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Expression of the Murine αB-Crystallin Gene in Lens and Skeletal Muscle: Identification of a Muscle-Preferred Enhancer

ROBERT A. DUBIN,[1]† RASHMI GOPAL-SRIVASTAVA,[1] ERIC F. WAWROUSEK,[2] AND JORAM PIATIGORSKY[1]*

Laboratory of Molecular and Developmental Biology, National Eye Institute, Bethesda, Maryland 20892,[1] and Research and Development, SmithKline Beecham, King of Prussia, Pennsylvania 19406[2]

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The αB-crystallin gene is expressed at high levels in lens and at lower levels in some other tissues, notably skeletal and cardiac muscle, kidney, lung, and brain. A promoter fragment of the murine αB-crystallin gene extending from positions −661 to +44 and linked to the bacterial chloramphenicol acetyltransferase (CAT) gene showed preferential expression in lens and skeletal muscle in transgenic mice. Transfection experiments revealed that a region between positions −426 and −257 is absolutely required for expression in C2C12 and G8 myotubes, while sequences downstream from position −115 appear to be determinants for lens expression. In association with a heterologous promoter, a −427 to −259 fragment functions as a strong enhancer in C2C12 myotubes and less efficiently in myoblasts and lens. Gel shift and methylation interference studies demonstrated that nuclear proteins from C2C12 myoblasts and myotubes specifically bind to the enhancer.

Crystallins comprise approximately 90% of the water-soluble proteins of the transparent eye lens and are important for its optical properties (52, 53). The three dominant classes of mammalian crystallins (α, β, and γ) contain two or more related polypeptides ranging in size from approximately 20 to 30 kDa and display regulated spatial and temporal expression within the lens (38, 52). Additional crystallins, the taxon-specific crystallins, exhibit a more restricted phylogenetic distribution; they are closely related or identical to common metabolic enzymes and are expressed at low concentrations outside the lens (11, 39).

The two α-crystallin proteins, αA and αB, have been the subject of extensive structural, biophysical, and evolutionary studies. αA- and αB-crystallin share approximately 55% sequence identity at the amino acid level (49), and mammalian α-crystallin genes exhibit conservation at the level of gene structure (12, 22, 42, 48). αA- and αB-crystallin are distantly related to an egg antigen of *Schistosoma mansoni* (35) and the small heat shock proteins of *Drosophila melanogaster* (19), and both the α-crystallins and small heat shock proteins are found associated with high-molecular-weight aggregates and may be phosphorylated (30). The two α-crystallin genes presumably arose from a gene duplication event, originating from an ancestor of a small heat shock gene.

Despite these similarities, the two α-crystallin genes are expressed differently. The αA-crystallin gene is expressed only in the lens (13, 37, 50). In contrast, the αB-crystallin gene, while expressed abundantly in lens, is also expressed at reduced levels in a variety of other tissues, including heart, skeletal muscle, kidney, lung, brain, retina, and iris (3, 13, 20, 21). αB-crystallin expression has been associated with a variety of pathological and experimental conditions, notably Alexander's disease (21) and Lewy body disease (31) in humans, scrapie infection in hamsters (14), and osmotic stress in certain cultured cells (10). In addition, it has recently been demonstrated that αB-crystallin is induced by oncogenes as well as heat shock in NIH 3T3 cells (24–26).

Characterization of the *cis*-acting elements and *trans*-acting nuclear proteins regulating expression of the αA-crystallin gene has demonstrated that a 134-bp fragment extending from positions −88 to +46 of the murine αA gene is sufficient for proper tissue-specific and developmental expression of a reporter gene in transgenic mice (50). Moreover, the murine αA-crystallin promoter contains an element between positions −66 to −57 which binds a zinc finger protein homologous to that involved in the regulation of the human β-interferon, major histocompatibility complex class I, and immunodeficiency virus genes in a transformed mouse lens cell line (34). A 319-bp fragment of the chicken αA promoter confers lens-specific expression in transgenic mice, and a region between −162 and −121 both is essential for expression in cultured chick lens cells and binds chick lens nuclear protein (23). We have been studying the murine and human αB-crystallin genes in an attempt to define the molecular basis underlying its complex expression pattern. It was previously demonstrated that a murine αB-crystallin minigene, containing 666 bp upstream of the transcription initiation site and approximately 2,400 bp of 3′ flanking sequences and lacking introns and some coding sequences, was expressed in a manner very similar to that of the endogenous αB gene in transgenic mice (13). In addition, transfection studies suggested that the murine αB promoter may be sufficient for expression in cultured lens cells (13), and the human αB promoter was preferentially, but not exclusively, functional in lens (12). We demonstrate here that the murine αB promoter (−661 to +44) confers lens- and skeletal muscle-preferred expression upon a reporter gene in transgenic mice. In addition, transfection experiments showed that sequences between positions −426 and −257 are essential for expression in two muscle cell lines, but not in lens cells, and that this upstream sequence functions as a muscle-preferred enhancer capable of binding nuclear proteins from a cultured muscle cell line.

* Corresponding author.
† Present address: Department of Pediatrics, Division of Genetics, New York University Medical Center, New York, NY 10016.

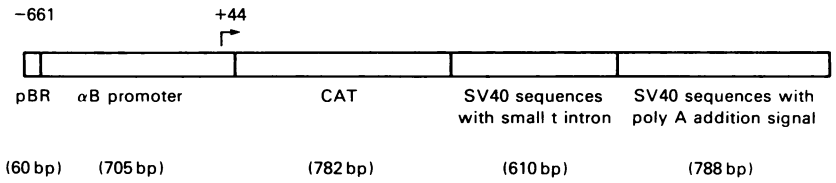


FIG. 1. The αB crystallin promoter (−661 to +44)/CAT chimeric transgene. An approximately 3,000-bp *Bgl*II-*Pst*I fragment isolated from pD96B2 containing the −661 to +44 fragment of the murine αB-crystallin gene linked to the bacterial CAT gene was used to generate transgenic mice (see Materials and Methods). Approximately 60 bp of pBR sequences are present 5′ to the CAT gene, and approximately 1,400 bp of simian virus 40 (SV40) sequence, including the small-t intron and poly(A) addition signal, are present 3′ to the CAT gene.

