Pax-6 and αB-crystallin/Small Heat Shock Protein Gene Regulation in the Murine Lens

INTERACTION WITH THE LENS-SPECIFIC REGIONS, LSR1 AND LSR2

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We have demonstrated previously that a transgene comprising the −164/+44 fragment of the murine αB-crystallin gene fused to the bacterial chloramphenicol acetyltransferase (cat) gene is lens-specific in transgenic mice. The −147 to −118 sequence was identified as a lens-specific regulatory region and is called here LSR1 for lens-specific region 1. In the present experiments, a −115/+44-cat transgene was also lens-specific in transgenic mice, although the average activity was 30 times lower than that derived from the −164/+44-cat transgene. The −115/+44 αB-crystallin fragment contains a highly conserved region (−78 to −46) termed here LSR2. A −68/+44-cat transgene, in which LSR2 is truncated, was inactive in transgenic mice. DNase I footprinting indicated that LSR1 and LSR2 bind partially purified nuclear proteins from either αTN4-1 lens cells or the mouse lens as well as the purified paired domain of Pax-6. Site-specific mutation of LSR1 eliminated both Pax-6 binding and promoter activity of the −164/+44-cat transgene in transgenic mice. Finally antibody/electrophoretic mobility shift assays and cotransfection experiments indicated that Pax-6 can activate the αB-crystallin promoter via LSR1 and LSR2. Our data strengthen the idea that Pax-6 has had a major role in recruiting genes for high expression in the lens.

The crystallins comprise approximately 90% of the water-soluble proteins of the transparent eye lens and contribute to its optical properties (1, 2). The αB-crystallin/small heat shock protein gene is expressed abundantly in lens and also in various other tissues, including skeletal muscle, heart, and to a lesser extent, lung (3, 4). Previously we demonstrated that the differential constitutive expression of the murine αB-crystallin gene is under transcriptional control (4, 5) and that the sequences between −426 and −259 of the murine αB-crystallin promoter functions as a muscle-preferred enhancer (5). Transgenic mice containing an αB-crystallin promoter/bacterial chloramphenicol acetyltransferase (cat) reporter transgene established that the sequences between −426 and −164 (which includes the enhancer) are required for expression in heart and skeletal muscle, while sequences downstream of −164 are sufficient to direct lens-specific gene expression (6). The −147/−118 sequence contains a putative lens-specific regulatory region called LSR (renamed here LSR1) (6). In the present investigation we have performed transient transfection and transgenic mouse experiments to identify another regulatory element at positions −78/−46, called here LSR2, which also contains lens-specific promoter activity. Thus, the regulatory elements required for lens specificity of the diversely expressed mouse αB-crystallin gene are located downstream from the enhancer required for expression in skeletal muscle and heart (5).

Recent experiments have indicated that Pax-6, a member of paired-domain (PD)1 family of transcription factors, is an essential factor for eye development (7–11). The Pax-6 gene also encodes an alternatively spliced variant (Pax-6-5a) (9, 12). This variant has a 14-amino acid insertion in N-terminal half of the PD changing its recognition specificity. During eye development, the expression pattern of Pax-6 indicates its direct role in the formation of lens, retina, and cornea (9). Mutations in Pax-6 are associated with distinct eye defects, including small eye in mouse and rat (13), aniridia in humans (8, 14–16), Peter’s anomaly in humans (17) and eyeless in Drosophila (18). We have demonstrated that Pax-6 is involved in lens-specific expression of chicken (19) and mouse (20) αA, chicken α1 (21) and guinea pig ζ (22) crystallin genes (23). In the present study, DNase I footprinting, antibody/electrophoretic mobility shift assay (EMSA), site-directed mutagenesis, and transient cotransfection experiments provide evidence that Pax-6 interacts at LSR1 and LSR2 to activate the αB-crystallin promoter in the lens.

