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Structural and functional evidence for differential promoter activity of the two linked δ-crystallin genes in the chicken

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There are two δ-crystallin genes (δ1 and δ2) in the chicken which are oriented with the same transcriptional polarity (5′ δ1-δ2-3′) and are separated by ~4.2 kb of DNA. Existing evidence indicates that δ1 is very active in the embryonic lens; in contrast, δ2 appears very inactive, if expressed at all. We have sequenced the 5′ regions of δ1 and δ2 and tested their ability to promote chloramphenicol acetyltransferase (CAT) activity using the pSVO-CAT expression vector in transfected embryonic lens epithelia. The sequence data establish that the previously reported δ-crystallin cDNAs were derived from mRNAs encoded in δ1 and not in δ2. The transfection experiments indicated that the δ1 promoter is appreciably stronger than the δ2 promoter. Interestingly, a consensus CCAAT sequence (position −71) and two consensus viral core-enhancer-like sequences (positions −308 and +350) are confined to the more active, δ1 gene. δ1 and δ2 have similar TATA boxes (TAAAA) at positions −28 and −27, respectively. Exon 1 in δ1 (35 bp) and in δ2 (34 bp) are extremely homologous, containing just two mismatches; exon 2 in δ1 (64 bp) and in δ2 (51 bp) are only 56% homologous and contain the putative translation initiating codon and three encoded amino acids. Unexpectedly, intron 1 of both genes has numerous pentameric repeats present in the murine immunoglobulin heavy-chain switch regions (GAGCT, GGGGT and GGGCT). Other direct repeats were found in δ1 and δ2, and short homologies were noted between the chicken δ-crystallin and murine αA-crystallin genes.

Key words: δ-crystallin genes/nucleotide sequence/gene promoter/lens/immunoglobulin switch regions/transfection/lens epithelia

Introduction

There are four major families of immunologically distinct structural proteins (α-, β-, γ- and δ-crystallin) of the vertebrate eye lens (Harding and Dilley, 1976; Bloemendal, 1977; Clayton, 1974). Each crystallin family comprises several related polypeptides which have been highly conserved during evolution (de Jong, 1981). The α- and β-crystallins are present in lenses of all vertebrate classes; δ-crystallin, however, replaces γ-crystallin in avian and reptilian lenses (Clayton, 1974; Williams and Piatigorsky, 1979; Piatigorsky, 1984). Crystallins are well suited for studying the regulation of eukaryotic gene expression since they constitute ~90% of the soluble protein of the lens and are differentially synthesized during development (Papaconstantinou, 1967; Zwaan and Ikeda, 1968; McAvoy, 1978; Piatigorsky, 1981).

δ-Crystallin is the first (Zwaan and Ikeda, 1968; Rabaey, 1962; Shinohara and Piatigorsky, 1976) and predominant (Yoshida and Katoh, 1971; Piatigorsky *et al.*, 1972) crystallin synthesized during development of the chicken lens. Synthesis of δ-crystallin decreases (Brahma and van der Starre, 1976; Bagchi *et al.* 1981) and δ-crystallin mRNA is lost 3–5 months after hatching (Treton *et al.*, 1982); δ-crystallin, however, remains the major protein in the center of the adult lens (Genis-Galvez *et al.*, 1968).

The molecular biology of δ-crystallin and its nucleic acids has been reviewed recently (Piatigorsky, 1984). There are two very similar δ-crystallin genes with numerous introns in the chicken genome (Bhat *et al.*, 1980; Jones *et al.*, 1980; Yasuda *et al.*, 1982a). The chicken δ-crystallin genes are tandemly arranged and separated by ~4.2 kb of DNA (Yasuda *et al.*, 1982b; Hawkins *et al.*, 1984). Only one species of δ-crystallin cDNA has been detected in the 15 day-old embryonic chicken lens which appears to be encoded in the δ1 gene (Nickerson and Piatigorsky, 1984; Yasuda *et al.*, 1984). It is not known whether the δ2 gene produces low levels of mRNA in the 15 day-old embryonic lens or whether it is expressed at a different time in development. We have compared the nucleotide sequence and the transcriptional strength of the 5′-flanking regions of the two δ-crystallin genes in the pSVO-CAT expression vector (Gorman *et al.*, 1982). The results indicate that the δ1 gene has a stronger promoter than the δ2 gene.

Results

Identification of the 5′ regions of the δ1- and δ2-crystallin genes

Initially we located the 5′ region of the δ1-crystallin gene. pδ1.1 was mapped with a series of restriction enzymes (Figure 1). The most 5′ exonic information of δ1 was putatively located on the 1.3 kb *Pst*I-*Kpn*I fragment, shown in Figure 1, by the following criteria. First, a *Hinf*I fragment containing the first 152 nucleotides of a full-length δ-crystallin cDNA, pδCr17 (Nickerson and Piatigorsky, 1984), hybridized to 4.2- and 0.7-kb *Msp*I fragments from pδ1.1, and not to the 1.3-kb *Msp*I fragment situated upstream from the 0.7-kb fragment (see Figure 1). The possibility that the 0.7-kb *Msp*I fragment (pδ1.4) contains the 5′ end of the δ1 gene was supported by the fact that pδ1.4 hybridized to δ-crystallin mRNA from the embryonic chicken lens in both dot-blot and Northern-blot hybridization analysis (data not shown). In contrast, neither the cloned 0.8-kb *Eco*RI-*Msp*I fragment (pδ1.2) nor the cloned 1.3-kb *Msp*I fragment (pδ1.3) hybridized to the δ-crystallin mRNA. We used pδ1.4 as a probe to identify the 5′ end of the δ2 gene, assuming that the 5′ ends of δ1 and δ2 would cross-hybridize as does the rest of the two genes (Jones *et al.*, 1980).

pδ2.1 was mapped by restriction digestion and Southern-blot hybridization using pδ1.4 as a probe (Figure 1). Digestion of pδ2.1 with a number of restriction enzymes narrowed our search for the 5′ end of the δ2 gene to a 0.6-kb fragment obtained by triple digestion with *Eco*RI, *Msp*I and *Bam*HI (data

