The EMBO Journal vol.4 no.10 pp.2589–2595, 1985

DNA sequences responsible for tissue-specific expression of a chicken α-crystallin gene in mouse lens cells

Kenji Okazaki, Kunio Yasuda, Hisato Kondoh and T.S.Okada

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan

Communicated by L.Saxen

We have studied the DNA sequences required for high-level expression of a cloned chicken α-crystallin gene by introducing a hybrid α/δ-crystallin gene into nuclei of mouse lens epithelial cells in primary culture. The level of transient expression of the hybrid gene consisting of the 5′ upstream promoter region of the α-crystallin gene fused to the structural portion of the δ-crystallin gene was determined by Western blot analysis using anti-δ-crystallin serum. The hybrid gene appears to be expressed in a tissue-specific manner, since it is active in mouse lens cells but not in fibroblasts or in L cells. The DNA sequences located 242 – 189 bp upstream from the transcription initiation site are required for high-level expression in lens cells. They are active when their orientation is reversed at the original site or when placed ~1.7 kbp downstream from the cap site in the second intron of the hybrid gene in either orientation. When these DNA sequences were replaced by the enhancer sequences of Moloney murine leukemia virus, the hybrid gene was expressed in both lens cells and fibroblasts.

Key words: α_A-crystallin/hybrid gene/microinjection/tissue-specific expression

Introduction

A specialized cell in eukaryotes selectively expresses only a small subset of many genes during differentiation and development. Lens cells of the eyes of vertebrates are terminally differentiated cells, which specifically synthesize a large amount of crystallins. Crystallins are very conserved proteins classified into four groups, α, β, γ and δ. α- and β-Crystallins are common to all vertebrates but δ-crystallins are present only in avians and reptilians and are replaced in other vertebrates with γ-crystallins (Piatigorsky, 1981). Crystallin genes are expressed specifically at a time and site during lens development (Harding and Dilley, 1976; Piatigorsky, 1981). Thus, the genes provide us with a suitable system to study the control mechanism of specific gene expression. Current evidence suggests that transcription is a key step in the control of gene expression and that each gene may have specific elements or signals which control its expression. Such control elements have been reported to exist in the 5′-flanking regions of the genes (Brinster *et al.*, 1982; Buetti and Diggleman, 1983; Ragg and Weissmann, 1983; Tavernier *et al.*, 1983; Walker *et al.*, 1983; Weidel and Weissmann, 1983; Otto *et al.*, 1984) or in the structural regions of genes (Bogenhagen *et al.*, 1980; Sakonju *et al.*, 1981; Charnay *et al.*, 1984; Merrill *et al.*, 1984; Wright *et al.*, 1984). For the analysis of these control signals, one useful strategy has been to introduce a specific gene and its mutant derivatives into cells and to measure the *de novo* synthesis of the gene products. In the case of eukaryotic genes encoding proteins, the detection of newly synthesized gene products has been frequently facilitated by the use of a variety of heterologous systems, that is, genes and cells from different organisms. For example, we have found a high level of expression of the chicken δ-crystallin gene introduced by microinjection into mouse lens cells (Kondoh *et al.*, 1983). Thus, the putative control element of the δ-crystallin gene has been identified (Hayashi *et al.*, in preparation).

Expression of the α- and δ-crystallin genes in the chicken appears to be controlled in a different manner (Piatigorsky, 1981). However, little is known about structural elements or signals which control expression of the α-crystallin gene. Here we describe a control element required for regulated high level expression of the α-crystallin gene in lens cells. This DNA sequence is located between −242 and −189 bp from the cap site. It acts in a tissue-specific manner like the enhancer found in the immunoglobulin heavy chain gene (Gillies *et al.*, 1983; Picard and Schaffner, 1984; Queen and Baltimore, 1983) but is less active on a gene which is a considerable distance away.

Results

Construction of hybrid α/δ-crystallin gene

There are two types of α-crystallins, α_A and α_B, which are common to all vertebrates and whose amino acid sequences are homologous to each other (de Jong, 1981; Harding and Dilley, 1976). α-Crystallins of vertebrates cannot be distinguished immunologically and migrate at almost the same mobility on gel electrophoresis. δ-Crystallin is absent in mouse lens cells and is identified immunologically using a specific antiserum. We have previously shown that the δ-crystallin gene is expressed much more efficiently in lens cells than in fibroblasts when introduced by microinjection into nuclei of various types of mouse cells in primary culture (Kondoh *et al.*, 1983). Recently we have cloned α_A-crystallin cDNA and α_A-crystallin gene in the chicken (designated α-crystallin gene) and determined their complete nucleotide sequences (Okazaki *et al.*, in preparation). The α-crystallin gene is 3467 bp in length and is composed of two introns and three exons. The first, second and third exons code 63, 41 and 69 amino acids, respectively.

Using the genomic clone, we investigated the tissue-specific expression of the chicken α-crystallin gene in mouse cells in the same way as we had investigated δ-crystallin gene. However, unlike the case of the δ-crystallin gene, we were not able to use anti-chicken α-crystallin serum to detect the chicken proteins in mouse cells, because mouse α-crystallin also cross-reacts with the serum. To distinguish the products of the injected chicken α-crystallin gene from the endogenous α-crystallin of mouse lens cells, we constructed a hybrid gene of α- and δ-crystallins, in which the 5′ promoter region of the chicken α-crystallin gene was linked to the structural portion of the chicken δ-crystallin gene. The hybrid α/δ gene contains a 2.4-kbp fragment which includes the 2.0 kbp of 5′ upstream promoter region, the first

