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The cooperative interaction between two motifs of an enhancer element of the chicken αA-crystallin gene, αCE1 and αCE2, confers lens-specific expression

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ABSTRACT

An 84 bp element located between nucleotides −162 and −79 of the chicken αA-crystallin gene exhibits lens-specific enhancer activity. Transient transfection experiments using 5′ deletion and linker scanner mutants has indicated that the 84 bp enhancer element is composed of three motifs, αCE1 (−162 to −134), αCE3 (−135 to −121) and αCE2 (−119 to −99). Neither αCE1 or αCE3 motif alone can exhibit enhancer activity even when trimerized, whereas together they can direct some degree of lens-specific expression. αCE2 alone shows low transcriptional activity when trimerized. A combination of αCE1 with αCE2 exerts full lens-specific enhancer activity comparable with that of the 84 bp enhancer element, indicating that αCE1 and αCE2 motifs are sufficient to confer lens-specific expression. Transcriptional activation by these two motifs from a distance required the additional presence of either or both motifs adjacent to the β-actin basal promoter. Gel shift experiments indicated that the αCE1, αCE2 and αCE3 motifs specifically bind nuclear proteins. αCE1 binds a protein predominantly present in lens cells, whereas αCE2- and αCE3-binding proteins differ between lens and lung cells. Mutations within the αCE1 and αCE2 motifs that failed to bind nuclear factors *in vitro* resulted in loss of transcriptional activation, indicating that these nuclear factors play a key role in controlling lens-specific expression.

INTRODUCTION

During developmental processes an undifferentiated cell progresses to a differentiated state. The differentiated phenotype presumably reflects the selective expression of a set of genes whose products specify the differentiated cell type. Selective gene expression is mostly controlled at transcriptional level (1). Transcriptional regulation is mediated through the interaction of nuclear factors with sequence-specific DNA elements (2, 3). Understanding the molecular mechanisms that underlie tissue-specific gene expression requires identification of both *cis*- (4) and *trans*-acting elements (1−3) and their interactions in the specialized cells (1, 5).

Crystallins are major structural proteins in vertebrate lens cells and are classified into four groups, α-, β-, γ-, and δ-crystallins (6, 7). One of α-crystallins, αA-crystallin, is highly conserved and its expression is restricted to lens cells (8), although another type, αB-crystallin, appears to be expressed in several tissue types, including lens, brain, kidney and heart cells (9, 10). Culture systems of lens cells, the neural retina and pigmented epithelium provide a suitable system to study the mechanisms regulating crystallin gene expression, since their expression is determined by culture conditions (11, 12) and , in particular, the neural retina and the pigmented epithelium are able to differentiate into lens cells *in vitro* (a review for 'transdifferentiation'; 13). The regulatory elements required for lens differentiation have been identified for several crystallin genes (5, 14−18).

Gene expression in mammalian cells is often controlled by enhancers that exert their effect on the promoter sequences (1). We have shown that an enhancer element located between −162 and −79 of the chicken αA-crystallin gene confers its high level of lens-specific expression (5). This 84 bp element functions as a strong lens-specific enhancer when oligomerized, and is large enough to potentially bind several nuclear factors. It has been shown that enhancer elements are generally composed of several motifs that interact with DNA-binding proteins (19−21). To determine the subunit composition of the 84 bp enhancer element and study the cell-specific activities of its elements, we tested tandem repeats of various regions of the 84 bp enhancer element upstream or downstream of a chicken β-actin basal promoter for transcriptional activity by transfecting them into lens and lung cells in culture.

We show here that the 84 bp enhancer element is composed of three distinct motifs, αCE1 (−162 and −134), αCE3 (−135 and −121) and αCE2 (−119 and −99). Both αCE1 and αCE2 motifs are necessary and sufficient to exert a high level of lens-specific enhancer activity. DNA-binding and transient expression experiments using mutations in these motifs indicate that the nuclear factors which recognize these motifs are essential for conferring the tissue-specific activity of the chicken αA-crystallin enhancer element.

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MATERIALS AND METHODS

Plasmid constructions

Recombinant plasmids containing a bacterial chloramphenicol acetyltransferase (CAT) gene were based on the parent vectors pBS0CAT and pBS0CAT2 (5). A series of 5′ deletion mutants of the chicken αA-crystallin promoter were constructed as follows: a 155 bp BglII/SmaI DNA fragment containing sequences −162 to −8 of the αA-crystallin promoter was inserted between the BglII/SmaI sites of pUC19. The resulting plasmid was cleaved with PstI and XbaI, digested with exonuclease III at 1 minute intervals as described (22), and the resulting deletion plasmids were ligated with BamHI linkers (CGGATCCG). The 5′ end-points of these deletion fragments were determined by DNA sequencing. DNA fragments with deletion end-points spanning regions of interest were inserted between the BamHI and SmaI sites of the large fragment of α162CAT (5).

Linker scanner (LS) mutants were constructed as follows: a common primer (−244 to −225) containing a SacI restriction endonuclease site (GAGCTC) and mutagenic primers containing the BamHI linker were synthesized using a DNA synthesizer (Applied Biosystems Inc., Co.). These primer sequences, in which the SacI and BamHI linker sequences are underlined, are as follows:

AGAGCTCTTGTTCATCCAGATG (−244/−225)
TGGATCCGGGATGCTGCCAGGCTGTG (−171/−188)
TGGATCCGGCCAGATCTCCTGGAGGG (−155/−172)
TGGATCCGACAGTCTGGTGGGAACCAG (−135/−153)
TGGATCCGCCTGGGGATGACAGTCTGG (−125/−143)
AGGATCCGGCGGAGACTGACCTGGGG (−114/−131)
TGGATCCGCAGCAGAAATGCGGAGACTG (−104/−123)
TGGATCCGAAGGCAACGTGGTCAGCAG (−91/−109)
TGGATCCGTCACGACGAAGGCAACGTG (−83/−101)
TGGATCCGGACATGATCTCACGACGAAG (−74/−93)
TGGATCCGTGGAAAGACATGATCTCAC (−68/−86)
TGGATCCGAGTGGGATTTCTCTGGAAAG (−55/−74)
TGGATCCGAAGGCATTAGTGGGATTTC (−47/−65)
TCCATCCGCGCAGAATGAAGGCATTAG (−38/−56)
TGGATCCGCTGCCCCTATATATACTGC (−16/−34)

Polymerase chain reactions (PCR) were performed using the common primer and each of the mutagenic primers. The PCR fragments cleaved with SacI and BamHI were inserted into the SacI-BamHI site of the respective 5′ deletion mutants. The 143/140 mutant was constructed using the mutagenic primer, 5′-GGAGATCTGGCGCTGGTTCCCATTCTACTGTCATCC-CCAGGTCA (−143/−140, mutated nucleotides are underlined). $N_3\beta$CAT was constructed as described previously (5). $N'_3\beta$CAT was constructed by inserting three tandem copies of the BglII/BamHI DNA fragment (−162 to −89) of the 90/83 LS mutant into the BamHI site of βCAT. Oligonucleotides αCE1 (−162 to −134) and αCE2 (−119 to −99) containing BamHI and BglII sites at both ends shown in Figure 4B and 4C, were separately ligated in the presence of BamHI and BglII. Trimers of αCE1 and αCE2 were inserted into the BamHI site of βCAT (5), generating $1_3\beta$CAT and $2_3\beta$CAT, respectively. Three copies of the double-stranded oligonucleotide αCE3 (−135 to −121; 5′-CCGCGGTCATCCCCAGGTCAG-3′) were inserted into the blunt-ended BamHI site of βCAT, generating $3_3\beta$CAT. The SacI/BamHI fragment of $1_3\beta$CAT was inserted into the SacI-BamHI site of $2_3\beta$CAT, yielding $1_3+2_3\beta$CAT. $1_3+3_3\beta$CAT was constructed by ligating the blunt-ended SacI/HindIII DNA fragment of $3_3\beta$CAT between the blunt-ended BamHI and HindIII sites of $1_3\beta$CAT. The BamHI/HindIII DNA fragment of $2_3\beta$CAT was inserted into the BamHI-HindIII site of $3_3\beta$CAT, generating $3_3+2_3\beta$CAT. The sequences of mutated αCE1 and aCE2 oligonucleotides are shown in Figure 4B. Plasmids containing the mutated αCE1 and aCE2 motifs were constructed according to the same procedure used to construct $1_3\beta$CAT, $2_3\beta$CAT and $1_3+2_3\beta$CAT.

