Tissue- and species-specific promoter elements of rat γ-crystallin genes

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ABSTRACT

The 5′ flanking regions of the six rat γ-crystallin genes (γA-γF) are all capable of conferring lens-specific expression to the bacterial chloramphenicol acetyl transferase (CAT) reporter gene in either transdifferentiating chicken neural retina cells or mouse lens epithelial cells. Deletion mapping of the most active γ-crystallin promoter region, the γD region, showed that at least three elements are required for maximal expression in mouse lens epithelial cells: element(s) located between −200 and −106, a conserved CG rich region around position −75, and a CG stretch around −15. The region between −200 and −106 was dispensable in transdifferentiating chicken neural retina cells, which instead required the region between −106 and −78. The maximal activity of the γE and γF promoters was also dependent upon the integrity of the conserved CG region located around −75. A synthetic oligonucleotide containing this sequence was capable of lens-specific enhancement of the activity of the tk promoter in transdifferentiating chicken neural retina cells but not in mouse lens epithelial cells. Our results further show that this region may contain a silencer element, active in non-lens tissues, as well.

INTRODUCTION

The γ-crystallin genes code for abundant water-soluble proteins found in the vertebrate eye lens. These proteins, together with the other crystallin protein families, are thought to be responsible for the unique optical properties of the lens (1). The rat genome contains six closely related γ-crystallin genes (γA through γF)(2). These genes all become active during early prenatal development of the lens. After birth a differential decrease in expression of these genes is observed and in the mature rat lens only the γB gene transcript can be detected (3). The γ-crystallin genes are evolutionally related to a second class of ubiquitous crystallin genes, the β-crystallin genes (for a recent review, see 4). This gene family shows the same mode of regulation as the γ-crystallin genes, in that a simultaneous activation of transcription occurs during prenatal lens development, while a non-coordinate shutdown is found postnatally (5). The evolutionary relation between the β- and γ-crystallin genes may thus well extend to a sharing of regulatory factors. The tissue and developmental specificity of the expression of the β- and γ-crystallin genes and the possibility that these two gene families use common regulatory mechanisms, make these two gene families an attractive model system for the study of gene regulation during terminal differentiation.

At present only the elements involved in the tissue-specific expression of one γ-crystallin gene, namely the mouse γ2 gene, have been studied. In this gene three regions have been identified that are involved in the tissue-specific expression of this gene: domain A at −68 to −18, domain B at −226 to −120 and an upstream enhancer located between −392 and −278 (6). It is of obvious importance to determine whether these results may be generalized to other members of the γ-crystallin gene family. We have therefore monitored the promoter activity of all six rat γ-crystallin genes and show that the 5′ flanking regions are sufficient to confer tissue-specificity. More precise mapping of one of these regions, namely that of the γD gene, showed that this gene lacks domain B as well as the upstream enhancer found in the mouse γ2 5′ flanking region. Instead, the maximal activity of this gene is dependent upon a region between the TATA box and the cap site.

The tissue-specific elements of the mouse γ2 gene as reported by Lok *et al.* (6) were identified using chicken lens epithelial cell explants. The use of chicken cells to study the expression of rodent genes which do not have an endogenous ortholog (chickens lack γ-crystallins) is questionable as there is accumulating evidence that even orthologous αA-crystallin promoters are recognized differently in chicken and mouse tissues (7). For comparative reasons we have therefore used both chicken and mouse lens-like cells in studying the rat γ-crystallin promoters. We show here that these two cell systems differ in the recognition of the rat γD-crystallin promoter. A crucial element in the expression of the γD gene was mapped to the region −70 to −80. This region is sufficient to confer lens-specificity to a heterologous promoter in chicken cells but not in mouse cells.

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MATERIALS AND METHODS

Materials

Restriction endonucleases, exonuclease III, T4 DNA ligase, alkaline phosphatase and acetyl-CoA were purchased from Boehringer-Mannheim. Mungbean nuclease was obtained from Pharmacia. D-threo-(dichloroacetyl-1-^{14}C) chloramphenicol (54 mCi/mMol) was from Amersham.

Construction of rat γ-crystallin CAT constructs

Fragments containing the 5′ upstream regions of the six γ-crystallin genes (ranging from 350 bp to 2 kb; see figure 4) were made blunt-end with mungbean nuclease and cloned in the alkaline phosphatase treated SmaI site of the polylinker directly upstream of the CAT coding sequence in the vector p$^-$CAT. Where possible (γC, γD, γE and γF), the NcoI restriction site in the first exon, overlapping the translation initiation site, was used for cloning. For the γA and γB gene, which lack this NcoI site, a more upstream restriction site was used in cloning their promoter regions (see figure 4). Clones were screened by restriction analysis for those constructs containing single inserts in either orientation.

Deletions in the γ-crystallin CAT constructs were made by cutting at unique restriction sites in the promoter region and the upstream polylinker SstI site. Sticky ends were blunted with mungbean nuclease and ligated. In case of the γD-CAT plasmid a series of deletion clones was made by cleaving γD-CAT with SstI/BglII or PstI/BamHI. After unidirectional digestion (3′→ 5′) of the insert with exonuclease III, leaving the 3′ protruding ends of SstI or PstI sticky ends intact, molecules were made blunt-end with mungbean nuclease and were closed (figure 1). The inserts of resulting clones were sequenced by the dideoxy method, after subcloning to M13mp vectors, to determine the precise length of the insert.

Primary cultures

Mouse lens epithelial (LE) cells. Lenses were isolated from newborn mice. Adhering tissue was carefully removed under a dissecting microscope. After two incubations with 0.15% trypsin (10 min. each at 37°C in HANKS' saline calcium and magnesium free), the lenses were immersed in M199 medium (Flow laboratories) without fetal calf serum. The epithelial cells were scraped off by pipetting the lenses up and down about five times. The fiber cell mass was allowed to settle and the supernatant containing the epithelial cells was transferred to collagen coated tissue culture dishes containing 2 ml of M199. The epithelial cells of about fifteen lenses were seeded per 35 mm dish (COSTAR). After 4 hrs of incubation fetal calf serum (Flow laborato-ries) was added to 10%.

