Tissue-specific regulation of a chicken δ -crystallin gene in mouse cells: involvement of the 5' end region

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A cloned δ -crystallin gene of the chicken is preferentially expressed in lens cells after introduction into various mouse tissues. The level of expression in the lens epithelium is 20 times higher than in fibroblasts. Taking advantage of this system, we attempted to define regulatory regions of the δ -crystallin gene using a variety of deletion and substitution mutants. The results indicate that tissue-specific regulation of the δ -crystallin gene is mediated by the 5' end region of the gene; sequences upstream from –93 are not required for expression and sequences downstream from +58 are not involved in tissue specificity. The high expression in lens cells requires 5' flanking sequences of 80-bp long from the cap site, whereas the low expression in fibroblasts requires an additional 12 bp upstream sequence. Expression of both types is lost in a mutant with only 51 bp of the 5' flanking sequence. Thus, fine deletion analysis demonstrated that expression in lens cells and expression in fibroblasts are distinct not only in level but in regulation.

Key words: deletion analysis/ δ -crystallin/dual regulation/micro-injection/tissue specificity

Introduction

Recent attempts at introducing cloned tissue-specific genes into animal cells revealed that such genes are preferentially expressed in the homologous cell type. For instance, we have shown that the lens-specific δ -crystallin gene of the chicken (Yasuda *et al.*, 1982; Piatigorsky, 1984) is expressed in lens cells at a much higher level than any other cell type when transferred by nuclear injection into mouse tissues in primary culture (Kondoh *et al.*, 1983). In addition, the δ -crystallin was expressed in the mouse lens epithelial cells at a high level comparable with the level in chicken lens epithelium. Such observations indicate that δ -crystallin and other tissue-specific genes are furnished with built-in signals in their base sequences for differential expression.

In mouse lens, δ -crystallin is totally absent and is replaced by γ -crystallin (Kondoh *et al.*, 1983). Thus expression of the exogenous δ -crystallin gene is clearly distinguished from endogenous mouse crystallins (Kondoh *et al.*, 1983). Taking advantage of this experimental system, we attempted to identify regulatory regions of the gene which are responsible for tissue-specific regulation. We introduced various deletions and substitutions into the δ -crystallin sequences and assessed the activity of the manipulated genes in mouse lens cells and fibroblasts. We pre-

sent evidence that the δ -crystallin expression can be classified into lens and non-lens types, which are distinct not only in their levels but also in their requirement for putative regulatory base sequences.

Results

The high-level expression of δ -crystallin gene is lens specific in mouse cells

We transferred a plasmid-cloned chicken δ -crystallin gene (δ C1A) and its derivatives (Figures 1 and 2) into mouse cells, either lens epithelial (LE) cells in primary culture or a fibroblast (FB) cell line, L801, by direct microinjection into the nucleus. We harvested the injected cultures after two days, since δ -crystallin synthesis reaches maximum around this time (Kondoh *et al.*, 1983).

To study the state of the injected DNA, we extracted DNA from subcellular fractions and carried out Southern-blot analyses. The majority of the p δ C1A sequences were found in the nuclear fraction and only trace amounts in the cytoplasmic fraction (data not shown). In the nucleus, p δ C1A sequences were present almost exclusively in the extrachromosomal fraction (Hirt, 1967) (Figure 3). The initially closed-circular DNA was recovered as either closed circular, open circular or linear forms as judged by its

Fig. 1. Structure of basic plasmids used. Thin line, open box and hatched box on an arc indicate pBR322-derived base sequence, the tetracycline resistance gene and SFFV LTR-derived base sequences, respectively. The filled box and thin lines on a horizontal line indicate transcribed and flanking regions, respectively, of the δ -crystallin gene carried by the plasmid. 5' and 3' ends of the transcribed region, and *Eco*RI and *Kpn*I sites relevant to the plasmid construction are indicated by 5', 3', E and K, respectively. The maps are drawn schematically and are not to scale. (a) pMSF5 carries a part of LTR sequence of SFFV at the original *Pst*I site of pBR322. (b) p δ KpF carries 3' part of δ -crystallin gene and 3' flanking sequences, from +677 to +8400, in an orientation for the δ -crystallin sequence to be transcribed from the SFFV promoter. (c) p δ H carries the 5' end portion of the δ -crystallin gene and 5' flanking sequences, and was used to construct deletion mutants. (d) p δ C1A carries the whole δ -crystallin gene and flanking sequences.

