

# Involvement of *Sox1*, *2* and *3* in the early and subsequent molecular events of lens induction

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## SUMMARY

Activation of the first lens-specific gene of the chicken, *δI-crystallin*, is dependent on a group of lens nuclear factors,  $\delta$ EF2, interacting with the *δI-crystallin* minimal enhancer, DC5. One of the  $\delta$ EF2 factors was previously identified as SOX2. We show that two related SOX proteins, SOX1 and SOX3, account for the remaining members of  $\delta$ EF2. Activation of the DC5 enhancer is dependent on their C-terminal domains. Expression of *Sox1-3* in the eye region during lens induction was studied in comparison with *Pax6* and *δI-crystallin*. *Pax6*, known to be required for the inductive response of the ectoderm, is broadly expressed in the lateral head ectoderm from before lens induction. After tight association of the optic vesicle (around stage 10-11, 40 hours after egg incubation), expression of *Sox2* and *Sox3* is activated in the vesicle-facing ectoderm at stage 12 (44 hours). These cells, expressing together *Pax6* and *Sox2/3*, subsequently give rise to the lens, beginning with formation of the lens placode and expression of  $\delta$ -crystallin at stage 13 (48 hours). *Sox1* then starts to be expressed in the lens-forming cells at stage 14. When the prospective retina area

of the neural plate was unilaterally ablated at stage 7, expression of *Sox2/3* was lost in the side of lateral head ectoderm lacking the optic cup, implying that an inductive signal from the optic cup activates *Sox2/3* expression. In the mouse embryonic lens, this subfamily of *Sox* genes is expressed in an analogous fashion, although *Sox3* transcripts have not been detected and *Sox2* expression is down-regulated when *Sox1* is activated. In ectodermal tissues of the chicken embryo,  $\delta$ -crystallin expression occurs in a few ectopic sites. These are always characterized by overlapping expression of *Sox2/3* and *Pax6*. Thus, an essential molecular event in lens induction is the 'turning on' of the transcriptional regulators SOX2/3 in the *Pax6*-expressing ectoderm and these SOX proteins activate crystallin gene expression. Continued activity, especially of SOX1, is then essential for further development of the lens.

Key words: Lens induction, *Sox*, *Pax6*, Chick

## INTRODUCTION

Lens cell differentiation from embryonic ectoderm is a classical example of tissue induction. Lenses arise in the areas of ectoderm apposed by the retina primordium, the optic cup. An earlier view held that the optic cup had an instructive power to direct lens differentiation in juvenile ectoderm. However, recent revisiting of this old problem revealed quite a different notion that only certain areas of the head ectoderm in a defined period are competent to be induced for lens differentiation (Grainger, 1992). Evidence suggests that one of the conditions for this competence is the expression of the transcription factor PAX6. *Pax6*-deficient homozygous mouse and rat embryos (*Sey*  $-/-$ ) fail to make any lens rudiment (Hogan et al., 1986; Fujiwara et al., 1994; Grindley et al., 1995), and a tissue recombination experiment has demonstrated that it is *Sey*  $-/-$  ectoderm rather than the optic cup that is responsible for the

lens defect (Fujiwara et al., 1994). On the other hand, ectodermal *Pax6* expression takes place correctly in the absence of apposition of the optic cup (Li et al., 1994). It thus appears that retina apposition must induce a second state in the *Pax6* positive ectoderm, which is critical for the initiation of lens differentiation.

In the chicken embryo, lens formation initiates in the period 40-50 hours after incubation. Earlier tissue interactions may be involved in regulation of the state of the ectoderm, as demonstrated for lens differentiation in *Xenopus* (Grainger, 1992), but definitive lens induction by the optic cup is considered to be initiated around stages 10-11 (40 hours after incubation of the egg), when the lateral head ectoderm becomes tightly associated with the optic cup (McKeehan, 1951; Piatigorsky, 1981). Morphological change of the ectoderm into lens placode and the first expression of lens-specific  $\delta$ -crystallin take place shortly after, at stage 13 (48

hours). Because of this early response, we have anticipated that the mechanism of *δ1-crystallin* gene activation is highly relevant to the lens induction process, reflecting the second state being met within the ectoderm.

We have found that *δ1-crystallin* gene is regulated by a lens-specific enhancer located in the third intron (Hayashi et al., 1987; Goto et al., 1990), and that the lens-specificity of the enhancer is determined by the 30 bp DC5 region in which binding sites for two transcription factors,  $\delta$ EF2 and  $\delta$ EF3 have been mapped (Kamachi and Kondoh, 1993). Binding of these factors is essential, since enhancer activity is destroyed by mutations in either one of the two binding sites.  $\delta$ EF2, as identified by gel mobility-shift assay, is composed of a group of proteins  $\delta$ EF2a-d, sharing the same binding specificity. The major components  $\delta$ EF2a and  $\delta$ EF2b in the lens appeared to be present only in the lens and the nervous system, among the tissues examined (Kamachi and Kondoh, 1993). Subsequently, efforts to characterize  $\delta$ EF2 identified SOX2 as one of the proteins,  $\delta$ EF2a (Kamachi et al., 1995).

SOX proteins comprise a family of transcription factors having DNA-binding HMG domains similar to that of Sry (Gubbay et al., 1990), of which more than 20 members have been found (for a review see Pevny and Lovell-Badge, 1997). It has generally been observed that SOX proteins by themselves do not activate transcription but require a partner factor, which binds a nearby site within the same enhancer (Kamachi and Kondoh 1993; Kamachi et al., 1995; Yuan et al., 1995; Lefevbre et al., 1997). In activation of the DC5 enhancer, SOX2/ $\delta$ EF2a needs the cooperation of the partner factor  $\delta$ EF3.

Crystallin composition varies among vertebrate species. The major avian crystallins are  $\alpha$ ,  $\beta$  and  $\delta$ , but  $\delta$ -crystallin is replaced by  $\gamma$ -crystallins in mammals. Nevertheless, there is evidence that the fundamental mechanism regulating lens-specific genes is conserved among the vertebrates. The chicken *δ1-crystallin* gene is regulated in the lens-specific manner in transgenic mice (Kondoh et al., 1987; Takahashi et al., 1988). Conversely, activity of the mouse  $\gamma$ -crystallin promoter is lens-specific in transfected chicken cells (Lok et al., 1989). Furthermore, it was demonstrated that  $\delta$ EF2/SOX binding is essential for the lens-specific promoter activity of the mouse *γF-crystallin* gene (Kamachi et al., 1995). Thus, participation of SOX protein function seems to be a critical part of the common mechanism of lens-specific gene regulation.

We show here that SOX1 and SOX3, in addition to SOX2, account for the  $\delta$ EF2 activity enriched in chicken lens cells. Expression of the *Sox1*, 2 and 3 genes during the early phase of lens development is analyzed in the chicken and mouse embryos. The results indicate that activation of *Sox2/3* expression is induced in the lateral head ectoderm already expressing *Pax6* by the retina primordium, and this activation seems to define the lens fate of the ectoderm.

## MATERIALS AND METHODS

### Isolation of Sox genes

*Sox*-type HMG box sequences were amplified by PCR from embryonic lens cDNA libraries using guess-mer primers (van de Wetering et al., 1993). The 30 randomly picked clones contained the HMG box sequences of *Sox2* (11), *Sox3* (15), *Sox5* (2) and *Sox9* (2), with the frequencies indicated in parentheses.

Screening of chicken lens cDNA libraries using the HMG box sequence of *Sox2*, 3, 5 and 9 as hybridization probes yielded only *Sox1*, 2 and 3 cDNA clones. Screening of cDNA libraries of the 14-day chicken embryonic brain and stage 14-17 whole embryos with the HMG box sequences of *Sox2*, 3, 5 and 9, isolated 27 *Sox* clones that included *Sox1*, 2, 5, 9, 11 and other *Sox* cDNAs. Some differences in the *Sox3* nucleotide and deduced amino acid sequences, involving patches of altered reading frames, were found compared to those reported by Uwanogho et al. (1995).

### Plasmid construction

To express *Sox* cDNAs in cultured cells, cDNA fragments were inserted into CMV-driven expression vector pcDNA1/Amp (Invitrogen).