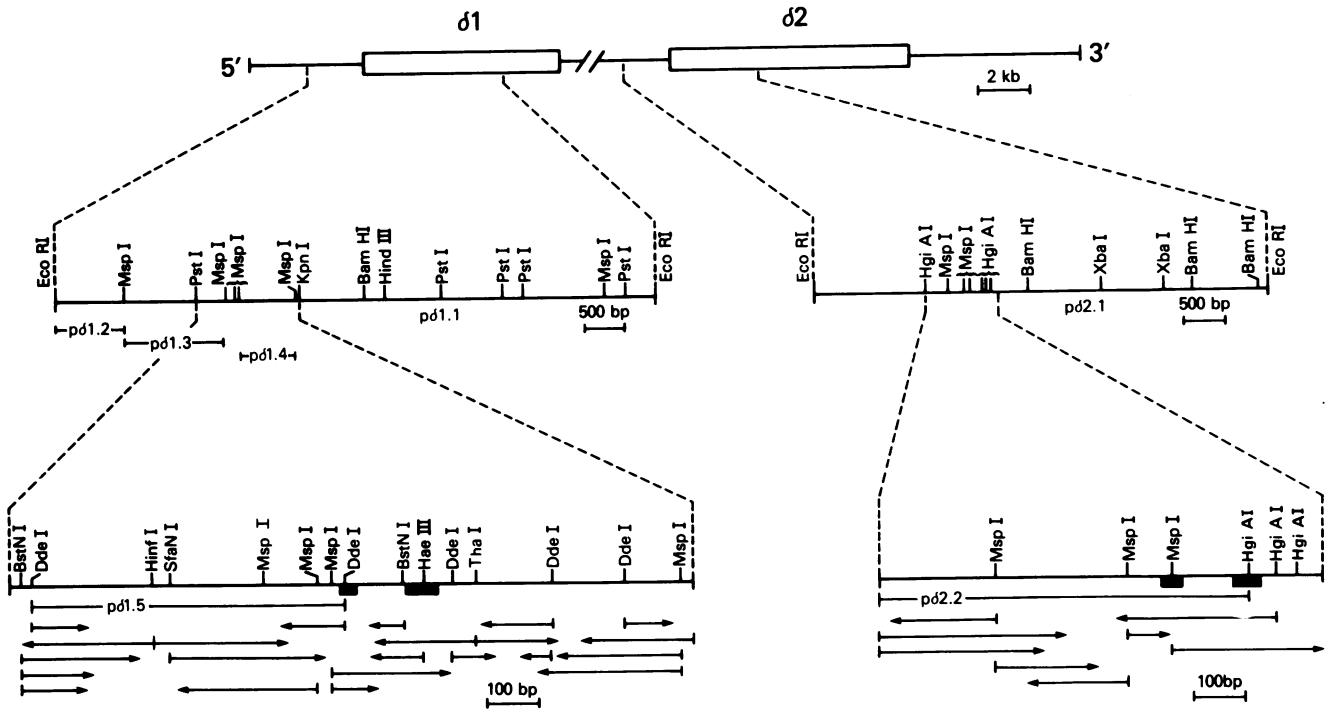

Fig. 1. Restriction map and sequencing strategy for the sequence of the 5′ flanking regions of δ1- and δ2-crystallin genes. The broken line in the spacer between δ1 and δ2 indicates that the clones analyzed were obtained from different genomic libraries (see Materials and methods). The *Hgi*AI sites given in pδ2.1 reflect only those present in the most 5′ *Eco*RI-*Bam*HI fragment. The exons (determined by sequencing) in the sequenced DNA fragments are depicted as solid black boxes; only the restriction sites used for sequencing are marked. The arrows indicate the direction and extent of the fragments sequenced. The 1.3-kb *Pst*I-*Kpn*I fragment sequenced from δ1 was not named since it was not subcloned in a plasmid. This figure shows several modifications in δ1 from previous reports. First, two extra *Msp*I sites are present just 5′ of exon 1 (Jones *et al.*, 1980, 1981; Yasuda *et al.*, 1982a). Second, the 0.7-kb *Msp*I fragment is 3′ to the 1.3-kb *Msp*I fragment (Jones *et al.*, 1980). Third, exon 1 is ~2.2 kb 3′ from the 5′ *Eco*RI site in pδ1.1 rather than 1.2 kb (Jones *et al.*, 1980) or 2.7 kb (Yasuda *et al.*, 1982a) from this site. Finally, sequencing has shown that exon 1, previously believed by electron microscopy to be 200–300 bp long (Bhat *et al.*, 1980; Jones *et al.*, 1980; Yasuda *et al.*, 1982a), is divided into two short exons (see text).

not shown). Analysis using *Eco*RI-*Xba*I and *Bam*HI alone allowed us to putatively identify the 0.6-kb fragment containing the sequences homologous to pδ1.4 within the *Hgi*AI fragment (pδ2.2), shown in Figure 1.

Sequence comparison of the 5′ regions of the δ1 and δ2 genes

The 1.3-kb *Pst*I-*Kpn*I fragment of δ1 and 0.8-kb *Hgi*AI fragment of δ2 were sequenced following the strategy presented in Figure 1 and the procedures given in Materials and methods. The nucleic acid sequences were aligned (Figure 2) following the computer program of Wilbur and Lipman (1983). The first two exons of δ1 were identified by comparison with the sequence of the δ-crystallin cDNA, pδCr17 (Nickerson and Piatigorsky, 1984). An exact match was obtained with the exception of one nucleotide (position +151, see legend to Figure 2). The comparable exons from δ2 were aligned with those from δ1 and are boxed and labelled in Figure 2. Exon 1 is 35 bp in δ1 and 34 bp in δ2; there are only two mismatches and one additional base in δ1 in this small, non-coding exon. The first intron is 57% homologous between δ1 and δ2 and is 110 bp in the former and 103 bp in the latter. Exon 2, which is 64 bp in δ1 and 51 bp in δ2, is only 56% homologous between the two genes. Intron 1 of both genes has the 5′ GT-AG 3′ splicing consensus sequence (Breathnach and Chambon, 1981). The putative protein coding sequence begins near the end of exon 2 in both genes, as judged by the closest ATG to the cap site (Kozak, 1983). It is interesting that the third encoded amino acid is threonine in δ1 and serine in δ2. If both genes are expressed, this provides the first sequence difference between two δ-crystallin polypeptides.

The TATA box of δ1 and δ2 is identical (boxed in Figure 2). This sequence is located 28 bp and 27 bp upstream from the initiation site of transcription (cap site) in δ1 and δ2, respectively. The cap site was established in δ1 by S1 and mung bean nuclease protection experiments and a primer extension experiment using δ-crystallin mRNA (Nickerson and Piatigorsky, 1984). δ1 has a consensus CCAAT box (Benoist and Chambon, 1981; Efstratiadis *et al.*, 1980) at position −71 which is not present in δ2. Both δ1 and δ2, however, have GC-rich sequences in this region. There are also G-rich regions on both sides of the TATA box in δ1 and δ2. It is noteworthy that a short stretch (7 bp) of alternating pyrimidines and purines borders the 5′ side of the TATA box in both δ-crystallin genes. Alternating pyrimidines and purines have been implicated in gene control due to their Z-DNA forming potential (Nordheim and Rich, 1983).

