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Functional redundancy of the DE-1 and αA-CRYBP1 regulatory sites of the mouse αA-crystallin promoter

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ABSTRACT

Previous studies have implicated the DE-1 (−111/−106) and αA-CRYBP1 (−66/−57) sites for activity of the mouse αA-crystallin promoter in transiently transfected lens cells. Here we have used the bacterial chloramphenicol acetyltransferase (CAT) reporter gene to test the functional importance of the putative DE-1 and αA-CRYBP1 regulatory elements by site-specific and deletion mutagenesis in stably transformed αTN4–1 lens cells and in transgenic mice. FVB/N and C57BL/6 × SJL F2 hybrid transgenic mice were assayed for CAT activiy in the lens, heart, lung, kidney, spleen, liver, cerebrum, and muscle. F0, F1, and F2 mice from multiple lines carrying single mutations of the DE-1 or αA-CRYBP1 sites showed high levels of CAT activity in the lens, but not in any of the non-lens tissues. By contrast, despite activity of the wild-type promoter, none of the mutant promoter/CAT constructs were active in the transiently transfected and stably transformed lens cells. The mice carrying transgenes with either site-specific mutations in both the DE-1 and αA-CRYBP1 sites or a deletion of the entire DE-1 and part of the αA-CRYBP1 site (−60/+46) fused to the CAT gene did not exhibit CAT activity above background in any of the tissues examined, including the lens. Our results thus indicate that the DE-1 and αA-CRYBP1 sites are functionally redundant in transgenic mice. Moreover, the present data coupled with previous transfection and transgenic mouse experiments suggest that this functional redundancy is confined to lens expression within the mouse and is not evident in transiently transfected and stably transformed lens cells, making the cultured lens cells sensitive indicators of functional elements of crystallin genes.

INTRODUCTION

The crystallins constitute 80–90% of the total soluble protein of the ocular lens, and are responsible for the optical properties of this transparent tissue (see 1 for review). Several crystallins (α, β, γ) are found in the lens of all vertebrates while others (enzyme-crystallins) are present only in selected species. The ubiquitous α-crystallins, αA and αB, are encoded by two evolutionarily-related genes (2). While αB-crystallin is present at high concentrations in the lens and at appreciable concentrations in other non-lens tissues (3, 4), αA-crystallin occurs at high concentrations in the lens and only at residual levels in some non-lens tissues and cell lines (5, 6). High-level lens expression of the mouse αA-crystallin gene is regulated at the level of transcription, as shown by the ability of the −366/+46, −111/+46, and −88/+46 promoter fragments to confer lens-specific activity upon a fused reporter gene in transgenic mice (7, 8).

Several lines of evidence have implicated both the DE-1 (−111/−106) and αA-CRYBP1 (−66/−57) sites in transcriptional regulation of the mouse αA-crystallin gene. Site-directed mutagenesis of these sites has eliminated promoter activity in transiently transfected lens cells (9, 10). Moreover, both sites specifically bind nuclear proteins *in vivo* and *in vitro* (11, 12). Moreover, a single copy of the mouse αA-CRYBP1 site can stimulate the heterologous HSV thymidine kinase (tk) promoter in transfected mouse lens cells (13). The DE-1 site is analogous to the functionally important DE1A/αCE2 site in the chicken αA-crystallin promoter (14, 15), and the αA-CRYBP1 site is similar to the binding site for the known human transcription factors PRDII-BF1 (16), MBP-1 (17), and NF-kB (for reviews see 18, 19). A cDNA encoding a protein (αA-CRYBP1) which binds to the αA-CRYBP1 site has been isolated (9); αA-CRYBP1 appears to be the mouse homologue of PRDII-BF1/MBP-1, and different forms of αA-CRYBP1 are present in cells which do and do not express αA-crystallin (Kantorow et al., submitted).

In the present study we have analyzed the role of the DE-1 and αA-CRYBP1 sites in mouse αA-crystallin promoter activity in the intact lenses of transgenic mice and in stably transformed lens cells in culture. As expected from earlier transfection experiments, mutation of either the DE-1 or αA-CRYBP1 sites eliminated activity of the αA-crystallin promoter-CAT fusion gene in stably transformed lens cells. Unexpectedly, however, the mutated promoter continued to function specifically in the lens of transgenic mice when at least one of these regions, DE-1 or αA-CRYBP1, was intact. Lens promoter activity was impaired in the transgenic mice when both sites were mutated or deleted, consistent with the DE-1 and αA-CRYBP1 sites being functionally redundant.

MATERIALS AND METHODS

Plasmid constructions

pαA111$_a$-CAT, pαA60$_a$-CAT (20), pDMD1, pDMD3, and pDMD9 (9) were originally described elsewhere. pαA111$_a$-CAT (wild type), pDMD1 (mutated at −111/−106, the DE-1 site), pDMD3 (mutated at −99/−94), and pDMD9 (mutated at −63/−58, the αA-CRYBP1 site) contain the −111/+46 sequence pαA60$_a$-CAT contains the −60/+46 sequence of the αA-crystallin gene fused to the bacterial CAT gene. pCMVβ contains the cytomegalovirus (CMV) immediate early promoter/enhancer fused to the bacterial β-galactosidase gene (Clontech). pMC1neo PolyA contains the HSV tk promoter fused to the neomycin-resistance gene (Stratagene). pαADM was constructed as follows: the 561 bp HaeIII fragment of pDMD1 containing the mouse αA-crystallin promoter linked to the CAT gene was subcloned into SmaI digested M13mp8 (pCS46); mutagenesis of the αA-CRYBP1 site to an XbaI site at positions −63/−58 (pCS47) was carried out using the oligodeoxynucleotide-directed *in vitro* mutagenesis system of Amersham (RPN.1523). The mutation was verified by dideoxy DNA sequencing. Oligodeoxynucleotides spanning −111/−84 (−111/−106 mutation included; 5′ primer) and +20/+46 (3′ primer) of the mouse αA-crystallin promoter, each containing a HindIII restriction site on the end, were used to polymerase chain reaction (PCR) amplify the doubly-mutated −111/+46 mouse αA-crystallin promoter region of pCS47. The amplified PCR product was digested with HindIII, subcloned into HindIII-digested pSV0-CAT (21), and the forward orientation of the promoter with respect to the CAT gene (pαADM) verified by dideoxy DNA sequencing.

