Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 7762–7766, December 1984
Biochemistry

γ-Crystallin family of the mouse lens: Structural and evolutionary relationships

(cDNA sequences/amino acid sequences)

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Communicated by Igor B. Dawid, August 24, 1984

ABSTRACT **The heterogeneity inherent among γ-crystallins of the mouse lens was investigated by sequence analysis of three γ-crystallin-specific cDNAs. Comparison of the nucleotide sequence of these cDNAs and one previously reported by us revealed that the four γ-cDNAs share 80–90% homology in nucleotide sequence. The entire 3′ half of the coding region shows more variability than the 5′ half, whereas the greatest variability is observed in the 3′ untranslated region where numerous base substitutions, deletions, and insertions seem to have occurred. Alignment of the amino acid sequences of the four mouse γ-crystallins according to the known four structural motifs of the major calf γ-crystallin, γ-II, suggests that all four mouse polypeptides are structurally very similar to calf γ-II. However, most of the mouse polypeptides differ from γ-II by the absence of one amino acid residue, resulting in a shorter connecting peptide between the two globular domains of the protein. Primary sequence alignment also revealed that the four mouse γ-crystallins are most divergent in the third structural motif of the polypeptide. The significance of these differences in terms of the structure and function of the γ-crystallins in the mouse lens is discussed.**

Approximately 90% of the soluble protein of the vertebrate lens consists of a group of structural proteins known as the crystallins (1, 2). In mammals, these lens-specific proteins can be divided into three antigenically distinct classes, α, β, and γ, each of which comprises several polypeptides of related primary structure. The importance of the structure and function of these polypeptides has long been indicated by the tight developmental regulation of the different crystallin classes and by their differential spatial distribution within the lens (3).

At present, little is known about the functional significance of the heterogeneity inherent among the polypeptides of each crystallin class. As a first step towards addressing this question, we have been involved in a study of the structure of the γ-crystallins of the mouse. In the mouse lens, up to seven different γ-crystallin polypeptides can be identified by two-dimensional gel electrophoresis (4) and a similar number has been reported for several other animal species (5–7). A number of γ-crystallin-specific cDNAs have been isolated from a variety of animal species (5, 8–11) and these have been analyzed by DNA sequencing. We (12) and others (13, 14) have shown that the γ-crystallins of representative gene members of mice and rats are encoded by a family of genes with a similar structure. All of them contain a small 5′ exon, encoding three amino acids, followed by two larger exons of approximately equal size that encode the remainder of the ≈20-kilodalton polypeptide (12–14). There is a precise correspondence between the two major exons of the gene and the two similar structural domains of the polypeptide, suggesting that the γ-crystallins may have evolved by exon duplication and subsequent gene amplification (12–14).

The nucleotide sequence of one of the mouse γ-crystallin cDNAs and its corresponding gene were described in detail in an earlier paper (12). In this communication, we report the nucleotide sequence of three additional mouse γ-crystallin cDNAs.

MATERIALS AND METHODS

cDNA Clones and DNA Sequencing. All cDNAs were cloned by dG·dC tailing into the *Pst* I site of pBR322 (8) and propagated in *Escherichia coli* HB101 using standard procedures (15). Nucleotide sequencing of the cDNA inserts was performed using the chemical cleavage method of Maxam and Gilbert (16). The end-labeling and fragment isolation procedures have been described (12). Pair-wise nucleotide sequence alignment between different cDNAs was performed using the NUCALN computer program of Wilbur and Lipman (17).