An interesting feature of δ1 is the presence of two consensus core-enhancer sequences found in viral (Weiher *et al.*, 1983) and immunoglobulin (Gillies *et al.*, 1983; Queen and Baltimore, 1983; Banerji *et al.*, 1983) genes (see Gruss, 1984). The first is at position −308 and the second within intron 2 at position +350 in δ1 (boxed in Figure 2). The upstream consensus core-enhancer sequence is in reverse orientation. We did not find any such sequence in the fragment from δ2.

There are nine direct repeats (DR) in δ1 and five in δ2 which are at least 7 bp long (Table I). DR9 in δ1 is repeated four

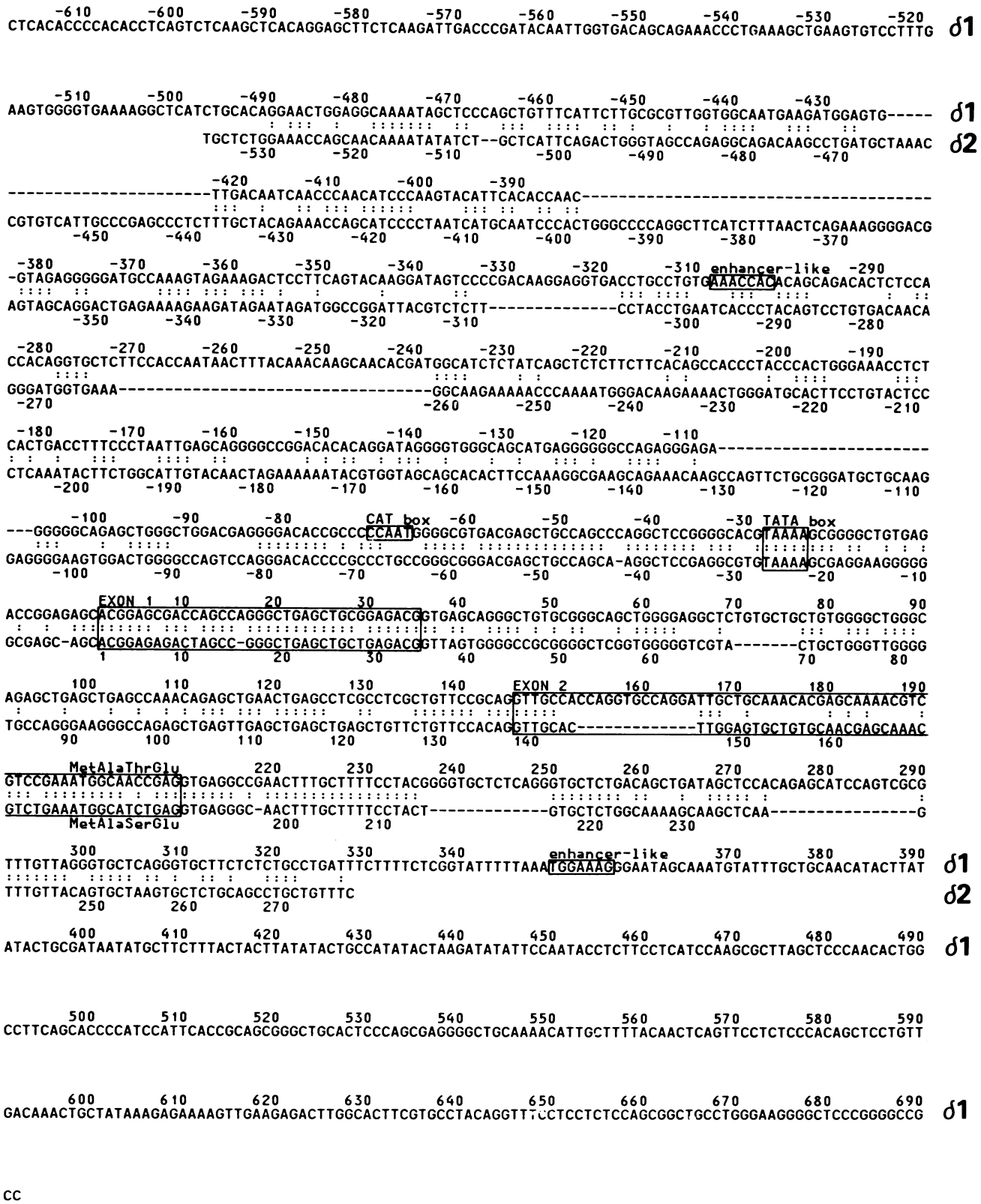

Fig. 2. Sequence and alignment of the 5′ and flanking regions of the δ1- and δ2-crystallin genes. The sequences were aligned following the computer program of Wilbur and Lipman (1983) using K-tuple size = 3, window size = 20 and gap penalty = 7 as parameters. The first two exons and the TATA sequence of each gene and the CCAAT and consensus viral core-enhancer-like sequences of δ1 are enclosed by boxes. The putative initiating ATG codon and coding sequences of the genes are indicated in the 3′ region of exon 2. Position +155 (GTGCAAC) of δ2 is cut unexpectedly by *Hgi*AI even though it has an extra A 5′ to the last C. The region corresponding to −103 to +299 of the δ1 gene has been reported recently by Yasuda *et al.* (1984). Our sequence differs from theirs as follows: insertions: C at −76, −74, −73 and +151; G at +239. Further data have lead to the following corrections. δ1: deletions: CA at −146 and −147. Insertions: G between −606 and −607, and between +89 and +90; CTGA between +265 and +266; T between +304 and +305. Inversion: GAT at −362 and −363. δ2: deletions: G at +251 and +258. Insertions: G between −63 and −64, and between +214 and +215; A between +196 and +197. Inversion: TG at +148 and +149. Substitution: G instead of A at +163. The present C in δ1 at +151 (in exon 2) modifies our cDNA sequence (Nickerson and Piatigorsky, 1984). This C was very clear by dideoxy sequencing, however, it was not resolved by Maxam-Gilbert sequencing.