$1_3(4)2_3\beta$CAT was constructed by cleaving, blunting and religating the BamHI site of plasmid $1_3+2_3\beta$CAT. $1_3(30)2_3\beta$CAT was constructed by inserting the SacI/blunt-ended SalI DNA fragment of $1_3\beta$CAT into the SacI and blunt-ended SacII sites of $2_3\beta$CAT. 2_3b1_3CAT was constructed by inserting the $1_3\beta$CAT blunt-ended NotI-SalI DNA fragment into the blunt-ended HindIII site of $2_3\beta$CAT. For constructions of $\beta CAT1_3$, $\beta CAT2_3$ and $\beta CAT1_3+2_3$, the plasmid vector PBS0CAT, which contains the BamHI site downstream of the CAT gene, was used. Blunt-ended SacI/PstI DNA fragments of $1_3\beta$CAT, $2_3\beta$CAT and $1_3+2_3\beta$CAT which contain 1_3, 2_3 and 1_3+2_3, respectively, were inserted into the blunt-ended BamHI site of pBS0CAT, generating plasmids $\beta CAT1_3$, $\beta CAT2_3$ and $\beta CAT1_3+2_3$, respectively.

Cell cultures and transfections

Brains and neural retina were isolated from 8-day-old chicken embryos. Lens, lung, fibroblast, liver, heart and kidney tissues were obtained from 15-day-old chick embryos. Cell cultures were prepared as previously described (5). After 2 days incubation, brain cells and neural retina cells were transfected with the relevant plasmid. When the other cell types reached confluence, they were harvested with trypsin and transferred to secondary cultures. By day 2 after secondary incubation, cells (30mm dish) were cotransfected with 1.8 μg of a test plasmid and 0.2 μg of a control plasmid pMiwZ containing the β-galactosidase gene (23), which was used to normalize transfection efficiency for each dish.

CAT and β-gal assays

The cultures were harvested 48 hours post-transfection and lysed by repeated-freeze and thawing. In CAT assays, [^{14}C]-chloramphenicol was separeted from its acetylated derivatives by thin layer chromatography (5, 24) and their radioactivities were determined using a Bio-Image Analyzer FUJIX BA-100 (Fuji Film Co., Ltd., Tokyo, Japan). All values of CAT activities are averages of two to three independent transfection experiments and their standard deviations indicate the statistical significance of the average values. β-Galactosidase activities were assayed by the method of Edlund *et al.* (25).

Preparation of nuclear extracts

Nuclear extracts from secondary cultures of lens and lung cells were prepared by the modified method of Dignam *et al.* (26, 27).

Electrophoretic mobility shift assay

The nucleotide sequences of oligonucleotides used for electrophoretic mobility shift assays are presented in Fig. 4B and 4C. Oligonucleotides αCE1 and αCE2 were end-labelled using Klenow fragment and [α-^{32}P] dATP. An oligonucleotide αCE3 was labelled with T4 polynucleotides kinase and [γ-^{32}P]ATP. Each probe (10000 cpm) was incubated with lens and lung nuclear extracts (10 μg) in 10 μl of a reaction mixture (40mM NaCl, 50mM KCl, 10mM Tris HCl (pH7.5), 1mM EDTA, 0.5 μg of poly dI:dC and 10%(v/v) glycerol) in the absence or presence of competitor DNA for 30 min at 20°C. The mixtures were electrophoresed on a native 4% polyacrylamide gel in 1/4 TAE buffer. Gels were dried and subjected to autoradiography.

RESULTS

5′ Deletion analyses of the regulatory sequences required for lens-specific expression of the chicken αA-crystallin gene

In a previous study we showed that an 84 bp element located between positions −162 and −79 of the chicken αA-crystallin gene is sufficient for lens-specific expression and acts as lens-specific enhancer for heterologous genes (5). We intended to further dissect this promoter region into smaller functional segments required for lens-specific expression. For this purpose, progressive (from −162 to −7) 5′ deletion fragments were generated by exonuclease III digestion and linked to the reporter gene CAT. These constructs were then tested for enhancer activity following transfection into lens and lung cell cultures along with an internal control plasmid, pMiwZ, which contains the lacZ gene under a control of a chicken β-actin promoter.

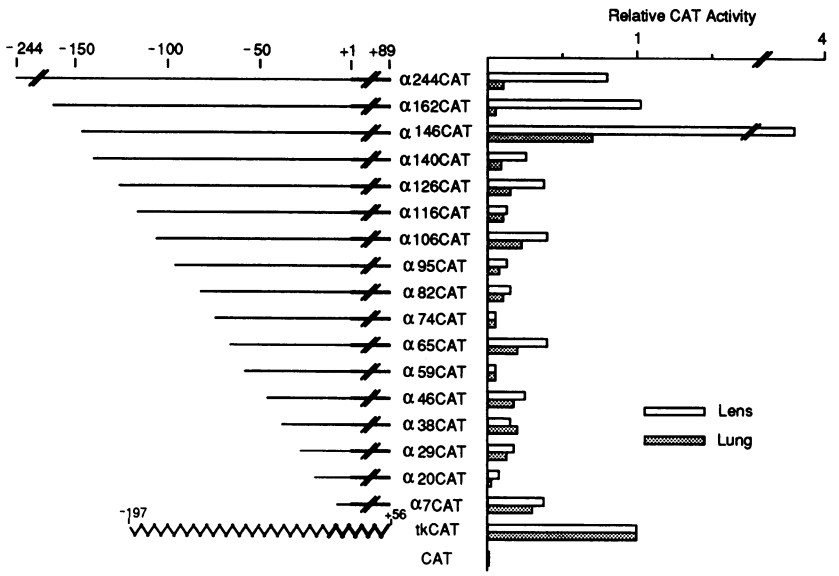

Figure 1. Deletion analyses of the 5′ regulatory region of the chicken αA-crystallin gene. 5′ Deletion mutants were constructed as described in Materials and methods. The numbers in plasmid names represent the nucleotide positions of the 5′ end point of the native sequences in the deletion mutants from the transcription start site. Relative CAT activities in lens and lung cultures are shown with the tkCAT activities in lens and lung cultures assigned as 1. Open and stippled bars represent the relative CAT activities of each plasmid in lens and lung cultures, respectively. The values represent the average of three independent experiments.

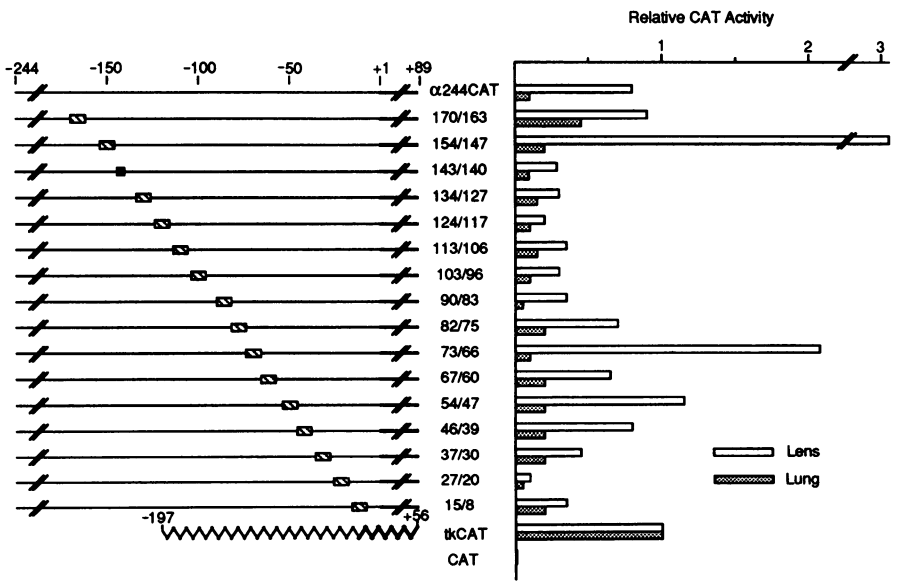

Figure 2. Linker scanner analysis of the 5′ regulatory region of the chicken αA-crystallin gene. Thin and thick lines represent the 5′ flanking and structural regions of the chicken αA-crystallin gene, respectively. Striped boxes represent the position of an 8 bp BamHI linker in each mutant and numbers in names of plasmids represent the nucleotide positions of both ends of the BamHI linker in each mutant from the transcription start site. Relative CAT activities in lens (open bar) and lung (stippled bar) cells are shown as described in the legend of the Figure 1. Each value represents the average of three independent experiments.