EXPERIMENTAL PROCEDURES

Isolation of DNA for Pronuclear Injections and Transgenic Mice Production—Approximately 2.7 kbp NdeI-PstI DNA fragments containing the mouse αB-crystallin gene were isolated from plasmids p11-3, p61-7 (5), and p9760 (6) by polyacrylamide gel electrophoresis followed by electroelution, phenol-chloroform extraction, and ethanol precipitation. Each fragment contained 60 bp of pBR322 sequence at its 5’ end, murine αB-crystallin promoter sequences, the bacterial cat gene, and SV40-derived sequences, including the small t antigen splice sites and polyadenylation signal. The αB115-cat fusion gene isolated from p11-3 contained the −115/+44 sequence of the αB-crystallin gene, the αB68-cat fusion gene isolated from p61-7 contained the −68/+44 sequence of the αB-crystallin gene, and the αBLSR1(Mu-9760)-cat fusion gene isolated from p9760 (−164/+44) contained mutated LSR1 sequence (143 to −129) (6) of the αB-crystallin promoter (Fig. 1). Linear DNA fragments were injected into one pronucleus of a single celled mouse embryo (FVB/N strain) (24). The embryos were obtained from superovulated FVB/N females. Injected embryos were transferred into FVB/N females made pseudopregnant by mating to vasectomized FVB/N males. Transgenic mice were created by the National Eye Institute Centralized Transgenic Facility.

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The abbreviations used are: PD, paired-domain; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; kbp, kilobase pair(s); bp, base pair(s).
Analysis of Transgenic Mice—DNA was isolated (25) from tails of founder Fo mice and analyzed by Southern blot and polymerase chain reaction analyses for the presence of the transgene. We used 5′ oligodeoxynucleotide primers (oligodeoxynucleotide 10660, 5′-CCCTGATCA-CAAGTCTCCATGAACT-3′, for αB115-cat and oligodeoxynucleotide 10661, 5′-ACCCCTGACCTCACCATTCCAGAAG-3′, for αB68-cat, and oligodeoxynucleotide 10662, 5′-TCTCTTTTCTTAGCTCAGTGTCGATAG-3′, for αBLSR1(Mu-9760)-cat), which were specific for the murine αB-crystallin promoter, and a 3′ oligodeoxynucleotide primer (oligodeoxynucleotide 7576, 5′-CGGTCTGGTTATAGGTACATTGAGC-3′) which was specific for the cat gene. Fo mice containing the transgene were mated to nontransgenic FVB/N mice to obtain F1 offspring, and sibling matings were used to establish homozygous mouse lines. The transgene copy number for each mouse was estimated by hybridization intensity in a slot blot analysis of the genomic DNA relative to the standard samples representing 0–50 copies of the transgene, using the

FIG. 1. Structure of the murine αB-crystallin-cat chimeric transgenes. Approximately 2.7-kbp NdeI/PstI fragments isolated from p11-3, p61-7, and p9760 (6) containing a -115/+44, a -68/+44, and a mutated -164/+44 fragment (6) of αB-crystallin gene, respectively, linked to the bacterial cat gene were used as transgenes in transgenic mice. Approximately 60 bp of pBR sequences are present 5′ to the αB fragments in the transgene and approximately 1,400 bp of simian virus 40 (SV40) sequences, including the small intron and poly(A) addition signal, were present 3′ to the cat gene. The site-specific mutation in αBLSR1(Mu-9760)-CAT is shown in Fig. 7A.

