Lens-specific enhancer in the third intron regulates expression of the chicken $\delta 1$ -crystallin gene

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We have previously shown that the tissue specificity determinant of the chicken $\delta 1$ -crystallin gene lies 3' of position -100 (Hayashi et al. 1985). Since the promoter of the gene ($\delta 1$ -crystallin promoter) did not show any tissue specificity, we examined various segments of the $\delta 1$ -crystallin gene for a tissue-specific enhancer activity by placing each segment downstream of a heterologous transcriptional unit coding for chloramphenicol acetyltransferase (CAT) and by transfecting chicken tissues in primary culture. We found that a segment spanning the third intron bears a strong lens-specific enhancer activity. This " $\delta 1$ -crystallin enhancer" activates transcription from the $\delta 1$ -crystallin promoter 20- to 40-fold in lens cells and to various degrees with other promoters. Deletion analysis of the enhancer region indicated that it covered nearly 1 kb but did not indicate clear-cut boundaries. For its enhancer effect the core region of 120 bp and associations with certain adjoining regions were required. Removal of the enhancer from the gene totally abolished $\delta 1$ -crystallin expression, and reinsertion of the enhancer in either upstream, internal, or downstream positions restored expression. We conclude that the $\delta 1$ -crystallin enhancer is an essential and major determinant for lens-specificity of $\delta 1$ -crystallin expression.

[Key Words: δ -Crystallin; tissue-specific enhancer; CAT assay; transfection]

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Since the initial observations that tissue-specific genes are preferentially expressed in the homologous cell type after molecular cloning and reintroduction into cultured cells (Renkawitz et al. 1982; Banerji et al. 1983; Gillies et al. 1983; Kondoh et al. 1983; Queen and Baltimore 1983; Walker et al. 1983), a number of efforts have been made using gene transfer techniques to identify and localize the DNA elements responsible for tissue-specific expression of the gene and to elucidate the molecular events taking place on the elements. Evidence provided by experiments along this line indicated that the nature of the elements for the tissue-specific regulation may be variable depending on the particular gene studied. However, the major regulatory control occurs at the transcriptional level.

We have focused on the δ -crystallin gene regulation (for review, see Kondoh et al. 1986). Differentiation of ocular tissues is one of the most conspicuous events in early embryogenesis, and the δ -crystallin gene(s) is the earliest among lens-specific crystallin genes to be turned on during ocular lens differentiation (for review, see Pia-

tigorsky 1981). δ -Crystallin is also the most abundantly synthesized class of crystallin in the embryonic period of the chicken, but its synthesis is turned off in the posthatching period (for review, see Piatigorsky 1984). Other crystallin classes follow quite different and unique time courses of expression. Molecular cloning analysis of the gene and the transcripts indicated that two nonallelic genes exist for δ -crystallin (Nickerson et al. 1985, 1986; Ohno et al. 1985), but only one of them, the $\delta 1$ -crystallin gene, is in fact active (Nickerson and Piatigorsky 1984; Yasuda et al. 1984; Borras et al. 1985).

Introduction of the molecular-cloned $\delta 1$ -crystallin gene into mouse cells in primary culture (Kondoh et al. 1983, 1986) and into transgenic mice (Kondoh et al. 1987) resulted in proper regulation of the gene with respect to tissue specificity, indicating that DNA elements essential for lens-specific gene regulation are in fact located within the introduced DNA sequence. We have also shown that the region upstream of position -100 is not necessary for lens-specific expression (Hayashi et al. 1985). Therefore, we examined various parts of the $\delta 1$ -crystallin gene to the 3' side of -100 for participation in tissue-specific expression. As we report here, we have discovered that a lens-specific enhancer element exists in the third intron of the $\delta 1$ -crystallin gene. Experimental evidence indicates that this enhancer element is the major determinant for lens specificity of $\delta 1$ -crystallin expression.

