γ-Crystallins of the Human Eye Lens: Expression Analysis of Five Members of the Gene Family

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While only two γ-crystallins have been identified in the human eye lens, molecular studies indicate that the human γ-crystallins are encoded in a multigene family comprising at least seven closely related members. Sequence analysis of five of these genes has suggested that three (γ1-2, G3, and G4) are potentially active, while two (G1ψ and G2ψ) correspond to closely related pseudogenes. Here we report on the detailed structure of a sixth γ-crystallin gene, G5, and our results obtained with transient expression assays to characterize both the promoter activity and translation products of five members of the gene family. We show that 5'-flanking sequences of G1ψ and G2ψ lacked detectable promoter activity, while the corresponding sequences of G3, G4, and G5 were able to direct high levels of expression of the bacterial chloramphenicol acetyltransferase gene in primary lens epithelia, but not in cultures of nonlens origin. Detailed sequence comparisons indicated that active genes contained several conserved sequence tracts 5' of the TATA box which may constitute functional elements of a lens-specific γ-crystallin promoter. Expression of the γ-crystallin coding sequences from the human metallothionein IIA promoter in nonlens cells facilitated characterization of the polypeptides encoded by individual γ-genes and, in future studies, should permit comparison of these proteins with distinct γ-crystallins in the human lens.

Crystallins are the major water-soluble structural proteins of the vertebrate eye lens. In mammals, these proteins are divided into three biochemically and immunologically distinct groups, α, β, and γ, each of which is differentially expressed during lens development (22, 23, 30). Although the exact function of these proteins is unknown, their high concentration and short-range order within the lens are generally believed to be critical to the maintenance of lens transparency (4).

The γ-crystallins, which account for up to 40% of soluble lens protein, are found exclusively in the terminally differentiated fiber cells of the lens (22). Six different γ-crystallins have been identified in the lenses of rats and mice (8, 31). These are encoded by a family of closely related genes (19, 25), each of which show characteristic differences in developmental regulation (27, 38a). While the functional significance of the heterogeneity among the γ-crystallins remains unclear, it has been suggested that structural differences among these proteins are important in maintaining different intermolecular interactions within the lens (2).

In contrast to the γ-crystallins of other mammalian species, relatively little is known about the complexity and regulation of the corresponding proteins in humans. The importance of developing such knowledge is underscored by the recent finding that a dominantly inherited cataract is closely linked to the locus encoding the human γ-crystallins (20), which has been localized to chromosome 2, region q33-36 (5, 34, 39). While only two distinct human γ-crystallin protein species have been described (41, 42), studies at the molecular level have provided evidence for at least seven closely related γ-crystallin genes (6, 24). Six of these genes have recently been isolated, and four have been determined to be clustered within 39 kilobases (kb) (6, 24). Sequence analysis of five of these genes has revealed that three of

them, γ1-2, G3, and G4, represent potentially functional members of the gene family, while the other two, G1ψ and G2ψ, correspond to highly related pseudogenes (6, 24).

In this paper, we report on the structure and nucleotide sequence of a sixth human γ-crystallin gene, G5, and our results obtained with transfection assays to characterize both the translation products and promoter activity of five different members of the gene family. We show that three of these genes, G3, G4, and G5, are preferentially active in lens cells and encode closely related polypeptides, while the other two, G1ψ and G2ψ, are both transcriptionally and translationally inactive. In addition to disclosing information on the elements regulating tissue-specific expression of these genes, our studies provide a framework for future investigations to characterize putative mutations within the human γ-crystallin genes that may be implicated as the underlying cause of hetediatory cataract.

MATERIALS AND METHODS

DNA sequencing. The isolation of five distinct phage clones containing human γ-crystallin sequences and the structure and organization of the γ-crystallin genes, G1ψ, G2ψ, G3, and G4, contained within four of these clones have been described previously (24). The nucleotide sequence of the γ-crystallin gene, G5, contained within the fifth phage clone, λ16G3, and the upstream sequences of G1ψ, G2ψ, G3, and G4 were determined by the chemical degradation method of Maxam and Gilbert (21).

Plasmid constructions. pSTK7 was constructed by inserting the 3.5-kb BamHI thymidine kinase fragment of herpes simplex virus into the BamHI site of pSV2gpt (26).

The expression vectors pSV0CAT and pRSVCAT were obtained from B. Howard (11). The restriction maps of the γ-crystallin genes G1ψ, G2ψ, G3, and G4 have been published (24) and the map of G5 is shown in Fig. 1. All of these
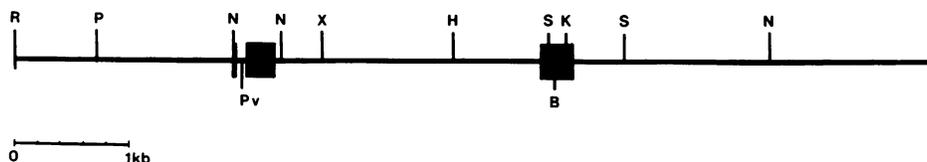
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FIG. 1. Gene structure and restriction map of G5. The three exons are indicated by shaded boxes and encode (left to right) 3, 81, and 90 amino acids, respectively. Abbreviations: R, *Eco*RI; P, *Pst*I; N, *Nco*I; Pv, *Pvu*II; X, *Xho*I; H, *Hin*dIII; S, *Sst*I; B, *Bgl*II; K, *Kpn*I.

genes contain a common *Nco*I site about their translation initiation codon which was used to separate the coding sequences from 5'-flanking regions. The putative promoter regions of the different genes were isolated as restriction fragments 5' to the *Nco*I site: a 0.46-kb *Hin*dIII-*Nco*I fragment for G1ψ, a 1.1-kb *Kpn*I-*Nco*I fragment for G2ψ, a 0.5-kb *Xba*-*Nco*I fragment for G3, a 0.6-kb *Taq*I-*Nco*I fragment for G4, and a 1.9-kb *Eco*RI-*Nco*I fragment for G5. Prior to cloning, the 5' protruding ends of these segments were blunted by S1 nuclease digestion to avoid regeneration of the γ-crystallin translation initiation codon. The DNA ends were then filled in with T4 DNA polymerase (New England Biolabs), ligated to *Hin*dIII linkers, and inserted into the *Hin*dIII site of pSV0CAT. Since the 1.1-kb *Kpn*I-*Nco*I fragment of G2ψ contained two internal *Hin*dIII sites, it was ligated with *Xho*I linkers and inserted into a derivative of pSV0CAT in which the *Hin*dIII site had been converted to an *Xho*I site. For all constructs, the absence of the γ-crystallin translation initiation codon at the 5' boundary of the chloramphenicol acetyltransferase (*cat*) gene was confirmed by DNA sequencing (data not shown). Clones were recovered that contained the 5'-flanking sequences of the different genes in either orientation.