| Table I | Expression of αB-crystallin promoter-CAT genes in F1 generation of FVB/N transgenic mice |
|---------|----------------------------------------------------------------------------------------------------------------------------------|
| Construct | Line/ Copy # Mouse # Sex Age | Cat Activity |
| αB115-CAT | 1 21–30 25 M 2 | 9.8 4.0 3.0 8.0 2.5 3.8 1.8 80.0 |
|           | 2 31–40 29 F 2 | 11.3 16.0 14.0 23.2 20.4 16.2 23.4 117.0 |
|           | 7 21–30 33 F 2 | 5.0 19.0 15.0 3.3 10.0 3.6 4.4 854 |
|           | 11 31–40 46 M 2.5 | 1.1 1.4 0.9 0.0 0.3 0.0 0.5 368 |
|           | 15 11–20 37 M 2.5 | 1.4 1.2 2.7 0.3 0.5 0.7 0.5 58 |
|           | 15 11–20 38 M 2 | 9.6 3.2 5.4 9.6 2.0 3.8 2.8 288 |
|           | 15 11–20 40 F 2 | 7.6 3.0 19.6 14.8 18.0 3.4 3.3 26 |
|           | 19 6–10 42 M 3.25 | 0.0 0.0 1.7 0.1 0.0 0.0 0.0 0.7 |
|           | 19 6–10 44 F 3.25 | 0.0 1.5 4.2 0.3 0.0 0.0 0.0 10.8 |
|           | 19 11–20 40 M 3 | 0.4 0.5 0.4 1.2 0.0 0.3 0.0 0.1 |
|           | 19 31–40 49 F 3 | 0.0 0.4 3.4 0.0 0.0 0.3 0.3 0.4 |
|           | 25 11–20 55 F 3.25 | 0.0 1.1 0.0 0.0 2.3 0.0 0.0 2490.0 |
|           | 25 21–30 57 F 3.25 | 0.0 0.0 0.2 0.0 0.0 0.0 0.0 0.0 |
|           | 25 21–30 54 F 3 | 0.0 0.5 5.0 1.5 0.0 1.5 0.0 0.0 |
|           | 25 21–30 58 F 3 | 0.4 0.8 5.2 0.5 0.8 0.3 0.7 0.0 |
|           | 24 21–30 30 M 2 | 11.2 6.1 4.4 18.2 2.7 1.5 5.6 7.0 |
|           | 24 21–30 31 M 2 | 6.1 0.0 2.8 0.0 0.0 3.0 10.4 6.6 |
| αB68-CAT | 6 21–30 62 M 2 | 8.2 3.0 2.9 11.6 3.6 7.7 6.4 0.2 |
|           | 6 11–20 66 F 2 | 0.7 1.5 2.4 0.0 0.0 1.2 6.6 1.8 0.0 |
|           | 6 21–30 66 M 2 | 0.0 0.0 0.1 0.2 0.0 0.0 0.0 5.5 0.0 |
|           | 20 11–20 52 M 2 | 0.1 0.0 0.0 0.0 2.3 0.0 0.0 0.0 3.0 |
|           | 25 31–40 39 F 2 | 0.5 13.9 13.5 7.3 1.8 1.3 6.0 1.7 |
|           | 25 31–40 41 M 2.25 | 0.1 1.4 0.1 1.2 1.5 1.3 0.3 9.0 |
|           | 25 31–40 39 M 2.25 | 1.5 3.4 3.1 3.3 1.7 1.8 3.4 2.3 |
|           | 25 46 M 2.50 | 2.3 5.1 1.3 2.6 1.1 2.9 3.0 12.0 |
| αBLSR1 (Mu-9760)-CAT | 6 21–30 52 M 2 | 8.2 3.0 2.9 11.6 3.6 7.7 6.4 0.2 |
| Nontransgenic | 25 46 M 2.50 | 2.3 5.1 1.3 2.6 1.1 2.9 3.0 12.0 |
| αB164-CAT | 20 6–10 54 M 2 | 8.2 3.0 2.9 11.6 3.6 7.7 6.4 0.2 |
|           | 20 11–20 52 M 2 | 0.0 0.0 0.1 0.2 0.0 0.0 0.0 5.5 0.0 |
|           | 25 31–40 39 F 2 | 0.5 13.9 13.5 7.3 1.8 1.3 6.0 1.7 |
|           | 25 31–40 41 M 2 | 0.1 1.4 0.1 1.2 1.5 1.3 0.3 9.0 |
|           | 25 31–40 39 M 2 | 1.5 3.4 3.1 3.3 1.7 1.8 3.4 2.3 |
|           | 25 46 M 2.50 | 2.3 5.1 1.3 2.6 1.1 2.9 3.0 12.0 |

Separate lines of transgenic mice were derived from different founder mice; all examined were F1 transgenic mice.

Age in months.

CAT activity: expressed as counts/min [3H]acetylchloramphenicol produced per min/μg of total protein. Boldface numbers indicate average CAT activity.

This number is not included for calculating average CAT activity because it is an outlier. When included the average CAT activity in the lens is 26.3.