Chicken neural retina (NR) cells. Neural retina cells from 5 days old embryonic chickens were isolated essentially as described (8). Cells were cultured in 35 mm dishes (primaria, FALCON) containing 2 ml of M199 medium supplemented with 10% fetal calf serum.

Other cell types used. Newborn mice skin, brain and retina cells and embryonic chicken skin, brain and liver cells were obtained by trypsinization of the various organs. Cells were plated on 35 mm tissue culture dishes containing 2 ml of M199 with 10% fetal calf serum.

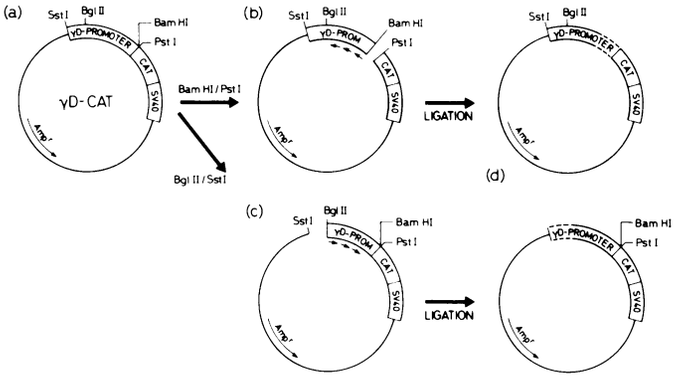


Figure 1. Construction of γD-CAT deletion clones. The γD-CAT construct (a) was digested with either PstI and BamHI (b) or with SstI and BglII (c). After unidirectional digestion with exonuclease III (indicated by → →), the DNA fragments were blunt endend with mungbean nuclease and religated (d).

DNA transfection

One hour before transfection cells were refed with 0.9 ml of fresh medium containing 10% fetal calf serum. Calcium phosphate-DNA precipitates were made by mixing 25 μl DNA solution (2 μg in 1mM Tris.HCl/0.1 mM EDTA) with 50 μl of 2×HBS (0.28 M NaCl/0.05 M HEPES/0.75 mM Na_2HPO_4/0.75 mM NaH_2PO_4, pH 7.05). After 10 min. 25 μl of 0.5 M $CaCl_2$/0.1 M HEPES (pH 7.05) was added with constant agitation (9). Precipitates were allowed to form for 30 min. before the suspension was added dropwise to the cells. After 5 hrs of incubation, cultures were exposed to 25% DMSO in medium for 5 min. in order to enhance DNA uptake. All transfections were done in duplo and at least two DNA preparations of each construct were tested in several independent experiments. Variations in transfection efficiency were monitored by transfecting parallel cultures with pSV2CAT.

CAT assay

Cells were harvested 48 hrs after transfection by washing once with HANKS' saline and scraping the cells into 1.5 ml of HANKS' saline. After collection by centrifugation, the cells were resuspended in 200 μl of 0.25 M Tris.HCl (pH 7.8) and lysed by repeatedly freezing and thawing. Cell debris was pelleted by centrifugation. The supernatant was incubated for 5 min. at 65°C to prevent deacetylation of acetylchloramphenicol and hydrolysis of acetyl-CoA (10). CAT activity was assayed by adding 4 μl of ^{14}C-chloramphenicol and 20 μl of 8 mM acetyl-CoA to 100 μl of cell extract. After 1.5 hrs of incubation at 37°C, reaction mixtures were extracted once with 800 μl of ice cold ethylacetate. The organic layer was evaporated and taken up in 20 μl of ethyl acetate (11). After thin-layer silica gel chromatography run in chloroform-methanol (95:5 v/v) and autoradiography, the amount of acetylated chloramphenicol was determined either by densitometric scanning or by cutting out the labelled spots followed by scintillation counting. CAT activity was corrected for differences in cell density by measuring protein content of the cell extracts (Bio-Rad protein assay). The variation between different experiments was usually within 20%.

Preparation of plasmid DNAs

Plasmid DNAs were prepared by the method of Birnboim and Doly (12) and further purified by RNase A treatment and centrifugation through 1 M NaCl (13).

