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Binding of a factor to an enhancer element responsible for the tissue-specific expression of the chicken αA-crystallin gene

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Summary

We have characterized a regulatory region of the chicken αA-crystallin gene using transfection assays, which revealed that a 84 base pair element (−162 to −79) in the 5′ flanking sequence is necessary and sufficient for lens-specific expression. A multimer of this element functions as lens-specific enhancer and synergistically activates transcription from chicken αA-crystallin or β-actin basal promoters fused to the CAT gene. *In vivo* competition experiments demonstrated that DNA sequences containing the 84 bp element reduced αA-crystallin–CAT fusion gene expression. A nuclear factor present exclusively in lens cells binds to the 84 bp element in the region between positions −165 and −140. Southwestern blot analysis showed that 61 000 M_r ($61\times10^3 M_r$) lens nuclear protein exhibited DNA-binding activity specific to the 84 bp element. Our data suggested that the $61\times10^3 M_r$ nuclear protein, and the 84 bp element that it interacts with, may be involved in regulating the αA-crystallin gene expression *in vivo*.

Key words: gene expression, transcriptional regulation, αA-crystallin, lens, enhancer, DNA-binding protein, CAT assay.

Introduction

Crystallins are structural proteins specific to vertebrate lens cells and their gene expression is regulated spatially and temporally during vertebrate lens development (Bloemendal, 1981; Clayton, 1974; Piatigorsky, 1989; Yasuda and Okada, 1986). Lens placode is induced from ectoderm by a close interaction with the optic cup, resulting in initiation of δ-crystallin gene expression in the lens placode (Shinohara and Piatigorsky, 1976). Invagination of the lens placode forms a lens consisting of lens epithelial and fiber cells. After this stage of lens development, the α-crystallin gene is transcribed and its mRNA accumulates in lens epithelial and fiber cells.

During very early stages of development, ectopic expression of crystallin mRNAs is observed temporally in non-lens tissues such as the neural retina, pigmented epithelium (Agata *et al.* 1983; Eguchi and Okada, 1973; Yasuda *et al.* 1983) and pineal (Watanabe *et al.* 1985). When these tissues are cultured *in vitro*, they change their differentiated state into that of lens tissue, forming lentoid bodies and accumulating large amounts of crystallin gene transcripts in these structures (Yasuda *et al.* 1983, 1984; Okada, 1983; Eguchi, 1986). This process is called 'transdifferentiation', and lens formation from chicken embryonic neural retina is a typical example of this process. This is a very attractive system to study the molecular mechanisms by which lens development and the expression of crystallin genes are regulated, since it has been shown that culture conditions determine whether neural retina and pigmented epithelial cells differentiate into their original state or into lens (Yasuda, 1979; Itoh and Eguchi, 1986).

We have shown by transfection of the chicken αA-crystallin gene into mouse lens cells that the sequence from −242 to −189 upstream of the transcription start site is responsible for lens-specific expression (Okazaki *et al.* 1985). The mouse, rat and human γ-crystallin genes, which are not formed in chicken, were shown to be expressed in a lens-specific manner in chicken lens cells and their regulatory regions have been identified (Lok *et al.* 1989; Peek *et al.* 1990; Yu *et al.* 1990). The distal region (−118 to −88) and the proximal region (−88 to +43) of the mouse αA-crystallin gene are required for lens-specific expression in chicken lens cells (Chepelinsky *et al.* 1987). However, sequences up to −88 are sufficient for lens-specific expression in transgenic mice (Wawrousek *et al.* 1990). Thus, species-specific differences exist in the regulatory sequences used by the chicken and mouse, despite the apparent species independence of lens-specific expression of crystallin genes.

To reveal the molecular mechanisms by which lens-specific expression of the chicken αA-crystallin gene is

regulated in a homologous system, we examined which regions of the αA-crystallin gene are required and sufficient for specific expression in chicken lens cultures during transient transfections (Yasuda *et al.* 1988). The data that we present here show that the sequence from −162 to −79 (the 84 bp element) of the αA-crystallin gene exhibits lens-specific enhancer activity and that a DNA-binding protein that interacts specifically with the sequence −162 to −128 plays a key role in regulating lens-specific expression.

Materials and methods

Plasmid constructions

pBS0CAT and pBS0CAT2 were used as reporter genes (Fig. 1). A short *Xmn*I–*Hin*dIII fragment of pSV0CAT (Gorman *et al.* 1982) was exchanged with a corresponding *Xmn*I–*Hin*dIII fragment of pUC18 (pUSV0CAT). pBS0CAT was constructed by exchanging a short *Xmn*I–*Xba*I fragment of Bluescript with a corresponding *Xmn*I–*Xba*I fragment of pUSV0CAT. pBS0CAT2 was constructed by cutting, blunting and religating the *Bam*HI site of pBS0CAT and then inserting a *Bam*HI linker into the *Xba*I site. Thus, pBS0CAT2 has a *Bam*HI site in front of the CAT gene. DNA fragments containing the promoter sequences of the chicken αA-crystallin gene were obtained from pCryα4 containing the *Hin*dIII fragment spanning from -2.5×10^3 to +378 region of the chicken αA-crystallin gene (Okazaki *et al.* 1985). α373CAT, α242CAT, α162CAT, and α82CAT contain the promoter sequences −373 to +89, −242 to +89, −162 to +89 and −82 to +89, respectively (Fig. 1B). Basal promoters, α74, β55 and tk197, contain the sequence of the chicken αA-crystallin gene from −74 to +89, the sequence of the chicken β-actin gene from −55 to +53 and the sequence of the *Herpes simplex* virus thymidine kinase gene from −197 to +56, respectively. To obtain plasmids containing from monomeric to tetrameric repeats of the 84 bp element upstream or downstream of the

