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Characterization of γ-Crystallin from the Eye Lens of Bullfrog: Complexity of γ-Crystallin Multigene Family as Revealed by Sequence Comparison Among Different Amphibian Species

Shao-Fan Lu,[1] Fu-Ming Pan,[2] and Shyh-Horng Chiou[1,3]

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γ-Crystallin is the major and most abundant lens protein present in the eye lens of lower vertebrates such as amphibian and piscine species. To facilitate structural characterization of γ-crystallins isolated from the lens of the bullfrog (*Rana catesbeiana*), a cDNA mixture was synthesized from the poly(A)$^+$mRNA isolated from fresh eye lenses. cDNA encoding γ-crystallin was then amplified using polymerase chain reaction (PCR) based on two primers designed according to the relatively conserved N- and C-terminal sequences of known γ-crystallins from teleostean fishes. PCR-amplified product corresponding to γ-crystallin isoforms was obtained, which was then subcloned in pUC18 vector and transformed into *Escherichia coli* strain JM109. Plasmids containing amplified γ-crystallin cDNAs were purified and prepared for nucleotide sequencing by the dideoxynucleotide chain-termination method. Sequencing several clones containing DNA inserts of about 0.54 kb revealed the presence of two isoforms with an open reading frame of 534 base pairs, covering two γ-crystallins each with a deduced protein sequence of 177 amino acids including the translation-initiating methionine. These γ-crystallins of pI 6.364 and 6.366 contain a low-methionine content of 2.81%, in contrast to 11–16% obtained for those γ-crystallins with high-methionine content from most teleostean lenses. Pairwise sequence comparison of bullfrog γ-crystallins with those published sequences of γ-crystallins from carp, shark, *Xenopus* and another *Rana* frog, bovine, and human lenses indicates that there is only 46–63% sequence similarity among these species, revealing that amphibians possess a very complex and heterogeneous group of γ-crystallins even from closely related species of *Rana* frogs. The sequence analysis and comparison of various isoforms of the frog γ-crystallin family provide a firm basis for identifying these lens proteins as members of a multigene family more complex than that reported for mammalian γ-crystallins.

KEY WORDS: γ-Crystallin; amphibian lens; polymerase chain reaction (PCR); sequence comparison; multigene family; phylogenetic tree.

1. INTRODUCTION

More than 90% of the water-soluble proteins in lenses of various animal species consist of several families of proteins (called crystallins), which are assumed to possess structural roles in the cytoplasm of lens fiber cells for the maintenance of transparency and optical clarity. Three major families of crystallins, classified as α-, β-, and γ-crystallins, are found in almost all vertebrates (Harding and Dilley, 1976; Bloemendal, 1982; Chiou, 1986), and other taxon-specific crystallins

[1] Laboratory of Crystallin Research, Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan 10098.
[2] Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan.
[3] To whom correspondence should be addressed.

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with enzymatic functions, e.g., δ-, ε-, λ-, and ρ-crystallins, etc., have been found to be present in specific species of various classes of animals (Wistow and Piatigorsky, 1988; Bloemendal and de Jong, 1991). Several crystallins that have been characterized both structurally and enzymatically are found to be related to conventional metabolic enzymes, especially noteworthy being ε- and δ-crystallins isolated from avian and reptilian lenses possessing genuine catalytic activity and defined kinetic mechanisms of lactate dehydrogenase and argininosuccinate lyase, respectively (Chiou *et al.*, 1990; Lee *et al.*, 1992).

The purification and protein characterization of γ-crystallin from the mammalian lenses were initiated early (Bjork, 1961, 1964), and were found to be more concentrated in the lens nucleus than in the cortex (Harding and Dilley, 1976; Bloemendal, 1982). The protein was found to be homogeneous in molecular size when examined by ultracentrifugation, but heterogeneous in charge by electrophoresis (Harding and Dilley, 1976; Bloemendal, 1982; Chiou *et al.*, 1988*a*). Unlike the other two major classes of α- and β-crystallins, γ-crystallin is monomeric in solution and possesses relatively high sulfhydryl content as compared with other classes of lens crystallins. The X-ray structural determinations of the representative β- and γ-crystallins (Blundell *et al.*, 1981; Bax *et al.*, 1990) have indeed provided great insights into the polypeptide packing and assembly of some unusual domains present in these two major classes of crystallins. These X-ray crystal studies revealed an essentially similar and symmetrical structure shared by these two classes of crystallins, corroborating the suggestion (Driessen *et al.*, 1981) that they may form a β/γ superfamily. In contrast to α-crystallin, which has been shown to be structurally related to heat-shock proteins and possess chaperone activity as well (Ingolia and Craig, 1982; Horwitz, 1992; Merck *et al.*, 1993), no specific assignment for the structural and functional roles of β- and γ-crystallins regarding their highly symmetrical polypeptide folding has been reported (Slingsby, 1985).