Cells and transfections

αTN4-1 (22), N/N1003A (23), and chicken primary lens epithelial cells (24) were grown in DMEM, 10% fetal calf serum, and 0.1 mg/ml gentamicin. For transient transfections, 10 μg of promoter-CAT plasmid was co-transfected with 3 μg of internal control pCMVβ via the calcium phosphate precipitation method (25) as previously described (13). 48 hours post-transfection the cells were harvested by scraping and disrupted by repeated freezing on dry ice and thawing at 37°C in 100 μl of 0.25 M Tris−HCl, pH 7.8. For stable transfections, 20 μg of promoter-CAT plasmid was co-transfected with 7 μg of pMC1neo PolyA as above. 48 hours post-transfection the cultures were fed with media containing 0.5 mg/ml Geneticin (G418 sulfate, GIBCO-BRL; 26), grown in culture in the continuous presence of Geneticin for 20 days, and subsequently harvested as above.

Isolation of DNA for pronuclear injections and creation of transgenic mice

pDMD1, pDMD3, pDMD9, pαADM, and pαA60$_a$-CAT were each digested with NdeI and BamHI. The appropriate linear 1.6 kb fragments were isolated by electrophoresis in agarose gels, followed by electro-elution, multiple phenol-chloroform extractions, and two ethanol precipitations of the DNA. Each DNA fragment contained from 5′ to 3′: 57 bp of pBR322 sequence, mouse αA-crystallin promoter sequence, the bacterial CAT gene, and SV40-derived sequences including the small t-antigen splice sites and polyadenylation site. DNA fragments were injected into one pronucleus of a single-celled mouse embryo as described by Gordon et al. (27). C57BL/6×SJL F2 hybrid (28) transgenic mice were created by the National Transgenic Development Facility at DNX, Inc. under U.S. Patent #4,873,191. FVB/N transgenic mice were created by the National Eye Institute Centralized Transgenic Facility, as described by Wawrousek et al. (8).

Analysis of transgenic mice

For screening the transgenic mice, tail DNA was isolated (29) and the presence of the transgene was established by PCR analysis using a 5′ oligodeoxynucleotide primer specific for the mouse αA-crystallin promoter (Fig. 1, #7279 for pαA60$_a$-CAT, #4076 for all other transgenes) and a 3′ oligodeoxynucleotide primer specific for the CAT gene (Fig. 1, #4077). The genomic DNAs of transgenic mice were also subjected to Southern blot analysis by digestion either with BglII, which does not cut within the transgene, in order to determine the number of integration sites or with NcoI, which cuts once within the transgene, in order to verify the proper unit length of the majority of the integrated transgenes. The transgene copy number of each mouse shown in Tables 2 and 3 was assessed by slot-blot analysis of the genomic DNAs relative to standard samples representing 0−50 copies of the transgene. Mice were sacrificed by CO_2 asphyxiation and the lung, heart, liver, spleen, kidney, thigh muscle, cerebrum, and lenses isolated. The lenses were homogenized on ice in 100 μl of 0.25 M Tris−HCl (pH 7.8) in an eppendorf microfuge tube; the other tissues were homogenized in 800 μl of the same buffer in a Duall tissue grinder (Kontes, size 0020). The cell debris was pelleted in a microfuge, the supernatant fraction heated at 65°C for 15 min, the ensuing precipitates pelleted in a microfuge, and the final supernatant fraction assayed for total protein content and CAT activity (see below).

PCR analysis of transgene promoters and structural CAT gene

PCR reactions were performed using the GeneAmp kit (N801-0055, Perkin-Elmer) and 1 μl of genomic tail DNA isolated from mice carrying either the pαADM or pαA60$_a$-CAT transgene (variable μg amounts for each sample), 1 μM 5′ primer, and 0.5 μM 3′ primer. To assess promoter integrity a 5′ oligodeoxynucleotide primer complementary to vector sequences at the 5′ end of the transgene (Fig. 1, #7723, 12 bp from the 5′ NdeI site used to isolate the original linear transgene fragment) and a 3′ oligodeoxynucleotide primer specific to the mouse αA-crystallin promoter (Fig. 1, #7724) were used. PCR reactions were carried out as follows: an initial 3 min 94°C denaturation, 30 cycles of 1.5 min at 94°C, 2 min at 55°C, and 3 min at 72°C, followed by a 10 min 72°C extension step. To assess CAT gene integrity a 5′ oligodeoxynucleotide primer specific to the mouse αA-crystallin promoter (Fig. 1, #7184) and a 3′ oligodeoxynucleotide primer (Fig. 1, #7971) complementary to and immediately flanking the stop codon of the CAT gene were used. PCR reactions were carried out as above except that the annealing step was performed at 65°C. PCR amplified samples were size-fractionated on a 1% agarose gel, blotted onto Nytran (Schleicher & Schuell) as recommended by the manufacturer, and UV-crosslinked to the membrane. The identity of the PCR products was verified by probing the blots using the ECL 3′-Oligolabelling and Detection System (RPN 2130/2131, Amersham) and the appropriate non-radioactively-labelled internal oligodeoxynucleotide: #7184 (Fig. 1) to assess promoter integrity or #4077 (Fig. 1) to assess CAT gene integrity. Following the final ECL detection reaction, blots were exposed to XAR-5 X-ray film at room temperature for 10 sec to 3 min with an intensifying screen.