A set of N- and C-terminal deletions in the SOX2-coding sequence was produced by treatments with exonuclease III/mung bean nuclease of the *Sox2* cDNA and by ligation of the *EcoRI* linker to the deletion termini. In the case of C-terminal deletions, the *EcoRI* site was followed by a linker carrying stop codons in all three frames. In the SOX2 $\Delta$ N35 deletion, a Kozak sequence (Kozak, 1989) and a methionine codon were placed to the 5' of the *EcoRI* site. The deletions are indicated by the amino acid residues that were removed.

GAL4 fusion constructs were made by joining the *HindIII-EcoRI* fragment encoding the GAL4 DNA binding domain (DBD) (aa 1-147) of pSGVP (Sadowski et al., 1988) with the cDNA fragments encoding the C-terminal domains of SOX proteins in expression vector pCMV/SV2 (Y. Kamachi et al., unpublished).

The luciferase reporter plasmid with the octamerized DC5 sequence has been described previously (Kamachi and Kondoh, 1993). The reporter to assay GAL4 fusion proteins contained the tetramerized GAL4 binding site sequence in the upstream of the *δ1-crystallin* minimal promoter of p $\delta$ 51LucII (Kamachi and Kondoh, 1993).

### Northern analysis of chicken Sox gene expression

Total RNA was isolated using Ultraspec RNA reagent (Biotecx). 10  $\mu$ g of total RNA was electrophoresed in 1% agarose containing 2.2 M formaldehyde in 1 $\times$  MOPS buffer and transferred onto Hybond N+ membrane (Amersham) with 7.5 mM NaOH. Blots were hybridized in QuikHyb hybridization solution (Stratagene) according to the manufacturer's instructions and washed in 0.2 $\times$  SSPE, 0.1% SDS at 65°C. The <sup>32</sup>P-labeled probes were made using the DNA fragments devoid of the HMG box sequence: *Sox1*, a 513-bp *MboII-MboII* of the 3' non-coding region (position 1502-2015); *Sox2*, a 552 bp (position 462-1013, Kamachi et al., 1995); *Sox3*, a 707 bp *NdeI-SacII* (position 676-1387); *Sox5*, ca. 700 bp 3' of the *NsiI* site; *Sox9*, a 610-bp *PstI*; *Sox11*, a 650 bp 3' of the *PstI* site. Transcript size was determined using an RNA ladder (0.24-9.5 kb, Gibco-BRL).

### Transfection

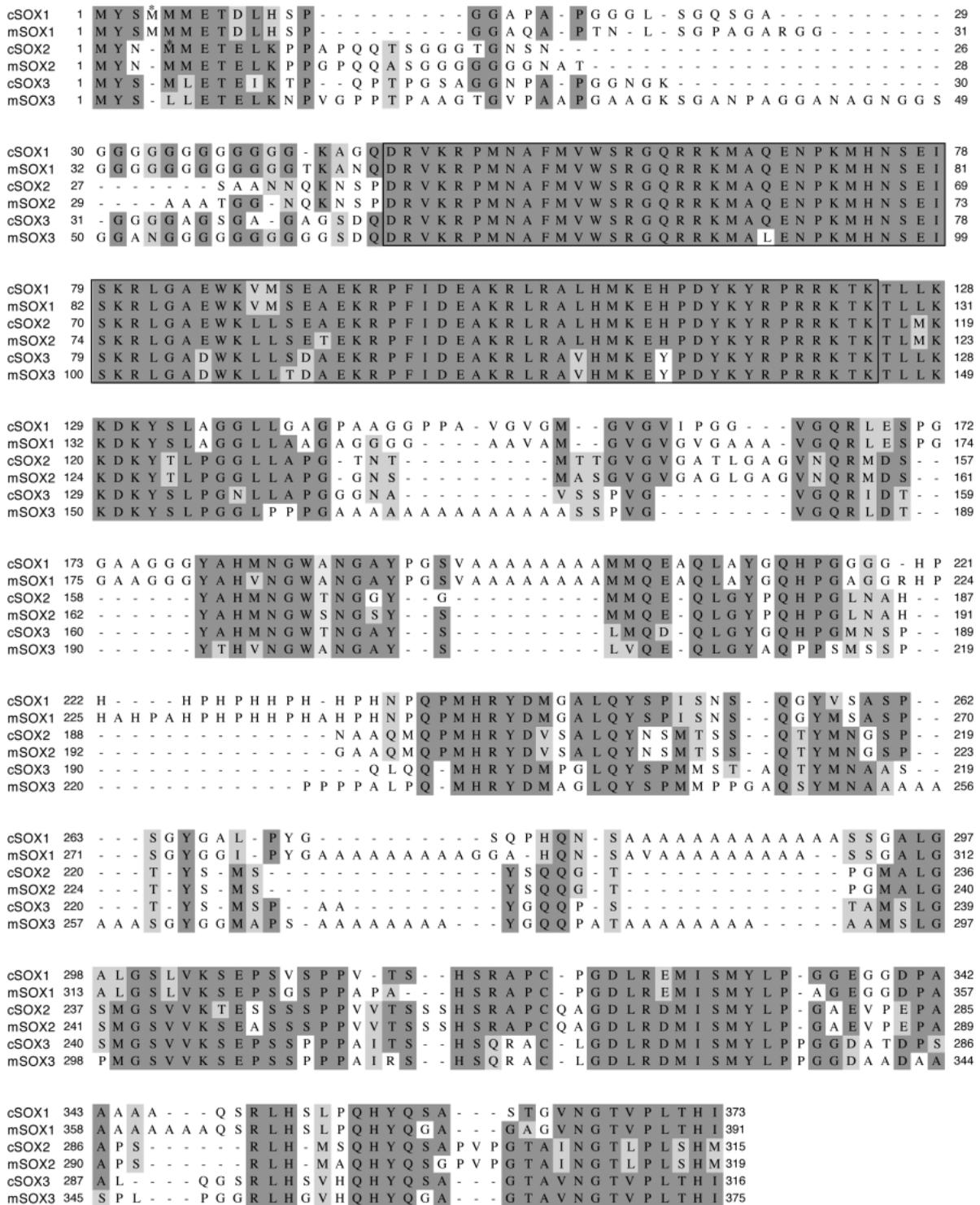
Chicken embryo lens cells or fibroblasts cultured in a 35 mm diameter dish were transfected with 1.5  $\mu$ g plasmid DNA containing 1.3  $\mu$ g of luciferase reporter, total 0.1  $\mu$ g of *Sox* expression vector/empty vector mixture and 0.1  $\mu$ g of  $\beta$ -galactosidase reference reporter pMiwZ (Suemori et al., 1990) or pSV- $\beta$ -galactosidase (Promega), as described by Kamachi and Kondoh (1993). Luciferase activity after 48 hours (Kamachi and Kondoh., 1993) was normalized with  $\beta$ -galactosidase activity, determined using 4-methylumbelliferyl- $\beta$ -D-galactopyranoside (Fiering et al., 1991). Transfections were carried out at least three times and values are averages plus standard deviations.

