Sox1 directly regulates the \( \gamma \)-crystallin genes and is essential for lens development in mice

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\( \gamma \)-Crystallins are major structural components of the lens fiber cells in amphibians and mammals. Many dominant inherited cataracts in humans and mice have been shown to map within the \( \gamma \)-crystallin gene cluster. Several transcription factors, including PAX6 and SOX proteins, have been suggested as candidates for crystallin gene regulation. Here we show that the targeted deletion of Sox1 in mice causes microphthalmia and cataract. Mutant lens fiber cells fail to elongate, probably as a result of an almost complete absence of \( \gamma \)-crystallins. It appears that the direct interaction of the SOX1 protein with a promoter element conserved in all \( \gamma \)-crystallin genes is responsible for their expression.

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The normal differentiation of the lens into a transparent structure involves changes in cell organization and shape as well as the synthesis of several classes of abundant lens-specific proteins, the crystallins. The anterior cells of the lens vesicle constitute a monolayer germinal epithelium. The posterior cells of the lens vesicle—those that are closer to the retina—stop dividing, elongate throughout most of the diameter of the lens, and fill the cavity of the lens vesicle.

Crystallins constitute 90% of the soluble protein in lens fiber cells (de Jong 1981) and they are divided into several classes. These include the conserved \( \alpha - \) and \( \beta - \)crystallins, which are found in all major vertebrate species, and those that are taxon specific (Wistow and Piatigorsky 1988), such as \( \gamma - \)crystallins, which are found in amphibians and mammals, and \( \delta - \)crystallin, which is found in birds and reptiles. Although there are many conserved and diverse crystallin genes, they are all expressed in the lens with spatial and temporal expression patterns characteristic for each class (McAvoy 1978; Wistow and Piatigorsky 1988). It has been postulated that the genes are controlled by common factors. Several transcription factors have been suggested as candidates for crystallin gene regulation, including PAX6 and SOX proteins (Cvekl and Piatigorsky 1996).

The mammalian Pax6 gene and its Drosophila homolog eyeless have been shown to play a critical role in vertebrate and invertebrate eye development. Misexpression of Pax6 or eyeless leads to the development of ectopic eyes in Drosophila (Halder et al. 1995). Mutations in Pax6 cause Aniridia in humans and Small eye syndrome in mice (Hill et al. 1991; Ton et al. 1991; Glaser et al. 1994). Recently, more genes involved in eye development have been identified and shown to be conserved in diverse species (Oliver and Gruss 1997).

The testis determining gene Sry is the founder member of the Sox family of genes, which encode transcription factors containing an HMG box type of DNA-binding domain (Gubbay et al. 1990; for review, see Pevny and Lovell-Badge 1997). The subfamily group B genes Sox1, Sox2, and Sox3 encode proteins with a considerable degree of overall sequence similarity, especially within their HMG box domains, which are 96% identical to each other (Collignon et al. 1996). They are expressed from early embryonic stages in a largely overlapping manner within the CNS, but in addition, each gene is expressed uniquely in some sites (Collignon 1992; Uwanogho et al. 1995; Collignon et al. 1996; Streit et al. 1997). In mice, Sox2 is expressed in all sensory placodes, including that which gives rise to the lens. However, Sox2 expression is down-regulated once the lens vesicle has formed and is replaced by Sox1 in this site (Collignon 1992). It has been shown that in chicken lens explants SOX protein binding is essential for lens-specific activity of the chicken \( \delta 1 - \)crystallin enhancer as well as the mouse \( \gamma F - \)crystallin promoter (Kamachi et al. 1995).

We deleted the Sox1 gene in mice by gene targeting and found that mice homozygous for the mutation have small eyes with opaque lenses. Although Sox1-deficient mice are viable, they suffer from spontaneous seizures; this aspect of the phenotype is not yet understood. We show that in the mutant eyes, the elongation of lens fiber cells is impaired and \( \gamma - \)crystallin gene expression is severely down-regulated. We also show that Sox1 protein binds to a promoter element that is conserved in all \( \gamma - \)and \( \delta - \)crystallin genes and that has been shown to be essential for expression in vitro. Sox1 is therefore a critical gene in mammalian lens development, at least partly through its direct action on \( \gamma - \)crystallin gene expression.

