Regulation of Expression within a Gene Family

THE CASE OF THE RAT γB- AND γD-CRYSTALLIN PROMOTERS*

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The six closely related and clustered rat γ-crystallin genes, the γA- to γF-crystallin genes, are simultaneously activated in the embryonic lens but differentially shut down during postnatal development with the γB-crystallin gene, the last one to be active. We show here that developmental silencing of the γD-crystallin promoter correlates with delayed demethylation during lens fiber cell differentiation. Methylation silencing of the γD-crystallin promoter is a general effect and does not require the methylation of a specific CpG, nor does methylation interfere with factor binding to the proximal activator. In later development, the γD-crystallin promoter is also shut down earlier by a repressor that footprints to the −91/−78 region. A factor with identical properties is present in brain. Hence, a ubiquitous factor has been recruited as a developmental regulator by the lens. All γ-crystallin promoters tested contain upstream silencers, but at least the γB-crystallin silencer is distinct from the γD-crystallin silencer. The γ-crystallin promoters were found to share a proximal activator (the γ-box; around −50), which behaves as a MARE. The γB-box is recognized with much lower avidity than the γD-box. By swapping elements between the γB- and the γD-crystallin promoter, we show that activation by the γB-box requires a directly adjacent −46/−38 AP-1 consensus site. These experiments also uncovered another positive element in the γD-crystallin promoter, around −10. In the context of the γD-crystallin promoter, this element is redundant; in the context of the γB-crystallin promoter, it can replace the −46/−38 element.

The mammalian genome contains a large number of gene families, which encode related proteins with similar structure and function, yet are optimized for a particular developmental and differentiation stage. The pattern of expression of gene family members varies between families. For example, in the β-globin gene family, the paradigm of a clustered gene family, expression switches between members such that only one or two genes are active at the same time (for review, see Ref. 1). In contrast, the six clustered and closely related members of the γ-crystallin gene family (the γA- to γF-crystallin genes), which encode abundant structural proteins of the vertebrate lens, are all simultaneously active in the embryonic lens but switched off individually during postnatal development (2–5). In the rat, at 3 months of age the γB-crystallin mRNA is still present at 90% of the level at birth, whereas the transcript level from the γD-crystallin gene has dropped to 60%, and those of the γE- and γF-crystallin genes have dropped to 5% of the level at birth (4). As lens cells do not die and as the younger lens cells overlay the older cells, the consequence of this pattern of gene expression is the creation of a γ-crystallin gradient across the eye lens, which correlates inversely with the water gradient. This gradient in turn sets the gradient of refraction across the lens and thereby prevents optical aberration.

The mechanism of the developmental regulation of the γ-crystallin gene expression is not known. There is a strong negative correlation between the methylation state of a γ-crystallin gene promoter region and gene activity, suggesting that DNA methylation, or rather lack of DNA demethylation, is involved in silencing the genes (6). Differential expression or availability of transactivating factors is also likely to be causally involved in developmental regulation of expression. It is generally assumed that the closely related γA–F-crystallin genes share a common regulatory element that specifies the lens specificity of these genes. The prime candidate for such an element is the palindromic sequence (here denoted the γ-box) located upstream of the TATA box (Fig. 1). Mutations of this sequence abolish promoter activity in transfection studies (7, 11, 12). Furthermore, Goring et al. (13) have shown that a pentamer of the mouse γF-crystallin γ-box sequence directs lens-specific expression in transgenic mice. The expression of this construct, however, was restricted to the embryonic lens nucleus, and it was suggested that the wider range of developmental expression of the mouse γF-crystallin promoter is determined by upstream enhancers (13, 14).

The study of the regulatory mechanisms of crystallin gene expression is complicated by the peculiar mode of growth of the lens; the lens epithelial cells differentiate to lens fiber cells at the equator of the lens. The fiber cells of a late developmental stage but at an early differentiation state thus overlap fiber cells of an earlier developmental stage but at a later differentiation state. The lens is thus a mixture of cells at different developmental and differentiation stages. To obtain a fiber cell at a specific developmental and differentiation stage, we have made use of an in vitro differentiation system. In this system, the monolayer of epithelial lens cells, still attached to the lens capsule, is cultured in the presence of bFGF,1 which induces the differentiation of lens epithelial cells to lens fiber cells (Ref. 15; for review, see Ref. 16). The lens fiber cells follow the course of differentiation also seen in vivo, including the typical

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1 The abbreviations used are: bFGF, basic fibroblast growth factor; MARE, Maf recognition element; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; tk, thymidine kinase; HSV, herpes simplex virus.
changes in morphology and the accumulation of the various crystallins. The lens epithelial cells are aware of their developmental age and differentiate to fiber cells corresponding to that developmental age (17, 18). When explants are taken from newborn rats, copious accumulation of γ-crystallin is seen after about 10 days of in vitro culture (19–21). Lens explants isolated from older rats differentiate more slowly in vitro than those from younger rats and accumulate less γ-crystallin mRNA and protein (18, 21). In differentiating explants from 10-day-old rats, the γ-crystallin mRNA levels are only 1% of that seen in newborn explants, and below the level of detection in explants from 14-day-old rats (21).