MATERIALS AND METHODS

Plasmid constructions. Plasmid pRD25 (13), containing a *Pst*I-*Bam*HI fragment extending from −666 to +76 of the murine αB-crystallin gene, was restricted with *Bam*HI, treated with Bal 31 (Bal 31-slow form; International Biotechnologies, Inc., New Haven, Conn.), repaired by using T4 DNA polymerase (Bethesda Research Laboratories, Gaithersburg, Md.) and deoxynucleoside triphosphate, and ligated (T4 DNA ligase; Bethesda Research Laboratories) in the presence of *Bam*HI (8-mer) linkers (Pharmacia, Piscataway, N.J.). Transformants were characterized, and one plasmid (pRD25BAL23) containing a *Bam*HI linker after nucleotide +44 (just upstream of the initiating ATG codon) was identified. This new plasmid was restricted with *Pst*I, the ends were repaired as described above, the plasmid was ligated in the presence of *Bam*HI linkers, and pRD25BAL23P+L, containing a 721-bp fragment extending from −661 to +44 of the murine αB-crystallin gene flanked by *Bam*HI linkers at both ends, was isolated. A series of 5′ unidirectional deletion fragments was created by restricting pRD25BAL23P+L with *Kpn*I and *Eco*RI (which cut just upstream of −661), treating the plasmid with exonuclease III and S1 nuclease (Promega, Madison, Wis.), repairing the ends, and ligating the plasmid in the presence of *Bam*HI linkers. Previously, a modified version of pSV0CAT (pRD30A) that could accept *Bam*HI fragments upstream of the chloramphenicol acetyltransferase (CAT) gene was constructed. Briefly, pRD30A was constructed by deleting a *Bam*HI site located downstream of the CAT gene and inserting a *Bam*HI linker at a *Hin*dIII site located just upstream of the CAT gene (12, 17). αB promoter-CAT plasmids were constructed by ligating *Bam*HI promoter fragments (extending from multiple upstream sites and all ending at +44) into pRD30A. Plasmid pD96 contained the largest promoter fragment, extending from −661 to +44, linked to the CAT gene. Plasmid pD96B2 was constructed by restriction of pD96 at a unique *Nde*I site (located approximately 50 bp upstream of the αB promoter), end repair, and ligation in the presence of *Bgl*II (8-mer) linkers. The reasons for originally requiring a *Bgl*II restriction site at this position are not germane to this work; however, its construction is described here since the αB-CAT fragment used to generate transgenic mice was derived from pD96B2.

Progressive 5′ or 3′ nested deletion fragments of this promoter were isolated as *Hin*dIII fragments by using pRD25BAL23P+L and pRD25, respectively, and methods (exonuclease III-S1) described above. 5′ deletion fragments extended variable lengths 5′ from −41, and 3′ deletion fragments extended variable lengths 3′ from −666; the 3′ deletion fragments were followed by an 18-bp polylinker from pBluescript just upstream of −666, which provided one *Hin*dIII site. By ligation of carefully chosen 5′ and 3′ deletion fragments at a unique *Sph*I site (located between −331 and −326), a fragment extending from −427 to −259, with *Hin*dIII linkers on both ends, was isolated. Plasmid pTKGH (45; Nichols Institute, San Juan Capistrano, Calif.) contains the human growth hormone (hGH) gene driven by the herpes simplex virus thymidine kinase (TK) promoter and has a unique *Hin*dIII site approximately 15 bp upstream of the promoter. Plasmids pTKGHΔHSAC2+H and pTKGHΔHNSI28+H are derivatives of pTKGH and were constructed by first removing the *Hin*dIII site within pTKGH (by restriction, fill-in, and ligation), followed by the addition of *Hin*dIII linkers at either the unique *Sac*I site within intron II (pTKGHΔHSAC2+H) or the unique *Nsi*I site located approximately 220 bp downstream from the end of the hGH transcript (pTKGHΔHNSI28+H). *Hin*dIII fragments isolated from the αB upstream region were tested for enhancer activity after being ligated, in both orientations, into the three hGH reporter gene plasmids at the unique *Hin*dIII site. All constructs were confirmed by restriction digestion and sequencing of the junctions performed as previously described (13) or by using Sequenase (U.S. Biochemical Corp., Cleveland, Ohio) and [α-^{35}S]dATP (1,000 Ci/mmol; Amersham).

Transgenic mice. An approximately 3,000-bp *Bgl*II-*Pst*I fragment containing the murine αB-crystallin promoter (−661 to +44)/CAT chimeric gene (isolated from pD96B2; Fig. 1) was isolated and injected into fertilized mouse egg pronuclei from strain FVB/N; transgenic mice identified by hybridization of tail DNA to transgene-specific probes (13) and nine founders were chosen for further study. F_1 hemizygous offspring were obtained by mating to nontransgenic FVB/N mice. From each line (except line 13, which produced only one transgenic offspring), two transgenic F_1 mice were analyzed further for CAT activity. Mouse tissues were extracted and treated as described by Wawrousek et al. (50) except that the extraction buffer was at pH 8.0.