In this report our major interest is on a species above the piscine class, *i.e.*, the bullfrog, which is a representative species of the amphibian class and is easily obtained locally from amphibian farms. We have amplified and sequenced cDNAs constructed from the lenses of the bullfrog using PCR methodology to aid in the structural analysis of several γ-crystallin isoforms. The extensive molecular characterization of various γ-crystallins from different species of the evolutionarily lower vertebrates such as the bullfrog may provide some insight into the mechanism underlying the evolution of this multigene γ-crystallin family.[4]

2. MATERIALS AND METHODS

2.1. Isolation and Protein Characterization of Frog γ-Crystallins

The bullfrogs (*Rana catesbeiana*) used for this study were obtained from a local frog farm. The decapsulated lenses were homogenized in 10–20 ml of 0.05 M ammonium bicarbonate buffer, *p*H 7.5, containing 5 mM EDTA similar to that described before (Chiou, 1987). The supernatant from 27,000 × *g* centrifugation was adjusted to give a concentration of about 20–30 mg/ml and the 5.0-ml aliquot was applied to Fractogel TSK HW-55 (Superfine Grade, Merck) (Chiou, 1987; Choiu *et al.*, 1988*b*). SDS–Polyacrylamide slab gel (SDS–PAGE, 5% stacking/14% resolving gel) was as described (Laemmli, 1970) with some modifications. Isoelectric focusing in 5% polyacrylamide gel containing 2.8% (v/v) carrier ampholytes of *p*H 3.5–10 was carried out on a slab gel with the incorporation of 0.1% 2-mercaptoethanol and 6 M urea. The gel was fixed in 12.5% trichloroacetic acid, washed several times with 20% methanol/7% acetic acid, and stained with Coomassie blue.

Amino acid analysis and N-terminal sequence analysis by automated Edman degradation with a pulsed-liquid phase sequencer (Model 477A, Applied Biosystems, Foster City, CA) of lyophilized γ-crystallin fraction from a gel-filtration column were carried out as described before (Chiou *et al.*, 1992).

2.2. Preparation of mRNA from Frog Lenses and Cloning by PCR Amplification

Frog lenses were removed and stored in a liquid nitrogen container immediately after they

[4] The sequence data of γ-crystallin mRNA have been deposited in the EBML Data Library under the accession numbers X86072 (γ-M_{I-1}) and X86081 (γ-M_{I-2}), respectively.

were dissected and before the processing for mRNA isolation. Two deep-frozen lenses from one frog were homogenized and RNA was extracted according to the standard procedures (Maniatis *et al.,* 1989). To obtain a full-length crystallin cDNA, poly(A)$^+$RNA was purified using QuickPrep mRNA preparation kit (Pharmacia, Uppsala, Sweden) and then subjected to the synthesis of cDNA mixture by cDNA Synthesis System/Plus kit (Amersham, England).

Two oligonucleotide primers of sense and antisense orientations, covering 5′- and 3′-nucleotide coding regions for short N- and C-terminal amino acid segments of piscine γM_1-crystallins (Pan *et al.,* 1994), were synthesized for PCR amplification of γ-crystallins, with the forward sequence 5′-CATGGGCAAG(A/G)TCA(T/C)-CTT(C/T)-3′(19-mer) and the reverse sequence 5′-(T/C)TAACA(G/C)ATATC(A/C)(G/A)TGA-(T/C)ACG-3′(21-mer) with slashes indicating the use of degenerate codons in the primers. The PCR reactions were subjected to 40 cycles of heat denaturation at 94°C for 1.0 min, annealing of the primers to the DNAs at 45°C for 1.5 min, and running DNA chain extension with *Taq* polymerase at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The PCR products were treated with Klenow Fragment and T4 polynucleotide kinase and separated on a 1.2% agarose gel and electroeluted according to standard protocols. The DNA fragments were subcloned into pUC18 previously digested with *SmaI*/BAP, and then transformed into *Escherichia coli* strain JM 109. Plasmids purified from positive clones were prepared for nucleotide sequencing by a dideoxynucleotide chain-termination method (Sanger *et al.,* 1977). The DNA sequence determined by the conventional isotope-labeled manual method was double checked by the automatic fluorescence-based sequencing of PCR-amplified templates using the model 373A DNA Sequencing System with a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, CA).