In a previous study (7), we analyzed the course of activation of the γD-crystallin promoter during the in vitro differentiation of rat lens explants isolated from newborn rats. Demethylation of this promoter occurs within the first 2 days of in vitro differentiation, long before activation of the endogenous gene. The pulse of activity of the endogenous gene, between days 10 and 12 (21), was suggested to be regulated by the balance of activity of a transactivating factor binding the γ-box, first detected around day 6, and of a silencing factor, which appears around day 10 (7). To investigate developmental changes in these regulatory interactions, we have now followed the activation of the γD-crystallin promoter during in vitro differentiation of lens epithelial explants isolated from 10-day-old rats. We show here that in explants from these older rats, promoter demethylation is delayed, whereas the silencing factor appears earlier. We have further compared the γD-crystallin promoter with the γB-crystallin promoter, the promoter with the most extended developmental expression. We show that the γ-boxes of the γD- and γB-crystallin promoters, which resemble a Maf recognition element (MARE; Refs. 22 and 23), are recognized by the same factor, possibly a Maf protein, but that the affinity of the γB-box for this factor is much lower than that of the γD-box. Activity of the γB-crystallin promoter requires interaction with an AP-1 binding site directly downstream of the γB-box. Like the γD-crystallin promoter, the expression of the γB-crystallin promoter is subject to silencing, but the silencing factor differs from the one that represses the γD-crystallin gene.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Lens epithelial explants from newborn or 10-day-old (as indicated) Wistar rats were obtained essentially as described (24). Rat lenses were isolated in Medium 199 (Life Technologies, Inc.). The lens capsule together with the anterior monolayer of epithelial cells were peeled off the fiber cell mass and pinned down on a 3.5-cm Petri dish. Explants (three per dish) were cultured as described previously (7). Basic PGF (a kind gift from Scios, Inc., Mountain View, CA) was added to a final concentration of 25 ng/ml, and the cells were cultured for the indicated period prior to transfection.

**Isolation of Chromosomal DNA and Ligation-mediated Polymerase Chain Reaction (PCR)—**Isolation of chromosomal DNA from lens explants and ligation-mediated PCR was performed as described previously by Dirks et al. (7). In Vitro Methylation of DNA—GpC-methylation of DNA using SssI methylase was essentially according to the manufacturer’s protocol (New England Biolabs). Total reaction time was 4 h, whereby addition of enzyme and S-adenosylmethionine to the reaction mix was repeated after 2 h. Completeness of methylation was tested by a pilot digestion using Tth1 and electrophoresis through an agarose gel.

**Mutagenesis and Reporter Gene Constructs—**The template for mutagenesis was single-stranded DNA from a pBluescript II (SK+) (Stratagene) construct containing the γD-crystallin −1087/−48 fragment. Oligonucleotides for mutations were DB1 (5′-GCC GCT TTT GCT TGT AAG GCA GCA GC-3′ at position −64/−45), DB2 (5′-GCT TCT CCT GAC ACG GCA GCA GAC-3′ at position −61/−41), and DB3 (5′-CTG TCT CTG TCG AGG CAG CAG-3′ at position −63/−43) to obtain the mutants γD1, γD2, and γD3, respectively. Oligonucleotide DB4 (5′-GCC GCA GTC ATG ACA GCT ATA TAT ATA GAT C-3′ at position −46/−15) was used to mutate both the wild type γD-crystallin promoter and the mutated γDB3 promoter to obtain γD4 and γDB4, respectively, and oligonucleotide BD1 (5′-TGG AGG CAG GAC ACC TGC TGC TAT TAT GAG CAG TAC CAG CAC-3′ at position −55/−21) to obtain γDB1. Mutations were introduced using the Sculptor oligonucleotide mutagenesis kit (Amersham, UK). The BglII (−375)/FokI (+10) promoter fragment of each mutant was cloned into pEUCAT (25). Subsequently, replacement of the γD −10 region by its γF equivalent was performed by using the oligonucleotide DF (5′-TGT AGG GCT GGC AGG GTC TAT A-3′), representing the sequence at position −23 to +1 of the γF promoter, and the appropriate DNA fragment, GCT TGC TGT G-3′, priming upstream from the pEUCAT multiple cloning site, in a PCR reaction using wild-type and mutant γD promoter constructs as templates. PCR products were cloned into pEUCAT. All mutants were checked by digest/equilibrium analysis (26). To delete the upstream region, promoter constructs were truncated at the −73 ApaI site. Other promoter constructs used have been described previously (12).

**γD-Crystallin Silencer Promoter Constructs—**Oligonucleotides DS1 s (5′-TGG AGT GCC CTC CCC CCC GGC G-3′) and DS1a (5′-TGG ACC GCG GGG GGC AGG GCA C-3′) were annealed (27) and cloned into the SalI site of pBLCAT2 (28) to obtain construct pBLCAT2γDS1. The ApaI (−33)/BamHI fragment of pBLCAT2γ (8) was exchanged with that of pBLCAT2γDS1 to obtain pBLCAT2-DS1. The 5′-GC-5′ (−73), 5′-C-TGC TGT G-3′, 5′-AhXI(−183)/ApaI (−69), 5′-D-PuII (−193)/ApaI (−73), and γF-crystallin SpeI (−280)/ApaI (−70) blunt-ended/sticky fragments were inserted into pBLCAT2β by replacement of its HindIII (blunt)/ApaI fragment to obtain pBLCAT2β, pBLCAT2γ, pBLCAT2βγ, and pBLCAT2γF, respectively. Construct pBLCAT2βBS was obtained by deletion of the −414/−110 fragment from pBLCAT2β using the SalI site at position −10.

