An everted repeat mediates retinoic acid induction of the γ F-crystallin gene: evidence of a direct role for retinoids in lens development

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The vertebrate lens is a classical system for examining mechanisms of tissue determination and differentiation, yet little is known about the signaling molecules controlling its development. Here, we report that retinoic acid (RA), a substance known for its teratogenic effects on the eye and as a natural endogenous morphogenetic agent, acts as a regulator of gene expression in the lens. We have identified a novel type of RA response element (RARE) within the lens-specific mouse γ F-crystallin promoter, consisting of two (A/G)GGTCA motifs in an everted arrangement spaced by 8 nucleotides. This element (γ F-RARE) mediates activation of the γ F-crystallin promoter by ligand-activated endogenous lens cell RA receptors (RARs) and confers RA responsiveness when linked to a heterologous promoter. γ F-RARE is bound in vitro by RAR/RXR heterodimers, and both receptors cooperate in vivo to *trans*-activate this element. These observations demonstrate a direct effect of RA on lens-specific gene expression and reveal a novel role for retinoids in the development and homeostasis of the mammalian eye.

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The ocular lens is an encapsulated transparent tissue that performs a critical role in vision by focusing light onto the retina, where photoreceptors transduce the light signal. It consists of a layer of mitotically active epithelial cells at the anterior surface and differentiated fiber cells in the remainder of the lens. The transparent and refractive properties of the lens are dependent on the ordered packing of a number of abundant proteins referred to as crystallins (Delaye and Tardieu 1983). In mammals, there are three major classes of crystallins (α , β , and γ), which are temporally and spatially differentially expressed within the lens (McAvoy 1978; Van Leen et al. 1987a; Wistow and Piatigorsky 1988). In particular, there are six closely related, chromosomally linked γ -crystallin genes in the murine genome (Lok et al. 1984; Moormann et al. 1985), whose expression is differentially regulated and restricted to fiber cells (Murer-Orlando et al. 1987; Van Leen et al. 1987b; Breitman et al. 1989). During the final stage of lens development, epi-

thelial cells differentiate into fiber cells in response to factors secreted from the retina (Coulombre and Coulombre 1963; Yamamoto 1976; Reyer 1977). Although a number of hormones and growth factors have been shown to influence growth and differentiation of lens cells in culture (Piatigorsky 1973; Beebe et al. 1987; Chamberlain and McAvoy 1987; Brewitt and Clark 1988), it is not known what signals are required in vivo. Thus, the γ -crystallin genes provide an excellent opportunity to investigate the molecules and mechanisms that regulate differentiation during lens development.

It has been known for decades that retinoids, the family of biologically active derivatives of vitamin A, are essential for vision. In the retina, 11-*cis* retinal functions as the chromophore component of the photoreceptors, and vitamin A deficiency can lead to night blindness and eventually causes irreversible ocular dysfunction (Goodman 1984). However, retinoids may play a more comprehensive role in eye physiology. Retinoids regulate developmental processes at various stages of vertebrate embryogenesis (Tabin 1991) and exposure of pregnant mammals to high levels of retinoic acid (RA) results in a wide range of malformations in the fetus including mi-

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crophthalmia and anophthalmia (Lammer et al. 1985; Rosa et al. 1986). Relatively high levels of RA are synthesized in the retina through the oxidation of retinaldehyde (McCaffery et al. 1992), suggesting that RA may be an important regulator of eye development and homeostasis.

The effects of retinoids on cellular activity result from the activation of two distinct classes of nuclear receptors, referred to as retinoic acid receptors (RARs) (Giguère et al. 1987, 1990; Petkovich et al. 1987; Benbrook et al. 1988; Brand et al. 1988; Zelent et al. 1989) and retinoid X receptors (RXRs) (Mangelsdorf et al. 1990, 1992). RARs and RXRs belong to a subgroup of the superfamily of steroid and thyroid hormone receptors that recognizes hormone response elements (HREs) composed of direct repeats of the core half-site motif (A/G)GGTCA. It has been proposed recently that specificity of DNA binding and transcriptional activation of these receptors is dictated by the spacing between the repeats: HREs with spacers of 3, 4, and 5 bp confer specific response to vitamin D₃, thyroid hormone, and RA, respectively (Näär et al. 1991; Umesono et al. 1991). The specificity of the retinoid response is further imposed by a series of complex interactions between the two types of receptors and their ligands. RARs respond to both all-*trans* RA and 9-*cis* RA, whereas RXR is activated specifically by the 9-*cis* isomer (Heyman et al. 1992; Levin et al. 1992). Furthermore, RARs can bind RA response elements (RAREs) and regulate transcription efficiently only when forming heterodimeric complexes with RXRs (Yu et al. 1991; Kliwer et al. 1992a; Leid et al. 1992; Zhang et al. 1992a) while RXRs, in the presence of 9-*cis* RA, can form homodimers that bind a subset of RAREs with high affinity (Zhang et al. 1992b).

The demonstration that RAR and RXR function as retinoid-activated transcription factors suggests that if retinoids control lens development, they must activate the expression of a particular subset of lens specific genes. As discussed above, lens differentiation is associated with the synthesis of the structural proteins, γ -crystallins, that serve as molecular markers of lens cell differentiation. Previous characterization of the mouse γ F-crystallin promoter has established that 5' flanking sequences -226 to +47 are sufficient for optimal promoter activity in cultured lens epithelial cells (Lok et al. 1989). This segment contains a proximal element responsible for lens specificity and an enhancer element essential for full activity of the promoter (Lok et al. 1989; Liu et al. 1991). Transgenic studies have also indicated that while sequences -171 to +47 are sufficient for lens fiber-specific expression of the *lacZ* reporter gene, upstream sequences (-759 to -171) are required for maximal expression (Goring et al. 1987; Yu et al. 1990). Here, we show that an element composed of an everted repeat of the (A/G)GGTCA motif located within the enhancer interacts specifically with RAR/RXR heterodimers in vitro and functions as a RARE in the context of the γ F-crystallin promoter. These results demonstrate a direct effect of RA on lens-specific gene expression through an unusual RARE and suggest a novel role for retinoids in vision.