2.3. Sequence Comparison of γ-Crystallins and Homology Search

In the comparison and analysis of the deduced amino acid sequences from determined cDNA sequences coding for various γ-crystallins, a multiple-sequence alignment software program (DNASTAR, Madison, WI) was used for the estimation of sequence homology based on percent sequence identity.

2.4. Construction of a Phylogenetic Tree for γ-Crystallins of Vertebrate Species

A software package of LaserGene for the Apple Macintosh computer from DNASTAR was used for the estimation of sequence homology based on percent similarity and divergence among different protein sequences. Percent divergence is calculated by comparing sequence pairs in relation to the phylogenetic tree. On the other hand, the percent similarity is estimated by comparing sequences directly without accounting for phylogenetic relationships. The phylogenetic or evolutionary tree was then constructed using the algorithm of Hein (1990) in the MegAlign programs of the package. It is a multiple-sequence alignment program that builds trees as it aligns DNA or protein sequences using a combination of distance matrix and approximate parsimony methods. This method constructs multiple alignment by imposing restrictions based on evolutionary relatedness of the aligned sequences, which is useful for aligning highly evolved gene families that have clear evolutionary relationships such as the γ-crystallins reported here.

3. RESULTS AND DISCUSSION

Understanding the mechanism for the evolution of functionally or structurally related proteins from different species remains a general biological problem. The recent rapid development of PCR methodology for DNA amplification has indeed provided a crucial tool to enrich specific crystallin genes so that cDNA sequence analysis may be performed easily without the need for time-consuming protein sequencing or cDNA library construction and screening. Previous studies have revealed that γ-crystallins purified and characterized from lenses of different teleostean species (Chiou *et al.,* 1987; Chang *et al.,* 1988; Pan *et al.,* 1995) possess an amino acid composition which is very high in methionine content, in contrast to that of the mammalian γ-crystallin class with typically low methionine content (Bloemendal, 1982; Choiu, 1986). Therefore it is reasonable to assume that there are two subclasses of γ-crystallin existing in

this major γ-crystallin family of vertebrates. In this report we have extended our structural characterization of lens crystallins to the molecular analysis of γ-crystallins from the bullfrog of amphibian class, an intermediate vertebrate species evolving between lower teleostean and higher mammalian classes.

3.1. Isolation and Characterization of Frog γ-Crystallins

Figure 1A shows an elution pattern of the crude extract of bullfrog lenses on a TSK gel-permeation column. Four major peaks were obtained, in contrast to three for the piscine and five for the mammalian lens extracts (Chiou, 1986; Chiou *et al.*, 1987). Similar to our previous reports on frog crystallins (Chiou, 1987; Chiou *et al.*, 1988*b*), the first peak I contains α/β-crystallin aggregates as judged from the subunit analysis by SDS–gel electrophoresis (Fig. 1B), possessing the characteristic 20-kD subunit bands corresponding to αA and αB subunits of α-crystallin and multiple bands for β-crystallins of 23–35 kD. Peaks II, III, and IV contained βH-, βL-, and ρ/γ-crystallins, respectively (Chiou, 1987). It is noteworthy that the purified crystallin fractions from the above-mentioned peaks except peak IV (γ-crystallin) are all shown to be N-terminally blocked after subjecting them to sensitive microsequencing by the protein sequencer. We have further purified the crude γ-crystallin (peak IV) into more than six γ-crystallin subfractions on cation-exchange chromatography (Chiou *et al.*, 1988*b*; and data not shown). Amino acid analysis of each γ-crystallin fraction revealed essentially similar compositional data with regard to most amino acid contents, indicating that they are probably very similar in protein sequence as well. N-Terminal sequencing of these proteins has indicated the presence of several heterogeneous sites with more than two amino acids being identified along the amino-terminal segments despite the fact that N-terminal partial sequences are in general very similar to those of carp γ-crystallins (Chiou *et al.*, 1986). The fact that γ-crystallin possesses multiple charge isomeric forms is further shown by isoelectric focusing of the crude γ-crystallin of peak IV, indicating clearly the exixtence of at least ten protein species with pI spreading in a range of 6.3 to about 9.3 (Fig. 2).