Cell culture and transfection. Primary patched lens epithelial cells (PLE) were prepared from 14-day-old chicken embryos, and both PLE and the rabbit lens cell line N/N1003A (43) were propagated in Dulbecco's modified Eagle's medium (DMEM; GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum (GIBCO) and 50 μg of gentamicin per ml (regular medium) in 10% CO_2. Muscle cell lines C2C12 (4) and G8 (8; American Type Culture Collection, Rockville, Md.) were maintained and transfected as myoblasts by growth in DMEM plus 20% fetal bovine serum and 50 μg of gentamicin per ml (proliferative medium) in 5% CO_2. Myotube differentiation was induced by replacing the medium with DMEM containing gentamicin and 5% horse serum (differentiating medium). PLE were propagated on collagen-coated plastic, and other cells were grown on plastic. For CAT plasmid and hGH plasmid

transfections, 10 and 4 μg, respectively, of test plasmid and 2 μg of an internal control plasmid were cotransfected as $CaPO_4$ precipitates as described in Dubin et al. (13). Internal control plasmids containing the β-galactosidase gene (pTB.1 for chicken cells and pCH110 for other cells) are described by Dubin et al. (12). Following DNA removal, myoblasts were glycerol shocked for 1 min at room temperature in 15% glycerol–proliferative medium; after a recovery period of about 2 h in proliferative medium, they were refed with either differentiating medium (for CAT assays) or fresh proliferative medium (for hGH assays). For CAT transfections, lens and muscle cells were harvested approximately 31 and 55 h, respectively, following DNA removal. Specific growth conditions used for transfections with hGH plasmids are described in the legends to Fig. 5 and 6. All transfection data represent the means of three or more experiments except those presented in Fig. 5, which are from assays performed in duplicate.

Gel mobility shift and methylation interference analyses. Nuclear extracts were prepared from 11- to 14-day-old embryonic chicken lenses and C2C12 myotubes as described by Sommer et al. (46). Complementary oligonucleotides were synthesized (Model 380A synthesizer; Applied Biosystems, Foster City, Calif.), purified by polyacrylamide gel electrophoresis (23), and annealed at a 1:1 molar ratio in 100 mM Tris-HCl (pH 7.5)–300 mM KCl–1 mM EDTA for 2 min at 100°C and then for 15 min each at 68°C, 37°C, room temperature, and finally 4°C. Double-stranded oligonucleotides extended from −426 to −371 (E1), −370 to −315 (E2), and −314 to −258 (E3) and were labelled on one strand by using polynucleotide kinase and [α-^{32}P]ATP (7,000 Ci/mmol; ICN, Costa Mesa, Calif.). A *Hin*dIII fragment extending from −427 to −259 was labelled with Klenow fragment and [α-^{32}P]dGTP (3,000 Ci/mmol; Amersham) and purified by electrophoretic transfer to DEAE-paper (9). Gel mobility shift assays were performed as described previously (46), using 0.5 to 1 ng of labelled DNA, 1 μg of poly(dI-dC), 20 mM KCl, 1 mM $MgCl_2$, 2 mM dithiothreitol, and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9). For methylation interference, end-labelled double-stranded E2 was partially methylated at G residues as described previously (32) except that the dimethyl sulfate reaction was for 5 min and was quenched with 0.26 M sodium acetate (pH 7.0)–0.17 M 2-mercaptoethanol–10 μg of yeast tRNA. Binding, mobility shift electrophoresis to separate free from bound fragments, and analysis of piperidine cleavage products were performed according to Sommer et al. (46) except that DNA was eluted from the gel by electrophoretic transfer to DEAE-paper.

Enzyme assays. CAT activity was determined by the methods of Gorman et al. (17), using [^{14}C]chloramphenicol (60 mCi/mmol; Amersham), and Neumann et al. (36), using [^{3}H]acetyl coenzyme A (200 mCi/mmol; New England Nuclear, Boston, Mass.). Transfected cells were analyzed for CAT by the [^{3}H]acetyl coenzyme A technique, and transgenic mice were analyzed by both methods. When the method of Gorman et al. was used, CAT activity was quantitated by cutting out the spots from the thin-layer chromatogram and subjecting them to scintillation counting. To control for variation in transfection efficiencies, CAT and hGH levels were normalized to β-galactosidase levels. hGH was measured by using a commercially available radioimmunoassay kit (Nichols Institute) as instructed by the manufacturer. β-Galactosidase activity and total protein (measured by using a Bio-Rad kit; Bio-Rad Laboratories, Richmond, Calif.) were determined as described by Dubin et al. (12) and Bradford (5), respectively.

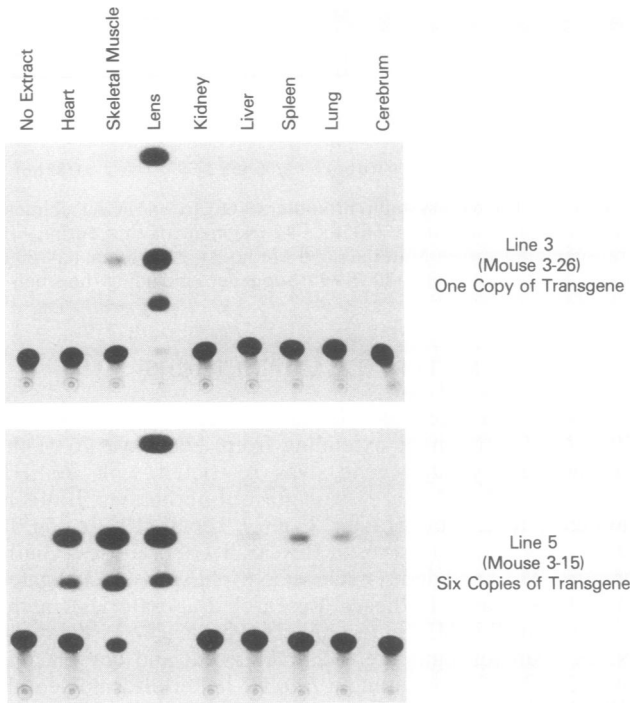


FIG. 2. Analysis of CAT activity in transgenic mice. Tissues from F_1 hemizygous transgenic mice were extracted, and either 5 μg (for lines containing multiple copies of the transgene) or 20 μg of protein was assayed for CAT activity (17). Representative samples from lines 3 and 5 are shown.