Results

Interaction of lens proteins with the γ F enhancer

Transfection studies have established that sequences -226 to +47 of the mouse γ F-crystallin promoter are sufficient for maximal promoter activity in chick lens epithelial cells (Lok et al. 1989). Within this segment two domains have been identified that are necessary for maximal promoter activity: an enhancer element located between -226 and -123 and a proximal lens-specific element (LSE) located immediately upstream of the TATA box (Fig. 1) (Lok et al. 1989; Liu et al. 1991). The activity of the enhancer is highly dependent on sequences -226 to -171, as deletion of this segment results in a sharp decrease in promoter activity. To further increase our understanding of the molecular mechanisms regulating γ F-crystallin gene expression, we decided to characterize the interaction of endogenous lens proteins with this region by performing electrophoretic mobility shift assays (EMSAs) using an enhancer fragment and chick lens nuclear extracts. A restriction fragment encompassing sequences -226 to -151 yields two major retarded bands that can be competed with promoter segment -226 to +47 [γ F(-226)] (Fig. 2A, lane 3) but not with a nonspecific segment derived from pUC18 (Fig. 2A, lane 4). Formation of this complex could also be prevented by inclusion of 5' flanking sequences -529 to +33 of the mouse γ A-crystallin gene (Fig. 2A, lane 5). 5'-Flanking sequences of the γ F- and γ A-crystallin genes display a high degree of sequence identity in the proximal domain (Fig. 1, sequence -67 to -25) and increasing divergence upstream of this region (Murer-Orlando et al. 1987; Lok et al. 1989). The ability of the γ A-crystallin segment to compete for binding suggests that a similar binding site is located within this promoter.

To further characterize this binding site we used the methylation interference assay to identify guanine residues that are in direct contact with nuclear proteins interacting with this region. Asymmetrically end-labeled segment -226 to -164 was partially methylated and incubated with lens nuclear extracts to identify guanine positions which, when methylated, interfere with complex formation. This analysis identified seven guanine contacts, located between -207 and -190, that cluster at or near (A/G)GGTCA repeats (Fig. 2B).

To confirm that the major retarded bands observed by EMSA with the -226/-151 fragment represent interac-

Figure 1. Schematic representation of the γ F-crystallin promoter showing regulatory regions. The γ F enhancer is essential for maximal expression of the gene, whereas the LSE targets its expression in a tissue-specific manner. Numbers relate to the positions of nucleotides within the γ F promoter relative to the transcription start site.

Figure 2. Lens nuclear proteins interact with γ F enhancer (A/G)GGTCA motifs. (A) EMSA with enhancer segment -226 to -151. A radiolabeled restriction fragment (20,000 cpm) was incubated with 45 μ g of lens nuclear extract. Bound and free probe were separated on a 5% native polyacrylamide gel. Competitors were used at 50-fold molar excess. Competitors are γ F(-226), 5'-flanking sequences -529 to +33 of the γ A-crystallin gene [γ F(-529)], and a 322-bp *Pvu*II restriction fragment of pUC18, which was used as a nonspecific competitor (N.S.). Arrowheads indicate retarded bands. (B) Methylation interference analysis. A coding or noncoding strand of enhancer segment -226 to -164 was labeled by end-filling with Klenow, using either the *Sal*I site at position -226 or the *Xho*I site at position -164. Partially methylated radiolabeled probe was used in binding reactions with chick lens nuclear extracts. Bound and free probe were separated by PAGE, recovered from the gel, and cleaved at methylated residues with piperidine. Guanine residues, which interfere with binding when methylated (arrowheads), were identified by comparison of cleavage products of bound (B) and free (F) probe on 12% denatured polyacrylamide gels. The experiment was performed at least three times for each strand. (C) Mutations within putative HRE disrupt the binding of lens nuclear factors. Oligonucleotide (~0.1 ng) containing sequences -210 to -185 of the γ F promoter, radiolabeled with polynucleotide kinase, was incubated with lens nuclear extract and products were fractionated by 6% PAGE. Competitors used are γ F(-226) (lanes 3,4), γ F(-226)_{m1} (lanes 5,6), and γ F(-226)_{m2} (lanes 7,8). Molar excesses are indicated. Arrowheads indicate sequence-specific complexes.

tions with sequences defined by methylation interference analysis, we synthesized an oligonucleotide consisting of sequences -210 to -185 of the γ F enhancer [γ F(-210/-185)] that comprises the nucleotides making contact with lens nuclear proteins. When used as a probe, the γ F(-210/-185) oligonucleotide generates four specific complexes, designated B1-B4 (Fig. 2C, lane 2), and competes efficiently with the larger enhancer segment for binding of all complexes generated with lens nuclear extracts. Formation of complexes B1-B4 could be prevented by inclusion of cold probe in the binding reaction but not by inclusion of a nonspecific competitor (data not shown). Complex B2 could be competed with each complementary single strand of γ F(-210/-185), indicating that this complex is generated by interactions involving single-stranded DNA (data not shown). This complex often obscured complex B3. To confirm the importance of the DNA-protein contacts identified by methylation interference studies, we mutated guanine residues located at positions -204 and -205 to adenine residues, thus creating a promoter mutant referred to as γ F(-226)_{m1}. We also tested a previously described mu-

tant [γ F(-226)_{m2}] in which the two proximal (A/G)GGTCA-like repeats were deleted. When these mutant promoters were used as competitors in EMSA, neither generated significant reductions in binding when present at 10-fold molar excess in the binding reaction; whereas the same molar excess of γ F(-226) promoter fragment prevented formation of complexes B1, B3, and B4 (Fig. 2C, cf. lanes 3, 5, and 7). Therefore, mutations in the first (A/G)GGTCA repeat or deletion of the second and third repeats disrupt interactions between lens nuclear proteins and γ F(-210/-185).