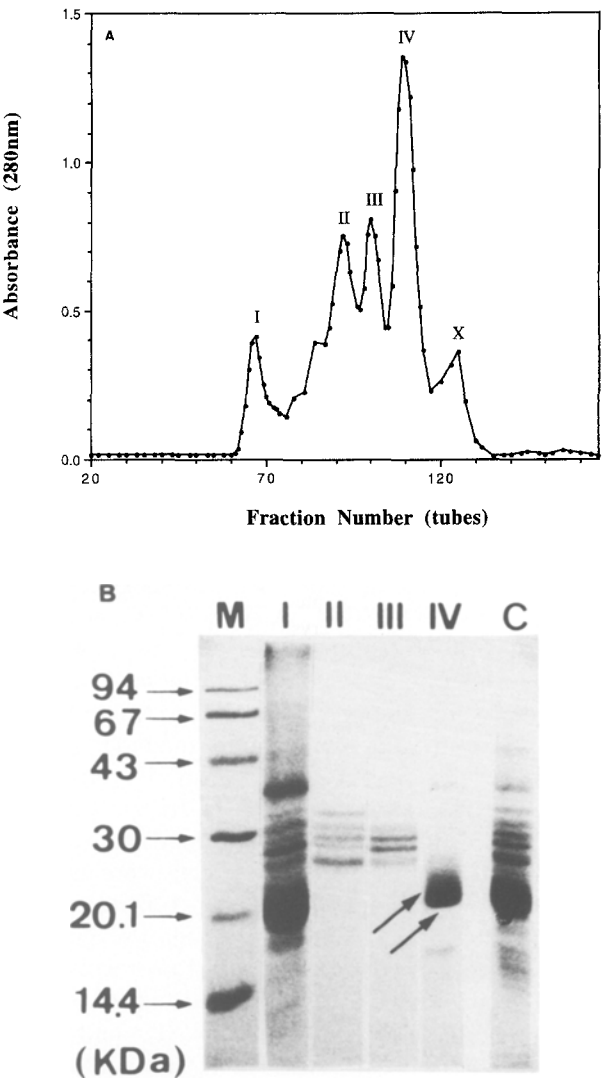

Fig. 1. (A) Gel-permeation chromatography of the crude extract of bullfrog lenses on Fractogel TSK HW-55(S) (2.5 × 115 cm column). The column eluates (3.0 ml/tube per 5.0 min) were monitored for absorbance at 280 nm. The peak fractions I–IV correspond to α/β-, βH-, βL-, and ρ/γ-crystallins respectively. The small peak (indicated by X) after peak IV is composed of nonprotein components of low molecular masses. (B) Gel electrophoresis of fractionated bullfrog crystallins under denaturing conditions (SDS–PAGE) in the presence of 5 mM dithiothreitol. Lane M, standard proteins used as molecular mass markers with positions indicated by arrows on the left (in kD): phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20), and lysozyme (14). Lane C, crude lens extract; lanes I–IV are the four peak fractions in part A. The gel was stained with Coomassie blue. Note that lane I is the aggregated crystallins composed of mainly α/β-crystallin subunits and lane IV crude γ-crystallins of 20 kD (arrows).

Due to the complexity of these γ-crystallins, we have resorted to the recent powerful cloning and sequencing tool of PCR for the convenient

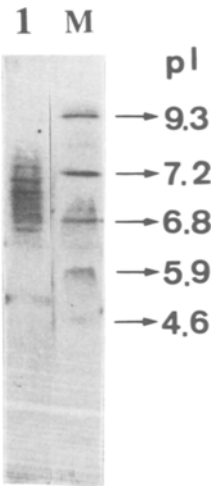

Fig. 2. Isoelectric focusing of bullfrog γ-crystallins under denaturing conditions. Lane M, pI-calibration standard of five proteins with pI 4.6–9.3 indicated on the right (Sigma). Lane 1, rechromatographed crude γ-crystallin from peak IV in Fig. 1A. The slab gel contained 6 M urea and 0.1% 2-mercaptoethanol and the electrophoresis was run at an initial voltage of 200 V for 6 hr until a final current of less than 0.5 mA was reached. The isoelectric points (pI) of various γ-crystallin isoforms ranged from 6.3 to 9.3. The gel was stained with Coomassie Blue.

determination of cDNA sequences coding for these multiple isoforms.

3.2. cDNA Amplification by PCR and Sequence Determination

The previous molecular cloning studies of amphibian γ-crystallins by Tomarev *et al.* (1982, 1984) provided incomplete cDNA sequences coding for two γ-crystallins, which due to some unspecified cloning errors, lacked the 5′-nucleotide segment of about 15 and 120 base pairs corresponding to missing amino-terminal sequences of 5 and 40 amino acids, respectively.

