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Distal Sox Binding Elements of the αB-Crystallin Gene Show Lens Enhancer Activity in Transgenic Mouse Embryos

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αB-Crystallin, a member of the small heat shock protein (sHSP) family, is expressed in various tissues including lens, heart, and skeletal muscle. Previously we identified the gene of HSPB2, another member of the sHSP family, located 1-kb upstream of the αB-crystallin gene in a head-to-head manner. In the present study, we found a highly conserved region of 220 bp approximately 2.4-kb upstream of the αB-crystallin gene and examined its role in expression of the αB-crystallin gene. Transgenic mice containing 3 kb of the upstream sequence of the αB-crystallin gene showed *lacZ* reporter gene expression in the lens as well as the myotome and heart on embryonic day 12.5. Deletion analysis revealed that the –2656/–2267 region including the conserved region with four putative Sox binding elements (E1–E4) exhibits lens enhancer activity toward the αB-crystallin promoter. Gel shift assays showed that the Sox1 and Sox2 proteins preferentially bound to E2 and E4. Moreover, disruption of E2 and E4 abolished the reporter gene expression in the lens. These results indicate that the newly identified enhancer with Sox elements activates the αB-crystallin promoter in the lens, although they are separated by the entire HSPB2 gene.

Key words: crystallin, enhancer, lens, transcription, transgenic-mouse.

Crystallins are structural proteins of the lens and account for approximately 90% of the total water-soluble protein of the vertebrate lens (*1*). These proteins contribute to the transparent, refractive properties of the lens and are encoded by several different families of genes (*2*, *3*). The α-, β- and γ-crystallins are most common in the lenses of major vertebrates, and exhibit a spatial and temporal expression pattern characteristic for each class during lens development (*3*, *4*). The expression of α-crystallin begins first at the lens placode stage, and persists through the lens vesicle stage (*5*). Thereafter, fiber differentiation in the lens is accompanied by expression of β- and γ-crystallins (*4*).

αB-Crystallin is a subunit of α-crystallin expressed abundantly in the lens but is also expressed in a number of non-lenticular tissues, including the heart, skeletal muscle, kidney, lung, brain, and placenta (*6–9*). αB-Crystallin is now recognized as a member of the small heat shock protein (sHSP) family (*10*) and possesses molecular chaperone activity (reviewed in 11). αB-Crystallin accumulates in the brain in a number of pathological conditions (*9*, *12–14*). In cultured cells, αB-crystallin expression is induced by a variety of stimuli, including heat shock, oxidative stress and hypertonic stress (*15–17*). The proximal heat shock element (HSE, –53/–39) is essential for transcriptional activation of the αB-crystallin gene by different stimuli and binds to two different heat shock factors in glial cells (*18*, *19*). Under non-stress conditions, the transcriptional regulatory regions of the αB-crystallin gene in the lens, heart and muscle have been studied. The sequence between –426 and –257 is required for expression in the heart and skeletal muscle, while the sequence downstream of –164 directs lens-specific gene expression (*20*, *21*). However, since most of the previous studies on tissue-specific regulatory elements were performed using the mouse –661/+44 fragment of the αB-crystallin gene, little is known about the function of the further upstream sequence.

By comparing human and rodent upstream sequences of the αB-crystallin gene, we previously identified the gene of HSPB2, a new member of the sHSP family, which is located approximately 1-kb upstream of the αB-crystallin gene and transcribed in the opposite direction (*22*). Suzuki *et al*. have also identified this protein as a myotonic dystrophy protein kinase-binding protein (MKBP) (*23*). The αB-crystallin and HSPB2/MKBP genes are thus closely located on human chromosome 11q23 in a head-to-head manner, although the genes for other human sHSPs, such as hsp27, αA-crystallin, p20, HSPB3, HSPB7/cvHSP, HSPB8/HSP22/H11, and HSPB9, are solely located at different chromosomes (see http://www.ensembl.org/). The HSPB2 gene is expressed in the heart and muscle, but not in the lens, while the neighboring αB-crystallin gene is highly expressed in all three tissues (*22*).

To identify the regulatory elements involved in the differential expression of these two closely linked genes, we used transgenic mice because we can examine the role of such elements in gene expression in multiple tissues simultaneously. In this study we examined the biological function of a newly identified conserved sequence be-

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tween human and mouse located approximately 2.4-kb upstream of the αB-crystallin gene. Here we show that the conserved region directs the lens-specific expression of the lacZ reporter gene in transgenic mouse embryos and that Sox elements are essential for the enhancer activity.

EXPERIMENTAL PROCEDURES

Plasmid Construction—A mouse genomic DNA clone, λM16, was obtained by screening a 129Sv mouse genomic library in the λFIXII vector (Stratagene) with rat αB-crystallin cDNA (*22*) as a hybridization probe. A 6.8-kb *Eco*RI fragment containing exons 1 and 2 of the αB-crystallin gene and the entire HSPB2 gene was cloned into pBluescript II SK (+) (Stratagene), and the resulting plasmid was designated as pMXG1. Sequencing was performed with a dye terminator cycle sequencing kit (Applied Biosystems) and an Applied Biosystems Model 3100 DNA sequencer. The sequence of the mouse HSPB2 gene has been deposited in the GenBank/EMBL Data Libraries under Accession No. AF126248.