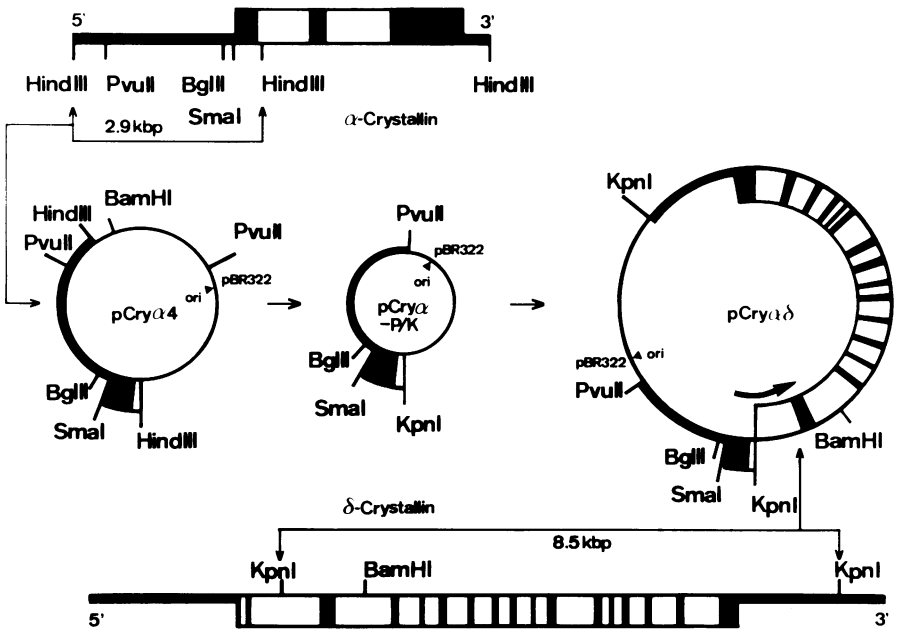

Fig. 1. Structure and construction of plasmid pCryαδ containing the hybrid chicken α/δ-crystallin gene. The hybrid gene was constructed from the chicken α- and δ-crystallin gene subclones as described in Materials and methods. The hybrid α/δ-crystallin gene comprises the 5′ region, containing the 2.0-kbp 5′-flanking region, the first exon and 98-bp 5′ sequences of the first intron of the α-crystallin gene fused to the structural portion, containing 630 bp 3′ sequences of the second intron, the 3rd to 17th exons and ~1 kbp 3′-flanking region of the chicken δ-crystallin gene. Therefore, the gene product comprises the fusion protein of the N-terminal 1st to 63rd α-crystallin amino acid sequences joined to the 5th to 465th δ-crystallin amino acid sequences.

exon and 98 bp of 5′ sequences of the first intron of the α-crystallin gene, and a δ-crystallin gene fragment including the 630 bp of 3′ sequences of the second intron, the 3rd to the 17th exons and ~1 kbp of the 3′-flanking region of the gene. The construction of the plasmid pCryαδ containing the hybrid α/δ gene is shown in Figure 1 and described in Materials and methods. The first exon of the α-crystallin gene encodes 63 amino acids of N terminus and the 3rd to the 17th exons of the δ-crystallin gene encode 461 out of a total 465 amino acids (Ohno *et al.*, 1985), which cross-react with anti-δ-crystallin serum. If the chimeric second intron of the hybrid gene is spliced correctly, the frame of translation from the first Met codon in the first exon of the hybrid gene does not change between its first and second exons. Therefore, a fusion protein from the hybrid gene is expected to consist of a total of 524 amino acids, in which the N-terminal 63 amino acids of α-crystallin are fused to the 461 amino acids (5th to 465th) of δ-crystallin; its mol. wt. is expected to be 56 K.

Expression of hybrid gene in mouse lens cells

About 100 copies of closed circular plasmid pCryαδ DNA containing the hybrid gene were introduced into nuclei of mouse lens epithelial cells in primary culture obtained from 3-week-old mice. 48 h after injection when transient expression of the introduced genes was expected to be maximum, the cultures were fixed and the gene products were examined. The analysis of the products was carried out immunohistochemically by the peroxidase anti-peroxidase (PAP) method using anti-δ-crystallin serum. Figure 2a shows two cells stained immunohistochemically. The percentage of cells stained by the PAP method relative to the total mouse lens cells injected with the plasmid was ~30%, varying slightly between experiments.

To characterize further the gene products we analyzed them by SDS-polyacrylamide gel electrophoresis (Laemmli and Favre, 1973) followed by Western blotting (Towbin *et al.*, 1979) using anti-δ-crystallin serum and [^{125}I]protein A. Figure 2b shows an autoradiogram of the Western blot. As described previously (Kondoh *et al.*, 1983), a plasmid pδC1A carrying the entire δ-crystallin gene produces a protein which corresponds to a band of authen-

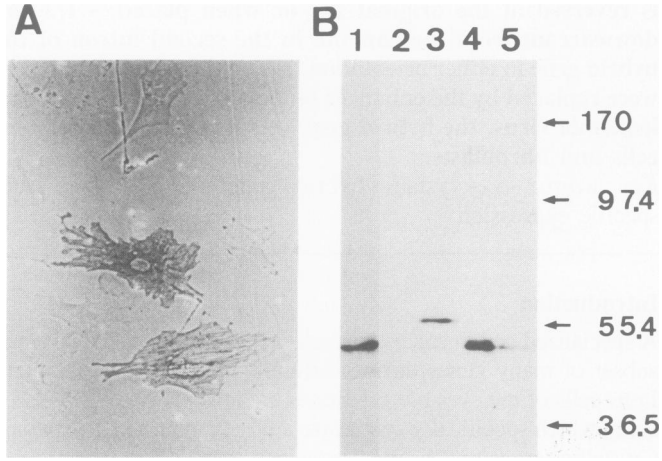

Fig. 2. Detection of products of the hybrid gene introduced into mouse lens epithelial cells in primary culture. **(A)** About 100 copies of the plasmid pCryαδ were introduced into nuclei of mouse lens cells. 48 h after injection the cells were fixed and examined for the expression of the hybrid genes using anti-δ-crystallin serum. Two cells were stained by the PAP method. **(B)** Western blot analysis of the hybrid gene products in mouse lens cells. Cells were solubilized in SDS solution 48 h after injection. The soluble fraction was subjected to an electrophoresis on a SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose filter and fixed to it. Bands cross-reactive to anti-δ-crystallin serum and [^{125}I]protein A were visualized by autoradiography. **1:** 200 cells injected with ~100 copies of the plasmid pCδ1 containing the entire chicken δ-crystallin gene (Kondoh *et al.*, 1983); **2:** Cells without injection of DNA; **3:** 500 cells injected with ~100 copies of the hybrid gene; **4** and **5:** 1 ng and 0.1 ng of purified δ-crystallin, respectively; M: mol. wt. markers (10^{-3} × mol wt.); bovine plasma α_2-macroglobulin (170 K), rabbit muscle phosphorylase (97.4 K), bovine liver glutamic dehydrogenase (55.4 K), porcine muscle lactic dehydrogenase (36.5 K).