To generate vectors containing the coding sequences of the γ-genes coupled to the promoter of the human metal-lothionein (MT) II_A gene, a 0.8-kb *Bam*HI fragment containing the MT_{IIA} promoter was subcloned from $pHMT_{IIA}$ (kindly provided by D. Hamer [14]) and inserted into the *Bam*HI site of pSV2gpt (26). The resulting vector, pSGM1, contains the MT_{IIA} promoter in the same transcriptional orientation as the *Escherichia coli gpt* gene and served as the basic cloning vector (see Fig. 6A). The 5.3-kb *Nco*I-*Eco*RI fragment of pSGM1 was then purified and ligated to *Nco*I-*Eco*RI fragments spanning the coding sequences and poly(A) addition signal of the different γ genes. These segments corresponded to 7.0 kb for G2ψ, 3.9 kb for G3, 3.0 kb for G4, and 6.25 kb for G5. The length of each fragment was dictated by the nearest available *Eco*RI site downstream of the poly(A) addition signal. For G2ψ and G3, the *Eco*RI site corresponded to the linker at the attachment site of the genomic sequence in the phage clone (24). Since the *Nco*I site in pSGM1 spans the translation initiation codon of the MT_{IIA} gene, all of the hybrid constructs maintained the integrity of both the MT_{IIA} transcription initiation site and 5' untranslated sequences. Transcripts expressed from the hybrid constructs are therefore predicted to contain 72 base pairs (bp) of 5' untranslated sequence derived from the MT_{IIA} gene. A hybrid construct was not generated for G1ψ because sequence data suggested that this pseudogene suffers perturbations at the level of both RNA splicing and translation (24). Test constructs were transiently expressed in COS-1 cells for 48 h in the presence of 5 μM cadmium chloride, after which cell lysates were prepared and assayed for γ-crystallin protein by immunoblotting.

Plasmid pRSVγ2, kindly provided by S. Lok, was constructed by isolating the coding region of the mouse γ2 gene

as a 3.0-kb *Nco*I-*Bam*HI fragment from λML2 (18) and ligating it to the 3.4-kb *Hin*dIII-*Bam*HI fragment of pRSVCAT (11) via synthetic *Hin*dIII linkers.

All restriction enzymes were purchased from Boehringer Mannheim or New England Biolabs and used according to conditions recommended by the supplier. T4 DNA ligase was purchased from Boehringer Mannheim, and S1 nuclease was from Sigma Chemical Co.

Cell cultures and DNA transfection. All tissue cultures were grown at 37°C in α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum and 50 μM gentamicin. The medium used for chicken cells was buffered with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.0). Chicken embryo fibroblasts (CEFs) were prepared from 7-day-old white Leghorn embryos and used as recipients for DNA transfection within 10 to 14 days. Culture dishes (100 mm) were seeded with 5 × 10^5 COS-1 cells (9), TK⁻ L cells, or CEFs and 20 to 24 h later were transfected with 20 μg of plasmid DNA (without carrier) by the calcium phosphate coprecipitation method of Graham and van der Eb (12). Four hours after exposure to DNA, cultures were treated with 15% glycerol to enhance the efficiency of DNA uptake (29). Following a rinse with phosphate-buffered saline (PBS), 10 ml of fresh medium was added, and the cells were harvested 48 h later.

Chick lens epithelia explants were dissected from 14-day-old white Leghorn embryos and cultured on collagen-coated (Vitrogen; Flow Laboratories) 35-mm dishes as described by Lok et al. (19). For assay of γ-crystallin promoter activity, six explants per 35-mm dish were transfected with 12 μg of plasmid DNA and glycerol shocked as described above.

Cell lines which contain the pMT-γ-crystallin constructs stably integrated were derived by transfecting the pMT-fusion genes with pSTK7 (at a 10:1 molar ratio) into TK⁻ L cells as described above. The following day, cells were trypsinized and seeded 1/5 in HAT medium [α-MEM supplemented with hypoxanthine (15 μg/ml), thymidine (10 μg/ml) and (+)-amethopterin (2 μg/ml)]. Fresh medium was added every 2 to 3 days, and individual HAT-resistant colonies were picked 10 days later and assayed for γ-crystallin expression by immunoblotting.

CAT assays. Cells were scraped from tissue culture plates with a rubber policeman into 1.0 ml of PBS, collected by brief centrifugation, and suspended in 40 μl of sterile water. Packed cells were frozen at −20°C for several weeks without apparent loss of activity. Cell extracts were prepared by three freeze-thaw cycles, followed by gentle homogenization. The CAT assay was performed as described previously (19).

Immunodetection of γ-crystallin proteins. Cytoplasmic proteins of transfected COS-1 cells were recovered following a 2-min lysis of the plasma membrane with 1.0 ml of buffer containing 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 1% Triton X-100. Samples were lyophilized and stored at −20°C until use. At that time they were reconstituted in 100 μl of PBS and an equal volume of sample buffer

(10% [vol/vol] glycerol, 0.1% bromophenol blue, 3% sodium dodecyl sulfate, 0.7 M β-mercaptoethanol, 0.06 M Tris, pH 6.8). The protein mixture was then denatured by heating at 100°C for 5 to 10 min. Samples (50 μl; 1/4-plate equivalents) were separated on a 10% polyacrylamide-sodium dodecyl sulfate gel with the discontinuous buffer system of Laemmli (16). The proteins were electrophoretically transferred to nitrocellulose at 100 V for 2 h at 4°C with a Hoefer Scientific Transfor Cell and an electrode buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 20% (vol/vol) methanol as described by Towbin et al. (37). The nitrocellulose filters were air dried and then incubated in 50 ml of a blocking solution (3% [wt/vol] bovine serum albumin, 10 mM Tris hydrochloride [pH 7.4], 0.9% NaCl) at 37°C for 1 h to prevent nonspecific protein interactions. The γ-polypeptides were detected by incubating the filters for 4 to 5 h at room temperature in 10 ml of binding buffer (150 mM NaCl, 20 mM Na2HPO4, 3% [wt/vol] bovine serum albumin) containing a 1/500 dilution of a rabbit anti-calf low-molecular-weight γ-crystallin serum or a 1/200 dilution of a rabbit anti-human low-molecular-weight γ-crystallin serum (kindly provided by S. Zigler, National Eye Institute). Unbound antibodies were removed with three to four 15-min washes in 150 mM NaCl–20 mM Na2HPO4–0.25% Triton X-100 (pH 7.3). The bound antibody was then detected by incubating the filters with 3 × 10⁶ to 4 × 10⁶ cpm of ¹²⁵I-protein A (New England Nuclear Corp.) in 25 ml of washing buffer for 5 h at room temperature. Excess label was extensively washed off until the spent wash fluid contained counts comparable to background. Air-dried filters were exposed to Kodak XAR-5 film with Du Pont Cronex Lightning-Plus intensifying screens at −70°C.