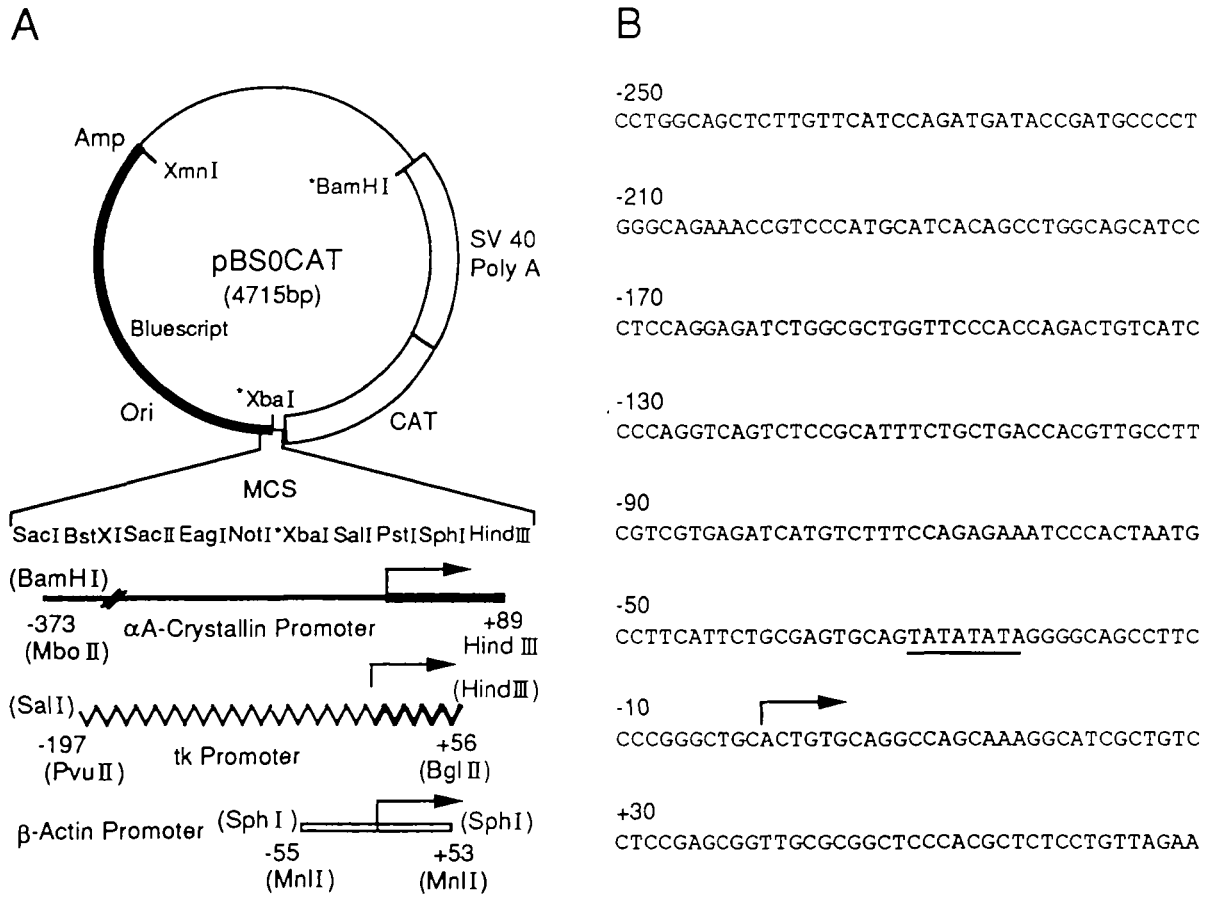


Fig. 1. Schematic representation of expression vectors and sequence of the chicken αA-crystallin promoter. (A) Schematic representation of expression vectors. The expression vectors were constructed from Bluescript (Stratagene) and pSV0CAT (Gorman *et al.* 1982) as described in Materials and methods. The CAT indicator gene and DNA fragment containing SV40 poly(A) addition site are represented by open boxes. The portions derived from Bluescript and pSV0CAT are indicated by thick and thin lines, respectively. Ori, poly(A) and Amp show the replication origin, poly(A) addition site and ampicillin resistance-gene, respectively. To obtain pBS0CAT2, pBS0CAT was digested with *Bam*HI, blunted and religated and then a *Bam*HI linker was inserted into the *Xba*I site. Thus, pBS0CAT2 contains one *Bam*HI site within multi cloning site (MCS). Basal promoters containing the *Sac*I–*Hin*dIII fragment of the chicken αA-crystallin gene, the *Pvu*II–*Bgl*II fragment of the chicken β-actin gene and the *Mnl*I fragment of the *Herpes simplex* virus thymidine kinase genes were inserted between the *Sac*I and *Hin*dIII sites, between the *Sal*I and *Hin*dIII sites and at the *Sph*I site of pBS0CAT, respectively, as shown below the map. (B) Sequence of the chicken αA-crystallin promoter. The arrow indicates the site of transcription initiation and the TATA box is underlined.

CAT gene, these units were inserted into the *Sac*II or *Bam*HI sites of α74CAT constructed from pBSOCAT2, respectively.

Cell cultures

Tissues were isolated from 15-day-old chick embryos. Lenses were dissected from eyes and the pigmented cells adhered to the lenses were removed by rolling them on Whatmann 3MM sheet. They were cut into pieces and incubated in Hanks' saline solution containing 0.1% collagenase for 15 min at 37°C. Cells were dispersed by gentle pipetting, collected by a brief centrifugation and suspended in a fresh medium [Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Flow Laboratories)]. They were cultured in fresh medium at a density of 10^5 per 60 mm dish (Primaria; Falcon Co.). When the cells reached confluence, they were harvested with trypsin and cultured at a density of 1.5×10^5 per 30 mm dish to obtain secondary cultures. 3 days after establishment of the secondary cultures the cells were transfected. Brains, lungs, kidneys and thighs were collected separately, cut into pieces with a razor blade and dispersed with 0.25% trypsin in calcium- and magnesium-free Hanks' saline for 10 min at 37°C. After this time cells were collected by centrifugation and cultured as described for lens cells above.

Transfection

The DNA–calcium phosphate coprecipitation method of Wigler *et al.* (1979) was used for transfection. In a typical assay, cultures per 30 mm Falcon dish were cotransfected with 2 μg of test plasmid and 0.5 μg of pmiwZ, which contains the chicken β-actin promoter and the RSV LTR sequence (Suemori *et al.* 1990), and served as an internal control for normalyzing transfection efficiencies. Fresh media was added 6 h after transfection and the cultures were washed three times with, and harvested in, phosphate-buffered saline (PBS, pH 7.4) 48 h later. The cells were collected by brief centrifugation and resuspended in 50 μl of 250 mM Tris–HCl (pH 7.8). Extracts were prepared by freezing–thawing and gentle homogenization and cell debris was removed by brief centrifugation. Aliquots of the lysate with or without incubation at 60°C for 5 min were assayed for CAT and β-galactosidase activities, respectively.

CAT and β-gal assays

CAT assays were performed essentially according to the procedure of Gorman *et al.* (1982) as modified by Hayashi *et al.* (1987). β-galactosidase activities were assayed by the procedure of Edlund *et al.* (1985). Protein concentration was measured by a Biorad protein assay (Biorad Co.).

In vivo *competition experiment*

The specific (p16S) and unspecific (p20N) competitor plasmids contain a 16-mer of the sequence −253 to −90 and a 20-mer of the sequence −373 to −162 at the *Bam*HI site of pUC18, respectively. A constant amount of plasmid α162CAT (0.5 μg per 30 mm dish) was transfected into lens cultures along with a constant amount of pmiwZ (0.1 μg) and increasing amounts of specific or unspecific competitor DNAs as shown in Fig. 4. By day 2 after transfection, the cultures were harvested and the CAT activities of their lyzates were assayed.

Preparation of nuclear extracts

Tissues were isolated from 1-day-old chickens and washed twice with PBS. Nuclear extracts were prepared according to the procedure of Dignam *et al.* (1983). The procedure is principally as follows. Minced tissues were homogenized in a lysis buffer [20 mM Hepes (pH 7.9), 0.6 M KCl, 1.5 mM MgCl, 0.2 mM EDTA, 1 mM DTT, 0.2 mM PAMSF and 25% (v/v) glycerol] by 10 strokes with a B-pestle in a Dounce homogenizer. Nuclei were pelleted by centrifugation, suspended in lysis buffer at a concentration of 10^8 nuclei ml^{-1} and extracted by a gentle stirring for 30 min at 4°C. Nuclei were pelleted by centrifugation and the supernatant was dialyzed against a buffer containing 10 mM Hepes (pH 7.9), 0.1 M KCl, 3 mM MgCl, 1 mM DTT, 0.2 mM EDTA, 0.2 mM PAMSF and 10% (v/v) glycerol. Aliquots of the nuclear extract were quickly frozen in liquid nitrogen and stored at −80°C until use.

Gel mobility shift assay

Fragments to be used for gel mobility shift assay were obtained by digesting AluI–*Sma*I DNA fragment spanning from −243 to −7 with one or two of the restriction endonucleases *Bgl*II, *Mva*I, and *Sau*3AI as shown in Fig. 3. The resulting DNA fragments were separated by agarose gel electrophoresis, isolated and end-labelled with Klenow enzyme using [α-^{32}P]dNTP. Probes (10 000 cts min^{-1} ng^{-1} DNA) were reacted with nuclear extracts (10 μg) in 10 μl of a binding buffer containing 0.1 M NaCl, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, poly dI:dC (100 μg ml^{-1}) and 10% (v/v) glycerol for 30 min at room temperature. These reaction mixtures were immediately loaded on a 4% polyacrylamide gel, which had been prerun for 2 h at a constant voltage of 180 V, and run for 2 h at room temperature at a constant voltage of 150 V (10 V cm^{-1}) with a circulation of a buffer (1/4 TAE buffer). The gels were transferred to DEAE papers, dried and autoradiographed.