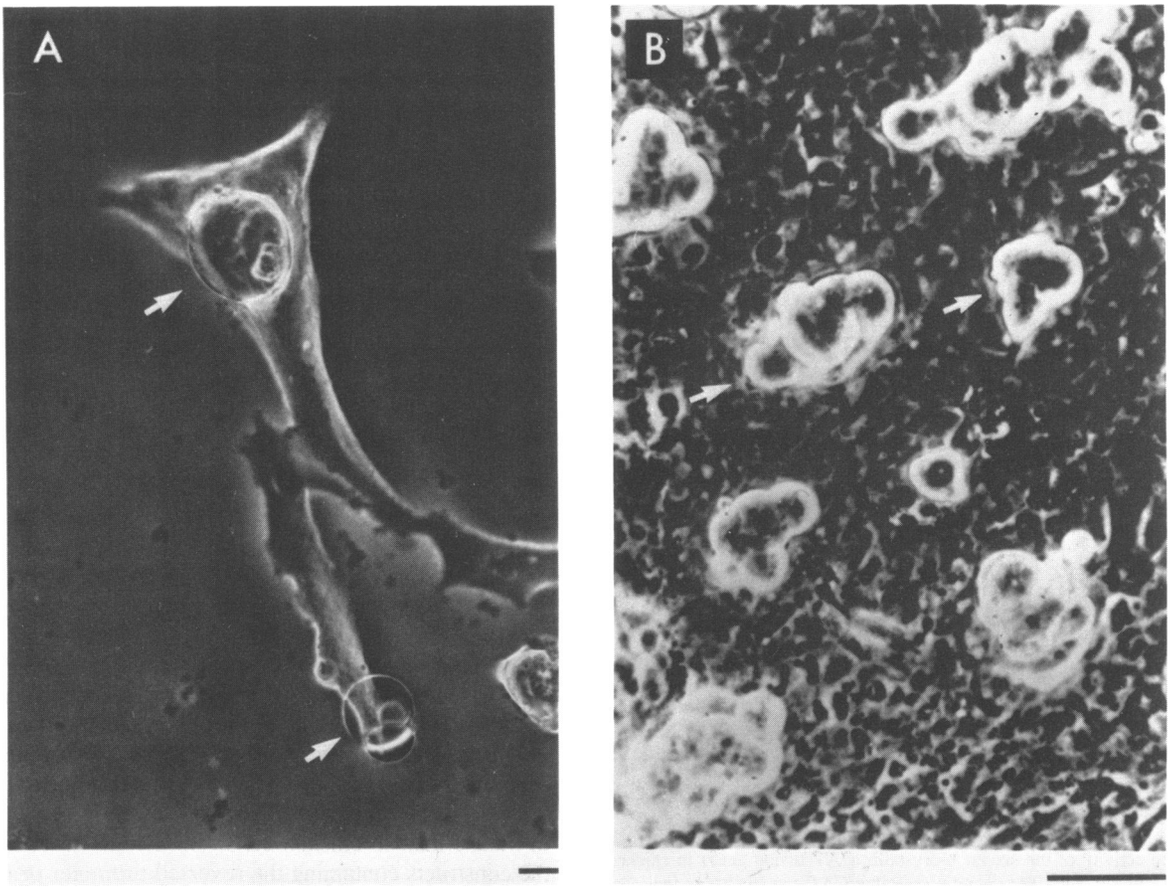


Figure 2. In vitro differentiation of primary cultures. Phase-contrast micrographs of mouse LE cells after 5 days in culture (A); chicken NR cells after 20 days in culture (B). Arrows point to the transparent multicellular structures (lentoid bodies). Bars represent 25 μm.

RNA isolation and analysis

RNA was isolated by washing the cells once with HANKS' saline and scraping the cells into 1 ml of 7.6 M Guanidinium chloride/0.1 M KAc (pH 4.5;14). Chromosomal DNA was sheared by passing the solution several times through a narrow (0.5 mm) needle. RNA was precipitated by adding 0.6 volumes of ethanol. After centrifugation RNA was dissolved in 150 μl 10 mM Tris.HCl/ 100 mM NaCl/1 mM EDTA/0.1% SDS (pH 7.9) and extracted twice with two volumes of phenol/chloroform. RNA was reprecipitated with ethanol and dissolved in water. RNA was separated on a 1% formaldehyde agarose gel, blotted to nitrocellulose and hybridized with a SP6 γ-crystallin antisense RNA probe, as described (15).

RESULTS

In vitro differentiation of the two lens-like cultures

To study lens-specific transcription elements cultured lens-like cells are required. Since none of the lens derived cell lines described so far retain their state of differentiation, as indicated by lack of synthesis of the endogenous crystallins (16–19), we used primary cultures as a source of either mouse or chicken lens cells.

Mouse lens cells were obtained by culturing newborn mouse lens epithelial (LE) cells. These were viable *in vitro* for about one week. During culture cells tended to elongate and the formation of lentoid bodies was observed (figure 2A). These morphological changes were accompanied by an accumulation of endogenous γ-crystallin RNA (figure 3). Although the differentiating mouse LE cells seem ideal for studying the activity of γ-crystallin promotor regions, the paucity of mice cells made this cell system only useful in validating results obtained with the second cell system.

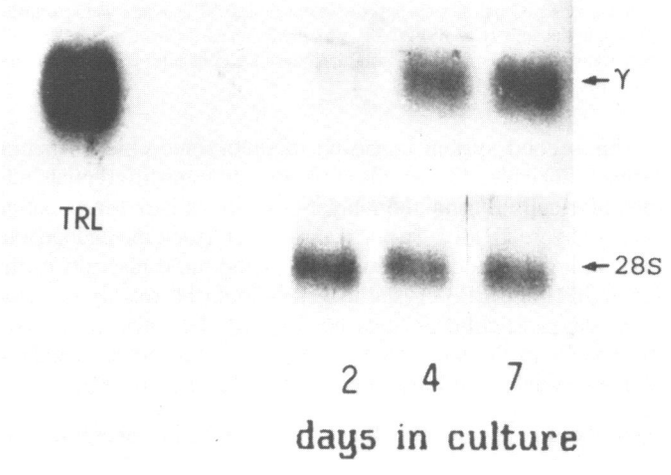


Figure 3. γ-crystallin transcripts in mouse LE cultures. RNA was isolated from mouse LE cells after the culture periods indicated. Approximately 1 μg was Northern blotted and hybridized with a rat γ-crystallin probe as described in Materials and Methods. As a quantitative control hybridization to the ribosomal 28S RNA is shown. For comparison 0.1 μg RNA isolated from 5 weeks old rat lenses (TRL) was used. Only the relevant parts of the autoradiogram are shown.