Total protein, CAT, and β-galactosidase assays

The total protein content of tissue/cell extracts was determined using a bovine serum albumin standard and the Protein Assay Dye Reagent Concentrate (BioRad). CAT assays (30) were carried out using 50 μl of tissue culture cell extract or 20 μg total protein from transgenic mouse tissues. β-galactosidase activities (31) in transfected cells were determined using 10−40 μl of cell extract.

RESULTS

Transfection of promoter-CAT constructs into lens and fibroblast cell lines

Previously, we showed that mutations of either the DE-1 or αA-CRYBP1 site in the mouse αA-crystallin promoter fused to the bacterial CAT gene eliminated promoter activity when transiently transfected into the SV40 T-antigen transformed mouse lens cell line αTN4-1 (9), the untransformed rabbit lens cell line

Table 1. Transfection of mouse αA-crystallin promoter-CAT plasmids into lens cells

Plasmid	Stable Transformation[a]	Transient Transfection[b]			Transient Transfection[c]		
	αTN4-1	αTN4-1	N/N1003A	PLEα	TN4-1	N/N1003A	PLE
pαA111$_a$-CAT	1.0	1.0	1.0	1.0	1.0	1.0	1.0
pDMD1	0.50 ± 0.10	0.26	0.20	0.24			
pDMD9	0.60 ± 0.06	0.23	0.20	0.14			
pαADM	0.50 ± 0.04	0.30 ± 0.02	0.18 ± 0.08	0.12 ± 0.04			
pSV0-CAT	0.48 ± 0.08	0.24 ± 0.10	0.18 ± 0.06	0.13 ± 0.06	0.34 ± 0.08	0.20 ± 0.06	0.18 ± 0.08
pαA60$_a$-CAT					0.42 ± 0.04	0.24 ± 0.10	0.12 ± 0.06

[a] Stable co-transformation of promoter-CAT plasmids and pMC1neo PolyA was carried out as described in Materials and Methods. The values shown represent the CAT activity (femtomoles of [^{3}H]acetylchloramphenicol produced per minute per μg total protein) of each plasmid relative to that of the pαA111$_a$-CAT (wild type promoter) control. Stable transformations were carried out twice and standard deviations are shown.
[b] The results of transient transfection of pDMD1 and pDMD9 were previously reported (9, 10). Values shown represent relative CAT activities as in a.
[c] Promoter-CAT plasmids were transiently co-transfected with pCMVβ as described in Materials and Methods. CAT activities (femtomoles of [^{3}H]acetylchloramphenicol produced per minute per unit of β-galactosidase) of the mutants are relative to that of pαA111$_a$-CAT. Transfections were carried out three times, and the average and standard deviations are shown.

Table 2. Expression of mutant αA-crystallin promoter-CAT genes in C57BL6×SJL F2 hybrid mice

Mutation[a]	Strain	Line[b]	Mouse	Gen[c]	Sex	#Sites[d]	Copy#	Age[e]	CAT Activity[f]								Average CAT Activity	
									Lens	Lung	Heart	Liver	Spleen	Kidney	Muscle	Cerebrum	Lens	Non-Lens
DE-1	187	1	K2237	F0	F	1	1-2	11.00	1107	1.7	3.5	6.3	2.1	1.9	1.6	2.9		
		2	K2239	F0	M	1	3-5	11.00	294	1.0	1.8	0.9	1.0	1.2	1.9	2.5		
		3	K2942	F1	F	2	3-5	3.25	745	1.1	1.6	1.1	1.2	1.3	1.0	5.8		
		3	X0053	F1	F	2	3-5	2.75	120	1.3	0.6	0.8	1.5	0.7	0.6	1.9		
		4	X0044	F1	M	1	1-2	3.00	1545	1.4	2.3	10.5	1.2	3.2	1.3	1.4	762±583	2.1±1.9
αA-CRYBP1	65	1	P3701	F2	M	1	11-20	3.50	1130	4.0	3.9	4.2	4.3	3.4	3.4	4.3		
		1	P3895	F2	F	1	11-20	4.50	2373	2.9	3.1	4.3	2.8	2.9	2.7	3.2		
		3	P3159	F1	F	1	1-2	7.00	1600	3.1	3.7	4.6	4.3	2.7	4.0	4.0		
		4	P1572	F1	F	1	1-2	3.25	5000	3.9	3.9	4.5	3.5	4.4	3.9	4.0		
		5	6-3	F0	M	1	1-2	3.50	7388	1.0	1.5	1.4	1.2	1.2	1.2	-	3498±2639	3.2±1.1
DE-1 + αA-CRYBP1	270	1	8031	F0	M	1	>50	5.00	0.4	0.3	0.3	1.0	0.4	0.6	0.3	0.6		
		2	8032	F0	F	1	21-30	7.50	3.5	4.5	5.9	11.7	4.8	5.0	3.9	3.7	2.0±2.2	3.1±3.3
Truncated -60/+46	188	1	X0074	F1	M	1	1-2	3.75	0.4	0.4	0.6	0.9	0.4	0.5	0.6	0.7		
		1	X0088	F1	M	1	1-2	3.00	0.6	1.6	3.6	12.3	2.0	5.5	1.0	1.6		
		2	K2407	F0	F	1	1-2	7.50	1.2	1.5	2.0	5.2	1.3	1.3	1.5	1.4		
		3	Q0883	F0	F	2	1-2	7.50	1.3	1.3	1.7	4.6	1.2	1.8	1.6	1.7	0.9±0.4	2.1±2.4
-99/-94 control	59	1	P1368	F1	M	1	1-2	2.75	34	2.8	3.7	9.5	3.8	4.4	2.2	3.3		
		1	P3854	F2	M	1	1-2	4.75	388	3.1	4.2	4.0	3.1	2.7	2.7	3.4		
		1	P3857	F2	F	1	1-2	4.75	514	2.1	2.7	2.2	2.5	2.6	3.1	2.6		
		2	P1561	F1	F	2	6-10	2.75	34	2.1	2.1	5.1	1.7	3.1	2.1	3.0		
		2	P3141	F1	M	1	6-10	2.50	1061	3.6	3.3	3.6	3.6	3.7	3.5	3.7		
		3	P1331	F1	M	2	1-2	9.50	529	3.9	4.6	5.1	3.6	4.0	4.1	4.2	426±382	3.4±1.3
non-transgenic		1	1	-	M	0	0	2.00	0.6	0.9	1.1	3.2	1.0	0.6	0.5	1.9		
		1	2	-	F	0	0	2.00	0.9	1.0	2.7	4.1	0.8	0.7	0.8	1.3		
		2	3	-	M	0	0	2.00	0.4	0.8	0.9	2.1	1.9	1.4	1.3	0.8		
		2	4	-	F	0	0	2.00	1.0	1.7	1.9	4.4	0.7	1.6	0.5	0.6	0.7±0.3	1.5±1.0