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Figure 1. Structure of the plasmids bearing the CAT gene. Boxes and lines indicate transcribed and nontranscribed sequences, respectively. (A) pSV0-CAT (Gorman et al. 1982) drawn as a linear form. Solid, stippled, and open boxes indicate CAT-coding sequence, CAT-flanking bacterial sequence, and SV40-derived intron poly(A) addition sequences, respectively, which altogether constitute a transcriptional unit. Thick and thin lines indicate untranscribed sequences of SV40 and pBR322 origin, respectively. Various promoters or enhancer-promoter complexes shown in B were inserted into the *Hind*III site (H) and $\delta 1$ -crystallin segments shown in Fig. 2 into the *Bam*HI site (B) to test for an enhancer activity. (B) Promoter and enhancer-promoter complexes to construct the plasmids. p δ CAT carries the region -459 bp to $+57$ bp of the $\delta 1$ -crystallin gene [Nickerson et al. 1985; Ohno et al. 1985; Hayashi and Kondoh 1986], shown to be sufficient for full promoter activity [Borrás et al. 1985; Hayashi et al. 1985; Das and Piatigorsky 1986; Hayashi et al. 1985; K. Goto, S. Hayashi, Y. Ueda, and H. Kondoh, in prep.]. The 57-bp-long transcribed sequence includes the 5' terminus of exon I at position $+35$, but this site is probably not used for the splicing reaction because joining the CAT sequence within exon I at position $+23$ did not significantly affect CAT expression. ptkCAT carries the region -197 to $+56$ of the HSV-tk gene [McKnight 1980]. pMoCAT carries the env-LTR region of MLV [from -1067 to $+30$ relative to the cap site (Shinnick et al. 1981)]. Solid wedges represent viral enhancers, and the open triangle indicates the 5' boundary of MLV LTR. pMR-CAT was constructed by removing two of the *Eco*RV fragments from pMoCAT. pSV2-CAT and pSV1-CAT are described by Gorman et al. (1982). Left termini of open boxes indicate transcriptional initiation sites. Restriction sites: (B) *Bam*HI, (Bg) *Bgl*II, (Bs) *Bst*EII, (H) *Hind*III, (Hp) *Hpa*I, (P) *Pvu*II, (R) *Eco*RV, (Sm) *Sma*I, (Sp) *Sph*I, (X) *Xba*I.

Results

$\delta 1$ -Crystallin promoter is not tissue specific

Our first attempt was to examine whether the $\delta 1$ -crystallin promoter itself bears tissue specificity. To this end, we linked the $\delta 1$ -crystallin promoter to the CAT coding sequence on the vector pSV0-CAT (Gorman et al. 1982) to create p δ CAT. We similarly constructed CAT coding genes, ptkCAT, pMR-CAT, and pSV1-CAT bearing promoters of the herpes simplex virus (HSV) thymidine kinase (tk) gene, Moloney murine leukemia virus (MLV) LTR and SV40 early gene, respectively (Fig. 1). The $\delta 1$ -crystallin sequence used to construct p δ CAT, i.e. -459 to $+57$, contained a surplus of about 350 bp in addition to the essential promoter sequence defined as being within 100 bp 5' of the transcription initiation site [Borrás et al. 1985; Hayashi et al. 1985; Das and Piatigorsky 1986]. However, this surplus sequence does not significantly contribute either to promoter activity or to specificity of gene expression as assayed in mouse cells [Hayashi et al. 1985] and in chicken cells [K. Goto, S. Hayashi, Y. Ueda, and H. Kondoh, in prep.]. To construct

viral promoter-driven CAT genes, known enhancer sequences were removed so that we could evaluate basic promoter activities.

We transfected these CAT coding genes to chicken lens epithelial cells (LE) and dermal fibroblasts (FB) from 15-day embryo, and compared CAT activities 24 hr after transfection. The results of one experiment are summarized in Table 1, columns 3 and 6 (with "none" enhancer). The CAT genes with different promoters were expressed to a comparable level in each cell type, and expression in LE culture was three- to fivefold higher than in FB culture (Table 1, legend). This consistent difference in CAT expression between the cell types appears primarily due to higher transfection efficiency of lens epithelial cells. Immunostaining of the cultures transfected with ptkCAT-SV (see Fig. 3, below) using anti-CAT antibodies revealed that 5.7% and 1.5% of LE and FB populations, respectively, expressed CAT. Essentially the same results were obtained in repeated experiments using different sets of cultures and different plasmid preparations. Thus, the $\delta 1$ -crystallin promoter was similar to viral promoters in strength and in nonse-

lectivity of cell type. We conclude from this observation that the $\delta 1$ -crystallin promoter itself is not tissue specific.

Third intron of $\delta 1$ -crystallin gene bears a lens-specific enhancer activity

Although the $\delta 1$ -crystallin promoter is nonspecific, the $\delta 1$ -crystallin gene is regulated in a lens-specific manner in cultured mouse cells (Kondoh et al. 1983; Hayashi et al. 1985; Kondoh et al. 1986) and in transgenic mice (Kondoh et al. 1987). Therefore, assuming that transcriptional regulation is the basis for lens-specificity, we suspected the presence of a control element that is separate from the promoter and functions in a way analogous to viral enhancers (Serfling et al. 1985). To detect such a putative element, we cleaved the $\delta 1$ -crystallin gene into segments of 1–2 kb by restriction enzyme digestion such that altogether the segments span the entire length of the gene (Fig. 2A). Each segment was inserted downstream of the tkCAT gene at the *Bam*HI site (Fig. 1) in the plus orientation (see Fig. 2, legend) and assayed for expression of tkCAT on the plasmid by transfecting LE and FB cells. The results are shown in Figure 2B. A significant activation (20- to 40-fold) of tkCAT expression was reproducibly observed only when the segment B-bearing plasmid was transfected to LE culture. A slight activating effect (1.5- to 2-fold) by segment E was also observed, but this effect was not cell type specific. Larger non-B segments (H and I, including A–B and B–C boundaries) did not activate tkCAT gene expression in either cell type. Segment B slightly activated expression in FB (Fig. 2), hepatocytes, gizzard smooth muscle, and brain cells (data not shown), but the activation in these cells was no higher than twofold. Thus, segment B appears to activate expression of the neighboring tkCAT gene in a manner basically specific for LE cells. The same extent of activation in LE was also observed with normal (+) and inverted (–) orientation of segment B (Fig. 2).