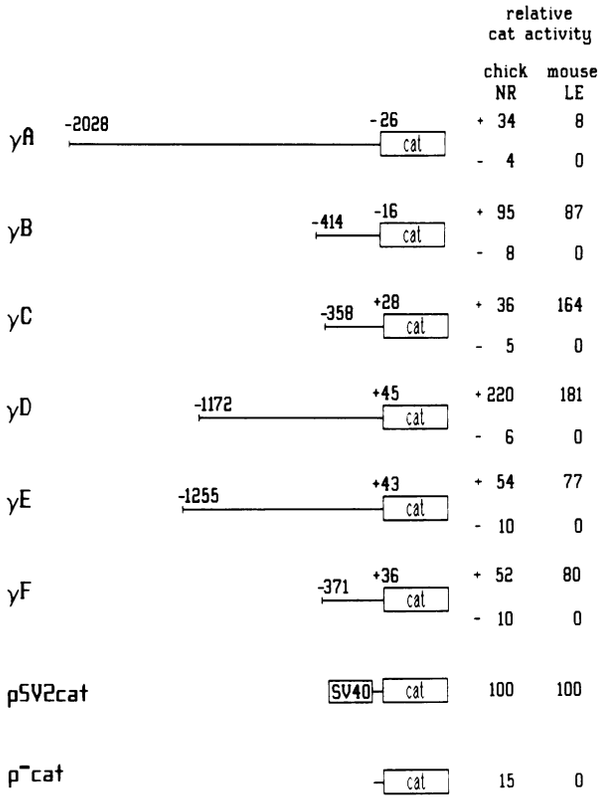


Figure 4. The activity of the six rat γ-crystallin CAT fusion genes in chicken NR or mouse lens LE cells. The six rat γ-crystallin/CAT con-structs are indicated schematically, the extent of the 5′ region cloned in front of the CAT gene is numbered relative to the γ-crystallin gene cap site. Note that for the γA and γB gene, which lack a NcoI site overlapping the translation initiation codon, a more upstream restriction site, respectively at position −26 and at position −16, was used in cloning their promoter regions. The positive orientation of the 5′ region is shown as +, − indicates the opposite orientation. CAT activities were calculated relative to the activity of pSV2CAT, which was set as 100%. Chicken NR cells were transfected after 8 days in culture, mouse LE cells after 2 days in culture.

This second system consisted of embryonic chicken neural retina (NR) cells. These cells are known to transdifferentiate into lens-like cells during monolayer growth and to form lentoid bodies (figure 2B)(8). During transdifferentiation the endogenous crystallin genes become active (8,20) and the transcripts of the αA-, βB1- and βB3-crystallin genes could be readily detected in 30 days old cultures (data not shown). This system has two drawbacks in the study of the rat γ-crystallin genes: chickens lack γ-crystallin and are not closely related to rodents.

Activity and tissue-specificity of γ-crystallin promoters in primary cultures of chicken NR and mouse LE cells

In order to test the ability of the rat γ-crystallin promoters to confer tissue-specificity to a reporter gene, all six promoter regions were fused with the chloramphenicol acetyl transferase (CAT) coding region (figure 4). The constructs were then transfected into embryonic chicken NR cells. As shown in figure 4 all six rat γ-crystallin CAT constructs yielded CAT activities significantly above background (as measured by transfecting the promoterless p⁻CAT vector). The constructs containing the reversed orientation of the γ-crystallin promoter region showed between 2 and 20% of the activity of the plus orientation, indicating that synthesis of CAT depends on a proximal γ-crystallin promoter. The range of activity of the γ-CAT constructs was 10 to 200% of the CAT activity yielded by pSV2CAT, with the γA promoter having the lowest and the γD promoter the highest activity. The low activity promoted by the γA-crystallin 5′ flanking region could be due to absence of sequences between −26 and the translation initiation site. As described below these sequences are essential for maximal activity of the γD promoter.

The correlation between crystallin promoter activity and transdifferentiation to lens-like cells was tested by comparing the activity of the γD-promoter with that of the SV40 promoter at different stages of this process. Expression of both constructs drops substantially during transdifferentiation, probably due to a decrease in transfection efficiency. However, relative to the activity of pSV2CAT, the rat γD-CAT construct shows an about tenfold increase in activity during transdifferention (figure 5A). Hence, although transdifferentiation is not a prerequisite for the recognition of the rat γ-crystallin promoters, it does improve the efficiency of expression of these promoters. Similar results were obtained by Kondoh *et al.* (21) using a δ-crystallin CAT construct.

In order to test the tissue specificity of the expression of the rat γ-CAT constructs in chicken cells, transfection experiments were performed with primary cultures derived from other tissues. Embryonic chicken fibroblasts, liver and brain cells showed no measurable expression of γC-CAT, while the expression of γD-CAT in these tissues varied from 5 to 10% of the activity found in the transdifferentiating cultures (data not shown).

The relative activities of the γ-CAT constructs in mouse LE cells are similar to those in chicken NR cells (figure 4). Only the γC-CAT construct appears to be considerably more active in mouse than in chicken cells. No activity was detectable for the constructs containing the reversed promoter orientation. As also observed in the chicken NR cells, the absolute amount of CAT activity obtained from the γD-CAT construct transfected into mouse LE cells decreased during differentiation. However, when the results were corrected for transfection efficiency by setting the activity of pSV2CAT at 100%, the γD-CAT shows a five fold higher activity in one week old cultures than in two days old cultures (figure 5B).

The tissue-specificity of the expression of the γ-CAT constructs in mouse cells was checked by transfecting mouse retina cells (which do not transdifferentiate) and fibroblasts. These cells did not yield significant CAT activity when transfected with any of the six γ-CAT plasmids (data not shown).

These results indicate that the rat γ-crystallin 5′ flanking sequences are sufficient to confer lens-specificity in both chicken NR cells and mouse LE cells and thus must contain the cis-acting sequences involved in lens-specific expression.