Table I. Expression of the hybrid α/δ gene injected into cultured cells of different tissues

	Cell type	% Expression
Primary culture	Lens epithelium	82/250 (33%)
	Lung epithelium[a]	7/100 (7%)
	Lung fibroblast	0/250 (0%)
	Dermis fibroblast	0/200 (0%)
Cell line	L-cell fibroblast	0/500 (0%)

Lens epithelium was obtained from 3-week-old mice. Lung epithelium, lung fibroblast and dermis fibroblast were obtained from 14-day-old fetus. About 100 copies of pCryαδ were introduced into the nuclei of cells. 48 h after injection cells were stained by the PAP method and positive cells were counted. The second row indicates number of positive cells/number of injected cells.
[a]Positive lung epithelium was stained more faintly than positive lens cells. In these cells δ-crystallin-related polypeptides were not detected by Western blot analysis.

tic δ-crystallin in mobility and is cross-reactive with anti-δ-crystallin serum. In contrast, the hybrid gene comprising the 5′-flanking region of the α-crystallin gene fused to the structural portion of the δ-crystallin gene produced a protein which is immunologically detectable by anti-δ-crystallin serum and has much slower mobility than authentic δ-crystallin. Its mol. wt. estimated from the mobility on SDS-PAGE gel is 56 K, which is consistent with the mol. wt. of the predicted α/δ chimeric protein. No other bands cross-reactive to anti-δ-crystallin serum were seen in the autoradiogram. These results indicate that the hybrid gene is expressed in mouse lens cells and synthesizes a fusion protein with an antigenicity to anti-δ-crystallin serum.

Tissue-specific expression of the hybrid gene

We have shown that the δ-crystallin gene is efficiently and specifically expressed in mouse lens cells (Kondoh *et al.*, 1983). To examine the possibility of specific expression of the α-crystallin in mouse lens cells, we introduced 100 copies of the hybrid gene into nuclei of cells in primary culture from several different tissues. Their expression in cells was examined immunohistochemically by the PAP method and the number of stained cells was counted. The efficiency of their expression in the different tissues is summarized in Table I and indicates clearly that the hybrid gene (pCryαδ) is expressed preferentially in mouse lens cells, but is not expressed at a detectable level in other types of cells.

There could be a possibility, however, that although the hybrid gene is expressed in tissues other than the lens, either the transcripts or the translation products in these tissues are rapidly degraded. To examine this possibility we constructed another plasmid pMLαδ bearing the hybrid gene with the 5′-flanking region consisting of the promoter and the cap site of the long terminal repeat (LTR) of Moloney murine leukemia virus (MoMLV; Shinnick *et al.*, 1981) fused to the structural portion of the hybrid gene. This LTR also contained an enhancer that remarkably stimulated expression of any gene whose promoter was several kilobases away from the enhancer sequence. The construction of the hybrid gene is shown in Figure 3A. Transcription of this hybrid gene should be controlled by the LTR promoter. This plasmid pMLαδ was introduced into mouse cell L-cells, and its expression was examined immunohistochemically (Figure 3C). L-cells injected with the plasmid pMLαδ were heavily stained by the PAP method but those with the plasmid pCryαδ were not (Figure 3B). When the plasmid pMLαδ was injected

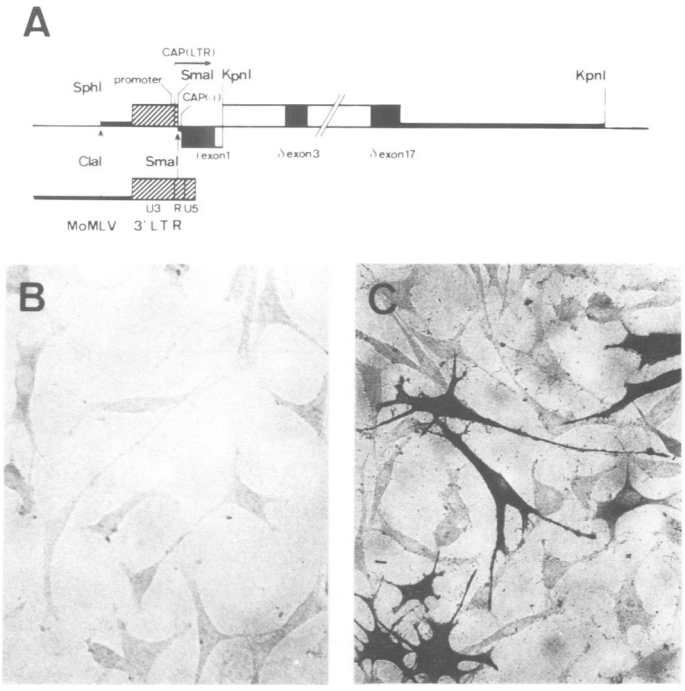

Fig. 3. Structure and expression of the hybrid gene plasmid pMLαδ containing the promoter sequence of Moloney murine leukemia virus. This plasmid was constructed by replacing the promoter region of the hybrid gene (pCryαδ) with the long terminal repeat (LTR) promoter sequences of MoMLV at the *Sma*I site (between −8 and −7) located between the promoter and the transcription initiation site of the α-crystallin gene (Figure 6). Therefore, the hybrid structural gene was transcribed under the direction of the LTR promoter. The construction procedure of the plasmid and its detected gene products expressed in injected cells are described in Materials and methods. **(A)** Structure of the hybrid gene in the plasmid pMLαδ. Solid and open portions of the bar represent the exons and introns of the α- and δ-crystallin genes, respectively. The hatched portion of the bar represents the LTR of MoMLV. Thin and thick lines represent the vector plasmid, and the flanking sequences of the MoMLV and the hybrid crystallin gene, respectively. **(B)** L-cells injected with the hybrid gene pCryαδ were stained by the PAP method. No cells were stained, showing no expression of the hybrid gene pCryαδ in L-cells. **(C)** L-cells injected with the plasmid pMLαδ were stained by the PAP method. The presence of the stained cells shows the expression of the plasmid pMLαδ in L-cells.