RESULTS

Expression of a murine αB-crystallin promoter (−661 to +44)/CAT chimeric gene in transgenic mice. Previously, expression of the murine αB-crystallin gene was examined by using minigene constructs containing both 5′ and 3′ αB flanking sequences (13). To restrict our analysis to regulatory elements present only within the promoter, an approximately 3,000-bp chimeric gene containing a promoter fragment extending from −661 to +44 of the murine αB-crystallin gene linked to the CAT gene was introduced into the germ line of mice by microinjection (Fig. 1). Transgenic mice were identified, and nine founders were chosen for further study. Southern blot analysis of DNA isolated from transgenic, F_1 hemizygous progeny revealed that lines 3 and 49 contained 1 copy of the transgene, lines 13, 46, and 113 carried 1 intact and 1 partial, linked transgene, lines 5 and 1 carried, respectively, 6 and 12 (apparently tandem) copies, and two lines contained rearranged transgenes (data not shown).

Transgene expression was determined after extraction of tissues from approximately 2-month-old transgenic F_1 hemizygous progeny and analysis for CAT activity. The level of CAT activity produced was taken as an indirect measure of promoter strength. As expected, no CAT activity was detected in mice with rearranged transgenes (data not shown). In the seven lines with an intact transgene(s), very high CAT activity was always observed in lens (Fig. 2 and Table 1). In the five mice lines containing only one complete copy of the

TABLE 1. CAT activity[a]

Tissue	CAT activity (U)						
	Line 1	Line 5	Line 3	Line 13	Line 46	Line 49	Line 113
Lens	750	224	261	291	116	92	134
Skeletal muscle	6	89	2	0.3	2	2	1
Heart	29	30	0	0.04	0.04	0.02	0.02
Kidney	0.5	6	0	0.03	0	0.8	0
Liver	0.3	1	0	0	0	0.07	0
Spleen	0.3	4	0	0	0.1	0.6	0.02
Lung	2	2	0	0.06	0	0.2	0
Cerebrum	0.2	0.6	0	0	0	0	0
Cerebellum	ND	1	0	ND	ND	ND	ND
Medulla	ND	0.8	0	ND	ND	ND	ND

[a] For all tissues except lens, CAT activity was determined by scintillation counting of excised thin-layer chromatography spots (17). For each tissue, 5 μg of protein for lines 1 and 5 and 20 μg of protein for the other lines were used. Since CAT activity in lens had passed the linear range for this assay, lens and skeletal muscle extracts were reassayed by the method of Neumann et al. (36) and used to estimate the CAT level in lens. ND, not determined.

transgene (lines 3, 13, 46, 49, and 113), low CAT activity was consistently observed in skeletal muscle; in the two multicopy mice lines (lines 1 and 5), significant CAT activity was detected in both skeletal and cardiac muscle (Fig. 2 and Table 1). Low or trace levels of CAT were also detected in some other tissues of some of the mouse lines (Table 1). For example, low CAT levels were observed in all other tissues of mice from line 5; in mice from line 49, low activity was observed in kidney, spleen, and lung (Table 1). While transgene expression in tissues other than lens and skeletal muscle was sometimes observed, these patterns were apparently unique to each line. It was concluded that although the murine αB-crystallin promoter (−661 to +44) does not contain sufficient information for proper regulation of this gene, it is sufficient to confer preferential expression of a reporter gene to lens and skeletal muscle.

5′ deletion analysis of the αB-crystallin promoter in transfected lens and skeletal muscle cells. Results from the transgenic experiments described above led to further analysis of the αB promoter in cultured lens and muscle cells. Progressive 5′ deletion fragments (all ending at +44) were generated within this promoter, linked to the CAT gene, and introduced by transfection into the mouse myogenic cell lines C2C12 and G8, the rabbit lens cell line N/N1003A, or primary embryonic chicken lens cells (PLE). Cultured primary chicken lens cells and the rabbit lens cell line synthesize αB-crystallin RNA (12). We have also demonstrated that while C2C12 myoblasts accumulate only very low levels of αB RNA, this level increases significantly in myotubes (Fig. 3); also, αB RNA in N/N1003A lens cells and C2C12 myotubes initiates at the same site as in mouse lens (data not shown).

Plasmids were introduced into C2C12 or G8 myogenic cells, myotube differentiation was induced, and the cells harvested and analyzed for CAT activity. As seen in Fig. 4, sequences between positions −426 and −257 are essential for expression in myotubes. Deletion between −426 and −339 reduces promoter activity 10-fold, and further deletion between −314 and −257 results in an additional 3- to 4-fold decrease, suggesting that this region contains two separate elements controlling expression in myotubes. In contrast, progressive deletion of this region has only a slight effect on promoter activity in lens. While upstream sequences may be required for maximal expression of the αB promoter in lens, a fragment as small as −115 to +44 appears to function, albeit at low levels, in cultured lens cells.

A muscle-preferred enhancer lies between positions −427 and −259. Since deletion analysis of the αB promoter defined an upstream region essential for expression in muscle but not lens, we chose to examine whether this region shared characteristics with transcriptional enhancers. Progressive 5′ or 3′ deletion fragments from the upstream region of the αB gene were placed just upstream of the herpes

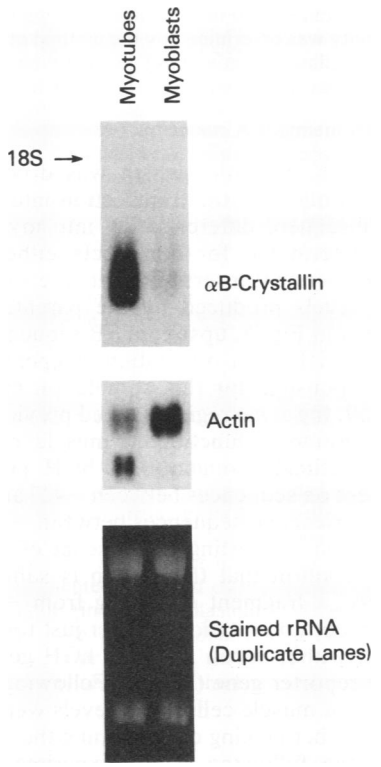


FIG. 3. Induction of αB-crystallin RNA during myogenesis. Total RNA was isolated from C2C12 myoblasts and myotubes, and 20 μg from each was fractionated by electrophoresis through a formaldehyde-agarose gel, transferred to Nytran, and hybridized to a murine αB-crystallin exon 3-specific probe (pRD22) as previously described (12, 13). RNA integrity was confirmed by reprobing the filter with a human β-actin cDNA and examination of ethidium bromide-stained RNA electrophoresed on duplicate lanes. Additionally, methylene blue staining of the Nytran filter subsequent to hybridization further confirmed RNA integrity and transfer (not shown).