Mapping of the rat γD-promoter

Because of its high expression in both lens-like primary culture systems, the γD-CAT construct was chosen for a more detailed analysis of the cis-acting sequences necessary for tissue-specific expression. To this end deletion clones of the original 1217 bp γD insert were made either by cutting at unique restriction sites in the insert or by unidirectional digestion with exonuclease III (see Materials and Methods). The results of these experiments are summarized in figure 6 and show that deletion of 5′ flanking sequences up to −106 had no effect in chicken NR cells. Further truncation up to −78 lowered activity with 50%. Removal of another 5 bp to −73 had a dramatic effect on promoter function by reducing it to about 20% of the activity of the parental clone. Subsequent deletion of TATA box and surrounding sequences lowered promoter activity to background levels in these cells

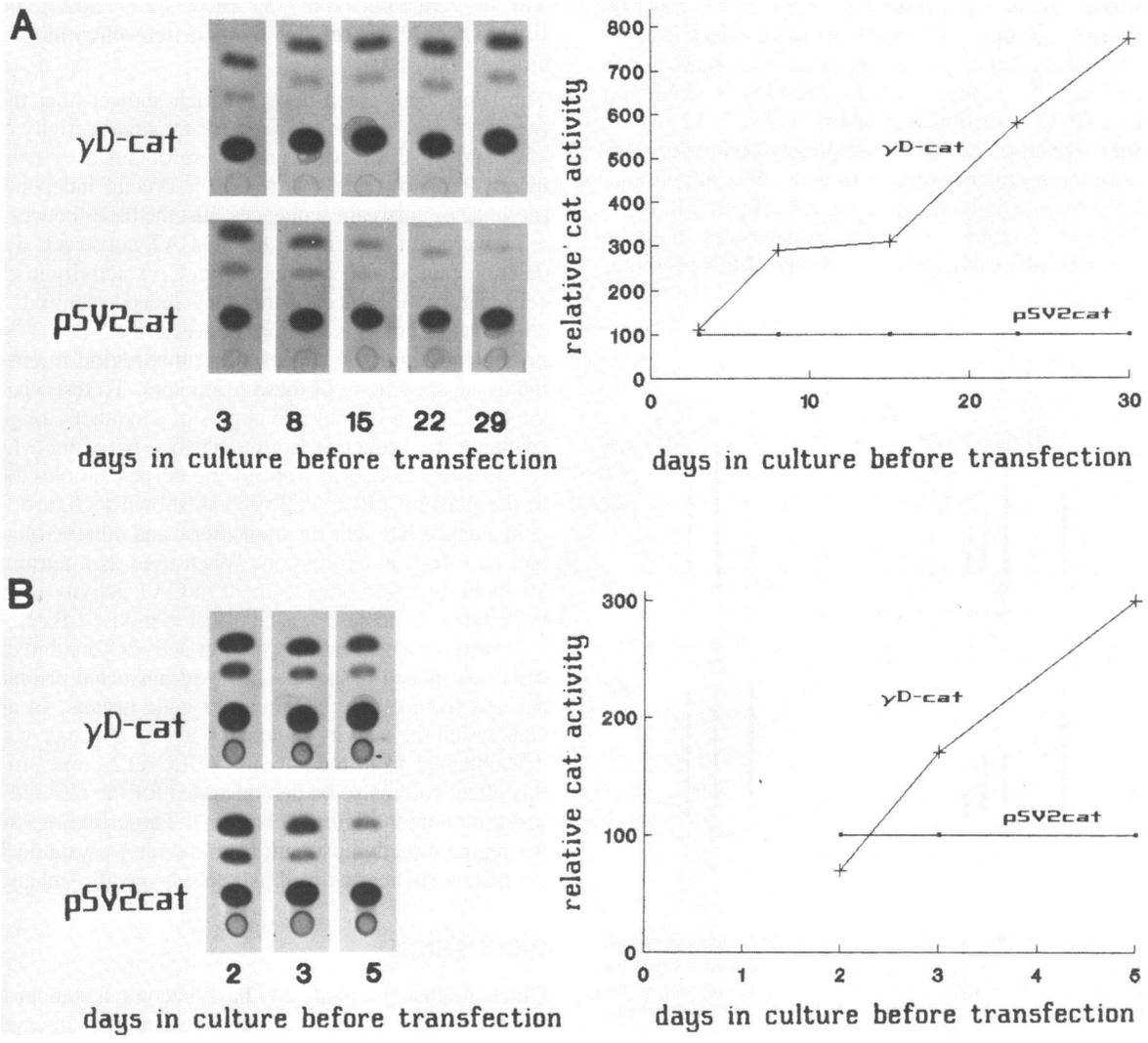


Figure 5. Relation between (trans)differentiation and expression of the γD-CAT construct. (A) The activity of γD-CAT and pSV2CAT was assayed in chicken NR cells after the time of culture indicated (left). The values shown in the right panel are relative to the activity of pSV2CAT, which was set at 100%. (B) The activity of rat γD-CAT and pSV2CAT in mouse LE cells after a period of *in vitro* culture as indicated (right). The values shown in the right panel are relative to the activity of pSV2CAT, which was set at 100%.

(figure 6A, left). The state of transdifferentiation did not affect the sequence requirement (data not shown).

The effect of progressive removal of sequences downstream from the TATA box starting at the junction between the γD promoter and the CAT gene are shown in figure 6A (right). In chicken NR cells deletion to +20 had no effect, but deletion to −9 diminished promoter activity to 20%. This result is rather puzzling, since, as will be shown below, the region between −85 and −67 is sufficient to drive a heterologous promoter in chicken NR cells.

In mouse LE cells deletion of the region between −200 and −106, which had no effect in chicken NR cells, already lowered promoter activity with 50%. Subsequent deletion to −78 did not affect activity, again in contrast to chicken NR cells, but further truncation to −73 had the same severe effect in mouse cells as in chicken cells and lowered promoter activity with another 50% (figure 6B, left). The downstream deletion of the region between +45 and −9, which virtually abolished activity in chicken NR cells, did not influence the expression in mouse LE cells (figure 6B, right). Hence, at least three regions of the γD promoter are differentially recognized by chicken NR and mouse LE cells: the region between −200 and −106 is recognized only in mouse LE cells while deletion of the region between −106 and −78 or between +45 and −9 affects activity in chicken NR cells only.