We compared segment B with the SV40 enhancer placed in the same *Bam*HI site to confirm cell type specificity of the effect. As shown in Figure 3 (lanes 3 and 12), the SV40 enhancer augmented expression of tkCAT

in both LE and FB cells, indicating a nonspecific enhancing effect (rate measurement indicated more than 40-fold, see Table 1), in sharp contrast to the effect of $\delta 1$ -crystallin segment B which was lens specific (Fig. 3, lanes 2 and 11). Thus, segment B exerted an enhancer effect on the heterologous gene in both orientations in a lens-specific manner. The putative tissue-specific enhancer element(s) present in segment B is referred to as the $\delta 1$ -crystallin enhancer.

The effect of the $\delta 1$ -crystallin enhancer was not limited to tkCAT expression (Fig. 3; Table 1). When segment B was placed at the same *Bam*HI site of p δ CAT, pMR-CAT, and pSV1-CAT, CAT expression was activated, although to different degrees. $\delta 1$ -Crystallin enhancer activated δ CAT expression in LE to an extent comparable to tkCAT with experimental variation between 20- and 40-fold. S1 nuclease analysis indicated that this was due to increased transcription from the correct initiation site (data not shown). The effect of the $\delta 1$ -crystallin enhancer on pMR-CAT was in the range of a 10- to 16-fold activation. Increasing expression from the MLV promoter threefold with its authentic enhancer (pMoCAT) or lowering expression fivefold by removing the *Eco*RV–*Xba*I region (pMX-CAT) (see Laimins et al. 1984) did not significantly alter the relative activation rate caused by the $\delta 1$ -enhancer (10–16-fold). The effect on expression from the SV40 early promoter was much less dramatic with a two- to sixfold activation. This range of activation by the $\delta 1$ -crystallin enhancer was also observed with pSV2-CAT by superposing its effect on the 20-fold activation caused by the SV40 enhancer. In any case, the $\delta 1$ -crystallin enhancer exerted its effect only in LE and not significantly in FB, in sharp contrast to the SV40 enhancer which functioned in both cell types (Table 1).

$\delta 1$ -Crystallin enhancer is essential for $\delta 1$ -crystallin expression

Enhancer activity of fragment B was discovered by its effect on the expression of the heterologous CAT gene. To see whether the activity is essential for $\delta 1$ -crystallin gene expression, we deleted a region between position +1409 (*Eco*RV site in the third exon) and +3273 (*Bss*HII

Table 1. CAT expression driven by various promoters

Promoter	(Plasmid)	LE			FB		
		Enhancer			Enhancer		
		none	δ	SV	none	δ	SV
None	(pSV0-CAT)	<0.1	—	—	<0.1	—	—
δ	(p δ CAT)	1.1	20.6 (18.7) ^a	53 ^b (48 ^b)	0.6	1.4 (2.3)	54 ^b (90 ^b)
tk	(ptkCAT)	1.0	19.7 (19.7)	50 ^b (50 ^b)	1.0	1.1 (1.1)	140 ^b (140 ^b)
MLV	(pMRCAT)	0.2	2.5 (12.5)	—	0.3	0.2 (0.6)	—
SV40	(pSV1-CAT)	0.8	5.0 (6.2)	16.1 (20.1)	0.4	0.5 (1.5)	16.2 (46.3)

A set of data from a single experiment. CAT expression in each cell type was normalized to that of ptkCAT. Transfection with ptkCAT of LE and FB yielded CAT activity of 170 and 36 pmole/hr per dish, respectively. Each plasmid was examined in at least three different experiments, which gave results essentially the same as this one but with some variation of enhancer effects as described in the text. (LE) Chicken lens epithelial cells; (FB) dermal fibroblasts.

^a The values in parentheses indicate multifold activation of CAT expression by the enhancer.

^b The values are underestimated since conversion of [¹⁴C]chloramphenicol to acetylated forms exceeded 60%, in contrast to less than 30% in other cases (see Materials and methods).