RESULTS

Structure and nucleotide sequence of human γ-crystallin gene G5. To establish the primary structure of the sixth human γ-crystallin gene, G5, an 8.0-kb EcoRI fragment from phage clone λ16G3 (24) was subcloned, and the nucleotide sequence of regions hybridizing with a mouse γ2-crystallin cDNA was determined (35) (Fig. 1 and 2). Alignment of the human sequence with that of the mouse cDNA revealed that G5 spanned approximately 3.0 kb and contained a three-exon gene structure similar to other γ-crystallin genes: a small 5′ exon, encoding three amino acids, was followed by two larger exons that encoded the two related structural domains of the 174-residue polypeptide. The first intron was relatively small at 101 bp, while the second intron was approximately 2.2 kb. Both introns were flanked by consensus splice signals and interrupted the coding sequence at positions that, for the most part, were invariant among the different γ-crystallin genes (6, 24). A TATA box was located 56 bp upstream of the translation initiation codon, and a poly(A) addition signal (AATAAA) was present 36 bp downstream of the translation termination codon (TAA). The coding sequences of G5 predicted a polypeptide with a calculated molecular mass of 20,893 daltons.

Figure 3 shows the primary structure of the γ-crystallin encoded by G5 in comparison with the polypeptides (or hypothetical proteins) encoded by γ1-2, G3, and G4 and the two pseudogenes G1ψ and G2ψ. The amino acid sequences are shown aligned by the four structural motifs of the major calf γ-crystallin, γ-II, whose three-dimensional structure is known. Calf γ-II corresponds to a β-pleated protein comprising two highly homologous structural domains, which are in turn folded into two very similar "Greek key" motifs
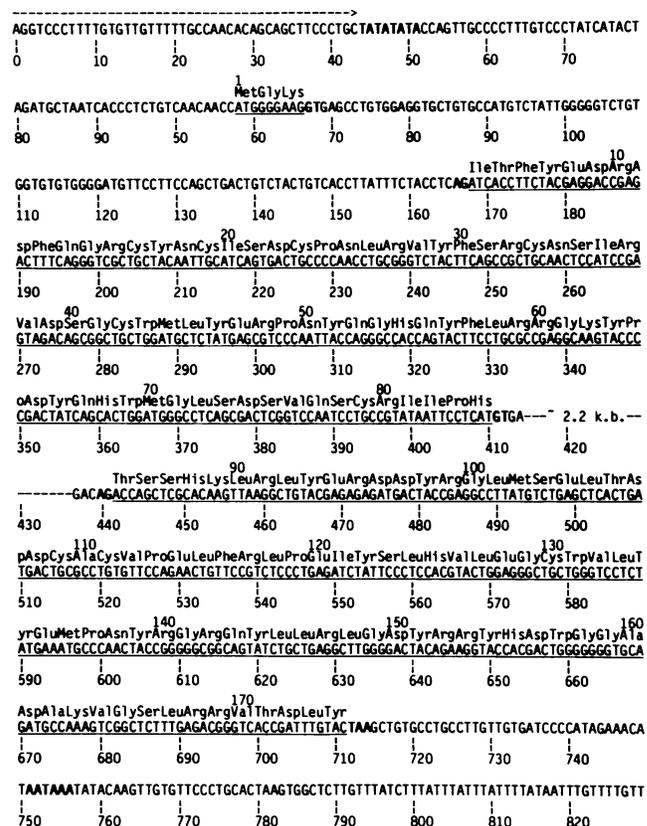

FIG. 2. Nucleotide and amino acid sequence of G5. Coding sequences are underlined, and the TATA box, splice donor/acceptor sites (GT/AG), translation termination codon (TAA), and poly-adenylation signal (AATAAA) are shown in boldface type. The horizontal arrow marks a highly conserved 44-bp sequence immediately preceding the TATA box (see Fig. 5).