### In situ hybridization

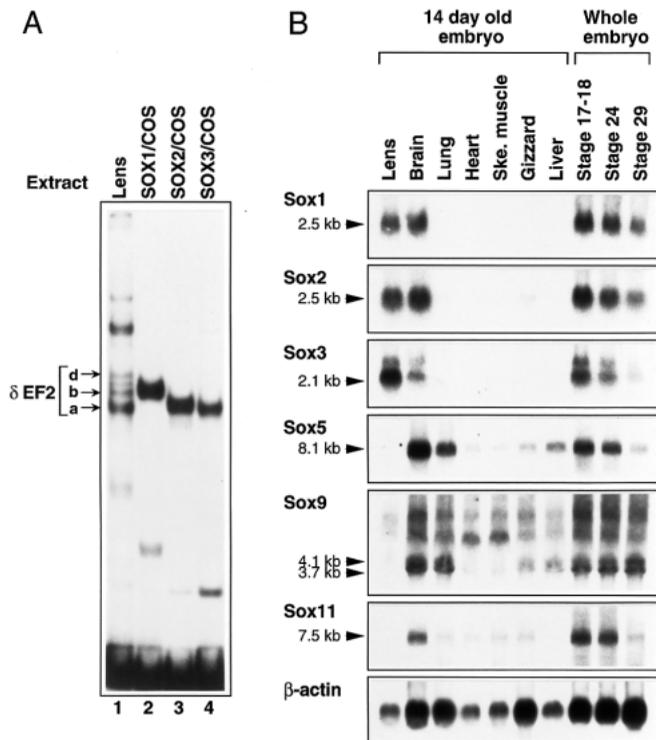
To detect *Sox*, *Pax6* and *δ1-crystallin* mRNAs, single-stranded, antisense RNA probes were produced by in vitro transcription of subcloned gene-specific fragments with T7 RNA polymerase (Promega) or T3 RNA polymerase (Pharmacia). Chicken *Sox* probes were digoxigenin-labeled transcripts of the same fragments as used

for northern blotting, *Pax6* probe, a 692 bp *PstI-ScaI* from cDNA (position 33-725; Li et al., 1994), and  $\delta$ -*crystallin* probe, a 738 bp cDNA of  $\delta$ 1-*crystallin* (position 300-1038; Yasuda et al., 1984). The procedure for whole-mount specimens was according to Rosen and

Beddington (1993) and that for sectioned specimens was according to Uwanogho et al. (1995). Hybridization signals were produced by treatment with alkaline phosphatase-conjugated anti-digoxigenin and by reaction with NBT-BCIP or BM-Purple (Boehringer-Mannheim).



**Fig. 1.** Alignment of predicted amino acid sequences of chicken (c) SOX1, SOX2 and SOX3, and their comparison with mouse (m) SOX proteins. The DNA-binding HMG domains are boxed. Identical amino acid residues have dark shading while similar residues are lightly shaded. Because the first ATGs of SOX1 and SOX2 have only a poor fit to the Kozak consensus sequence (Kozak, 1989), the second ATG codons with better match to the Kozak sequence (asterisks) may be used as the functional initiator codons. DDBJ/EMBL/GSDB/NCBI data base accession numbers: *Sox1*, AB011802; *Sox2*, D50603 (Kamachi et al., 1995); *Sox3*, AB011803.



**Fig. 2.** *Sox1-3* expressed in the lens encode the  $\delta$ EF2 proteins. (A) Gel mobility-shift analysis of SOX1, SOX2 and SOX3. The  $\delta$ EF2 proteins in a lens nuclear extract are compared with individual SOX proteins expressed in COS-7 cells by a gel mobility-shift assay using the  $\delta$ 1-crystallin DC12 probe, a subfragment of DC5 (Kamachi and Kondoh, 1993).  $\delta$ EF2a, b and d are indicated on the left. Other bands are not sensitive to competition by the unlabeled wild-type DC5 DNA (Kamachi et al., 1995). (B) Northern analysis of *Sox* expression in tissues of 14-day chicken embryos and in whole embryos at early stages. The sizes of the major transcripts are indicated on the left. A filter was re-hybridized with a  $\beta$ -actin probe.

Mouse *Sox* probes were  $^{35}$ S-UTP labeled transcripts of the following: *Sox1*, a 340 bp *StuI-XhoI* from the *Sox1* cDNA (position 1694-2046); *Sox2*, a 440 bp *EcoRI-ApaI* from the *Sox2* cDNA (position 1-460), as described in Collignon et al. (1996). The procedure of hybridization was as described (Wilkinson and Green, 1990). After hybridization the slides were subjected twice to a high-stringency wash in 50% formamide,  $2\times$  SSC, 10 mM DTT at 65°C for 30 minutes. Typically, slides were exposed for 6 days.

## Gel mobility-shift assays

Gel mobility-shift assays of nuclear extracts were done as described (Kamachi and Kondoh, 1993; Kamachi et al., 1995).

## Chicken embryos

Chicken embryos were staged based on somite numbers according to Hamburger and Hamilton (1951). Embryo operations and culture were done according to Li et al. (1994), except that the supporting medium was RPMI1640 in 0.3% Agar Noble (Difco) and embryos were incubated in humidified atmosphere containing 5% CO<sub>2</sub>.

## RESULTS

### Chicken *Sox1*, *Sox2* and *Sox3* encode $\delta$ EF2 subspecies

We have previously shown that an essential element of the DC5 enhancer is bound by a group of nuclear factors of the lens,  $\delta$ EF2a, b and d (Kamachi and Kondoh, 1993), and that SOX2 protein corresponds to  $\delta$ EF2a (Kamachi et al., 1995). It seemed possible that other  $\delta$ EF2 subspecies were also SOX proteins, since they shared the same DNA binding specificity. We then extensively screened lens cDNA libraries for *Sox* cDNAs, and successfully cloned cDNAs of only *Sox1*, *Sox2* and *Sox3*, suggesting that these three are the major *Sox* genes expressed in the lens. The *Sox1* cDNA sequence is 2312 bp long and contains a single open reading frame encoding a predicted protein of 370 or 373 aa, depending on choice of the initiator codon (Fig. 1, see figure legend). The overlapping *Sox3* clones reconstitute a 1824 bp cDNA that contains a single open reading frame encoding a predicted protein of 316 aa. The deduced amino acid sequences of chicken SOX1, SOX2 and SOX3 are aligned and compared with mouse SOX proteins in Fig. 1. These three proteins not only had the highly conserved HMG domain but also showed significant sequence similarity throughout the length of the polypeptide. The conserved amino acid sequence was interrupted into blocks by insertions of amino acid repeats. Poly(alanine) stretches present in mouse SOX1 and SOX3 (Collignon et al., 1996) are found only in SOX1 of the chicken. A PRD-type repeat (His-Pro) is present in SOX1 of both species (Fig. 1; Collignon et al., 1996).

We produced SOX1, SOX2 and SOX3 proteins of the chicken in COS-7 cells and compared them with  $\delta$ EF2 proteins present in lens nuclear extract by gel mobility-shift assays. SOX1 produced in COS-7 cells gave a probe-protein complex that migrated to the same position as  $\delta$ EF2b, while both SOX2 and SOX3 produced complexes showing the same mobility as

**Table 1.** Gene expression in the lateral head ectoderm in embryos with unilateral ablation of the retina primordium

Expression	Embryos analyzed	Ablation of an optic vesicle					
		Complete			Partial		
		Bilateral	Unilateral	Ambiguous	Bilateral	Unilateral	Ambiguous
<i>Sox2</i>	20	0	13	3*	3†	0	1*
<i>Sox3</i>	12	0	8	0	2†	2‡	0
<i>Pax6</i>	8	6	0	0	2	0	0

Total number of embryos analyzed, 40; those with complete ablation of an optic vesicle, 30; those with partial ablation, 10.

\*Because of deformation of embryos, it was hard to distinguish between ventral expression and possible occurrence of additional weak lateral expression on the operated side. However, in no case was the strong lateral expression characteristic of lens induction observed.

†The operated side with a small optic vesicle was weaker (e.g. Fig. 6D).

‡Optic stalks without bulging of the optic vesicle were formed and apposed to the ectoderm, but no *Sox3* expression occurred.

$\delta$ EF2a (Fig. 2A). Thus,  $\delta$ EF2a was a mixture of SOX2 and SOX3, and  $\delta$ EF2b corresponded to SOX1. The nature of the minor subspecies  $\delta$ EF2d, distributed widely among tissues (Kamachi and Kondoh, 1993), has not been clarified.

### Sox1, 2 and 3 are the major Sox genes expressed in the lens

We compared expression patterns of *Sox1*, 2 and 3 with those of *Sox5*, 9 and 11 by northern analysis of RNAs from various tissues of 14-day chicken embryos. All these *Sox* genes are expressed in the brain at this embryonic stage. Transcripts of *Sox1*, 2 and 3 were detected in the lens and brain. *Sox2* expression was also observed in the gizzard at a low level. In contrast, *Sox5*, 9 and 11 transcripts were barely detectable in the lens, confirming that *Sox1*, 2 and 3 are the major *Sox* genes expressed in the lens (Fig. 2B). When whole embryo RNAs of earlier developmental stages (17-18, 24 and 29) were analyzed, all *Sox* genes listed here were expressed at high levels up to stage 24.