**DNA Transfection, Chloramphenicol Acetyltransferase (CAT) Assay, and β-Galactosidase Assay—**Plasmid DNA was isolated according to the alkali lysis procedure (27) in conjunction with either the Wizard Maxiprep System (Promega) or CaCl2 gradient centrifugation (27). DNA was transfected to the lens cells using either Lipofectamine Reagent (Life Technologies, Inc.) or the PDS-1000/He Biolistic Particle Delivery System (Bio-Rad). When cells were transfected with Lipofectamine per dish 2.0 μg of CAT reporter construct and 0.25 μg of CMV/lacZ construct (29) were transfected to the cells according to the manufacturer’s protocol. Using the Biologic Particle Delivery System, 0.5 μg of CAT reporter construct and 0.125 μg of CMV/lacZ construct was coated on 1-μm gold particles and bombarded on the cells at 450 psi helium. At least 3–5 rounds were used in the presence of 25 ng/ml forskolin. The cells were harvested in 100 μl of reporter lysis buffer (25 mM bicine, pH 7.8, 0.05% Tween 20, 0.05% Tween 80) per dish, and vigorously shaken for 10 min. The cell debris was pelleted in an Eppendorf centrifuge. To determine transfection efficiency, 20 μl of the supernatant was used to assay for β-galactosidase activity (29). The remainder of the supernatant was heated for 15 min at 65 °C to inactivate cellular deacetylases. 10 μl of supernatant, 20 μl of 1 M β-mercaptoethanol, and 20 μl of β-galactosidase assay solution (29) were incubated overnight at 37 °C as described by Gorman et al. (30) or using the Quan-T-CAT system (Amersham). Transfections were done in duplo or triplo, and two DNA isolates from each construct were tested in independent experiments.

**Electrophoresis Mobility Shift Assay (EMSA)—**Nuclear extracts were prepared as described previously (12). EMSAs were performed essentially as described (31, 32). DNA restriction fragments were size-fractionated through a native 6% polyacrylamide gel and isolated by electro-elution using a Bio-Trapp apparatus (Schleicher and Schuell), according to the manufacturer’s protocol. Approximately 0.1–0.5 ng of end-labeled probe (10,000–20,000 cpm) was added to 5–10 μl of nuclear extract (5 μl, final concentration of 100 μM NaCl) and either 1.0 μg of poly(dIdC)·poly(dIdC) or 1.0–3.0 μg of poly(dIdC)·poly(dIdC), as indicated, in binding buffer (final concentrations 20 μM HEPES, pH 7.9, 10–50 μM KCl as indicated, 1 mM EDTA, 1 mM DTT, 4% (v/v) Ficoll) in a total volume of 20 μl. The reaction mixture was left for 10 min at room temperature, loaded on a pre-run 4% (v/w) polyacrylamide gel in 0.25 × TBE (1 × TBE = 89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA), which then was run for 2 h at 100 volts/cm. The gel was dried and exposed to a Fujix AX film overnight with one intensifying screen.

In Vitro Footprint Analysis—The γD-promoter fragment was 32P-labeled at one end. An aliquot (600,000 cpm) was methylated using dimethylsulfate (DMS) essentially as described (33, 34). The methylated probe was incubated with 150–200 μl of nuclear extract (150–400 μg of protein) for a preparative gel retardation assay (analytical assay 30–40-fold scaled up). The complexed and the free probe were visualized by autoradiography overnight. The DNA was cut out of the gel,
isolated by electro-elution as described above, cleaved by piperidine (final concentration 10% (v/v)), and size-fractionated in a 15% sequencing gel. The gel was dried, and exposed to a Fuji AX film for 18–72 h.

RESULTS

Demethylation of the γ-D-Crystallin Promoter Region—In explants from newborn rats, the γD promoter region is fully demethylated between day 1 and 2 of in vitro differentiation (Ref. 7; see also Fig. 2A). To test whether promoter demethylation still occurs in 10-day-old rat explants, in which the level of expression of the γD promoter is about 50-fold lower, the state of methylation of the genomic Thal site at position −13 of the γD promoter was followed during in vitro differentiation. In a parallel experiment, the methylation state of this Thal site in explants from newborn rats was tested. In newborn rat explants, virtually complete demethylation of the Thal site in γD promoter region was found after 2 days of culture, in agreement with the results of Dirks et al. (7). In contrast, demethylation of this site was significantly slower during differentiation of explants from 10-day-old rats (Fig. 2A). Even after 5 days of differentiation, demethylation was only 65% complete.

To determine the effect of DNA methylation on γD promoter activity, a γD(−73/+10)CAT fusion gene was methylated using CpG methylase and transfected into explanted lens cells. We found a strongly reduced activity of the methylated construct; activity was only 1% of that of the unmethylated promoter and within background levels (Fig. 2B). Although the CAT coding region is also methylated in these experiments, other studies have shown that DNA methylation does not impede elongation (35–37) and that methylation of the CAT coding sequence does not affect transient expression (6, 38). Hence, the effect of CpG methylation is likely to block γD promoter activity, although from our own experiments, we cannot rule out an aspecific effect.

The γD promoter contains a CpG site in its proximal activator, the γ-box, located around −50 (see also Fig. 1). To test whether methylation of the γ-box element is sufficient to block binding of the cognate activating factor in vitro, the binding of rat lens nuclear extract factors to a methylated promoter fragment was compared with that to a nonmethylated fragment in an EMSA. Complex formation with the methylated fragment was reduced when compared with that of the unmethylated fragment, but not abolished (complex D1; Fig. 2C). This was confirmed by the fact that the methylated fragment competed for the activator complex as efficiently as the unmethylated fragment itself. In the EMSAs using the methylated γD promoter fragment, an additional band is seen (complex D2; note that this complex migrates slower than the faint aspecific complex seen in some of the other lanes), this band could represent binding to the methylated DNA by general McpG binding proteins.

These data suggest that methylation of the γ-box is not sufficient to suppress promoter activity. We therefore tested the effect of methylation on the activity of mutant constructs, lacking either the CpG site at −5 to −20 or the A promoter sites between −20 and −10. The activity of these mutant γD promoters, when methylated, was also in the background range (Fig. 2B). Similarly, the activity of the γC promoter was also very low when methylated. Together, our results indicate that the reduction in γD-crystallin promoter activity by methylation is a general effect and not due to methylation of a specific site.