(1, 40). Hydrogen bonds within and between motifs confer thermodynamic stability to the protein as well as resistance to proteolytic degradation (36, 40). Detailed sequence comparisons indicated that the polypeptide encoded by G5 showed the least homology (67 to 74%) with the other human γ-crystallins but was generally conserved for residues determined to be critical to the three-dimensional conformation of calf γ-II (1, 40). However, a few amino acid alterations in G5 were noteworthy. For example, the substitution in G5 of aspartic acid for glycine at position 10 may disrupt the intradomain hydrophobic interaction that normally occurs between Gly-10 and Pro-63 in calf γ-II (40). Similarly, the substitution of lysine for aspartic acid at position 61 may eliminate a hydrogen bond that normally occurs in calf γ-II between Asp-61 and Gln-12. Because the protein concentration in the lens may approach the crystal form of calf γ-II and the above regions have been identified as important lattice contacts (40), it is possible that the observed substitutions in G5 could alter the stability of the polypeptide. However, the effects of these substitutions may be marginal since Asp-10 and Lys-61 in G5 are predicted to be in proximity and may therefore interact to preserve an intradomain contact and the stability of the protein. Finally, relative to the other γ-crystallins, G5 showed a slight increase in the number of surface hydrophobic residues (e.g., Val-27, Ala-110, and Leu-148) and exposed sulfhydryl groups (e.g., Cys-15 and Cys-111). It is possible that these changes in G5 favor the
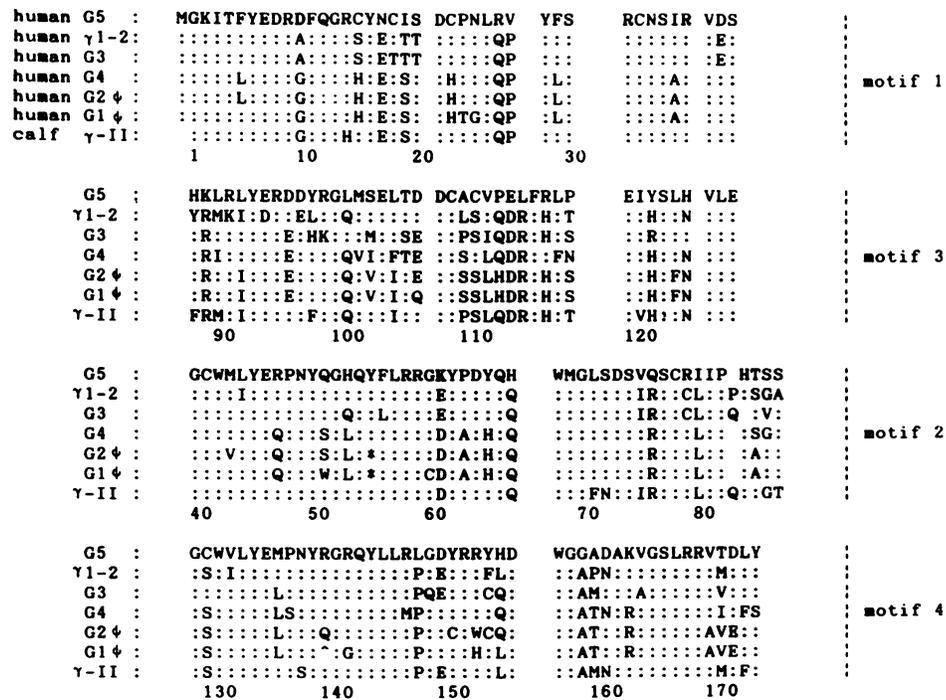
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human  G5   :  MGKITFYEDRDFQGRCYNCIS DCPNLRV  YFS   RCNSIR VDS       :
human  γ1-2:  ::::::::::A::::S:E:TT  :::::QP   :::   :::::: :E:       :
human  G3   :  ::::::::::A::::S:ETTT  :::::QP   :::   :::::: :E:       :
human  G4   :  :::::L::::G::::H:E:S:  :H:::QP   :L:   ::::A: :::       :  motif 1
human  G2 ♦ :  :::::L::::G::::H:E:S:  :H:::QP   :L:   ::::A: :::       :
human  G1 ♦ :  ::::::::::G::::H:E:S:  :HTG:QP   :L:   ::::A: :::       :
calf   γ-II:  :::::::::G:::H::E:S:  :::::QP   :::   :::::: :::       :
                1      10      20             30
```

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G5   :  HKLRLYERDDYRGLMSELTD DCACVPELFRLP  EIYSLH VLE       :
γ1-2 :  YRMKI:D::EL::Q::::::  ::LS:QDR:H:T  ::H::N :::       :
G3   :  :R:::::::E:HK:::M::SE  ::PSIQDR:H:S  ::R::: :::       :
G4   :  :RI:::::E::::QVI:FTE  ::S:LQDR::FN  ::H::N :::       :  motif 3
G2 ♦ :  :R::I:::E::::Q:V:I:E  ::SSLHDR:H:S  ::H:FN :::       :
G1 ♦ :  :R::I:::E::::Q:V:I:Q  ::SSLHDR:H:S  ::H:FN :::       :
γ-II :  FRM:I:::::F::Q:::I::  ::PSLQDR:H:T  :VH::N :::       :
          90      100     110            120
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G5   :  GCWMLYERPNYQGHQYFLRRGKYPDYQH  WMGLSDSVQSCRIIP HTSS       :
γ1-2 :  ::::I:::::::::::::::::E:::::Q  :::::::IR::CL::P:SGA       :
G3   :  ::::::::::::::Q::L::::E:::::Q  :::::::IR::CL::Q :V:       :
G4   :  ::::::Q::S:L:::::::D:A:H:Q  :::::::R:::L:: :SG:       :  motif 2
G2 ♦ :  :::V:::Q:::S:L:*:::::D:A:H:Q  :::::::R:::L:: :A::       :
G1 ♦ :  ::::::Q:::W:L:*::::CD:A:H:Q  :::::::R:::L:: :A::       :
γ-II :  :::::::::::::::::::::D:::::Q  :::FN::IR::L::Q::GT       :
          40      50      60             70      80
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G5   :  GCWVLYEMPNYRGRQYLLRLGDYRRYHD  WGGADAKVGSLRRVTDLY       :
γ1-2 :  :S:I::::::::::::::P:E:::FL:  ::APN:::::::::M:::       :
G3   :  :::::::::L:::::::::::PQE:::CQ:  ::AM:::A::::::V:::       :
G4   :  :S:::::LS::::::::::MP::::::Q:  ::ATN:R:::::::I:FS       :  motif 4
G2 ♦ :  :S:::::L:::Q:::::::P::C:WCQ:  ::AT::R::::::AVE::       :
G1 ♦ :  :S:::::L:::^:G::::::P::::H:L:  ::AT::R:::::AVE::       :
γ-II :  :S:::::::S:::::::::P:E::::L:  ::AMN::::::::M:F:       :
          130     140     150            160     170
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FIG. 3. Comparison of the amino acid sequences of the γ-crystallins or hypothetical proteins encoded by six members of the human γ-crystallin gene family. The amino acid sequences deduced from the nucleotide sequences of G1ψ, G2ψ, G3, and G4 were taken from Meakin et al. (24) and of γ1-2, from den Dunnen et al. (6). Calf γ-II was included for alignment of the four structural motifs, and the numbering system follows that of Wistow et al. (40). The termination codons at position 55 of G1ψ and G2ψ are indicated by asterisks, and the single base deletion resulting in the frameshift mutation at position 140 in G1ψ is marked with a caret (^). Relative alignment of the human sequences is shown according to the known or postulated order of their corresponding genes within the genome.

aggregation of the protein or increase its susceptibility to oxidative processes within the aqueous environment of the lens. Such processes presumably account for the observation that cataractous human lenses contain significantly more disulfides in the insoluble crystallin fraction than normal lenses of equivalent age (13, 17, 38).