Fig. 2. (A) Structure of the injected plasmid pC1A and its deleted derivatives. The detailed structure of the δ -crystallin gene (gene 1) in the plasmid is presented by Ohno *et al.* (1985). The circular DNA is drawn as a linear form in the map locating the *SalI* sites in the vector sequence at both ends. Vector sequences are indicated by thin lines, chicken sequences by a thick line, and exons by solid rectangles. The region -646 to +677 flanked by *PstI* and *KpnI* sites is enlarged below. Exons I and II are shown as boxes and the translated region is filled. Restriction sites for *AhaIII* (A), *EcoRI* (E), *KpnI* (K), *PstI* (Ps) and *PvuII* (Pv) are indicated. The bars below the map represent sequences in the representative deletion mutants. **(B)** Base sequences around the cap site of the δ -crystallin gene (Yasuda *et al.*, 1984; Ohno *et al.*, 1985). The inverted repeat structure in the 5' flanking sequence is indicated by arrows. Note that there is a mismatch between positions -60 and -76. The CAAT and TATAA box sequences are underlined. **(C)** SFFV LTR-derived sequences in pMSF5. The 5' end of the R region is denoted as +1. pBR322-derived sequences with their positions (Sutcliffe, 1979) are in small letters. The CAAT and TATAA boxes, and the *KpnI* recognition site used to construct pCKpF are underlined.

mobility in agarose gels (Figure 3). The amount of pC1A DNA in these forms, if combined, accounts for at least half the estimated amount of injected DNA. Thus, the observations indicate that the injected circular DNA molecules remained fairly

stable in the nucleus without appreciable integration into mouse chromosomes. In the experiment shown in Figure 3, a larger amount of the injected pC1A DNA was apparently recovered from FB than from LE cells; however, this appears to be due

to experimental variation. Anyway, the result indicates that the injected p δ C1A DNA is no more stable in LE than in FB cells.

We assessed gene expression by Western-blot analysis of synthesized δ -crystallin and related polypeptides. Injection of LE cells with p δ C1A (each at 125 copies), resulted in the synthesis of authentic δ -crystallin polypeptides with a mol. wt. of 50 kd, at the rate of 5 pg/cell in two days [Figure 4A(b)]. In FB cells, however, expression of the δ C1A gene was reduced at least

Fig. 3. Fate of the injected DNA. 10–20 fl of p δ C1A DNA at 1 mg/ml was injected in 2000 nuclei each of LE and FB cells. After two days, DNA in the nuclei was fractionated according to Hirt (1967), and analyzed by Southern blotting using 32 P-labelled p δ C1A probe. Lane (a) contained 4 pg of closed circular p δ C1A DNA used for injection. In the other lanes, extrachromosomal fractions of (b) injected LE nuclei, (c) uninjected LE nuclei, (d) injected FB nuclei, (e) uninjected FB nuclei, and chromosomal fractions of (f) injected LE nuclei and (g) injected FB nuclei were analyzed. Positions of open circular (O), linear (L) and closed circular (C) forms of p δ C1A DNA are indicated.

20-fold compared with LE cells [Figure 4A(f)], confirming the immunohistological observation that the injected δ -crystallin gene was very efficiently expressed in the LE cells (Figure 5a), but rather poorly in FB (Figure 5c) and other non-lens murine cells (Kondoh *et al.*, 1983). We found a linear response between the amount of synthesized δ -crystallin and gene dosage up to 500 copies/injection in both cell types (data not shown, see also Xie, 1984).