To generate the *lacZ* reporter DNA, a DNA fragment containing the sequence from –5128 to +75 of the αB-crystallin gene was fused to a 3.4 kb *Bam*HI fragment of the *lacZ* reporter gene after filling in with the Klenow fragment. The resulting plasmid, pMGL03, was designed to produce β-galactosidase containing ten amino acids of the N-terminus of mouse αB-crystallin at the N-terminal end. The β-galactosidase activity of this chimeric protein was confirmed by X-gal staining of C2C12 cells transfected with pMGL03. A plasmid, pMGL06, was generated by deletion of a 0.5-kb *Sac*I–*Bam*HI fragment from pMGL03.

The introduction of mutations into putative Sox binding sites was achieved by mismatched primer mutagenesis (*24*). For example, to prepare MA6m3, three DNA fragments were first generated by PCR with three pairs of primers, MX5 (*Xho*I) (5′-GGCTCGAGTACAAATGACTAAAAGGAAACC-3′) and M4F (5′-GAGACTCTTAAGCTTGGGTCTCCAGGCTGTCTG-3′), M4R (5′-ACCCAAGCTTAAGAGTCTCTAATCGCCAGCTCA-3′) and M3F (5′-CTCAGCATGCAAGCTTAGAGAGAGTGATCCTGGG-3′), and M3R (5′-CTCTAAGCTTGCATGCTGAGAAAATTATGGCGAGGAAT-3′) and HX19 (5′-TTCGTGTCCCGAGAGTTCTG-3′), using pMGL06 as a template. The sites of mutations are underlined. Second, two PCR products, MX5 (*Xho*I)-M4F and M4R-M3F fragments, were annealed, extended by mutually primed synthesis, and amplified by PCR using primers MX5 (*Xho*I) and M3F. Finally, the resulting DNA fragment and M3R-HX19 fragment were annealed, extended, and amplified by PCR using primers MX5 (*Xho*I) and HX19. The amplified fragment was digested with *Bam*HI and *Xho*I, and then ligated into pMGL07 digested with *Bam*HI and *Xho*I. The resulting plasmid was designated as pMGL14, and MA6m3 was prepared by digesting pMGL14 with *Xho*I and *Not*I.

Production of Transgenic Mice and X-Gal Staining—Fertilized one-cell eggs were obtained from B6C3 (C57BL/6×C3H) F1 female mice, microinjected with about 350 copies of the transgene fragments, and then transplanted into the oviducts of pseudopregnant MCH (ICR) females. The day a vaginal plug was first observed was defined as 0.5 days post coitum (d.p.c.). Mice were sacrificed by cervical dislocation. Whole embryos (E12.5) were isolated and fixed with 1% formaldehyde, 0.2% glutaraldehyde and 0.02% Nonidet P-40 (NP-40) in phosphate-buffered saline (PBS, pH 7.3) at 4°C for 1 h, washed extensively in PBS, and then stained for β-galactosidase activity in a solution comprising 0.4 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, and 1 mM $MgCl_2$ in PBS (pH 7.3) at 30°C for 48 h. Genomic DNA was isolated from the yolk sac and subjected to PCR for detection of the β-galactosidase gene using a pair of primers, P3 (5′-GTCACACTACGTCTGAACGTCG-3′) and P4 (5′-ACGATTCATTGGCACCATGCCG-3′). Copy numbers were determined by Southern blotting of the genomic DNA using an α^{32}P-labeled *lacZ* probe.

Preparation of Mouse Sox1 and Sox2 Expression Vectors—To prepare the mouse Sox1 expression vector, a DNA fragment was generated by PCR with a pair of primers, mSox1-F (*Bam*HI) (5′-CGGGATCCAGATGTACAGCATGATGAT-3′) and mSox1-R (*Eco*RI) (5′-TAGAATTCGCTAGATGTGCGTCAGGG-3′), using the B6C3F1 mouse genomic DNA as a template, digested with *Bam*HI and *Eco*RI, and then cloned into pcDNA3 (Invitrogen). The resulting plasmid was designated as pcDNA-Sox1. The mouse Sox2 expression vector was prepared in a similar manner using primers mSox2-F (*Bam*HI) (5′-TAGGATCCGCGCGCATGTATAACATGA-3′) and mSox2-R (EcoRI) (5′-TAGAATTCAGCCCTCACATGTGCGACA-3′). The resulting plasmid was designated as pcDNA-Sox2.