[a] The mouse αA-crystallin promoter was mutated and fused to the bacterial CAT gene. −111/+46 promoter mutations: DE-1 (−111/−106 mutated; pDMD1), αA-CRYBP1 (−66/−58 mutated; pDMD9), DE-1 + αA-CRYBP1 (−111/−106 and −66/−58 mutated; pαADM), −99/−94 (control; pDMD3). 'Truncated −60/+46' denotes pαA60$_a$-CAT which contains only the −60/+46 promoter. 'Nontransgenic' denotes nontransgenic mice that were assayed as background controls.
[b] Separate lines of transgenic mice derived from separate founder mice.
[c] Mouse generation: FO (founder), F1, or F2.
[d] The number of transgene integration sites determined from BglII digestion of genomic DNA and Southern blot analysis (data no shown).
[e] Age of mice, in months, at the time of sacrifice.
[f] CAT activities are expressed as cpm [^{3}H]acetylchloramphenicol produced per minute per μg total protein.

Table 3. Expression of mutant αA-crystallin promoter-CAT genes in FVB/N transgenic mice

Mutation[a]	Strain	Line[b]	Mouse	Gen[c]	Sex	#Sites[d]	Copy#	Age[e]	CAT Activity[f]								Average CAT Activity	
									Lens	Lung	Heart	Liver	Spleen	Kidney	Muscle	Cerebrum	Lens	Non-Lens
DE-1	DB01	1	63	F1	M	1	1-2	1.75	2891	2.8	5.8	10.9	3.9	7.0	2.9	1.9		
		1	75	F1	F	1	1-2	1.75	3131	1.8	3.8	9.1	3.7	4.3	2.7	3.4		
		2	59	F1	M	1	1-2	2.50	3065	1.5	4.9	9.1	1.3	4.0	1.2	0.9		
		3	84	F1	M	1	3-5	2.25	2089	1.2	2.6	3.5	1.3	3.3	1.2	1.3		
		3	90	F1	F	1	3-5	3.00	4540	0.3	0.5	5.0	0.6	0.3	2.4	0.5	3143±884	3.2±2.6
αA-CRYBP1	DB02	1	60	F1	M	1	11-20	1.50	4050	3.7	2.6	8.0	3.3	2.2	2.1	2.7		
		1	66	F1	F	1	11-20	1.75	2983	2.0	4.2	7.2	3.1	9.2	2.9	2.8		
		2	70	F1	M	1	21-30	1.50	4361	4.2	5.1	13.6	4.2	5.5	1.6	2.7		
		2	77	F1	F	1	21-30	1.75	838	3.0	7.0	12.9	3.1	9.4	3.5	3.3		
		3	83	F1	M	1	11-20	1.50	3160	2.5	2.9	13.3	2.2	3.9	2.6	1.9		
		3	88	F1	F	1	11-20	1.75	3106	4.6	4.5	11.1	5.4	7.7	3.4	4.5		
		4	91	F1	M	1	3-5	2.25	151	1.1	2.9	5.5	1.3	4.2	1.1	1.0		
		4	102	F1	F	1	3-5	2.25	149	1.5	3.2	6.2	1.4	3.4	1.2	0.7	2349±1712	4.3±3.1
DE-1 + αA-CRYBP1	DB07	1	30	F1	M	1	1-2	2.50	0.2	0.7	1.8	4.0	0.5	1.9	0.4	0.5		
		1	38	F1	F	1	1-2	2.50	0.6	1.2	1.9	6.9	1.7	3.4	0.8	1.0		
		2	58	F1	M	1	1-2	2.50	0.7	0.9	2.4	7.1	1.3	2.9	1.0	1.9		
		2	64	F1	F	1	1-2	2.50	0.8	1.2	2.2	6.3	1.6	3.9	0.9	0.9		
		3	84	F1	M	1	3-5	3.00	1.5	1.2	2.3	2.7	1.4	1.5	1.4	1.0		
		3	94	F1	F	1	3-5	3.00	1.4	1.6	1.9	1.7	0.9	1.6	0.8	1.3	0.9±0.5	2.0±1.6
Truncated -60/+46	DB09	1	76	F1	M	1	3-5	1.50	0.4	0.8	1.9	6.3	1.4	3.5	0.7	1.4		
		1	82	F1	F	1	3-5	1.50	2.3	3.4	3.6	9.5	3.0	5.4	2.4	2.6		
		2	93	F1	F	1	1-2	1.50	1.8	2.8	2.9	10.2	3.1	5.7	2.9	2.6		
		3	101	F1	F	1	1-2	1.50	1.7	2.6	5.4	6.5	2.2	3.9	2.3	2.7		
		4	106	F1	F	1	6-10	1.75	0.6	0.9	2.6	11.1	0.9	2.1	0.5	11.4	1.4±0.8	3.8±2.9
non-transgenic		1	1	-	M	0	0	1.00	0.9	3.4	2.6	8.8	3.7	2.5	2.3	1.5		
		1	2	-	F	0	0	1.00	0.5	1.6	3.5	3.9	4.1	4.3	1.5	1.3		
		2	3	-	M	0	0	1.00	1.1	2.8	1.6	9.7	3.1	1.4	2.4	1.6		
		2	4	-	F	0	0	1.00	0.8	1.5	2.7	6.8	2.9	2.6	1.6	1.5	0.8±0.3	3.1±2.1