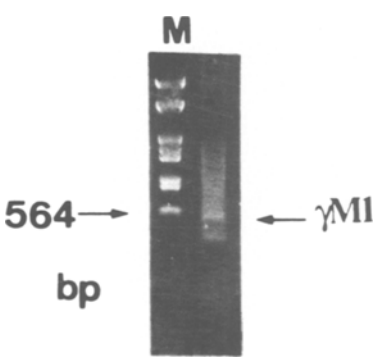

Fig. 3. Identification and size determination of the PCR-amplified γ-crystallin cDNA. Electrophoresis was carried out in 1.2% agarose gel. Lane M, DNA size markers of the *Eco*RI and *Hind*III digestion products of λDNA, ranging from 564 to 21,227 bp; right lane indicates the amplified PCR product of about 540 bp (arrow) encoding bullfrog γM1-crystallin isoforms.

This has prompted us to reexamine the cDNA sequences coding for these crystallins by a convenient PCR cloning and sequencing technique in order to acquire complete structural information for these γ-crystallins. PCR amplification of total lens cDNA mixtures with the designed primers corresponding to the relatively conserved N- and C-terminal 5- and 6-amino acid segments of known sequences determined from cDNAs of teleostean γ-crystallins (Pan *et al.*, 1994; Chang *et al.*, 1988) achieved the isolation of one PCR fragment encoding γ-crystallins of the amphibian species.

Figure 3 shows the size determination of PCR-amplified cDNA coding for this crystallin subunit. The DNA band was estimated to be about 540 bp, in agreement with a γ-crystallin polypeptide of about 170–180 amino acid residues. The PCR-amplified DNA fragment was subcloned into pUC18 previously digested with *SmaI*/BAP and then transformed into *E. coli* strain JM 109. It is noteworthy that similar to our previous characterization of piscine γ-crystallin with multiple isoforms (Pan *et al.*, 1994), there are more than ten positive clones identified and all of them possess very similar cDNA sequences. This would also indicate the presence of a multiplicity of γ-crystallin isoforms in this amphibian species.

The two deduced protein sequences together with their nucleotide sequences of frog γ-crystallins are shown in Fig 4A, 4B. They were found to consist of 534 nucleotides, which covers a full-length protein of 177 amino acids including the initiating methionine plus the stop codon. It is worth noting that the frog γ-crystallin sequences determined here possess the same number of amino acid residues as that of carp γM_1 (Chang *et al.*, 1988), whereas the reported sequences from the other *Rana* frog (Tomarev *et al.*, 1982, 1984) contain only about 168 and 136 residues, indicating that they are incomplete sequences lacking the amino-terminal segments plus some deletions in other parts of the protein chains. From the deduced protein sequences, the isoelectric points (pI) of these two γ-crystallins were calculated to be 6.364 and 6.366, corresponding to the acidic species shown in Fig. 2. They were also found to belong to