Replacement of the 5' end region of the δ -crystallin gene with a Friend SFFV LTR segment alters the tissue specificity in expression

We first examined the possible involvement of the promoter and its surrounding regions in the tissue-specific expression of the gene. This was achieved by replacing the transcriptional initiation signals with those from long terminal repeats (LTR) of a murine retrovirus, Friend spleen focus-forming virus (SFFV). We excised an 8.4-kb long *KpnI* fragment from the δ -crystallin gene (Yasuda *et al.*, 1982; Kondoh *et al.*, 1983) and inserted this into the unique *KpnI* site of an SFFV LTR segment in the plasmid pMSF5. This *KpnI* fragment contained the whole transcribed base sequence of the δ -crystallin gene except for the first 677 bp, with the break point in the middle of the second intron (Figure 2A). In the orientation of the insert found in plasmid p δ KpF, the δ -crystallin sequence was so joined to the promoter sequence of SFFV LTR as to be transcribed from this new promoter. Expression of this novel promoter-replaced chimeric gene, designated as δ KpF, was compared with the original gene δ C1. Immunochemical staining of the cultures indicated that the gene δ KpF is not only expressed efficiently in LE cells but also in FB cells (Figure 5b, d), suggesting that the

Fig. 4. Expression of substitution and deletion mutants of δ -crystallin gene, as analyzed by Western blotting. Positions of the normal δ -crystallin (50 kd), and 52 kd and 48 kd polypeptides are indicated by arrows. (A) Comparison of p δ C1A and p δ KpF in LE and FB cells. Proteins from LE cells (a) without injection, (b) injected with p δ C1A, (c) injected with p δ KpF, and (d) injected with the mixture of p δ C1A and p δ KpF; and proteins from FB cells (e) without injection, (f) injected with p δ C1A, (g) injected with p δ KpF, (h) injected with the mixture of p δ C1A and p δ KpF, and (i) *BglI*-digested p δ KpF. Samples were prepared from 200 cells injected at a DNA concentration of 250 μ g/ml. (B) Effect of 5'-starting deletions on the δ -crystallin expression. (a) 1 ng of purified δ -crystallin. (b) Uninjected LE cells. Others are LE cells injected with an equimolar mixture of p δ KpF and the plasmids, (c) p δ C1A, (d) p δ C1 Δ -646, (e) p δ C1 Δ -406, (f) p δ C1 Δ -220, (g) p δ C1 Δ -186, (h) p δ C1 Δ -174, (i) p δ C1 Δ -166, (j) p δ C1 Δ -106, (k) p δ C1 Δ -51, (l) p δ C1 Δ +5, and (m) p δ C1 Δ +55, and (n) p δ KpF alone. Samples were prepared in the same way as in A, except that the DNA concentration in (n) was 125 μ g/ml. (C) Effect of deletions of the -51 to -106 region on the expression in LE. Mixture of p δ KpF and (a) p δ C1 Δ -106, (b) p δ C1 Δ -80, (c) p δ C1 Δ -65, and (d) p δ C1 Δ -51. Conditions were the same as in A. (D) Effect of the deletions on the expression in FB. Each deletion plasmid was injected into 500 cells at 250 μ g/ml. p δ KpF was not included in the mixture. (a) p δ C1 Δ -106, (b) p δ C1 Δ -92, (c) p δ C1 Δ -88, (d) p δ C1 Δ -80, (e) p δ C1 Δ -65, (f) p δ C1 Δ -51, and (g) without injection. (h) and (i), 50 and 500 pg, respectively, of purified δ -crystallin. The bands other than 50 kd are due to non-specific binding of iodinated protein A to cellular proteins which became apparent by longer exposure of this blot compared to others. (E) Effect of deletions in the transcribed sequences on the expression in LE. (a) No injection, (b) p δ C1 Δ -106, (c) p δ C1 Δ PvC, (d) p δ C1 Δ PvB, and (e) p δ C1 Δ Ah. The result of p δ C1 Δ PvA, not shown here, was identical to that of p δ C1 Δ PvC. Conditions were the same as in A.

specificity of the gene expression was altered by the gene manipulation. The inverted position of the 8.4-kd fragment in the vector pMSF5 did not permit any detectable expression in either cell type, indicating that gene expression is dependent on transcriptional initiation outside of the 8.4-kb *KpnI* δ -crystallin fragment, i.e., in the vector pMSF5 sequences.

