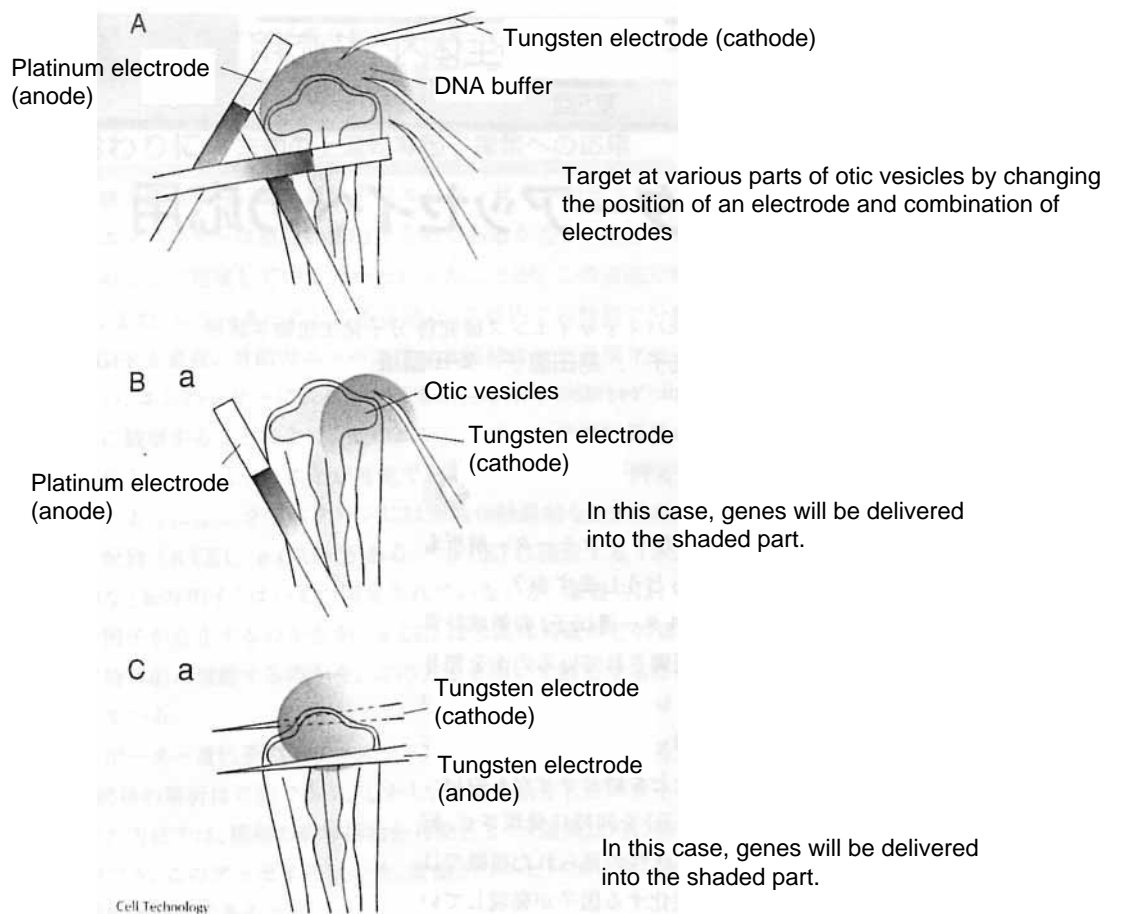
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Electroporation parameter : Pulse length : 50msec Voltage: 7mV No of pulse: 3 times