[a] The mouse αA-crystallin promoter was mutated and fused to the bacterial CAT gene. −111/+46 promoter mutations: DE-1 (−111/−106 mutated; pDMD1), αA-CRYBP1 (−66/−58 mutated; pDMD9), DE-1 + αA-CRYBP1 (−111/−106 and −66/−58 mutated; pαADM), −99/−94 (control; pDMD3). 'Truncated −60/+46' denotes pαA60$_a$-CAT which contains only the −60/+46 promoter. 'Nontransgenic' denotes nontransgenic mice that were assayed as background controls.

[b] Separate lines of transgenic mice derived from separate founder mice.

[c] Mouse generation: FO (founder), F1, or F2.

[d] The number of transgene integration sites determined from BglII digestion of genomic DNA and Southern blot analysis (data no shown).

[e] Age of mice, in months, at the time of sacrifice.

[f] CAT activities are expressed as cpm [^{3}H]acetylchloramphenicol produced per minute per μg total protein.

N/N1003A, and chicken primary lens epithelial cells (PLE) (10). In order to confirm and examine further the DE-1 and αA-CRYBP1 sites for αA-crystallin promoter function, we stably transformed the αTN4-1 cell line using the same mutant promoter-CAT constructs as in the transient transfection experiments. The integration of the promoter-CAT gene fusion in the stably transformed cells was confirmed by slot-blot analysis (data not shown). When the DE-1 (pDMD1), αA-CRYBP1 (pDMD9), or doubly mutated (pαADM) sites were used, CAT activity was no higher than that in the cells stably transformed with the promoterless vector (Table 1). Since the expression of the wild type promoter in the αA111$_a$-CAT gene decreases in the transformed cells with time in culture (data not shown), the loss of promoter activity of the mutants relative to that of pαA111$_a$-CAT in the stable transformants was less striking than that observed in the transiently transfected cells (Table 1; 9, 10). Nonetheless, mutation of either site impairs promoter activity in transfected cells regardless of whether or not the plasmid construct is integrated into the genome.

To establish the minimal αA-crystallin promoter elements of the mouse αA-crystallin gene required for expression, the −60/+46 promoter fused to CAT (pαA60$_a$-CAT) was transiently transfected into a variety of lens cells. This construct lacks the DE-1 site and most of the αA-CRYBP1 site. As expected (20), the −60/+46 promoter-CAT fusion gene did not function when transiently transfected into the mouse αTN4-1, rabbit N/N1003A, or chicken PLE cells (Table 1). These results confirm that transcriptional regulatory sequences between −111 and −60 are critical for activity of the mouse αA-crystallin promoter in transfected lens cells. Moreover, the similar expression pattern observed in lens cells derived from mouse, rabbit, and chicken argues for a conservation of regulatory mechanisms, consistent with the conserved promoter sequences in this region (32).

Expression of mutant αA-crystallin promoter-CAT genes in transgenic mice

We next analyzed the activity of the site-specific mutant and truncated promoter-CAT fusions in the intact lenses of transgenic mice. A series of transgenic mice carrying either pDMD1, pDMD3, pDMD9, pαADM, or pαA60$_a$-CAT were created. FVB/N and C57BL/6×SJL F2 hybrid mice were employed to test for possible differences in expression pattern between different strains of mice. Multiple independent (different founder) lines of mice from each construct were examined; the copy number, age, and number of integration sites varied between lines. In some cases founder mice were sacrificed and assayed for CAT activity, despite the potential for mosaicism, because they would not breed. As a control, we created transgenic mice carrying a promoter mutation (Table 2, strain 59), which did not eliminate promoter activity in transfected lens cells (Nakamura et al., 1990; Donovan et al., 1992). As expected, this transgene exhibited high levels of CAT activity in the lens but not in any of the non-lens tissues assayed. In addition, CAT activities were