Southwestern blot analysis

To make the probes for southwestern analysis, specific (−162 to −83) and non-specific sequences (−242 to −163) were labelled using the Klenow fragment of *E. coli* DNA polymerase and [α-^{32}P]dNTP. Nuclear proteins were separated by 10% SDS–polyacrylamide gel electrophoresis and blotted electrophoretically onto nitrocellulose membranes (Schleicher & Schuell). The membranes were soaked in 5% skim milk solution containing 10 mM Tris–HCl (pH 7.4) for 1 h at a room temperature and then reacted with specific or non-specific DNA probes in the binding buffer used for gel mobility shift assay in the presence of competitor DNA (poly dI:dC, Pharmacia). Subsequently, the membranes were washed 3 to 4 times in a binding buffer 10 min each at room temperature, dried and autoradiographed.

DNAase I footprinting analysis

pUαE/H (Okazaki *et al.* 1985) was cleaved with *Eco*RI and *Hin*dIII and the DNA was electrophoretically separated on an agarose gel. The DNA fragment containing the sequences from −242 to +10 of the αA-crystallin gene was recovered from the gel and endlabelled at both ends using Klenow enzyme. Endlabelled DNA fragments were obtained by cleaving either with *Sal*I or *Kpn*I. The standard reaction consisted of the following components in a final volume of 10 μl: 10 mM Hepes (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 50 ng of carrier DNA (poly dI:dC), 1–3 ng of end labelled DNA fragment and up to 5 μl of nuclear extracts. Extracts were preincubated with the carrier DNA in the reaction mixture for 10 min at 0°C, after which time the end-labelled DNA fragment was added and the mixture incubated for an additional 10 min at 20°C. Freshly diluted DNAase I (10 u ml^{-1}; DPRF, Worthington Co.) was added and incubated for 60 s at 37°C. Reactions were stopped by the addition of 2 volumes of 50 mM EDTA, 0.2% SDS,

100 $\mu g\,ml^{-1}$ of yeast tRNA and 100 $\mu g\,ml^{-1}$ of proteinase K and incubation for 30 min at 37°C. Nucleic acids were extracted with an equal volume of phenol:chloroform (1:1), ethanol precipitated, dissolved in 99% formamide, 10 mM EDTA, with tracking dyes and heated at 90°C for 3 min. The DNA fragments were separated on 6% polyacrylamide/7.5 M urea gels. The gels were transferred to Whatman 3MM paper, dried and exposed for autoradiography.

Results

Identification of a cis*-element required for lens-specific expression of the chicken αA-crystallin gene*

To assess the role of the immediate 5′ upstream sequence of the chicken αA-crystallin gene in tissue-specific expression, the gene promoter (−373 to +89) was fused to the chloramphenicol acetyl transferase coding region (CAT gene) of a bacterial gene. This recombinant DNA (α373CAT) was cotransfected into cultures prepared from various tissues of 15-day-old chick embryos with a plasmid pmiwZ, which served as an internal control for transfection efficiency. CAT activities were normalized to β-galactosidase activity for each transfection.

The CAT activity of lens cultures containing plasmid α373CAT was about 50 times higher than in lung, liver and muscle cultures transfected with the same plasmid (Fig. 2A). However, it should be noted that CAT activity in brain cultures was about 5–10% of that of lens cultures and was always much higher than in other non-lens tissues. α373CAT was also efficiently expressed in neural retina cultures (data not shown). These results demonstrate that expression of this fusion gene is controlled in a tissue-specific manner and that the DNA sequence from −373 to +89 is essential for the regulation of lens-specific expression of the αA-crystallin gene.

To determine more precisely the DNA sequence required for lens-specific expression, we constructed mutant genes with a series of 5′ upstream deletions

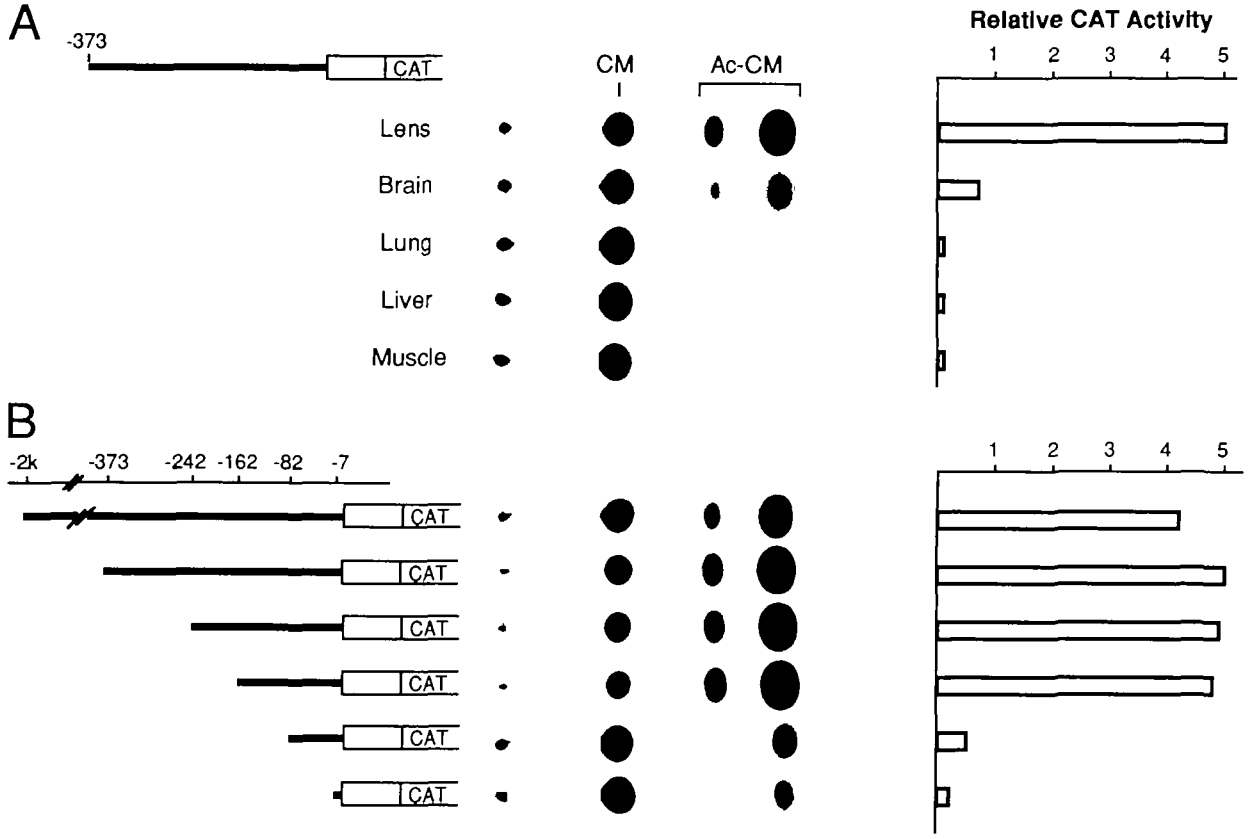


Fig. 2. Identification of a *cis*-element in the αA-crystallin gene promoter which is responsible for lens-specific expression. Tissue-specific expression of the αA-crystallin gene. α373CAT was cotransfected with a plasmid pmiwZ into primary cultures (lens, brain, lung, liver and muscle) by the calcium phosphate precipitation method. After 48 h the cells were harvested, and CAT activities in soluble fractions of transfectants were assayed using thin layer chromatography. The percentages of conversion of labeled chloramphenicol (CM) to its acetylated derivatives (Ac-CM) in CAT assays were calculated directly by measuring the radio activities of spots corresponding to those of CM and Ac-CM on thin layer chromatograms using a Fuji Bioimage Analyzer BA-100. CAT activities were normalized to β-galactosidase activities for each transfection. Histograms show the relative levels of CAT activity in cultures. Each value represents the average from two independent experiments. (B) 5′ deletion analysis of the αA-crystallin promoter region responsible for lens-specific expression. Deletion mutants were constructed using the restriction enzyme cleavage sites shown at the top. Transfections and detection of CAT activities were performed as described above. Histograms show the relative CAT activities of these 5′ deletion mutants in lens cultures. The values represent the average of two independent experiments.

using restriction endonuclease cleavage sites. Fig. 2B shows the CAT activities of these deletion mutants in lens cultures. A recombinant plasmid containing about 2 kb upstream region was as efficiently expressed as α373CAT, indicating the absence of additional important elements upstream nucleotide −374. Deletion of nucleotides −242 to −162 gene did not diminish transcription level significantly, whereas further deletion to nucleotide −82 resulted in a 10-fold decrease in stimulating activity. These results suggested that the DNA sequence between positions −162 and −83 (termed the 84 bp element, since filling in both ends of the *Bgl*II–*Sau3*AI DNA sequence between −162 to −83 generates an 84 bp DNA sequence between −162 and −79) is involved in regulating lens-specific expression of the αA-crystallin gene.