Figure 2. Examination of various segments of the δ 1-crystallin gene for enhancer activity. (A) δ 1-crystallin gene and its segments. The gene is drawn with its 5' end to the left. Exons are indicated by boxes, introns and flanking sequences by lines. (B) Effect of δ 1-crystallin segments inserted at the *Bam*HI site on tkCAT expression. An autoradiogram to show the extent of acetylation of 14 C-chloramphenicol. Positions of the 1-, 3-, and 1,3-acetylated forms are indicated. The segments at the *Bam*HI site are those shown in A; (-) no insertion. Orientation: (+) polarity of the segment relative to the direction of transcription is the same as found in the δ 1-crystallin gene; (-) reverse orientation. Reproducibility of relative CAT activity was confirmed in at least three separate experiments for each plasmid.

site in the sixth exon) from the δ 1-crystallin gene on p δ C1C-107 (which carried the 5'-flanking sequence of 107 bp). The remaining gene sequences were rejoined so as to restore the same open reading frame as in the intact gene (called p δ C1E). The internal deletion in p δ C1E was designed to remove segment B and to reduce simultaneously the size of the encoded polypeptide from 50 kD to 39 kD so that exogenous δ -crystallin expression could be assessed in chicken LE cells. We also constructed pRS δ C1E in which the enhancer-promoter complex of RSV LTR is placed at -234 bp from the δ C1E initiation site to force δ C1E expression to occur.

We transfected p δ C1E and pRS δ C1E into LE and FB cells and analyzed the products by immunoblotting (Fig. 4). By transfection of pRS δ C1E, the 39-kD polypeptide was synthesized in both LE and FB cells at comparable efficiencies (Fig. 4B, lanes 2 and 7). Therefore, the δ C1E gene was expressed nonspecifically under direction of the RSV LTR. In contrast, p δ C1E did not express any detectable amount of the 39-kD polypeptide (Fig. 4B, lanes 3 and 8), even though 107 bp of the 5'-flanking sequence sufficient for promoter function was present (Hayashi et al. 1985). This indicated that a region essential for expression other than the δ 1-crystallin promoter

Figure 3. Effect of δ 1-crystallin (δ) and SV40 (SV) enhancers on expression of tkCAT, δ CAT, and SV1-CAT genes. The enhancer elements were placed in + orientation at the *Bam*HI site shown in Fig. 1 and transfected to LE and FB. ptkCAT with SV40 enhancer (ptkCAT-SV), which expressed the highest CAT activity in both cell types, was used to estimate the transfection efficiency of cultures by immunohistology.

Figure 4. Effect of $\delta 1$ -crystallin enhancer on expression of enhancer-deleted $\delta 1$ -crystallin gene. (A) Structure of the plasmid used. Exons are indicated by boxes, introns, and other chicken sequences by thick lines, enhancer-containing segment B by a thin line flanked by open boxes, vector sequences by thin lines, RSV LTR by an arrowhead. Relevant restriction sites are indicated: (Pv) *PvuI*; (R) *EcoRV*; (Bss) *Bss*HIII; (Xb) *XbaI*; (Xh) *XhoI*. In the lowermost diagram, three different insertion positions for segment B are indicated with notation of the gene construct. (B,C) Immunoblot analysis of the products of the gene constructs shown in A transfected to LE and FB cells. The cell type and transfected plasmid are indicated above the lanes. (–) Mock transfection. 50k and 39k are positions of endogenous δ -crystallin in LE and the product of the δ C1E gene, respectively. Other bands also originate from molecular microheterogeneity of endogenous δ -crystallin (Piatigorsky 1984; Y. Ueda, in prep.). In repeated experiments with segment B at the *PvuI* (upstream) or *XhoI* (downstream) sites, we sometimes observed a higher activation by + orientation, as shown here, but in other experiments activation was comparable between the two orientations.

was missing from p δ C1E. Placement of the $\delta 1$ -crystallin enhancer (segment B) at the *XhoI* site located about 1.2 kb downstream of the poly(A) addition site of p δ C1E elicited expression in LE irrespective of its orientation (Fig. 4B, lanes 4 and 5), but not in FB (lanes 9 and 10). This LE-specific activation of the δ C1E gene by the $\delta 1$ -crystallin enhancer was also observed when the enhancer was inserted at other locations of the gene, upstream [*PvuI* site, –234; Fig. 4C, lanes 5 and 6] or in the middle of an intron [*XbaI* site, +3339; Fig. 4C, lanes 7 and 8]. The lower activation seen with the $\delta 1$ -crystallin enhancer in the intron in its plus orientation (Fig. 4C, lane 7) may be due to introduction of extra donor and

acceptor sites for transcript splicing in the intron or due to an altered stability of the nascent transcript.

Dissection of $\delta 1$ -crystallin enhancer

Segment B (+1409 to +2480), which includes the $\delta 1$ -crystallin enhancer, spans the entire region of the third intron (924 bp long) and extends beyond boundaries to exons 3 and 4. To delimit the sequence responsible for the enhancer activity, we examined various parts of segment B. Restriction sites *ScaI* (+1555), *HgiAI* (+1861), *BamHI* (+1960), *NcoI* (+2080), and *PstI* (+2205) were used to define subsegments of B as shown in Figure 5.