RESULTS AND COMMENTS

Characterization of the Mouse γ-Crystallin cDNAs. The mouse γ-crystallin cDNAs analyzed in the present study were isolated by Shinohara *et al.* (8). By using hybrid-selection, four of the cDNA clones, pMγ$_1$, pMγ$_2$, pMγ$_3$, and pMγ$_4$, were previously shown to correspond to lens mRNAs encoding electrophoretically distinct γ-crystallin polypeptides (8). By using these cDNAs under stringent hybridization conditions, discrete genomic segments were detected preferentially in mouse DNA (12). A fifth cDNA clone, pMγ$_1$a, was subsequently isolated from the same cDNA library and shown by hybrid-selection experiments to correspond to the same γ-crystallin species as pMγ$_1$ (T. Shinohara, personal communication). Subsequent sequence analysis (see below) showed that pMγ$_1$ and pMγ$_1$a are probably cDNA clones derived from mRNA transcripts of the same gene. The nucleotide sequences of pMγ$_4$ and its corresponding gene have been reported by us in an earlier communication (12). We have now determined the nucleotide sequences of pMγ$_1$, pMγ$_2$, and pMγ$_3$ by using the strategy outlined in Fig. 1.

The longest cDNA clone, pMγ$_2$, contains an insert of 614 base pairs (bp) in length, excluding the dG·dC homopolymer tails (see Fig. 2). Translation of the major open reading frame in this cDNA results in a polypeptide of 174 residues initiated from a methionine codon at base number 5. Since the nu-

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Abbreviation: bp, base pair(s).

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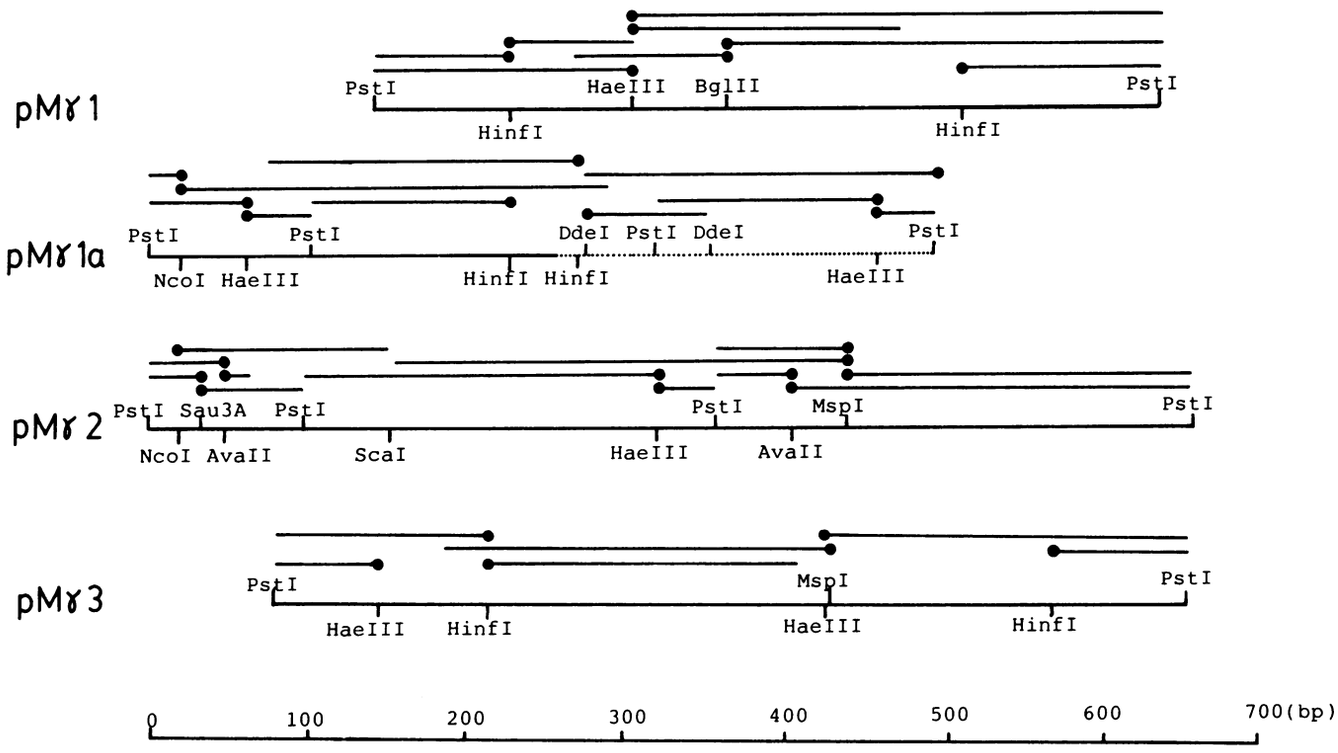