Analysis of the nucleotide sequence of the γ F enhancer element revealed considerable identity with known HREs. γ F(-210/-185) contains two copies of the hexamer (A/G)GGTCA and one highly related sequence, which are arranged as direct and everted repeats (Fig. 3). The (A/G)GGTCA motif has been described as the consensus half-site structure of thyroid hormone, RA, and vitamin D₃ receptor-binding sites, whereas orientation and spacing of the two motifs within the HRE confer receptor specificity (Näär et al. 1991; Umesono et al. 1991). The structure of some of the known RAREs and

Figure 3. Sequence comparison of $\gamma F(-210/-185)$ with characterized HREs. Sequences -210 to -185 of the γF -crystallin promoter are displayed showing two (A/G)GGTCA repeats (thick arrows) and a highly related sequence (thin arrow). Bases within this degenerate repeat that do not fit the core-binding motif are indicated ($\times$). Repeats are arranged in a direct configuration spaced by 2 bp (repeats 1 and 2), unspaced everted (repeats 2 and 3), and everted spaced by 8 bp (repeats 1 and 3). TREpal is a synthetic idealized element derived from the rat growth hormone gene (Glass et al. 1988), which is responsive to both RAR and T_3R (Umesono et al. 1988). TRElys was identified within a silencer of the chicken lysozyme gene (Baniahmad et al. 1990) and responds preferentially to T_3R . β RARE was identified in the promoter of the β_2 RAR gene and responds only to RAR (de Thé et al. 1990; Sucov et al. 1990). RARE-mCRBP-I was identified from the promoter of the CRBP-1 gene and mediates specific response to RAR (Smith et al. 1991). Boxed sequences represent the spacers between repeats.

thyroid hormone response elements (TREs) are shown in Figure 3. Within the γF enhancer element, the repeats are arranged in direct orientation spaced by 2 bp (repeats 1 and 2), everted configuration spaced by 8 bp (repeats 1 and 3), and unspaced everted (repeats 2 and 3). The overall structure of this element does not correspond to previously described HREs; however, similarities in the orientation and spacing of the individual repeats are apparent. The direct repeats are similar in spacing to the RARE found in the cellular retinol-binding protein-I (CRBP-I RARE) gene (Smith et al. 1991), and the everted configuration spaced by 8 bp is reminiscent of that of the lysozyme TRE (TRElys), which has two everted repeats spaced by 6 bp (Baniahmad et al. 1990).

The results of the DNA-binding studies suggest that the mouse γF -crystallin promoter contains a putative HRE and therefore might be under hormonal control. In view of the critical role that retinoids play in vision, we first explored the possibility that the binding activities present in the lens extracts might represent RAR/RXR

complexes. We thus performed EMSA using as competitors a natural RARE identified within the promoter of the β RARE gene (β RARE) (de Thé et al. 1990; Sucov et al. 1990)—and shown to bind with high specificity to RAR/RXR heterodimers (Yu et al. 1991; Kliewer et al. 1992a)—and TREpal, which confers responsiveness to both thyroid hormone and RA (Glass et al. 1988; Umesono et al. 1988). Relatively small amounts of cold HRE competitors were sufficient to prevent formation of specific complexes. When 10-fold molar excess of competitor was used, both β RARE and TREpal completely prevented the formation of complex B1 (Fig. 4, lane 5,7), whereas some binding could still be detected when the same molar excess of $\gamma F(-210/-185)$ competitor was used (Fig. 4, lane 3). β RARE was five- to sevenfold less efficient at competing complex B4 compared with the γF enhancer element. Complex B3 could not be monitored accurately owing to the subtle nature of this interaction. The differential sensitivity of complexes B1 and B4 to competition with β RARE suggests that distinct proteins with different binding affinities are involved in the formation of these specific complexes. The ability of β RARE and TREpal to compete with $\gamma F(-210/-185)$ for binding indicates that lens proteins recognize common sequence motifs present in these elements and that endogenous RAR/RXR complexes in lens cells interact with the γF enhancer.

RA activates the γF -crystallin promoter in chick lens cells

To examine whether the γF enhancer element can function as a RARE in vivo, we first transfected primary cultures of chick lens epithelial cells with reporter gene

Figure 4. Band-shift competition analysis of $\gamma F(-210/-185)$, β RARE, and TREpal for binding of lens nuclear factors. Radio-labeled $\gamma F(-210/-185)$ was incubated with lens nuclear proteins in the presence of the indicated molar excess of the competitors cited above. (NS) Nonspecific oligonucleotide.