Table I. Direct repeats (DR) 7 bp or longer present in the δ-crystallin gene sequences presented in Figure 4

δ1	5′→3′ sequence	Position 1(5′)	Position 2(5′)
DR1	TAGCTCCCA	−474	+475
DR2	GAGGGGG	−380	−126
			−109
DR3	AGGATAG	−343	−146
DR4	GGGCAGAGCTG	−105	+ 87
DR5	GCTGAGCTG	+ 19	+ 94
DR6	CTGAGCC	+100	+120
DR7	ACAGAGC	+109	+273
DR8	GCTGCAAA	+168	+541
DR9	GGGTGCT	+237	+248
			+298
			+307
DR10	TACTTATATACTGC	+384	+416
δ2	**Sequence**	**Position 1(5′)**	**Position 2(5′)**
DR1	TGCTCTGG	−537	+216
DR2	AAACCAGCA	−529	−429
DR3	GACTGGG	−498	− 97
DR4	GCTGAGCTG	+ 18	+111
DR5	TGAGCTG	+108	+118

times; DR4 in δ1 is repeated a third time in seven out of 11 positions (+110). DR5 in δ1 is identical to DR4 in δ2, and their relative positions are similar in the two genes (middle of 1st exon and 1st intron).

Comparison with other crystallin genes

The sequences of δ1 and δ2 (Figure 2) were compared with the published sequences from other crystallin genes using the dot-matrix computer program of Maizel and Lenk (1981). No obvious homologies were found between these δ-crystallin sequences and those from the murine αA- (King and Piatigorsky, 1983), β23- (Inana *et al.*, 1983) and γ4- (Lok *et al.*, 1984) crystallin genes, and a rat γ-crystallin (Moormann *et al.*, 1983) gene. We also looked for the presence of the direct repeats in the δ-crystallin genes (noted in Table I) in the other crystallin genes using the SEQ computer program (Brutlag *et al.*, 1982). Interestingly, 6 bp (CTCCCA) of DR1 from δ1 are repeated at positions +382 and +514 in the murine αA-crystallin gene (King and Piatigorsky, 1983; the α-crystallin gene nucleotide numbers have been adjusted relative to the cap site). In addition, 6 bp (TGAGCT) of DR5 from δ1 and DR4 from δ2 (which are identical) are also repeated at positions −126 and −84 in the murine αA-crystallin gene. Ten other short homologies were found between the δ-crystallin genes and the murine αA-crystallin gene. These include AAAGGCT (−507 in δ1, +933 in αA), TGTGGGG (+78 in δ1, +655 in αA), GGGGCTGGGCAG (+81 in δ1, −142 in αA), CCACAGA (+271 in δ1, +1018 in αA), GGAAACC (−531 in δ2, +566 in αA), CTTTGCT (−440 in δ2, +832 in αA), CAGTCCT (−290 in δ2, +1394 in αA), TGACAAC (−282 in δ2, +1220 in αA), GGGTTGGG (+74 in δ2, +660 in αA), and GAGCTGAGCT (+109 in δ2, −88 in αA). No one sequence was found in all the crystallin genes given above.

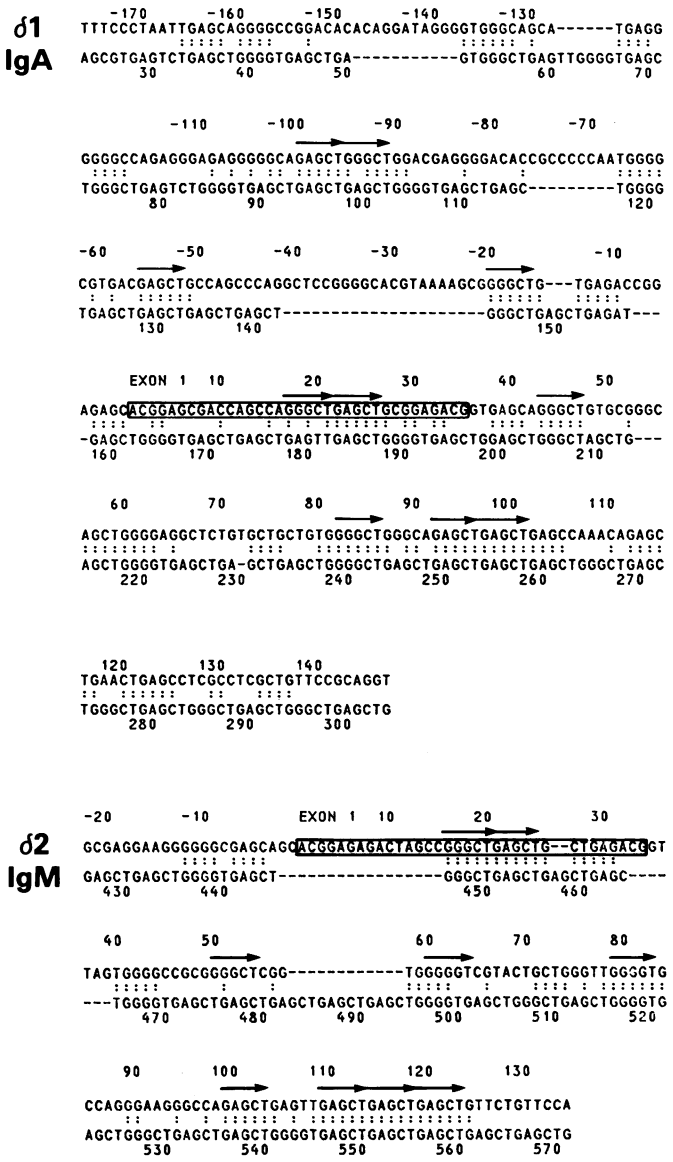

Fig. 3. Homology between the δ1- and δ2-crystallin gene sequences and the murine immunoglobulin heavy-chain switch regions. This homology was found during a search of the National Institutes of Health Genetic Sequence Data Bank (1983) using the program of Wilbur and Lipman (1983), using the following parameters: K-tuple size = 4, window size = 20 and gap penalty = 4. The arrows indicate the pentameric repeats characteristic of the immunoglobulin heavy-chain switch region (see text).