Comparison of γD, γE and γF promoter regions

The activity of the γD promoter in mouse LE as well as chicken NR cells is strongly dependent upon the sequence in the region around −75 (figure 6A,B). This region is conserved in all six rat γ-crystallin promoters and is even identical in the γD, γE and γF genes (figure 7). In spite of this sequence identity, the γD promoter is twofold (mouse cells) or fourfold (chicken cells) more active than the γE and γF promoters. To test whether upstream regions are responsible for the enhanced activity of the γD promoter, the activity of deletion clones of similar length of all three promoter regions was measured. As shown in figure 8, γD constructs containing only sequences up to the SstII site at position −78 or the ApaI site at position −73 were still two- to fourfold more active than the equivalent γE or γF constructs. These results indicate that the sequence responsible for the higher

promoter activity of the γD versus the γE/γF genes must be located between −73 and +45. Nucleotides in which the γD sequence differs from that of γE and γF in this region are boxed in figure 7. The γD region contains, besides 8 single bp substitutions, a GCG inser-tion at position −15 to −12, which creates another stretch of 12 nucleotides solely composed of C and G. Because the region between +45 and −9 is dispensable for maximal promoter activity in mouse LE cells (figure 6B, right) this CG rich region around −15 in the γD promoter must be responsible for the enhancement of the activity of this promoter in these cells.

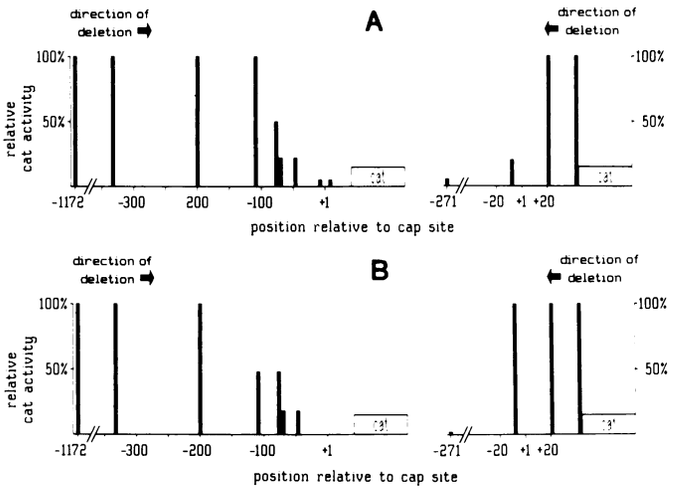


Figure 6. Deletion mapping of the rat γD promoter region. The activity of various deletion clones of the γD promoter region, constructed as described in Materials and Methods, is indicated by the height of the bars, while the position of the bars correspond to the end point of the deletion. Values are expressed relative to the activity of the parental γD-CAT construct (see figure 4), which was set at 100%. The activity of the clones was tested in chicken NR (A) as well as in mouse LE (B) cells.

The sequence between −85 and −67 is capable of inducing tissue-specific expression from a heterologous promoter in chicken NR cells

The results presented above, which showed that the CG rich region around −75 is required for maximal activity of the γD-, γE-, and γF-CAT constructs, were obtained by truncating the insert. To show that this region is crucial independent of the presence of upstream sequences, just the bases between −76 and −71 were removed from the γF-CAT construct. This five bp deletion caused a 60% reduction in CAT activity in chicken NR cells (figure 8). Hence this region is indeed required. The strong conservation of this region between the six rat γ-crystallin promoters (figure 7) suggests that this region might determine the tissue-specificity of these promoters. To test whether this is the case, single or multiple copies of a synthetic oligonuceotide spanning the sequence from −85 to −67 of the γD promoter region were cloned in front of the herpes simplex tk promoter in the plasmid pBLCAT2 (23) as shown in figure 9 (top).

In chicken NR cells the monomeric and dimeric oligonuceotide had no effect on expression. When used as a tetramer though, an about threefold enhancement in CAT activity is seen (figure 9, bottom). In no case was an effect in mouse LE cells detected.

To test if the increased promoter activity found in chicken NR cells was indeed tissue-specific we transfected primary cultures derived from other chicken embryonic organs. In all non-lens cells tested the activity obtained with the tetramer was less than that obtained from the parental pBLCAT2 clone. In liver cells this effect could also be demonstrated for the plasmid containing just a monomer (figure 9, bottom). These findings suggest that the region directly upstream from the rat γ-crystallin TATA box contains a silencer as well as a tissue-specific enhancer region.

DISCUSSION

Chicken lens epithelial cells have become a standard system to assay the sequence elements driving mammalian crystallin gene promoters (6,24−27). In agreement with these earlier results, we find that the transdifferentiating chicken NR cells specifically