Corrected relative CAT activities of the deletion mutants in lens and lung cells are shown in Fig 1.

As shown in Fig. 1, the CAT expression of both αA244CAT and αA162CAT containing promoter region sequences up to positions −244 and −162, respectively, was 10- to 20-fold higher in lens cells than in lung cells, indicating that both constructs exhibited a high level of lens-specific expression. Surprisingly, a deletion to −147 resulted in a remarkable activation in both cell types. CAT activities of αA147CAT in lens and lung cells were 3.6- and 14-fold greater, respectively, than the respective activities of αA162CAT. There could be two possible explanations for this unexpected transcriptional activation: a negative element such as a silencer is located between −162 and −147; alternatively a site for a ubiquitous transcription-activating factor is generated by the insertion of a BamHI linker to link the 5′ ends of deletion mutants with the vector plasmid. The latter possibility is highly likely, since full activation of enhancer function requires the sequence −162 to −134, and the sequences −147 to −134 are not sufficient for full enhancer activation, as will be shown later (Fig. 3; $1_3+2_3\beta$CAT, Fig. 4; mt1g). Moreover, electrophoretic mobility shift experiments using DNA fragments containing sequences from −147 to −140 with BamHI linkers yield a new shifted band, not observed with the wild type sequences from −162 to −140 (data not shown).

A deletion to −140 (αA140CAT), however, specifically caused markedly reduced CAT expression in lens cells alone but did not suppress expression to a basal level, resulting in no cell type preference for transcriptional activity. This result suggested that the sequences from −162 to −140 contain an element controlling lens cell expression. Progressive deletions to −106 had only a slight effect on the promoter activity in both cell types. However, further deletion to −95 resulted in an additional 3- to 4- fold decrease and reduced expression to a basal level comparable with that of the construct containing only the TATA box (αA29CAT), suggesting that a region between −140 and −96 contains a *cis*-element controlling expression in lens and lung cells. Taken together, these results suggest that the region between −162 and −96 contains two functionally separable elements controlling lens-specific expression.

Further deletion to −20 resulted in no preference of transcriptional activities in lens and lung cells. However, αA65CAT showed a significant increase in CAT expression in both lens and lung cells, suggesting the presence of a negative element or the generation of a site for a transcription activating factor within the joining region of this deletion mutant. Deletion of the TATA box between −29 and −20 resulted in a complete loss of transcriptional activity. However, a further deletion to −7 resulted in a significant activation of CAT activities in lens and lung cells, suggesting the presence of an initiator (28). The definitive identification of a negative element and an initiator awaits further investigation.

Analyses of regions required for lens-specific expression by site-directed mutagenesis

5′-Deletion mutant analysis can identify a unique *cis*-element but not multiple *cis*-elements required for expression, since this procedure eliminates multiple elements that might be present upstream of the 5′ end of deletion mutants. To exclude this possibility, we constructed 8 bp BamHI linker scanner (LS) mutants using the polymerase chain reaction (PCR) method as described in Materials and Methods. A 4 bp linker mutation was employed for mutant 143/140. These LS mutations covered a region between −170 and −8 of the αA-crystallin promoter. Figure 2 shows the results of CAT activities of these LS mutant constructs in lens and lung cultures. An LS mutation between positions −170 and −163 gave no effect on expression in lens cells, but significantly increased transcription in lung cells. This is likely to be due to destruction of a negative element between positions −170 and −163, since this mutation increased CAT expression in all non-lens cultures so far tested (data not shown). LS mutant 154/147 showed a 3-fold activation in transcription in lens cells, consistent with the result of a plasmid αA146CAT. In these two constructs, 8 bp sequences upstream of position −147 are exactly the same, since the same 8 bp BamHI linker was used for construction of 5′ deletion and LS mutants. Therefore, this up regulation is likely to be due to an artificial generation of a transcription factor binding site.

A 4 bp mutation between −143 and −140 drastically decreased transcriptional activity in lens cells. This confirmed the result of 5′ deletion mutants (αA140CAT), indicating that sequences from −162 to −140 are essential for lens-specific expression. The results of 5′ deletion mutants suggest the presence of another element between positions −140 and −96 controlling expression in lens and lung cells. LS mutants between −143 and −83 still retained promoter activities and similar expression patterns were observed both in lens and lung cells. In these mutants the element −162 to −140, which is essential for expression, is present, so might confer partial transcriptional activity. LS mutants between −82 and −39 showed promoter activities comparable to wild type αA244CAT, indicating that the sequences −82 to −39 are not important for lens cell expression, and consequently that the sequences between −162 and −79 are essential for lens-specific expression. The increase of transcription with mutant 73/66 might be due to generation of a site for a ubiquitous transcription factors, since a cooperative interaction between the element −162 to −79 and a site for ubiquitous factors such as Sp1 and AP1 resulted in a drastic transcriptional activation in lens cells (Matsuo and Yasuda, in preparation). Plasmid 27/20 containing a mutation in the TATA box sequence completely lost promoter activity. Taken together, these results suggest that at least two positive elements, sequences between positions −162 and −130, and between positions −130 and −84, are required for lens-specific expression.

Cooperative interactions between αCE1 and αCE2 motifs confers lens-specific expression

The results described above suggest that the 84 bp enhancer element is composed of at least two elements, sequences from −162 to −130 and from −130 to −84. To explore the multiple functional elements within this enhancer element which are sufficient for lens-specific expression, we divided the 84 bp enhancer element into three motifs, αCE1 (−162 to −134), αCE2 (−119 to −99) and αCE3 (−135 to −121) (Fig. 3A). In order to easily detect the CAT activities of different constructs containing various combinations of these motifs, we obtained three tandem copies with each segment and linked them alone or in combination with each other to the CAT gene under the control of the chicken β-actin basal promoter. This promoter (−55 to +53) contains only a TATA box and shows no transcriptional activity (Fig. 3; βCAT). A schematic representation of the constructions and sequences of each motif are presented in Fig. 3A.

As reported in a previous study, $N_3\beta$CAT which contains three tandem copies of the original 84 bp enhancer element