Analysis of Transgenic Mice—DNA was isolated (25) from tails of founder Fo mice and analyzed by Southern blot and polymerase chain reaction analyses for the presence of the transgene. We used 5′ oligodeoxynucleotide primers (oligodeoxynucleotide 10660, 5′-CCCTGATCACAAGTCTCCATGAACT-3′, for αB115-cat and oligodeoxynucleotide 10661, 5′-ACCCCTGACCTCACCATTCCAGAAG-3′, for αB68-cat, and oligodeoxynucleotide 10662, 5′-TCTCTTTTCTTAGCTCAGTGTCGATAG-3′, for αBLSR1(Mu-9760)-cat), which were specific for the murine αB-crystallin promoter, and a 3′ oligodeoxynucleotide primer (oligodeoxynucleotide 7576, 5′-CGGTCTGGTTATAGGTACATTGAGC-3′) which was specific for the cat gene. Fo mice containing the transgene were mated to nontransgenic FVB/N mice to obtain F1 offspring, and sibling matings were used to establish homozygous mouse lines. The transgene copy number for each mouse was estimated by hybridization intensity in a slot blot analysis of the genomic DNA relative to the standard samples representing 0–50 copies of the transgene, using the
ECL 3'-Oligo Labeling Detection System (RPN 2130/2131, Amersham) and the 1.6-kbp NdeI/BamHI fragment from pSVO-CAT as the labeled probe. Blots were exposed at room temperature for 1 min.

**Nucleic Acid Isolation**—Plasmid DNA was prepared by the alkaline lysis method (27) followed by ultracentrifugation banding in CsCl-ethidium bromide. For transfection experiments plasmid DNA was isolated and purified using the Qiagen plasmid kit (Qiagen Inc., Chatsworth, CA). Mouse tail DNA was isolated by modification of the procedure of Hogan et al. (25).

**Proteins, Nuclear Extracts, Antiserum, EMSA, and DNase I Footprinting Assays**—Nuclear extracts were prepared from αTN4-1 mouse lens cells (28), L929 fibroblasts (29), N/N1003A rabbit lens cells (30), and adult mouse lenses as described by Shapiro et al. (31). Complementary oligodeoxynucleotides were synthesized (model 380A synthesizer; Applied Biosystems) and annealed at 1:1 molar ratio as described previously (32). Double-stranded oligodeoxynucleotides were labeled on one strand, and EMSAs were performed as described previously (32). Wild-type and mutated versions of an oligodeoxynucleotide containing the sequence −152 to −121 of the αB-crystallin promoter were used for EMSA. For footprinting lens extract was partially purified on a heparin ultrogel A4R column and eluted with 400 mM KCl as described previously (33). Anti-quail-Pax-6 antibody was a gift from Dr. Simon Saule (34).

DNase I footprinting was performed by using the EcoRI-BamHI restriction fragment of pD96 (5) spanning positions −666 to +76 of the αB-crystallin promoter. A polymerase chain reaction-generated fragment corresponding to the −190 to +40 sequence of the αB-crystallin promoter was used for footprinting experiments with the paired domain of purified mouse Pax-6 (PD5 and its alternatively spliced form, PD5a) (12). The Pax-6 GST expression vectors were gifts of Dr. Richard Maas (Harvard Medical School, Boston, MA). End labeling, EMSA, and DNase I footprinting were performed as described previously (32).

**Site-directed Mutagenesis**—Mutations that were generated previously (6) within the −164/+4 EcoRI/PstI fragment of the mouse αB-crystallin gene obtained from pRD30A (5) were used for transfection experiments and EMSAs. In brief, site-specific mutations (Mu-9760 and Mu-9761) (6) were introduced by using an oligodeoxynucleotide-directed mutagenesis kit (Sculptor in vitro mutagenesis kit, Amersham). Mutagenic oligodeoxynucleotides contained the substitution sequence TCTAGA (XbaI site) and 20 bases on each side complementary to the αB-crystallin promoter sequence. The resulting mutated restriction fragment of pRD28 was digested with BamHI and the lower strand was 5' end-labeled with [γ-32P]dATP, using T4 polynucleotide kinase. Partially purified nuclear extracts were used from αTN4-1 lens cells (28) or adult mouse lenses, as indicated in the figure legend. The level of CAT activity was taken as an indirect measure of promoter strength.