Western blots revealed that the plasmid p δ KpF directs synthesis of two major classes of δ -crystallin-related polypeptides with mol. wts. of 48 kd and 52 kd in both LE cells and FB cells (Figure 4A). When the TATAA box sequence of the SFFV promoter was removed from p δ KpF using the flanking *SstI* sites (GAGCTC) (see Figure 2C), the 48 kd polypeptide was not synthesized while the 52 kd polypeptide continued to be synthesized (data not shown). Thus, synthesis of the 48 kd polypeptide is most probably due to transcription from the SFFV promoter. The polypeptide size of 48 kd is predictable if translation initiates at the second methionine codon located in exon III after removal of the authentic initiator codon by gene manipulation. The 52 kd polypeptide, however, appears to depend on transcriptional initiation in pBR322-derived base sequences which we tentatively designate here as pBR promoter. By cleavage of p δ KpF with *BglII* in the pBR322 sequence 252 bp upstream from the SFFV cap

site (Figure 2C), synthesis of the 52 kd polypeptide was totally abolished, but synthesis of the 48 kd polypeptide remained unaffected [Figure 4A(i)].

In LE cells, expression of the δ KpF gene, as estimated from synthesis of 48 kd and 52 kd polypeptides, appeared less efficient than expression of the original δ C1 gene, with 7% and 20% activities for 48 kd and 52 kd polypeptides, respectively. This difference in efficiency of gene expression between authentic and retroviral promoters was confirmed by injecting LE cells with an equimolar mixture of p δ C1A and p δ KpF [Figure 4A(d)]. In FB cells, on the other hand, gene expression using the authentic promoter was reduced at least 20-fold, but expression using SFFV and pBR promoters was increased some 4-fold [Figure 4A(f) to (h)]. Thus, cell-type specificity of δ -crystallin gene expression was altered by replacing the 5' end region with different sets of base sequences having promoter functions. This result strongly suggests that the replaced region of the δ -crystallin gene contained the regulatory base sequences for expressing tissue-specificity.

Deletion of the 5' flanking sequences

We introduced upstream-starting deletions in the region between positions -2200 and +677 of the δ -crystallin gene, i.e., the region that was totally replaced in p δ KpF, and studied the effect of these deletions on gene expression. In expression in LE cells, the deletion mutant genes were mixed with an equimolar amount of p δ KpF to control the efficiency of gene transfer. Western-blot analysis using independent samples was performed at least four times for each deletion mutant, and the results were highly reproducible. A representative set of results is shown in Figure 5B–D, and quantitative data are summarized in Figure 6.

We first removed the furthest upstream *PstI*-*EcoRI* fragment (-646 to -2200) and replaced it with the 752 bp *PstI*-*EcoRI* fragment of pBR322. This gene, δ C1 Δ -646, was indistinguishable from the original gene δ C1A in its efficiency of expression in both LE and FB cells. Thus, the region upstream of -646 is dispensable for δ -crystallin expression. We constructed sequential deletion mutants from δ C1 Δ -646 using BAL31 exonuclease up to position +55, leaving the same *EcoRI*-*PstI* fragment of pBR322 as a common upstream sequence.

The results of expression of BAL31-generated mutants are shown in Figures 4B and 6. There was no significant variation

Fig. 5. Immunocytochemical staining of δ -crystallin in injected cultures. (a) and (b) are phase contrast micrographs of LE cultures each with three cells injected with p δ C1A and p δ KpF, respectively. (c) and (d) are FB cultures each with four expressing cells derived from a pair of cells injected with p δ C1A and p δ KpF, respectively. The bar indicates 20 μ m.