into the different types of tissues shown in Table I, Western blot analysis detected bands which cross-reacted with anti-δ-crystallin serum and had an electrophoretic mobility of 56 K in all tissues and the intensity of the bands was equal to that in lens cells (data not shown). These results indicate that the 5′-flanking region upstream of the cap site is involved in the regulated tissue-specific expression of the α-crystallin gene in mouse lens cells and that an undetectable level of expression of the α-crystallin gene in fibroblasts is not due to the unstable nature of mRNA and fusion proteins. The tissue-specific expression is thus controlled at the transcription level.

Identification of DNA sequences responsible for tissue-specific expression

To locate DNA sequences required for tissue-specific expression within the 5′-flanking region of the α-crystallin gene, we constructed a series of 5′ deletions extending from the *Mbo*II site at position −373 towards the cap site of the α-crystallin gene by restriction with specific enzymes (Figure 4A and Figure 6). Figure 4B shows the expression of these deletions in lens cells examined by Western blot analysis. Deletion mutants which still

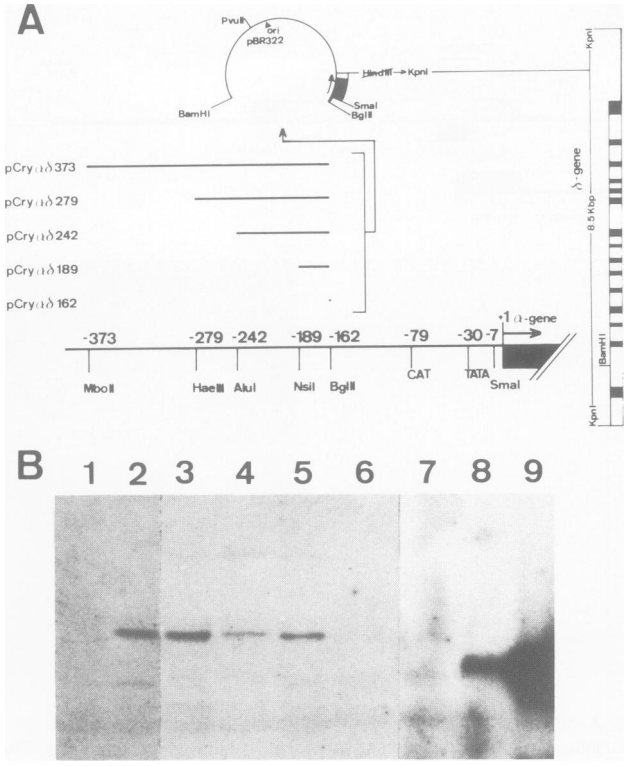

Fig. 4. Structure and expression of the hybrid genes with the deletion of the 5′-flanking sequences of the α-crystallin gene. **(A)** Schematic representation of deletion mutants. The construction procedure of deletion mutants is described in Materials and methods. The deletions in the 5′ to 3′ direction are referred to as pCryαδn where n is the nucleotide present at the deletion end point within the 5′-flanking region of the α-crystallin gene. **(B)** Expression of deletion mutant genes. About 100 copies of the plasmid DNA were injected into 500 mouse lens cells. Cell lysates were analyzed by Western blotting. **1:** no DNA; **2:** pCryαδ; **3:** pCryαδ373; **4:** pCryαδ274; **5:** pCry242; **6:** pCry189; **7:** pCry162; **8** and **9:** 1 ng and 10 ng of purified δ-crystallin.