**Lens Expression of αB-crystallin Gene.** Footprinting is shown for the lower (antisense) strand of the EcoRI-BamHI fragment (−666 to +76) of pRD28 (5). pRD28 was digested with BamHI and the lower strand was 5' end-labeled with [γ-32P]dATP, using T4 polynucleotide kinase. Partially purified nuclear extracts were used from αTN4-1 lens cells (28) or adult mouse lenses, as indicated in the figure legend (26). The level of CAT activity was taken as an indirect measure of promoter strength.

**Cell Culture, Transfection, and Enzyme Assay**—Mouse COP-8 fibroblasts (35) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum and 5 μg/ml of gentamicin in 10% CO2. The cells were propagated on 60-mm diameter
RESULTS

Production of Transgenic Mice—Three constructs of αB-crystallin promoter-cat fusion genes (Fig. 1) were used as transgenes in transgenic mice. The αB115-CAT construct does not contain the αB-crystallin muscle-preferred enhancer (–426/–259) and LSR1 (–147/–118). The αB68-CAT construct contains a severely truncated αB-crystallin promoter (–68/–44) lacking any of the previously identified αB-crystallin cis-regulatory elements (32, 36). The αBLSR1(Mu-9760)-CAT construct contains 164 bp of 5′-flanking sequence and a mutated LSR1 sequence (6) (see Fig. 7A).

Polymerase chain reaction analyses of tail DNA were performed from the transgenic mice containing αB115-CAT, αB68-CAT, and αBLSR1(Mu-9760)-CAT constructs, to establish the presence of the transgene. Five of the 21 Fo mice carried the αB115-cat transgene in their genome, 3 out of 29 Fo mice contained the αB68-cat transgene, and 5 out of 37 Fo mice contained the αBLSR1(Mu-9760)-cat transgene. Three lines carrying one integration site (all had multiple copy numbers) of each construct were chosen for further analysis. The transgene data are summarized in Table I.

Expression of the αB-crystallin Promoter-cat Fusion Genes in Transgenic Mice: Evidence for a Proximal Lens-specific Regulatory Region—To investigate the possibility that there are sequences downstream of LSR1 (6) that confer lens-specific αB-crystallin promoter activity, we analyzed the αB115-CAT and αB68-CAT transgenic mice generated above. The transgenic mice were sacrificed and numerous tissues (heart, lung, liver, spleen, kidney, skeletal muscle, brain, and lens) were assayed for CAT activity. The transgenic data are summarized in Table I. Average CAT activities for αB115-CAT, αB68-CAT, and αBLSR1(Mu-9760)-CAT in various tissues are shown in Fig. 2A. All the mice from multiple independent lines (different founders) containing the αB115-cat transgene expressed CAT activity exclusively in the lenses at significantly higher level than the background CAT activity observed in the lenses of nontransgenic mice (see Table I and Fig. 2A). In contrast to the results obtained with the αB115-cat transgene, the mice containing the αB68-cat transgene had no CAT activity in the lens or any other tissue examined (Table I and Fig. 2A) except for mouse #55 in which CAT activity in the lenses was above the background. The low lens activity of the αB68-cat transgene in mouse #55 may be due to its different integration site, which may have reconstructed an adequate binding site for a positive transcription factor. Unexpectedly, αBLSR1(Mu-9760)-cat transgene containing mice did not exhibit CAT activity in any of the tissues analyzed, including the lens (Table I and Fig. 2A). With LSR2 still intact in the αBLSR1(Mu-9760)-CAT construct, a low CAT activity was expected in the lenses of the transgenic mice. Several possibilities to explain this observation are discussed below.

Fig. 2B depicts the average CAT activities in lenses of all the transgenic mice for the present and earlier experiments carrying the αB426-cat (6), αB164-cat (6), αB115-cat, and αB68-cat transgenes. The transgenic mice containing the αB426-cat transgene (which includes the muscle-preferred enhancer) had an average of seven times more CAT activity in the lens than the transgenic mice containing the αB164-cat transgene (which lacks the enhancer). For the αB115-cat transgene (which lacks both enhancer and LSR1) there was an additional 50-fold decrease in average CAT activity in the lens. Finally there was no CAT activity in the lenses of the transgenic mice harboring the αB68-cat transgene. These data indicate that the sequences downstream of –115 contribute to the lens expression of the αB-crystallin gene. Sequences downstream of –115 contain a motif highly conserved in the human, mouse, and duck αB-crystallin promoter, previously called Block 2 (37), which we rename here LSR2 for lens-specific region 2. Taken together, these experiments demonstrate that the minimum lens-specific promoter for the αB-crystallin gene is sequence –115 to +44 and that the –164/–115 sequence enhances activity of the αB promoter in a lens-specific fashion.