Homology with immunoglobulin switch regions

We searched through the Genetic Sequence Data Bank (National Institute of Health, 1983) for homologies between the δ-crystallin gene sequences in Figure 2 and gene sequences of other eukaryotes and viruses. The data base comprised ~1.5 x 10^6 bp in 2119 loci at the time of the search. The strongest homology observed was between the δ-crystallin genes and the switch region sequences for the murine immunoglobulin heavy-chain genes. As indicated by the arrows in Figure 3, the pentameric repeats (GAGCT, GGGGT and GGGCT) composing the immunoglobulin heavy-chain switch regions are prevalent in the δ1 gene (positions −166 to +137) and the δ2 gene (positions −12 to +124). The homology with the δ1 gene is greatest with the murine IgA switch region (Davis *et*

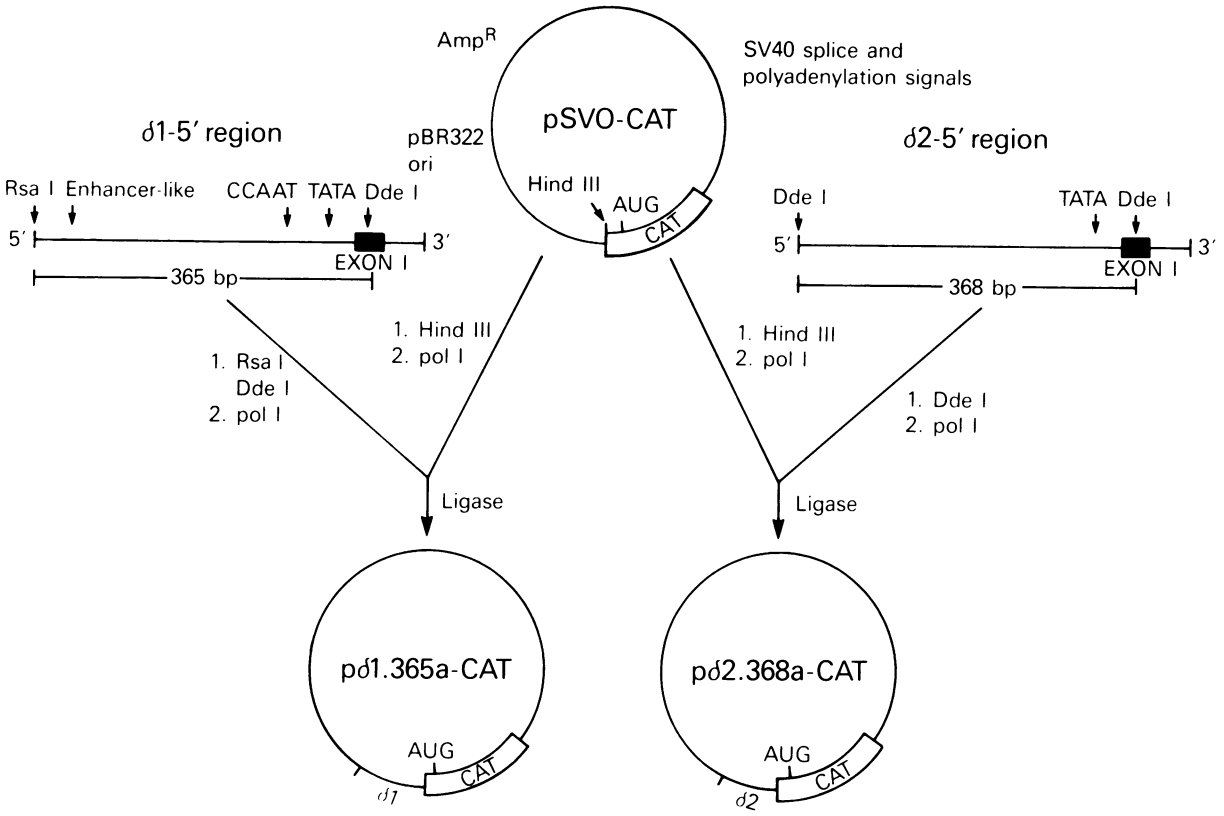

Fig. 4. Construction of CAT expression vectors containing the 5′ regions of the δ-crystallin genes. Blunt-end ligation was accomplished by mixing 0.02 pmol of pSVO-CAT with 8 pmol of each of the fragments in a final volume of 10 μl and in the presence of 12 units of T4 ligase. The mixture was incubated at 14°C for 18 h and transfected in HB 101 competent cells.

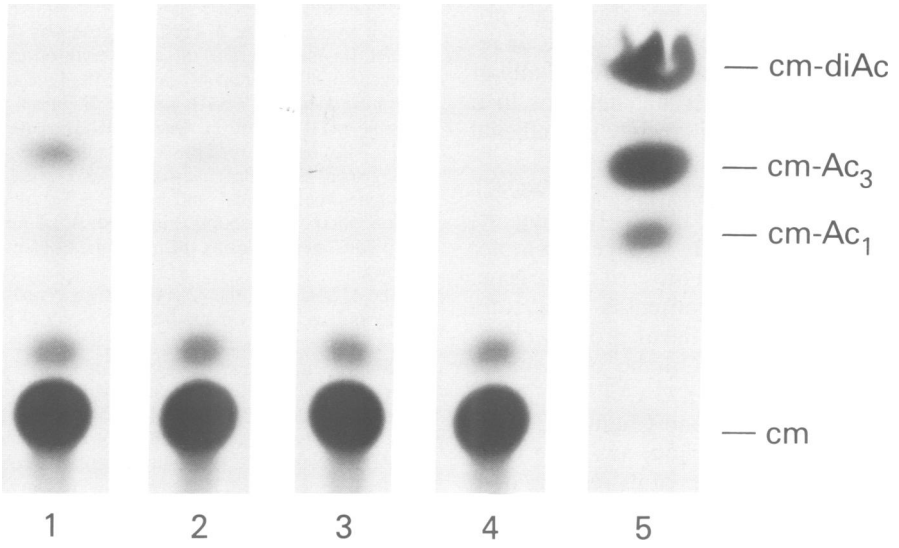

Fig. 5. Assay of CAT activity in explanted embryonic lens epithelia transfected with δ-crystallin-CAT vectors. Cells were transfected with 10 μg of plasmid DNA and 60 h later assayed for CAT activity as indicated in the text. The four radioactive species are cm:[^{14}C]chloramphenicol; cm-Ac^1:1-acetate-chloramphenicol; cm-Ac$_3$: 3-acetate-chloramphenicol; cm-diAc: 1,3-acetate-chloramphenicol. **Tracks 1 – 3** show lysates from cell transfected with pδ1.365a-CAT (**track 1**), pδ2.368a-CAT (**track 2**), and pSVO-CAT (**track 3**). Enzyme controls with (**track 5**) or without (**track 4**) purified CAT.

al., 1980) while that with the δ2 gene is greatest with the murine IgM switch region (Nikaido *et al.*, 1981). We observed 154 matches out of 261 nucleotides for δ1 and 77 out of 125 nucleotides for δ2. The computer-derived homology scores were 27.2 for δ1 and 16.7 for δ2. These values indicate the number of standard deviations higher than the mean value for the homology between the δ-crystallin gene sequence and each of the other individual gene sequences examined in the data base.