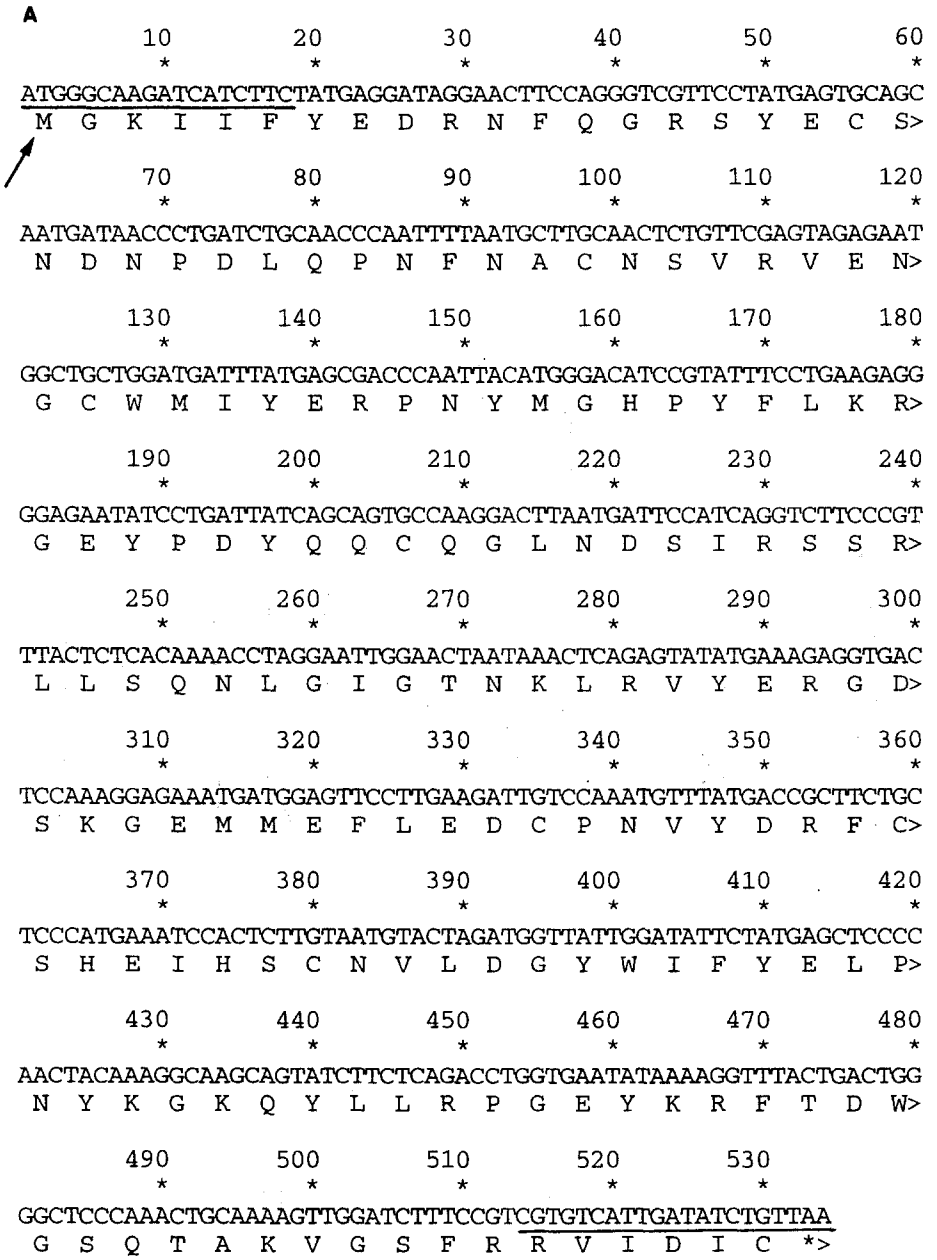

Fig. 4. Nucleotide and deduced protein sequences of bullfrog (A) $\gamma M_{1\text{-}1}$- and (B) $\gamma M_{1\text{-}2}$ crystallin cDNAs. The nucleotide sequences of 534 base pairs for both crystallins are shown above the amino acid sequences of 177 residues, which include the translation initiation methionine (arrow) as the first amino acid. Asterisks at the end of sequences indicate the stop codon *TAA*. cDNA sequences are marked (*) in every ten-nucleotide segment for easy tracing of sequence contents. Amino acids are denoted by one-letter symbols. The 5′- and 3′-nucleotide segments used as primers for PCR reactions are underlined. The sequence identity of the deduced protein sequences between the two is about 97%.

mammalian-type γ-crystallins with low methionine (2.81 mol %), in contrast to 11–16% reported for teleostean γ-crystallins such as those present in carp and catfish lenses (Chang *et al.*, 1988; Pan *et al.*, 1995).

3.3. Structural Comparison of γ-Crystallins Among Various Vertebrate Species

In the comparative analysis of multiple sequence alignment for the deduced amino acid