Multimers of the 84 bp element synergistically enhance lens-specific expression

In a wide range of cell types, tissue-specific gene expression is regulated by enhancers that can activate genes independent of orientation and distance (Serfling *et al.* 1985). For crystallin genes, enhancers have been found for the mouse αA- (Chepelinsky *et al.* 1987) and γF-crystallin (Lok *et al.* 1989) and the chicken δ1-crystallin genes (Hayashi *et al.* 1987; Goto *et al.* 1990).

To test for enhancer activity of the 84 bp element in lens cells, we inserted up to 4 copies of this sequence in its native or reverse orientation immediately 5′ to an αA-crystallin basal promoter (−74 to +89) fused to the CAT gene and transfected the resulting constructs into lens or lung cells (Fig. 3). In this paper, lung cultures were employed as a control culture representative of non-lens tissues. Nα74CAT and Rα74CAT, which contain one copy of the 84 bp sequence in normal and reverse orientations, respectively, showed a CAT activity comparable to that of α162CAT in lens cells. However, they were inactive in lung cultures. Thus, these indicate that the 84 bp element exhibits lens-specific enhancer activity.

Increasing the number of copies of the 84 bp element placed immediately 5′ to the crystallin basal promoter in either orientation synergistically activated transcriptional activity in lens cells. Multimerization to a trimer or tetramer of the 84 bp element led to a remarkable activation, by a factor of 28 or 96, respectively, in lens cells, but only to a 2- to 3-fold activation in lung cells (Fig. 3A). An effect of the orientation of insertion was found. In lens cells, multimers in reverse orientation gave about 1.5-fold greater activation than those in the normal one. In contrast, in lung cells multimers in normal orientation gave a basal level of expression but those in reverse orientation completely inhibited CAT activities in this tissue.

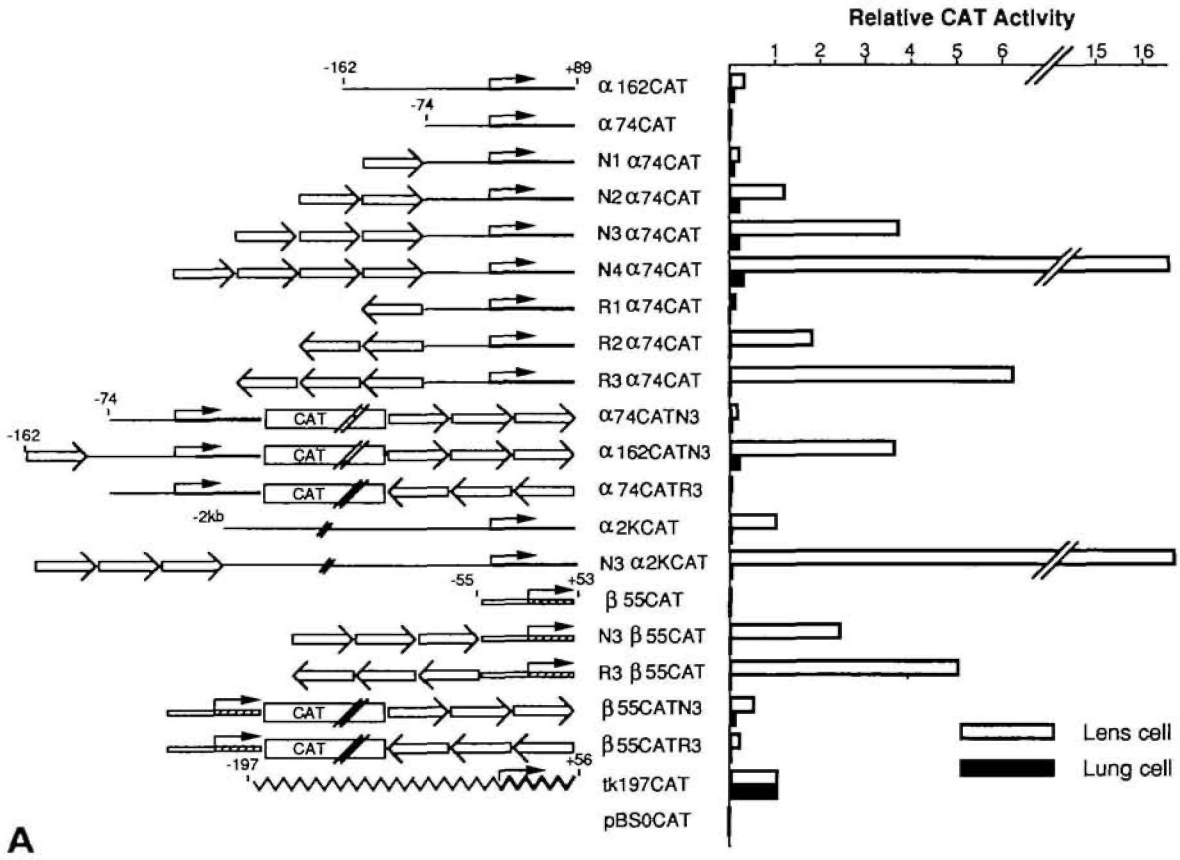
To determine whether the 84 bp element can function at a distance, a trimer of the 84 bp element was inserted in either orientation into the *Bam*HI site of α74CAT, 1.6 kb downstream of the transcription start site. These constructs promoted transcription but only weakly, suggesting a distance-dependency of the enhancer activity. However, a trimer of the 84 bp element activated the transcriptional level in lens cells when placed in its normal orientation 2.0 kb 5′ of the αA-crystallin gene bearing −2.0 kb of the 5′ flanking region, which contains one 84 bp element in the original site. These results suggest that a close association of at least one 84 bp element 5′ to TATA box is essential for the 84 bp element to exhibit enhancer activity from a distance. To test this possibility, we inserted a trimer of the 84 bp element 1.6 kb downstream from the start site at the *Bam*HI site of α162CAT, which contains one 84 bp element close to the TATA box. The resulting construct α162CATN3 showed an enhancing activity in lens cells comparable to that of N3α74CAT and a 30-fold greater activity than α74CATR3. α162CATN3 did not function at all in lung cells. Taken together, these results indicate that the 84 bp element of the chicken αA-crystallin gene has an enhancer activity and must be involved in regulating lens-specific expression.