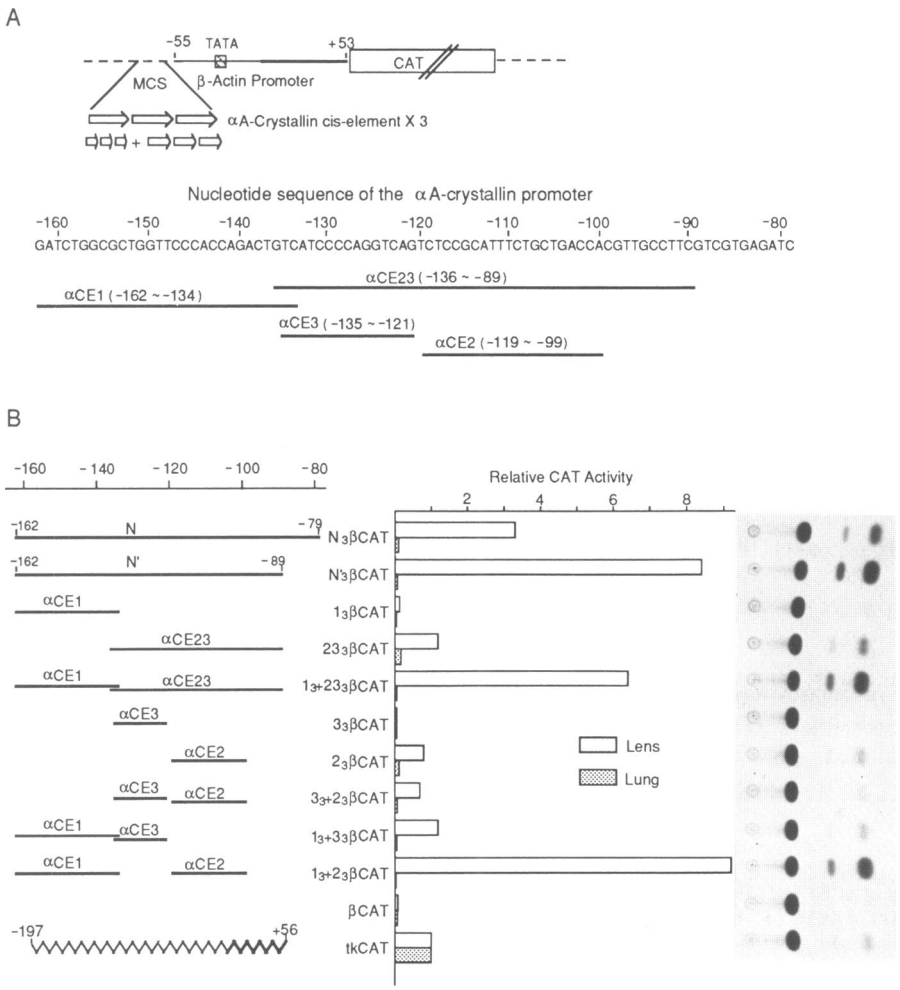

Figure 3. Dissection of the 84 bp enhancer element into three motifs. **(A)** A schematic representation of the vector plasmid and the nucleotide sequence of the 84 bp enhancer element. Three tandem copies of sequences (αCE1, αCE2, αCE3, αCE23) from the 84 bp enhancer element were inserted alone or in combination with either of the other two motifs as shown in the left hand-side of the panel B into the BamHI site upstream of a chicken β-actin basal promoter (−55 to +53) in the same orientation as the original one. **(B)** Transcriptional activity of constructs containing one or two motifs. The combination of motifs are shown on the left. Relative CAT activities of each construct in lens (open bar) and lung (shaded bar) cells are presented in the middle and an autoradiogram of the CAT assays in lens cultures at the right. The values represent the average of three independent experiments.

activated transcription at high levels only in lens cells (5). Deletion of 10 nucleotides from the 3′ end of this enhancer element ($N'_3\beta$CAT) resulted in a 2.5-fold activation of transcription in lens cells. 5′ Deletion experiments indicated that a sequence −162 to −134, termed αCE1, is essential for high level expression in lens cells. However, even three tandem copies of this motif itself failed to activate transcription both in lens cells and in lung cells (Fig. 3B, $1_3\beta$CAT), indicating that this element alone is not sufficient for expression. In contrast, αCE23 (−135 to −89) exhibited lens-specific expression ($23_3\beta$CAT), although the level of expression was 8 times lower than that of $N'_3\beta$CAT. However, when both elements were combined adjacent to the TATA box, the resulting construct $1_3+23_3\beta$CAT showed an activity comparable with that of the original construct $N'_3\beta$CAT. These results indicate that two elements are required for full activation and that the enhancer element is composed of functionally distinct motifs.

To further examine the functional motifs within the region between −135 and −89, this region was separated into two fragments, αCE3 (−135 to −121) and αCE2 (−119 to −99) as shown in Fig. 3A. αCE3 alone failed to activate transcription in both cell types, even when trimerized ($3_3\beta$CAT). In contrast, αCE2 showed detectable transcriptional activities in lens and lung cells as a single copy, and significantly increased transcription when trimerized (Fig. 3 and 6, $2_3\beta$CAT). αCE2 alone exhibited expression some extent of enhanced expression in lens cells and a detectable level of expression in non-lens tissues such as lung ($2_3\beta$CAT) and liver cells (Fig. 5, $2_3\beta$CAT).

Surprisingly, combination of the αCE1 motif with the αCE3 motif, either of which alone showed no transcriptional activity in either lens or lung cells, resulted in an increase in lens cell specific transcriptional activity ($1_3+3_3\beta$CAT), this transcription level being comparable to that of $23_3\beta$CAT but much less than $N'_3\beta$CAT. Full enhancer activity, comparable with that of the original plasmid $N'_3\beta$CAT, was recovered only when αCE1 and αCE2 were combined ($1_3+2_3\beta$CAT). Taken together, these results strongly indicate that the 84 bp enhancer element is composed of three motifs, αCE1, αCE2 and αCE3, and that αCE1 and αCE2 together are both necessary and sufficient for lens-specific expression.

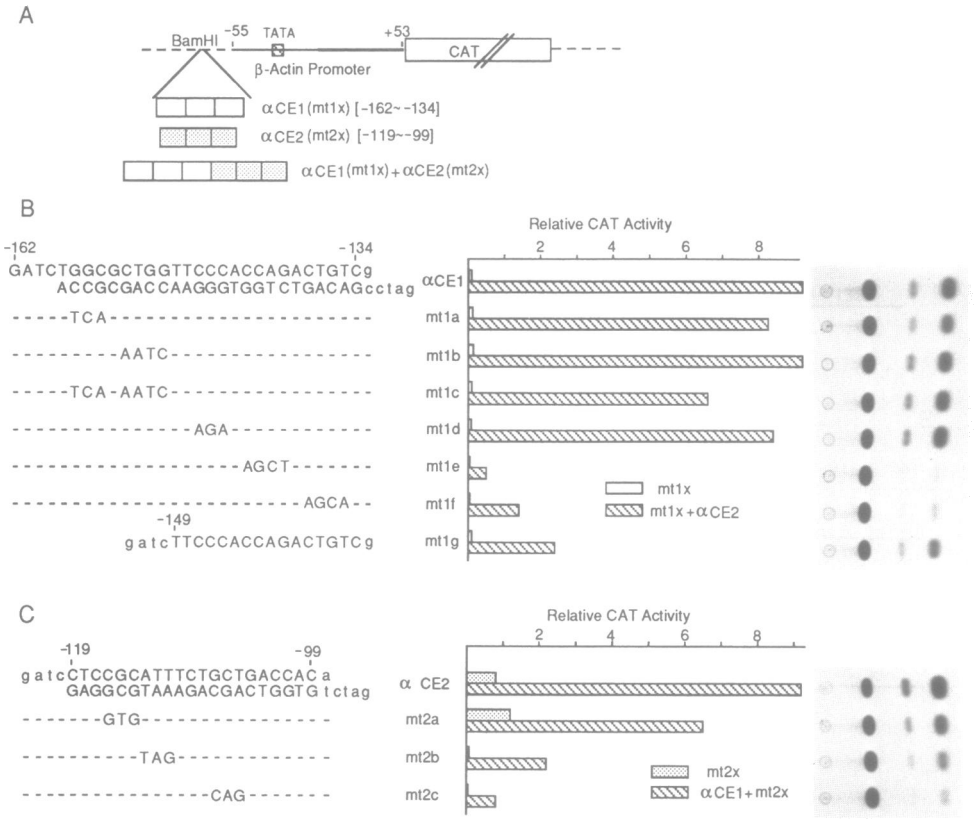

Figure 4. Mutational analysis of αCE1 and αCE2 motifs for lens-specific expression. **(A)** A schematic representation of reporter plasmid constructs. Three tandem copies of mutated αCE1 (mt1x) or αCE2 (mt2x) motifs together with or without the other motif were inserted into the BamHI site upstream of the β-actin promoter (−55 to +53). The nucleotide sequences of the upper and lower strands of the wild type αCE1 (B) and αCE2 (C) motifs are shown along with the upper strand of the mutations, relative location being shown below the wild type. The name of each mutant oligonucleotide is indicated to the right. **(B)** Three tandem repeats of each mutant oligonucleotide (mt1x) were inserted into the BamHI site of 2_3βCAT or βCAT. A histogram indicates relative CAT activities of each construct with (striped bar) or without (open bar) αCE2 after assigning the activities of tkCAT in lens cells as 1. **(C)** Three tandem repeats of each mutant oligonucleotide (mt2x) were inserted into the BamHI site of 1_3βCAT or βCAT. A histogram indicates the relative CAT activities of each construct with (striped bar) or without (stippled bar) αCE1 with the activities of tkCAT in lens cells assigned as 1. Autoradiograms indicate a representative CAT assay of each construct. Each value represents the average of three independent experiments.