FIG. 1. Restriction maps and sequencing strategy for γ-crystallin-specific cDNAs. Regions sequenced are indicated by lines above the restriction maps and labeled ends are indicated by dots. Only restriction sites used for end-labeling are indicated. Dashed line for γ_1a corresponds to sequences derived from intron 2 of the mouse γ_1-crystallin gene. Sequence determinations have led to the modifications (see ref. 18) of the preliminary restriction maps of the cDNAs (8).

cleotide sequence of this cDNA corresponds perfectly to the amino acid sequence that was partially determined for the NH_2 terminus of mouse γ_2-crystallin (see ref. 8), clone pMγ_2 encodes a complete γ-crystallin polypeptide. It is of interest that the amino acid sequence of this polypeptide is identical to that which was deduced for rat γ_3-crystallin by analysis of its corresponding cDNA (9). There are only seven nucleotide differences between the two cDNAs, including the 3′ untranslated region, suggesting that mouse γ_2 and rat γ_3 are probably corresponding members of the γ-crystallin gene families in these two closely related animal species.

Clone pMγ_3 contains a 530-bp cDNA insert. As shown in Fig. 3, translation of the single open reading frame of this cDNA leads to a polypeptide highly homologous to calf γ-II crystallin (19). Moreover, alignment of the latter protein and the predicted mouse γ_3-crystallin suggests that pMγ_3 lacks coding information for the first 37 amino acids of the mouse γ_3-polypeptide. The amino acid sequence of mouse γ_3-crystallin has been partially determined for the NH_2-terminal 34 residues (8). Comparison of the sequence of our cDNA with this amino acid sequence also supports the conclusion that the cDNA insert in pMγ_3 is incomplete.

The cDNA insert contained in pMγ_1 is only 463 bp in length and, from comparison with the other cDNAs, probably lacks coding information for most of the NH_2-terminal half of the putative γ_1-crystallin polypeptide. However, a fifth γ-crystallin clone isolated from the same cDNA library, pMγ_1a, encodes the missing portion of this protein. The nucleotide sequence of this latter cDNA encodes the amino acid sequence reported for the NH_2 terminus of mouse γ_1-crystallin (8) and, in addition, overlaps for 101 bp with the sequence at the 5′ end of the insert in pMγ_1. Interestingly, the cDNA insert in pMγ_1a also contains a 198-bp sequence at its 3′ end that is not represented in pMγ_1. Subsequent analyses revealed that the latter sequence was derived from the second intron of the mouse γ_1-crystallin gene (data not shown). Hence, the insert in pMγ_1a was presumably generated by reverse transcription of an RNA molecule that had spliced out the first but not the second intron. In any event, we were able to use the composite nucleotide sequences of the two overlapping cDNAs to generate the complete coding sequence of mouse γ_1-crystallin as shown in Fig. 2.

The γ_4 cDNA described previously (12) is also incomplete. However, since the sequence of its corresponding gene had also been determined (12), it was possible to reconstruct the complete coding sequence for mouse γ_4-crystallin as was done for γ_1.

Nucleotide Sequence Comparisons. The four mouse γ-crystallin cDNA sequences, either obtained directly or reconstructed as given above, were aligned to compare their evolutionary relationships (Fig. 2). Since the coding region for γ_3 was incomplete, its comparison with the other cDNAs was limited to the 3′ half of the cDNA.