Transfection and Preparation of Nuclear Extracts—COS-1 cells were grown in Dulbecco's modified Eagle's medium (Nissui) containing 10% fetal bovine serum, 1 mM glutamine, and penicillin-streptomycin. The cells in a 100-mm diameter dish were transfected with 4 μg of pcDNA-Sox1, pcDNA-Sox2 or pcDNA using 20 μl of LipofectAMINE reagent and 30 μl of LipofectAMINE PLUS reagent (Gibco BRL). Nuclear extracts were prepared by the method of Schreiber *et al*. (*25*), with some modifications. Cells were harvested and centrifuged. The pellet was suspended in buffer A, comprising 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES, pH 7.8), 10 mM KCl, 0.1 mM ethylenediamine-tetraacetic acid (EDTA), 0.1% NP-40, 1 mM dithiothreitol (DTT), and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride [PMSF], 2 μg/ml aprotinin, 2 μg/ml pepstatin A, and 2 μg/ml leupeptin), mixed briefly and then centrifuged. The pellet was resuspended in buffer C, comprising 50 mM HEPES-KOH (pH 8.0), 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2% glycerol, and protease inhibitors, and then gently mixed at 4°C for 30 min. After centrifugation, the supernatant was used for gel mobility shift assays. The protein concentrations of the extracts were measured using the Bradford assay (Bio Rad).

Gel Mobility Shift Assay—A typical binding reaction was carried out in a 20-μl volume comprising 0.3 μg of nuclear extract, 50 fmol ^{32}P-labeled oligonucleotide probe, 0.15 μg poly(dI-dC), and 0.15 μg BSA, 25 mM HEPES (pH 8.0), 105 mM KCl, 0.05 mM EDTA, 6% glycerol, 0.5 mM DTT, 0.25 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin at room temperature for 20 min. For the competition experiment, a 25- or

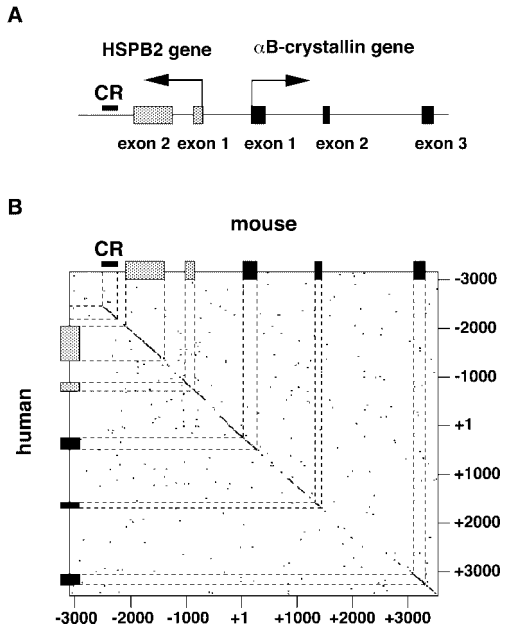

Fig. 1. **Identification of the conserved region between mouse and human downstream of the HSPB2 gene.** (A) The schematic structures of the αB-crystallin and HSPB2 genes. The αB-crystallin and HSPB2 genes are composed of three and two exons, respectively, and arranged in a head-to-head manner. The exons of the αB-crystallin and HSPB2 genes are shown as black and gray boxes, respectively. Arrows indicate the transcriptional direction of each gene. The location of the conserved region (CR) is indicated by a black bar. (B) Dot matrix comparison of the αB-crystallin and HSPB2 region sequences between mouse and human. The homology plot program DNASIS v3.0 (Hitachi Software Engineering) was used to align the mouse (*x* axis) and human (*y* axis) sequences. The stringency of comparison is 9 matches of 10.

50-fold molar excess of the unlabeled oligonucleotide was added for each reaction mixture. The reaction products were separated electrophoretically on a 4% polyacrylamide gel for 2 h at 200 V and detected by autoradiography. In supershift assays, anti-chicken Sox1 antiserum (3162-4) and anti-chicken Sox2 antiserum (3159-4), which were kindly provided by Drs. H. Kondo and Y. Kamachi (Osaka University), were added to the nuclear extracts for each reaction.

RESULTS

A Highly Conserved Region is Located Upstream of the αB-Crystallin Gene and Downstream of the HSPB2 Gene—To clarify the genomic organization of the αB-crystallin and HSPB2 genes in mice, we determined the sequence of the 5′-flanking region of the mouse αB-crystallin gene. The mouse HSPB2 gene is composed of two exons and located 863-bp upstream of the αB-crystallin gene in a head-to-head manner (Fig. 1A). Homology plot analysis revealed a highly conserved region (CR) between mouse and human of 220 bp in length located 130-bp downstream of the HSPB2 gene and approximately 2.4-kb upstream of the αB-crystallin gene (Fig. 1B). The sequence identity of the newly identified conserved region was 95% between mouse and human, whereas those of exon 1, exon 2, and intron 1 of the HSPB2 gene were 92%, 90%, and 61%, respectively, suggesting that this region has some important biological function.