Appearance of Trans-acting Factors during in Vitro Lens Cell Differentiation—We have previously proposed that the demethylation stage-specific expression of the γD-crystallin gene during fiber cell differentiation was regulated by the phased appearance of first an activating and then a silencing factor (7). The reduced activity of the γD promoter in explants of 10-day-old rats could be due to a changed expression profile of these factors. Therefore, the activities of the γD(−73/+45)CAT fusion gene and of a silencer-tkCAT construct were followed during the course of differentiation of 10-day-old explants. The γD(−73/+45)CAT construct was active at all stages of differentiation, with a maximum around day 12 (Fig. 3A). The timing of up-regulation of the γD activating factor in these explants is very similar to that in explants from newborn rats (see Ref. 7). However, the activity of the γD(−73/+45)CAT construct in 10-day-old explants was around 50% of that in explants from newborn animals (data not shown), indicating that the level of the activating factor is decreased in the older explants.

Rather different results were obtained when the presence of the silencing factor was assayed; the construct containing four copies of the silencing element in front of the HSV tk promoter was inactive even in early differentiated cells from 10-day-old rats (Fig. 3B), indicating that the silencing factor is present throughout differentiation of these cells. In contrast, in explants from newborn rats, silencing activity was maximal only after 10 days of in vitro differentiation (Ref. 7; see also Fig. 2B). The level of silencing in fiber cells from 10-day-old rats was not significantly different from that during late differentiation of cells from newborn animals. The earlier appearance of the silencing factor might well explain the reduced activity of the γD gene in the 10-day-old explants.

The γD-Crystallin Silencing Factor—The γD silencer was originally found by chance, when testing the effect of the −84/−71 G/C-rich region conserved among the γ-crystallin promot-
Control of γ-Crystallin Gene Expression

Fig. 2. Demethylation of the γD-crystallin promoter during development. A, analysis of the methylation state of the genomic Thal site at position −13. Lens explants from both newborn and 10-day-old rats were cultured in the presence of bFGF and harvested at several stages during differentiation. Chromosomal DNA was isolated as described previously (7) and digested with both Thal (cuts at −13) and Sau3A (cuts at −23). The DNA was amplified by ligation-mediated PCR using three primers in succession (from +85/ +67, +42/ +21, and +42/ +19; for details see Ref. 7) and visualized by electrophoresis and autoradiography. PCR products from methylated and unmethylated DNA are indicated. Both the autoradiograph and the quantitated data are shown. B, activity of methylated (mutant) γD- or γC-crystallin promoters. Wild-type and mutant −73/+10 γD-crystallin promoter constructs or the −70/+28 γC promoter construct were in vitro methylated using CpG methylase (Sso1 methylase). Methylated and mock-methylated constructs were transfected to explants pre-cultured for 10 days in the presence of bFGF. The explants were cultured for three more days before harvesting (see “Experimental Procedures” for details). Activities of the non-methylated γD and γC promoter constructs were set at 100%; the activity of γDB2 or γDF is given relative to that of γD. The bars indicate the standard deviation. C, factor binding to the CpG-methylated γ-box. Methylated and unmethylated γD (−73/+45) fragments were used as probes, as indicated. Completeness of DNA methylation was tested by digestion (Thal), and factor binding was examined in the absence (−) and presence (+) of nuclear extract from newborn rat lenses. Binding was in the presence of 50 mM KCl and 50 ng/μl poly(dG:dC-dG:dC). Complex D1 represents the γ-crystallin activator complex (γ-box complex), as confirmed by methylation interference footprint analysis using the −73/+45 fragment (results not shown). Complex D2 might represent binding of methylation interference footprint analysis using the −73/+45 fragment (results not shown). Complex D2 might represent binding of Methyl CpG-binding protein, as it is found only with the methylated probe. Specific competitor DNA was added in a 100-fold molar excess (right two lanes).

Fig. 3. Activities of the proximal γD-crystallin promoter and its silencer element during differentiation of explants from 10-day-old rats. Transfections were done as described under “Experimental Procedures.” The dotted lines represent the activities obtained using newborn rat lens explants as reported previously (7). A, activity of γD(−73/+45)/CAT transfected into explants from 10-day-old rats at several stages of bFGF-directed differentiation. Activities are shown relative to that of the maximum level (100%). The bars indicate the standard deviation. B, activity of pBLCAT2-γD, containing four copies of the −85/-67 silencer region in front of the tk promoter, transfected to explanted lens cells from 10-day-old rats at several stages of differentiation. Activities are shown relative to that of the parental construct pBLCAT2, which was set at 100% (not shown). The bars indicate the standard deviation.

A synthetic copy of the −91/−78 element silenced the heterogeneous tk promoter by about 65% (Fig. 5A; γDS1), which corresponded to the silencing activity of a larger promoter fragment (γD), whereas the conserved G/C-rich region (−84/−68; γ) silenced by about 25%. Thus, the −91/−78 element is the silencer element within the γD promoter. The in vivo ac-
is as reported by Den Dunnen et al. (9) was \(^{32}\)P labeled at either end and used to bind nuclear factors from either newborn rat lens or brain. Results using both free (F) and bound (B) DNA are shown. Sites of protein contacts are indicated by asterisks.