### $\delta$ 1-crystallin DC5 enhancer is activated by SOX1, SOX2 and SOX3

We have previously shown that overexpression of SOX2 increases the activity of the DC5 enhancer in lens cells, but this does not occur in fibroblasts (Kamachi et al., 1995). To assess transactivation by SOX1 and SOX3, we co-transfected lens cells and fibroblasts with the luciferase reporter gene carrying the DC5 enhancer and with various amounts of effector vectors expressing either SOX1, 2 or 3 (Fig. 3A). These exogenous SOX proteins augmented the DC5 enhancer activity in lens cells, the levels being highest with SOX1, followed by SOX2 and then SOX3 (Fig. 3B). In fibroblasts lacking  $\delta$ EF3, none of the three activated the DC5 enhancer, resulting in the basal level expression of the reporter gene. Mouse SOX1, 2 and 3 also activated the DC5 enhancer similar to its chicken counterparts (data not shown).

### Requirement of the C-terminal domain for activation of DC5

SOX1/2/3 are composed of N-terminal, HMG and C-terminal domains. We explored which parts of the proteins are involved in activation of the DC5 enhancer, taking SOX2 protein as an example.

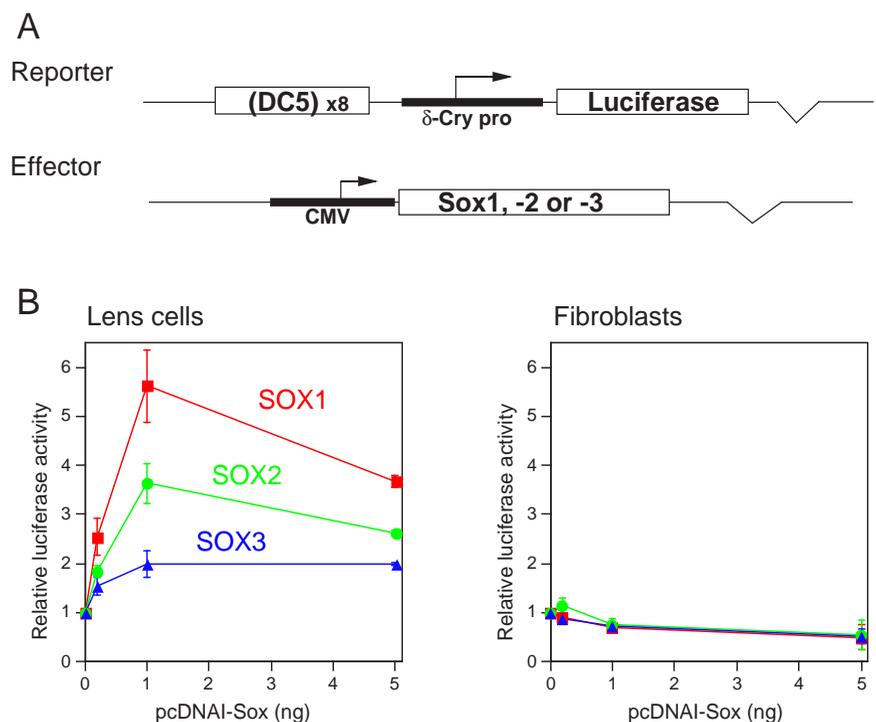
A series of progressive C-terminal truncations was introduced into SOX2 and activation of DC5 by these mutant forms was examined. As shown in Fig. 4Aa, removal of 41 amino acids from the C terminus ( $\Delta$ C275) greatly reduced the activation of the DC5 enhancer. The residual activity was lost by extension of the truncation to position 187. Thus, the C-terminal domain is essential for activation of the DC5 enhancer. Requirement of a domain proximal to the C terminus was also reported for mouse SOX2 in activation of the *Fgf4* enhancer (Yuan et al., 1995).

In contrast, the N-terminal domain was dispensable for activation of DC5. Deletion of most of the N-terminal domain slightly increased activation (Fig. 4Ab), suggesting an inhibitory effect of this domain.

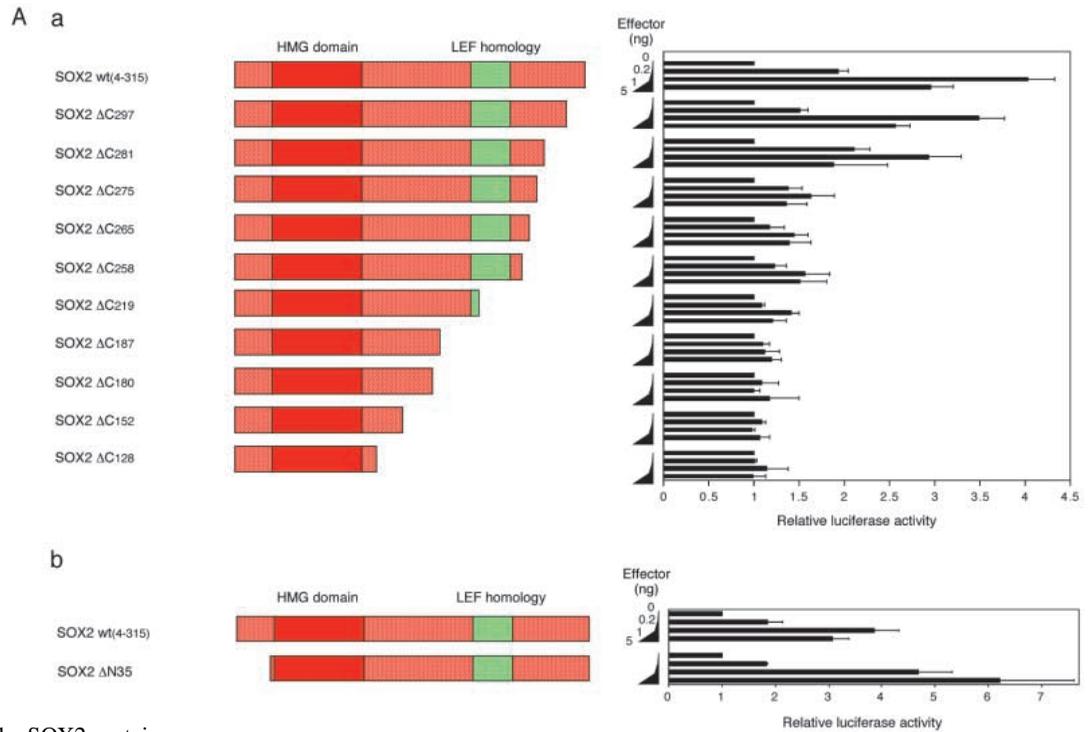
These truncated mutant proteins were expressed in COS-7 cells and their DNA binding activity was examined by gel mobility-shift assay (Fig. 4B). The mutant proteins bound to the DC12 probe, a subfragment of DC5, comparably to wild-type (wt) SOX2. The band intensity had no correlation with the activation potential shown in Fig. 4A.

The intrinsic transactivation potential of the C-terminal domain of SOX2 was examined using the fusion protein with the DNA-binding domain of GAL4 (GAL4DBD). The expression vector for the fusion protein and the luciferase reporter plasmid, carrying GAL4-binding sites, were transfected into lens cells and fibroblasts. In lens cells, while GAL4DBD alone had no effect on luciferase expression, GAL4-SOX2 fusion protein stimulated the expression 40-fold (Fig. 4C). Essentially the same results were obtained in the fibroblasts (data not shown). These indicate that the C-terminal domain of SOX2 carries the activity of transactivation, and when tethered to GAL4DBD this activity becomes expressed independent of the partner factor  $\delta$ EF3.

To delineate the activation domain of SOX2 further, several portions of the C-terminal domain were fused to the GAL4DBD (Fig. 4C). The subdomains, 116-280 and 176-315, activated transcription to levels comparable to that of the full



**Fig. 3.** Activation of the DC5 enhancer by exogenous SOX1, 2 and 3. (A) Structure of reporter and effector plasmids. In the reporter the octamerized DC5 fragment is placed upstream of the  *$\delta$ 1-crystallin* minimal promoter (–51 to +57;  $\delta$ -Cry pro) plus the luciferase coding sequence. The effector plasmids had the relevant insert corresponding to a chicken *Sox* cDNA to be transcribed from the CMV promoter. (B) Varying amounts (0, 0.2, 1 and 5 ng) of effector plasmids to express one of the SOX proteins were transfected into lens cells and fibroblasts simultaneously with the reporter plasmid. The amount of luciferase activity generated by the reporter in the absence of exogenous SOX was taken as 1.