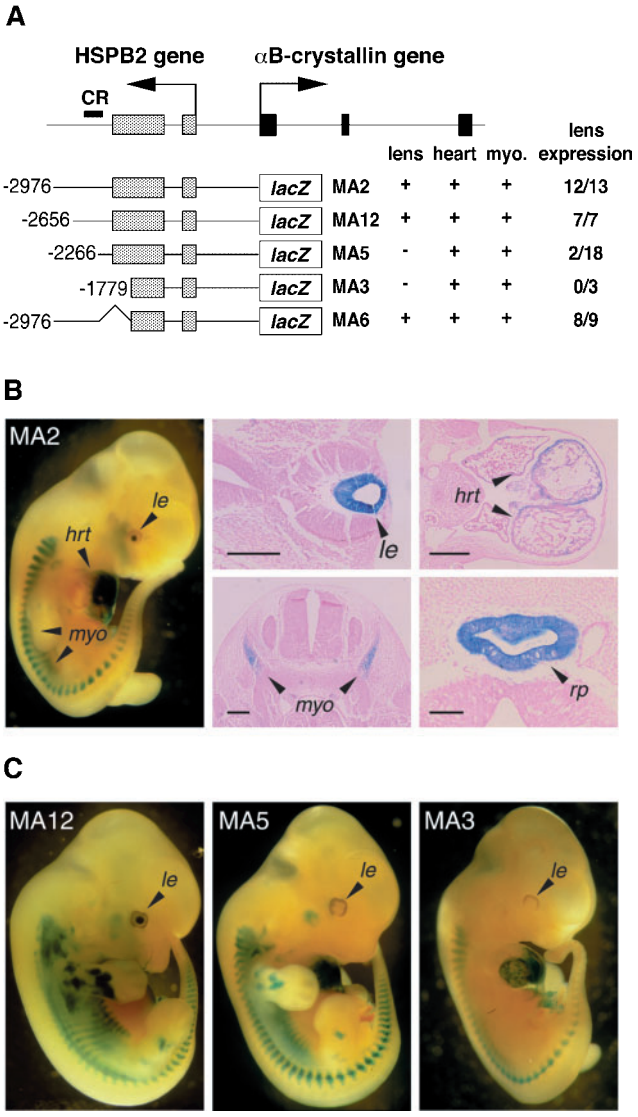

Fig. 2. **The reporter constructs and expression patterns of the reporter genes in transgenic mice.** (A) Various portions of the upstream region of the mouse αB-crystallin gene were fused with the *lacZ* gene. The expression patterns of the reporter gene in the lens, heart and myotome (myo) in transgenic mice are summarized: + indicates expression, – indicates undetectable expression. Lens expression indicates the number of embryos that showed expression of the reporter gene in the lens versus the total number of embryos which showed expression of the reporter gene. (B) Staining of whole-mount embryos and transverse sections of MA2 for β-galactosidase activity. Strong X-gal staining is observed in the lens (le), heart (hrt), myotome (myo), and Rathke's pouch (rp). The scale bar in each section represents 200 μm. (C) Whole-mount staining of MA12, MA5 and MA3 embryos. In MA12, strong X-gal staining was observed in the lens, heart and myotome, although the heart tissue is behind the forelimb in this picture. In MA5 and MA3, expression of the reporter gene was detected in the heart and myotome but not in the lens.

The Region Including the Conserved Sequence Shows Lens-Specific Enhancer Activity—The functional significance of the conserved region was examined by mouse transgenic reporter analysis (Fig. 2A). The –2976/+75