**Results and Discussion**

We used targeted mutagenesis in embryonic stem (ES) cells to delete the major part of the Sox1 gene that encodes for the HMG box and the entire carboxy-terminal domain (Fig. 1). Mice heterozygous for the Sox1 deletion did not exhibit any obvious abnormalities and were fertile. Mice homozygous for the mutation were born with
the expected Mendelian frequency in both an inbred 129/Sv and hybrid (mainly 129/Sv × MF1) background, indicating that Sox1 is not essential for embryonic life. However, homozygous mice are microphthalmic, suffer from spontaneous seizures, and show high mortality in both genetic backgrounds. The surviving homozygotes of both sexes rarely mate and are unfit to care for their offspring.

Consistent with the expression pattern of Sox1 during eye development, histological analysis of Sox1 mutant eyes at different stages revealed abnormal lens development (Fig. 2). Normally, the posterior cells of the lens vesicle, those that are closer to the retina, stop dividing, elongate throughout most of the diameter of the lens, and fill the cavity of the lens vesicle (Fig. 2A,C,E). In the mutant lens, induction and fiber cell differentiation occurred but the fiber cells fail to elongate all the way toward the anterior epithelial wall, leaving a cavity within the lens vesicle (Fig. 2B,D,F). The size of the mutant lens is reduced by ∼20%; at 15.5 days postcoitum (dpc) the diameter of the lens was 550 ± 20 μm (mean ± s.d.) in the wild-type (n = 4) and 430 ± 50 μm in the mutant (n = 4). This correlates with a similar reduction of cell number in the mutant lens. As Sox1, Sox2, and Sox3 are mainly expressed in the dividing neural epithelium in the CNS (Collignon et al. 1996), it is possible that they may be involved in mitotic activity. To explain the reduction in size and cell number of the mutant lens we performed BrdU incorporation assays at 12.5 and 15.5 dpc (Fig. 2G–K). We did not find any significant difference in the mitotic activity of cells in the anterior germinal epithelium between wild-type and mutant lenses; at 15.5 dpc the proliferative index, which is the ratio of proliferating cells (BrdU-positive nuclei) to the total number, was the same in wild-type (0.222 ± 0.023 in three embryos) and mutant (0.223 ± 0.016 in three embryos) anterior germinal epithelium. Surprisingly, we observed some (1%–2%) dividing fiber cells, as indicated by significant BrdU incorporation, in the 15.5 dpc mutant lens (Fig. 2J,K). We conclude that the absence of Sox1 is not important for proliferation in the anterior germinal epithelium. Although Sox1 may play a role in survival of lens cells, we cannot exclude the possibility that the difference in size and cell number between mutant and wild-type lenses is a secondary event (cell death or degeneration). Mice heterozygous for the Sox1 deletion do not show any lens defects or late onset cataracts.

The time when the lens defect first becomes apparent correlates with the commencement of crystallin gene expression seen in normal lens at 12.5 dpc (Goring et al. 1992). This coincides with the up-regulation of Sox1 expression and concomitant down-regulation of Sox2 (Fig. 3). We examined the expression of most crystallin genes in the eyes of the Sox1 mutant embryos using an RT-PCR assay (Fig. 4). We did not find any significant difference in expression of both α-class genes (αA and αB) and of the two genes from the β-class we have examined. However, this is not the case for the γ-cristallin gene cluster. This consists of six very homologous genes: γA, γB, γC, γD, γE, and γF. The time of activation of the cluster is ∼12.5 dpc in normal lenses (Goring et al. 1992) although we found that γC is activated a day earlier (data not shown) and γD later than 12.5 dpc. At 12.5 dpc, we found that γA and γB gene transcripts are undetectable while γF is present at a low level in the mutant lens. However, by 15.5 dpc, all the γ-crystallin genes are down-regulated, including γC and γE, that were unaffected at 12.5 dpc. At this stage, this down-regulation is not a secondary event due to the small size of the lens or lens-cell degeneration as the expression of the other lens-specific crystallin classes α- and β- in the mutant eye is unaffected. Within the limits for quantitation of the RT-PCR assay, we do not see a reduction in γ-cristallin gene expression in heterozygous mice. This is in contrast with other SOX mutations that show haploid insufficiency, such as in human SOX9, where homozygotes invariably show campomelic dysplasia (Foster et al. 1994).