Mutational analyses of two motifs

To determine the nucleotide sequences of αCE1 and αCE2 motifs responsible for full activation, we introduced 3 to 7 nucleotide substitution mutations within each motif (Fig. 4B and 4C), and serially replaced one motif of the constructs 1_3βCAT, 2_3βCAT or 1_3+2_3βCAT with a mutated motif. The resulting constructs $(mt1x)_3$βCAT, $(mt2x)_3$βCAT, $(mt1x)_3+2_3$βCAT or $1_3+(mt2x)_3$ βCAT were transfected into lens cells and their CAT activities assayed (Fig. 4B and 4C).

First, in order to identify the important sequences within the αCE1 motif we examined the functions of mutated αCE1 motifs alone or in combination with three tandem copies of the wild type αCE2: e. g. $(mt1a)_3$βCAT and $(mt1a)_3+2_3$βCAT (left side of Fig. 4B). Their relative CAT activities are demonstrated on the right side of Fig. 4B. Substitutions of 3 to 4 nucleotides between positions −157 and −145 of the αCE1 sequence exhibited no effect on transcriptional activity (Fig. 4B; mt1a, mt1b and mt1d). However, substitution of 7 nucleotides within this region led to a 30% reduction $[(mt1c)_3+2_3$βCAT]. In contrast, a 4 bp mutation spanning nucleotides −143 to −140 $[(mt1e)_3+2_3$βCAT] completely abolished expression in lens cells and mutating between positions −138 and −135 resulted

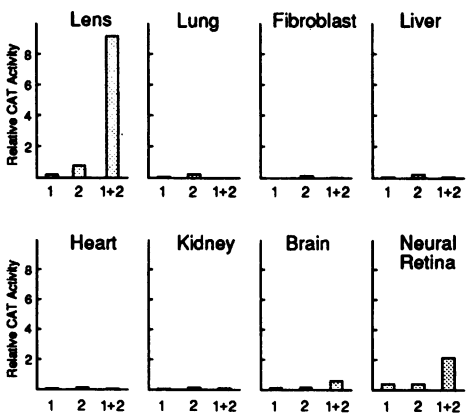

Figure 5. Both αCE1 and αCE2 motifs are sufficient to confer lens-specific expression. Three copies of αCE1 (1; 1_3βCAT) or αCE2 (2; 2_3βCAT) or both motifs (1+2; 1_3+2_3βCAT) were inserted into the BamHI site upstream of the β-actin promoter (−55 to +53) linked to the CAT gene. Each construct was transfected into cells cultured from lens, lung, fibroblast, liver, heart, kidney, brain and neural retina tissues. CAT activities are shown to relative to the respective CAT activities of tkCAT which is assigned as 1. Each value represents the average from two independent experiments.

in a substantial reduction in promoter activity [$(mt1f)_3+2_3\beta CAT$]. These results demonstrated that nucleotides −143 to −135 are important for full activity in lens cells. To examine whether the sequences from −143 to −135 are sufficient to activate transcription in lens cells, the sequences from −149 to −134 were trimerized, ligated with three tandem copies of αCE2 and linked them to the CAT reporter gene. The resulting construct [$(mt1g)_3\beta CAT$] showed 4 folds lower activity than wild type. These results indicate that sequences from −143 to −135 are not enough and that at least nucleotide sequences between −162 and −135 are important for full lens cell transcriptional activity.

Secondly, we examined the importance of the αCE2 sequence in the similar constructs to those described above. Mutations of an αCE2 sequence at positions −116 to −114 did not affect basal activity and exhibited enhancer activity when combined with αCE1 (Fig. 4C, mt2a, stippled bar). Mutation between nucleotides −113 and −111 reduced expression but still retained transcriptional activity in lens cells (mt2b, stippled bar). In contrast, mutation between positions −107 and −105 resulted in complete abolition of promoter activity (mt2c; stippled bar). These results indicates that αCE2, especially the sequences from −107 to −105, together with αCE1, play a key role in activating transcription in lens cells.

Two motifs confer crystallin gene expression

To address how αCE1 and αCE2 alone or together function in various types of cultured cells, we examined the promoter activities of these three types of constructs, $1_3\beta CAT$, $2_3\beta CAT$ and $1_3+2_3\beta CAT$ in various cell types, including lens, lung, fibroblast, liver, heart, kidney, brain and neural retina. The results of transient expression experiments in various types of cultured cells are shown in Fig. 5. αCE1 alone functioned solely in neural retina, in which CAT activity was detectable and always significantly higher than in other tissues. αCE2 alone activated low level transcription in lens cells, lower levels in neural retina, lung and liver cells and none in other tissues. A construct combining αCE1 with αCE2, however, completely failed to function in lung, fibroblast, liver, heart and kidney cells. In contrast, transcription was activated to a high level in lens cells, a middle level in neural retina and a low level in brain cultures. Low levels of transient expression in neural retina and brain cultures of the αA-crystallin gene have been reported previously (5, 29), indicating that αCE1 and αCE2 together function as well as the original 84 bp enhancer element. This ectopic expression of crystallin gene was explained by the observations that during early development of chick embryos crystallin genes are transiently expressed at low levels in brain and neural retina and that these tissues can transdifferentiate into lens cells when cultured *in vitro* (8, 12, 13). Levels of transient expression of construct $1_3+2_3\beta CAT$ in brain and neural retina correlate well with the capacities of these cell types to transdifferentiate into lens cells.

Effects of position and number of two motifs on expression

For functional analyses of the two motifs, αCE1 and αCE2, we used the plasmid $1_3+2_3\beta CAT$ which contains three tandem copies of αCE1 directly linked to three tandem copies of αCE2 immediately 5′ to the TATA box and found that it acts as a lens-specific enhancer. To examine whether this highly lens-specific enhancer activity is dependent on or independent of the distance between the two motifs, we separated them by various distances and tested enhancer activity. These results are represented in Fig. 6. Insertions of 4 and 30 nucleotides between two motifs in $1_3+2_3\beta CAT$, generating constructs $1_3(4)2_3\beta CAT$ and $1_3(30)2_3\beta CAT$, respectively, caused 20 and 60% reduction, respectively, in CAT activities in comparison with the original plasmid $1_3+2_3\beta CAT$, but retained the ability to activate transcription in lens cells. These results indicate that separating two motifs by 30 bp had no significant effect on lens-specific enhancer activity. However, when they were placed 140 bp apart across the TATA box (2_3b1_3CAT), enhancer activity was drastically reduced to a level comparable with that observed when only three tandem copies of one αCE1 motif are placed immediately 5′ to the TATA box ($1_3\beta CAT$). Further separation by 1.6 kbp completely lost CAT expression ($1_3\beta CAT2_3$ and $2_3\beta CAT1_3$). These results suggest that motif spacing of less than 140 bp is important to exert enhancer activity.

To determine whether the positions of two motifs relative to the TATA box are important, we placed αCE1 motif immediately 5′ to the TATA box and the αCE2 motif 30 bp upstream of the αCE1 motif. The resulting plasmid $2_3(30)1_3\beta CAT$ showed enhancing activity comparable to that of $1_3(30)2_3\beta CAT$, indicating that the relative position of the two motifs is not essential for enhancer activity. As we have used trimers of both motifs for functional analyses, we tested whether one motif alone can synergize with three tandem copies of the other motif for transcription activation. To this end, we inserted one αCE2 motif between three tandem copies of αCE1 and the TATA box ($1_32\beta CAT$). Insertion of one αCE2 motif into plasmid $1_3\beta CAT$ drastically increased transcription (50 times) compared with that of the original plasmid $1_3\beta CAT$. These results indicate that at least one αCE2 motif, together with three copies of αCE1, is sufficient for enhancer activity.