constructs containing the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the mouse γ F-crystallin promoter. Cultured chick lens epithelial cells support mouse γ -crystallin promoter activity (Lok et al. 1985) and have been shown to be responsive to RA (Patek and Clayton 1986, 1990). These cells were transfected with reporter constructs consisting of wild type or mutated sequences of the γ F promoter (–226 to +47) linked to the CAT gene (Fig. 5A), and expression vectors directing the synthesis of mouse RAR α_1 or RAR β_2 . We found that in the absence of RAR expression vectors, γ F promoter activity is induced ~3- to 10-fold by RA treatment (Fig. 5B). RA-dependent induction of promoter activity confirms that the retinoid signaling pathway is functional in lens epithelial cells. Upon cotransfection of RAR expression vectors, basal promoter activity increases ~8- and 27-fold with RAR α_1 and RAR β_2 , respectively (Fig. 5B). Treatment with RA leads to an additional 5-fold increase in CAT activity in cells transfected with RAR α_1 and a further 2.5-fold activation when an RAR β_2 expression vector is transfected (Fig. 5B). In two representative experiments the basal activity of the wild-type γ F promoter was increased an average 40- and 60-fold in the presence of both RA and transfected RAR α_1 and RAR β_2 , respectively. Mutation of two residues in the distal half-site motif [γ F(–226)_{m1}] or deletion of the two proximal half-sites [γ F(–226)_{m2}] resulted in the complete loss of RA response mediated through endogenous RARs (Fig. 5C) while decreasing

considerably the RA-dependent induction obtained with transfected RAR α (Fig. 5D). Mutation of the first (A/G)GGTCA repeat has little effect on basal promoter activity without transfected receptor (Fig. 5C) but decreases basal activity by approximately fourfold in the presence of transfected RAR α (Fig. 5D). As demonstrated previously (Lok et al. 1989), deletion of sequences –202 to –185 reduces γ F basal promoter activity by several-fold (Fig. 5D), and this mutant promoter can no longer be *trans*-activated by transfected RARs.

γ F–RARE confers RA responsiveness to a heterologous promoter

To confirm that γ F(–210/–185) confers RA responsiveness, we linked one and three copies of this element in front of the herpes simplex thymidine kinase (TK) promoter (sequence –105/+51) and the luciferase reporter gene. These reporter constructs were tested by transfection in the embryonal carcinoma line P19, which possesses a relatively high level of endogenous RARs. A luciferase reporter construct containing a single copy of the γ F enhancer element, hereafter referred to as the γ F–RARE, could be induced approximately threefold with RA without cotransfected receptor (Fig. 6). When expression vectors synthesizing the mouse RAR α_1 , RAR β_2 , or RAR γ_2 receptor are cotransfected, five- to sixfold induction with RA is observed. Higher levels of RA respon-

Figure 5. RA responsiveness of the γ F-crystallin promoter is mediated by an enhancer element. (A) γ F promoter–reporter constructs used in this study are outlined. γ F(–226)CAT contains sequences –226 to +47 linked to the CAT gene; γ F(–226)_{m1}–CAT reporter contains replacements at positions –205/–204 (indicated by arrows); and γ F(–226)_{m2} contains a deletion between nucleotides –202 and –185. (B) Activation of the transfected γ F-crystallin promoter by RAR. Chick lens primary cultures were transfected with 10 μ g of γ F(–226)CAT reporter, 0.5 μ g of RAR expression vector (pRSmRAR α_1 or pRSmRAR β_2), and 1 μ g of plasmid RSV– β -gal, which was used to normalize transfection efficiency. Following transfection, cells were treated with RA (100 nM) for ~48 hr before harvesting. CAT assay was performed with extracts normalized for β -galactosidase activity. (C,D) Effects of mutations within putative HRE on RA responsiveness. Cells were transfected with wild-type and mutant γ F reporter constructs outlined in the absence (C) or presence (D) of RAR α_1 expression vectors and treated with RA (100 nM) for 48 hr. (B–D) (Open bars) Control; (solid bars) +RA.

Figure 6. Analysis of RA responsiveness of γ F-RARE-TKLUC constructs in P19 cells. One and three copies of γ F-RARE were introduced in front of the TK promoter of the luciferase reporter plasmid pTKLUC, using the *SalI*-*Bam*HI sites in the polylinker. The three copy reporter contains three tandem copies of γ F-RARE. Cells were transfected with 2 μ g of reporter, 1 μ g of RSV- β -gal, and 0.5 μ g of RAR expression vector (mouse RAR α_1 , RAR β_2 , RAR γ_2). Following transfection, cells were treated for 20–24 hr with RA (100 nM) in charcoal-stripped serum. Luciferase readings were normalized to β -galactosidase activity. (Open bars) Control; (solid bars) +RA.

siveness (30- to 60-fold) are observed when three copies of the γ F-RARE are placed in front of the TK promoter (γ F-RARE₃TKLUC). Apparent differences in the activity of receptors on γ F-RARE were observed using this sensitive reporter construct. Transfection of RAR α_1 results in repression of basal promoter activity to ~30% of that obtained in the absence of transfected receptor. This repression is relieved by treatment with RA. This is in

contrast to the constitutive activation that we observed in chick lens cells transfected with γ F-crystallin promoter-CAT reporter constructs and RAR expression vectors. These differences could reflect the involvement of cell-specific factors and/or be dependent on promoter context. Transfection of either RAR β_2 or RAR γ_2 did not alter basal promoter activity and activated this reporter in the presence of RA five- to sixtimes more efficiently than transfection of RAR α_1 . These observations suggest that although RAR isoforms are highly homologous proteins, they may not be functionally identical. Specificity of the hormonal response was further investigated in transfection studies using estrogen, glucocorticoid, and vitamin D₃ receptors. We found no evidence of transcriptional activation by these related receptors (data not shown).

γ F-RARE is bound and trans-activated by RAR/RXR heterodimers

We then investigated whether the γ F-RARE could interact directly with the RARs in vitro. EMSA was performed using in vitro-synthesized RAR α and RXR β and radiolabeled oligonucleotide encoding γ F-RARE. As reported recently for various RAREs (Yu et al. 1991; Bugge et al. 1992; Kliewer et al. 1992a; Leid et al. 1992; Marks et al. 1992; Zhang et al. 1992a), neither RAR α or RXR β alone is sufficient to bind to γ F-RARE (Fig. 7A, lanes 2,3). However, high affinity binding can be readily detected in the presence of both RAR α and RXR β (Fig. 7A, lane 4). The specificity of this interaction is confirmed by the fact that cold γ F-RARE competes for binding while a nonspecific competitor does not (Fig. 7A, lanes 5,6).