Fig. 6. Effect of deletions in the 5' flanking sequences on the expression of the δ -crystallin in LE and FB. Gene activities in LE ($\blacktriangle$) and FB ($\triangle$) were plotted against deletion termini. The averaged data are taken from two independent sets of Western-blot analysis including the one shown in Figure 4, where high enough radioactivities were localized on the bands (>5000 c.p.m./ng δ -crystallin). Expression of δ -crystallin in LE was normalized against the expression of the 52 kd polypeptide from the coinjected p δ KpF, on the assumption of synthesis of 200 pg of the 52 kd polypeptide in 100 LE cells, and gene activity was presented as δ -crystallin synthesis two days after injection of 100 cells with the plasmid at 125 μ g/ml, taking into account the proportional relationship of the rate of δ -crystallin synthesis to DNA concentration and to the number of injected cells.

in gene expression after deletion to 106 bp upstream from the cap site in either LE or FB cells. However, by extension of the deletion to -51, which still left the TATAA box homology, gene activity was lost in both cell types simultaneously. Thus, essential regulatory elements for gene expression seem to reside in the region -51 to -106.

We derived a series of finer deletion mutants for the region between -106 and -51, starting from p δ C1 Δ -106, and measured the mutant activity. Results are shown in Figure 4C and 4D, and are summarized in Figure 6. There was a dramatic difference between LE and FB cells. In LE cells, the deletion to -80 did not significantly affect the gene activity, progression of the deletion to -65 reduced activity to 3% of the original, and further deletions totally abolished activity. By contrast, a low level of gene activity characteristic of FB cells was maintained by the deletion of -92, decreased by deletion to -88 and completely lost by deletion to -80 which had no effect on the activity in LE. These results indicate that low-level expression of the δ -crystallin gene in FB cells requires the region -92 to -80, but the lens-characteristic high-level expression does not. Thus, the deletions from upstream in the non-transcribed 5' flanking sequence defined the 5' border of the putative regulatory sequences as around -80 bp for expression in LE and around -90 bp for expression in FB.

Deletion of the transcribed sequences.

We constructed a series of internal deletion mutants in the first 677 bp transcribed region from δ C1 Δ -106 using *Pvu*II, *Aha*III and *Kpn*I sites (Figure 2). Deletion in the second intron, Δ PvB (+258 to +677) and Δ Ah (+347 to +677), did not affect the synthesis of the 50 kd δ -crystallin polypeptide [Figure 4E(d) and (e)], indicating transcription and processing of transcripts proceed normally in these mutants. The deletion of the second exon along with its flanking intron sequences [Δ PvA (+58 to +260)] (data not shown) and Δ PvC (+58 to +677) [Figure 4E(c)] reduced the size of the coding polypeptide from 50 kd to 48 kd, as expected from the loss of the authentic initiator codon in the second exon. However, these deletions did not significantly affect the efficiency of gene expression itself, either in LE cells (Figure 4E) or in FB cells (data not shown). These results indicate that transcribed sequences between positions +58 and +677 are not involved in regulating the efficiency and specificity of the gene expression. They also indicate that transcription of the injected δ -crystallin genes initiates between the nucleotides -80 and +58.

Discussion

We have attempted to identify the regulatory region of the chicken δ -crystallin gene responsible for tissue-specific expression. To this end, we introduced various deletions and substitutions in the sequence, and assessed the activity of the manipulated genes in mouse lens cells and fibroblasts. This heterologous combination of gene and recipient cells has the advantage of providing a clear distinction between exogenous δ -crystallin expression and expression of the endogenous mouse crystallins, but has the obvious limitation that the complete pattern of regulation occurring in the chicken may not be fully reproduced in mice. Nevertheless, the high preference of lens cells over other mouse tissues (Kondoh *et al.*, 1983), indicates that an essential part of the mechanism directing lens-specific δ -crystallin expression is reproduced, and thus can be studied in this experimental system.

Mouse LE cells supported a level of δ -crystallin expression comparable to that in chicken LE cells (Kondoh *et al.*, 1983). Mouse FB cells also expressed exogenous δ -crystallin, albeit at

a low level, approximately 5% of that in LE cells. This apparently relaxed tissue-specificity may be partly ascribed to the heterologous experimental system, but most probably reflects an intrinsic property of the δ -crystallin gene itself. Expression of δ -crystallin in chicken embryo is not strictly lens specific, either, in the sense that its expression at a low level is not infrequently observed in certain tissues other than lens (for review, see Okada, 1983). Thus, in studying the effect of deletions we compared two cell types, LE and FB, to see if expression could be distinguished by criteria other than its level.