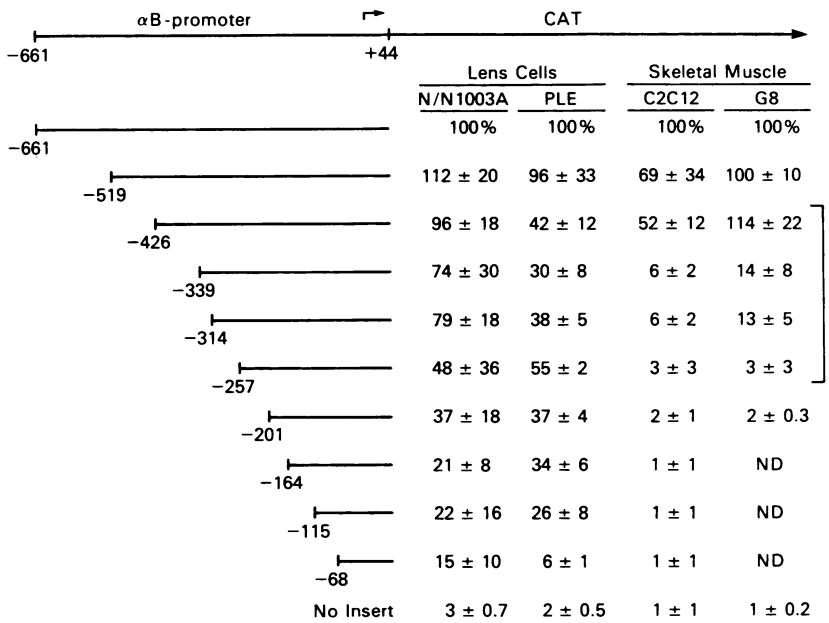


FIG. 4. 5′ deletion analysis of the αB-crystallin promoter in lens and skeletal muscle. Progressive 5′ nested deletion fragments of the murine αB-crystallin gene (all extending to +44) were linked to the CAT gene (in pRD30A) and introduced by transfection into the rabbit lens cell line N/N1003A, primary chicken embryonic lens cells (PLE), and skeletal muscle cell lines C2C12 and G8. Lens cells were harvested 31 h following DNA removal. Muscle cells were transfected as myoblasts in proliferative medium; following DNA removal and a brief recovery period, cells were induced to differentiate by being transferred to differentiation medium, and myotubes were harvested approximately 55 h later. CAT activity was determined by the method of Neumann et al. (36) and normalized to the activity of the cotransfected, internal control marker β-galactosidase. Relative CAT levels (± standard deviations) are expressed as a percentage of the levels expressed by the plasmid containing the largest promoter fragment (−661 to +44). ND, not determined.

simplex virus TK promoter which was driving the hGH reporter gene. Following the transfection into C2C12 myoblasts and subsequent differentiation into myotubes, hGH levels were determined for constructs either lacking or containing αB fragment inserts. Results are expressed relative to hGH levels produced by the parental (insertless) vector. As seen in Fig. 5, upstream αB sequences stimulate production of hGH in an orientation-independent manner. The region responsible for this stimulation maps between −427 and −259, the same region defined previously as being essential for promoter function in muscle cells (Fig. 4). Additionally, optimal stimulation of hGH production appears dependent on sequences between −427 and −335 (Fig. 5; 5′ deletion series) and sequences between −259 and −316 (3′ deletion series), suggesting the presence of two separable elements. To confirm that this region is sufficient for enhancer activity, a fragment extending from −427 to −259 was placed, in both orientations, either just upstream of the TK promoter, within intron II of the hGH gene, or downstream of the reporter gene (Fig. 6). Following transfection into either lens or muscle cells, hGH levels were determined for constructs either lacking or containing the −427 to −259 fragment. In the following set of experiments, we took advantage of the fact that the hGH gene product is secreted into the growth medium. It was thus possible to examine hGH secretion rates at early and late time points following DNA transfection, which proved particularly useful in studying differential effects during myogenesis. The new growth and collection protocol is fully described in the legend to Fig. 6.

Analysis of hGH levels at an early time point following transfection into C2C12 myoblasts revealed that whereas a significant amount of product was secreted by cells containing the parental plasmid (generally 50 to 250 ng/ml/24 h), only a two- to eightfold stimulation was produced by plasmids containing the insert. Following differentiation into myotubes, the rate of hGH secretion by the parental plasmid was substantially reduced (approximately 1 ng/ml/24 h), whereas that of the insert-containing plasmid remained high (30 to 180 ng/ml/24 h). While the reason for the sharp decline in secreted hGH by myotubes transfected with pTKGH remains unknown, it may be related to TK promoter function at this stage of muscle differentiation, a suppressor activity associated with the hGH gene, a decreased ability of myotubes to secrete protein, or a combination of these factors. Whatever the reason, these results strongly suggest that the αB upstream fragment has only a slight effect in myoblasts but a significant stimulatory effect in differentiated myotubes. Similar results were obtained independent of the orientation or position of the insert (Fig. 6), suggesting that this fragment shares properties with transcriptional enhancers.

In contrast, the −427 to −259 fragment had only a slight stimulatory effect in lens cells. Stimulation by this fragment at early (not shown) and late (Fig. 6) time points was about the same, approximately 5- to 10-fold in N/N1003A cells and even less in chicken PLE. Further, transfection and analysis of N/N1003A cells following propagation in the proliferative and differentiative media used for C2C12 cells similarly revealed relatively low (fivefold) enhancer stimulation (not shown).