DNase I Footprinting with Lens Nuclear Extract and Pax-6 PD—We next performed DNase I footprinting experiments in order to localize lens nuclear protein binding sites in the αB-promoter. A DNase I footprint obtained with a partially pu-
indicate matching of the nucleotides with the consensus binding site for Pax-6.

Positions -106/-142 of the upper (sense) strand were footprinted by PD5 (Fig. 4). Positions -164 to -120 (LSR1), -72 to -28 (LSR2) and -70 to -7 of the αB-crystallin 5’-flanking region were protected from DNase I digestion (Fig. 3). Since we noted sequence similarities between the footprinted regions and binding sites for Pax-6 (23), we examined the possibility that the paired domain of Pax-6 (PD5) can bind to the DNase I protected regions. A DNase I protection assay of the 190/140 fragment revealed that the regions covering LSR1 (-160/-131), LSR2 (-97/-27), and a sequence in exon 1 (+2/+29) on the upper (sense) strand were footprinted by PD5 (Fig. 4). Positions -164 to -120 (LSR1), -72 to -28 (LSR2) and +2 to +27 (in exon 1) of the lower (antisense) strand were also protected from DNase I digestion by PD5. There were some differences in the footprinted regions obtained with PD5 and its alternatively spliced form, PD5a (PD of Pax-6 that contains 14 extra amino acids) (12). PD5a did not create a DNase I footprint in either the LSR1 or the LSR2 regions (data not shown). These complexes were almost abolished by self competition (Fig. 6B, lanes 4–6, respectively). These data suggest that the lens nuclear protein, which binds to αB-crystallin promoter via LSR1, is Pax-6 or an antigenically related protein.

A double-stranded radiolabeled LSR2 oligodeoxynucleotide comprising the -78/-28 sequence of the αB-crystallin promoter produced several complexes when incubated with N/N1003A and αTN4-1 nuclear extracts (Fig. 6C, lanes 2 and 9, respectively). These complexes were almost abolished by self competition (Fig. 6C, lanes 3 and 8) and by cross-competition with the LSR1 oligodeoxynucleotide (Fig. 6C, lanes 4 and 7). The formation of several specific complexes with the LSR2 oligodeoxynucleotide is consistent with the presence of multiple Pax-6 binding sites within LSR1. Preincubation of N/N1003A and αTN4-1 nuclear extracts with anti-Pax-6 antibody significantly reduced the formation of the complexes (Fig. 6C, lanes 5 and 6, respectively).
and 10). In a control test, preincubation of N/N1003A lens nuclear extract with anti-E12 antibody (from Pharmingen, San Diego, CA) did not significantly affect complex formation (Fig. 6C, lane 6). GST fused prox-1 (recombinant protein) did not complex with either LSR1 or LSR2 oligodeoxynucleotides (data not shown), confirming the specificity for binding of Pax-6 to these regions.

**Cotransfection with a Pax-6 cDNA Expression Vector**—To test whether Pax-6 can activate the αB-crystallin promoter, transient cotransfection experiments were performed using a Pax-6 cDNA expression vector, pKW10-Pax-6 (39), in COP-8 cells. Site-specific mutations Mu-9760 and Mu-9761 (Fig. 7A) that were generated previously in the −152 to −121 of αB-crystallin promoter was used. The arrow indicates the probe complexed with nuclear protein. B, the binding reactions included αT4-1 nuclear extract (lanes 1–6) plus an αB-crystallin promoter LSR1 oligodeoxynucleotide (−152/−121) (lane 2), 9718/19 oligodeoxynucleotide (lane 3) (19), chicken αA-crystallin oligodeoxynucleotide (−60/−27) (lane 4) (19), H2B2.2 oligodeoxynucleotide (lane 5) (38), or chicken αA-crystallin oligodeoxynucleotide (−177/−133) as a competitor (lane 6). C, 0.1 ng of labeled LSR2 binding site oligodeoxynucleotide containing sequence from positions −78 to −28 was used. The binding reactions included N/N1003A nuclear extract (lanes 1–6) or αT4-1 nuclear extract (lanes 7–10) plus an unlabeled αB-crystallin promoter LSR2 oligodeoxynucleotide (lanes 3 and 8) or unlabeled LSR1 oligodeoxynucleotide (lanes 4 and 7) as competitors.