The 84 bp element has a lens-specific enhancer activity

To determine whether the 84 bp element could activate expression from a heterologous promoter and is also sufficient for lens-specific expression from such a promoter, we constructed chimaeric genes containing a trimer of the 84 bp element upstream or downstream of the chicken β-actin basal promoter (−55 to +53) coupled to the CAT coding region. This promoter contains a TATA element, a transcription start site, and is an equally very poor promoter in any cell type; fibroblasts, lung and lens cells (Fig. 3; β55CAT). As three copies of the 84 bp element have shown a very strong lens-specific activity in promoting transcription from the chicken αA-crystallin promoter, one would be expected to be able to detect easily their effect on CAT activity from a heterologous promoter, the chicken β-actin basal promoter. Thus, using constructs containing a trimer of the 84 bp element, their enhancer activity was examined. N3β55CAT and R3β55CAT, in which three copies of the 84 bp element were placed in normal and reverse orientation, respectively, immediately 5′ to the chicken β-actin basal promoter showed an enhancing activity comparable to those of N3α74CAT and R3α74CAT in lens cells. However, N3β55CAT and R3β55CAT showed no activation at all in lung cells. Insertion of a trimer of the 84 bp element 1.6 kb downstream of the transcription initiation site (β55CATN3 and β55CATR3) led to a lower enhancing activity in lens cells compared to N3β55CAT and R3β55CAT. This tissue-specific enhancer activity of the 84 bp element was also observed when a *Herpes simplex* virus thymidine kinase promoter (−197 to +56; tk197CAT) was used as the basal promoter (data not shown). Taken together, these results indicate that the 84 bp element of the αA-crystallin gene confers lens-specific expression independent of orientation, distance and promoters, indicating that the 84 bp element functions as a lens-specific enhancer.

In vivo *competition experiment*

The results mentioned above suggest the presence of a *trans*-acting factor(s), which binds to the 84 bp element



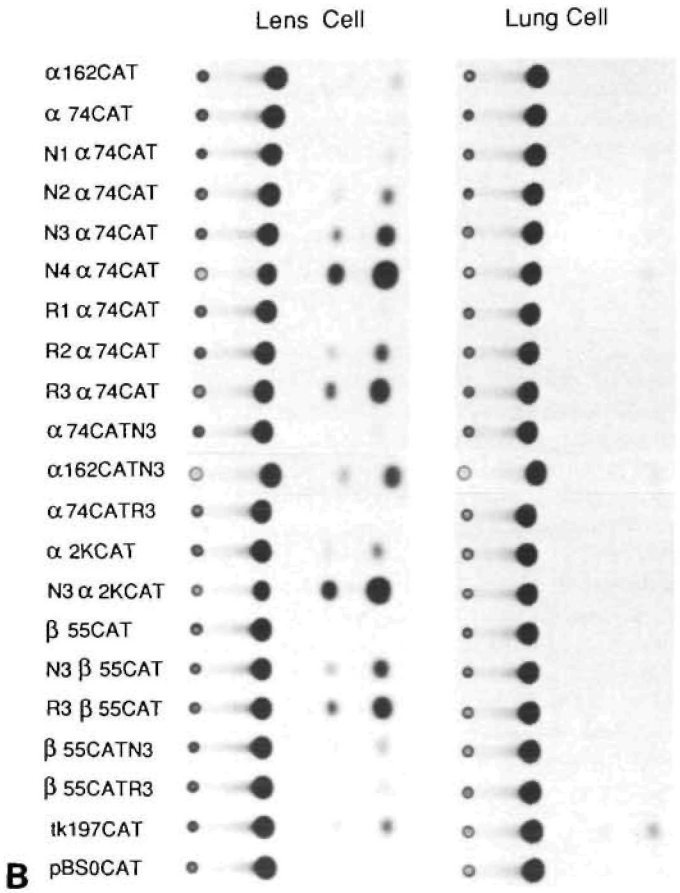


Fig. 3. Lens-specific enhancer activity of the 84 bp element. Enhancer activity of the 84 bp element was examined in lens and lung (as a control) cultures in transient transfections. (A) Schematic representation of plasmid constructs and histograms showing the relative levels of their CAT activities in lens and lung cells. The thin line, thin open box and zigzag line represent basal promoters derived from the chicken αA-crystallin, chicken β-actin and the *Herpes simplex* virus thymidine kinase genes, respectively. Numbers above the lines represent the nucleotide positions relative to the transcription initiation site (arrows). The open box with arrowhead represents the 84 bp element (−162 to −79) and its relative orientation. The open box represents the CAT gene. Plasmid construction, transfection and measurement of CAT activity are described in Materials and methods section. Relative CAT activities of the various constructs in lens (open bar) and lung cells (closed bar) are shown with the CAT activities of tk197CAT in lens and lung cells arbitrarily assigned as 1. Each value represents the average from three independent experiments. (B) Autoradiograms of CAT assays. DNA constructs correspond to those in the A.

thus activating αA-crystallin gene expression in a tissue-specific manner. In order to explore the nature of the factor(s) that binds to the 84 bp element of the αA-crystallin gene, we carried out an *in vivo* competition experiment (Baldwin and Sharpe, 1987; Goto *et al.* 1990). Plasmids α162CAT and α74CAT, which differ by the presence or absence, respectively, of the 84 bp element, were transfected separately into lens culture cells along with various amounts of a competitor plasmid. Two competitor plasmids were used in the experiment. Plasmids p16S and p20N contained 16 tandem copies of the sequence −253 to −90 and 20 tandem copies of the sequence −373 to −162, respectively, of the αA-crystallin promoter at the *Bam*HI site of pUC18. To examine the effect of the competitor DNAs on CAT activity in lens cells, a constant amount of α162CAT was transfected into lens cells along with varying amounts of competitor plasmid. The amount of α162CAT was fixed at 0.5 μg/dish and that of competitor DNAs was increased up to 5 μg/dish. Fig. 4 showed that an increasing amount of the non-specific competitor DNA had no effect on CAT activity even when present at 300-fold molar excess over the reporter gene. In contrast, the levels of CAT activity were proportionally reduced with an increasing amounts of the specific competitor DNA and were inhibited to about 50% in 150-fold molar excess. Neither competitors, however, affected the expression of α74CAT (data not shown). These results indicate the presence of a *trans*-acting factor in lens cells, which interacts with the 84 bp element and stimulates the αA-crystallin promoter function in a lens-specific manner.

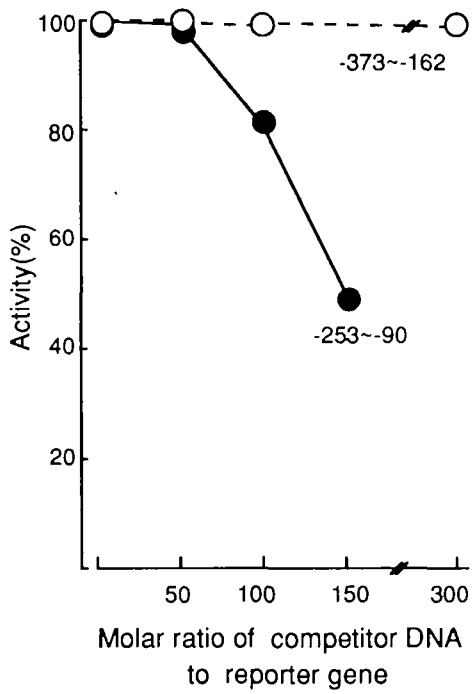


Fig. 4. Competition *in vivo* for a factor that interacts with the αA-crystallin promoter. Plasmids α162CAT (0.5 μg per dish) and pmiwZ (0.1 μg) were transfected into lens cultures alone or with 1 to 7 μg of plasmids that contain a unidirectional 16-mer of DNA sequence −253 to −90 (p16S) or a unidirectional 20-mer of DNA sequence −373 to −162 (p20N) at the *Bam*HI site of pUC18 CAT activities were determined and normalized to β-galactosidase activity as described in the Materials and methods. The relative CAT activities of α162CAT are shown with specific (closed circle) and unspecific (open circle) competitors with the CAT activity of α162CAT in the absence of competitors assigned as 100%. The molar ratio represents the relative concentration of sequences −373 to −162 or −253 to −90 contained in competitors to that of sequence −253 to −90 in the reporter gene.