Examination of the nucleotide sequence of the muscle-preferred enhancer fragment reveals a number of sequence motifs that may potentially bind nuclear proteins regulating transcription of the αB-crystallin gene (Fig. 7A). A nearly exact heat shock consensus element lies between positions

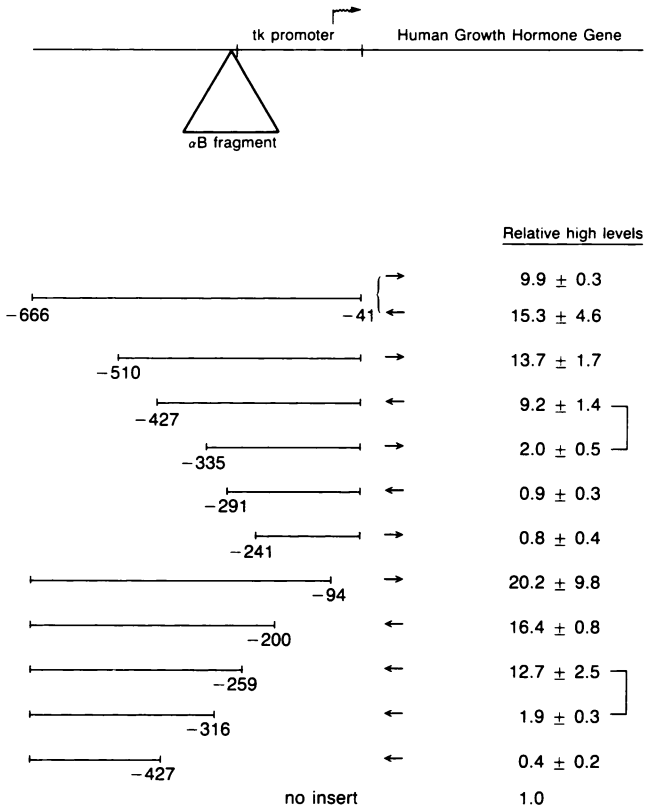
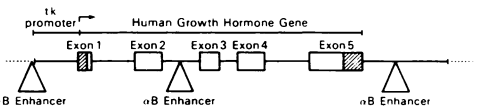


FIG. 5. Enhancer activity within upstream sequences of the αB-crystallin gene. A fragment extending from −666 to −41 or unidirectional 5′ or 3′ deletion fragments of the murine αB-crystallin gene were inserted at the *Hin*dIII site 12 bp upstream of the TK promoter in pTKGH; the αB-crystallin TATA box between −28 and −22 is not on these insert fragments. Plasmids were introduced into C2C12 myoblasts by transfection; following DNA removal and a brief recovery period, cells were refed with 5 ml of differentiative medium to stimulate myotube formation. Approximately 53 h later, both medium and myotubes were separately collected. Medium was directly assayed for hGH levels; myotube lysate was analyzed for β-galactosidase activity per microgram of protein and used to normalize for transfection efficiency. Relative hGH levels (± standard deviations) are presented as a fold over levels produced by the parental plasmid pTKGH. Arrows indicate insert orientation.



cell type	insert			
N/N1003A	←	8 ± 2	7 ± 3	4 ± 1
(Lens)	→	9 ± 3	5 ± 1	5 ± 2
	none	1	1	1
PLE	←	4 ± 2	2 ± 2	
(Lens)	→	3 ± 1	0.4 ± 0.4	ND
	none	1	1	
C2C12	←	3 ± 1	8 ± 3	2 ± 1
(myoblasts)	→	3 ± 1	6 ± 4	3 ± 2
	none	1	1	1
C2C12	←	58 ± 9	226 ± 26	17 ± 11
(myotubes)	→	85 ± 24	141 ± 54	67 ± 38
	none	1	1	1

FIG. 6. Analysis showing that the −427 to −259 fragment functions as a muscle-preferred enhancer. A fragment isolated from the murine αB-crystallin gene and extending from positions −427 to −259 was inserted, in both orientations, just 5′ to the TK promoter in pTKGH, within intron II of the hGH gene in pTKGHΔHSAC2+H, or downstream of the hGH gene in pTKGHΔHNSI28+H and introduced by transfection into either lens or muscle cells (C2C12 myoblasts). Following DNA removal and a brief recovery period, cells were refed with 5 ml of either fresh regular medium (lens cells) or fresh proliferative medium (C2C12 myoblasts); 24 h later, the medium was removed and stored at −20°C and the cells were fed with either fresh regular medium (lens cells) or differentiation medium (C2C12 cells). After an additional 48 h (by which time C2C12 cells had differentiated into myotubes), the medium was removed and cells were refed with 5 ml of either fresh regular medium (lens) or differentiation medium (C2C12 myotubes); both the medium and cells were separately collected after an additional 24-h incubation. Medium from both 24-h collection periods was assayed for hGH, and transfection variation was normalized to the level of β-galactosidase activity per microgram of protein. Relative hGH levels are presented as a fold over the level produced by the appropriate parental (insertless) plasmid (± standard deviations). For C2C12 cells, both early (myoblast) and late (myotube) time points are presented; for lens cells, the levels for early and late time points were very similar and only the late time point is presented.

−388 and −374, and an AP2-like sequence can be found between positions −338 and −330 (12). With respect to our results, it is notable that a potential binding site for the muscle regulatory factors (MRFs) MyoD1, myogenin, Myf-5, and MRF4 (6, 27, 29, 33, 40, 47) resides within this region between positions −300 and −287.