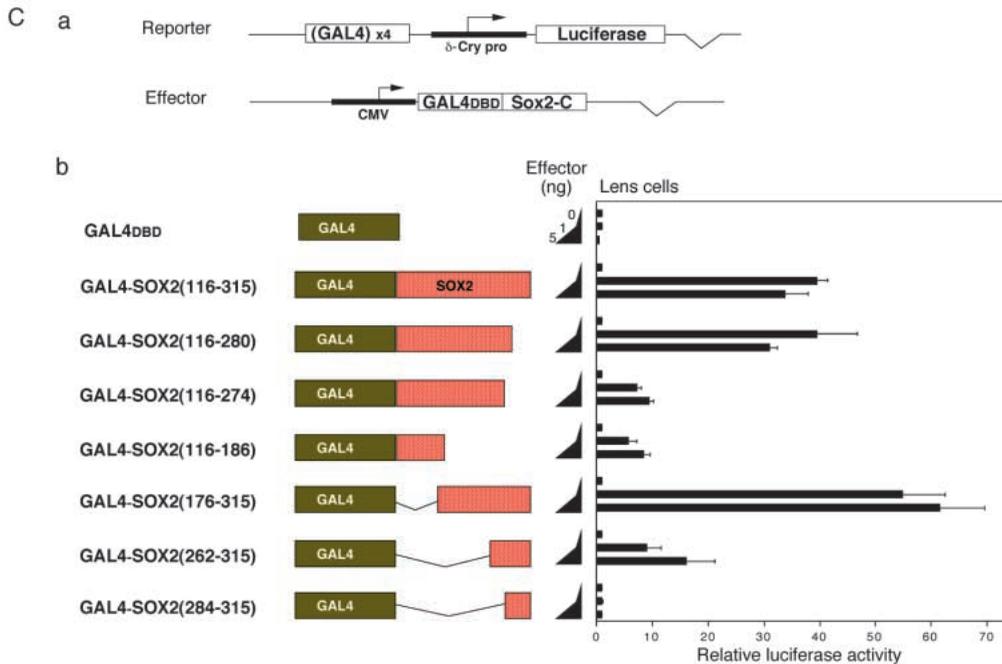
**Fig. 4.** Mutational analysis of the SOX2 protein. (A) SOX2 protein and its C-terminal (a) and N-terminal (b) deletion forms are schematically shown on the left. The LEF homology region has been described in Kamachi et al. (1995). Lens cells were transfected with various amounts of pcDNAI/Amp-based SOX2 expression vectors (0, 0.2, 1 and 5 ng), producing either wt SOX2 or the mutant proteins, together with the luciferase reporter plasmid containing the octamerized DC5 fragment (Fig. 3). (B) Expression and DNA binding activity of the SOX2 mutant proteins with the C-terminal (a) and N-terminal (b) deletions. Nuclear extracts of transfected COS-7 cells expressing SOX2 mutant proteins were analyzed by gel mobility-shift assay using DC12 probe. (C) Activation potential of the C-terminal domain of the SOX2 protein. (a) The luciferase reporter plasmid had a tetramerized GAL4 binding sequence that is placed upstream of the *δ1-crystallin* minimal promoter (–51 to +57;  $\delta$ -Cry pro). Various C-terminal regions of SOX2 protein (SOX2-C) were fused in-frame to GAL4DBD in the expression vector. (b) Varying amounts of expression vector plasmids (0, 1 and 5 ng) for the GAL4-SOX2 fusion proteins were transfected into the lens cells together with the luciferase reporter plasmid. The amount of luciferase activity generated by the reporter in the absence of GAL4 fusions was taken as 1.

C-terminal domain, suggesting that the region 176–280 is important for transactivation. Removal of six amino acids from the C terminus of the subdomain 116–280 greatly reduced transactivation (Fig. 4C, compare 116–280 with 116–274), consistent with the result in Fig. 4A (much lower activity of  $\Delta$ C275 compared to  $\Delta$ C281). Small subdomains 116–186 and 262–315 showed a low activity of transactivation. Taken together with the data in Fig. 4A, it is concluded that the distal portion of the C-terminal domain of SOX2 carries the potential of transactivation. This activation domain is composed of multiple subdomains with a modest activating potential, among

which the subdomain including the amino acid residues 275–280 appears to have the greatest effect on the overall transactivation. Contribution of the LEF homology region (Kamachi et al., 1995) to transactivation seems small in the assay.

#### Activation of *Sox2* and *Sox3* expression at the initial step of lens induction

We examined how the genes for *Sox1* to *Sox3* are regulated during lens induction and in subsequent lens development. *Sox* expression in the head region was examined by comparison



with *Pax6* and  $\delta$ -*crystallin* expression. The whole-mount specimens of in situ hybridization and their cross sections are shown in Fig. 5A,B.

*Pax6* expression in the prospective head ectoderm was already recognizable at the head fold stage (late stage 5, data not shown; Li et al., 1994), and at stage 11 covered a large area of the lateral head ectoderm (Fig. 5A). *Pax6* expression continued in the head ectoderm and its derivatives after the onset of lens differentiation (Fig. 5B).

Expression of *Sox3* appeared to respond to the optic vesicle apposition. At stage 11 or before, there was no expression of *Sox3* in the head ectoderm. However, at stage 12, only 4 hours after stage 11, expression of *Sox3* was turned on in the ectoderm just in the region apposed by the optic vesicle (Fig. 5A,B). This region develops into the lens placode (stage 13) and into lens vesicle. In the case of *Sox2*, strong expression in the ventral surface of the head occurred from earlier stages, and this extended to the lateral ectoderm and then decayed off toward the dorsal side (Fig. 5B, stage 11). Analogous to *Sox3* expression, strong *Sox2* expression commenced at stage 12 in the lateral region facing the optic vesicle and is maintained in the forming lens placode (Fig. 5A, stage 12,13), while in other regions of the lateral head ectoderm *Sox2* expression gradually became extinguished (Fig. 5A, stage 13). At stage 13 when the lens placode was formed, the first  $\delta$ -*crystallin* expression was detected. Expression of *Sox1* in the lens-forming ectoderm began at stage 14 (data not shown). After this, the lens placode invaginates to form the lens pit and then gives rise to the lens vesicle, and the cells in the internal compartment of the vesicle elongate to become the lens fibers. Throughout these and subsequent stages of lens development, expression of *Sox1*, 2 and 3 continued (Fig. 5B,C). In stage 24 (4 day) lenses, there was an interesting difference of expression pattern between the *Sox* genes and *Pax6* in the lens. While *Sox* expression appeared stronger in the equatorial region and in the fibers than in the lens epithelium, *Pax6* expression was stronger in the epithelium and the equatorial region (Fig. 5C).

In the retina the expressions of *Sox1-3* and *Pax6* were also slightly different from each other (Fig. 5C). At stage 15, those *Sox* genes analyzed were expressed in the optic stalk, but were almost absent in the prospective pigment layer. *Sox2* was expressed rather uniformly in the neural retina compartment, but expression of *Sox1* and *Sox3* was in a gradient which was decreasing toward the dorsal end. *Sox3* expression in the neural retina was relatively low. Expression of *Pax6* was low in the optic stalk, strong in the internal layer to become the pigmented retina, and in the neural retina it showed an increasing gradient toward the dorsal end (Fig. 5C).