In vitro experiments using chicken lens explants showed that SOX2 protein binding is required for lens-specific activity of the chicken α1-cristallin enhancer as well as the mouse γF-crystallin promoter (Kamachi et

Figure 1. Targeted disruption of the Sox1 gene. (A) The structure of the wild-type allele, targeting vector, and targeted allele are shown together with the restriction sites. The open box represents Sox1 coding sequence. The sizes of DNA fragments from the wild-type and mutated allele detected by the 5′ and 3′ probes are indicated. The positions of the PCR primers for genotyping are shown as arrows above the gene. (B) BamHI; (R) EcoRI. (B) Southern blot analysis of BamHI-digested DNA from ES cell clones using 5′ and 3′ probes. (Lanes 1,3) Wild-type ES cells; (lane 2) targeted ES cells. (C) PCR analysis of offspring derived from a mating of mice heterozygous for Sox1 deletion.
Figure 2. Histological analysis and cellular proliferation of wild-type and mutant lens. Hematoxylin and eosin staining of embryonic lens: (A,C,E) Wild-type lens, (B,D,F) mutant lens at 12.5 dpc (A,B), 15.5 dpc (C,D), and postnatal day zero (P0) (E,F). Loss of Sox1 causes impaired posterior lens fiber cell elongation and small, hollow lens. The nuclei in the mutant lens fiber cells are located closer to the retinal side at 12.5 dpc, whereas at P0, they are located closer to the cavity of the lens. BrdU incorporation assays: (G,I) Wild-type lens; (H,J,K) mutant lens at 12.5 dpc (G,H) and 15.5 dpc (I–K). Arrows indicate BrdU-positive nuclei in the lens fiber cells. Bar, 50 μm in A, B, G, and H; 100 μm in C and D; 140 μm in E and F. 120 μm in I and J; 50 μm in K.

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Sox1 expression persists in the developing mouse lens long after the Sox2 gene is silent (Fig. 3). Furthermore, there is evidence that mouse Sox1 activates the γF-crystallin promoter better than Sox2 in chicken lens explants (Y. Kamachi and H. Kondoh, pers. comm.). Sox2 binds to an element that is essential for activation of the γF-crystallin promoter in chicken lens explants (Kamachi et al. 1995). This element is conserved in all γ-crystallin promoters (Kamachi et al. 1995) and contains a consensus SOX binding site (Lok et al. 1989; Pevny and Lovell-Badge 1997). We therefore performed EMSAs using recombinant SOX1 protein to show that SOX1 binds specifically to this site (Fig. 5B). Incubation of SOX1 with the mouse γA-crystallin promoter element produced a protein–DNA complex. This binding is sequence-specific as it was competed by an excess of wild-type γA-crystallin oligonucleotide but not by a mutated one (γAM), in which the consensus SOX binding site sequence is disrupted (Fig. 5B). In addition, the specific signal is no longer detected in the EMSA when anti-SOX1 antiserum abFB43 or ab791 is added. We conclude from both our in vitro and in vivo data that γ-crystallin genes are direct targets for SOX1.

Our data show that SOX1 is essential for activation of some γ-crystallin genes and for maintaining expression of all of them. The two genes, γC and γE, that are expressed at 12.5 dpc in the Sox1 mutant lens may have been activated by SOX2. This is possible as SOX1 and SOX2 proteins have similar properties in vitro (Kamachi et al. 1995; Collignon et al. 1996). In addition, we find that SOX2 protein is present in the lens-pit nuclei at 10.5 dpc and in some nuclei of the lens fiber cells at 12.5 dpc (Fig. 3). However, by 15.5 dpc, we cannot detect SOX2 protein in the nuclei of the fiber cells (Fig. 3). Therefore, in the Sox1 mutant lens, all genes within the entire γ-crystallin gene cluster are turned off coincident with the loss of SOX2 protein within lens fiber nuclei. The notion that SOX1 and SOX2, and presumably SOX3 may be functionally redundant with respect to each other is also supported by the finding that they can bind to the same DNA sequences (Pevny and Lovell-Badge 1997), and it is consistent with our observation that loss of SOX1 does not have any obvious consequences during early development of the CNS. Another example of functional redundancy within the SOX gene family is provided by the Sox4 gene disruption in mice. This causes major abnormalities only in cardiac and pro-B-lymphocyte development although the gene is expressed in many other sites in the embryo (Schilham et al. 1996).