Transient expression of pδ1.365a-CAT and pδ2.368a-CAT in explanted lens epithelia

We performed functional tests for promoter activity of the 5′-flanking region of δ1 and δ2 by constructing the expression

vectors pδ1.365a-CAT and pδ2.368a-CAT, as shown in Figure 4. pδ1.365a-CAT contains 342 bp and pδ2.368a-CAT contains 349 bp upstream from their respective cap sites in the δ-crystallin genes (see Nickerson and Piatigorsky, 1984). These vectors were used to transfect explanted embryonic chicken lens epithelia (Chepelinsky *et al.*, 1984). The 5′-flanking region of both genes promoted CAT activity in the transfected epithelia (Figure 5). The pSVO-CAT vector, lacking a gene promoter, expressed negligible, if any, CAT activity. The acetylated chloramphenicol spots (CM-Ac-1 and CM-Ac-3) were cut from the chromatogram and assayed for radioactivity in two separate experiments. The δ1 promoter was five times stronger than the δ2 promoter in these tests.

Discussion

The present sequence data establish that the previously reported (Nickerson and Piatigorsky, 1984; Yasuda *et al.*, 1984) full-length δ-crystallin cDNAs were derived from mRNAs encoded by the δ1 gene and not by the δ2 gene. The δ1 cDNA codes for a 48-K δ-crystallin polypeptide, which is the size of the most prevalent δ-crystallin polypeptides in the lens (Reszelbach *et al.*, 1977). Since restriction analysis suggests that all δ-crystallin cDNAs examined so far were derived from the δ1 gene (Jones *et al.*, 1980; Yasuda *et al.*, 1982a, 1984; Nickerson and Piatigorsky, 1984), it seems established that the δ1 gene is much more actively expressed in the embryonic lens than is the δ2 gene. Strong expression of the δ1 gene is consistent with the production of the 48-K δ-crystallin polypeptide by the δ1 gene after microinjection into cultured murine lens cells (Kondoh *et al.* 1983). The presence of nuclear RNA in the lens which hybridizes with an intron probe specific for δ2 (Jones *et al.*, 1981) provides evidence for δ2 transcription in the embryonic chicken lens, but does not show that the δ2 gene produces a functional mRNA. It remains possible that the δ2 gene is expressed differentially at different times of development or after hatching, or in a regionally specific location in the lens (i.e., only in epithelial cells).

Our transfection experiments using the pSVO-CAT plasmid provide direct evidence that the 5′-flanking sequences of the δ1 gene have significantly stronger promoter activity in the cultured lens epithelia than those of the δ2 gene. Possibly the CCAAT sequence and consensus viral core-enhancer-like sequences associated with the δ1 promoter contribute to its relatively high activity. On the other hand, although some evidence implicates the CCAAT box in the regulation of gene expression (Benoist and Chambon, 1981; Grosschedl and Birnstiel, 1980; Mellon *et al.*, 1981; McKnight *et al.*, 1981), altering a number of its residues does not necessarily affect transcription accuracy or efficiency (McKnight and Kingsbury, 1982).

The results of transfection experiments are consistent with the above conclusion that the δ1 gene is more active than the δ2 gene in the embryonic lens. It should be noted, however, that despite their lower promoter strength, the 5′-flanking sequences of the δ1 gene displayed more ability to stimulate transcription than can account for the consistent failure to identify a cDNA derived from a δ2 mRNA in the chicken lens. Post-transcriptional events, such as altered splicing or polyadenylation for example, may limit mRNA formation by the δ2-crystallin gene. Previous experiments using the electron microscope have suggested that the first exon of the δ-crystallin genes is 200 – 300 bp in length (Jones *et al.*, 1980; Yasuda *et al.*, 1982a; Hawkins *et al.*, 1984). The present data show that an intron divides this exon in both genes into a short 5′ exon containing only untranslated sequences and another exon containing mostly 5′-untranslated sequences followed by the putative translation initiation codon. A similar result has been reported recently for the δ1 gene (Yasuda *et al.*, 1984). The marked sequence conservation of the 5′ regions of the two δ-crystallin genes reinforces the idea that they were derived by gene duplication (Jones *et al.*, 1980; Hawkins *et al.*, 1984).

We do not know the meaning of the unexpected homology between the murine immunoglobulin heavy-chain switch regions and intron 1 of the δ-crystallin genes. Possibly these sequences played a role in recombinational events in the evolution of the δ-crystallin genes. Another possibility is that an undescribed δ-crystallin gene rearrangement occurs during development, but there is no evidence to support this idea at the present time.

Finally, the short sequences shared by the chicken δ-crystallin and murine αA-crystallin genes deserve further attention with regard to the expression of these genes. It is also interesting that the direct repeats shared by the two δ genes (DR5 in δ1 and DR4 in δ2) are located in similar relative positions in both genes (exon 1 and intron 1), and that this repeat sequence is also present in the murine αA-crystallin gene (both in intron 1). Our ability to use the pSVO-CAT expression vector to study crystallin promoters, demonstrated here and elsewhere (Piatigorsky *et al.*, 1984; Chepelinksy *et al.*, 1984), will allow us to test whether these and other DNA sequences affect the transcription of the crystallin genes.

Materials and methods

Recombinant plasmids

The 5′ region of the δ1-crystallin gene was derived from a cloned 7.5-kb *Eco*RI fragment (gδCr1) of adult erythrocyte DNA from white Leghorn chickens (Cofal Marek, gs$^-$, SPAFAS, Lancaster, PA) (Bhat *et al.*, 1980). We subcloned this *Eco*RI genomic fragment into pBR322 and called the plasmid pδ1.1 (Figure 1). One *Eco*RI-*Msp*I (0.8-kb) and two *Msp*I (1.3-kb and 0.7-kb) fragments of pδ1.1 were subcloned in the bacterial plasmid pUC9 and grown in *Escherichia coli* JM83 (Vieira and Messing, 1982). These subclones (pδ1.2, pδ1.3 and pδ1.4, see Figure 1) were used to identify the region where transcription is initiated. A 1.3-kb *Pst*I-*Kpn*I fragment was gel purified from pδ1.1 using the technique of Hansen (1981) and sequenced (see below).

The 5′ region of the δ2-crystallin gene was derived from a recombinant bacteriophage library made from the DNA of pooled 6-day-old, embryonic, white Leghorn chickens (line 6, subline 3, US Department of Agriculture, Lansing, MI) (Jones *et al.*, 1980). An 11.9-kb DNA insert was obtained by hybridization to pδCr17, a full-length δ-crystallin cDNA (Nickerson and Piatigorsky, 1984). Southern blot hybridization (Southern, 1975) and restriction mapping indicated that this DNA fragment contained the δ2-crystallin gene (Hawkins *et al.*, 1984). In particular, it contained a portion of a 5.4-kb *Eco*RI fragment which is present only in δ2 and is situated in the 5′ region of the gene (Hawkins *et al.* 1984). This 5.4-kb *Eco*RI fragment was subcloned in pBR322 and called pδ2.1. A 0.8-kb *Hgi*AI fragment from pδ2.1 was subcloned in the *Pst*I site of pUC9 (pδ2.2) and sequenced (see below).