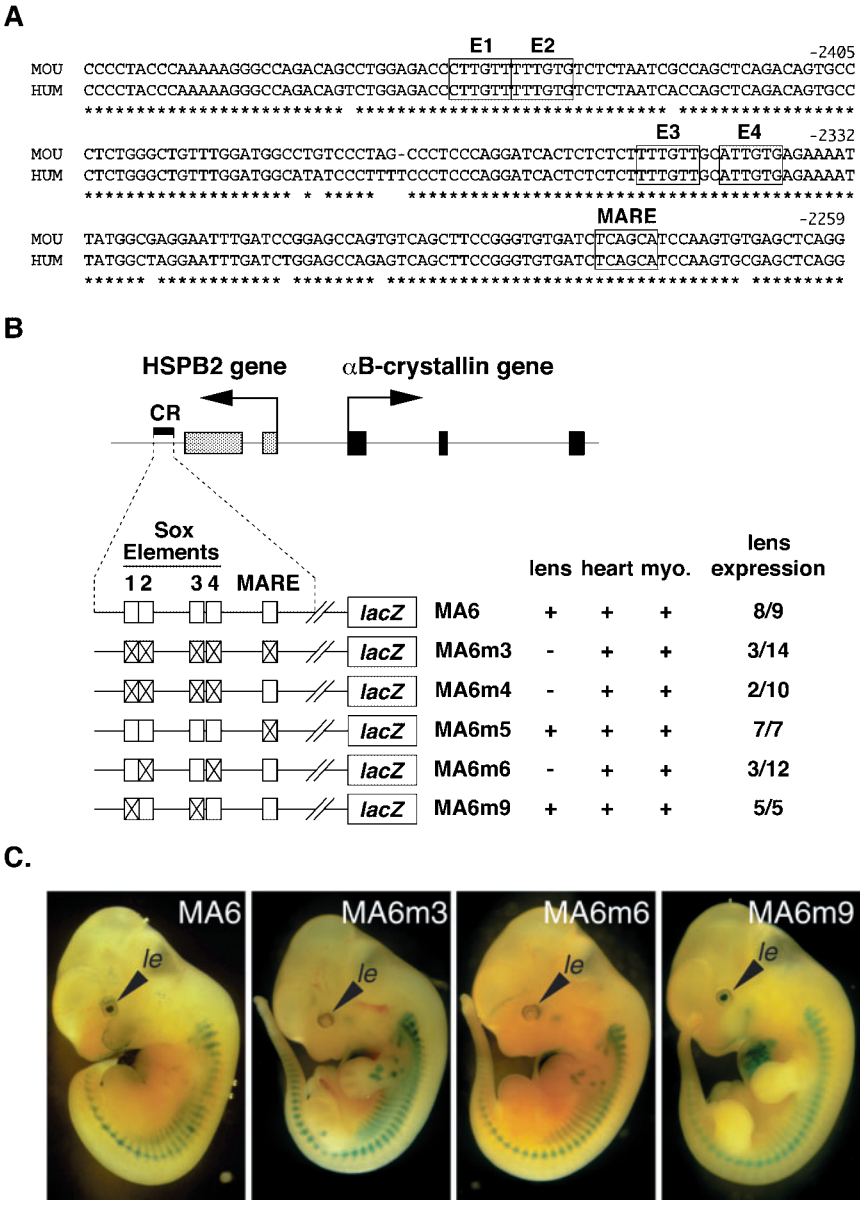

Fig. 3. **Sox elements in the newly identified conserved region are required for the expression of the reporter gene in the lens in transgenic mice.** (A) Alignment of the sequences of the conserved region between mouse and human. The nucleotides that are identical between the two species are indicated by asterisks. The overall identity of the conserved region (CR) is 95%. Putative Sox elements (E1–E4) and a Maf recognition element (MARE) are boxed. (B) The reporter constructs containing the site-specific mutations used for generating transgenic mice are schematically represented. Putative Sox elements (E1–E4) and MARE are shown by open boxes. The elements containing site-specific mutations are indicated by crossed boxes. The sequences of the Sox elements with the mutations are shown in Fig. 4A. The expression patterns of the reporter gene in transgenic mice are summarized in the same way as described in the legend to Fig. 2. Lens expression indicates the number of embryos that showed expression of the reporter gene in the lens versus the total number of embryos which showed expression of the reporter gene. (C) Whole-mount staining of MA6, MA6m3, MA6m6 and MA6m9 embryos. Disruption of all four Sox elements (MA6m3), and of E2 and E4 (MA6m6) led to a loss of expression of the reporter gene in the lens, but not in the heart or myotome. On the other hand, disruption of E1 and E3 (MA6m9) had no effect on the expression pattern. Strong X-gal staining of the heart in MA6, MA6m3 and MA6m6 was observed, although the heart tissue is behind the forelimb in these pictures.

region of the αB-crystallin gene, including the conserved region (–2477/–2259), the entire HSPB2 gene and the intergenic sequence, was fused to a *lacZ* reporter gene, and the resulting hybrid gene fragment was microinjected into fertilized eggs. Transgenic mouse embryos were analyzed for β-galactosidase activity on embryonic day (E) 12.5. Expression of the reporter gene was observed in the lens, heart, myotome and Rathke's pouch in twelve independent transgenic mouse embryos (see MA2 in Fig. 2B). This expression pattern of the reporter gene was consistent with that of the endogenous αB-crystallin gene (*26*), and that of two lines of mice harboring a transgene consisting of an about 4-kb promoter-containing region of the αB-crystallin gene fused to the *lacZ* reporter (*27*).

Next, a series of deletion constructs was prepared to examine the role of the conserved region in the expression of the αB-crystallin gene (Fig. 2A). Expression of the reporter gene in the lens, heart and myotome was also observed in MA12 mice carrying the –2656/+75 region in the embryos (Fig. 2C). Interestingly, deletion of the –2656/–2267 region resulted in a complete loss of expression of the reporter gene in the lenses of 16 of 18 independent transgenic mouse embryos, while the expression in the heart, myotome and Rathke's pouch remained (see MA5 in Fig. 2C). Further deletion of the –2266/–1780 region had no effect on the expression pattern in these tissues (see MA3 in Fig. 2C). We always analyzed multiple independent F0 embryos generated with each transgene fragment to avoid misinterpretation of the results with a limited number of transgenic mouse lines. Although a few transgenic embryos showed a different expression pattern, probably because of position-dependent variegation in transgene expression, most of the transgenic mice embryos containing the same reporter fragment showed a similar expression pattern. There was slight variation in the intensity of the β-galactosidase activity between different embryos. The results of