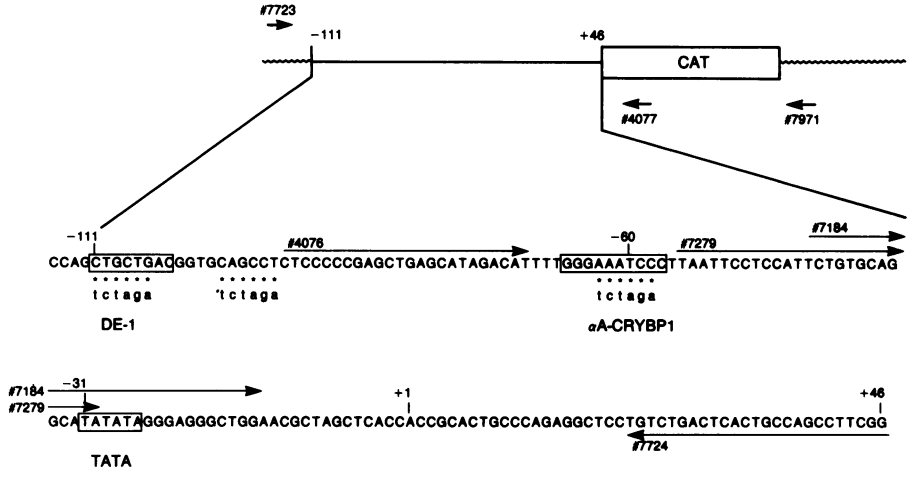

Figure 1. Structure of the mouse αA-crystallin promoter-CAT fusion plasmids. The top schematic shows the structure of the promoter-CAT constructs used: wavy line denotes vector sequences, solid line denotes the mouse αA-crystallin promoter, the open box denotes the bacterial CAT gene. Shown is the promoter sequence of wild type pαA111$_a$-CAT. The 5′ promoter boundary of pαA60$_a$-CAT is at −60. The DE-1 (−111/−106), αA-CRYBP1 (−66/−57), and TATA (−31/−26) regions are boxed. The promoter sequence of the mutant constructs differs from what is shown by the substitution of an XbaI restriction site (5′-TCTAGA-3′) at −111/−106 (pDMD1, pαADM), −66/−58 (pDMD9, pαADM), or −99/−94 (pDMD3); these positions are marked by *. The position of oligodeoxynucleotides (see Materials and Methods) used as PCR primers and as probes in southern blot analysis (Figure 3) are shown: 4076 (−93/−70), 4077 (CAT codons 29−36; 5′-CGGTCTGGTTATAGGTACTTTGAGC-3′), 7184 (−43/−14), 7279 (−55/−31), 7723 (vector sequence, 5′-CAGTACAATCTGCTCTGATG-3′), 7724 (+22/+46), 7971 (immediately flanking the CAT stop codon, 5′-ACCAGGCGTTTAAGGG CACCAAT-3′).

compared to the lens CAT activity in previously prepared FVB/N transgenic mice carrying the wild type −111/+46 promoter fused to CAT (range of 15 to 18,892 units of CAT activity; 8).

The mice carrying either the DE-1 (Table 2, strain 187; Table 3, strain DB01) or αA-CRYBP1 (Table 2, strain 65; Table 3, strain DB02) promoter mutation exhibited high levels of CAT activity in the lenses of all mice assayed. This observation differs from the inactivity of these mutated promoters in transfected lens cells (Table 1; 9, 10). The average lens CAT activity in the transgenic mice were significantly different from the average level of background CAT-like activity observed in the lenses of the nontransgenic mice (P=0.05; Aspin−Welch test; 33). The average CAT activity in the lenses of transgenic mice carrying the control promoter mutation (Table 2, strain 59) was not significantly different from the average CAT activity observed in the lenses of C57BL/6×SJL F2 hybrid mice carrying either the DE-1 (strain 187) or αA-CRYBP1 (strain 65) mutation (P=0.025; Aspin−Welch test). The expression pattern of each construct between the two strains of mice used in this study was similar; however the amount of lens CAT activity observed varied considerably among the mice of the same and different strains. A calculation of the correlation coefficient between the level of lens CAT activity and the transgene copy number, number of transgene integration sites, generation, sex, and age of the mice did not indicate a relationship between lens CAT activity and any of these variables. The observed variation may be due to differences in CAT mRNA and/or protein stability, as well as to the influence of the sequences surrounding the integration site on transcriptional activity of the transgene.

The transgenic mice carrying the singly mutated promoters did not exhibit appreciable CAT activity in any of the non-lens tissues analyzed. The average non-lens CAT activities of the transgenic mice were not significantly different from background CAT activity levels observed in the non-lens tissues of non-transgenic mice (P=0.05; Aspin−Welch test). In addition, mutation of