Comparison of all four cDNAs revealed that γ_2 was the most conservative species, showing the least number of nucleotide changes relative to the other cDNAs. It was therefore used as the prototype for sequence alignment. Fig. 2 shows that all four cDNAs share extensive sequence homology, with the percentage of matched nucleotides between different cDNAs ranging from 80% to 90%. Sequence comparisons also revealed that γ_1 and γ_2 are more closely related to each other than to γ_3 and γ_4, which, in turn, match in nucleotide sequence at a large number of positions that are divergent from γ_1 and γ_2. These observations suggest that γ_1,γ_2 and γ_3,γ_4 correspond to different evolutionary groups and that their corresponding genes may have evolved by stepwise duplication. The greatest variability among the four cDNAs was observed in the 3′ untranslated region where extensive base substitutions, deletions, and insertions seem to have occurred. In addition, the entire 3′ half of the coding region showed more variability than the 5′ half: the nucleotide sequence conservation for the 3′ half was calculated to

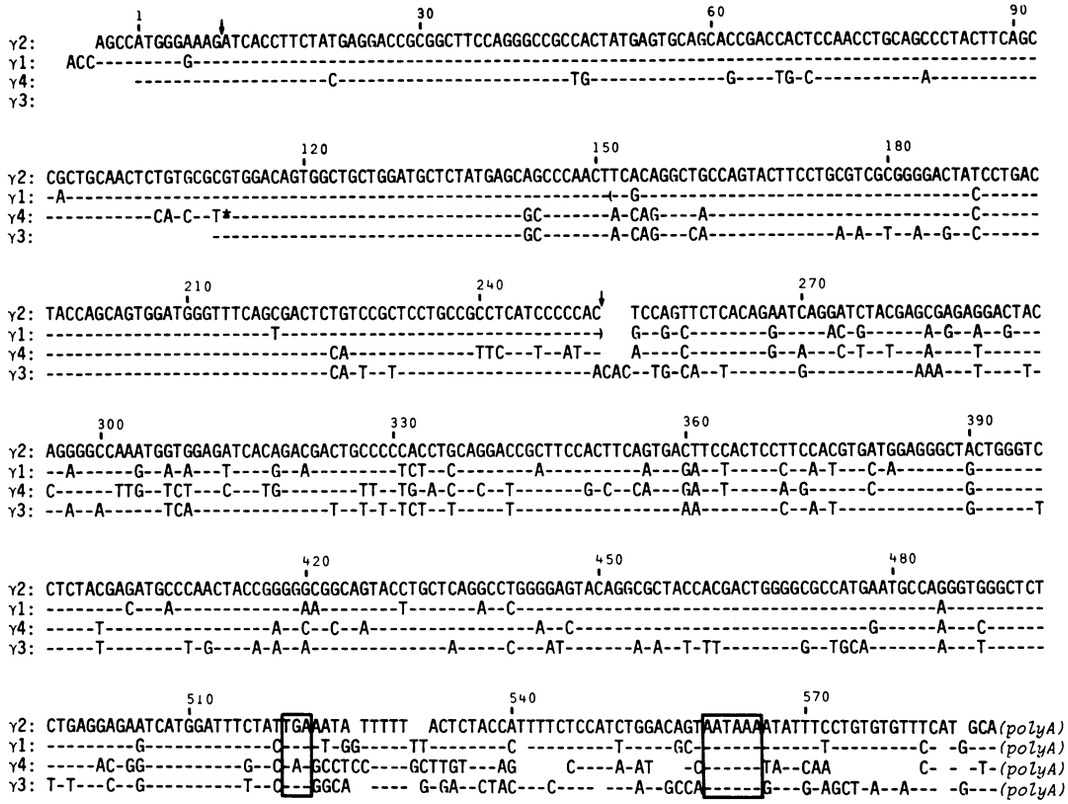

FIG. 2. Nucleotide sequence comparison of four mouse γ-crystallin cDNAs. The nucleotide sequences of γ_2 and γ_3 were determined by sequencing the cDNA inserts of pMγ_2 and pMγ_3, respectively. The γ_2 cDNA was used as the prototype for sequence alignment and the first nucleotide of the initiation codon ATG was marked as base number 1. The portion of the γ_1 sequence enclosed in parentheses was found in the two overlapping clones pMγ_1 and pMγ_1a as described in the text. The sequence of γ_4 was described previously (12) and the asterisk (*) denotes where genomic sequence (12) was used 5′ to this point. The termination codons and the poly(A) addition signal A-A-T-A-A-A are boxed. Arrows indicate splice junctions for γ_1, γ_2, and γ_4 (ref. 12; unpublished results).