A. Methylation interference footprint analysis of the \(\gamma\)-D-crystallin \(-80\) region complexed by either lens or brain nuclear factors. \(\gamma\)-D-crystallin \(-106/+45\) promoter fragment containing mutation \(-46G\rightarrow T\), thus abolishing factor binding to the \(\gamma\)-box; see Ref. 7) was \(^{32}\)P labeled at either end and used to bind nuclear factors from either newborn rat lens or brain. Results using both free (F) and bound (B) DNA are shown. Sites of protein contacts are indicated by brackets. B, summary of footprint analyses shown in A. Residues involved in factor binding are specified by asterisks. Nucleotide sequence is as reported by Den Dunnen et al. (9). C, estimate of the molecular weights of gel-retarded DNA/protein complexes as described by Orchard and May (39). A cloned \(^{32}\)P-labeled oligonucleotide containing the \(-91/-78\) \(\gamma\)-D-crystallin silencer element (\(\gamma\)DS1; see Fig. 5) was bound to either lens or brain nuclear extracts. EMSAs were run together with native standard proteins in a series of gels with increasing polyacrylamide concentration. The relative migration of the DNA/protein complexes was determined.

activity of the original tetramer silencer construct (Ref. 7; see Fig. 3B), although containing only part of the silencer element is probably due to the fact that the multimerization of the \(-84/-68\) sequence by chance partially provided the missing part of the element.

The Common Proximal Activator of the \(\gamma\)-Crystallin Promoter—The alignment of the proximal promoters of the \(\gamma\)-crystallin genes shows that the \(\gamma\)-box is a well conserved element (Fig. 1) and predicts that all \(\gamma\)-crystallin promoters, with the possible exception of the \(\gamma\)B promoter, bind the same factor. Indeed, nuclear factor binding to the \(\gamma\)D promoter is efficiently competed for by the \(\gamma\)C and the \(\gamma\)F promoter (data not shown). The \(\gamma\)B promoter fragment, however, competed poorly for binding (Fig. 6A). As the \(\gamma\)B promoter is also the one with the most extended developmental expression, we analyzed the \(\gamma\)B promoter element in more detail.

A promoter fragment containing the \(\gamma\)B-box yielded two complexes in an EMSA. The lower complex (B2) was competed for by the \(\gamma\)B but not by a \(\gamma\)D fragment and thus appeared to be \(\gamma\)B-specific (Fig. 6B). The upper complex (B1) comigrated with the single complex formed by the equivalent \(\gamma\)D promoter fragment (not shown) and was also competed for efficiently by this \(\gamma\)D fragment (Fig. 6D), suggesting that this complex represents the \(\gamma\)B \(-57/-46\) activator (\(\gamma\)B-box) complex. Competition by the \(\gamma\)D fragment for either the \(\gamma\)D-box (Fig. 6A) or the \(\gamma\)B-box complex (Fig. 6B) was very poor, confirming the relatively low affinity of this \(\gamma\)B promoter element for factor binding. To confirm the conclusion that complex B1 (see Fig. 6B) represents binding to the \(\gamma\)B \(-57/-46\) region, this complex was mapped by \textit{in vitro} footprinting. As shown in Fig. 6C (left panel), the B1 complex is indeed the result of factor interaction between positions \(-55\) and \(-46\). \textit{In vitro} footprinting of complex B2 showed that the binding site in this complex is the \(-46\) to \(-38\) region (Fig. 6C, right panel), directly adjacent to the \(\gamma\)B-box. The B2 footprint corresponds to a consensus AP-1 site (Fig. 6D). A second AP-1 site is located directly downstream, but no interaction with this site was seen \textit{in vitro}.

To understand the functional significance of the low affinity binding by the \(\gamma\)B-box, the \(\gamma\)D-box was exchanged for the corresponding \(\gamma\)B element. Mutating the \(\gamma\)D-box in the \(\gamma\)D\(-375/+10\)CAT construct successively to the equivalent \(\gamma\)B sequence led to a gradual decrease in promoter activity (Fig. 7A, left panel), showing that the \(\gamma\)B-box is a less activator than the \(\gamma\)D-box. The drop in activity is sharpest between mutant constructs \(\gamma\)DB1 and \(\gamma\)DB2, nicely corresponding to the \textit{in vitro} binding affinity of these mutants; \(\gamma\)DB1 still efficiently competes with the \(\gamma\)D sequence, but \(\gamma\)DB2 no longer does so (Fig. 7B). An even more dramatic effect of mutating the \(\gamma\)D-box to the \(\gamma\)B-box is seen when the upstream region is deleted from the \(-375/+10\) constructs (Fig. 7A, right panel). Deletion to \(-73\) in the wild-type \(\gamma\)D promoter is also the one with the most extended developmental expression, we analyzed the \(\gamma\)D promoter for the \(\gamma\)D-box (Fig. 6D). A second AP-1 site is located directly downstream, but no interaction with this site was seen \textit{in vitro}.

The functional significance of the AP-1 consensus site at \(-46\) to \(-38\) in the \(\gamma\)B promoter was tested by mutating this sequence to the corresponding \(\gamma\)D sequence (\(\gamma\)DB1; Fig. 7C). This mutation severely decreased promoter activity of the \(\gamma\)B promoter, showing that the AP-1 site acts as an activator (Fig. 7C). This was confirmed by the reciprocal construct, in which the \(-46\) to \(-38\) \(\gamma\)B element was introduced at the corresponding site in the \(\gamma\)D promoter (\(\gamma\)DB4); again, this element functioned as an activator, as a 2-fold increase in promoter activity was the result. Finally, we tested the effect of a combination of both the \(\gamma\)B \(-57\) to \(-46\) and \(-46\) to \(-38\) elements in the \(\gamma\)D promoter. As expected, this construct (\(\gamma\)DB4) had the same low activity as the \(\gamma\)B-promoter itself.