At least one of the motifs is required for enhancer function from a distance

In a previous study we showed that three copies of the 84 bp enhancer element drastically increase expression in lens cells when placed immediately 5′ to a basal promoter, but decrease it 100-fold when placed at a distance, 1.6 kbp downstream of the transcription start site. However, enhancer activity increased 20-fold when only one 84 bp element was inserted immediately 5′ to the basal promoter of the latter construct. These results indicate that the enhancer elements synergistically interact with each other to activate transcription. When two motifs αCE1 and αCE2 are placed 1.6 kbp downstream of the transcription start site, they fail to enhance transcription as well as the 84 bp enhancer element. To address the question of whether this cooperative interaction requires either αCE1, αCE2, or both, we inserted them immediately 5′ to the plasmid containing three copies of two motifs downstream of the CAT gene. As demonstrated in Fig. 6, either motif alone is enough to activate transcription in lens cells ($1\beta CAT1_3+2_3$ and $2\beta CAT1_3+2_3$), and both motifs adjacent to the TATA box ($1+2\beta CAT1_3+2_3$) further stimulate transcription to a level comparable with that of the construct $1_3+2_3\beta CAT$.

Identification of factors specifically binding to each motif

To see whether stimulation of transcription by these enhancer motifs is due to the presence of factors binding to each motif, electrophoretic mobility shift assays and binding competition experiments were performed using the three motifs as probes and nuclear extracts from cultured lens and lung cells. The results

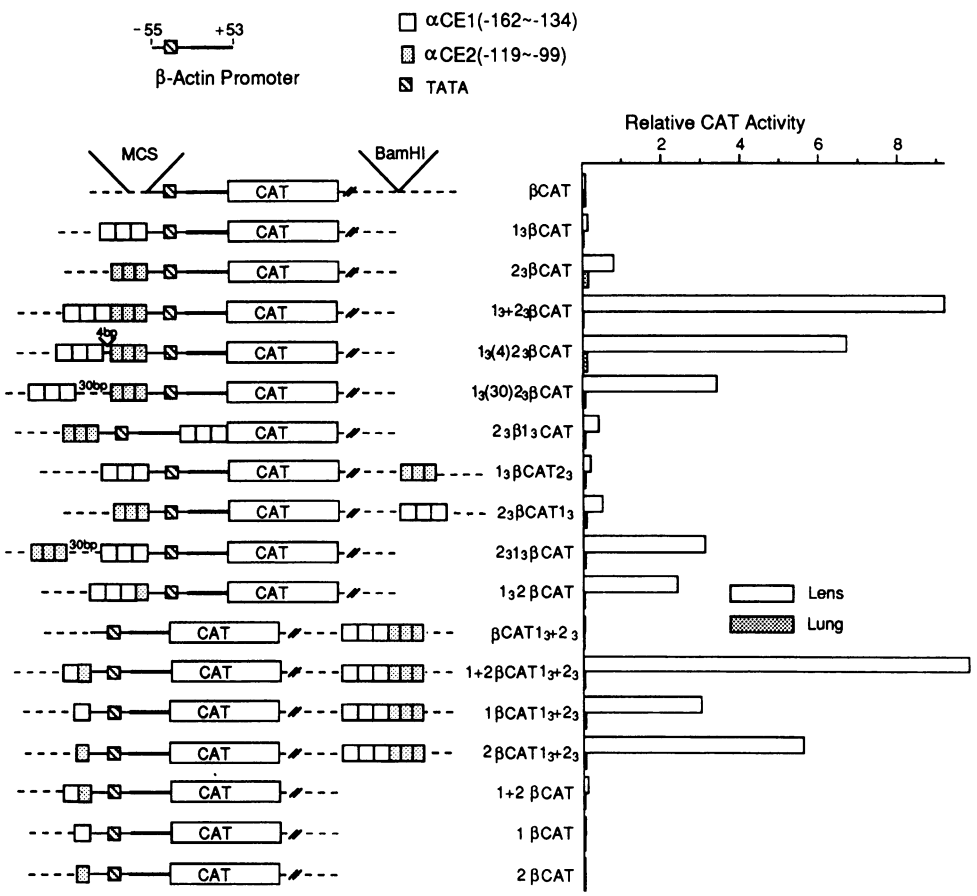

Figure 6. Effects of spacing between two motifs and their positions relative to the TATA box on CAT expression. The chicken β-actin basal promoter used is schematically represented at the top of the figure. Open, stippled and striped boxes represent αCE1 motif (−162 to −134), αCE2 motif (−119 to −99) and the β-actin TATA box, respectively. The dotted line indicates regions of the vector plasmid. Each construct is schematically shown on the left. The numbers between motifs indicate the distances in bp between the junctions of two motifs. Relative CAT activities of each construct in lens and lung cells are demonstrated by open and stippled bars, respectively, on the right-hand side. The values represent the average of three independent experiments.

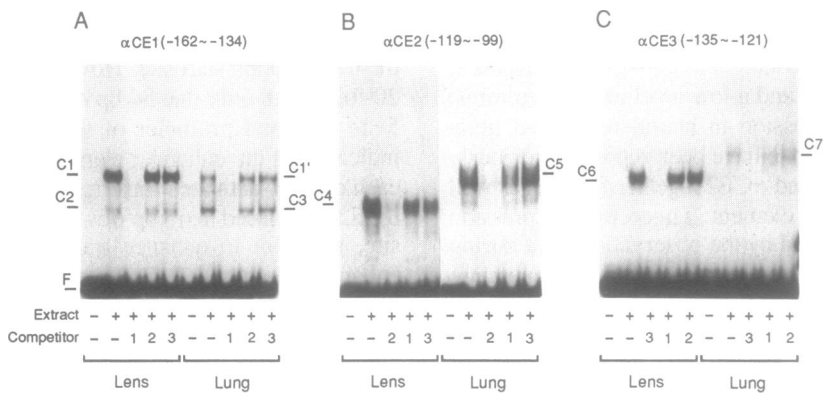

Figure 7. Electrophoretic mobility shift analysis of nuclear factors binding to αCE1, αCE2 and αCE3 motifs. Probes were incubated with lens (Lens) or lung (Lung) nuclear extracts in the absence (−) or presence (+) of 50-fold molar excess of unlabeled competitor oligonucleotides, αCE1 (1) αCE2 (2) and αCE3 (3). Probes in panel A, B and C are the αCE1, αCE2 and αCE3 motifs, respectively.

presented in Fig. 7A showed the presence of a specific αCE1-binding factor, termed αCEF1, in both lens and lung cells, since 50-fold molar excess of unlabeled competitor sequences, αCE2 and αCE3, failed to compete for complex formation (complex C1 and C2) but the same amount of specific competitor DNA (αCE1 sequence) completely inhibited binding. The αCE1-binding factor is 10- to 20-fold less abundant in lung cells than in lens cells, suggesting that this factor is ubiquitous and

its concentration differs in various tissues. There is a slight difference in mobility of complexes formed with lens and lung nuclear extracts.

The binding competition experiments shown in Fig. 7B indicate that factors which specifically bind to the αCE2 motif are also present in both cell types (Fig. 7B, C4 and C5). The mobilities of the specific complexes formed with lens and lung nuclear extracts are quite different, indicating that these DNA-binding factors have the same or very similar binding specificity but are of a quite different molecular nature. Therefore, this difference might result in a reversal function of the αCE2 motif when combined with the αCE1 motif. αCE2 drastically stimulates crystallin gene expression in lens cells but αCE2 completely abolished expression in lung cells.

Between positions −131 and −125 of the αCE3 motif an AP2-binding site consensus sequence CCCCAGG is present. Using an αCE3 probe, a single major complex (Fig. 7C; C6) was formed with lens nuclear extracts, only a minor and more slowly migrating complex (C7) being observed in lung nuclear extracts. Formation of both complexes was completely prevented with the specific competitor DNA (αCE3) but not with unlabeled non-specific competitor DNAs (αCE1 and αCE2), indicating that the lens and lung cell DNA-binding proteins have the same binding specificity but differ in molecular mass.