be 70–80%, whereas that for the 5′ half was >90%. Interestingly, the transition point between the two regions coincides roughly with the splice junction of the mRNA where the major intervening sequence (intron 2) is located (ref. 12; unpublished data). However, as discussed below, >50% of the nucleotide changes do not alter the encoded amino acid, indicating strong selection pressure for a large number of the residues in the γ-crystallin polypeptides. These observations are consistent with earlier protein studies that indicated that γ-crystallins vary more at the COOH-terminal than at the NH_2-terminal end (6, 20, 21).

The above data combined with present information on the tertiary structure of γ-crystallin polypeptides was also instructive in terms of crystallin gene evolution. The three-dimensional structure of the major γ-crystallin of the calf lens, γ-II, has been determined by high-resolution x-ray diffraction analysis (19). The polypeptide is organized into two similar globular domains and each domain consists of two "Greek key" motifs that contain predominantly β-pleated sheets (19). Assuming similar symmetrical structures exist for the γ-crystallin polypeptides of the mouse, the sequencing data in Fig. 2 support the idea that the coding information for γ-crystallins evolved by intragenic sequence duplication (9, 22). Considerable internal homology was observed when the mouse cDNA sequences corresponding to each of the four structural motifs of the polypeptides were aligned and compared. For example, in the case of γ_2, about 50% homology exists between the nucleotide sequences of motifs 1 and 3 and motifs 2 and 4 after a little adjustment is allowed for maximal sequence alignment. In addition, ≈40% of the nucleotides in each motif are conserved in at least two other motifs. This intragenic sequence homology suggests that the γ_2 coding information originated from a single primordial sequence that underwent two successive duplication events to form the present four structural motifs. Similar observations were made for γ_1, γ_3, and γ_4, indicating that the internal homology described above has been highly conserved during evolution of the individual members of this gene family.

Amino Acid Sequence Comparisons. The amino acid sequences of the four mouse γ-crystallin polypeptides inferred from the nucleotide sequence were aligned according to the known four structural motifs of the major calf γ-crystallin, γ-II (19). As revealed in Fig. 3, the four mouse γ-crystallin polypeptides are highly related in primary structure, showing 80–90% homology in their amino acid sequence. This alignment also suggests that all four mouse γ-crystallins are structurally very similar to calf γ-II. All of the structurally important residues of the characteristic Greek key fold (Tyr-6, Phe-11, Gly-13, Ser-34, and equivalents in all four motifs) are conserved, as are important hydrophobic core residues (such as Trp-42, Trp-68, and equivalents in all four motifs) and interdomain contact residues (Met-43, Phe-56, Val-132, Leu-145, Ile-81, with Val-170 conservatively varied). Although most of the amino acid changes are conservative, such as Val/Ile at position 35 and Glu/Asp at position 96, a small number of residues appeared to have changed types, such as leucine for Gln-101, methionine for Thr-106, serine for Pro-110, and serine for Leu-80 between γ_4 and γ-II. The major difference between most of the mouse γ-crystallins and γ-II was the absence and presence of one amino acid residue, Gln-83, in the connecting peptide between the two globular domains. Since this extra residue was common to both mouse γ_3 and γ-II, and the polypeptides exhibit >90% amino acid sequence homology, mouse γ_3 seems to be the

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mouse γ2:   M G K I T F Y E D R G F Q G R H Y E C S T   D H S N L Q P     Y F S       R C N S V R   V D S
mouse γ1:   . . . . . . . . . . . . . . . . . . . . .   . . . . . . .     . . .       H . . . . .   . . .
mouse γ4:   . . . . . . . . . . . . . . C . . . . S     . C P . . . T     . . .       . . . . . .   . . .     motif 1
mouse γ3:     . . - - . - - - - - - - - - - - - - . S   - C - - - - -     . . .       - . - . - -   - - -
calf γII:   . . . . . . . . . . . . . H C . . . . S     . C P . . . .     . . .       . . . . I .   . . .
            1                 10                20                          30