The \(\gamma\)D-Box Is a MARE—Kataoka et al. (22) first suggested that the \(\gamma\)B-box might be a Maf recognition element (MARE). This suggestion is supported by the experiments reported by
Ogino et al. (40). We have therefore tested whether the factor binding to the γD-box also binds the MARE consensus sequence. As shown in Fig. 8, in an EMSA using lens nuclear extract, a γD promoter fragment competes efficiently with binding to a consensus MARE. In addition, the mobility of the γD-box complex is the same as that of a MARE complex (data not shown), suggesting that the γD-box does indeed bind a Maf, at least in vitro.

Two Maf sequences have been reported thus far to be present in the rat lens, Maf-1 and Maf-2 (41, 42). Maf-2 is expressed in lens fibers but not in epithelium, whereas Maf-1 is also present in epithelial cells. Cotransfection of expression constructs for Maf-1 or Maf-2 and γD(-73/+10)CAT into differentiating lens explants showed that the γD promoter activity was stimulated about 2-fold by Maf-2 but not Maf-1 (data not shown). Hence, the factor binding the γD-box might well belong to the Maf family.

The γD -10 Element—The data presented above show that the γB-box can function as an activator only in conjunction with the downstream -46/-38 element. Yet, the γDB3 mutant, which lacks this activator, still retains activity, albeit low (see Fig. 7A). We therefore wondered whether an additional activating element is present in the -73/+10 γD promoter, which, in cooperation with the low affinity γDB3-box, drives promoter activity in this mutant. Genomic footprinting of the rat γD promoter had revealed a protected site downstream of the TATA box: the GC-rich -10 region (Ref. 7; see Fig. 1). The nucleotide sequence of this region is unique to the γD promoter and absent from the otherwise nearly identical γE and γF promoters. To test the effect of the γD -10 element, we constructed γD/γF promoter chimeras by replacing the γD TATA box and downstream region with the γF equivalent, causing the mutations -21(T→C), -18(C→T), and -15 to -12(CCGG→T—). The latter series of four mutations is situated within the in vivo footprint sequence mentioned above (see Fig. 1). In addition, for practical reasons, the 5’ noncoding sequence was truncated from -10 to +1.

The activity of the -375/+1 γDF construct was not significantly lower than that of the wild-type γD construct when transfected into explanted lens cells (Fig. 9). However, shortening to -73 caused a drop in activity to about 20% of the corresponding γD construct. Introduction of the γB-box in the -73/+1 γDF promoter inactivated it, whereas mutation of the G/C-region in construct γD4 (see also Fig. 7C) strongly decreased promoter activity. Hence, the γD -10 region acts as an activator. These data further show that the γD-box is a relatively poor activator and needs additional elements for full activity. In the γD promoter, such elements are located between -375 and -73 and around -10. In the context of our experiments, these elements are redundant. Finally, our results confirm the observation that the γB-box is inactive in the absence of other positive elements. However, the positive element does not need to be positioned closely to the γB-box, as is the -46/-38 element in the γB promoter, but can also be located at a distance, as is the -10 region in the γDB3 construct.

Differences between the γB- and γD-Crystallin Silencers—We have shown above that the γ-crystallin genes share the γ-box activator. The question arises whether they all have silencers, as predicted from the in vivo mRNA levels (see Ref. 4) and, if so, whether these silencers are common or specific. To examine the presence of silencer elements within the γ-crystallin promoters, we determined the silencing activity of the upstream regions of the γ-crystallin promoters (from position -69 on the heterologous HSV tk promoter. All promoter regions tested repressed activity of the tk promoter when transfected into explanted lens cells, indicating that in all of these promoters, a functional silencing element is present (not shown). Again the γB sequence is most divergent and was selected for further analysis.

The -414/-69 γB fragment, which showed silencing activity in transient transfections, could be deleted to -110 without loss of silencing activity in lens cells during terminal differentiation (not shown), indicating that the γB silencer element must be located between -110 and -69. To test whether the γB and the γD silencing regions bind the same or different factors, the mobility of the γB and γD complexes was compared (Fig. 10A). Two γB complexes were found, both with mobility higher than that of the single γD complex, suggesting the formation of different γB and γD complexes. The non-identity of the complexes was confirmed by competition assays; the γD fragment did not compete for the γB complexes nor did the γB fragment compete for the γD complex (Fig. 10A).

In ewbhorn explants, the γB silencer is active only in terminally differentiated fiber cells (Ref. 7; see also Fig. 5B). To determine whether the γB silencer shows the same differentiation-dependent expression, both the γB(-414/-69)-tkCAT and γB(-110/-69)-tkCAT fusion genes were transfected to in vitro differentiating lens fiber cells. Like the γD element, the γB silencer demonstrated differentiation-dependent recognition, as silencing activity was present only during a restricted period of differentiation (Fig. 10B). However, this silencing activity was apparent already between days 4-7 of differentiation and continued through the terminal stage of differentiation. The -110/-69 fragment demonstrated silencing activity only during terminal differentiation, similar to the
Control of γ-Crystallin Gene Expression

**FIG. 6.** The γB-crystallin γ-box region. A, EMSAs showing binding of the 32P-labeled γD-crystallin −73/+45 promoter fragment in lens nuclear extracts (+), competed with the equivalent γB-crystallin promoter fragment. Binding was in the presence of 50 mM KCl and 50 ng/μl poly(dGdC-dGdC). Specific competitor DNA was added in a 10–200-fold molar excess, as indicated. In the first lane (−), no extract was added. Only the region of the autoradiograph with bound probe is shown. See “Experimental Procedures” for details. B, reciprocal competitive EMSAs using the 32P-labeled γB-crystallin −73/−16 promoter fragment as a probe. See A for further details. Note the appearance of an additional specific complex (B2) migrating faster than B1. C, in vitro methylation interference footprints of the upper (coding) strand. The γB-crystallin −73/−16 fragment was methylated and bound to nuclear factors from newborn rat lenses. See “Experimental Procedures” for further details. Sequence ladders of both bound (B) and free (F) probes are shown. Positions of the G residues relative to the transcription start site are indicated. Sites of protein contact are marked by brackets. D, summary of the results shown in C. G residues involved in protein interaction are marked by asterisks. Binding sites in complexes B1 and B2 are indicated. AP-1 consensus binding sites are specified by arrows. Nucleotide sequence and numbering are according to Den Dunnen et al. (9).