### Induction of *Sox2* and *Sox3* by the retinal primordium

The chronological and spatial relationships between retinal apposition and the expression of *Sox2/3* in the head ectoderm strongly suggested that the activation of these *Sox* genes is induced by an effect of the optic vesicle. To test this, we adopted the technique of unilateral ablation of the retina primordium, described by Li et al. (1994). If activation of *Sox2/3* expression in the prospective lens areas is indeed induced by the apposed optic vesicle, then its removal will result in the loss of *Sox2/3* activation (Fig. 6A). One side of the presumptive retina areas in the head fold was removed at stage 7 (23 hours after incubation), and the operated embryos were cultured to allow further development. A total of 40 such operated embryos were analyzed (Table 1). The embryos with successful ablation of an optic vesicle did not show any sign of activation of *Sox2* or *Sox3*, nor the placodal structure in the ectoderm of the operated side, while development of the eye tissues in the unoperated side proceeded normally (Fig. 6B,C; Table 1). In some of the operated embryos small optic vesicles were still formed, and in these cases *Sox2/3* expression occurred in a small area of the head ectoderm exactly opposite the optic vesicle (Fig. 6D). However, apposition of remnant optic stalks did not induce *Sox2/3* expression (Table 1). These observations indicated that induction of *Sox2/3* activation is

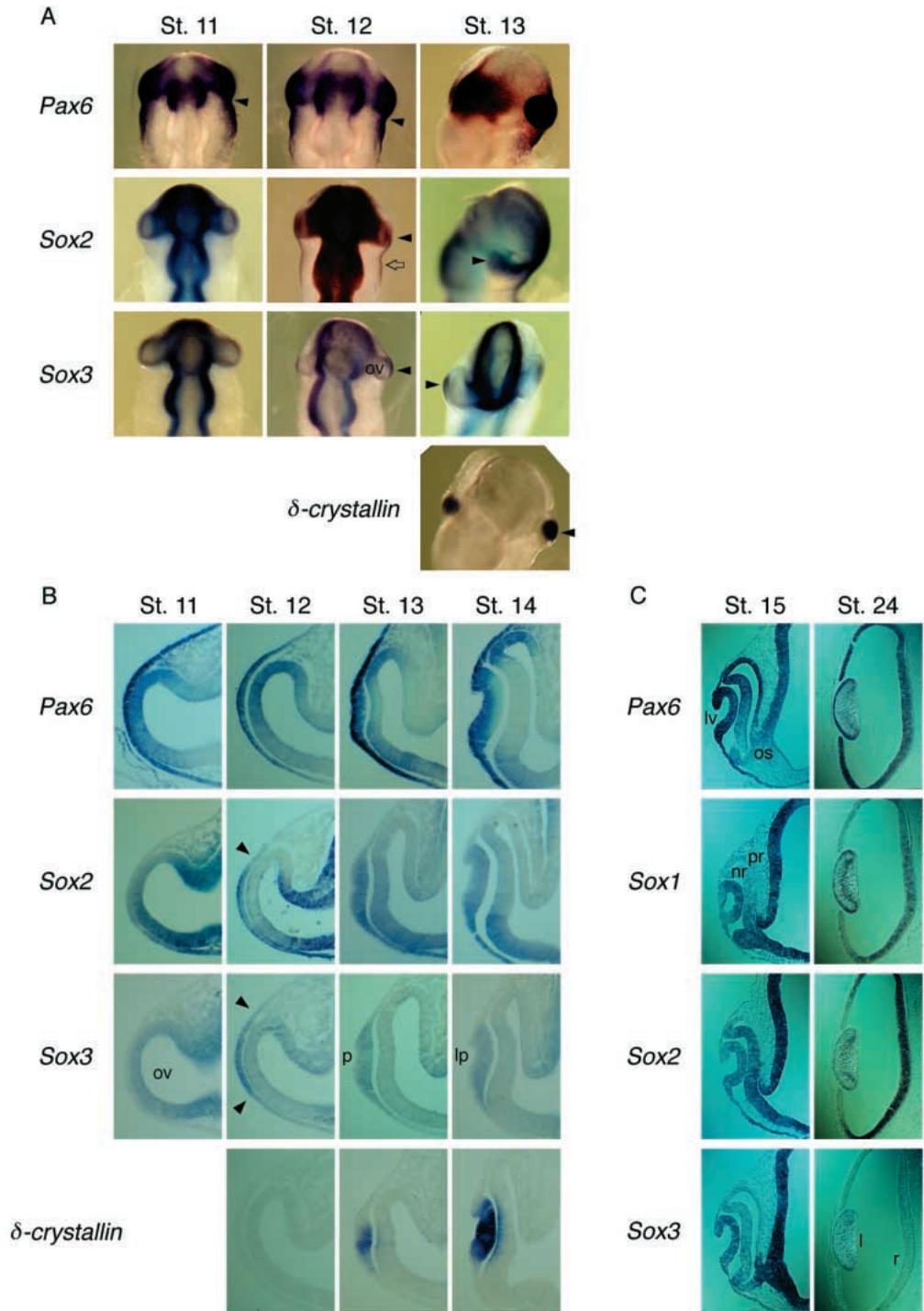
dependent on the effect of apposing optic vesicle. In contrast, *Pax6* expression was not significantly affected by optic vesicle removal (Table 1), confirming the report of Li et al. (1994).

**Sox expression in mouse lens development**

We compared mouse *Sox* expression in the early lens development with that of the chicken. Sections of mouse embryos were hybridized with radioactive *Sox* probes. Similar to the chicken embryo, mouse embryos just before the onset of lens differentiation (8.5-9.5 dpc; Pei and Rhodin, 1970) expressed *Sox2* in the ventral head ectoderm and this

expression extended to the lateral side, the dorsal limit roughly corresponding to the prospective lens region (Fig. 7A). *Sox1* was not expressed in the surface ectoderm at these stages. At 10.5 dpc, when the lens placode was indented and formed the lens pit (Fig. 7B,C), high *Sox2* expression was observed in the portion of the ectoderm that was in contact with the optic cup and was invaginated to form the lens vesicle. This invagination coincided with the onset of expression of *Sox1* in the lens placode. At 11.5 dpc, when the lens was composed of an external epithelial layer and an internal mass of elongated fiber cells, *Sox1* was expressed in both layers, whereas *Sox2* was

**Fig. 5.** *Sox1-3* expression in comparison with *Pax6* and  $\delta$ -*crystallin* during early lens development of the chicken. (A) Whole-mount in situ hybridization of *Pax6*, *Sox2*, *Sox3* and  $\delta$ -*crystallin* at stages 11-13. Expression of *Pax6* and *Sox* was observed in both the ectoderm and the CNS. The ectodermal signals are indicated by the arrowheads. At stage 11, *Pax6* expression covered a wide area of the lateral head ectoderm, but *Sox* expression in the area was low (*Sox2*) or entirely lacking (*Sox3*). At stage 12, strong expression of *Sox2* and *Sox3* was activated in the region apposed by the optic vesicle (ov). The *Sox2* signal of the surface ectoderm caudal to the eye region (open arrow) represented expression continuous from the ventral surface, and was distinct from the expression in the lens-forming area. At stage 13, lens placode was formed and  $\delta$ -*crystallin* expression began in the placode. High *Sox2* expression was confined to the surface area apposed by the retina rudiment (arrowheads). (B) Sections of stage 11-14 embryos hybridized in whole mount. ov, optic vesicle; p, lens placode; lp, lens pit. This series of specimens demonstrated that the dorsal border of *Sox2* expression and both borders of *Sox3* expression (arrowheads) are correlated with the apposition of the retina rudiment and delimit the region to follow the lens fate. (C) In situ hybridization of sections of embryos at stages 15 and 24 for expression of *Sox1-3*, *Pax6* and  $\delta$ -*crystallin*. lv, lens vesicle; os, optic stalk; nr, presumptive neural retina; pr, presumptive pigmented retina; l, lens; r, retina.



down-regulated and only weakly expressed (Fig. 7D). This pattern of *Sox1/2* expression was not changed at 13.5 dpc (Fig. 7E). Thus the order of expression, *Sox2* first followed by *Sox1*, is the same in the mouse and chicken lenses during development. Significant differences, however, were the sharp transition from *Sox2* to *Sox1* (Fig. 7) and the absence of *Sox3* expression in the mouse lens (Collignon et al., 1996). On the other hand, *Pax6* expression continued in the derivatives of the lateral head ectoderm during lens development similar to the chicken embryo (Fig. 5) (data not shown; Walther and Gruss, 1991; Grindley et al., 1995).