To examine possible interactions between RAR and RXR at the level of transcriptional activation, we performed transfection studies in CV-1 cells that have relatively low levels of endogenous RAR activity. Using the

Figure 7. RAR/RXR heterodimers bind γ F-RARE in vitro and cooperate in *trans*-activation in vivo. (A) Interaction of RAR/RXR with γ F-RARE in vitro. Approximately 0.1 ng of radiolabeled γ F-RARE was incubated with reticulocyte lysate (total 5 μ l) programmed with either human RAR α_1 or mouse RXR β_2 mRNA. Probe was also incubated with the same amount of unprogrammed lysate as a control. Cold γ F-RARE (lane 5) and a nonspecific competitor (NS) (lane 6) were used at 100-fold molar excess. (B) Cooperation of RAR and RXR in *trans*-activation of γ F-RARE reporter in CV-1 cells. Cells were transfected with 1 μ g of γ F-RARE₃TKLUC reporter, 100 ng of pRshRAR α_1 and/or pRshRXR α , and 2 μ g of RSV- β -gal. Cells were treated with 100 nM RA for 20–24 hr before harvesting. (Open bars) Control; (solid bars) +RA.

three-copy γ F-RARE₃TKLUC reporter, no induction of basal promoter activity by RA is detected in the absence of transfected RAR α (Fig. 7B). As expected, cotransfection of the RAR α_1 expression vector causes an RA-dependent increase in luciferase expression (Fig. 7B). When RXR α was transfected alone, an increase in RA-dependent activity was also observed, indicating that introduction of RXR α can enhance the activity of the low level of endogenous RAR present in CV-1 cells to a point where its activity becomes detectable. When RXR α is introduced along with RAR α_1 , the RA induction of luciferase activity is synergistically increased (Fig. 7B). These experiments demonstrate that the tripartite γ F-RARE behaves in a manner similar to classical RAREs composed of two direct (A/G)GGTCA half-sites and that RXR can also act as a strong coregulator of RAR on this composite element.

Proximal and distal everted repeats are sufficient for high affinity binding of RAR/RXR and RA response

To define the precise sequence requirements for interaction of the RAR/RXR heterodimer with γ F-RARE, we generated duplex oligonucleotides corresponding to the direct (repeats 1 and 2) and everted repeats (repeats 2 and 3) found within this element (Fig. 8A), and tested them by competition in EMSA performed with radiolabeled γ F-RARE probe and in vitro-translated RAR and RXR. The direct repeat (γ F-RARE_D) competed only weakly (Fig. 8B), whereas the everted repeat (γ F-RARE_I) did not compete even when present at 150-fold molar excess in the binding reaction. These results indicate that the entire -210/-185 region of the γ F promoter, which includes all three putative half-site motifs, constitutes the γ F-RARE. To further assess whether the middle half-

Figure 8. Structural analysis of γ F-RARE. (A) Sequence of oligonucleotides used in this study. Only the sense strand is shown. (B) Analysis of binding of RAR/RXR to the direct and everted repeats within γ F-RARE. Conditions are as described in Fig. 7. Molar excess of competitors are indicated. (C) Effects of mutation of the second repeat on binding of RAR/RXR. Mutants contained a replacement of two residues (lowercase letters) (γ F-RARE_{m3}) and a deletion of the same residues (γ F-RARE_{m4}). (D) Analysis of RA responsiveness of γ F-RARE oligonucleotides linked to the TK promoter in P19 cells. A single copy of each oligonucleotide was placed in front of the TK promoter in the luciferase reporter plasmid pTKLUC. Cells were transfected and treated as described in Fig. 6. (Open bars) Control; (solid bars) + RA.

site, which contains only 4 out of 6 bp of the consensus, plays any role in the activity of the γ F-RARE, two additional oligonucleotide mutants (Fig. 8A) were tested for their binding activity. These mutants contained either a replacement of two cytosines (γ F-RARE_{m3}) or a deletion (γ F-RARE_{m4}) within the second repeat. As shown in Figure 8C, these mutants compete as well as the wild-type γ F-RARE. To examine whether the binding activities displayed by each oligonucleotide containing a portion or a mutated form of the γ F-RARE correlate with their ability to be transcriptionally activated by RARs, a series of luciferase reporter constructs linked to the TK promoter and a single copy of each γ F-RARE oligonucleotide was cotransfected in P19 cells together with RAR α_1 expression vector. Neither the direct or everted repeats mediate RA responsiveness while mutation of the second repeat did not abolish RA responsiveness (Fig. 8D). These results demonstrate that the two distal half-sites, arranged in an everted orientation with a spacer of 8 bp, function efficiently as a RARE, thus defining a new type of natural RARE. In addition, it appears that the same configuration of repeats spaced by 6 bp (γ F-RARE_{m4}) also functions as a RARE. Thus, everted repeats spaced by 6 and 8 bp (TGACCCN_{6/8}AGGTCA) can bind RAR/RXR complexes in vitro and mediate RA response in P19 cells.

Discussion

The association of γ -crystallin expression with the differentiated lens phenotype and the ability of RA to trigger cell differentiation led us to investigate whether RA could activate γ F-crystallin gene expression. We have identified an element (γ F-RARE) within the 5'-flanking sequences of the γ F-crystallin gene that mediates RA-dependent *trans*-activation. This element is an unusual RARE composed of everted, rather than direct, repeats of the half-site core motif (A/G)GGTCA. The γ F-RARE can be transcriptionally activated by endogenous RARs present in lens cells and interacts with lens nuclear proteins. Although the precise identity of the lens proteins binding to this element has not been determined, we have shown that RAR/RXR heterodimers can bind and activate gene expression through this element. Overall, these observations demonstrate that RA can directly regulate gene expression in the lens and suggest that the role played by retinoids in vision is more comprehensive than their well-known function as chromophores in the transduction of the light signal.