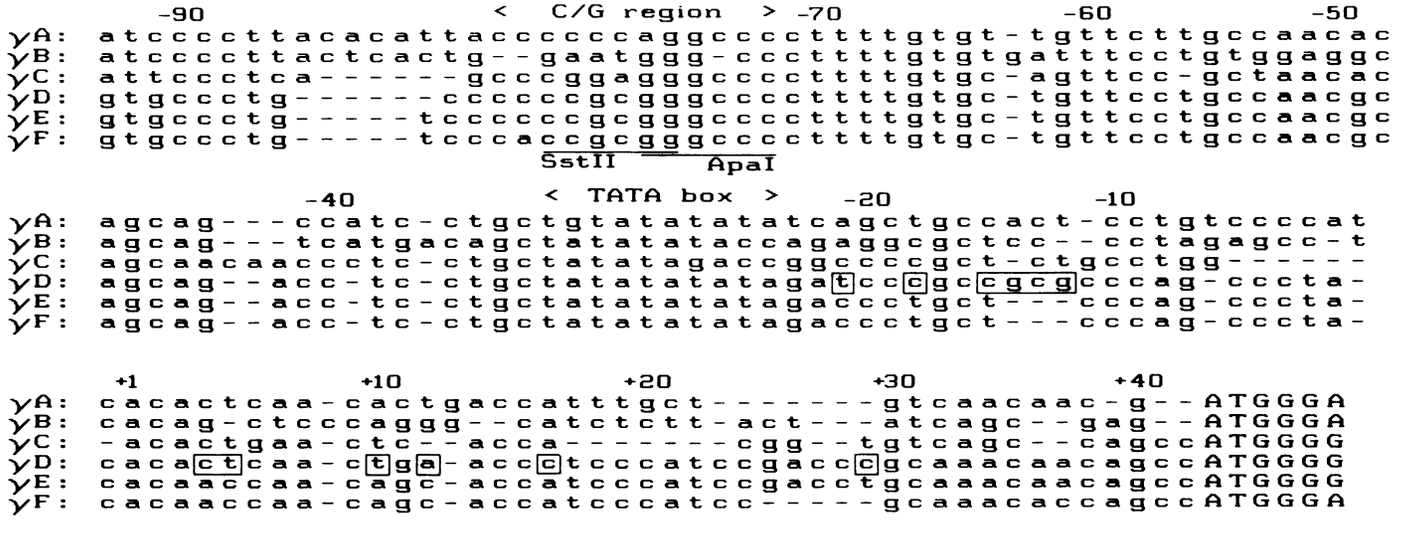


Figure 7. Comparison of the 5′ flanking regions of the rat γ-crystallin genes. The gaps introduced in the sequences for maximal alignment are shown by dashes. The ApaI and SstII restriction sites are underlined. The TATA box and CG region are indicated. Sequence numbering is relative to the cap site of the γD gene; the first digit indicates the base position. Differences between the γD and the γE/F sequences downstream from −70 are boxed. Gene flanking and non-coding regions are shown in lower case letters, the beginning of the coding region is capitalized. Sequences and their alignment were taken from Den Dunnen *et al.* (22).

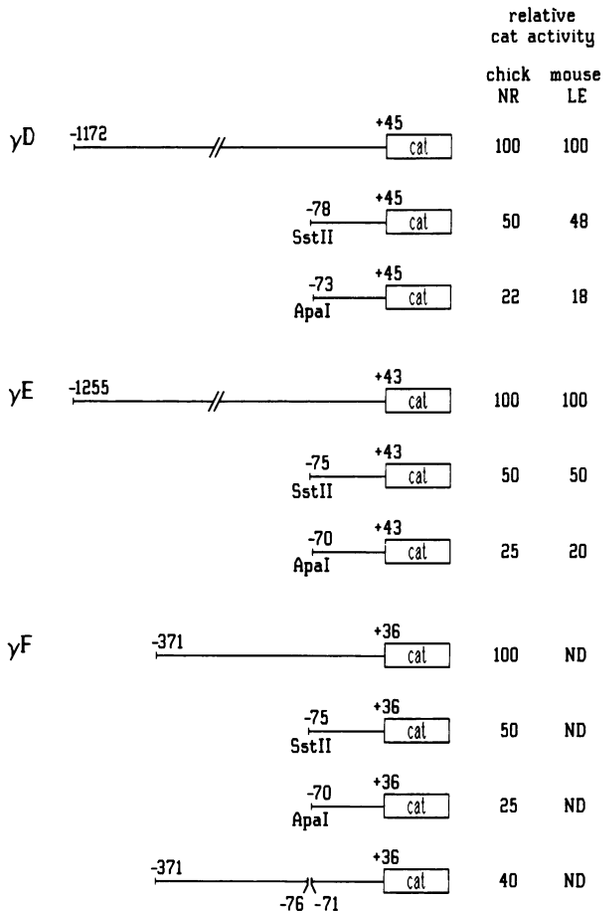
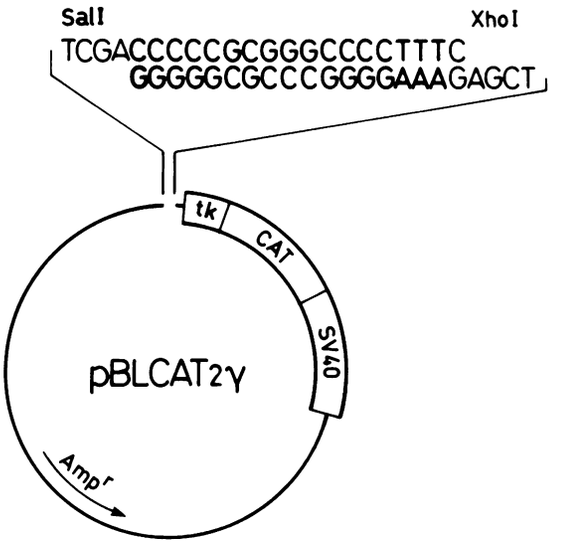


Figure 8. The relative activities of γD, γE and γF deletion clones. The activity of equivalent clones from the γD, γE or γF promoter regions was measured in chicken NR or mouse LE cells. Values are expressed relative to the activity of the parental clone (see figure 4).



cell type	insert	rel. CAT activity
mouse LE	→	100
	→→	100
	→←→	100
chicken NR	→	100
	←	100
	→←→	280
chicken skin	→	90
	→←→	40
chicken brain	→	110
	→←→	45
chicken liver	→	50
	→←→	40