**Competition EMSAs**—Competition EMSAs were performed to test whether the inability of Pax-6 to stimulate activity of the mutated αB promoters correlates with a corresponding decrease in the ability of the mutated promoters to bind lens nuclear factors. The wild type double-stranded LSR1 oligodeoxynucleotide (−152/−121) produced a complex when incubated with the αT4-1 nuclear extract (Fig. 8A, lane 1, arrow), which was greatly reduced by self-competition (Fig. 8A, lane 2). The mutated LSR1 sequences did not compete to the same extent as the unlabeled wild type LSR1 oligodeoxynucleotide for complex formation with the wild type oligodeoxynucleotide (Fig. 8A, compare lanes 3 and 4 with lane 2). Complex formation was also reduced significantly by competition with LSR2 oligodeoxynucleotide (−78/−28) (Fig. 8B, lane 2). These results provide a positive correlation between protein-DNA complex formation and functional activity of LSR1 and LSR2 within the αB-crystallin promoter.

**DISCUSSION**

Our recent experiments have shown that αB-crystallin gene sequences between −164 and +44 are sufficient to promote...
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FIG. 7. Transfection of αB-crystallin promoter-cat and Pax-6 cDNA constructs in the COP-8 fibroblast cell line. A, DNA sequence of the αB-crystallin promoter used for site-directed mutations. The location of the mutations (TCTAGA, an XbaI site) are shown below the sequence. B, relative CAT levels in COP-8 cells cotransfected with pKW10-Pax-6 (wild type Pax-6 cDNA) and either p65-7 (wild type −164/+44 αB-crystallin promoter fragment fused to cat gene) or the −164/+44 promoter fragment containing the Mu-9760 or Mu-9761 mutation indicated in A. The CAT levels are expressed as a relative response to that obtained by parallel cells cotransfected with pKW10-Pax-6 and pRD30A (the promoterless parent vector) (5). C, relative CAT levels in COP-8 cells cotransfected with pKW10-Pax-6 and p11-3 (wild type −115/+44 αB-crystallin promoter fragment fused to the cat gene) or the truncated Pax-6 cDNA constructs (p309C, p310A, and p312A, see “Experimental Procedures”). CAT levels are relative to parallel tests using pRD30A. Cells were harvested 48 h after DNA removal; CAT activity was determined by the biapptic assay (28) and normalized with respect to the activity of β-galactosidase, which resulted from cotransfection of pH110 (see “Experimental Procedures”).

lsen-specific expression of a heterologous reporter gene in transgenic mice (6). In addition, we have previously identified five distal cis-acting regulatory elements (αBE-1, αBE-2, αBE-3, MRF, and αBE-4) located within the muscle preferred enhancer (−427/−259) of the αB-crystallin gene (5). αBE-1, αBE-2, and αBE-3 are utilized for αB-crystallin enhancer activity in lens cells and in other tissues (32); MRF is essential for enhancer activity in heart and skeletal muscle, and αBE-4 is utilized selectively for enhancer activity in heart (36). Activity of fragment −164 to +44 in the lens depends at least on a regulatory region LSR1 (−147/−118), which is physcically separated from the distal enhancer control elements required for expression in non-lenticular tissues.