The delay of fiber cell elongation in the mutant coincides with the down-regulation of γ-crystallin genes. The γ-crystallin deficit is probably responsible for the incomplete elongation of the lens fibers. This could be explained by the theory that fiber cell elongation may be controlled osmotically by the protein concentration inside the cell (Parmelee and Beebe 1988). However, targeted disruption of the mouse αA-crystallin gene, which is also a major crystallin, does not affect fiber elongation and mice have normal but smaller lens (Brady et al. 1997). In this case, there may be an up-regulation of αB-crystallin gene expression that partially compensates for the loss of αA. Alternatively, crystallins may have additional nonrefractory functions, like architectural and intracellular functions important for the differentiation of the lens fiber cells (Piatigorsky and Wistow 1989; Brady et al. 1997). This theory has been supported by the discovery that α-crystallin functions as a chaperone in vitro (Horwitz 1992). Another example of the involvement of...
they function as architectural factors. Nevertheless, SOX proteins share several characteristics with classical transcription factors and putative transactivation domains have been mapped for several of them, including SOX1 (see Pevny and Lovell-Badge 1997 and references therein). Further analysis of the Sox1 mutant mice will allow us to understand the mechanism of its action and learn more about the functional interchangeability with other SOX proteins.

Finally, it is important to emphasize that the Sox1 mutant mice could be a useful tool in studying lens development and in investigating the role of γ-crystallins in lens development and in cataracts. The γ-crystallin gene cluster has been shown to be linked to the Coppel like cataract in humans (Lubsen et al. 1987) and the Cat-2', No, and Tol mutations in mice (Everett et al. 1994). We have cloned the human SOX1 gene and mapped it to chromosome 13q34 (Malas et al. 1997); this locus should now be examined for linkage with similar abnormalities in humans.

Materials and methods

Targeting vector and gene disruption

The replacement targeting vector 4PLV was designed to delete all of the coding region of Sox1 except for that encoding the first 56 amino acids. Smal and NotI sites of pgos1 (gift from P. Soriano, Fred Hutchinson Cancer Research Center, Seattle, WA) were converted to BglII and a 3.9-kb BglII-BglII fragment containing its own translation start site was cloned into a BglII-converted XhoI site of the Sox1 coding region. The 1.5-kb XhoI-XhoI fragment of this vector, which contains the Sox1 coding region, was replaced with a 1.1-kb XhoI-SaiI fragment from pMC1neo/polyA (Stratagene) in the forward direction. For 4PLV, the 8.0-kb KpnI-XbaI fragment of this vector, the 5.7-kb XbaI-XhoI fragment from pPNT (gift from V. Tybulewicz, MRC, NIMR, London, UK), and the 5.5-kb XhoI-KpnI fragment from the Sox1 5' homologous region were ligated. This targeting vector was linearized and introduced into CCE ES by electroporation. Double selection in G418 and Gancyclovir produced 465 clones that were analyzed by Southern blotting using probes from outside the 3' and 5' regions of the targeted area. Three clones bearing a disrupted Sox1 gene were injected into C57BL/6 embryos and passed into the germ line. LacZ activity was not detected, possibly because translation does not initiate from the translation start site of pgos1.

Histological analysis of mutant mice

Embryos and adult eyes were fixed in 4% paraformaldehyde in PBS overnight at 4°C, transferred to 50 mM sucrose, 50 mM glycine, and 100 mM phosphate buffer at 4°C until equilibrated, dehydrated, cleared in Histoclear, and embedded in paraffin. Embedded samples were sectioned at 7 µm. Embryos were genotyped by PCR of yolk-sac DNA using 5'-TACCCATGGGCTTTAAGCCG-3' (which binds the wild-type allele), neo2, 5'-CGTATCTCAGTCTCATCGCTTA-3' (which binds the mutated allele), and the 3' common antisense primer SX1 3'R, 5'-TGATGACATTGGGGTATCTC-3'. SX3Fnew and SX1 3'R detect a 373-bp fragment indicative for the wild-type allele, and