Figure 5. Dissection of δ 1-crystallin enhancer. Boxes and bar on top indicate exon and intron sequences, respectively, of segment B. Segment B was divided into subsegments B1–B6, and the fragments containing various subsegments indicated by horizontal bars were inserted into the *Bam*HI site of ptkCAT in either plus or minus orientations and transfected to LE and FB cells to assess their enhancer activity. Relative CAT expression is shown as multifold activation of tkCAT expression and is the average of the number of experiments indicated in parentheses. Restriction sites: (B) *Bam*HI; (Hg) *Hgi*AI; (Nc) *Nco*I; (Ps) *Pst*I; (R) *Eco*RV; (Sc) *Sca*I.

Various fragments consisting of sets of the subsegments were prepared and placed at the *Bam*HI site of ptkCAT.

When the enhancer effect of the fragments in the plus orientation was examined, full enhancer activity (>20-fold activation) in LE cells was observed if the fragment spanned at least three contiguous subsegments to include subsegment B4 (Fig. 5, lines 1–5). Fragments made of B4 and an additional subsegment B5 (line 6) exhibited a moderate enhancer activity (~10-fold activation), but B4 alone or B3 plus B4 did not show a significant enhancer activity (lines 7 and 8). On the other hand, no fragments devoid of B4 exhibited enhancer activity (lines 9–14), and, in fact, removal of B4 from a fragment with full enhancer activity totally abolished the enhancer activity (compare lines 2 vs. 9 and 3 vs. 11).

With regard to the enhancer effect in the minus orientation, the same dependence of the effect on B4 was observed, but the results were slightly different in that a full enhancer activity required the presence of B2 as well. Fragments B3-B4-B5 (line 4) and B4-B5-B6 (line 5), which exhibited full enhancer activity in the plus orientation, elicited only a partial activity in the minus orientation. It is to be noted that the effect of B3-B4-B5 and B4-B5-B6 in the minus orientation was comparable to the effect of B4-B5 (line 6), which did not depend on the orientation.

When the same set of plasmids was transfected to FB cells, a slight enhancing effect (sixfold at most) of tkCAT expression was reproducibly observed with certain fragments, often in an orientation-dependent manner. This effect was observed only with plasmids carrying a B2- and/or B3-containing fragment, but was

independent of subsegment B4 and hence likely to be different from that observed in LE cells (Fig. 5).

In summary, subsegment B4 constitutes an essential core for the lens-specific δ 1-crystallin enhancer. However, B4 by itself was not enough for enhancer activity and required association with additional subsegments for the activity. Association of the neighboring subsegment B5 partially restored the enhancer activity, and association of B2 plus B3, B3 plus B5, or B5 plus B6 was sufficient for maximum enhancer activity in the plus orientation. Full enhancer activity in the minus orientation required the B2 subsegment as well.

Discussion

In this report, we have demonstrated that the third intron of the δ 1-crystallin gene contains a lens-specific enhancer, δ 1-crystallin enhancer. This was the only region to have significant enhancer activity in the δ 1-crystallin gene and its flanking sequences spanning a total of 13.2 kb. The δ 1-crystallin enhancer activated heterologous genes from a remote position, independent of its orientation and in a lens-specific manner. It was established that the δ 1-crystallin enhancer activated transcription from the correct initiation site of the δ 1-crystallin promoter. The δ 1-crystallin enhancer was in fact essential for δ 1-crystallin expression, since removal of the enhancer from the gene totally abolished δ 1-crystallin expression, and readdition of the element in upstream, internal, or downstream positions restored the expression. Since we confirmed that the δ 1-crystallin enhancer exerted its lens-specific effect also in mouse

cells (K. Goto, S. Hayashi, and H. Kondoh, unpubl.), this enhancer effect would account for previous observations that the chicken $\delta 1$ -crystallin gene was correctly regulated in cultured mouse cells (Kondoh et al. 1983; Hayashi et al. 1985; Kondoh et al. 1986) and in transgenic mice (Kondoh et al. 1987).

These results clearly indicate that the $\delta 1$ -crystallin enhancer is the major determinant of tissue specificity in $\delta 1$ -crystallin expression. However, there may be subsidiary mechanisms in the $\delta 1$ -crystallin gene to tighten the tissue specificity of expression. The $\delta 1$ -crystallin enhancer effects a 20- to 40-fold activation of CAT expression which takes place, albeit at a low level, in its absence. On the other hand, expression of the $\delta 1$ -crystallin gene itself appears more dependent on the $\delta 1$ -crystallin enhancer and is more tissue specific. Expression of an enhancer-deleted gene ($\delta C1E$) in LE cells became detectable only after readdition of the $\delta 1$ -crystallin enhancer, and expression in FB cells was not detectable irrespective of the condition of the enhancer (Fig. 4B). This highly stringent effect of the $\delta 1$ -crystallin enhancer on $\delta 1$ -crystallin expression cannot be accounted for solely by the difference in sensitivity of the assay methods, since a 40-fold reduction of expression seen with $p\delta C1E$ -*Xho*⁺ was still in the detectable range for immunoblotting.