Figure 9. The tissue-specific effect of the direct upstream CG rich region. (top) A synthetic oligonucleotide with a SalI and a XhoI sticky end and spanning the γD region from −85 to −67 was cloned in the SalI site of pBLCAT2, in which the CAT coding sequence is placed behind the HSV tk promoter (23). The −85 to −67 sequence is shown in bold. The orientation and the copy number of the inserted oligonucleotides was determined by restriction mapping. (bottom) The activity of different pBLCAT2γ constructs in the primary culture cells listed is shown as percentage of the activity of the pBLCAT2 parental plasmid. Arrows indicate the orientation and number of the inserted oligonucleotide.6

recognize the rat γ-crystallin gene promoters even before endogenous crystallin expression can be detected. Since chickens do not contain γ-crystallin genes, the rat γ-crystallin gene promoters must share recognition elements with chicken lenticular genes. The most likely candidates are the regulatory elements of the chicken β-crystallin genes, which are, as mentioned above, evolutionally related to the γ-crystallin genes (4) and show the same mode of regulation in the rat lens (5). Alternatively, the rat γ-crystallin genes may be recogni-zed in chicken cells by cellular factors involved in the expression of a distant member of the γ-crystallin gene family, the γS crystallin gene, which is present in chicken as well as in rats (28).

Since there is some doubt about the identity of the chicken lens transcription factors that recognize the rat γ-crystallin gene promoters, it is crucial to test and compare the expression of the γ-crystallin genes in a more homologous system, i.e. mouse LE cells. Our results provide strong evidence for species specific recognition of at least two regions of the γD promoter: the region between −200 and −106 which is required in mouse cells only, and the region between −106 and −78 of which deletion influences activity only in chicken cells. Whether the differences observed with more downstream deletions reflect significant differences in the way sequence motifs are recognized in mouse and chicken cells is more questionable. It is possible that the requirement for the region between −9 and +20 in the chicken cells only is an artifact of the assay system. The activity of the γB-CAT construct, in which the cap site is also deleted, shows that the presence of the authentic cap site is not an essential requirement for expression in chicken NR cells. The fact that the −85 to −67 γD region is capable of activating the tk promoter also shows that sequences downstream from the TATA box are not essential for expression. The deletion to −9 in the γD construct requires initiation of transcription within the CAT gene. It is possible that the sequence requirements for cap site

selection are more stringent in chicken cells than in mouse cells.

An apparently more serious discrepancy between the two systems is the lack of ability of the tetramer of the −85 to −67 region to drive the tk promoter in mouse LE cells, while it does so in chicken NR cells. First of all, it must be noted that a single copy of the oligomer is not sufficient to drive the tk promoter, while it is sufficient in case of the γ-crystallin promoter. This may be due to the close apposition of the oligomer to the distal SP1 binding site of the tk promoter. A similar observation has been made in another system, where it was proposed that steric hindrance due to binding at this site influenced the activity of an upstream enhancer (29). It may well be that the spatial requirements for interaction between presumptive cellular factors, the −85 to −67 region and the TATA box differ between chicken NR cells and mouse LE cells.

All the mapping data point to the importance of the CG rich sequence directly upstream from the TATA box in the expression of the γ-crystal-lin promoter in chicken NR as well as mouse LE cells. As the sequence correspondence between the six rat γ-crystallin gene promoters is high in this region, it is logical to assume that in all γ-crystallin gene promoters the same sequence is used. As shown in the alignment of the direct upstream sequence (figure 7), the only sequence motif shared by all six promoters is -GGCCC-. This sequence is part of the ApaI recognition site in the γF promoter. The removal of this site decreases activity of this promoter by 60%, strongly supporting the suggestion that -GGCCC- exerts a crucial function in the regulated expression of the γ-crystallin genes. The CG stretch around −15 in the γD promoter, which is shown to be indispensable for maximal activity in these same cells (figure 6), contains a -CGCCC- sequence which deviates from the -GGCCC- motif by a single base substitution. It is possible that these CG motifs are recognized by the same regulatory factors. Surprisingly Lok *et al.* (6) found that the region −122 to −30 of the mouse γ2 gene, which also contains this -GGCCC- sequence, was not sufficient to drive a heterologous promoter. This discrepancy could be due to their use of the relatively strong heterologous SV40 promoter, while we used the weak HSV tk promoter.

The inhibitory effect of the −85 to −67 region on the tk promoter activity in non-lens tissues further suggest that this regions contains a negative as well as a positive regulatory sequence. Similar silencer regions have been found in the promoters of other tissue-specific genes like the immunoglobulin heavy chain gene (30), the β-interferon gene (31) and the insulin gene (32,33).

In our mapping studies we found no evidence for the contribution of far upstream regions to the activity of the γD, γE and γF promoter in chicken NR cells. In contrast, Lok *et al.* (6) found that maximal activity of the mouse γ2 promoter, the ortholog of the rat γF promoter (34), in chicken lens explants is dependent upon sequences in the region −226 to −120. Apparently the few sequence changes in this region between rat and mouse are sufficient to abolish an enhancer effect of the corresponding rat region. This difference in promoter activity between these orthologous genes correlates well with the difference in the *in vivo* expression of these genes: the γF transcripts constitute only 15% of the γ-crystallin transcripts in the newborn rat lens (3), while, according to Murer-Orlando *et al.* (35), in the newborn mouse lens 50% of the γ-crystallin transcripts are γ2 transcripts.

The data presented here, together with those obtained by Lok *et al.* (6), indicate that the tissue-specificity and basal level of expression of the γ-crystallin genes are determined by conserved direct upstream sequences while further modulation is achieved through gene-specific enhancers such as found for the mouse γ2 gene in more upstream regions and for the rat γD gene between the TATA box and the cap site.

The results described in this report define transcriptional regulatory sequences within the 5′ flanking regions of the rat γ-crystallin genes. Although these sequences have proven to be sufficient for tissue-specificity, it can not be excluded that additional regulatory elements are present in inter- and intragenic regions, which could contribute to the time and site specific expression of the γ-crystallin genes in the eye lens. Our future studies will focus on these regions.

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