### Ectopic $\delta$ -crystallin expression correlates with the presence of *Sox2/3* and *Pax6* expression

The above observations indicate that lens cells differentiate from specific areas of the ectoderm where expression of *Sox2/3* overlaps with that of *Pax6*.  $\delta$ -Crystallin is known to be expressed in a few embryonic tissues other than the lens, although at a much lower level, notably in Rathke's pouch (Barabanov, 1982; Ueda and Okada, 1986) and ventromedial bands in the spinal cord (Takahashi et al., 1988). We investigated expression of *Sox* and *Pax6* genes in these ectopic sites of  $\delta$ -crystallin expression. In the oral ectoderm from which Rathke's pouch invaginates, there was a strong and widespread expression of *Sox2* (Figs 7B, 8A). There was also expression of *Sox3* and *Pax6* which was confined to the pouch (Fig. 8A and data not shown). The domain of the oral ectoderm with overlapping expression of *Pax6*, *Sox2* and *Sox3* also expressed  $\delta 1$ -crystallin (Fig. 8A). In the spinal cord, *Sox2/3* are expressed throughout the ventricular zone (Fig. 8B; Uwanogho et al., 1995; Streit et al., 1997; Rex et al., 1997) and *Pax6* is strongly expressed in the medial portion of the zone (Fig. 8B; Li et al., 1994). Ectopic  $\delta 1$ -crystallin expression is seen in roughly the ventral two-thirds of the medial zone showing *Sox2/3* and *Pax6* expression (Fig. 8B; Takahashi et al., 1988). Thus, these  $\delta$ -crystallin-expressing non-lens tissues co-express *Sox2/3* and *Pax6*.

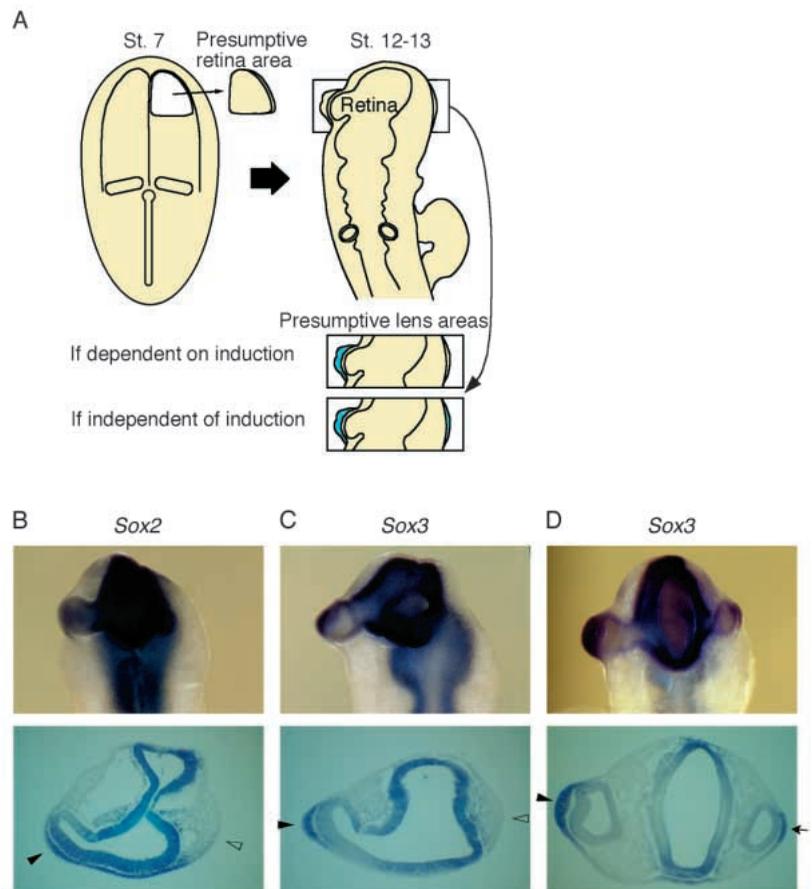
## DISCUSSION

### SOX1, SOX2 and SOX3 are the major SOX proteins in the lens and account for the $\delta$ EF2 activity

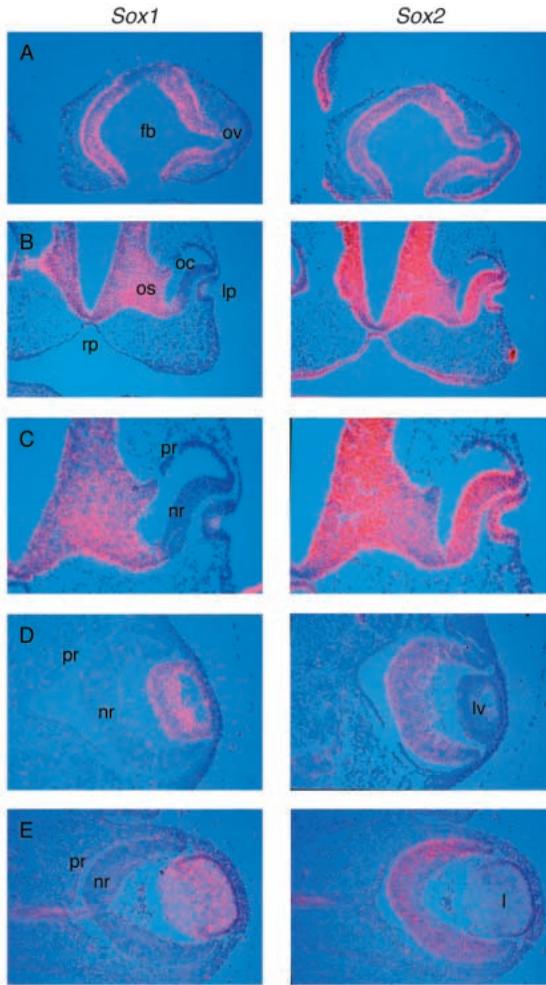
In this report, we show that *Sox1*, 2 and 3 are a unique group of *Sox* genes highly expressed in the lens of the chicken embryo (Figs 2, 5). In addition to the lens, they are broadly co-expressed in the central nervous system (Figs 5C, 8B; Uwanogho et al., 1995; Streit et al., 1997; Rex et al., 1997; M. Uchikawa, unpublished result). Their encoded proteins, SOX1, 2 and 3 form a distinct SOX subfamily. Their HMG domains are more than 92% identical in amino acid sequence to one another, and a significant degree of similarity exists throughout the lengths of the amino acid sequences

(Fig. 1). These proteins alone account for the  $\delta$ EF2 activity of the lens cells, i.e. they activate  $\delta 1$ -crystallin DC5 enhancer in lens cells in situations where the partner factor  $\delta$ EF3 is available at a nearby binding site.

Other SOX proteins (e.g. SOX9, 11) failed to activate the DC5 enhancer (Kamachi et al., unpublished). The specificity of SOX1, 2 and 3 in activation of DC5 is primarily ascribed to their ability to interact with  $\delta$ EF3 (Kamachi et al., unpublished result). When this interaction is achieved, SOX1/2/3 activate the DC5 enhancer, owing to their intrinsic transactivation potential associated with the distal portion of the C-terminal domain. The transactivation potential was demonstrated when this portion of the C-terminal domain of SOX2 was fused to GAL4DBD (Fig. 4C). Deletion analysis indicated that multiple



**Fig. 6.** *Sox2/3* expression is activated in lens-forming head ectoderm in response to induction by the retina primordium. (A) The scheme of unilateral removal of the prospective retina region of the head fold in the chicken embryo, and interpretation of the results. (B-D) Representative embryo samples at stage 13 with unilateral ablation of the retina rudiment. Ventral view of whole-mount specimens (top) and their cross sections (bottom). (B) An embryo with complete removal of an optic cup and hybridized with *Sox2* probe. *Sox2* was not activated in the operated side of the lateral head ectoderm above the basal level (open arrowhead). In this embryo the optic cup of the unoperated side was displaced slightly toward the ventral direction, and the activation of *Sox2* (arrowhead) and placode development were also shifted ventrally (compare with Fig. 5). (C) Similar embryo but with normal positioning of the unoperated optic vesicle and hybridized with *Sox3* probe. Ectodermal expression of *Sox3* occurred only on the optic cup-apposed side. (D) An analogous embryo but with development of a tiny optic vesicle on the operated side where *Sox3* was expressed in a corresponding small region of the ectoderm (small arrow).