Analysis of the activity of subsegments of the $\delta 1$ -crystallin enhancer region indicates that the $\delta 1$ -crystallin enhancer is not a single element with clear boundaries but is made up of unequal elements, some of which are functionally redundant. Subsegment B4 constitutes an essential core for the lens-specific activity of the $\delta 1$ -crystallin enhancer, since any enhancer fragment having an activity in LE cells contained B4, but B4 alone is not sufficient for any enhancer effect. B4–B5 is the minimum enhancer fragment to show some activity, and the addition of neighboring subsegments augmented the enhancer effect, which saturates with about a 30-fold activation of CAT expression. The regions neighboring the enhancer core thus appear to assist the lens-specific enhancer effect generated by the core, since they themselves fail to show a significant enhancer effect in LE cells. However, the fragments containing subsegment B2 and/or B3, irrespective of the presence of the core region, often showed a weak but appreciable augmenting effect in FB cells (Fig. 5). This result may indicate that the $\delta 1$ -crystallin enhancer contains nonspecific elements, represented by activity in FB, which in concert with lens-specific elements generate a high activating effect in LE cells.

Comparison of the nucleotide sequence of the $\delta 1$ -crystallin enhancer region with those of known enhancers of viral and cellular origins did not indicate any significant homology. Instead, we found a repeated appearance of the sequence ACAAT (and its counterstrand homolog ATTGT) within the $\delta 1$ -crystallin enhancer region (Fig. 6A). The ACAAT sequence occurs randomly (on average once in 500 bp) in other parts of the $\delta 1$ -crystallin gene and in other genes including α - and γ -crystallins, but it occurs 12 times in the third intron, at least once in each of subsegments B1–B5, and most significantly, it is con-

centrated in the region that crosses the boundary between subfragments B4 (core) and B5 at a frequency of 7 repeats per 140 bp (the expected value for this occurrence is 10^{-4}) (Fig. 6B). It is interesting to note that palindromic nucleotide sequences bracket the ACAAT-rich core region (Fig. 6B). Experimental evidence for involvement of the ACAAT sequence in the enhancer function remains to be provided. However, strong correlation between its occurrence and contribution of the subsegment to the lens-specific enhancer activity indicates the possibility that ACAAT sequences provide sites for tissue-specific interactions between nuclear transcription factors and the $\delta 1$ -crystallin gene. Indeed, there is a precedent where a repetitive short sequence is part of a regulatory element, as was found in the upstream region of the β -interferon gene (Fujita et al. 1985). As we have previously reported (Kondoh et al. 1987), parts of subsegments B1 and B2 have a certain degree of homology to a middle-repetitive sequence found in an intron of the rat growth hormone gene (Barta et al. 1981; Page et al. 1981). However, ACAAT is not found in the repetitive sequence.

An additional, a nonallelic gene for $\delta 1$ -crystallin, $\delta 2$, is located 4 kb downstream of the $\delta 1$ -crystallin gene (Piatigorsky 1984; Nickerson et al. 1986). However, cumulative evidence indicates that the $\delta 2$ -crystallin gene is scarcely expressed in the chicken lens (Piatigorsky 1984) in spite of a gene organization similar to that of the $\delta 1$ -crystallin gene and in spite of its coding potential (Nickerson et al. 1986). Comparison of nucleotide sequences indicated that high homologies lie in the exons and some of the introns, including the third intron where the $\delta 1$ -crystallin enhancer is located. Especially, the region from +1812 to +2248 of the $\delta 1$ -crystallin enhancer, covering the subsegments B3–B5 and sufficient for full enhancer activity (Fig. 5), is highly conserved in the $\delta 2$ -crystallin gene with only a single transitional nucleotide exchange at position +1886. It is thus likely that the third intron of the $\delta 2$ -crystallin gene also bears a lens-specific enhancer activity and hence that the defect of $\delta 2$ -crystallin gene expression is to be ascribed to other factors. In fact, Borrás et al. (1985) have shown that the promoter of the $\delta 2$ -crystallin gene is significantly weaker than that of $\delta 1$ -crystallin gene.

Tissue-specific enhancer elements have been found in the vicinity of a number of genes: immunoglobulin genes (Banerji et al. 1983; Gillies et al. 1983; Queen and Baltimore 1983; Picard and Schaffner 1984; Queen and Stafford 1985), the HLA E β gene (Gillies et al. 1984), pancreas-specific genes (Edlund et al. 1985; Boulet et al. 1986), pituitary-specific genes (Nelson et al. 1986), liver-specific genes (Ciliberto et al. 1985; Godbout et al. 1986; Muglia and Rothman-Denes 1986), and the lysozyme gene (Theisen et al. 1986). With the exception of the immunoglobulin genes, tissue-specific enhancers were located 5' of the transcribed region. The $\delta 1$ -crystallin enhancer provides a second exception. However, since the most routinely employed strategy in the search for regulatory elements of gene expression is to fuse the 5'-flanking sequence to a heterogeneous coding sequence, such as the CAT gene, intragenic control elements may