Binding of muscle nuclear proteins to the muscle-preferred enhancer. Using C2C12 nuclear extracts combined with gel mobility shift and methylation interference analyses, we have identified nuclear factors interacting with the upstream, αB-crystallin muscle-preferred enhancer. As seen in Fig. 7B, incubation of a radiolabelled fragment (−427 to −259) with myotube nuclear extract followed by electrophoresis revealed a more slowly migrating band, suggesting binding of some factor(s) to the fragment. Binding was specifically localized to the middle of the fragment, since the more slowly migrating species was successfully competed for with excess, unlabelled, double-stranded oligonucleotide E2 (−370 to −315) but not with double-stranded E1 or E3 (−426 to −371 or −314 to −258, respectively) or single-stranded DNA. Complementary results were obtained by demonstrating specific binding of myotube nuclear extract to labelled region E2 but to neither E1 nor E3 (Fig. 7C). Further, binding to E2 was sensitive to proteinase K, suggesting a protein component to this binding factor (Fig. 7C). The same retarded complex was formed when oligonucleotide E2 was incubated with a nuclear extract derived from the C2C12 myoblasts (data not shown). Methylation interference analysis performed with labelled E2 revealed that five methylated G's on the coding strand and three methylated G's on the noncoding strand interfered with the binding of one or more myotube nuclear factors (Fig. 8), and most of the G's were centered around position −354. Additionally, gel shift analysis with labelled E2 demonstrated the presence of a specific binding activity in chicken lens nuclear extract; however, the shifted band had a mobility different from that produced by the mouse myotube extract (Fig. 7B).

DISCUSSION

The αB-crystallin gene is abundantly expressed in lens and at lower levels in many (but not all) tissues, including skeletal and cardiac muscle, kidney, lung, and brain. Results presented above demonstrate that the murine αB-crystallin

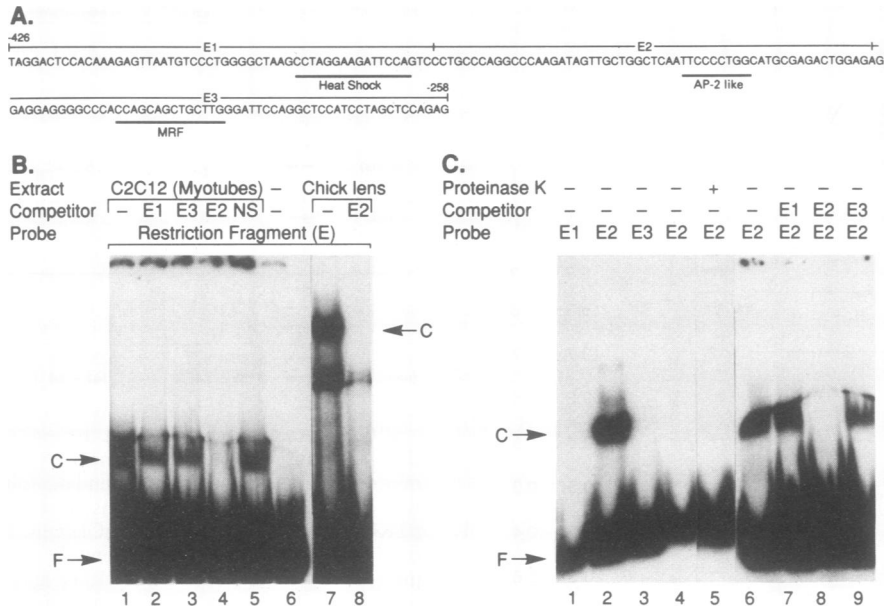
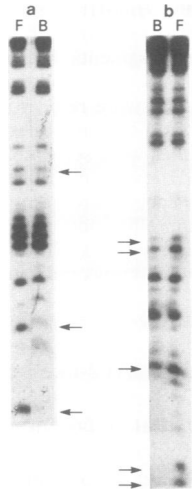


FIG. 7. Gel mobility gel shift analysis of the αB enhancer. (A) Sequence of the murine αB-crystallin enhancer. The potential regulatory sequence motifs are underlined, and the boundaries of oligonucleotides E1, E2, and E3 are indicated above the sequence. A potential MRF binding site is shown. (B and C) Gel mobility shift analyses. In panel B, the labelled enhancer fragment (−427 to −259; E) was incubated in the presence of C2C12 myotube or chicken lens nuclear extracts. Free (F) and protein-complexed (C) species were resolved by electrophoresis. Competitions were performed by using unlabelled, double-stranded oligonucleotide E1, E2, or E3 or a double-stranded nonspecific oligonucleotide, NS (5′-ATGCGCATCCAAGTATCGCGTA-3′). In panel C, gel mobility shift analysis was performed with labelled, double-stranded oligonucleotide E1, E2, or E3 in the presence of C2C12 myotube nuclear extract and either with or without excess, unlabelled competitor DNA or proteinase K, as indicated. Lanes 1 to 3 and 5 to 9 have nuclear extract; lane 4 lacks nuclear extract.

promoter fragment extending from positions −661 to +44 contains sufficient information to confer preferential and reproducible expression of a CAT reporter gene to the lens and skeletal muscle in transgenic mice. CAT activity was significantly higher in lens than in skeletal muscle in all seven lines examined. Since CAT activity was sometimes detected in some other tissues, the promoter was not completely restricted to lens and skeletal muscle. However, since CAT expression patterns in tissues other than lens and skeletal muscle were generally very low and nonreproducible between lines, it is likely that this promoter may be extremely susceptible to influences associated with the integration site or loss of 3′ flanking sequences (such as silencer elements). We did note transgene expression in cardiac muscle in two mouse lines; these two lines contained multiple copies of the transgene, however, and CAT expression in heart was high. This finding suggests that interactions between two or more αB promoter elements (as would exist in the case of multiple, linked transgenes) can expand the repertoire of expressing tissues to include heart, and our identification of a skeletal muscle enhancer within this region may be relevant to this observation.

While the −661 to +44 αB promoter fragment contains regulatory elements capable of directing preferential expression to lens and skeletal muscle, it is insufficient to confer the normal pattern of αB-crystallin expression upon a reporter gene. Since expression of an αB-crystallin minigene containing both 5′ and 3′ flanking sequences and lacking introns more closely paralleled that of the endogenous gene in transgenic mice (13), our present results strongly suggest the involvement of 3′ flanking sequences in the regulation of this gene, at least in some tissues.