Activity of γ-crystallin promoters. Although, as indicated above, considerable information is emerging on the structure and organization of the human γ-crystallin gene family, relatively little is known about the expression of these genes in vivo or of the molecular mechanisms regulating their expression. We therefore set out to determine the activity of different cloned gene members in transient expression assays. Since previous studies in our laboratory established that sequences immediately upstream of the mouse γ2-crystallin gene contain lens-specific promoter activity (18), we constructed vectors in which putative 5' promoter regions from five of the six characterized γ-crystallin genes were cloned in both the positive (+) and negative (−) orientation in front of the bacterial cat gene in pSV0CAT (11). The resultant vectors, designated G(+) or G(−), were transfected into dissected chicken lens explants as well as nonlens cells such as CEFs and COS-1 cells.

Figure 4 shows representative CAT levels detected in 5 to 10 independent transfections in both cultured lens explants and CEFs. Each panel is shown relative to the positive (pRSVCAT) and negative (pSV0CAT) controls for that experiment. The Rous sarcoma virus (RSV) long terminal repeat (LTR) routinely expressed CAT at a high level in both cell types, while the promoterless parental vector generally expressed the bacterial gene at very low levels. In lens cultures, G3(+), G4(+), and G5(+) all produced CAT activ-

ity significantly higher than the background level obtained with the promoterless control vector pSV0CAT. In CEFs, on the other hand, G3(+) and G5(+) generated levels of CAT activity that were insignificant relative to the parental control vector, while cultures transfected with G4(+) invariably contained levels of activity that were marginally higher than those obtained with pSV0CAT. However, G3(+), G4(+), and G5(+) all failed to produce levels of CAT activity above
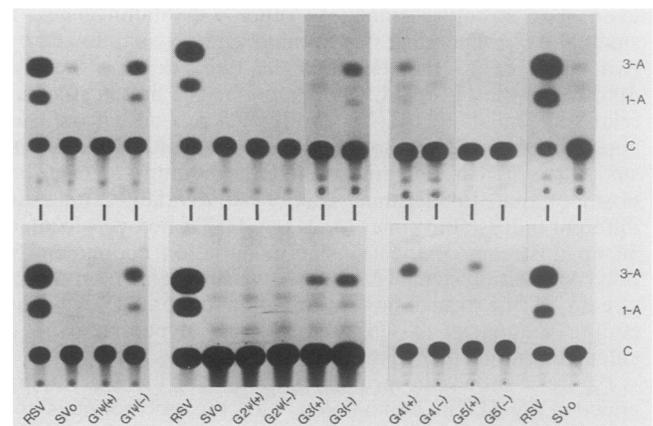

FIG. 4. Analysis of human γ-crystallin promoter activity in CEFs (top row) and primary chicken lens epithelia explants (bottom row). Cultures were transfected with the indicated plasmids, and CAT activity was determined 72 h later by monitoring the conversion of [14C]chloramphenicol to its acetylated forms. C, Native chloramphenicol; 1-A, 1-acetoxy chloramphenicol; 3-A, 3-acetoxy chloramphenicol. RSV, RSV LTR construct.
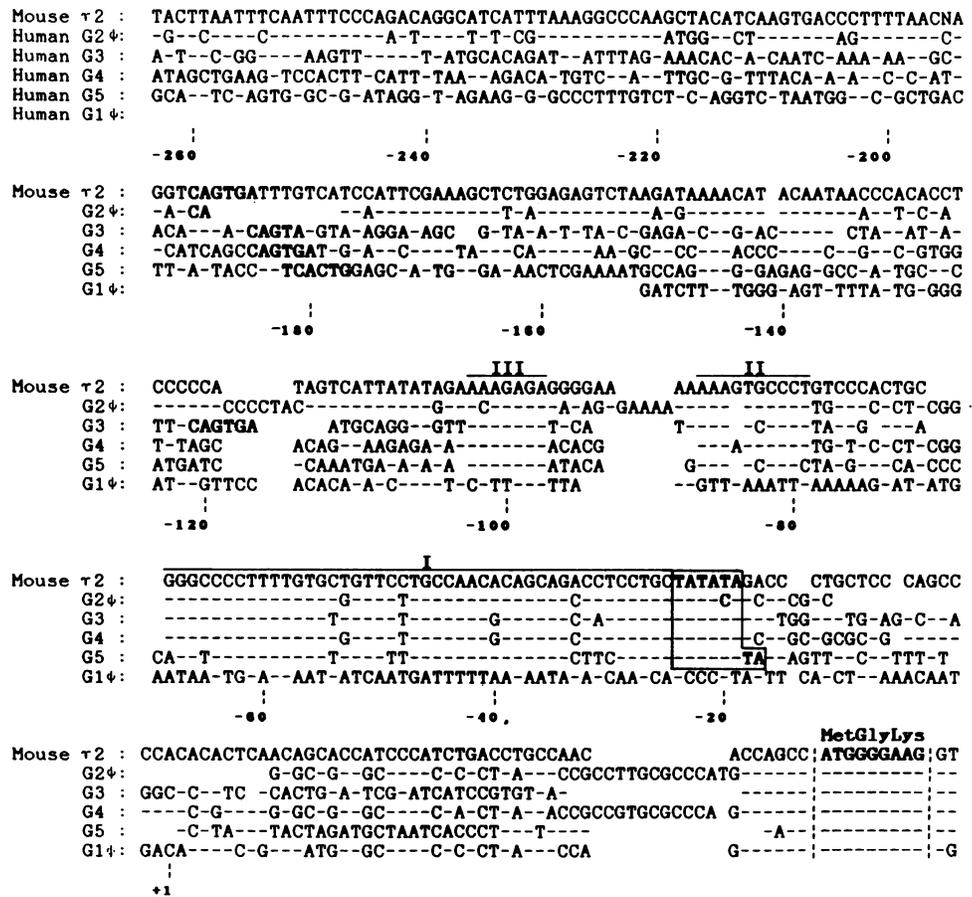
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Mouse τ2 : TACTTAATTTCAATTTCCCAGACAGGCATCATTTAAAGGCCCAAGCTACATCAAGTGACCCTTTTAACNA
Human G2ψ: -G--C----C---------A-T----T-T-CG-----------ATGG--CT-------AG-------C-
Human G3 : A-T--C-GG----AAGTT-----T-ATGCACAGAT--ATTTAG-AAACAC-A-CAATC-AAA-AA--GC-
Human G4 : ATAGCTGAAG-TCCACTT-CATT-TAA--AGACA-TGTC--A--TTGC-G-TTTACA-A-A--C-C-AT-
Human G5 : GCA--TC-AGTG-GC-G-ATAGG-T-AGAAG-G-GCCCTTTGTCT-C-AGGTC-TAATGG--C-GCTGAC
Human G1ψ:

              -260           -240           -220           -200

Mouse τ2 : GGTCAGTGATTTGTCATCCATTCGAAAGCTCTGGAGAGTCTAAGATAAAACAT ACAATAACCCACACCT
G2ψ:       -A-CA          --A-----------T-A--------A-G------- ----A--T-C-A
G3 :       ACA---A-CAGTA-GTA-AGGA-AGC  G-TA-A-T-TA-C-GAGA-C--G-AC----- CTA--AT-A-
G4 :       -CATCAGCCAGTGAT-G-A--C----TA---CA-----AA-GC--CC---ACCC----C--G--C-GTGG
G5 :       TT-A-TACC--TCACTGGAGC-A-TG--GA-AACTCGAAAATGCCAG---G-GAGAG-GCC-A-TGC--C
G1ψ:                                                     GATCTT--TGGG-AGT-TTTA-TG-GGG

              -180           -160           -140

                             ___III___          ___II___
Mouse τ2 : CCCCCA    TAGTCATTATATAGAAAAGAGAGGGGAA    AAAAAGTGCCCTGTCCCACTGC
G2ψ:       ------CCCCTAC----------G---C------A-AG-GAAAA----- ------TG---C-CT-CGG ·
G3 :       TT-CAGTGA    ATGCAGG--GTT-------T-CA    T---- -C----TA--G ---A
G4 :       T-TAGC    ACAG--AAGAGA-A--------ACACG    ---A------TG-T-C-CT-CGG
G5 :       ATGATC    -CAAATGA-A-A-A -------ATACA    G--- -C---CTA-G---CA-CCC
G1ψ:       AT--GTTCC    ACACA-A-C----T-C-TT---TTA    --GTT-AAATT-AAAAAG-AT-ATG

              -120           -100           -80

                         ___I___
Mouse τ2 : GGGCCCCTTTTGTGCTGTTCCTGCCAACACAGCAGACCTCCTGTATATAGACC  CTGCTCC CAGCC
G2ψ:       -------------G----T-------------C-------C-C--CG-C
G3 :       -------------T----T-------G------C-A------------TGG---TG-AG-C--A
G4 :       -------------G----T-------G------C--------C--GC-GCGC-G -----
G5 :       CA--T---------T----TT------------CTTC----TA-AGTT--C--TTT-T
G1ψ:       AATAA-TG-A--AAT-ATCAATGATTTTTAA-AATA-A-CAA-CA-CCC-TA-TT CA-CT--AAACAAT

              -60            -40            -20

                                                    MetGlyLys
Mouse τ2 : CCACACACTCAACAGCACCATCCCATCTGACCTGCCAAC    ACCAGCC ATGGGGAAG GT
G2ψ:                   G-GC-G--GC----C-C-CT-A---CCGCCTTGCGCCCATG------ --------- --
G3 :       GGC-C--TC -CACTG-A-TCG-ATCATCCGTGT-A-             ------ --------- --
G4 :       ----C-G----G-GC-G--GC----C-A-CT-A--ACCGCCGTGCGCCCA G------ --------- --
G5 :         -C-TA---TACTAGATGCTAATCACCCT---T----              -A-- --------- --
G1ψ:       GACA----C-G---ATG--GC----C-C-CT-A---CCA        G------ --------- -G

              +1
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FIG. 5. Comparison of the 5'-flanking sequences of human G2ψ, G3, G4, G5, and G1ψ and mouse γ2. Sequences are shown aligned relative to mouse γ2 and have been positioned to maximize homology. Position +1 corresponds to the transcription initiation site of mouse γ2 (18). The three highly conserved sequence tracts I, II, and III are indicated, and the hexamer CAGTGA and its reverse complement TCACTG are shown in boldface type. TATA sequences are boxed, and the coding sequences of the first exon are demarked by vertical dashes. The upstream sequence of mouse γ2 was taken from Lok et al. (18), and the nucleotide sequences extending from the first exon to tract I of G2ψ, G3, G4, and G1ψ have been reported previously (24).