Figure 6. (A) High-frequency occurrence of ACAAT sequence in the δ 1-crystallin enhancer region. Positions of ACAAT in the coding and noncoding strands are indicated on the δ 1-crystallin gene map by downward and upward arrowheads, respectively. Exons are indicated by boxes. Base sequence data used are from Ohno et al. (1985) and Nickerson et al. (1985). (B) Nucleotide sequence of the enhancer core and surrounding region. Positions of nucleotides are from the transcriptional initiation site. ACAAT and complementary ATTGT are boxed. Note that ACAAT-related sequences AC(A/T)(A/T)(A/T) also occur six times in this region. Occurrences of palindromic sequences are indicated by converging arrows in which noncomplementarity of the nucleotides is indicated by thin lines and presence of an extranucleotide by a gap.

have been overlooked in many cases. The δ 1-crystallin enhancer sequence shows no significant homology with any of the above-mentioned enhancers or with viral enhancers, but shares some general features with immunoglobulin enhancers. For instance, immunoglobulin enhancers also have a core structure plus associated sequences (Banerji et al. 1983; Queen and Stafford 1984), and in addition, they are made of tissue-specific as well as nonspecific components which interact with respective nuclear factors (Mercola et al. 1985; Sen and Baltimore 1986).

Among other crystallins, α _A-crystallin of mouse and chicken and γ -crystallin of mouse have been studied for expression of the cloned gene in cultured cells (Chepelinsky et al. 1985; Okazaki et al. 1985; Lok et al. 1985) and in transgenic mice (Overbeek et al. 1985; Goring et al. 1987). In all of these cases, the 5'-flanking sequence of the genes directed lens-specific expression of linked genes, and it was found that the sequences upstream of -100 were essential for the lens specificity. However, it has not been established whether these sequences are categorized as enhancers. Comparison of the regulatory region of the α - and γ -crystallin genes with that of the δ 1-crystallin enhancer region did not reveal mutual homologies of any significant extent. This indicates that the genes for different crystallin classes are not necessarily regulated by the same *cis*- and *trans*-acting elements, which may partly account for the spatio-temporal

expression patterns unique to each crystallin class (Piatigorsky 1981).

Participation of promoter elements in cell type-specific gene expression has been demonstrated for immunoglobulin genes (Grosschedl and Baltimore 1985; Mason et al. 1985; Picard and Schaffner 1985) and suggested for insulin (Edlund et al. 1985), and α -fetoprotein genes (Godbout et al. 1986; Muglia and Rothman-Denes 1986). However, we have clearly shown that the δ 1-crystallin promoter is in no way tissue specific (Table 1). Previously, we have suggested that the promoter region of the δ 1-crystallin gene may be involved in generating tissue-specific expression, on the basis of the observation with mouse cells that replacement of the promoter region with rodent retroviral LTRs resulted in apparent loss of lens specificity (Hayashi et al. 1985; Kondoh and Okada 1986). We now regard the apparently nonspecific expression of LTR-driven genes even in the presence of the δ 1-crystallin enhancer as the result of poor performance of the δ 1-crystallin enhancer in combination with the LTR elements in the mouse cell system.

In conclusion, the δ 1-crystallin enhancer appears to be the major and essential element for lens-specific δ 1-crystallin expression. The effect of the δ 1-crystallin enhancer is in all likelihood transcriptional. Tissue-specific activation of transcription by the δ 1-crystallin enhancer presumably involves specific interactions between nuclear factors of lens cells and the enhancer.

Experiments in these areas are under way. How such interactions activate gene transcription is still an open question, as is the case with other enhancers.