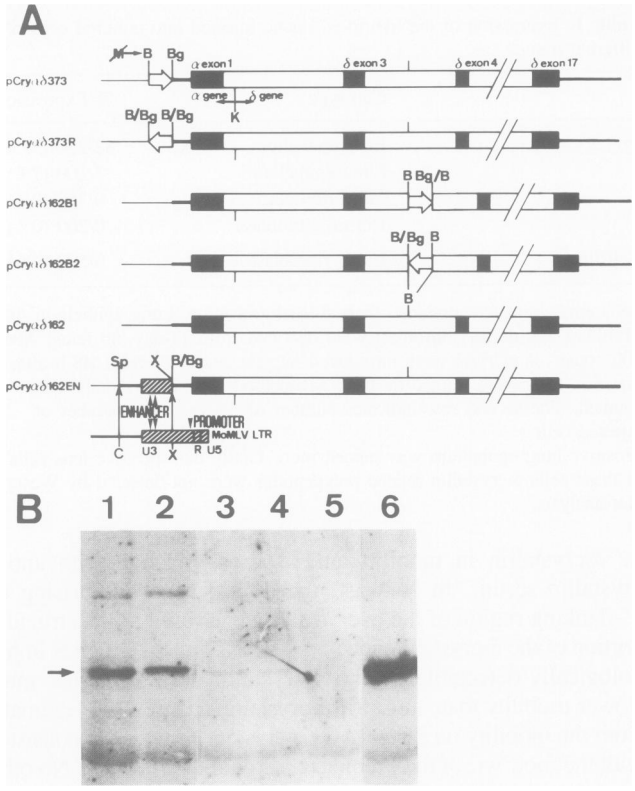

Fig. 5. Orientation and position effects of the *Mbo*II-*Bgl*II DNA fragment (−373 to −162) on expression of the hybrid gene. **(A)** Schematic representation of mutant hybrid genes. The solid and open portions of the bar represent the exons and introns of the crystallin genes, respectively. The open arrows represent the 212-bp *Mbo*II-*Bgl*II DNA fragment (see Figure 4) and the direction of the arrows shows the direction of the DNA fragment in the original α-crystallin gene. This DNA fragment is reversed in the original site (pCryαδ373R) or is inserted at the *Bam*HI site located in the second intron of the hybrid gene in normal (pCryαδ162B1) or reversed orientation (pCry162B2). The plasmid pCryαδ162 is lacking in this DNA fragment. The plasmid pCryαδ162EN has an insertion of the enhancer sequences of MoMLV at the *Bgl*II site on the 5′-flanking sequences of the hybrid gene. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; K, *Kpn*I; Sp, *Sph*I; M, *Mbo*II. *Mbo*II site was destroyed and changed to *Bam*HI. **(B)** Expression of hybrid gene mutants. 500 mouse lens cells injected with ~100 copies of the mutants were analyzed according to the procedure described in Materials and methods. The arrow indicates the band of the fusion protein. The upper band in **lanes 1** and **2** appeared non-specifically depending on the lot of [^{125}I]protein A used. **1**; pCry373, **2**; pCry373R, **3**; pCry162B1, **4**; pCry162B2, **5**; pCry162. **6**; pCry162EN.

contain 242 bp or more of the 5′-flanking region of the α-crystallin gene are efficiently expressed to almost the same extent. Those having <189 bp of the 5′-flanking region of the α-crystallin gene, however, showed markedly little expression. This indicates that at least 242 bp of the 5′-flanking region of the α-crystallin gene are required for their expression in mouse lens cells. These results led us to conclude that the sequences responsible for tissue-specific expression of the α-crystallin gene is located within the 53-bp region, the *Alu*I-*Nsi*I DNA sequences, between −242 and −189 bp from the transcription initiation site.

Effect of position and orientation of the MboII-BglII fragment on expression of the hybrid gene

Viral enhancers stimulate the transcription of homologous or heterologous promoters either upstream or downstream as long as the distance between the enhancer and the promoter is within several kilobases and in either orientation with respect to transcription (Banerji *et al.*, 1981; de Villiers and Schaffner, 1981; Levinson *et al.*, 1982; Weiher *et al.*, 1983). The enhancer element found in the intron of the immunoglobulin heavy chain gene behaves in a manner analogous to the viral enhancers but is tissue-specific (Gillies *et al.*, 1983; Picard and Schaffner, 1983; Queen and Baltimore, 1983).

To investigate how the *Alu*I-*Nsi*I DNA sequences in the 5′-flanking region of the α-crystallin gene affect the expression when the orientation of the sequences is reversed or is inserted at another site, we isolated the 212-bp *Mbo*II-*Bgl*II DNA fragment (−373 to −162 bp) containing the *Alu*I-*Nsi*I DNA sequences and constructed four mutants (Figure 5A). The first one contains the 212-bp sequences in the reversed orientation at the original position (pCryαδ373R). The second and third contain these sequences at the *Bam*HI site (1664 bp downstream from the cap site) of the second intron of the hybrid gene (pCryαδ) in the normal (pCryαδ162B1) or reversed orientation (pCryαδ-162B2). The fourth contains the *Cla*I-*Xba*I fragment which has only the enhancer sequences of the LTR of MoMLV (not including the promoter and cap site) at the same position in place of the *Mbo*II-*Bgl*II fragment. The expression of these mutant genes was analyzed by introducing them into mouse lens cells followed by Western blotting (Figure 5B). The insertion of the *Mbo*II-*Bgl*II fragment into the original position in the reversed orientation restored an expression comparable with the normal hybrid gene. The insertion of the sequences into the second intron of the hybrid

Table II. Effect of 'enhancer' and promoter on the expression of the hybrid gene

'Enhancer'			Promoter	Plasmid	Activity (%)	
Origin	Position	Orientation			Lens	Fibroblast
	−162 bp	N	α-gene	pCryαδ373	100	<2
α-gene	−162 bp	R	α-gene	pCryαδ373R	98	<2
(*Mbo*II-*Bgl*II)	+1664 bp	N	α-gene	pCryαδ162B1	12	<2
	+1664 bp	R	α-gene	pCryαδ162B2	11	<2
−			α-gene	pCryαδ162	<2	<2
MoMLV	−162 bp	N	α-gene	pCryαδ162EN	270	220
(*Cla*I-*Xba*I)	−186 bp	N	MoMLV	pMLαδ	280	290

Positions represent the nucleotide numbers from the cap site to the positions, at which the 'enhancer' sequences were inserted into the hybrid gene. N and R represent the direction of the 'enhancer' sequences in mutant genes in the normal (N) and reversed (R) orientation relative to the direction of transcription of the original genes, respectively.
Activity was determined by densitometric scanning of the autoradiograms. The relative activities are expressed as a percentage of the activity of the plasmid pCryαδ373. Numbers represent the average values of two independent experiments.

gene in either orientation did not restore the expression of the normal hybrid gene but retained it at a level ~10% that of the normal hybrid gene. Plasmid pCryαδ162EN containing the enhancer sequences of MoMLV exhibited approximately three times higher levels of fusion protein production than did the normal hybrid gene pCryαδ373. This indicates that the viral enhancers stimulate transcription of the α-crystallin gene more strongly than the 'enhancer-like element' of the α-crystallin gene. The expression of these mutant genes was also examined in mouse L-cells. The mutant gene containing the enhancer of MoMLV showed a high level of expression both in lens cells and in L-cells. These results and some of the previous results are summarized in Table II.