**Fig. 7.** *Sox1* and *Sox2* expression during early eye development of the mouse. (A,B) Frontal sections through the head of 9.5 and 10.5 dpc embryos, respectively. (C) Higher magnification of B. In the lateral head ectoderm *Sox2* expression becomes restricted to and augmented in the area overlying the optic vesicle and forming the lens placode. As the lens invaginates, *Sox1* begins to be expressed. (D,E) Transverse sections of 11.5 and 13.5 dpc embryos, respectively. As *Sox1* expression progressively increases, *Sox2* is down-regulated. fb, forebrain; ov, optic vesicle; os, optic stalk; oc, optic cup; lp, lens pit; pr, presumptive pigmented retina; nr, presumptive neural retina; lv, lens vesicle; l, lens; rp, Rathke's pouch.

subdomains of the C-terminal domain contribute to the transactivation potential (Fig. 4A,C), among which amino acids 275-280 seem to have a relatively large effect. The C-terminal domains of SOX1 and SOX3 also had analogous activities (Y. Kamachi et al., unpublished; L. Pevny and R. Lovell-Badge, unpublished data).

When chicken SOX1, -2 and -3 are compared with their mouse counterparts (Collignon et al., 1996), there is extremely high sequence conservation between the orthologues except for the number of insertions of poly(alanine) repeats: chicken SOX3 is devoid of the repeats, while mouse SOX3 contains four repeats; chicken SOX1 has three repeats, while mouse SOX1 has four (Fig. 1). Activation of the DC5 enhancer by mouse SOX1, 2 and 3 was indistinguishable

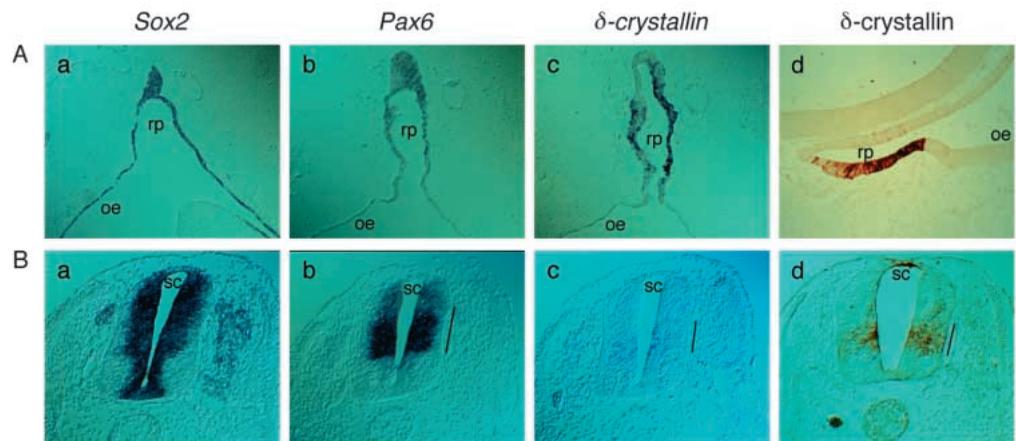
from the respective chicken counterparts. The order of *Sox* expression in lens development, *Sox2* followed by *Sox1*, was conserved between the chicken and mouse, but *Sox3* is not expressed in the mouse lens (Collignon et al., 1996). This may suggest that *Sox2* and *Sox3* are functionally redundant in the chicken lens.

### Induction of *Sox2/3* activation by the retinal primordium

In the development of the chicken eye, it was found that strong expression of *Sox2* and *Sox3* is induced in the *Pax6*-positive ectoderm by apposition of the optic vesicle. Activation of *Sox2/3* probably determines the future lens fate (Fig. 5).

It was demonstrated that the induction of *Sox* expression in the head ectoderm requires apposition by the retina primordium. When the retina primordium on one side of an embryo was surgically removed at stage 7, it resulted in failure of the optic vesicle to develop, in the loss of *Sox* expression in the head ectoderm, and ultimately in the absence of lens formation on the operated side (Fig. 6). Partially ablated optic cup induced weaker *Sox2/3* expression in smaller areas but remnant optic stalk failed to do so (Fig. 6D, Table 1). These observations imply that an inductive signal emanating from the retina primordium activates expression of *Sox2* and *Sox3* in the head ectoderm.

Once SOX2 and SOX3 are synthesized, they then activate  $\delta$ -crystallin expression and possibly other target genes which



**Fig. 8.** Ectopic  $\delta$ -crystallin expression is correlated with overlapping expression of *Pax6* and *Sox2*. (A) Oral ectoderm (oe) and Rathke's pouch (rp) of a 4-day chicken embryo in cross section was analyzed by in situ hybridization for expression of *Sox2* (a), *Pax6* (b) and  $\delta$ -crystallin (c). A sagittal section was immunohistologically stained for  $\delta$ -crystallin (d). (B) Spinal cord (sc) of a 3-day chicken embryo in cross section was analyzed by in situ hybridization for expression of *Sox2* (a), *Pax6* (b) and  $\delta$ -crystallin (c), and by immunostaining for  $\delta$ -crystallin (d). The region of high *Pax6* expression in (b) and  $\delta$ -crystallin-positive regions in c and d are indicated by bars.

may include those for cytoskeletal components in the placodal cells.

*Sox1* expression is initiated later in both the chicken and mouse lenses, and becomes the major *Sox* gene expressed in the lens. SOX1 shows the highest ability to activate the DC5 enhancer. SOX1 expression may be correlated with the synthesis of new cytoskeletal components, which are involved in the transition of cellular architecture, from the epithelium to the fiber cells. In the mouse, *Sox1* is clearly shown to be essential for maintenance of  $\gamma$ -crystallin gene expression and for later lens development (Nishiguchi et al., 1998).

### Co-expression of *Pax6* and *Sox2/3* are required for lens differentiation

At the beginning of normal lens development, a *Sox2/3*-expression domain is formed within the wider domain of *Pax6*-expression in head ectoderm and defines the origin of lens cells. This observation supports the model that co-expression of *Pax6* and *Sox2/3* provides appropriate conditions for lens cell differentiation. There are a few tissues besides lens which ectopically express low levels of  $\delta$ -crystallin, Rathke's pouch (Fig. 8A; Barabanov, 1982; Ueda and Okada, 1986) and the ventromedial bands of the ventricular zone of the spinal cord (Fig. 8B; Takahashi et al., 1988). It was demonstrated that these  $\delta$ -crystallin-expressing cells coincide with those that locally express *Pax6* and *Sox2/3* together. Of course, not all the tissues of *Pax6-Sox2/3* co-expression showed  $\delta$ -crystallin synthesis, probably because SOX2/3 requires the partner factor  $\delta$ EF3 to activate the  $\delta 1$ -crystallin enhancer, and because  $\delta$ EF3 activity is presumably limited in its tissue distribution.

It is important to note that the embryonic neural retina, which has been demonstrated to have the potential to transdifferentiate into lens (Okada, 1980), also expresses both *Pax6* and *Sox2/3* (Fig. 5C). Presumably because *Pax6* and *Sox2/3* are already expressed, the retina tissue is prepared to differentiate into lens if additional requirements are met in the culture conditions.

The discovery that *Sox2* and *Sox3* are involved in the initial steps of authentic lens induction, and the notion of possible involvement of the action of these genes in lens differentiation through non-authentic pathways, have opened up an avenue to full understanding of how lens tissue is induced to differentiate. In particular, determining the nature of the signal required for lens induction may now, at least partly, be reduced to finding the signal that induces *Sox2/3* expression in head ectoderm. *Sox2/3* and *Pax6* are not sufficient by themselves to elicit lens cell differentiation, as other transcription factors must be involved in the process. A strong candidate for such a factor participating in lens differentiation is  $\delta$ EF3, the partner of SOX proteins in activation of the  $\delta 1$ -crystallin DC5 enhancer. Thus, elucidation of the entire mechanism responsible for activation of the first lens-specific gene,  $\delta 1$ -crystallin, is crucial to a full understanding of lens induction.

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