Binding of a factor to the 84 bp element

To examine whether nuclear proteins in lens cells bind to the 84 bp element, we used an electrophoretic gel mobility shift assay. We reacted lens nuclear extracts with the endlabelled 84 bp element at 20°C for 30 min and then separated the DNA–protein complexes from free probes by agarose gel electrophoresis. Fig. 5A shows that a major shifted band was observed with this probe, indicating the presence of lens nuclear proteins which specifically bind to the 84 bp element. Then, the 84 bp element was cleaved into two fragments with *Mva*I to test whether either of them would yield DNA–protein complex. Fig. 5A shows that only the sequence −162 to −128 showed the binding activity. These results indicate that a lens nuclear factor(s) binds to the sequence from −162 to −128. To test the sequence-specificity of the DNA–protein complex formation with lens nuclear extracts, a 50-fold molar excess of unlabelled specific or unspecific oligonucleotides were added to the reaction mixtures (Fig. 5B). The DNA–protein complex formation was competed away efficiently by specific oligonucleotides containing the sequence from −162 to −128 (Fig. 5B; a, b) but not by oligonucleotides that do not contain this sequence (Fig. 5B; c, d, e). These results confirm the hypothesis that a lens cell nuclear protein specifically interacts with the sequence from −162 to −128, which had previously been shown to be essential for lens-specific expression.

To test whether the specific DNA-binding activity exhibits a tissue-specific distribution pattern, we reacted an equal amount of nuclear extracts from different tissues with a specific probe, sequence −162 to −128, and examined the ability to form a DNA–protein complex by electrophoretic mobility shift assay (Fig. 5C). The specific DNA–protein complex was formed efficiently with nuclear proteins from lens cells; however, no complex formation was observed with kidney and heart nuclear extracts, in which no crystallin expression was detected. A weak shifted band was, however, detected with brain nuclear extracts. These results indicate that levels of DNA-binding activity amongst nuclear extracts from various tissues correlates well with the expression levels of the αA-crystallin gene transfected into these tissues (Fig. 2A).

Southwestern blot analysis of nuclear proteins

We reacted lens nuclear protein blots with specific (−162 to −82) or unspecific probes (−242 to −163) to determine if we could detect the protein responsible for

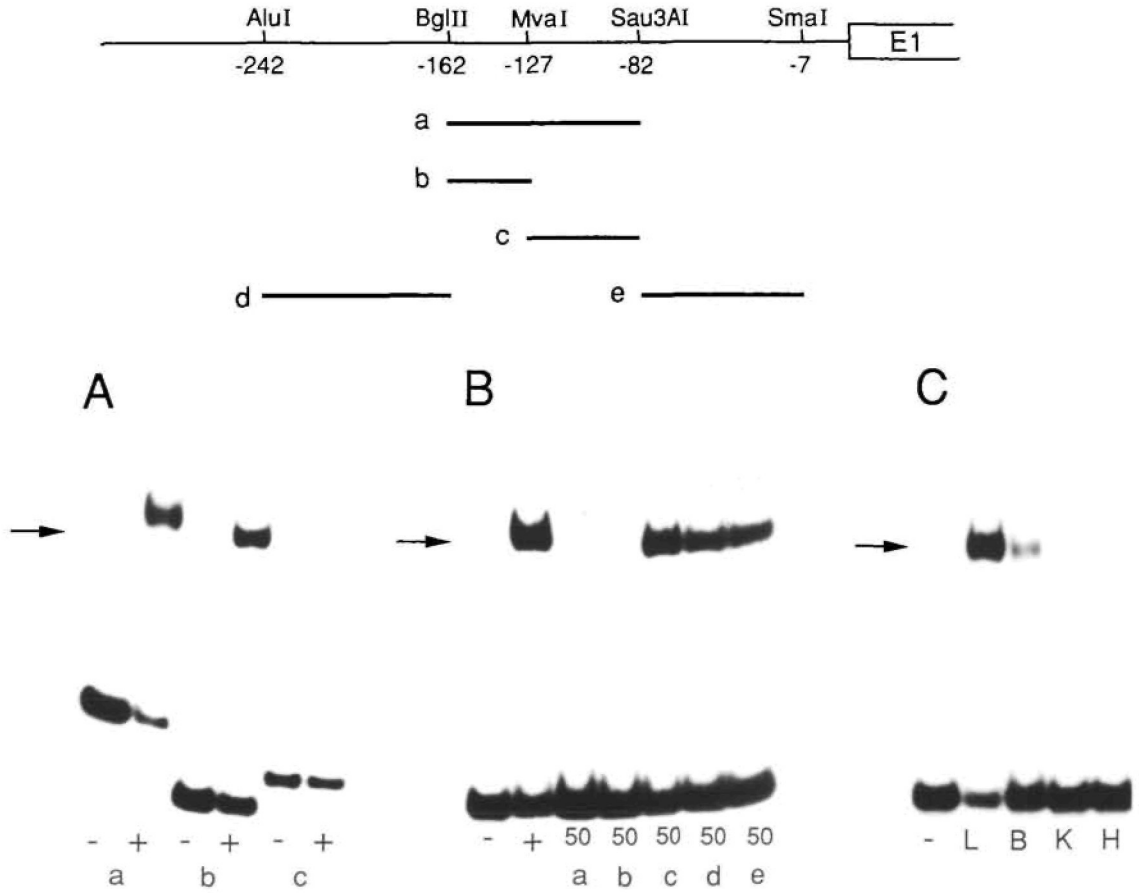


Fig. 5. Analyses of DNA-binding activity by gel mobility shift assay. DNA fragments (a: −162 to −83; b: −162 to −128; c: −127 to −83) were obtained by cleaving α242CAT at the restriction enzyme sites shown at the top and labelling by filling in the ends using Klenow fragment. Nuclear extracts were prepared from various tissues of 1-day-old chickens according to the procedure of Dignam *et al.* (1984). The formation of DNA–protein complexes was carried out as described in Materials and methods. After incubation for 30 min at 20°C, the complexes were separated from the free DNA probe by electrophoresis in a low ionic buffer. The gels were dried and autoradiographed. (A) Nuclear proteins from lens cells bind to the 84 bp element. Probes (b, c and d) are shown at the top. Probes were reacted with (+) or without (−) lens nuclear extracts. The arrow shows specific DNA–protein complexes. (B) Competition of various promoter regions for specific DNA–protein complex formation. Sequence −162 to −128 (a) was used as a probe. Reactions were carried out in the absence (+) or presence of 50 molar excess of unlabelled competitor DNAs (a,b,c, d and e). Specific DNA–protein complex is shown by the arrow. (C) Tissue-specific distribution of a factor that interacts with the 84 bp element. Specific probe −162 to −128 was reacted without (−) or with an equal amount of nuclear extracts from various tissues (L, lens; B, brain; K, kidney; H, heart) and the complexes were separated from free probe as described in Materials and methods. The arrow shows specific DNA–protein complex.

the sequence-specific DNA-binding activity observed by the gel mobility shift assays. We prepared nuclear protein gel blots separated by SDS–polyacrylamide gel electrophoresis and then reacted them with the specific or unspecific probes. Fig. 6A shows the results obtained with lens, brain, heart and lung nuclear extracts. In lens nuclear extracts, the specific probe binds to 110×10^3 M_r and 61×10^3 M_r proteins, even in the presence of large amounts of carrier DNAs. In other nuclear extracts, the specific probe binds only to the 110×10^3 M_r protein and not to the 61×10^3 M_r protein, even if under less-stringent conditions. The non-specific DNA probes interacted with 110×10^3 M_r protein in all nuclear extracts, suggesting that this 110×10^3 M_r protein may be a non-specific DNA-binding protein. Together, these results indicate that the 61×10^3 M_r protein might be a candidate for the 84 bp element-specific DNA-binding protein, since this is the only protein that has tissue and DNA-sequence specificities similar to those revealed by gel retardation assay. This is also supported by our recent results using DNA affinity chromatography, by which an 84 bp element DNA-binding protein was purified to homogeneity from brain nuclear extracts and appeared to be consist of $61–63\times10^3$ M_r proteins (Kitamura and Yasuda, unpublished observations).