To further define promoter-associated regulatory elements



CTGCCCAGGCCCAAGATAGTTGCTGGCTCAATTCCCCTGGCATGCGAGACTGGAGA
GACGGGTCCGGGTTCTATCAACGACCGAGTTAAGGGGACCGTACGCTCTGACCTCT

AP-2 like

FIG. 8. Methylation interference analysis of region E2 and myotube nuclear extract. Double-stranded oligonucleotide E2 (−370 to −315), end labelled at a single 5′ terminus, was partially methylated, incubated in the presence of C2C12 myotube nuclear extract, electrophoresed to separate free (lanes F) and protein-bound (lanes B) probes, and treated with piperidine; an equal number of radioactivity counts for each probe was resolved on a denaturing sequencing gel. The G residues involved in protein binding are indicated on the E2 sequence with a dot, and AP-2-like site is underlined. (a) Noncoding strand; (b) coding strand.

responsible for preferential expression of αB-crystallin in lens and skeletal muscle, 5′ deletion fragments linked to the CAT gene were studied by transfection in cultured cells. A fragment extending from positions −115 to +44 continued to be partially active in transfected primary chicken lens cells and the rabbit N/N1003A lens cell line, suggesting that sequences upstream of −115 control the quantitative level of expression in lens while sequences between −115 and +44 encode regulatory elements essential for lens-specific expression. It is possible that the enhancer fragment, which functioned at low levels in N/N1003A cells, serves this quantitative function in this cell line. Our inability to more adequately define a lens-specific element may reflect both the complexity of this promoter and the limitations of our in vitro assay system. Analysis of additional αB promoter-CAT constructs in transgenic mice may provide the sensitivity required to further localize a lens-specific element. We previously demonstrated that sequences between positions −222 and −167 of the murine αB-crystallin gene were important in controlling expression of an αB minigene in cultured chicken lens cells (13); however, our promoter-CAT constructs did not reveal this region to be as significant in controlling expression in the transfected lens cells (Fig. 4). Although we are presently unable to resolve this difference, it remains possible that the presence of αB downstream sequences on the minigene constructs influences promoter behavior.

In contrast to our results in lens, the region between positions −426 and −257 is absolutely required for high-level expression of the αB promoter in myotubes. A fragment extending from −427 to −259 is sufficient to confer muscle-preferred enhancer activity in myotubes and appears to be activated during myogenesis, since enhancer activity is low in myoblasts. An increase in both enhancer activity and the steady-state levels of αB RNA during myoblast-to-myotube differentiation suggests that this enhancer may be the major positive determinant regulating αB-crystallin expression in skeletal muscle. 5′ deletion analysis (Fig. 4) suggested the presence of two regions required for promoter function in cultured muscle cells, a distal element between −426 and −339 and a proximal element between −314 and −257. 5′ and 3′ deletion analyses also revealed that both proximal and distal elements are required for optimal enhancer activity in C2C12 cells (Fig. 5). We have demonstrated specific binding of myoblast and myotube nuclear protein to within and bordering the distal element (Fig. 6 and 7). Additionally, the proximal element contains a potential MRF binding site (Fig. 7A). This sequence motif, minimally defined as CANNTG, has been identified in a variety of muscle-specific enhancers (7) and interacts with at least four known muscle-specific DNA-binding proteins, MyoD1, myogenin, Myf-5, and MRF4, perhaps as heterodimers with the ubiquitously expressed protein E12 (6, 27, 29, 33, 47, 51). All four muscle-specific factors appear to play important roles in skeletal muscle determination (40). Our inability to detect nuclear extract binding to the potential MRF binding site was unexpected, since C2C12 cells express MyoD1 (40). Perhaps our nuclear extract is deficient in MyoD1 binding activity because of the method of extract preparation (see discussion in reference 18); alternatively, surrounding sequences may inhibit binding in vitro. A detailed series of site-specific mutations is required to further define the proximal and distal elements within the muscle-preferred enhancer as well as to demonstrate whether the binding activity detected in C2C12 cells plays any role in enhancer function. Gel retardation analysis with myoblast extract suggests that nuclear protein binding to oligonucleotide E2 is already present in undifferentiated cells.

Our results indicating that the αB muscle preferred-enhancer contains two separate elements required for optimal enhancer activity, one of which appears to be an MRF binding site, is significant in light of recent results by others. Lin et al. (29) have shown that optimal activity of the quail troponin I gene muscle-specific enhancer is dependent on both an MRF site and two adjacent elements that bind ubiquitous protein factors. Additionally, Weintraub et al. (51) have demonstrated that MyoD1 can stimulate expression of a reporter gene containing two or more MRF sites but not a single site. These results confirm the complex nature of muscle enhancers.

We also detected binding of chicken lens nuclear factors to the central region E2 of the αB-crystallin enhancer; the gel shift migration pattern suggests, but does not prove, that this factor may be different from that found in the C2C12 myotubes. Since our studies (Fig. 4 and 6) did not suggest any functional involvement for this sequence in the regulation of αB expression in transfected chicken lens cells, no potential role for the chicken lens DNA-binding factor can be assigned at this time.

It is clear that the regulation of the αB-crystallin gene is complex. We have shown that the −661 to +44 fragment preferentially functions in lens and skeletal muscle in transgenic mice. The region between positions −426 and −257 is essential for expression in muscle and functions as an enhancer in this tissue. In contrast, the determinants for expression in lens appear to be between −115 and +44. Separable control elements have been described in a number of genes that are expressed in more than one tissue (1, 2, 15, 16, 28, 41, 44), and we believe that further studies of this gene, particularly its 3′ flanking region, will lead to the identification of additional regulatory elements. Clearly, further work characterizing these elements and their *trans*-acting regulators is necessary.

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