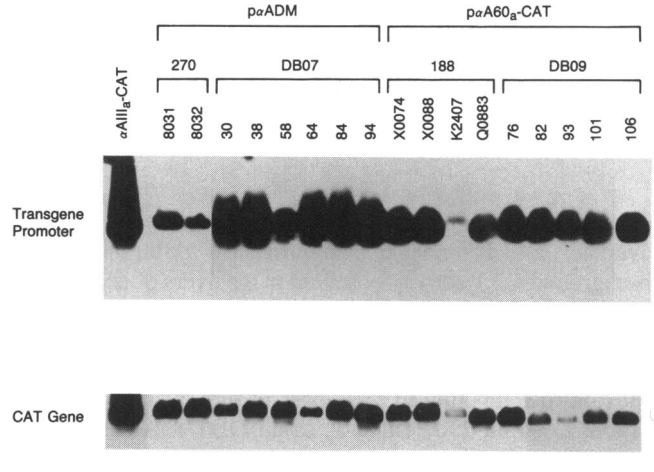

Figure 2. Analysis of doubly-mutated or truncated αA-crystallin promoter-CAT transgenes. The integrity of the transgene promoter and structural CAT gene was verified in genomic DNA isolated from mice of strains 270 and DB07 (pαADM, DE-1 and αA-CRYBP1 sites mutated) as well as strains 188 and DB09 (pαA60$_a$-CAT, DE-1 and αA-CRYBP1 sites deleted). Genomic DNA samples were subjected to PCR, size-fractionated on a 1% agarose gel, blotted onto Nytran, and probed with an internal oligodeoxynucleotide. Transgene promoter integrity (top panel) was checked using oligodeoxynucleotides #7723 and #7724 (Fig. 1) as PCR primers, and the identity of the resulting PCR product verified by Southern blotting using oligodeoxynucleotide #7184 (Fig. 1) as a probe. CAT gene integrity (bottom panel) was checked using oligodeoxynucleotides #7184 and #7971 (Fig. 1) as PCR primers, and the identity of the resulting PCR product verified by southern blotting using oligodeoxynucleotide #4077 (Fig. 1) as a probe.

either site did not promote expression above background levels in transfected mouse L929 fibroblasts (data not shown). These results suggest that no simple repressor system is acting at the DE-1 or αA-CRYBP1 sites in non-lens cells and tissues. While the level of CAT activity in the livers of these transgenic mice

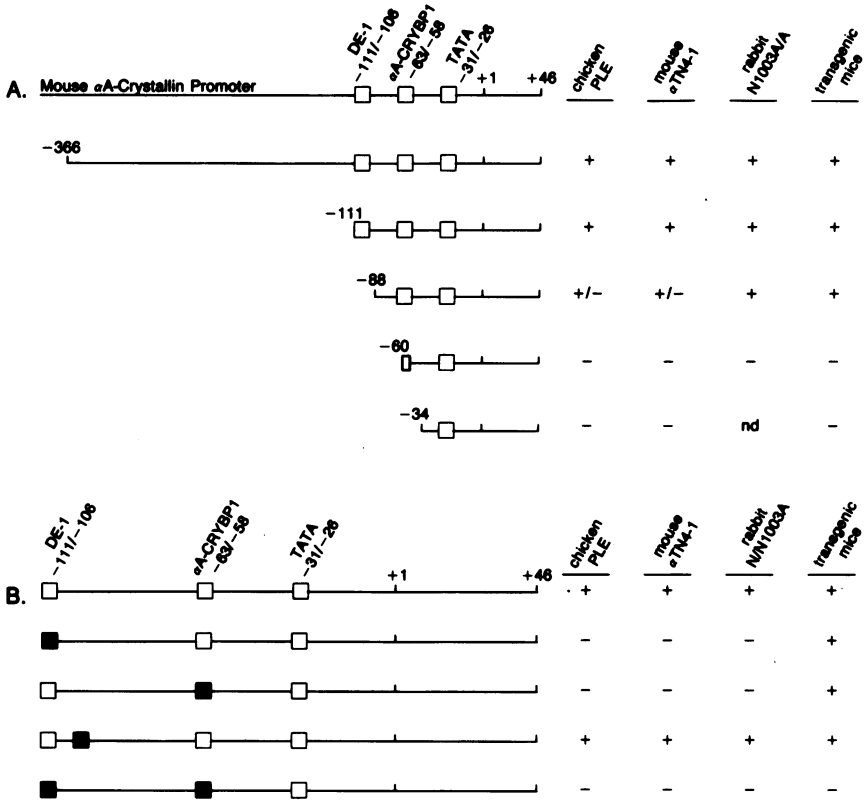

Figure 3. Expression of promoter-CAT constructs in transfected lens cells and transgenic mice. The DE-1, αA-CRYBP1, and TATA regions are marked by boxes: open boxes denote wild type sequences, solid boxes denote mutant sequences. **A.)** Deletions of the mouse αA-crystallin promoter fused to the CAT gene and transiently transfected into lens cells or used to create transgenic mice (present study; 7, 8, 9, 10, 20, 23, 38, 39). **B.)** Site-specific mutation of the mouse αA-crystallin promoter fused to the CAT gene and transiently transfected into lens cells or used to create transgenic mice (present study; 8, 9, 10, 20, 39).

appeared to be higher than in the other non-lens tissues, the average activity was not significantly different from the background CAT activity observed in the livers of non-transgenic mice (P=0.05; Aspin−Welch test).

In contrast to the activity of the singly mutated promoters, trangenes carrying mutations in the DE-1 and the αA-CRYBP1 sites did not function in the lenses of any of the mice assayed (Table 2, strain 270; Table 3, strain DB07). As expected, the promoter constructs with double mutations also exhibited background CAT activity in transfected αTN4-1, N/N1003A, and PLE cells (Table 1). The average CAT activity in the lens was not significantly higher than background CAT activity observed in the lenses of nontransgenic mice or the non-lens levels of CAT activity observed in the same transgenic mice (P=0.05; Aspin−Welch test). Mice carrying the truncated −60/+46 promoter ($p\alpha A60_a$-CAT), in which the entire DE-1 and most of the αA-CRYBP1 site were deleted, did not exhibit significant CAT activity in any of the tissues examined, including the lens (P=0.05; Aspin−Welch test). As with the single mutations, the average CAT activity in the liver was not significantly different from the background CAT activity observed in the livers of non-transgenic mice (P=0.05; Aspin-Welch test).