Identification of binding sites by DNAase I footprinting

To determine more precisely the binding sites, DNAase I footprinting assays were used. For this experiment, we obtained *Eco*RI–*Hin*dIII fragment of pUαE/H con-

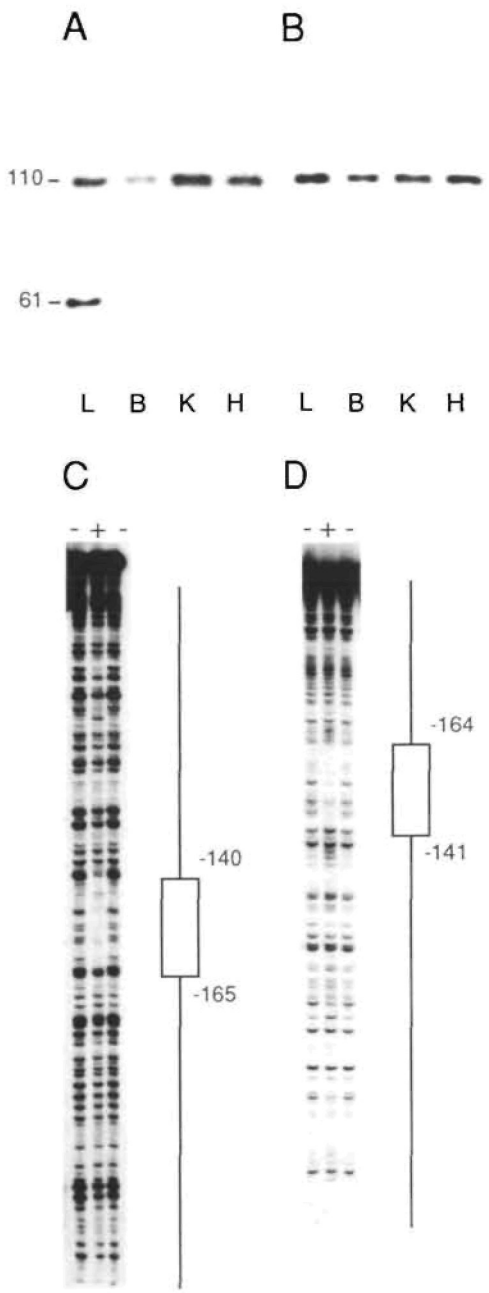


Fig. 6. Southwestern and DNAase I footprinting analyses of binding sites of a factor present in lens nuclear extract. (A, B) Southwestern analysis. Proteins in nuclear extracts from various tissues (L, lens; B, brain; K, kidney; and H, heart) were separated on SDS–polyacrylamide gel electrophoresis and blotted electrophoretically onto nitrocellulose membranes. The filters were treated with 5% non-fat skim milk and then reacted with double-stranded DNA probes −162 to −83 (A) or −242 to −163 (B) in the presence of carrier DNA (sonicated salmon sperm DNA). Fragments were labelled at both ends by filling in the ends with Klenow fragment. Molecular mass are indicated in $\times 10^3$ at the left. (C, D) DNAase I footprinting analysis. DNA fragment containing the sequence −242 to +10 at the *Bam*HI site of pUC18 was cleaved at both the *Eco*RI and *Hin*dIII sites. The fragments were labelled by filling in the ends and cleaving at the *Sal*I or *Kpn*I sites to obtain one end-labeled probes. The probe was incubated with lens nuclear extracts (+) or BSA (−) as a control at 20°C for 30 min and then treated with DNAase I at 37°C for 1 min. The resulting DNA fragments were extracted with phenol–chloroform, precipitated, resolved and then separated on sequencing gel in the presence of 7.5 M urea. The gels were dried and then autoradiographed. Regions protected from DNAase I cleavage of the coding (C) and noncoding (D) strands are shown as the open boxes at the right side of the panel and the numbers represent the nucleotide positions from the transcription start site.

taining αA-crystallin DNA, from the *Alu*I site at − 242 to the *Hae*III site at +10, labelled them by filling in the ends and then obtained endlabelled probes by digesting them with *Sal*I or *Kpn*I. We reacted the end-labelled DNA fragments with lens nuclear proteins or bovine serum albumin, as a control, for 30 min at 20°C and then treated them with DNAase I at 37°C for 1 min. Fig. 6B shows the results of running these digested DNA fragments separately on a sequencing gel containing 10% acrylamide and 7.5 M urea. On the coding sequence, a region stretching from −165 to −140 was protected from DNAase I cleavage. Weak protection was also observed between −60 and −80 and this is supported by the appearance of a hypersensitive site at position −70. On the other hand, on the non-coding sequence the only protected region stretched from −164 to −141. These results confirm those of gel mobility shift assay, which revealed the presence of lens nuclear protein binding to the sequence −162 to −128.

Discussion

The study of the regulation of eucaryotic gene expression has focused on both the sequences required for gene expression and the nuclear factors that interact specifically with these regulatory elements. In this study, we have identified a regulatory element between nucleotides −162 and −79 (the 84 bp element) of the 5′ flanking sequence of the chicken αA-crystallin gene that is required for lens-specific expression (Fig. 2A). We have also demonstrated that this element can function to stimulate transcription from a heterologous promoter. Insertion of three copies of the 84 bp element immediately 5′ of the chicken β-actin basal promoter stimulated expression from this promoter (Fig. 3). Furthermore, we have identified a nuclear factor that binds to the 84 bp element (Fig. 5 and 6). Recognition of this sequence is important for gene expression *in vivo* since cotransfection with an excess of plasmids containing multiple copies of the binding site effectively reduced the CAT activity of a reporter gene whose expression is under the control of the binding site (Fig. 4). The DNA-binding protein exists exclusively in lens cells (Fig. 5C), suggesting that the binding protein identified here is a lens-specific transcription factor. This factor is a polypeptide with a relative molecular mass of 61×10^3 and binds to the region between nucleotides −165 and −140 (Fig. 6). These results show

that the $61 \times 10^3 M_r$ lens nuclear protein and the 84 bp element that it interacts with have a direct role in regulating the lens-specific expression of the chicken αA-crystallin gene.

Regulatory regions

Deletion mutations of the αA-crystallin gene promoter define a sequence element between −162 and −79 (the 84 bp element) that directs lens-specific gene expression (Fig. 2B). We have also demonstrated that this 84 bp element can stimulate a heterologous promoter. Fusion of this element to the chicken β-actin basal promoter (−55 to +53) stimulated expression from this promoter but only in lens cells, therefore the 84 bp element is sufficient for lens-specific expression (Fig. 3). In a previous paper, by transfecting mouse lens cells with 5′ deletions of the chicken αA-crystallin gene, we revealed that lens-specific expression required a sequence between nucleotides −242 and −189 (Okazaki *et al.* 1985). These results show that different, non-overlapping regions of the chicken αA-crystallin gene contribute to lens-specificity of gene expression in chick and mouse lens cells. Thus, the regulatory regions required for lens specificity in a homologous and a heterologous system differ.

This difference in regulatory mechanisms is in agreement with previous findings with other crystallin genes. A regulatory region of the mouse αA-crystallin gene was located between positions −118 and +46 when tested in explanted chicken lens epithelia (Chepelinsky *et al.* 1987) but the sequence between −88 and +46 was sufficient for lens specificity in transgenic mice (Wawrousek *et al.* 1990). The 5′ flanking regions of the six rat γ-crystallin genes that confer lens specificity have been examined in transdifferentiating chicken neural retina cells and mouse lens cells (Peek *et al.* 1990). Deletion mapping of the most active γ-crystallin gene, the γD region, showed that at least three elements, a region between −200 and −106, and two GC rich regions around −75 and −15, were required for maximal expression in mouse lens cells. However, the region between −200 and −106 was dispensable in transdifferentiating chicken neural retina cells, which required the region between −106 and −78 instead.