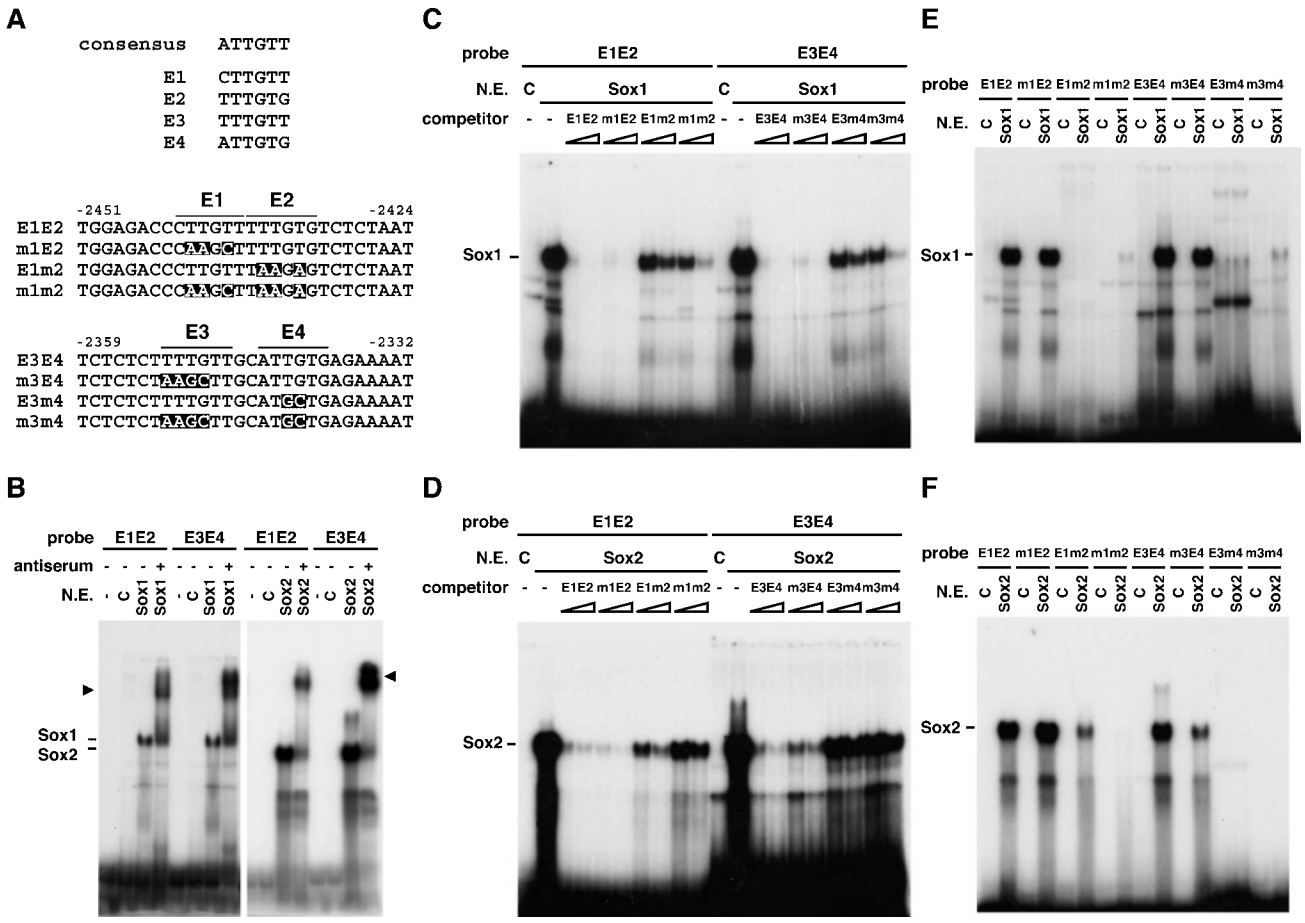

Fig. 4. **Mouse Sox1 and Sox2 proteins bind to E2 and E4, but not E1 and E3, *in vitro*.** (A) The sequences of the putative Sox binding elements, E1–E4, are aligned together with the consensus sequence on the top. The sequences of oligonucleotides used as probes and competitors in gel mobility shift assays are shown below. The substituted nucleotides in the Sox elements are boxed. (B) Gel mobility shift assays were performed using 0.3 and 0.1 μg of nuclear extracts (N.E.) of COS-1 cells expressing mouse Sox1 and Sox2, respectively, with (+) or without (–) anti-Sox1 and anti-Sox2 antisera. It was confirmed by western blotting that almost the same amount of each Sox protein was present in each nuclear extract (data not shown). A nuclear extract prepared from COS-1 cells transfected with the empty vector (C) was used as a negative control. The positions of the bands corresponding to the Sox1-DNA complex (Sox1) and Sox2-DNA complex (Sox2) are indicated. The positions of the shifted complexes are indicated by arrowheads. Both Sox1 and Sox2 bound to the E1E2 and E3E4 probes. However, the binding affinity of Sox2 to DNA appeared to be much stronger than that of Sox1. The mobility of the Sox1-DNA complexes was slower than that of the Sox2-DNA complexes, probably because the molecular weight of Sox1 is slightly higher than that of Sox2. (C, D) Competition assays were performed using 25- and 50-fold excesses of each unlabeled oligonucleotide. The Sox1- and Sox2-DNA complexes competed with the oligonucleotides containing the intact E2 or E4 sequence. (E, F) Each oligonucleotide containing site-specific mutations at one or two Sox elements was ^{32}P-labeled and used as a probe. The Sox1- and Sox2- DNA complexes were observed when the E2 or E4 site was intact.

the deletion analysis suggest that the –2656/–2267 region is essential for the expression of the αB-crystallin gene in the lens, but not in the heart or myotome.