those obtained with pSV0CAT in COS-1 cells (data not shown). From these results, we conclude that the 5'-flanking sequences of G3, G4, and G5 contain functional promoters that are preferentially active in lens cells. Similar analyses carried out with G1ψ(+) and G2ψ(+) revealed that the 5'-flanking sequences of the two pseudogenes failed to promote the expression of significant levels of CAT activity in both lens cells and CEFs. It would therefore appear that G1ψ and G2ψ lack functional promoters and that these two pseudogenes are probably transcriptionally inactive within the lens. Finally, the lens-specific promoter activity scored in the above assays was dependent on the appropriate orientation of the 5'-flanking gene sequences (Fig. 4). Thus, the negative orientation vectors G2ψ(−), G4(−), and G5(−) failed to promote the expression of significant levels of CAT activity in lens cultures as well as CEFs. While G1ψ(−) and G3(−) produced substantial levels of CAT activity in lens cells, these vectors also generated comparable levels of activity in CEFs. Although the basis for the activity of the latter two constructs remains unknown, the above analyses established that none of the 5'-flanking sequences of the five human γ-crystallin genes supported lens-specific promoter activity in the opposite orientation.

Promoter sequence comparisons. We next determined the 5'-flanking sequences of the different γ-crystallin genes in an attempt to identify conserved domains that may be important for the expression of these genes within the lens. Figure 5 shows the upstream sequences of the G1ψ, G2ψ, G3, G4, and G5 genes extending 202 to 320 bp 5' of their respective translation initiation codons. The human sequences are shown aligned by the corresponding sequences of the mouse γ2-crystallin gene (18), whose promoter element has been analyzed in greater detail. For mouse γ2, it has been determined that sequences extending from −226 to the initiation codon are sufficient for lens-specific promoter activity and that deletion of sequences between −226 and −171 completely abolishes promoter function (18; S. Lok, unpublished data). Detailed comparison of the different upstream sequences revealed that, with the exception of G1ψ, mouse γ2 and all of the human γ-crystallin genes contained three highly conserved sequence tracts which were located within the 5' boundary of the mouse γ2 promoter as defined by deletion analysis. The largest of these, tract I, spanned a 44-bp segment immediately 5' of the TATA box. Tract II corresponded to a 10-bp sequence located between −77 and −88 of the mouse γ2 gene, while tract III was a 7-bp polypurine sequence, AAAGAGA, located between −97 and −103 of mouse γ2. In G4 and G5 the latter sequence was also present 3 and 43 bp, respectively, further upstream. While the functional significance of
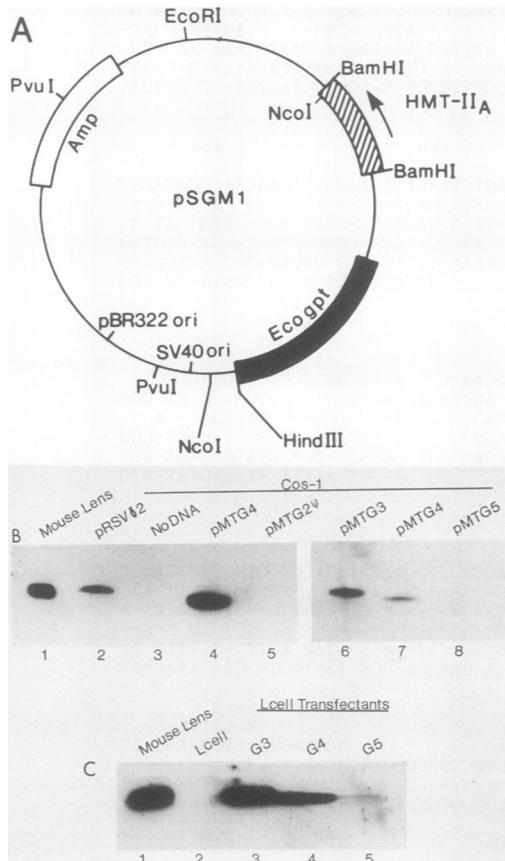
FIG. 6. (A) Expression vector pSGM1. (B) Western blot (immunoblot) analysis of γ-crystallins produced by COS-1 cells from the human MT$_{IIA}$ promoter. Lanes: 1, soluble extract from mouse lens (positive control); 2 to 8, equivalent cell lysates from COS-1 cells transfected with pRSVγ2, no DNA, and the MT$_{IIA}$-γ-crystallin hybrid gene constructs pMTG4, pMTG2ψ, pMTG3, pMTG4, and pMTG5, respectively. Lanes 1 to 5 and 6 to 8 represent separate transfection experiments. Immunoreactive proteins were detected with rabbit anti-calf γ-crystallin serum. (C) Western blot of γ-crystallins produced in stable L cell transfectants. Lanes: 1, soluble extract from mouse lens; 2, 3, 4, and 5, equivalent cell lysates from control L cells and transfectants expressing G3, G4, and G5, respectively. Immunoreactive proteins were detected with rabbit anti-human γ-crystallin serum.

these tracts is presently unclear, their strong conservation and strategic location suggest that they may correspond to essential elements of a functional γ-crystallin promoter. This idea is consistent with our observation that the 5'-flanking region of G1ψ lacked the above sequence tracts, as well as a TATA box, and showed no detectable promoter activity. The basis for the inactivity of the G2ψ promoter, however, was less apparent. Since this pseudogene contained all three highly conserved sequence tracts, it is possible that the defect in its promoter resides in more distal 5' sequences, which in the case of mouse γ2 are also essential for promoter activity (see also below). Alternatively, G2ψ may contain a subtle mutation in its TATA box since, in contrast to the other γ genes, it contains the sequence TATACA rather than TATATA. Further studies will be required to address these possibilities.

Although the different human genes generally show little sequence identity upstream of tract III, mouse γ2 and the transcriptionally active gene members G3, G4, and G5 all