Materials and methods

Construction of recombinant plasmids

All the recombinant DNAs were handled according to standard methods (Maniatis et al. 1982). Unless otherwise noted, DNA fragments were ligated after making their ends blunt by T4 DNA polymerase. pSV2-, pSV1-, and pSV0-CAT were described by Gorman et al. (1982). pδCAT, ptkCAT, and pMoCAT were constructed inserting the *Hind*III linker-attached promoter region of the δ1-crystallin gene (−459 to +57; Borrás et al. 1985; Hayashi et al. 1985; Nickerson et al. 1985; Ohno et al. 1985), the promoter region of the HSV-tk gene (−197 to +56; McKnight et al. 1981) and the LTR region of MLV (−1067 to +30; Shinnick et al. 1981), respectively, into the *Hind*III site of pSV0-CAT. To make pMR-CAT, an *Eco*RV fragment between −561 and −230 of the MLV LTR sequence including the major part of its enhancers was removed from pMoCAT. For enhancer assays, the following fragments of the δ1-crystallin gene were prepared from pδC1A+5 (Hayashi et al. 1985): segment A, *Pst*I (+5)–*Eco*RV (+1412); B, *Eco*RV (+1410)–*Eco*RV (+2480); C, *Bgl*III (+2420)–*Xba*I (+3339); D, *Xba*I (+3336)–*Bgl*III (+4947); E, *Bgl*III (+4944)–*Hind*III (+5728); F, *Hind*III (+5725)–*Hind*III (+7123); H, *Pst*I (+5)–*Sca*I (+1557). Segments G [*Hind*III (+7120)–*Xho*I (+9000)] and I [*Nco*I (+2081)–*Xho*I (+9000)] were prepared from pδC1C in which a *Kpn*I site of pδC1B (Kondoh et al. 1984) located 3' of the gene was converted to a *Xho*I site. These fragments were cloned into the *Bam*HI site of ptkCAT. Segment B was dissected by digestion with combinations of restriction enzymes to cleave at +1555 (*Sca*I), +1861 (*Hgi*AI), +1960 (*Bam*HI), +2080 (*Nco*I), and +2205 (*Pst*I), then cloned into the *Bam*HI site of ptkCAT. *Xho*I linker-attached SV40 enhancer in pβGX_{SV}512 (Banerji et al. 1981) was inserted into the *Bam*HI site of pδCAT, ptkCAT, and pSV1-CAT to construct pδCAT-SV, ptkCAT-SV, and pSV1-CAT-SV, respectively. pδC1E was constructed by deleting the *Eco*RV (+1409)–*Bss*HII (+3273) segment extending from exon 3 to exon 6 in pδC1C-107. To make pRSδC1E, a fragment containing RSV-LTR (from −496 to +34) on pSR1 (obtained from T. Yamamoto through the Japanese Cancer Research Resources Bank; Yamamoto et al. 1980) was inserted into the *Pvu*I site in the vector sequence located 234 bp to the 5' end of the δ1-crystallin gene. Fragment B was inserted into the *Pvu*I (−234), *Xba*I (+3339), or *Xho*I (+9000) sites to make pδC1E-Pv, pδC1E-Xb, and pδC1E-Xh, respectively.

Primary cell culture

Cultures of LE cells and other tissues were prepared from 15-day-old chicken embryos. Isolated lens were disrupted by forceps in 0.1% collagenase in Hanks' saline and incubated for 30 min. The dissociated cell clumps were cultured in D10F, which is Dulbecco's modified Eagle's minimal essential medium containing 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml Fungizone. After 2 days in culture, the cells were dissociated to single cells using 0.05% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA) in calcium-magnesium-free Hanks' saline (CMF), and 2×10^5 cells were replated on a 3.5-cm-diameter dish in H10F, which is Ham's F12 medium containing 10% fetal calf serum and the antibiotics. Cells were cultured for 48 hr before transfection. FB cells [dermal fibroblasts of dorsal trunk], hepatocytes, and giz-

zard smooth muscle cells were obtained by dissociating tissues with 0.1% each of collagenase and trypsin in CMF and cultured in D10F as above, except that they were replated at a density of 10^5 cells per dish.

Transfection

DNA–calcium phosphate coprecipitates were prepared as described by Xie (1984). For CAT enzyme assay, 1 μg of DNA in a 75 μl suspension of DNA–calcium phosphate coprecipitates was added to a 3.5-cm dish. The cells were incubated with the precipitates for 4 hr, then fed with fresh H10F and incubated for another 20 hr. Transfection efficiencies were estimated by immunohistochemically staining the transfected cultures for the CAT antigen, using antibodies raised in a rabbit according to Kondoh et al. (1983).

CAT assay

The CAT assay was performed essentially as described by Gorman et al. (1982). Transfected cells were suspended in 40 μl of 250 mM Tris-HCl (pH 7.8), ruptured by three freeze-thawings in liquid N₂ and 37°C incubation, and heated at 60°C for 5 min. Extracts were prepared by pelleting the cell debris, the reaction was carried out in 100 μl of solution containing 20 μl of the extract, 250 mM Tris-HCl (pH 7.5), 0.2 μCi of ¹⁴C-labeled chloramphenicol (Amersham 53 mCi/mmol), 0.8 mM acetyl-CoA at 37°C for 2 hr. Acetylated and nonacetylated forms were separated by thin-layer chromatography (Gorman et al. 1982). After autoradiographic visualization of the spots, radioactivity of 3-acetylated chloramphenicol was measured by liquid scintillation counting. To measure rates of acetylation, extracts were appropriately diluted so that the reaction did not exceed 30% conversion of chloramphenicol to its acetylated forms.

Immunoblotting

Protein of transfected cells in a 3.5-cm dish was solubilized in 40 μl of sample buffer, and 10 μl were electrophoresed through a slab of 12.5% polyacrylamide gel (Laemmli and Favre 1973), followed by electrophoretic transfer to a nitrocellulose filter (Towbin et al. 1979). The filter was blocked against nonspecific antibody binding with 5% skim milk (Difco) in 50 mM Tris-HCl, 150 mM NaCl, and 10 mM EDTA (pH 7.5) (TBS), then serially treated with anti-δ-crystallin from rabbit and ¹²⁵I-labeled protein A (Amersham) at 0.1 μCi/ml. At each interval, the filter was washed with TBS containing 0.1% Tween-20. The filter was dried and exposed to X-ray film.

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