To examine whether the *in vivo* function of the normal or mutated αCE2 and αCE1 motifs correlate with their DNA-binding activities, we performed binding competition experiments using an αCE1 and αCE2 probes and unlabeled DNA fragments from plasmids used for functional analysis of each motif. These plasmids containing three tandem copies of mutated and normal motifs are cleaved with SacI and PstI, generating a roughly 200 bp DNA fragment containing two motifs, e. g. $(mt1a)_3+(\alpha CE2)_3$. Thus, for example, competitor 1a in Fig. 8A represents 20-molar excess of unlabeled DNA fragment $(mt1a)_3+(\alpha CE2)_3$. This correlates to a 60 fold molar excess of binding sites since each DNA fragment contains three tandem copies of this motif. These competitor DNA fragments and nuclear extracts from cultured lens cells were used for binding competition experiments. The results presented in Fig. 8A demonstrate that unlabeled normal (αCE1) and the mutated αCE1 motifs (mt1a and mt1d), which were shown to stimulate transcription *in vivo*, competed efficiently for complex C1 and C2 formation, indicating that they retain DNA-binding activity. In contrast, addition of mutated oligonucleotides which failed to stimulate transcriptional activity (mt1e and mt1g) in binding mixtures exerted no significant effect on complex formation, indicating loss of their ability to form DNA-protein complexes. Together, these results strongly indicate that a factor(s) which specifically binds to αCE1 participates in the stimulation of transcription in lens cells. However, there is one exception in that the mutated αCE1 (mt1c) which still retain 70% CAT expression compared with that of the wild type αCE1 lost its inhibitory effect on DNA-binding. This shows that whilst mt1c can not bind a nuclear factor but retains its ability to stimulate transcription. mt1f was able to bind αCEF1 but lost the ability to stimulate transcription. These discrepancies could be due to differences between *in vivo* and *in vitro* αCEF1 binding activity, since a single αCE1 motif was used as a probe for *in vitro* binding assays but multiple copies of αCE1 and αCE2 were used for the transient expression experiments. Potentially a single αCE1 motif may not bind nuclear factors *in vitro* but could bind them *in vivo* when multimerized or combined with αCE2 motifs. It

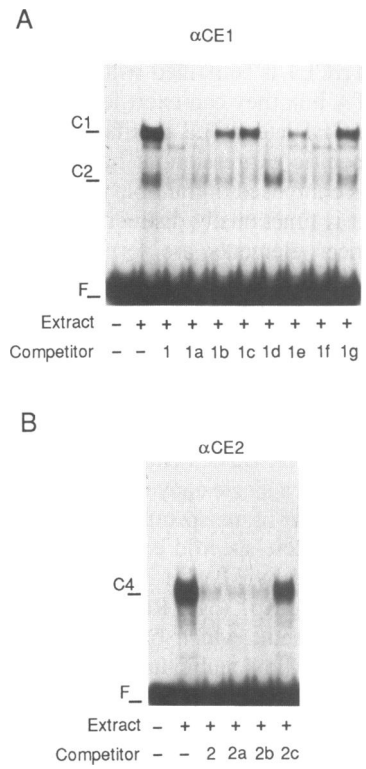

Figure 8. Binding competition experiments using mutated oligonucleotides of αCE1 and αCE2 motifs. **(A)** Probes αCE1 were incubated with lens nuclear extracts in the presence of a 20-fold molar excess of unlabeled competitor DNAs. Competitor DNA 1x corresponds to the SacI/PstI DNA fragments $[(mt1x)_3+(\alpha CE2)_3]$ of plasmid $(mt1x)_3+2_3\beta CAT$. The nucleotide sequences of mt1x are shown on the left-hand side of Fig. 4B. **(B)** Probes αCE2 were incubated with lens nuclear extracts in the presence of a 20-fold molar excess of unlabeled competitor DNAs. Competitor DNA 2x corresponds to the SacI/PstI DNA fragments $[(\alpha CE1)_3+(mt2x)_3]$ of plasmid $1_3+(mt2x)_3\beta CAT$. The nucleotide sequences of mt2x are shown on the left-hand side of Fig. 4C.

is likely that a protein involved in the formation of the C2 complex is not critical for expression, since mt1d was able to compete out C2 complex formation.

A good correlation was observed between binding activity and transcription stimulatory activity with mutated αCE2 motifs with the exception of one mutation. Fig. 8B indicates that the wild (αCE2) and mutated αCE2 (mt2a) motifs efficiently prevented complex formation and the mutant mt2c completely failed to compete for binding. These mutations were shown to stimulate transcription (Fig. 4B), indicating that the binding protein(s) (αCEF2) plays a important role in stimulating transcription in lens cells. The mutant mt2b can bind lens nuclear factor as efficiently as wild type but reduced 4 times CAT activity level in comparison with the wild type, suggesting a possible requirement of an unidentified factor(s) for transcriptional activation by αCE2 in *in vivo* lens cells.

DISCUSSION

Modular structures of crystallin enhancers

In this study we clearly demonstrated that the chicken αA-crystallin gene 84 bp enhancer element is composed of three motifs, αCE1 (−162 to −134), αCE3 (−135 to −121) and αCE2 (−119 to −99). αCE1 and αCE3 motifs alone fail to stimulate transcription from a TATA promoter (Fig. 3, $1_3\beta$CAT

and $3_3\beta$CAT). However, the αCE2 motif alone stimulated a low transcriptional activity when oligomerized (Fig. 6, 2βCAT and $2_3\beta$CAT). When αCE1 is combined with either αCE2 or αCE3 close to the TATA box they can exert lens-specific expression. It is noted that αCE1 and αCE2 motifs together are sufficient to confer lens-specific expression. It has been shown that the SV40 enhancer is composed of multiple functional elements and that each element is functionally distinct (19–21, 30). Subunits that form enhancer elements are termed as enhansons and correlate with sequence motifs and protein binding sites (21). These enhansons are classified into three classes (19). Based on this classification, αCE1 and αCE3 motifs of the 84 bp crystallin enhancer are class B enhansons as they cannot enhance transcription when oligomerized as a tandem repeats but whose association with a second motif results in transcription activation after oligomerization. αCE2 is a class C enhanson as it exhibits enhancer activity when a single copy of the motif is oligomerized.

Modular structures of tissue-specific enhancers have also been reported (1, 30). Muscle-specific expression of the cardiac α-actin gene is mediated by MyoD1, CArG-box binding factors and a ubiquitous factor Sp1 (31). Rat albumin gene expression requires a distal element, which binds a liver-specific factor HNF1, and a CCAAT-box, a ubiquitous factor binding site (32). A hepatitis B virus promoter can be activated in a liver-specific manner through cooperative interaction between HNF1 and Oct-1 (33). Several crystallin genes also require multiple elements to activate transcription in a lens-specific manner. An enhancer of the mouse αA-crystallin gene is composed of a proximal (−111 to −88) and distal element (−88 to −60) (14). The mouse γF-crystallin enhancer comprised of three enhancer elements and duplication of the sequences from −67 to −25 acts as a strong transcriptional activator (34). In the chicken δ1-crystallin gene an enhancer appeared to be present in the third intron and contains two functionally distinct elements: one functions only in lens cells and the other in any cell types (35). These results demonstrate that tissue-specificity of most gene expression is determined not by a single tissue-specific factor but, rather, by combinations of sites for tissue-specific and ubiquitous transcription factors.

It has been shown that the SV40 enhancer can not stimulate transcription from a reporter gene with a minimal promoter when placed at a distance from that promoter (36–38). Long range enhancer activity requires one or more sites for ubiquitous factors, such as Sp1 or CREB, adjacent to the promoter. Three copies of αCE1 and αCE2 motifs function as a strong lens-specific enhancer when placed adjacent to the basal promoter (Fig. 6, $1_3+2_3\beta$CAT) but fail to stimulate transcription when placed downstream of the CAT gene (βCAT1_3+2_3). However, when either or both motifs are placed adjacent to the TATA box in the latter construct, (1βCAT1_3+2_3, $_2\beta$CAT1_3+2_3 and $1+2\beta$CAT1_3+2_3), the ability to stimulate transcription in lens cells is obtained. Thus, it is likely that the motif(s) close to the TATA box can interact with basal transcriptional machinery and thus could mediate long range interaction between this machinery and the enhancer elements downstream of the CAT gene to stimulate transcription.