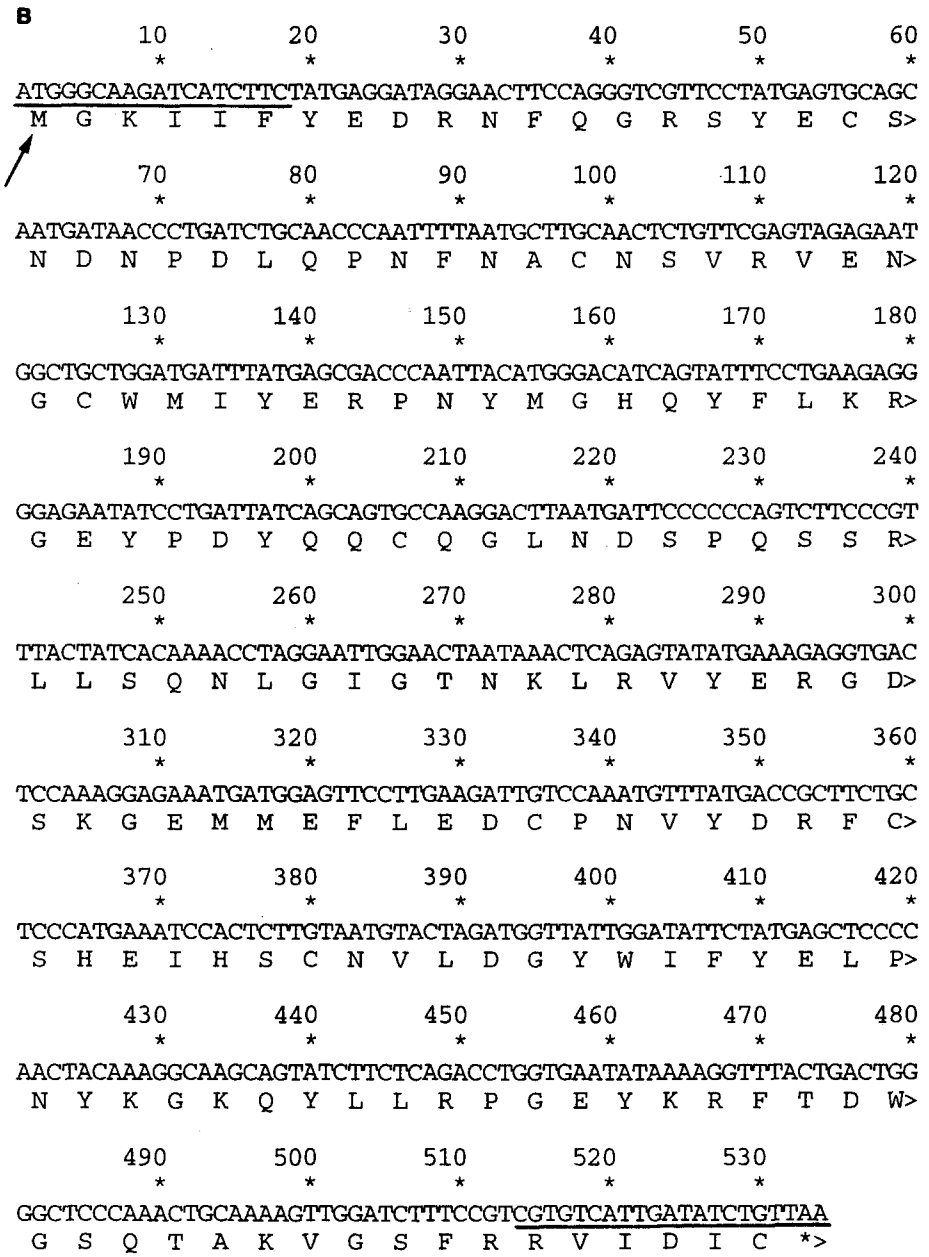

Fig. 4. Continued.

sequences of bullfrog γ-crystallins and the homologous sequences reported for another *Rana* frog, *Xenopus,* shark, carp, bovine, and human γ-crystallins (Fig. 5), it was found that γ-crystallins of the bullfrog show about 46–63% sequence identity to the γ-crystallins of these species. It is of interest to note that they show only about 60–62% sequence identity (about the same percent identity as that between bullfrog and bovine γ-crystallins) between bullfrog and two other phylogenetically related species of frogs, *Rana temporaria* and *Xenopus laevis.* In general the orthologous γ-crystallin sequences from the same class of vertebrates show more than 70% sequence similarity. This certainly underlines the unusual evolutionary relationship of these three species of amphibian frogs and deserves further study on other classes of crystallins in these amphibian species.

In Fig. 5 we show the optimal alignment by

Fig. 5. Multiple sequence alignment and sequence comparison of nine crystallin sequences. The identical amino acid residues among various sequences are expressed in white letters with black-background blocks. The gaps were introduced for optimal alignment and maximum homology among the sequences. Note that the sequence of *Rana temporaria* γ2-crystallin (Tomarev *et al.*, 1984) lacks an N-terminal segment of five amino acids and bovine βBp-crystallin shows greater sequence variation than those γ-crystallins themselves, even though they are supposed to belong to a β/γ superfamily based on three-dimensional structures. The sequences compared here were taken from published sequences of *R. temporaria* (Tomarev *et al.* 1984), *Xenopus* γ1 (Smolich *et al.*, 1993), shark $\gamma M_{1\text{-}1}$ (Chuang, 1994), carp γM_1 (Chang *et al.*, 1988, 1991), bovine γII (Hay *et al.*, 1987), human γ5 (Meakin *et al.*, 1985, 1987), and bovine βBp (Driessen *et al.*, 1981).