Sox Elements in the Conserved Region Are Essential for the Enhancer Activity—Several transcription factors including the Pax6, Maf, and Sox proteins have been shown to be involved in lens development (*28–31*). We expected that *cis*-acting regulatory elements would exist in the conserved region and thus searched for binding sequences of the transcription factors using the MatInspector v2.2 program in the TRANSFAC v4.0 database (*32*). Consequently, we found four elements in which more than 4 bp of 6 bp were homologous to the consensus sequence, 5′-ATTGTT-3′, for Sox HMG domains, and designated them as Sox Element 1 (E1), E2, E3, and E4 in the 5′ to 3′ direction (Fig. 3A). E1 and E2 are adjacent, and E3 and E4 are only 2 bp apart. In addition, we found one potential Maf recognition element (MARE) that perfectly matched the half site of the consensus sequence 5′-TCAGCA-3′ (*33*).

To determine whether or not these elements are involved in the lens enhancer activity, we introduced site-specific mutations into the putative Sox and Maf binding sequences, and analyzed the effects of the mutations on the expression of the reporter gene in transgenic mice (Fig. 3B). MA6 embryos containing the lens enhancer but lacking the –2266/–1780 region (Fig. 2A) showed the same reporter gene expression pattern as MA2 embryos (Fig. 3C). Disruption of all four Sox elements and the MARE resulted in a complete loss of the reporter gene expression in the lens (see MA6m3 in Fig. 3C). Expression of the reporter gene in the lens was observed in

MA6m5 embryos, in which only the MARE was disrupted, but not in MA6m4 embryos, in which all four Sox elements were disrupted (Fig. 3B). These results suggest that some or all of the four Sox elements, but not the MARE, are required for the expression in the lens.

Sox1 and Sox2 Proteins Bind to E2 and E4 In Vitro—Next, using a gel mobility shift assay, we examined the binding ability of these four Sox elements to Sox1 and Sox2 proteins, both of which are known to be expressed in the developing mouse lens (*34*). The nucleotide sequences of the probes used in this assay are shown in Fig. 4A. Nuclear extracts were prepared from COS-1 cells transfected with mouse Sox1 and Sox2 expression vectors. As shown in Fig. 4B, the E1E2 and E3E4 probes produced DNA-protein complexes with both Sox1 and Sox2. The bands of these complexes supershifted when anti-Sox1 and anti-Sox2 antisera were added. These data suggest that Sox1 and Sox2 can bind to both the E1E2 and E3E4 probes *in vitro*.

To determine which element in the E1E2 and E3E4 probes binds to the Sox1 and Sox2 proteins, probes with the same Sox element mutations as for the functional analysis were prepared (Fig. 4A), and then subjected to the gel mobility shift assay. Sox1- and Sox2-DNA complexes competed with excess E1E2, m1E2, E3E4 and m3E4 (Fig. 4, C and D). Gel mobility shift assays with the ^{32}P-labeled mutant probes revealed that Sox1 preferentially binds to E2 and E4, but not E1 and E3 (Fig. 4E). The preferential binding of Sox2 to the E2 and E4 sites was also observed (Fig. 4F). Therefore, E2 and E4 may be critical for the enhancer activity of the conserved region toward the expression of the αB-crystallin gene in the lens.

Disruption of Both E2 and E4 Abolishes the Lens Expression of the Reporter Gene in Transgenic Mice—Finally, we generated transgenic mice containing mutations at both E2 and E4 (see MA6m6 in Fig. 3). As we expected, introduction of the mutations at both E2 and E4 completely abolished the expression of the reporter gene in the lens of nine independent transgenic mice embryos, while disruption of both E1 and E3 did not affect the reporter gene expression (see MA6m9 in Fig. 3). Together with the fact that Sox1 and Sox2 preferentially bind to E2 and E4 *in vitro*, these results indicate that E2 and E4 in the enhancer region play a critical role in the αB-crystallin gene expression in the lens through interaction with Sox1 and/or Sox2.

DISCUSSION

In the present study, we identified a lens enhancer (–2656/–2267) upstream of the αB-crystallin gene, and provided several lines of evidence indicating that Sox binding elements E2 (–2437/–2432) and E4 (–2344/–2339) are essential for its enhancer activity. First, the sequence around the two Sox elements is highly conserved between mouse and human, suggesting functional significance of this region (Fig. 1). Second, functional analyses revealed that the two Sox elements regulated the expression of the reporter gene in the lenses of transgenic mice. Deletion or disruption of the Sox elements resulted in a loss of the reporter gene expression in the lens (Figs. 2 and 3). Third, gel mobility shift assays showed that Sox1 and Sox2, members of the Sox protein family expressed in the mouse lens, bound to the two elements *in vitro* (Fig. 4). Therefore, we propose that Sox1 and/or Sox2 may be the regulators of transcription of the αB-crystallin gene in the lens.