αCE1 and αCE2 motifs in regulatory regions in crystallin genes

The αA-crystallin gene is highly conserved between species. Regulatory regions of the murine and chicken αA-crystallin genes have been determined in homologous and heterologous systems. Both promoters show preferred expression in lens cells but their

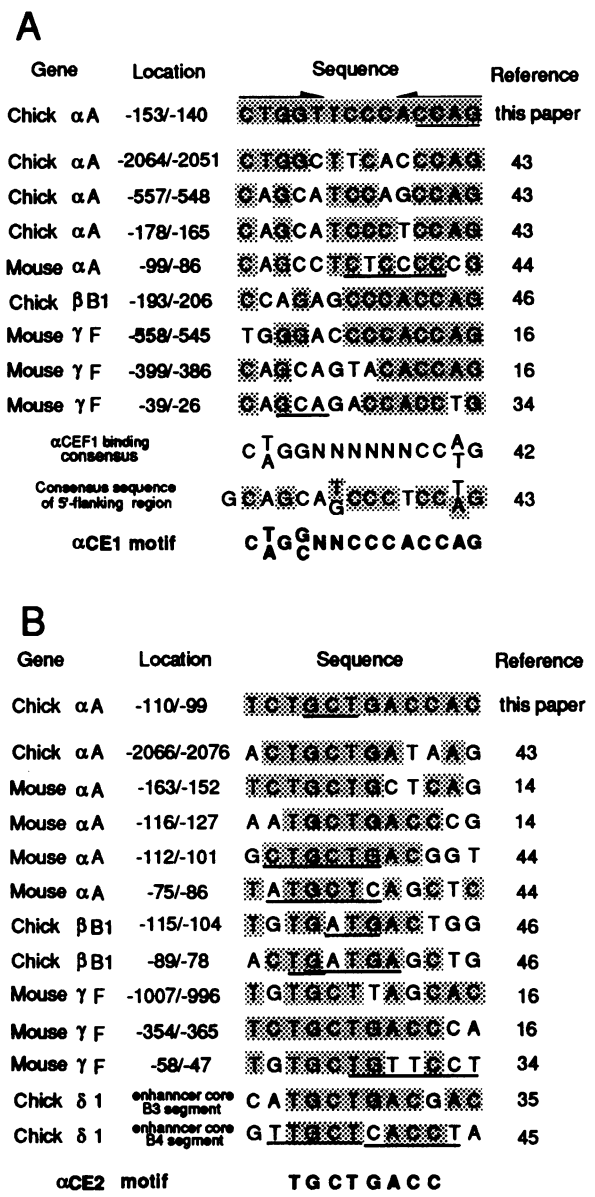

Figure 9. Comparison of the sequences of αCE1 and αCE2 motifs with the regulatory regions of several crystallin genes. Nucleotide sequences of the regulatory regions of several crystallin genes are grouped into αCE1 (**panel A**) and αCE2 motifs (**panel B**). The sequences whose mutations reduced transcription are underlined and are taken from the references listed on the right-hand side. Nucleotides which are conserved with each motif of the chicken αA-crystallin enhancer element are shaded.

regulatory regions differ. Sequences from −242 to −178 of the chicken αA-crystallin promoter are required for lens-specific expression in cultured mouse lens cells (17) and transgenic mice (39), but sequences from −162 to −79 are sufficient in chicken lens cells (5). Lens-specific expression of the mouse αA-crystallin promoter required both the distal and proximal elements in cultured chicken lens cells (14), but only the proximal element in transgenic mice (18). The functional differences in the murine and chicken αA-crystallin promoters probably reflect the numerous sequence differences (5, 39). In addition, although the mouse does not contain a homologue of the chicken δ1-crystallin

gene and the chicken does not contain a mouse γF-crystallin gene homologue, the promoters of these genes contain regulatory elements for lens specificity in both species. The chicken δ1-crystallin gene is expressed in cultured mouse lens cells (15) and transgenic mice lens cells (40). Conversely mouse γF-crystallin promoters determine lens specificity in transgenic mice (41) and in cultured chicken lens cells (16, 34). Therefore, it is highly likely that common regulatory sequences and transcription factors are involved in regulating crystallin gene expression in these divergent species.

Recently we identified the binding consensus sequence of the αCE1 motif by electrophoretic mobility shift assays using site-directed mutagenesis (42). On the basis of the consensus sequence, several potential αCEF1-binding sites were identified in the control regions of crystallin genes. The consensus sequence, C(T/A)GGN$_6$CC(A/T)G, is a 4 bp inverted repeat separated by 6 bp, which is presented together with the regulatory sequences in Fig. 9A. Nucleotide sequences similar to this αCE1 motif were found in the 5′ flanking regions of several crystallin genes (43). These regulatory sequences were shown to be essential for transcriptional activation in lens cells, since mutations in these regions (underlined in Fig. 9A) abolished expression.

Next, we compared homologous sequences of αCE2 motif with regulatory regions of several crystallin genes and found the homologous sequences shown in Fig. 9B. Some of these sequences were shown to be essential for transcriptional activation in lens cells, since mutations in them (underlined in Fig. 9B) abolished expression (44–46). Mutations between positions −111 and −106, and between positions −81 and −76 of the mouse αA-crystallin promoter abolished transcriptional activity in mouse lens cell lines (44). The sequences up to −88 of the mouse αA-crystallin promoter are sufficient to confer lens-specific expression in transgenic mice (18). Promoter sequences −58 to −47 of γ-crystallin genes of several species are highly conserved, and deletions and mutations within this region of the mouse γF-crystallin promoters result in a complete loss of enhancer function in chicken lens cultures (34). Chicken δ1-crystallin enhancer core sequences in the third intron lost enhancer activity in chicken lens cultures when 5 bp-substitutions were introduced within the sequence shown in Fig. 9B (45). These results indicate that αCE2 motifs in these crystallin genes are further candidates for regulatory elements that confer lens specificity of expression.

DNA-binding proteins

We identified factors which bind to each motif using electrophoretic mobility shift assays. Binding competition experiments indicate that they are sequence-specific. An αCE1-binding protein, termed αCEF1, is predominantly present in lens cells and at a reduced level in lung cells. Transfection experiments indicated that αCE1 and αCE2 together function in brain and neural retinal cells (Fig. 5), indicating the presence of this DNA-binding protein in these tissues. We purified αCEF1 from brain nuclear extracts to homogeneity and silver-staining of SDS-gels indicated that this purified protein is composed of six polypeptides with molecular masses of 61- to 66-kDa (Kitamura and Yasuda, unpublished results). Western blot experiments using antiserum raised against purified αCEF1 and nuclear extracts from various tissues suggested post-translational modifications account for the observed heterogeneity. Modifications might be required for αCEF1 functions to efficiently stimulate transcription in lens cells.

αCE2-binding protein, termed αCEF2, also appeared to exhibit sequence-specificity (Fig. 8B). The αCE2-protein complexes formed with lens nuclear extract (C4) migrated much faster than those with lung nuclear extract (C5), indicating that αCEF2 binding activity in lens cells differs from that in lung cells. Thus, it is likely that lens, neural retina and brain cell αCEF2 functions differently from αCEF2 in other tissues. Since αCEF1 and αCEF2 are indispensable for transcriptional activation in lens cells, it is important to clarify whether αCEF1 and αCEF2 can bind the regulatory sequences presented in Fig. 9A and 9B, respectively. We will be able to reveal any common mechanisms involved in regulating tissue-specific expression of these crystallin genes by cloning DNA-binding proteins which bind to the consensus motifs proposed here and by examining the DNA/protein interactions with these binding motifs.

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