contain a highly conserved 6-bp sequence located between positions comparable to −177 to −190 of mouse γ2. In G4 and mouse γ2, this hexamer contained the sequence CAGTGA, whereas in G5 it was present in the reverse complement, TCACTG. G3 only contained an incomplete copy of this sequence (CAGTA) in this region but carried the sequence CAGTGA further downstream, 13 to 18 bp 5' of tract III. Two different lines of evidence suggest that this sequence may be important for promoter function. First, in mouse γ2, the hexamer CAGTGA is located between −190 and −185. Since sequences 5' of −171 have been determined to be critical for promoter activity (18), the location of the hexamer in mouse γ2 is consistent with the idea that this sequence plays a role in gene expression. Second, as shown in Fig. 5, mouse γ2 and the transcriptionally inactive pseudogene G2ψ showed extensive identity in their 5'-flanking sequences upstream of tract III. However, relative to mouse γ2, G2ψ contained an apparent 6-bp insertion, CCCCTA, at position −118 of mouse γ2. This insertion is unlikely to account for the inactivity of G2ψ since a comparable sequence, CCCCCA, was also present in the transcriptionally active rat γ gene, γ3-1, which is otherwise closely related to mouse γ2 (7). In comparison with mouse γ2, however, G2ψ also appeared to contain a conspicuous 11-bp deletion, between −178 and −188, which spanned a portion of the hexamer CAGTGA. This observation raises the possibility that the inactivity of the G2ψ promoter may be due to an incomplete copy of the hexamer within the pseudogene. Experiments to test these hypotheses are presently in progress.

Immunodetection of human γ-crystallins produced in vitro. The studies described above established that three of the five human γ-crystallin genes we analyzed, G3, G4, and G5, contained functional promoters that were preferentially active in lens cells. Moreover, the nucleotide sequence of these three genes predicted closely related γ-crystallin polypeptides. However, to date, only two distinct γ-crystallin species have been identified in the human lens (41, 42). This apparent discrepancy suggests either that one of the above genes contains an undisclosed defect which renders it functionally noncoding or, alternatively, that at least one additional γ-crystallin is synthesized in the human lens. To provide a basis for characterizing the γ-crystallins encoded by individual cloned genes, we constructed a series of vectors in which the coding sequences of the different γ-crystallin genes were placed under the transcriptional control of the human MT$_{IIA}$ promoter. The MT$_{IIA}$ promoter allowed us to express the γ-crystallin coding sequences in a non-lens cell environment so that the individual gene products could be detected in the absence of any other cross-reacting lens proteins. The expression vector used for these studies, pSGM1, contains a simian virus 40 origin of replication, allowing amplification of the template after transfection into COS-1 cells (Fig. 6A). This feature of the vector and the ability to transcriptionally induce the MT$_{IIA}$ promoter both served to increase the amount of protein that could be produced in the transient expression assay. Since MT$_{IIA}$ and the human γ-crystallin genes contain a common NcoI site about their translation initiation codons, precisely constructed hybrid genes were generated by directionally cloning the coding sequences of G3, G4, G5, and G2ψ between the NcoI and EcoRI sites of pSGM1 (Fig. 6A). The resultant vectors, designated pMTG3, pMTG4, etc., were transfected into COS-1 cells, and 48 h later cell lysates were prepared and assayed for γ-crystallin protein by immunoblotting. As a positive control, COS-1 cells were transfected in parallel

with pRSVγ2, which contains the coding region of the mouse γ2 gene coupled to the LTR of RSV.

Both G3 and G4 encoded polypeptides that reacted strongly with antiserum raised against calf γ-crystallins and which migrated similarly to γ-crystallins isolated directly from mouse lens (Fig. 6B). The protein encoded by G3 showed a slightly slower mobility than the species encoded by G4, despite the fact that the latter protein was predicted from its gene sequence to have a slightly higher molecular weight (24). Analysis of comparable cell lysates also revealed that COS-1 cells transfected with pMTG3 consistently produced greater amounts of immunoreactive protein than parallel cultures transfected with pMTG4 (e.g., Fig. 6B, lanes 6 and 7). While this observation may simply reflect differences in the immunoreactivity of the two proteins, it could also reflect differences in protein stability or posttranscriptional regulation of the hybrid genes. No immunoreactive protein was detected in COS-1 cells transfected with pMTG5 (lane 8). This result was unexpected because the nucleotide sequence of G5 predicted that this gene encoded a polypeptide with 70 to 74% amino acid homology to the γ-crystallins encoded by G3 and G4. To maximize detection of the G5-encoded polypeptide, we established L cell lines which contained pMT-γ-crystallin constructs stably integrated and used an antiserum raised against human γ-crystallins for immunodetection. Immunoreactive proteins encoded by G3, G4, and G5 could be readily detected in the transfected L cells (Fig. 6C), supporting the idea that all three genes give rise to closely related γ-crystallins in the human lens. No immunoreactive protein was detected in COS-1 cells transfected with pMTG2ψ (Fig. 6B). This result was for the most part expected, since G2ψ contains an in-frame termination codon at position 55 of the 174-residue coding sequence (24) (see also Fig. 3). However, since G2ψ contains appropriate splice and polyadenylation signals, failure to detect an immunoreactive species of lower molecular weight suggests either that the putative truncated protein encoded by G2ψ is highly unstable or that it lacks significant reactivity with our antiserum.

DISCUSSION