Transcription start site of transcripts

To determine whether the hybrid gene was transcribed from the expected promoter of the α-crystallin gene, we analyzed total RNA of lens cells injected with hybrid genes by S1 mapping or primer extension analyses. We were not able to detect any positive band in autoradiograms, although we isolated total RNA from up to 2×10^3 injected cells. To map the precise transcription start site of the transcripts, many more injected cells would be needed. We therefore took another approach. We modified the distance between the TATA box and the cap site of the α-crystallin gene by an insertion of oligonucleotides or a 1-bp deletion at the unique *Sma*I site between them (Figure 6). Plasmids pCryαδ and pCry162EN with a deletion of one nucleotide at position −7 showed the same level of expression as normal hybrid gene pCry373. In contrast, the insertion of oligonucleotides (5′-CGCCC-3′) or oligonucleotides (5′-AATTCGAGGCTCGCCC-3′) into the *Sma*I site (−7) of plasmids pCryαδ373 and pCryαδ-162EN caused a dramatic reduction in expression of fusion proteins (data not shown). Western blot analysis showed that these four kinds of plasmids synthesized no fusion proteins in mouse lens cells. When the 2.9-kb *Hind*III fragment generated from the α-crystallin gene fragment (see Figure 1) was transcribed in the *in vitro* transcription system using whole-cell extracts of HeLa cells, we detected only one type of run-off transcript, the start site of which was identified as the cap site of the α-crystallin gene, and no other cap site was apparent in this DNA fragment (Yasuda, unpublished results). These results suggest that the expression of hybrid genes might be under the control of the expected promoter of the α-crystallin gene.

Discussion

The introduction of cloned genes into differentiated vertebrate cells in culture has been used to study regulatory elements of gene expression (Walker *et al.*, 1983; Charnay *et al.*, 1984; Melloul *et al.*, 1984; Merrill *et al.*, 1984). We have used the microinjection technique to probe the mechanisms involved in tissue-specific expression. Plasmid pCryαδ containing the 5′-flanking and structural sequences of the hybrid α/δ-crystallin gene exhibited high-level transient expression predominantly in mouse lens cells, but did not exhibit detectable levels of expression in non-lens tissues such as fibroblasts. This artificial system involving transient expression of an exogenous gene, mimics the expression of the α-crystallin gene in homologous lens cells.

In an attempt to map the regulatory element within the 5′-flanking region we constructed 5′ deletion mutants. Analysis of expression of these deletion mutants revealed that the *Alu*I-*Nsi*I DNA sequences (−242 to −189 bp) are crucial to a high-level expression of the α-crystallin gene in mouse lens cells. In other genes, the 5′-flanking regions upstream of the cap site have been shown to be crucial to their tissue-specific expression (Brinster *et al.*, 1982; Buetti and Digglemann, 1983; Ragg and Weissmann, 1983; Tavernier *et al.*, 1983; Walker *et al.*, 1983; Weidel and Weissmann, 1983; Otto *et al.*, 1984).

This regulatory element of the α-crystallin gene is active when reversed at the original site or when placed ~1.7 kbp downstream of the cap site in either orientation. Therefore, the regulatory element of the α-crystallin gene shares common features with the previously characterized viral enhancers (Banerji *et al.*, 1981; de Villiers and Schaffner, 1981; Levinson *et al.*, 1982) and immunoglobulin heavy chain gene enhancer (Gillies *et al.*, 1983; Picard and Schaffner, 1983; Queen and Baltimore, 1983). This regulatory element is 'enhancer-like', mediating activity relatively independent of distance and orientation with relation to a given promoter. Viral enhancers and the immunoglobulin heavy chain gene enhancer stimulate the expression of the promoters to almost the same level as long as the distance between them is within several kilobases. However, in the case of the 'enhancer-like' element of the α-crystallin gene, since the level of expression of this gene decreases to ~10% of its original level when the distance is 1.7 kbp from the cap site (originally 200 bp), the expression might be determined by the distance between this element and the promoter. This also might be explained by the insertion of DNA sequences into an intron reducing the efficiency of splicing *in vivo*. The regulatory element of the α-crystallin gene and immunoglobulin heavy chain gene enhancer differ from viral enhancers in that the former act in a tissue-specific manner. 'Enhancer-like' elements could be found in genes expressed specifically in differentiated cells.

α-Crystallin is very conserved between chicken and mouse (de Jong, 1981). Exogenous chicken α-crystallin gene is efficiently expressed in mouse lens cells, and therefore the functional regulatory element acting in gene expression should be conserved between them. Comparison of the 'enhancer-like' element of the chicken α-crystallin gene with nucleotide sequences in mouse α-crystallin gene (King and Piatigorsky, 1983) revealed the presence of stretches of sequence homology in the region between −288 and −169 bp of the mouse gene (Figures 6 and 7). The 53-bp region in the chicken gene has a 50% homology with the coincident sequences in the mouse gene. Analysis of expression of the