      γ2:    H R I R I Y E R E D Y R G Q M V E I T D   D C P H L Q D R F H F S       D F H S F H   V M E
      γ1:    . . . . L . . . . E . . . . V I . F . E   . . . S . . . . . . . N       E I Y . L N   . L .
      γ4:    . . . . L . . . D . . . . L V S . L M .   . . S C I H . . . R L H       E I Y . M .   . L .     motif 3
      γ3:    Y . M . . . . K D . F . . . . S . . . .   . . L S . . . . . . . .       E I . . L N   . . .
     γII:    F . M . . . . . D . F . . . . S . . . .   . . . S . . . . . . L T       E V . . L N   . L .
                 90                100                      110                       120
                                                                                     CONNECTING PEPTIDE
      γ2:    G C W M L Y E Q P N F T G C Q Y F L R R G D Y P D Y Q Q     W M G F S D S V R S C R L I P   H S S S
      γ1:    . . . . . . . . . . . A . . . . . . . . . . . . . . . .     . . . . . . . . . . . . . . .   . A G .
      γ4:    . . . . . . . R . . Y Q . Y . . . . . . . . . . . . . .     . . . . . . . I . . . . S . .   Y T . .   motif 2
      γ3:    . . . . . . . R . . Y Q . H . . . . . . . . E . . . . . .   . . . . . . . I . . . . . . . Q . . G T
     γII:    . . . . . . . R . . Y Q . H . . . . . . . . . . . . . . .   . . . N . . I . . . . . . . Q . T G T
             40                50                   60                      70                80

      γ2:    G Y W V L Y E M P N Y R G R/Q Y L L R P G E Y R R Y H D     W G A M N A R V G S L R R I M D F Y
      γ1:    . C . . . . D . T . . . . . . . . . . . . . . . . . . .     . . . . . . K . . . . . . V . . . .
      γ4:    . C . . . . . . . . . . . . . . . . . . D . . . . . . .     . . . . D . K . . . . . . V . . L .     motif 4
      γ3:    . C . . . . . . . S . . . . . . . . . . M . . . . L .       . . . A . . K . . . F . . V . . . .
     γII:    . S . . . . . . . S . . . . . . . . . . . . . . . L .       . . . . . . K . . . . . . V . . . .
                130                140                  150                    160               170
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FIG. 3. Amino acid sequence comparison for four mouse γ-crystallins. The amino acid sequences were deduced from their cDNA sequences. The calf γ-II crystallin was included for motif alignment and the amino acid numbering system followed that of Wistow *et al.* (19). These results are consistent with those determined by partial protein sequencing (8). The dots (·) represent amino acids identical to those in the prototype sequence and bars (-) denote unknown residues in γ_3. Residues corresponding to the connecting peptide are indicated.

murine counterpart of bovine crystallin γ-II.

Primary sequence alignment also revealed that the four mouse γ-crystallins are most divergent in motif 3, which, with motif 1, forms the most solvent-exposed pair of structural motifs. As shown in Table 1, amino acid sequence conservation between the different polypeptides is only about 65% in motif 3, whereas for the other three motifs it is considerably greater, ranging from 83% to 98%. It was also of interest that for some of the γ-crystallins, the relative divergence of motif 4 is less at the amino acid level than at the nucleotide level, whereas the reverse is true for the other three motifs (see Table 1), although these differences are small. This may reflect a relatively greater number of silent nucleotide changes in regions corresponding to motif 4 than in other regions.