The γ F-crystallin enhancer element defines a novel type of RARE

Recently, it has been proposed that spacing between direct repeats of the hexamer (A/G)GGTCA confers selective activation by RAR, T₃R, and vitamin D₃ receptor (Näär et al. 1991; Umesono et al. 1991). A spacer size of 3, 4, and 5 bp each configures the binding site to function as a vitamin D₃ response element (VDRE), TRE, and RARE, respectively (Umesono et al. 1991). This scheme

was later expanded to include elements with a spacer of 1 bp, which can be recognized by RXR homodimers (Mangelsdorf et al. 1991), and heterodimers of RXR and the peroxisome proliferator-activated receptor (PPAR) (Kliewer et al. 1992b). RA response can also be transduced through a RARE composed of two half-sites and a spacer of 2 bp (Smith et al. 1991), which shows that some flexibility in the spacing requirement of half-sites in RAREs is allowed. Although tandem repeat versions of HREs show selective response to T₃R or RAR, depending on the size of the spacer, palindromic arrangement without a spacer as found in the synthetic TREpal results in the generation of dual response to RAR and T₃R (Glass et al. 1988; Umesono et al. 1988). This observation suggests that natural HREs with configurations other than direct repeats may exist, albeit with a broader specificity of action.

In vitro-binding and *trans*-activation studies with the γ F enhancer element indicate that RAR/RXR heterodimers do not bind with high affinity to either the direct (repeats 1 and 2) or everted repeats (repeats 2 and 3), whereas the two distant everted half-sites (1 and 3) are sufficient for high affinity binding and transcriptional activation. These studies thus defined a novel type of RARE composed of everted repeats of the core half-site motif (A/G)GGTCA. In the γ F-RARE, the distal everted half-site repeats are in the opposite orientation (inside out) in relation to the repeats found in synthetic TREpal (see Fig. 3). The orientation is identical to that of TRElys, which has a 6-bp spacer. TRElys works very efficiently as a TRE (Baniahmad et al. 1990) and can mediate a weak RA response in Ltk cells, although apparently not in CV-1 cells (Baniahmad et al. 1992). These observations are consistent with our studies of the γ F-RARE in which the spacing requirements between the distal everted half-sites for RA response appear to be flexible, because both 6- and 8-bp spaced everted repeats generate a RA response in P19 cells (Fig. 8D). Conversely, preliminary experiments to explore the possibility that γ F-RARE might be *trans*-activated by T₃R have shown that the γ F-crystallin promoter can be weakly activated by T₃ in chick lens cells; although, in contrast to the RA response, cotransfection of T₃R expression vector is required to observe the T₃ response (M. Tini, unpubl.). Thus, it appears that HREs composed of 8- and 6-bp everted repeats may confer hormonal response to both RA and T₃, although the specificity and strength of the response may be dependent on the interaction of cell-specific factors with these elements. The middle repeat in the γ F enhancer element does not appear to be essential for RA responsiveness, but it may be required for interactions with other lens nuclear proteins, possibly novel members of the superfamily of nuclear receptors. We infer this conclusion from in vitro binding studies that detected at least two distinct interactions of lens nuclear proteins with this element (Fig. 4) and mapped one strong guanine contact within the second (A/G)GGTCA-like motif (Fig. 2B). Taken together with the preliminary data showing a weak T₃ response conferred by the γ F-RARE, these observations suggest that tripar-

tite HREs such as the γ F-RARE may be pleiotropic in nature and used to increase the complexity of hormonal response at the level of gene transcription.

Retinoids and their role in lens development

The eye is a rich source of retinoids, where they play a critical function in the vision process by acting as cofactors in photoreceptors of the retina. On the basis of the specific activation of the γ F-crystallin gene reported here, we propose a more comprehensive role for retinoids in lens development. During embryogenesis, the lens vesicle is derived from the head ectoderm as a result of inductive influences from the optic vesicle. Epithelial cells that comprise the lens vesicle differentiate posteriorly to generate elongated fiber cells that fill the lens cavity. Differentiation occurs in response to factors apparently secreted by the retina into the vitreous humor [Coulombre and Coulombre 1963; Yamamoto 1976; Reyer 1977]. In the mature lens, epithelial cells at the equator undergo terminal differentiation to generate fiber cells, as the lens continues to grow at a reduced rate throughout life. Factors stimulating proliferation and differentiation of lens cells in culture have been identified in extracts from ocular tissues, including the retina and vitreous humor (Arruti and Courtois 1978; Barritault et al. 1981; Beebe et al. 1987). Although the precise identity of these factors remains unknown, a number of previously characterized growth factors and hormones have been shown to promote proliferation and differentiation of lens cells in vitro (Piatigorsky 1973; Beebe et al. 1987; Chamberlain and McAvoy 1987; Brewitt and Clark 1988). These include insulin and insulin-like growth factor-I (IGF-I), whose activity has been identified in chicken vitreous humor (Beebe et al. 1987). Presently, it is not known whether these signals are operational in vivo. However, it is known that the retina is a site of synthesis and storage for retinoids, and relatively high levels of RA synthesis have been detected in this tissue (McCaffery et al. 1992). These observations, coupled with our finding that the γ F-crystallin gene can be regulated by RA, lead us to suggest that RA or related retinoids synthesized locally in the retina may be released into the vitreous humor to act on the lens. Alternatively, RA may be synthesized in situ in the lens.