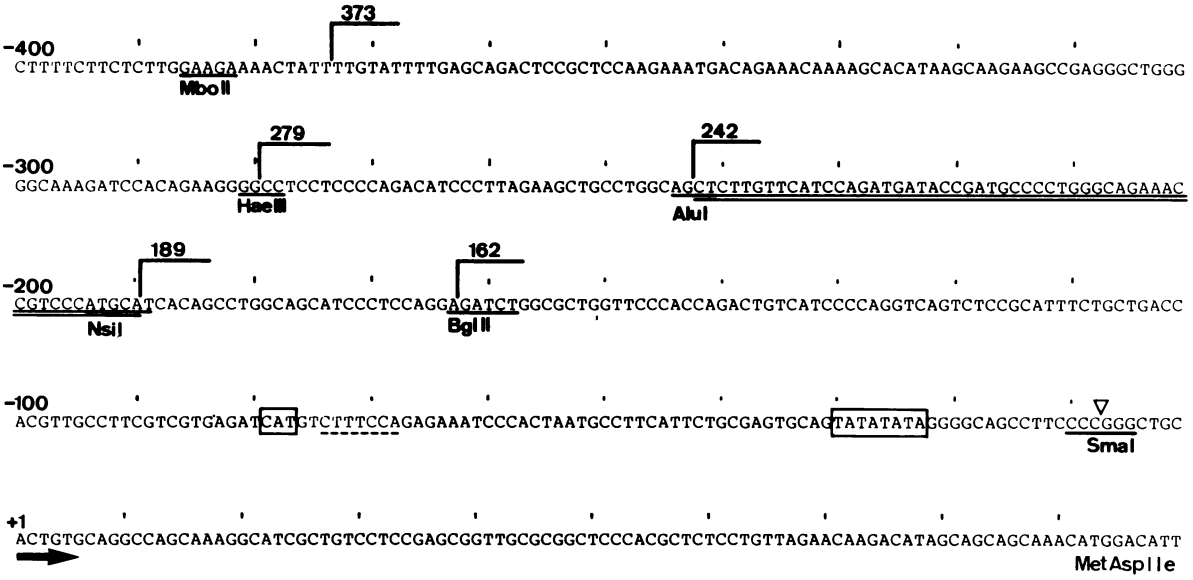

Fig. 6. Nucleotide sequences of the 5′-flanking region and 5′ structural region of the chicken α-crystallin gene. The nucleotide sequences of the α-crystallin gene will appear elsewhere (Okazaki *et al.*, in preparation). The 5′ ends of the flanking sequences of the hybrid genes are shown by numbers above the nucleotide sequences. DNA sequences responsible for tissue-specific expression of the α-crystallin gene are double-underlined. The CAT and TATA sequences are boxed. The arrow indicates the transcription initiation site and orientation transcription of the α-crystallin gene. The sequences homologous to the 'core' element common to viral enhancers are shown by the dashed underline.

Fig. 7. Comparison of 'regulatory sequence' of the chicken α_A-crystallin gene with comparable sequence of the mouse α_A-crystallin gene. The regulatory sequence of the chicken α_A-crystallin gene (Okazaki *et al.*, in preparation) and a comparable sequence of the mouse α_A-crystallin gene (King and Piatigorsky, 1983) are shown. Numbers shown above and below the sequences indicate the nucleotide numbers from the cap sites of the chicken and the mouse crystallin gene, respectively.

hybrid gene consisting of the 5′-flanking region of mouse α-crystallin gene fused to the structural sequences of the CAT gene suggests that this region is responsible for high-level expression (Piatigorsky *et al.*, 1984).

It is interesting to compare the regulatory sequences of the α-crystallin gene required for specific gene expression with that of the δ-crystallin gene, since expression of the two genes is seen only in lens cells but is controlled differently temporally and spatially. During development, the δ-crystallin gene is expressed at the early stage of lens placode prior to lens formation. Lens tissue consists of lens epithelial cells and lens fiber cells. In epithelial cells the two genes show the same level of expression but in fiber cells the δ-crystallin gene is expressed preferentially compared with the α-crystallin gene. No homologous sequences were found between DNA sequences required for high-level expression of the two genes (Hayashi *et al.*, in preparation). This is consistent with the speculation that there might be different factors interacting with the regulatory elements of the two genes which specify their spatially and temporally differential expression.

Previously we showed that the δ-crystallin gene is expressed efficiently in mouse lens cells, but that a low level of expression was observed in brain or lung cells (Kondoh *et al.*, 1983). In contrast, the α-crystallin gene is never expressed in tissues other than lens cells. This leads us to conclude that the α-crystallin gene is regulated in lens cells more strictly than is the δ-crystallin gene. This is consistent with observations of the ectopic expression of the δ-crystallin gene in adenohypophysis of early chick embryo or when adenohypophysis or brain tissues are cultured *in vitro*, but the α-crystallin gene is not expressed at all in these tissues (Takagi *et al.*, 1983).

Materials and methods

Chemicals and enzymes

Chemicals were from Nakarai Chemicals Ltd. and Wako Pure Chemical Industries Ltd. Nucleotides and synthesized linkers were from Pharmacia P-L Biochemicals. Protein mol. wt. markers for SDS-PAGE gel electrophoresis and calf intestinal alkaline phosphatase were from Boehringer Mannheim. Other enzymes were from New England Biolabs Inc. and Takara Shuzo Ltd.

Construction of plasmids

pCryαδ4. The 2.9-kb *Hind*III fragment containing the 5′ upstream promoter region, the first exon and 98 bp of the 5′ region of the first intron of the α-crystallin gene (−2.5 kb to +378 bp) was obtained from λC10α (Okazaki *et al.*, in preparation). This fragment was introduced into pBR322 by ligation at the *Hind*III site to produce a plasmid pCryα4. pCryα-P/K was constructed by a deletion of the small *Pvu*II fragment from pCryα4 and by a change of the *Hind*III site into the *Kpn*I site using the *Kpn*I linker followed by ligation. The 8.5-kbp *Kpn*I fragment containing the structural sequence (+630 to ~1 kbp downstream from the 3′ end) of the δ-crystallin gene was obtained from pδC1A (Kondoh *et al.*, 1983) and was introduced into the plasmid pCryα-P/K by ligation at the *Kpn*I site. The *Kpn*I site on the first intron of the α-crystallin gene was joined to the *Kpn*I site on the second intron of the δ-crystallin gene. Therefore, the δ-crystallin structural gene was transcribed under the direction of the α-crystallin gene promoter. Since the hybrid structural gene comprises the first exon of the α-crystallin gene, the 3rd to 17th exons of the δ-crystallin gene and since the frame of codons encoded in the first exon of the α-crystallin gene and in the third exon of the δ-crystallin gene matches, the fusion protein of the gene product comprises the 1st to 63rd amino acids of α-crystallin fused to the 5th to 465th amino acids of δ-crystallin.

pMLαδ. The 4.2-kbp *Sma*I/*Sph*I fragment was obtained from pCryα4. The 0.7-kbp *Cla*I/*Sma*I fragment extending from the *Cla*I site 0.3 kbp upstream from the 5′ end of the 3′ LTR to the *Sma*I site 30 bp downstream from the cap site of the 3′ LTR of MoMLV was obtained from a plasmid pMoMLV (Shinnick *et al.*, 1981). The 4.2-kbp *Sma*I/*Sph*I fragment was ligated to the 0.7-kbp *Cla*I/*Sma*I fragment and circularized. The *Hind*III site of this plasmid was changed into the *Kpn*I site by *Kpn*I linkers and the 8.5-kbp *Kpn*I fragment of the δ-crystallin gene (Figure 1) was inserted into the *Kpn*I site as shown in Figure 3a to obtain the

plasmid pMLαδ. Thus, pMLαδ has the promoter and cap site of LTR of MoMLV in place of those of the α-crystallin gene.