The present transgenic mouse experiments demonstrate that even in the absence of LSR1 (for αB115-CAT construct) CAT activity still occurs specifically in lens, while further deletion to −68 of the αB promoter (for αB68-CAT construct) abolishes CAT expression in the lens. Thus, the sequence −115 to +44 is sufficient to generate lens-specific activity of the αB-crystallin promoter. The overall levels of CAT activity were approximately 30-fold lower in the lenses of the αB115-CAT lines than in the lenses of the transgenic mice harboring the αB164-αB-crystallin transgene (6), indicating that LSR1 increases αB-crystallin promoter activity in the lens. In the present studies using DNase I footprinting, transient transfection, and transgenic mice we have identified a lens-specific regulatory region (LSR2) between positions −78 and −46 of the αB-crystallin promoter. LSR2 is located in a highly conserved region of the mouse αB-crystallin promoter (37), and truncation of this site eliminates promoter activity in transgenic mice. Mutations in LSR1 also strongly reduce or eliminate promoter activity in transfected lens cells (6) and transgenic mice. Taken together, these experiments indicate that LSR2 is sufficient for lens expression of αB-crystallin, but for high lens expression of the αB-crystallin gene, coupling between the enhancer elements (αBE-1, αBE-2, αBE-3), and lens-specific cis-regulatory regions (LSR1 and LSR2) is required. This coupling may be facilitated by the ability of Pax proteins to bend DNA (40).

Unexpectedly, the construct lacking LSR1 (αB115-CAT) exhibited CAT activity in the lenses of the transgenic mice, while the construct containing a mutation in LSR1, but with an intact LSR2, was not active in the lens. Several possibilities can be considered to explain this apparent inconsistency. First, it is possible that a repressor binding site was created in the LSR1-mutated construct that resulted in the binding of a transcription inhibitor. Second, there may be a transcription inhibi-

FIG. 8. A, EMSA and competition analyses of protein-DNA interaction using a nuclear extract of αTN4-1 lens cells and 32P-labeled LSR1 oligodeoxynucleotide (−152/−121). Free and protein-complexed species were resolved by 5% polyacrylamide gel electrophoresis; competitions were performed with unlabeled double-stranded self-oligodeoxynucleotide (lane 2) or mutant oligodeoxynucleotides (lanes 3–5). Mutants (Mu-9760 and Mu-9761) are described under “Experimental Procedures.” The arrow indicates the specific complex. B, competitions were performed with unlabeled double-stranded LSR2 oligodeoxynucleotide (lane 2) or with a self-oligodeoxynucleotide (lane 3).
tor binding site present between the sequences −164 and −115, normally deactivated by LSR1, which was deleted in the smaller construct αB115-CAT. Third, in addition to Pax-6, LSR1 may bind to another factor that acts as an activator when Pax-6 is also bound to LSR1 and as a repressor in the absence of Pax-6, making LSR1 a composite regulatory element (41). Further experiments are required in order to distinguish among these or other possibilities.

The present data add to the possibility that the use of Pax-6 for crystallin transcription may be a conserved mechanism to regulate gene expression in the lens (23). Pax development is a multistep process that starts at gastrulation and the major inductive events that determine the ectoderm to become lens occur during neurulation (42). Pax-6 is expressed in the presumptive lens cells of the ectoderm overlying the outgrowing optic vesicle (9), prior to the beginning of mouse lens formation and expression of the crystallins. The expression of Pax-6 in lens continues during development well after αB-crystallin expression commences (9, 43). In addition to the αB-crystallin gene, Pax-6 has been shown to bind and activate the chicken αA (20, 19), chicken αΔ (21) and guinea pig γ (22) crystalline gene regulatory regions. The exact mechanism of action of Pax-6 in mouse αB-crystallin expression is still unknown. One cannot exclude the possibility that in addition to Pax-6 other factors may be used for lens-specific expression of αB-crystallin (via LSR1 and LSR2) such as SOX proteins (44–46) and retinoic acid receptors (47–49). Indeed both SOX-2 (47) and Pax-6 (21) appear to interact to activate the δ1-crystallin enhancer in the lens, and both SOX and retinoic acid receptors (50, 51) are used for lens-specific activity of the murine γ-crystallin promoter. Our preliminary experiments suggest that retinoic acid receptors may also contribute to the regulation of the αB-crystallin promoter in the lens. Clearly further experiments are necessary to delineate the spectrum of transcription factors that are used for activating the αB-crystallin gene in the lens and to determine whether Pax-6 is used for expression of the multifunctional αB-crystallin/small heat shock protein gene in non-lens tissues or under conditions of physiological stress (52).

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