The plasmid pSVO-CAT was obtained from Dr.B.Howard (NCI, NIH, Bethesda, MD) and has been described elsewhere (Gorman *et al.*, 1982). pδ1.368a-CAT and pδ2.365a-CAT were derived by insertion of 368-bp or 365-bp fragments from the promoter region of δ1 or δ2, respectively, into the pSVO-CAT plasmid, as follows. The 600-bp *Dde*I-*Dde*I fragment from the 5′ region of pδ1.1 was subcloned into pUC9; the new plasmid was called pδ1.5 (see Figure 1). The *Pst*I-*Eco*RI fragment from pδ1.5 was polyacrylamide gel-purified, recut with *Dde*I, repurified and cleaved with *Rsa*I; finally, the resulting 368-bp *Rsa*I-*Dde*I fragment was obtained by electroelution from a polyacrylamide gel. The insert of pδ2.2 (see Figure 1) was isolated after cleavage with *Hind*III-*Bam*HI, recut with *Dde*I and the resulting 365-bp *Dde*I-*Dde*I fragment recovered by electroelution. The cohesive ends of the 365-bp δ1 and 368-bp δ2 fragments were blunt-ended with DNA polymerase I Klenow fragment. The pSVO-CAT vector was cleaved with *Hind*III and

filled-in using Klenow fragment. Each fragment was ligated to the pSVO-CAT vector to yield pδ1.365a-CAT and pδ2.368a-CAT, respectively. Each contained the insert in the same transcriptional orientation as in the CAT gene, as judged by restriction analysis using two enzymes for each construct.

Preparation of nucleic acids

Plasmid DNA was prepared by the method of Holmes and Quigley (1981) with the following modifications: STET buffer (8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-HCl pH 8.0) was used to suspend the bacteria and the nucleic acids were treated sequentially with 100 μg/ml of RNase A for 20 min followed by 100 μg/ml of Proteinase K for 30 min, both at 37°C, and finally extracted with phenol:chloroform (1:1) before centrifugation in CsCl containing ethidium bromide.

DNA sequencing

The 1.3-kb *PstI-KpnI* fragment of δ1 was digested with the indicated restriction enzymes and labeled either at the 5′ ends with T4 polynucleotide kinase and γ-labeled [^{32}P]dNTP or at the 3′ ends with an appropriate α-labeled [^{32}P]dNTP and Klenow DNA polymerase (Bethesda Research Laboratories, Gaithersburg, MD); the nucleotide sequence was determined by the method of Maxam and Gilbert (1980). The reaction products were analyzed on 0.4 mm, 8% and 20% polyacrylamide gels containing 8 M urea. The 0.7-kb *MspI* fragment from δ1 (pδ1.4) was subcloned in the phage M13 mp9 (Messing *et al.*, 1981; Messing and Vieira, 1982) and sequenced in both orientations by the dideoxy method of Sanger *et al.* (1977).

The 0.8-kb *HgiAI* fragment from δ2 (pδ2.2) was subcloned in the phage M13 mp9 and sequenced in both directions by the dideoxy method. In addition, the four fragments obtained by *MspI* digestion of the 0.8-kb *HgiAI* fragment of δ2 (pδ2.2) were further subcloned in M13 mp9 and sequenced by the dideoxy method. Sequencing reactions were done using [α-^{35}S]dATP (Amersham, 650 Ci/mmol). The reaction products were subjected to electrophoresis in 8% acrylamide gels (17 cm x 42 cm) containing a salt gradient buffer (Biggin *et al.*, 1983). A constant power of 40 W was used; each gel was loaded twice at a 2 h interval. All gels were examined by autoradiography using Kodak X-OMAT AR film for 20 h at −70°C.

Transfection of cultured lens cells

Whole lens epithelia from 14 day-old chicken embryo lenses were cultured as described in detail elsewhere (Piatigorsky *et al.*, 1984; Chepelinsky *et al.*, 1984). The transfection experiments were performed using 12 explants/35 mm collagen-coated plastic dishes. Calcium phosphate transfections using 10 μg of recombinant DNA were performed as given by Stow and Wilkie (1976), except that the explants were treated with 15% glycerol 4 h after transfection instead of with DMSO. Cultures were transfected 18 h after explantation and harvested 60 h later.

Assay of CAT activity

Chloramphenicol acetyltransferase (CAT) activity was determined as described before (Gorman *et al.*, 1982; Chepelinsky *et al.*, 1984). The tissue was resuspended in 80 μl of 0.25 M sucrose and 0.025 M Tris-HCl at pH 7.5, and homogenized in an Eppendorf tube. Duplicate CAT assays were performed. The assay mixture (in a final volume of 50 μl) contained 7 μl of 12 mM acetyl CoA and 0.4 μCi of [dichloro-acetyl-1,2-^{14}C]chloramphenicol (57.8 mCi/mmol; New England Nuclear Corp.). After 60 min at 37°C, the reaction mixtures were extracted with ethyl acetate as described (Gorman *et al.*, 1982). Control reactions contained *E. coli* CAT obtained from Sigma Chemical Company.