The removal of sequences to the 5' of position -92 did not affect the gene expression in either cell type (Figures 4B and 5). Thus, no inhibitory or activating mechanisms appear to operate through the base sequences between positions -93 and -2200, at least in this experimental system. As we extended the deletions and the break points neared the cap site, decrease of gene activity in the FB cells was first observed in the deletion to -88 and complete loss of the activity by deletion to -80 (Figure 4D). These deletions, however, did not at all affect the activity in LE cells (Figure 4C). Thus, the deletion mutant gene δ C1 Δ -80 has an all-or-none regulation with respect to lens specificity, and inclusion of an additional 12 upstream base pair sequences gives activity in FB cells. This clearly demonstrates that expression in LE cells and expression in FB cells are distinct from each other in their regulation and extent. Although a single FB cell line was chosen as the non-lens representative in this study, experiments with a variety of mouse cells have shown that this conclusion can be extended to other non-lens cells (H. Kondoh and S. Hayashi, in preparation).

Further extension of the deletion to position -51 totally abolished the gene activity in LE, although the TATAA box homology and some upstream sequences were left. Extension of the deletion into the normally transcribed region did not result in a recurrence of the gene expression.

These observations suggested that transcription starts close to the cap site in this experimental system. Although direct mapping of the transcriptional initiation site in the injected cells has not been successful, due to too small a sample size, it is possibly at position +1, since HeLa cell extract transcribes the p δ H-derived templates (Figure 1) from the normal cap site (K. Yasuda, unpublished data).

The region -51 to -80 contains a characteristic G-C rich inverted-repeat sequence (between -59 and -77). By deletion in p δ C1 Δ -65, which removes the 5' half of the inverted repeats, the activity of the δ -crystallin gene in LE was decreased to only a 3% level, which could represent a half-site activity of the repeat. The observation suggests interaction of the inverted-repeat base sequences with cellular factors for specific gene expression. The situation appears similar to some other animal protein genes which require upstream sequences 50–100 bp from the cap site for the promoter activity. It is especially intriguing that the inverted repeat contains the CCGCC sequence which participates as an essential element in the 5' flanking sequence to express the promoter activity of HSV-tk (McKnight *et al.*, 1984) and SV40 early genes (Myers *et al.*, 1981; Fromm and Berg, 1982; Byne *et al.*, 1983; Everett *et al.*, 1983).

The rest of the sequences (the major body of those transcribed) from +58 to the end of the gene, and also the 3' flanking sequences, do not appear to make any notable contribution to the tissue specificity of the gene. The removal of any part of the region between +58 and +677 had no substantial effect on efficiency of expression in either LE or FB cells. Sequences downstream from +677 were expressed by the SFFV promoter

with an altered specificity (Figures 4A and 5) and, in addition, we observed that the chimeric gene made with a Moloney murine leukemia virus LTR and the same δ -crystallin sequence from +677 was expressed more-or-less constantly in various mouse tissues in primary culture (H.Kondoh and S.Hayashi, in preparation).

An interesting observation was the efficient synthesis of the shorter polypeptide (48 kd) after removal of the normal initiator codon of δ -crystallin, either by promoter substitution (p δ KpF) or by internal deletions. From the polypeptide size it is likely that the second methionine codon was used as an efficient initiator for this polypeptide. The second methionine codon, as well as the authentic initiator codon is preceded by pyrimidine tracts characteristic of the initiator methionine codons (Hagenbuehle *et al.*, 1978) and both conform to the consensus PuXXAUGG sequence for the initiator codons (Kozak, 1981).