The Sox family of HMG domain proteins has been recognized as a key player in the regulation of embryonic development and in the determination of cell fate (reviewed in *35*, *36*). Twenty members of the mammalian Sox protein family have been identified to date (*37*), and can be divided into 10 subgroups on the basis of sequence similarity and genomic organization. In the developing mouse lens, two Sox proteins belonging to subfamily B1, Sox1 and Sox2, are expressed and their expression is developmentally regulated as follows: at E9.5–10.5, Sox2 mRNA is highly expressed in the head ectoderm overlying the optic vesicle and the lens placode; at E11.5, Sox2 mRNA is down-regulated and Sox1 mRNA progressively increases in the lens vesicle (*34*, *38*). During lens development, αB-crystallin mRNA is expressed in the lens placode at E9.5, in the lens cup at E10.5, in the lens vesicle at E11.5, and in the lens epithelium and lens fibers at E12.5 (*5*). The expression of the αB-crystallin gene, therefore, partially overlaps that of the Sox1 and Sox2 genes spatiotemporally, supporting the idea that Sox1 and/or Sox2 are involved in the αB-crystallin gene expression in the developing lens.

We showed that both Sox1 and Sox2 bound to two Sox elements, E2 and E4, in the lens enhancer *in vitro*, and found that the binding affinity of Sox2 to DNA was much stronger than that of Sox1 (Fig. 4B). This difference in binding affinity between Sox1 and Sox2 is consistent with a previous observation that the binding affinity of the HMG domain of Sox2 to the consensus sequence, 5′-AACAAT-3′, was much higher than that of Sox1 *in vitro* (*39*). Chromatin immunoprecipitation assay is necessary to determine whether Sox1 and/or Sox2 bind to Sox elements E2 and E4 *in vivo*.

Although it remains unclear whether one or both Sox proteins contribute to the αB-crystallin expression, we speculate that Sox2, rather than Sox1, is the main activator for the transcription of the αB-crystallin gene, because Sox1 is not expressed in the lens at E9.5–10.5 when αB-crystallin mRNA expression starts (*5*). Moreover, there was no significant difference in the expression levels of the αB-crystallin mRNA in the eye between the wild-type and Sox1-deficient mouse embryos in the lens at E12.5 when examined by semi-quantitative RT-PCR (*40*). Sox2 knockout mice were embryonic lethal, probably because Sox2 plays an important role in early embryogenesis, as well as lens differentiation (*41*). Therefore, conditional knockout of the Sox2 gene may provide direct evidence for the involvement of Sox2 in the αB-crystallin gene transcription in the lens.

Our data clearly demonstrated that the two Sox elements are essential for the enhancer activity in transgenic mouse embryos at E12.5. Previous studies by others involving the –661/+44 upstream region of the αB-crystallin showed that the –147/–46 sequence including two lens-specific regulatory regions (LSRs) interacts with Pax6 *in vitro* and is sufficient for the expression of the CAT reporter gene in the transgenic mouse lens at 2 months old (*20*, *21*, *42*). Recently, Swamynathan and

Piatigorsky proposed that there are lens-specific negative regulatory elements within the –919/–662 region (*43*). In the present study, expression of the lacZ reporter gene was undetectable in most of the E12.5 transgenic mice with LSRs and negative regulatory elements but not the newly identified Sox elements. Probably, the distal Sox enhancer elements upstream of the gene counteract the influence of the negative regulatory element and activate the αB-crystallin promoter.

It is of interest that the newly identified lens enhancer activates the distally located αB-crystallin promoter, but not the closely located HSPB2 promoter, because the expression of αB-crystallin mRNA is high in the lens, but HSPB2 mRNA is undetectable (*22*). This preferential activation may be explained by promoter competition. The αB-crystallin core-promoter contains a well-conserved TATA box, while the HSPB2 core-promoter has no TATA box but contains a GC box. The competition between two different core promoters, which were divergently transcribed and closely located, has been observed in the gene expression in *Drosophila*. The *Drosophila* AE1 and IAB5 enhancers, which are situated between the TATA-containing *even-skipped* and TATA-less *white* core promoters, preferentially activate transcription from the TATA-containing relative to the TATA-less promoters (*44*). Alternatively, a sequence proximal to the αB-crystallin promoter may recruit a distal lens enhancer. A mechanism of promoter-enhancer interaction involving a promoter-proximal tethering element was proposed for the regulation of the *Drosophila Antennapedia* gene complex. The tethering elements in the vicinity of the *scr* promoter can specifically recruit the distally located T1 enhancer to the *scr* promoter, bypassing the closely located *ftz* promoter. As a result, the T1 enhancer can activate the weak distal *scr* promoter but not the strong proximal *ftz* promoter (*45*). Further studies, on such as alteration of the core promoter elements and deletion of the elements outside of the core promoter, may provide insights into the mechanism underlying preferential activation of the expression of the αB-crystallin gene but not the HSPB2 gene in the lens.

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