The impetus for our present study was provided by the recent finding that a dominantly inherited cataract is closely linked to the human γ-crystallin gene family (20) and that, in contrast to the γ-crystallins of a number of species (8, 31, 40), the corresponding proteins in humans have been considerably less well characterized. This is primarily due to the fact that the human γ-crystallins undergo extensive posttranslational modification during aging, leading to significant protein microheterogeneity that complicates their separation and physical characterization (32, 33; P. Russell, D. Garland, J. S. Zigler, Jr., S. O. Meakin, L.-C. Tsui, and M. L. Breitman, FASEB J., in press). To generate knowledge of both the complexity of the human γ-crystallins and the molecular mechanisms regulating their expression, we determined the primary structure of a sixth human γ-crystallin gene, G5, and performed transient assays to monitor the activity of five different members of the gene family. In addition to providing information on the tissue specificity of the γ-crystallin promoters and on the proteins encoded by individual genes, the transfection assays, as opposed to Northern blot (RNA blot) analyses of lens RNA, circumvented the technical difficulties of having to distinguish among nonallelic transcripts derived from closely related genes.

The sixth human γ-crystallin gene, G5, was determined to contain a three-exon gene structure comparable to that reported previously for other γ-crystallin genes (5, 18, 19, 25). Moreover, determination of its primary structure provided clues to the possible linkage relationships among the different members of the human γ-crystallin gene family. Cloning studies have established that at least four of the human γ-crystallin genes are linked head to tail in the order 5'-γ1-2-G3-G4-G2ψ-3' (6, 24). As noted previously (24), the order of these genes is identical to that of the orthologous genes of the rat (25), except for G2ψ, which lacks a rodent counterpart. Since nucleotide sequence comparisons suggest that G5 may be the ortholog of rat γ1-1 (7), it is possible that G5 occupies a comparable position in the human gene cluster, generating the gene order of 5'-G5-γ1-2-G3-G4-G2ψ-3'.

Of the six human γ-crystallin genes that have been cloned and characterized, four (γ1-2, G3, G4, and G5) were predicted to encode closely related polypeptides (this study; 6, 24), while the other two (G1ψ and G2ψ) have been shown to contain identical in-frame termination codons at comparable positions in the second exon (24). By expressing the coding sequences of G3, G4, and G5 from the MT_{IIA} promoter in COS-1 cells as well as stably transfected L cells, we established that transcripts from three of the four potentially active genes have the ability to be appropriately spliced and encode the predicted polypeptides. Furthermore, because the 5'-flanking sequences of these genes were shown to direct the expression of the bacterial cat gene in lens explants, it is probable that G3, G4, and G5 are also transcriptionally active at some stage of development in the human lens. This is in fact supported by recent studies in which we observed that the translation products of G3, G4, and G5 show properties similar to distinct γ-crystallin proteins present in fetal lens (Russell and Meakin, unpublished observations).

The finding that the upstream sequences of G3, G4, and G5 contain lens-specific promoter activity coincides with our previous observations on the promoter region of the mouse γ2 gene (18). As lens-specific promoter activity has also been detected in the 5'-flanking sequences of the chicken δ-crystallin gene (15), the mouse and chicken αA-crystallin genes (3, 28) and, more recently, the human βA3/A1-crystallingene (S. O. Meakin and B. Hogg, unpublished observations), it would appear that the 5' localization of tissue-specific regulatory information is a general theme shared by these different classes of lens-specific genes. However, it is intriguing that the γ-crystallin genes, which as a class are expressed only in the terminally differentiated fibers cells of the lens, show significant divergence in their 5'-flanking regulatory sequences. In this regard, mouse γ2 and the human genes G3, G4, G5, and G2ψ contain three highly related sequence tracts which reside between the TATA box and positions comparable to −103 of the mouse γ2 gene. Related sequence tracts are also present in the 5'-flanking regions of other mouse and rat γ-crystallin genes that have been analyzed (7, 27). However, these regions would appear to be insufficient for lens-specific promoter activity because (i) they are present within G2ψ, which lacks detectable promoter function, and (ii) it has been shown that lens-specific promoter activity of the mouse γ2 gene is dependent on sequences extending further than 171 bp upstream of the transcription start site (18). Since there is very little homology upstream of these sequence tracts among mouse γ2 and the functional human genes G3, G4, and G5, with the possible exception of the hexamer

CAGTGA, it is possible that the divergence in these upstream sequences plays a role in defining the subtle differences that have been observed in the developmental regulation of different γ-crystallin genes (27; Van Leen et al., in press). We are presently investigating this possibility, particularly since recent studies in our laboratory have established that sequences within the 5'-flanking region of the mouse γ2 gene are sufficient to direct both tissue-specific and appropriate developmental expression of the E. coli lacZ gene in transgenic mice (10).

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