Plasmids with mutant genes

The 4.6-kbp *Bam*HI/*Bgl*II fragment from a plasmid pCryα4 was used as a recipient vector DNA for an insertion of the 5′-flanking sequence with deletion, followed by an insertion of the 8.5-kbp *Kpn*I fragment in the δ-crystallin gene DNA at the unique *Kpn*I site of the recipient vector DNA. For the construction of pCryαδ373 (pCryαδ279,pCryαδ242), the 2.9-kbp *Hind*III fragment of the α-crystallin gene was digested with *Mbo*II (*Hae*III, *Alu*I) and their ends were filled with T4 DNA polymerase and were ligated to *Bam*HI linkers, followed by digestion with *Bam*HI and *Bgl*II. A 218-bp (123 bp, 86 bp) *Bam*HI/*Bgl*II fragment eluted from an acrylamide gel was ligated to the 4.6-kbp recipient vector followed by an insertion of the 8.5-kbp *Kpn*I fragment of the δ-crystallin gene. During the preparation of pCry373, pCryαδ373R containing the 218-bp *Bam*HI/*Bgl*II fragment in a reversed orientation compared with that in a plasmid pCryαδ373 was also selected. pCryαδ189 (pCryαδ162) was constructed from plasmid pCryα4 by deleting a small *Bam*HI/*Nsi*I (*Bam*HI/*Bgl*II) fragment followed by an insertion of the 8.5-kbp *Kpn*I fragment of the δ-crystallin gene at the *Kpn*I site changed from the *Hind*III site. pCryαδ162B1 and pCryαδ162B2 were constructed by inserting the 218-bp *Bam*HI/*Bgl*II fragment into the *Bam*HI site of the second intron of the hybrid α/δ gene in a normal and a reversed orientation, respectively. To obtain an enhancer sequence of MoMLV in an attempt to insert it into pCryαδ162, a 0.5-kbp fragment extending the *Cla*I site to the *Xba*I site 0.2 kbp downstream from the 5′ end of the 3′ LTR of MoMLV was inserted into *Acc*I and *Xba*I sites of pUC19 (pUC19EN). pCry162EN was constructed by ligating the *Sph*I/*Bam*HI fragment from plasmid pUC19EN to the 4.4-kbp *Sph*I/*Bgl*II fragment from pCryα4 followed by inserting the 8.5-kbp *Kpn*I fragment of the δ-crystallin gene at the *Kpn*I site changed from the *Hind*III site. The site and orientation of the fragments inserted were determined by restriction analysis and DNA sequencing.

Cell culture

Lens epithelial cells were taken from 3-week-old mice (C57BL/J6). Cultures were maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 15% fetal calf serum.

Microinjection

An apparatus devised by Yamamoto and Furusawa (Injectoscope; Olympus Co. Ltd.) was employed for microinjection (Kondoh *et al.*, 1983). Closed circular DNA dissolved in 0.066 M phosphate buffer (pH 7.2) at a concentration of 0.2 – 1.0 mg/ml was centrifuged just before injecting to remove debris. This concentration roughly corresponds to 100 – 500 copies per cell assuming that the volume of DNA solution injected into the nuclei is 2×10^{-14} l (Cappechi, 1980).

Detection of products

For *in situ* detection, 45 – 50 h after injection, cultures were fixed with 100% methanol at −20°C overnight, treated with non-immune goat serum, with anti-δ-crystallin rabbit serum, with anti-(rabbit IgG) goat serum (Miles Inc.) and finally with horseradish peroxidase-anti-peroxidase rabbit IgG complex (PAP; DAKO Inc.). The peroxidase-catalyzed reaction was carried out in a solution containing 0.001 M 3-amino-9-ethyl carbazole, 0.01% H_2O_2 and 0.1 M sodium acetate (pH 5.2) at room temperature for 15 min to stain red the cells containing δ-crystallin-related polypeptides.

For electrophoretic detection, a sample was recovered 45 – 50 h after injection by dropping 20 μl of an electrophoretic sample buffer onto the injected cells and collecting the solution with a micropipette. The samples were subjected to a 10% SDS-polyacrylamide gel electrophoresis (Laemmli and Favre, 1973) and blotted on nitrocellulose filter (0.2 μm pore size; Toyo Roshi Co.) by an electrophoretic transfer method (Towbin *et al.*, 1979). The filter was coated with 3% bovine serum albumin (BSA; Sigma A 7888) solution (TPS) containing 0.02 M Tris-phosphate (pH 7.5), 0.15 M NaCl and 0.002 M EDTA, and treated with 0.5% anti-δ-crystallin serum diluted in the BSA solution. The filter was then washed three times with TPS containing 0.05% Tween 20, treated with 0.5 μCi/ml [^{125}I]protein A (Amersham) in the BSA solution followed by washing, drying and exposure to X-ray film (Fuji RX) with or without an intensifying screen (Fuji Hi-screen).

Acknowledgements

We thank Dr Y.Shimura for valuable suggestions and critical reading of the manuscript. This work was supported by Grant-in-Aid for Special Research Project from the Ministry of Education, Science and Culture of Japan to K.Y., H.K. and T.S.O.

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Received on 21 June 1985; revised on 23 July 1985

Note added in proof

A paper by Hayashi *et al.*, quoted as 'in preparation', is now in press (Hayashi,H., Kondoh,H., Yasuda,K., Soma,G., Ikawa,Y. and Okada,T.S. (1985) *EMBO J.*, **4**, 2201-2207.