The expression of the 52 kd polypeptide from p δ KpF appears to be due to transcription-initiating activity in the pBR322-derived base sequences, which we tentatively designate as pBR promoter, since the cleavage of p δ KpF with *Bgl*I at position 3481 of the pBR322 sequence and at 252 bp upstream from the SFFV cap site resulted in disappearance of the 52 kd polypeptide. It is thus likely that the pBR promoter is upstream from the *Bgl*I site. We suspect that this is around position 3200 of the pBR322 sequence where a number of TATAA box-like sequences are clustered. An open reading frame starting from the met codon at position 3424 of pBR322 (Sutcliffe, 1979) exists immediately upstream of the δ -crystallin sequence in p δ KpF. If one of the triplet junctions either at position 3561 or 3576 act as a splice junction to exon III, then the predicted polypeptide is compatible with the observed mol. wt. of 52 kd. There have been reports of abundant transcripts which may start in this region of pBR322 and provide donor site(s) for splice junction(s) (Fuhrman *et al.*, 1981; Grosveld *et al.*, 1982).

Overall our analyses indicate that the chicken δ -crystallin gene carries a putative regulatory region around its 5' terminus to achieve dual expression in lens and non-lens cells. The expression in LE cells requires the 5' flanking sequences 80 bp from the cap site, and expression in FB cells requires an additional 12 base pairs. The 3' border of the essential elements for expression is not precisely defined, but is shown to be upstream from position +58. Although direct mapping of the transcriptional initiation site in this experimental system has not been done, it is likely that both types of expression share the same sole promoter found in the regulatory region. Among the tissue-specific genes studied thus far, there are examples using multiple promoters which function at different efficiencies in different tissues, e.g., alpha-amylase genes (Schibler *et al.*, 1983), and the ϵ -globin gene (Allan *et al.*, 1982, 1983). In these cases, the promoters are spatially separated from each other, but the δ -crystallin gene may be regarded as a case where the functionally separate promoters have structurally fused. Thus, the tissue specific genes may not always be under an all-or-none regulation but may have evolved potential flexibilities to fulfill the various demands of cells and tissues.

The expression of δ -crystallin at a low level occurs in some embryonic tissues of the chicken (Clayton, 1982; Kodama and Eguchi, 1982; Okada, 1983; Agata *et al.*, 1983; Takagi *et al.*, 1983; Ueda *et al.*, 1983). In addition to their analogous levels of expression, non-lens expression in both chicken embryo and gene-transferred mouse cells is characterized by the absence of other lens phenotypes, including expression of other crystallins (Kondoh *et al.*, 1984; H.Kondoh, unpublished data). In the

chicken embryo, the variety of non-lens tissues expressing δ -crystallin is rather limited (Okada, 1983). In mouse tissues, on the other hand, the low-level expression of the exogenous δ -crystallin was demonstrated not only in FB cells (Kondoh *et al.*, 1983, this paper) but in a large variety of cell types (Kondoh *et al.*, 1983; H.Kondoh and S.Hayashi, in preparation) where the majority of exogenous gene copies remain unintegrated with the chromosome (Figure 2). However, we have previously shown that following integration of the exogenous δ -crystallin gene into a mouse chromosome, the expression was largely suppressed and, even at a low level, was restricted to certain defined cell types specific to each integration site (Kondoh *et al.*, 1984) in a way reminiscent of the ectopic δ -crystallin expression in the chicken embryo. Probably the ectopic expression of δ -crystallin observed in the chicken does not always imply lens differentiation but reflects the intrinsic potential of the δ -crystallin gene for a ubiquitous low-level expression which is usually suppressed by the dominant chromosomal effect.

Thus, mouse cells seem to regulate the chicken δ -crystallin gene in a way that mimics the chicken cells. Mouse LE cells supported a high level of δ -crystallin expression comparable to the level in chicken lens epithelium. Mouse FB cells expressed chicken δ -crystallin at a low level which may be analogous to that of ectopic δ -crystallin expression in chicken embryonic tissues. These deletion analyses suggest trans-acting positive regulation through 5' flanking sequences distinct between classes of δ -crystallin expression. However, to avoid the inherent limitation of an heterologous system we are now developing ways of introducing the cloned δ -crystallin gene into chicken cells.