Further evidences for a direct role of retinoids in lens development come from in situ hybridization studies during mouse embryogenesis. These studies showed that the gene for CRBP-I was strongly expressed in the lens and the retina (Dollé et al. 1990). Although the precise role of CRBP-I remains to be defined, it appears to be involved in retinol metabolism (Blomhoff et al. 1990); therefore, its expression is generally believed to be associated with tissues in which retinoids are synthesized and exert biological effects. Thus, strong expression of CRBP-I in the lens and the retina implies that retinoids synthesized in situ play important regulatory functions in the eye. On the other hand, the presence of RAR activity in the lens is deduced from the ability of the γ F

promoter to be induced by endogenous RARs in cultured lens cells and the detection of RAR transcriptional activity in the lenses of transgenic mice carrying RA-inducible indicator transgenes (Rossant et al. 1991; Balkan et al. 1992a). In addition, others have shown that RA treatment increases the rate of differentiation and crystallin accumulation in cultured chick lens epithelial cells (Patek and Clayton 1990). In culture, these cells differentiate in response to growth factors present in the media. Retinoids may work in conjunction with growth factors to trigger lens cell differentiation and concomitant γ -crystallin gene expression in the lens.

Vitamin A deficiency has profound effects on the ability of the retina and the lens to mediate normal vision (Pirie and Overall 1972; Goodman 1984). Exposure to RA early in *Xenopus* development has been shown to abate the formation of the eye, whereas treatment at later stages can generate microphthalmic eyes with multiple lenses and folded retinas (Manns and Fritsch 1991). These results are reminiscent of the pattern duplication that is observed in the developing chick limb bud and regenerating amphibian limb upon treatment with RA (Brookes 1989), and support the notion that retinoids may be involved in lens development. Furthermore, it has been shown recently that the expression of a constitutively active RAR mutant in the lens of transgenic mice mimics the teratogenic effects of retinoids in the eye (Balkan et al. 1992b). The RARE that we have described here may be present in other members of the mouse γ -crystallin gene family, as promoter sequences from the divergent γ A-crystallin gene compete with γ F-RARE for binding of lens factors in vitro (Fig. 2A). The apparent conservation of this element in different γ -crystallin promoters suggests that it may play a crucial role in the regulation of this family of lens-specific genes. This assertion is consistent with our transgenic studies, which indicate that sequences -226 to -171, containing the γ F-RARE, are required for maximal expression of the *lacZ* indicator gene in transgenic mouse lenses (D. Goring, M. Breitman, and L.-C. Tsui, unpubl.). The structural integrity of the lens is important to the general development of the eye, and ablation of the lens through the targeted expression of a bacterial toxin in transgenic mice results in microphthalmia (Breitman et al. 1987). Interestingly, vitamin A deficiency in mammals during development also causes microphthalmia (Hale 1937; Warkany and Schraffenberger 1946; Lammer et al. 1985).

Future direction

The results presented here open new avenues of investigation on the role of retinoids and their receptors in eye development and homeostasis. In particular, it will be of interest to determine whether other retinoids, in addition to RA, are involved in this process and whether the role of retinoids in controlling gene expression in the lens is limited to the final stages of cellular differentiation, or whether these signaling substances also influence the early stages of lens induction (Grainger 1992). Most intriguing, however, is the tripartite nature of the

γ F enhancer element and the fact that we have detected seemingly lens-specific interactions with this HRE. These observations suggest the distinct possibility that novel nuclear receptors are expressed in the lens. We are currently testing a number of so-called orphan nuclear receptors for their ability to bind and activate and/or cooperate with RARs and RXRs through the γ F enhancer element.

Materials and methods

Oligonucleotides

γ F-RARE oligonucleotides used in this study were designed with *Sall* and *Bam*HI cohesive ends. The sense and antisense strands consist of the following sequences: γ F-RARE, 5'-TCGACAGTGACCCTTTTAAACCAGGTCAGTGAG-3' and 5'-GATCCCTCACTGACCTGGTTAAAGGGTCACTGTG-3'; γ F-RARE_D, 5'-TCGACAGTGACCCTTTTAAACCAGG-3' and 5'-GATCCCTGGTTAAAGGGTCACTG-3'; γ F-RARE_L, 5'-TCGACTTTAAACCAGGTCAGTGAG-3' and 5'-GATCCCTCACTGACCTGGTTAAAG-3'; γ F-RARE_{m3}, 5'-TCGACAGTGACCTTTTAAAGAGGTCAGTGAG-3' and 5'-GATCCCTCACTGACCTCTTTAAAGGGTCACTG-3'; γ F-RARE_{m4}, 5'-TCGACAGTGACCCTTTTAAAGGTCAGTGAG-3' and 5'-GATCCCTCACTGACCTTTTAAAGGGTCACTG-3'.

Plasmids for transfection analysis

The γ F-crystallin promoter-reporter construct consisted of sequences -226 to +47, relative to the transcription start site, linked to the CAT gene of *Escherichia coli* [designated γ F(-226)CAT] (Lok et al. 1989). A mutant containing a deletion of sequences -202 to -185 [γ F(-226) _{Δ -202/-185}] has been described previously (Lok et al. 1989). For the sake of simplicity we have renamed this mutant γ F(-226)_{m2}. Site-directed mutagenesis was used to replace guanines at positions -205 and -204 of the γ F-crystallin promoter with adenines. This mutated promoter, designated γ F(-226)_{m1}, was introduced in front of the CAT gene in plasmid pSVoATCAT (Lok et al. 1989). Expression vectors for the mouse RAR α ₁, RAR β ₂, RAR γ ₂ and human RAR α ₁ and RXR α contain the appropriate cDNA under the control of the Rous sarcoma virus long terminal repeat (RSV LTR), and have been described elsewhere (Giguère et al. 1990; Mangelsdorf et al. 1991). Plasmid TKLUC contains sequences -105 to +51 of the TK promoter linked to the firefly luciferase gene. γ F-RARE oligonucleotides described above were cloned into the *Sall*-*Bam*HI sites of the polylinker as a single copy in the positive orientation. The nomenclature of these constructs is as described for the oligonucleotides (above). A reporter containing three tandem copies of γ F-RARE (γ F-RARE₃TKLUC) was also made in a similar manner. The three copies are arranged in the sense, antisense, and sense orientation. Constructs were confirmed by sequencing.