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References

Bagchi,M., Alcala,J.R. and Maisel,H. (1981) *Exp. Eye Res.*, **32**, 251-254.
Banerji,J., Olson,L. and Schaffner,W. (1983) *Cell*, **33**, 729-740.
Benoist,C. and Chambon,P. (1981) *Nature*, **290**, 304-315.
Bhat,S.P., Jones,R.E., Sullivan,M.A. and Piatigorsky,J. (1980) *Nature*, **284**, 234-237.
Biggin,M.D., Gibson,T.J. and Hong,G.F. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3963-3965.
Bloemendal,H. (1977) *Science (Wash.)*, **197**, 127-138.
Brahma,S.K. and van der Starre,H. (1976) *Exp. Cell Res.*, **97**, 175-183.
Breathnach,R. and Chambon,P. (1981) *Annu. Rev. Biochem.*, **50**, 349-383.
Brutlag,D.L., Clayton,J., Friedland,P. and Kedes,L. (1982) *Nucleic Acids Res.*, **10**, 279-294.
Chepelinsky,A.B., King,C.R., Zelenka,P.S. and Piatigorsky,J. (1984) *Proc. Natl. Acad. Sci. USA*, in press.
Clayton,R.M. (1974) in Davson,H. and Graham,L.T. (eds.), *The Eye*, Vol. **5**, Academic Press, NY/London, pp. 399-494.
de Jong,W.W. (1981) in Bloemendal,H. (ed.), *Molecular and Cellular Biology of the Eye*, Wiley, NY, pp. 221-387.
Davis,M.M., Kim,S.K. and Hood,L.E. (1980) *Science (Wash.)*, **209**, 1360-1365.
Efstratiadis,A., Posakony,J.W., Maniatis,T., Lawn,R.M., O'Connell,C., Spritz,R.A., DeRiel,J.K., Forget,B.G., Weissman,S.M., Slighton,J.L., Blechl,A.E., Smithies,O., Baralle,F.E., Shoulders,C.C. and Proudfoot, N.J. (1980) *Cell*, **21**, 653-668.
Genis-Galvez,J.M., Maisel,H. and Castro,J. (1968) *Exp. Eye Res.*, **7**, 593-602.
Gillies,S.D., Morrison,S.L., Oi,V.T. and Tonegawa,S. (1983) *Cell*, **3**, 717-728.
Gorman,C.M., Moffat,L.F. and Howard,B. (1982) *Mol. Cell Biol.*, **2**, 1044-1051.
Grosschedl,R. and Birnstiel,M.L. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 1432-1436.
Gruss,P. (1984) *DNA*, **3**, 1-5.
Hansen,J.N. (1981) *Anal. Biochem.*, **116**, 146-151.
Harding,J.J. and Dilley,K.J. (1976) *Exp. Eye Res.*, **22**, 1-73.
Hawkins,J.W., Nickerson,J.M., Sullivan,M.A. and Piatigorsky,J. (1984) *J. Biol. Chem.*, **259**, 9821-9825.
Holmes,D.S. and Quigley,M. (1981) *Anal. Biochem.*, **114**, 193-197.
Inana,G., Piatigorsky,J., Norman,B., Slingsby,C. and Blundell,T. (1983) *Nature*, **302**, 310-315.
Jones,R.E., Bhat,S.P., Sullivan,M.A. and Piatigorsky,J. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5879-5883.
Jones,R.E., DeFeo,D. and Piatigorsky,J. (1981) *J. Biol. Chem.*, **256**, 8172-8176.
King,C.R. and Piatigorsky,J. (1983) *Cell*, **32**, 707-712.
Kondoh,H., Yasuda,K. and Okada,T.S. (1983) *Nature*, **301**, 440-442.
Kozak,M. (1983) *Microbiol. Rev.*, **47**, 1-45.
Lok,S., Tsui,L., Shinohara,T., Piatigorsky,J., Gold,R. and Breitman,M. (1984) *Nucleic Acids Res.*, **12**, 4517-4529.
Maizel,J.V. and Lenk,R.P. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7665-7669.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499-560.
McAvoy,J.W. (1978) *J. Embryol. Exp. Morphol.*, **45**, 271-281.
McKnight,S.L. and Kingsbury,R. (1982) *Science (Wash.)*, **217**, 316-324.
McKnight,S.L., Gavis,R., Kingsbury,R. and Axel,R. (1981) *Cell*, **25**, 385-398.
Mellon,P., Parker,V., Gluzman,Y., Maniatis,T. (1981) *Cell*, **27**, 279-288.
Messing,J., Crea,R. and Seeburg,P.H. (1981) *Nucleic Acids Res.*, **9**, 309-321.
Messing,J. and Vieira,J. (1982) *Gene*, **19**, 269-276.
Moormann,J.M., Den Dunnen,J.T., Mulleners,L., Andreoli,P., Bloemendal,H. and Schoenmakers,J.G.G. (1983) *J. Mol. Biol.*, **171**, 353-368.
National Institute of Health (1983) Genetic Sequence Databank: Genbank (Research System Div., Bolt, Baranek, and Newman, Boston).
Nickerson,J.M. and Piatigorsky,J. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 2611-2615.
Nikaido,T., Nakai,S. and Honjo,T. (1981) *Nature*, **292**, 845-848.
Nordheim,A. and Rich,A. (1983) *Nature*, **303**, 674-679.
Papaconstantinou,J. (1967) *Science (Wash.)*, **156**, 338-346.
Piatigorsky,J. (1981) *Differentiation*, **19**, 134-153.
Piatigorsky,J. (1984) *Mol. Cell. Biochem.*, **59**, 33-56.
Piatigorsky,J., Webster,H. de F., Craig,S.P. (1972) *Dev. Biol.*, **27**, 176-189.
Piatigorsky,J., Chepelinsky,A.B., Hejtmancik,J.F., Borrás,T., Das,G.C., Hawkins,J.W., Zelenka,P.S., King,C.R., Beebe,D.C. and Nickerson,J.M. (1984) *Molecular Biology of Development, Cetus-UCLA Symposium*, Alan R.Liss, Inc., pp. 331-349.
Queen,C. and Baltimore,D. (1983) *Cell*, 741-748.
Rabaey,M. (1962) *Exp. Eye Res.*, **1**, 310-316.
Reszelbach,R., Shinohara,T. and Piatigorsky,J. (1977) *Exp. Eye Res.*, **25**, 583-593.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467.
Shinohara,T. and Piatigorsky,J. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 2808-2812.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503-517.
Stow,N.D. and Wilkie,M.N. (1976) *J. Gen. Virol.*, **33**, 447-458.
Treton,J.A., Shinohara,T. and Piatigorsky,J. (1982) *Dev. Biol.*, **92**, 60-65.
Vieira,J. and Messing,J. (1982) *Gene*, **19**, 259-268.
Weiher,H., Konig,M. and Gruss,P. (1983) *Science (Wash.)*, **219**, 626-631.
Wilbur,W.J. and Lipman,D.J. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 726-730.

Williams,L.A. and Piatigorsky,J. (1979) *Eur. J. Biochem.*, **100**, 349-357.
Yasuda,K., Kondoh,H., Okada,T.S., Nakajima,N. and Shimura,Y. (1982a) *Nucleic Acids Res.*, **10**, 2879-2891.
Yasuda,K., Kondoh,H., Okazaki,K., Shimura,Y. and Okada,T.S. (1982b) *Fifth International Congress of Eye Research*, Oct. 3-8, Eindhoven, The Netherlands, p. 38 (abstr.).
Yasuda,K., Nakajima,N., Isobe,T., Okada,T.S. and Shimura,Y. (1984) *EMBO J.*, **3**, 1397-1402.
Yoshida,K. and Katoh,A. (1971) *Exp. Eye Res.*, **11**, 184-194.
Zwaan,J. and Ikeda,A. (1968) *Exp. Eye Res.*, **7**, 301-311.

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