**FIG. 7.** γB-crystallin promoter elements. A, functional comparison of the γB- and γD-boxes and mutant intermediates. The γ-box sequence in a γD(−375/+10)CAT construct was successively mutated to its γB equivalent yielding constructs γDB1 to γDB3 (left panel). γB-like sequences are underlined. These constructs and a γB(−414/−16)CAT construct were transfected into explanted lens cells from newborn rats, following a preculture period of 10 days in the presence of bFGF. Cells were cultured for three more days before harvesting. Promoter activities were determined as described under “Experimental Procedures.” Similar experiments were performed with constructs in which the upstream sequences were deleted to −73 (right panel). All activities shown are relative to that of the parental γD(−375/+10) promoter construct (100%). The bars indicate the standard deviation. B, EMSA showing binding of 32P-labeled wild-type γD(−73/−10) fragment and lens nuclear extract (+) competed with the γD fragment, the equivalent γB fragment, or mutant fragments (see A). Binding was in the presence of 50 mM KCl and 50 ng/μl poly (dGdC-dGdC). Specific competitor DNA was used in 100-fold molar excess. In the first lane, no extract was added (−). Free (F) and bound (B) probes are indicated. C, functional analysis of the γB2(−46/−38) region. By in vitro mutagenesis, the γB−40 region was exchanged for its γD counterpart yielding construct γBD1. The reciprocal experiment yielded construct γD4. Subsequently, the γB-box was introduced in the latter construct, yielding construct γDB4. γB-like sequences are underlined. Arrows point to mutated nucleotides. Constructs were transfected into explanted lens cells from newborn rats, which had been precultured for 10 days in the presence of bFGF. After three additional days of culture, the cells were harvested, and promoter activities were assessed as described under “Experimental Procedures.” Activities are shown relative to the wild-type γD construct (100%). The bars indicate the standard deviation.
γD silencer. Although the extent of silencing by the −110/−69 fragment is similar to that of the larger −414/−69 fragment during the course of differentiation, surprisingly, in early differentiation it strongly activated the tk promoter. Hence the −414 to −69 region of the γB-crystallin gene must contain additional enhancers and silencing elements.

**DISCUSSION**

A simple mechanism for developmental regulation of the promoter activity of the γ-crystallin genes would be that the level of activity is determined by the affinity of a common activating factor for the proximal activator, the γ-box. One would then predict that a γ-crystallin promoter that is shut down early in development (e.g. γE, γF) would have a low affinity γ-box, whereas a gene of which expression continues until later in development would have a high affinity γ-box. Our data clearly show that this hypothesis is not correct; we find that the γ-crystallin gene with the most sustained expression during development, the γB gene, has the γ-box with the lowest affinity.

Comparison of the γB-box sequence with that of the γD-box shows that in the γB-box the C at −54, which shows a protein contact in vivo (7), has been replaced by a T. This suggests that it is the 5'-half of the binding site in the γB-box that is responsible for the low affinity. However, our results show that even such a scrambled site is sufficient to target the corresponding cognate factors to the promoter, providing that additional activating elements are present. The additional activating element can be either a closely linked AP-1 site, as in the γB promoter, or the more distant up- and downstream elements of the γD promoter. Hence, there is no constraint on either the nature or the distance of the additional activating element.

The γ-box resembles a MARE in sequence, and binding of a cognate factor in lens extracts is competed by a consensus MARE sequence. Furthermore, the γD promoter is slightly activated by cotransfection of an expression vector for Maf-2. Hence, the in vivo activator of the γ-crystallin promoter might well belong to the Maf family of transcription factors. Interaction of the mouse γF-box with a (chicken) zinc finger protein has also been reported (11). However, this protein acts as a transcriptional repressor rather than as an activator.

The Maf family is a diverse one with small and large members (for recent reviews, see Refs. 43–45). The large members, which include the founding member of this family, v-Maf, have an N-terminal acidic activation domain. The small members, such as MafF, MafG, and MafK, lack this activation domain and can activate transcription only as heterodimers with, for example, a large Maf member or a member of the AP-1 family (e.g. see Refs. 22, 23, 45). Two large Maf family members have thus far been shown to be present in the rat lens. Maf-1 is the rat homologue of the mouse MafB, and Maf-2 may be the homologue of the chicken c-Maf (41, 42). Maf-1 mRNA is primarily found in the lens epithelial layer; Maf-2 mRNA is prominent in the fiber cell mass, the site of expression of the γ-crystallin genes. Expression of Maf-2 is not limited to the lens, since Maf-2, as well as Maf-1, is also found in many other tissues of the body such as kidney, spleen, and liver. Hence, a role for Maf-2 in the expression of the γ-crystallin promoters is seemingly at odds with the lens specificity of these promoters in transgenic mice (13, 14) or even in transgenic *Xenopus laevis* (46). However, Maf-2 could partner a lens-specific protein, and the role of Maf-2 in directing lens-specific expression could be analogous to that of Mafk in erythroid-specific transcription; MafK acts as the partner of the erythroid-specific transcription factor NF-E2 (47). Alternatively, the "true" activator of the γ-crystallin promoters could be another Maf protein. A possible candidate would be the rat homologue of L-Maf, a lens-specific protein, which includes the LDL receptor promoter in vivo (12).