Preparation of lens nuclear extracts and EMSA

Lens nuclear extracts were prepared from 20- to 21-day embryonic chick lenses. Lenses were dissected and stored under liquid nitrogen before use. To prepare a crude nuclear extract, 300 lenses were washed in cold PBS buffer and homogenized in 10 ml of lysis buffer [10 mM HEPES (pH 8), 50 mM NaCl, 0.5 M sucrose, 0.1 M EDTA, 0.5% Triton X-100, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF] with 20 strokes of the B pestle in a Dounce glass homogenizer. Crude nuclei were washed in nuclei buffer

[10 mM HEPES (pH 8), 50 mM NaCl, 0.1 mM EDTA, 5 mM MgCl₂, 25% glycerol (vol/vol), 1 mM DTT, 1 mM PMSF] and extracted with 0.5 M NaCl in the same buffer containing 1 mM spermidine. The extract was dialyzed for 5 hr in nuclei buffer containing 50% glycerol.

EMSAs were carried out by incubating 45 μ g of lens extract with 20,000 cpm of end-labeled restriction fragments or double-stranded oligonucleotides. Specific activity ranged from 2×10^7 to 5×10^7 cpm/ μ g for restriction fragments and 5×10^7 to 2×10^8 cpm/ μ g for oligonucleotides. Competitor DNAs were purified using conventional methods (Maniatis et al. 1982) and quantified by fluorometry in a TKO fluorometer and by absorbance at 260 nm. PAGE analysis was carried out on 5–6% polyacrylamide gels cast in 0.25 $\times$ TBE buffer.

Methylation interference assay

Sequences -226 to -164 were derived from γ F-crystallin promoter mutant Δ -163/-162 (Lok et al. 1989) by digestion with *Sall* and *Xho*I. Each strand was uniquely end-labeled by digestion of the plasmid with either *Sall* or *Xho*I, followed by end-filling with Klenow fragment of DNA polymerase in the presence of isotopically labeled dNTPs. Following labeling, unincorporated dNTPs were removed by G-50 spin chromatography, and the fragment was released from the plasmid by digestion with either *Sall* or *Xho*I and separated by PAGE. Recovery of the labeled fragment was performed by crush elution. Half a million counts per minute of fragment was partially methylated with dimethyl sulfate in the presence of 10 μ g of poly[d(I-C)]/[d(I-C)] (Pharmacia), as described by Siebenlist and Gilbert (1980). Partially methylated template was used in binding reactions as described above except that the amount of labeled fragment was increased by threefold. Twelve reactions were done and fractionated as described above. The wet gel was exposed for at least 24 hr at 4°C. Bands representing bound and free fractions were excised and DNA was recovered by sequential electrophoretic transfer onto a single strip of NA45 ion exchange paper. The bound fraction contained at least two closely migrating bands (see Results). Recovery of DNA was as described by the manufacturer. DNA was cleaved by boiling in 1 M piperidine. Piperidine was removed by repeated lyophilization. Equal amounts (cpm) of DNA from bound and free fractions were displayed on 12% sequencing gels.

Cell culture and transfection

Primary lens cultures were prepared essentially as described by Borrás et al. (1988). Lenses were dissected and stored in modified Eagle media (α -MEM) containing 20 mM HEPES and 50 mM gentamycin before trypsinization. Fifty to sixty lenses were disrupted with forceps in a 60-mm tissue culture dish and trypsinized in 7 ml of 1 $\times$ trypsin-EDTA (GIBCO). The equivalent of five lenses was plated on 60-mm culture dishes coated with collagen. Three days after preparation, the medium was replaced several hours before transfection. Cells were transfected with 10 μ g of γ F-crystallin reporter construct and 1 μ g of plasmid RSV- β -gal, and 0.5 μ g of expression vectors directing the synthesis of RAR α , RAR β , and TR β . P19 and CV-1 cells were cultured on α -MEM containing 7% fetal calf serum. These cells were transfected with 2 μ g of TK promoter-based reporter plasmids, 1–2 μ g of RSV- β -gal, and 100–500 ng of appropriate expression vector. β -Galactosidase and luciferase assays were carried out as described elsewhere (Giguère et al. 1990). CAT assays were performed using equivalent amount of β -galactosidase activity as described by Gorman et al. (1982).

In vitro synthesis of RAR/RXR and EMSA

Plasmid pCMXhRAR α (Kliewer et al. 1992a) containing the human RAR α cDNA, and plasmid pSKmRXR β (Mangelsdorf et al. 1992) containing the mouse RXR β were linearized with *Bam*HI and *Acc*I, respectively. Capped RAR α_1 mRNA was synthesized in vitro using T7 RNA polymerase, whereas RXR β mRNA was synthesized with T3 RNA polymerase. These mRNAs were used to synthesize RAR α and RXR β protein in vitro using rabbit reticulocyte lysates (Promega). Probe for EMSA was radiolabeled by end-filling with Klenow. Approximately 0.1 ng of probe was used in each reaction with a total of 5 μ l of programmed reticulocyte lysate in a buffer containing 10 mM Tris-HCl (pH 8.0), 40 mM KCl, 6% glycerol, 1 mM DTT, and 0.05% NP-40 in a final volume of 20 μ l. To prevent single-stranded binding, 10 ng of the sense strand oligonucleotide γ F-RARE_D was included in the binding reaction. As a control, probe was also incubated with the same amount of unprogrammed lysate. Competitors and probes were added before the addition of lysate.

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