PCR analysis of doubly mutated and truncated αA-promoter-CAT transgenes

In order to test that the doubly mutated (pαADM) and truncated ($p\alpha A60_a$-CAT) transgenes were not functional in lens due to the appropriate mutation rather than to a gross rearrangement/deletion of the promoter or CAT gene upon genome integration, PCR analysis of the αA-crystallin promoter-CAT fusion transgene was performed for each of the mice in strains DB07, DB09, 270, and 188. Two sets of primers were employed (Fig. 1): 1) #7723 specific for the 5′ vector sequences in the transgene upstream of the mouse αA-crystallin promoter and #7724 specific for the +22/+46 region of the mouse αA-crystallin promoter, or 2) #7184 specific for the −43/−14 region of the mouse αA-crystallin promoter and #7971 specific for the 3′ end of the CAT structural gene. Following PCR amplification, the products were probed with an internal oligodeoxynucleotide (Fig. 1, #7184 for promoter integrity, #4077 for CAT gene integrity) to verify the identity of the PCR products. PCR and subsequent Southern blot analysis revealed the expected size product in all cases (Fig. 2; promoter integrity: 214 bp for strains DB07 and 270, and 163 bp for strains DB09 and 188; CAT gene integrity: 822 bp for all). The resolution of the 214 bp and 163 bp bands is not striking in this figure due to the use of a low-resolution agarose gel for southern blotting and over-exposure of the autoradiogram to visualize all hybridizing bands. The size difference in these PCR products was however resolved in acrylamide gel electrophoresis of the PCR products without southern blotting, as well as in shorter exposures of the autoradiograms shown in Figure 2 (data not shown). Mouse K2407 exhibits a slightly higher PCR product than the expected 163 bp band. This may indicate a rearrangement in the 1−2 copies of this transgene, or may represent an electrophoretic anomaly given the smaller quantity of PCR product loaded in this lane compared to that loaded in the other

lanes. Thus, both the transgene promoter and CAT gene appeared intact lacking any gross rearrangements or deletions. This is in agreement with Southern blot analysis of the transgenes in these mice (NcoI digestion; data not shown).

DISCUSSION

The functional importance of the DE-1 and αA-CRYBP1 sites was demonstrated previously by the elimination of promoter activity in transiently transfected lens cells upon mutation of either site (9. 10). It was therefore surprising when mutation of either the DE-1 or αA-CRYBP1 site did not eliminate lens promoter activity in transgenic mice in the present study. Our results point to an inherent difference between the lens *in situ* and cultured lens epithelial cells. The cultured cells appear more sensitive to promoter mutations, making them useful in the inital identification of regulatory regions. This difference in crystallin gene expression between the intact lens and cultured lens does not appear to depend upon integration of the transgene into the genome per se, since singly mutated promoters were also non-functional in stably transformed lens cells whose integration has occurred. The expression discrepancy between cultured lens cells and the intact lenses of transgenic mice may be due to differences in the presence, amounts, or modifications of trans-acting factors. For example, although the αA-CRYBP1 site is bound by the αA-CRYBP1 protein in αTN4-1 and whole lens nuclear extracts (Kantorow et al., submitted), at least one additional antigenically-dissimilar protein is bound to this site in whole lens extracts (Kantorow, unpublished).

Our studies indicate that DE-1 or αA-CRYBP1 must be intact to achieve αA-crystallin gene expression in the lens (Fig. 3). Mutation or deletion of both sites eliminates promoter activity in the lens. While the −111/+46 (containing both DE-1 and αA-CRYBP1) and −88/+46 (containing αA-CRYBP1) promoters conferred lens-specific activity in transgenic mice produced earlier (8), the doubly mutated (DE-1 and αA-CRYBP1) −111/+46 promoter and the truncated −60/+46 promoter (lacking the DE-1 and most of the αA-CRYBP1 sites) were inactive in the lenses of transgenic mice in the present study. Taken together, these findings suggest that the DE-1 and αA-CRYBP1 sites are functionally redundant *in situ*. Both the rat elastase I (34) and proopiomelanocortin (35) promoters contain similarly identified functionally redundant regulatory sequences. In practice, this redundancy may involve an interaction between either the DE-1 or αA-CRYBP1-bound proteins and another αA-crystallin transcription factor. Although the mutation of both the DE-1 and αA-CRYBP1 sites eliminates promoter activity in the lens of transgenic mice, it is not certain that these sequences themselves are solely responsible for the lens-specific expression of the αA-crystallin gene. It is possible that the DE-1 and αA-CRYBP1 sites interact with downstream sequences for lens-specificity, and further experiments are necessary in this connection.

The redundant DE-1 and αA-CRYBP1 elements within the mouse αA-crystallin promoter are structurally similar (although not identical) to their counterparts, αCE2/DE1A (14, 15, 32) and the αA-CRYBP1-like sequence (10, 13) in the chicken αA-crystallin promoter. Interestingly, however, there appear to be functional differences between these sequences in the mouse and chicken. The mouse DE-1 sequence does not compete with the chicken αCE2/DE1A sequence for complex formation using chicken lens nuclear extracts, suggesting that the chicken and mouse regulatory sequences bind different factors (15). Moreover, while the mouse αA-CRYBP1 sequence is able to drive the viral tk promoter in transfected lens cells, the chicken αA-CRYBP1-like sequence is not able to do so (13), and site-specific mutagenesis of the chicken αA-CRYBP1-like sequence has no effect on the activity of the chicken αA-crystallin promoter in transfection experiments (15). Finally, unlike the mouse αA-crystallin promoter (8, 20), the chicken αA-crystallin promoter depends on upstream sequences between positions −160 and −121 for activity (14, 15, 36, 37). These experiments indicate that there have been numerous evolutionary changes in the regulatory sequences of the αA-crystallin gene.

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