DISCUSSION

The close resemblance of the primary structure of the various mouse γ-crystallins (see Fig. 3) suggests that these polypeptides have a very similar role in the organization of macromolecules within the cells of the lens. The fact that all γ-crystallins exist in monomeric form when isolated from the lens (1, 23, 24) is consistent with this hypothesis. Due to the conservation of key amino acid residues, the mouse γ-crystallins are also predicted to have the same overall tertiary structure as that of calf γ-II (see Fig. 3), with most of the hydrophobic residues buried in the center of the globular domains and the charged residues located mainly on the surface. However, amino acid substitutions do occur in different γ-crystallin polypeptides, as already indicated by their different isoelectric points (8). These amino acid changes may provide slightly differing surface properties to allow for different intermolecular contacts in the lens. The differential expression of the crystallin genes during lens development (3) suggests that diversity in the structure of the γ-crystallins might be important for maintaining the transparency of the lens. The reason for the greater amino acid variability in motif 3, also observed in the rat (25), is unclear. One possibility is that this motif plays an important role in intermolecular interactions and has therefore been subject to greater selective pressure to evolve specialized surface properties for different functions in different γ-crystallins. A completely opposite view is that this motif is the least important part of the structure and is thus less constrained by selection and more susceptible to drift in sequence. Further insight into this question may be provided by determining the locations of the altered amino acid residues in polymorphic γ-crystallin species that have been detected in several mouse strains with apparently normal lenses (26). In addition, site-specific mutagenesis and expression of the γ-crystallin cDNAs provide an opportunity to study the structural role of different regions of the γ-crystallin sequence.

Table 1. Sequence conservation in mouse γ-crystallins

γ-Crystallin	Total coding region	Motif 1	Motif 2	Motif 3	Motif 4
γ_1 vs. γ_2	87 (91)	97 (98)	98 (98)	66 (76)	89 (92)
γ_1 vs. γ_3	—	—	86 (86)	66 (78)	85 (79)
γ_1 vs. γ_4	79 (84)	85 (88)	86 (90)	61 (63)	89 (86)
γ_2 vs. γ_3	—	—	86 (86)	71 (80)	83 (78)
γ_2 vs. γ_4	78 (84)	87 (90)	86 (90)	54 (72)	87 (88)
γ_3 vs. γ_4	—	—	93 (91)	51 (70)	85 (78)
Average	81 (86)	90 (92)	89 (90)	62 (73)	86 (84)

Numbers represent percentage of matched amino acids and nucleotides (in parentheses) in the indicated regions. The regions corresponding to the connecting peptides (see Fig. 2) are not included in the comparisons.

The presence or absence of a residue, Gln-83, located in the connecting peptide between the two globular domains represents a major difference among the various γ-crystallin polypeptides. This additional amino acid residue is found in mouse γ_3 as well as in calf γ-II, whose three-dimensional structure is known (19). The absence of this residue results in a shorter connecting peptide that might reduce intramolec-

ular flexibility, restricting the freedom of the domains to separate. Conceivably, the geometry of interdomain packing would be altered. In view of this, it is interesting that some of the γ-crystallins also have a cysteine residue, Cys-130, in the COOH-terminal domain (see Fig. 3). With some adjustment to the relative positions of the domains, this could form a disulfide bond with the conserved residue Cys-41 in the NH_2-terminal domain. Such an intramolecular interaction would pin the domains together, severely restricting the flexibility of the molecule. This may be related to the relatively greater hardness and lower water content of some rodent lenses as compared with those of other species. Raman spectroscopy has shown that crystallins of mouse and rat lenses undergo significant formation of disulfides with maturation (27, 28). Decreased molecular flexibility may be necessary to allow tighter packing and short-range ordering of crystallin molecules, raising the possibility that those γ-crystallins with Cys-130 might be found predominantly in the most central, most dehydrated regions of the lens. These features are shared by the closely related rat γ-crystallins (25) and some of the human γ-crystallins analyzed (ref. 25; unpublished observations).

We thank Dr. T. Shinohara for generously providing the cDNAs analyzed in this study. We also thank Roger Paterson for excellent technical assistance and Dr. L. Siminovitch for critical comments on the manuscript. L.-C.T. is a research scholar of the Canadian Cystic Fibrosis Foundation and M.L.B. is a research scholar of the Medical Research Council of Cananda. This work was supported by Medical Research Council Grant MA-7786.

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