Materials and methods

Plasmid construction

The plasmid p δ C1A carrying the whole length of a δ -crystallin gene and the 2.2-kb 5' flanking sequences was derived from p δ C1 (Kondoh *et al.*, 1983) by cleavage at a *Kpn*I site to the 3' of the gene followed by brief BAL31 digestion and recircularization to remove unnecessary SFFV-derived base sequences and to leave in the plasmid a unique *Kpn*I site of the δ -crystallin gene at +677. pMSF5 is a cDNA clone of SFFV containing part of the LTR sequences (175 bp long) with dG.dC tails inserted in *Pst*I-digested pBR322 (G.Soma, unpublished data). The base sequence of the insert was determined by the chain-termination method (Sanger *et al.*, 1977) with the *Aha*III-*Sma*I fragments cloned in M13mp9, and in part confirmed by the chemical degradation method (Maxam and Gilbert, 1980). The plasmid p δ H carrying the 5' flanking sequence and the first two exons of the δ -crystallin gene was constructed by cloning the 2.8 kb *Kpn*I-*Eco*RI fragment of p δ C1A into the *Kpn*I and *Eco*RI sites of pMSF5. The plasmid p δ KpF, containing the rest of the δ -crystallin sequence, was constructed by inserting the 8.4 kb *Kpn*I fragment of p δ C1 into the *Kpn*I site of pMSF5 located 31 bp downstream from the SFFV cap site in an orientation such that SFFV-started transcription could continue into the δ -crystallin sequences.

Deletion mutants

All the gene manipulations to create deletions in the region -2200 to +677 were carried out in p δ H. The manipulated fragments excised by *Pst*I and *Kpn*I digestions were subsequently ligated with the 1400-bp *Sal*I-*Pst*I fragment of pBR322 and the 11-kb *Sal*I-*Kpn*I fragment of p δ C1A to derive deletion mutants of p δ C1A. To make deletions in the 5' flanking sequences, p δ H was cleaved at the unique *Pst*I site at position -646 and digested by BAL31 exonuclease to various extents, then recircularized after converting the termini to *Pst*I sites using a synthetic linker sequence, CCTGCAGG (Boehringer). The deletion end-points between -174 and +55 were determined by sequencing (Sanger *et al.*, 1977) and are designated by their junction to the linker sequence. End points of other deletion mutants were estimated from the size of restriction fragments. To obtain deletions in the transcribed sequences, p δ HA-106 was partially digested with *Pvu*II or *Aha*III. The blunt ends at positions +58, +258 and +344 were converted to *Kpn*I sites by linker attachment, and the *Pst*I-*Kpn*I fragments were used to construct derivatives of p δ C1A-106 with one of the deletion termini at +677. Likewise, a product of partially digesting p δ HA-106 with *Pvu*II was directly circularized and the *Pst*I-*Kpn*I fragment was used to create the deletion p δ C1A Δ PvA.

Cell culture and microinjection

Primary lens epithelial cells (Kondoh *et al.*, 1983) and the fibroblast cell line L801 (Xie, 1984) have been described. Cultures were maintained in Dulbecco's modified Eagle's MEM supplemented with 10% fetal calf serum. Nuclear microinjection was performed using an Injectoscope (Olympus) according to Kondoh *et al.* (1983). Closed-circular plasmid DNA in 2 mM Tris-HCl (pH 8.0) and 0.2 mM EDTA was injected at the concentration of 200–1000 μ g/ml, which roughly corresponded to 100–500 copies/cell assuming an injected volume of 10 fl (Capecchi, 1980; Anderson *et al.*, 1980). To analyze the state of the injected DNA, the cultures were fractionated according to Hirt (1967) as modified by Graessmann and Graessmann (1983), and analyzed by Southern blotting carried out according to Wahl *et al.* (1979). To analyze δ -crystallin expression by Western blotting, the bulk proteins of 100–500 injected cells were recovered in 20 μ l of sample buffer with 1% SDS dropped directly on the injected area of a culture. The sample was electrophoresed, transferred to a nitrocellulose sheet, and treated with mono-specific anti δ -crystallin antibodies from rabbit and with 125 I-labelled protein A, according to Kondoh *et al.* (1984). 125 I-labelled bands were visualized by autoradiography on X-ray film and cut out of the sheets to measure radioactivity. An experiment using purified δ -crystallin indicated that the radioactivity increased proportionally to the amount of δ -crystallin in the range of 50 pg–10 ng/band.

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