Figure 3. Immunofluorescent analysis of the wild-type lens. Eye sections at 10.5 (A,B), 12.5 (C,D), and 15.5 (E,F) dpc. (A,C,E) SOX1 and (B,D,F) SOX2 expression. The left of each panel shows the DAPI nuclear counterstain (blue) with SOX protein expression patterns (green); the right shows protein expression only. SOX1 protein can be detected in the nuclei of the presumptive lens fibers later during day 10 of development (not shown). SOX2 protein is detected in the nuclei of the developing lens fibers and the anterior epithelium (not shown). At 12.5 dpc, SOX1 is present in the nuclei of the developing lens fibers and the anterior epithelium (C); anti-SOX2 predominantly stains the cytoplasm of the lens fibers (D), although the protein is sometimes detected in the nuclei of the less differentiated lens fiber cells around the equatorial region and in the anterior epithelium (not shown). At 15.5 dpc in the lens, SOX1 is still detected in fiber cell nuclei (E), but SOX2 protein is absent (F). (oc) Optic cup; (lp) lens pit; (ae) anterior epithelium. Bar, 50 µm.
zyme OY) using the following crystallin primers: 

RT–PCR amplification of hprt, αA, αB, βA3/A1, γA, γB, γC, γD, γE, γF-crystallins at 12.5 and 15.5 dpc. αB-crystallin expression in the Sox1 mutant eye at 15.5 dpc is up-regulated possibly due to hypertonic stress (Dasgupta et al. 1992).

Figure 4. Crystallin gene-expression study of wild-type and mutant lens. RT–PCR analysis of hprt, αA, αB, βA3/A1, γA, γB, γC, γD, γE, γF-crystallins at 12.5 and 15.5 dpc. αB-crystallin expression in the Sox1 mutant eye at 15.5 dpc is up-regulated possibly due to hypertonic stress (Dasgupta et al. 1992).

BrdU labeling of embryos

For BrdU assays, pregnant mice from heterozygote matings were injected with BrdU (0.1 mg/g body weight) 1 hr before caesarean section. Embryos were dissected and fixed in 4% paraformaldehyde overnight at 4°C, dehydrated, cleared, and embedded in paraffin. Sections were cut at 7 µm, incubated in 3% H2O2 for 10 min, denatured in 2N HCl for 30 min, digested with 0.1% trypsin for 5 min at 37°C, each followed by washing in PBS for 5 min, and stained with an anti-BrdU monoclonal antibody (Dako) diluted 1:50 for 1 hr at 37°C. A Vectastain Elite kit (Vector Laboratories) was used for final antigen detection using DAB and hydrogen peroxide. The sections were counterstained with hematoxylin.

Immunohistochemistry

Embryos were fixed overnight in 95% ethanol/1% acetic acid at 4°C, dehydrated, cleared, and embedded in paraffin. Embryos were dissected and fixed in 4% paraformaldehyde overnight at 4°C, dehydrated, cleared, and embedded in paraffin. Sections were cut at 7 µm, incubated in 3% H2O2 for 10 min, denatured in 2N HCl for 30 min, digested with 0.1% trypsin for 5 min at 37°C, each followed by washing in PBS for 5 min, and stained with an anti-BrdU monoclonal antibody (Dako) diluted 1:50 for 1 hr at 37°C. A Vectastain Elite kit (Vector Laboratories) was used for final antigen detection using DAB and hydrogen peroxide. The sections were counterstained with hematoxylin.

RT–PCR amplification

RT–PCR amplification of hprt, αA, αB, βA3/A1, γA, γB, γC, γD, γE, γF-crystallins at 12.5 and 15.5 dpc. αB-crystallin expression in the Sox1 mutant eye at 15.5 dpc is up-regulated possibly due to hypertonic stress (Dasgupta et al. 1992).

Figure 5. Binding of SOX1 protein to the mouse γA-crystallin promoter. (A) Complementary strand of SOX binding consensus sequence, wild-type (γA), and mutated (γAM) oligonucleotide sequence from the γA-crystallin promoter that was used for EMSA, promoter–sequence alignment of the six mouse γ-crystallin genes. Sequences identical to the consensus SOX binding site are shown. (B) EM SA with recombinant SOX1 protein. γA oligonucleotide probe incubated without recombinant protein (in vitro transcription/translation reaction mixture incubated without DNA template (lane 1) or with SOX1 recombinant protein (lanes 2–7). Assays were done in the presence of nonspecific competitor (lane 2), nonradioactive γA oligonucleotide (lane 3), nonradioactive mutated γAM oligonucleotide (lane 4), normal rabbit serum (NRS) (lane 5), SOX1 rabbit antiserum abFB46 (lane 6), or ab791 (lane 7) as shown. (→) The position of SOX1 complex.
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