**FIG.8.** The γD-box resembles a MARE. EMSA showing binding of a double-stranded MARE consensus oligonucleotide (TGCTGACTCAG) and lens nuclear extract from newborn rats (+) competed for with a γD(−73/+10) fragment. As control, competition with the MARE sequence is also shown. Binding was in the presence of 50 mM KCl and 50 μg/μl poly(dGdC·dGdC). Specific competitor DNA was added in 10- and 100-fold molar excess. In the first lane, no extract was added (−). Free (F) and bound (B) probes are indicated.

**FIG.9.** The γD-crystallin −10 region acts as an activator. In the γD-crystallin constructs indicated (see also Fig. 7), the TATA box and downstream region were exchanged for the γF equivalent, and the −375/−73 region was deleted. The γD-box is denoted by γ-box, in γDB3 the γD-box has been mutated to the γB-box sequence, B2 represents the AP-1 consensus sequence as found in the γB promoter. Constructs were transfected into newborn rat lens explants, which had been precultured for 10 days in the presence of bFGF. After three additional days of culture, the cells were harvested, and promoter activities were assessed as described under "Experimental Procedures." Activities are shown relative to the wild-type γD(−375/+10) construct (100%). The bars indicate the standard deviation.
The γ-crystallin promoters contain an invariant sequence (CCCTTTTGTG) located −35 base pairs upstream from the TATA box (−73 to −63 in the γD promoter). The TTTG region in this sequence has been shown to be a binding site for Sox-2, a member of the Sry family of transcription factors (Ref. 48; see Fig. 11). The CCC are contacted by a factor in vivo, as they are detected on the in vivo footprint of the γD promoter (7). In that paper, the assumption was made that these CCC formed part of the silencer element. However, we show here that the silencer is located further upstream and contacts the bases −90 to −78. The close proximity of the CCC footprint at −73 to −71 to the Sox-2 target site suggests that this footprint belongs to a factor that forms a heterodimeric complex with Sox (note that Sox binds in the minor groove, whereas in vivo DMS footprinting detects only major groove G contacts). We have not studied the effect of this site on γ-crystallin promoter activity here as the sequence is invariant. We have previously shown that deletion of −77 to −71 in the γF promoter caused a 60% drop in activity (35).

Together, our studies show that there is a plethora of positive and negative factor interactions in the proximal γ-crystallin promoter (see Fig. 11). During development, the balance between these factors shifts toward repression. At least for the γD-crystallin promoter, it is the developmental change in the expression of the silencing factor that is the primary cause of promoter repression. Lack of demethylation is probably a secondary cause. However, the rate of promoter demethylation progressively decreases during development, and at even later developmental stages the rate of promoter demethylation may well be too slow to allow transcriptional activation, even if the cognate transactivating factors are present. Note that demethylation of the γD promoter region cannot be passive, i.e. due to lack of maintenance methylation following DNA replication, but must be active as there is no cell division coincident with promoter demethylation in differentiating lens explants.2 The

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**Fig. 10.** Comparison of the γB- and γD-crystallin silencers. A, the γB- and γD-crystallin silencer regions bind different factors in vitro. 32P-labeled fragments of the γB−110/−69, (BS) and γD-crystallin (−93/−76, γDS1) promoters were incubated with rat lens nuclear extract and electrophoresed through a native polyacrylamide gel (+). Fragments were bound in the presence of 10 mM KCl and either 50 ng/pmol poly(dIdC-poly(dGdC) (in the case of the γB probe) or 50 ng/pmol poly(dIdCdIdC) (in the case of the γD probe). Specific competitor DNA was added in a 100-fold molar excess. In the first and fifth lanes, no extract was added (−). Free (F) and bound (B) probes are indicated. See “Experimental Procedures” for details. B, functional analysis of the γB-crystallin silencer region. γB−114/−60tkCAT and γB−110/−69tkCAT constructs were transfected to newborn rat lens explants, precultured with bFGF for the time as indicated. The cells were harvested 3 days later, and promoter activities were determined as described under “Experimental Procedures.” Activities are shown relative to that of the tk promoter (pBLCAT2; 100%). The bars indicate the standard deviation.

**Fig. 11.** Regulatory elements in the γD- and γB-crystallin promoters. The transcription start sites are shown by open arrows. The γ-box elements and surrounding sequences are fully shown, whereas for the consensus binding sites of known transcription factors only differences are indicated. Nucleotide sequences and numbering are according to Den Dunnen et al. (9). The nucleotide sequence of the γ-box elements resembles the phorbol-12-O-tetradecanoate-13-acetate-responsive element-type MARE (T-MARE; consensus sequence TGCTGACTCAG; see Refs. 44 and 45). Also, the consensus binding sequences of Sry/Sox-2 (48–50) and AP-1 (e.g. Refs. 51 and 52) are shown.

The γ-crystallin gene expression is controlled by extracellular factors, i.e., the growth factor signals to the transcriptional apparatus. The transcription factors, well known for their role in transmitting the signals, seem to be involved in regulating the γ-crystallin gene expression. The γF-crystallin promoter is regulated by extracellular factors. This could play a role in the control of the γ-crystallin gene expression. However, it is not yet known whether such a rat homologue of the chicken lens (40).3 However, it is not yet known whether such a rat homologue indeed exists. Clearly, further experiments are required to elucidate the role of Maf proteins in the regulation of γ-crystallin gene expression.
sequence elements that signal αD promoter demethylation are unknown. Mapping these elements has thus far been precluded by the low transfection efficiency of lens explants at early stages of differentiation. Our efforts are now directed at overcoming this practical problem.

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