It is interesting that these crystallin genes are specifically expressed even in chicken lens cells that do not have endogenous orthologs. Many crystallin genes show lens specificity even when introduced into the lens cells of species lacking that particular crystallin. Examples of lens-preferred expression of such foreign crystallin genes include the expression of the chicken δ1-crystallin gene when introduced into mouse lens cells (Hayashi *et al.* 1985) or transgenic mice (Kondoh *et al.* 1987*a*) and that of the mouse (Chepelinsky *et al.* 1988) or human γ-crystallin promoters (Lok *et al.* 1989) in cultured chicken lens cells. These results suggest that crystallin genes probably contain one or more elements preferentially recognized in lens cells. The presence of these multiple elements might explain the differences observed for regulatory regions mentioned above. One or some of the multiple elements could be recognized preferentially by lens-specific and/or ubiquitous transcription factors in lens cell nuclei. It seems that these interactions between *cis*- and *trans*-acting elements differ in the lens tissues of each different species.

Enhancers

Enhancers are found in many genes and their functions in activating gene expression are independent of orientation and position (Serfling *et al.* 1985). Some function in any types of cells, whilst others only in specialized tissues. The results in this paper demonstrated that the 84 bp element of the chicken αA-crystallin gene exhibits a lens-specific enhancer activity, since it is able to stimulate CAT gene expression from a heterologous promoter, the chicken β-actin basal promoter, and functioned in either orientation when placed immediately 5′ to the basal promoter (Fig. 3). Multiple copies of this element synergistically activate expression at the same site. Three copies of this element, however, fail to enhance transcription from a position far downstream of the gene (Fig. 3). Distance-dependency of tissue-specific enhancers has also been observed for an enhancer of the murine gene encoding the delta-subunit of the acetylcholine receptor (Baldwin and Burden, 1989). Nucleotides −148 to −95 from the delta-subunit gene were sufficient to confer muscle specificity. This 54 bp element activated transcription equally well in either orientation when positioned immediately 5′ of the c-*fos* basal promoter (FBP). This FBP contains nucleotides −107 to +24 and is composed of a TATA element and a transcription initiation site and on its own shows no activity in any cells. A trimer of the 54 bp element failed to promote transcription from the FBP when positioned about 1.7 kb 3′ to the FBP. The β-actin basal promoter used in the present study is similar to the FBP in that it also contains a TATA element and a transcription initiation site and no other binding sites. These are in agreement with previous findings that in mammalian cells an enhancer cannot induce substantial transcription from a promoter that only contains a TATA box and no other upstream factor binding site (Kuhl *et al.* 1987; Schatt *et al.* 1990; Westin and Schaffner, 1988).

The situation for the activation by downstream 84 bp elements is dramatically improved, however, if one copy of the 84 bp element is placed immediately 5′ to the promoter (Fig. 3). It is likely that enhancing activity from a distance requires at least one enhancer motif or factor binding site close to a TATA element to mediate interactions of the TATA element with the distant enhancers (Schatt *et al.* 1990).

Enhancer or enhancer-like elements are also found amongst other crystallin genes. The distal element of the mouse αA-crystallin gene functioned in either orientation only when linked to the proximal element as a basal promoter (Chepelinsky *et al.* 1987). Two enhancer-like elements were found in the 5′ flanking sequence of the mouse γF-crystallin gene (Lok *et al.* 1989). These functioned independently in chick lens explants when fused to the SV40 early promoter. An enhancer activity of the *Bam*HI–*Hin*dIII DNA frag-

ment from the third intron of the chicken δ1-crystallin gene has been well characterized (Hayashi *et al.* 1987; Goto *et al.* 1990). The enhancer is about 1 kbp long and its activity is mediated through two elements. One is a 120 bp core segment which does not function by itself, but can function in a lens-specific fashion when multimerized. The other is an adjoining segment that functions in any type of cells but only when multimerized. This is in agreement with previous findings that the SV40 enhancer is composed of multiple distinct elements (Fromental *et al.* 1988; Ondek *et al.* 1988).

DNA-binding protein

The regulation of differential gene expression is mediated through interactions of sequence-specific binding proteins and their target sites located within the promoter and enhancer sequences (Mitchell and Tjian, 1989). In the present study, we have demonstrated the presence of a lens nuclear factor that binds to the 84 bp element required for the lens-specific expression of the chicken αA-crystallin gene (Fig. 5). Use of DNA fragments of the 84 bp element as probes limited the binding site to a region nucleotides −165 and −128 (Fig. 5A). It appeared from DNAase I footprinting analysis that the binding site is located between −165 and −140 (Fig. 6C and D). The binding is sequence-specific, since an unlabelled competitor DNA fragment containing nucleotides −162 to −128 competed with a labelled version of this sequence for factor binding (Fig. 5B).

The factor that binds to the 84 bp element of the chicken αA-crystallin promoter is cell type specific as it is found exclusively in lens cells, and not in other tissues; kidney, heart (Fig. 5C), liver and lung cells (data not shown). The tissue-specificity of the binding activity was confirmed by southwestern blot experiments (Jofuku *et al.* 1987), in which a double-stranded probe (−162 to −79) specifically bound to a polypeptide with a relative molecular mass of 61×10^3 which was present only in lens cells. Recently our studies also confirmed that the relative molecular mass of the binding protein is $61–63\times10^3$ (Kitamura and Yasuda, unpublished observations). As brain cells contain the 84 bp element DNA-binding protein, although at low abundance (Fig. 5C), and it is much easier to obtain large amounts of nuclear extracts from brain tissues than from lenses, we have attempted to purify the 84 bp element binding protein from brain nuclear extracts by DNA affinity chromatography using a synthetic oligonucleotide corresponding to nucleotides −165 to −130. Silver staining showed that the binding protein purified to homogeneity corresponds to a band with a relative molecular mass of $61–63\times10^3$.

Transdifferentiation

Transient transfection experiments in this paper showed that the αA-crystallin promoter (α373CAT) functioned in lens cells and also in brain cells though with less activity (Fig. 2A). This is intriguing, since the endogenous αA-crystallin gene is not active in chicken brain tissues. The ectopic expression of the αA-crystallin gene in brain and neural retina cells might be explained by the following observations. During the early stages of normal development very low levels of the αA- and δ1-crystallin gene transcripts are detected in neural retinal, pigmented epithelial and brain cells (Agata *et al.* 1983; Takagi, 1986). Immunological and northern blot analyses indicated that levels of crystallin transcripts increased up to 10 days in brain cell cultures and subsequently decreased. Therefore, crystallin genes must be transiently expressed in these cultures, indicating the presence of their transcription factors or DNA-binding proteins. This observation was confirmed by gel retardation experiments (Fig. 5C). These results show that a nuclear factor from brain cells binds to the 84 bp element, although the binding activity in brain was much lower than in lens cells. Neural retina also possessed this DNA-binding activity (data not shown). No binding activity was detected in kidney and heart nuclear extracts (Fig. 5C).

These results support the notion that the tissue specificity of crystallin expression correlates well with the capacity to transdifferentiate into lens cells (Agata *et al.* 1983; Clayton *et al.* 1979). It has been well established that several embryonic non-lens tissues transdifferentiate into lens cells (Okada, 1983). These include chick embryonic neural retina cells (Itoh and Eguchi, 1986), retinal pigmented epithelial cells (Eguchi and Okada, 1973; Yasuda *et al.* 1984), brain cells and pineal body cells (Watanabe *et al.* 1985). All of these cells are derived from the neuroectoderm. In all cases, transdifferentiation is accompanied by the formation of lentoid bodies (Moscona and Degenstein, 1980), which are morphologically and biochemically identical to authentic lens (Eguchi and Okada, 1973; Yasuda *et al.* 1983, 1984). Recently, Eguchi (1986) established conditions by modifying culture media that either maintain the original state of the pigmented epithelial cells or cause them to completely differentiate into lens cells. Therefore, these culture systems are suitable ones in which to study lens development and crystallin gene expression (Kondoh *et al.* 1987*b*; Peek *et al.* 1990). The molecular mechanisms behind such transdifferentiation remains to be revealed before the regulation of crystallin gene expression can be clarified.

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