introducing a minimum number of gaps along the entire lengths of eight determined sequences of γ-crystallins plus one βBp-crystallin subunit chain, encompassing some representative species of three major classes of vertebrates. With the exception of bovine βBp-crystallin—showing long stretches of mismatches and lower than 35% sequence identity with other members of the authentic γ-crystallin family—the eight γ-crystallin sequences from various species of different classes of vertebrates exhibit 46–63% sequence identity. Homology comparison based on structural motifs as revealed by the X-ray three-dimensional structure (Blundell *et al.*, 1981) indicates that the motif 3 of the

carboxyl-terminal domain possesses higher sequence variability than any other motif in the γ-crystallin molecule. The variation in this region appears to account for the lower sequence homology observed between the bullfrog and two other amphibian species. In this respect, the rate of evolutionary change in γ-crystallin is much faster than that for αA-crystallin among different species in the lens of amphibian class (Lu, 1995; unpublished result). We cannot exclude the possibility that the high divergence in γ-crystallin sequences observed between bullfrog and the other two amphibian species is the result of comparing paralogous products of multiple-copy γ-crystallin genes in these three amphibian species (Stapel *et al.*, 1984).

3.4. Sequence Divergence and Construction of Phylogenetic Tree

In our systematic pairwise sequence comparison of crystallin genes and their deduced protein sequences from varied species of the animal kingdom, higher sequence homology is generally found between cDNA sequences than protein sequences (Chiou, 1988; Chiou *et al.*, 1995). It seems to be more sensitive to detect sequence divergence based on protein sequences than cDNA sequences when comparing highly homologous protein families. We have therefore constructed a phylogenetic tree based on the nine γ-crystallin sequences shown in Fig. 5 using the algorithm of Hein (1990), which is a multiple sequence alignment program that builds trees as it aligns DNA or protein sequences using a combination of distance matrix and approximate parsimony methods. It has been shown to be useful to align highly evolved gene families and their corresponding protein sequences that have clear evolutionary relationships as in the case of the γ-crystallin family. The results (Fig. 6) indicate that the phylogenetic tree based on the sequence divergence among these crystallin sequences indeed exemplifies the close relatedness between the γ-crystallin pair of the mammalian class (human and bovine γ-crystallins) or that of the piscine class (carp and shark γ-crystallins). It is also of interest to find that two γ-crystallins from the bullfrog and those of *Rana temporaria* and *Xenopus laevis* are indeed located at different branching points of the tree. This leads to the supposition that *Xenopus* γ-crystallin diverged from the common ancestral γ-crystallin earlier than the γ-crystallins of *Rana temporaria* and bullfrog. There is only a slight discrepancy between trees constructed based on DNA (not shown) and protein (Fig. 6), attesting to the general applicability of the tree construction based on either DNA or protein to shed light on the phylogenetic relationship among different species.

4. CONCLUSION

In contrast to recent studies regarding the structural similarity of α-crystallin with heat-shock proteins and the associated chaperone activity observed for both proteins, no functional or physiological roles of β- or γ-crystallins have been assigned despite the fact that closely related polypeptide folding of this β/γ superfamily has been revealed through X-ray crystallography. Insights into the evolutionary history of different protein families are being discovered through the analysis of molecular sequences. As sequence data become increasingly plentiful, our ability to unravel and reconstruct various phylogenetic relationships based on protein or nucleotide sequence information is limited only by the availability of biological samples of exotic species situated at the most distant evolutionary branching positions of phylogenetic trees. Therefore we have undertaken extensive characterization of various classes of crystallin sequences from diverse species by cDNA cloning and sequencing coupled with recent protocols of PCR methodology.

In this study we have enriched specific crystallin genes by a convenient protocol of PCR cloning and sequencing so that cDNA sequence analysis may be performed easily without the need for time-consuming cDNA library construction and screening. We have complemented the structural information for two nonallelic isoforms of γ-crystallins by a simple and reliable PCR method. The sequence analysis and comparison of various isoforms of the frog γ-crystallin family reveal a complex γ-crystallin family in the amphibian lens that is more complex and heterogeneous than that reported for mammalian γ-crystallins.

ACKNOWLEDGMENTS

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Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8	9		
1		97.0	62.0	60.0	50.0	47.0	63.0	57.0	34.0	1	Bullfrog rM1-1
2	1.0		62.0	60.0	48.0	46.0	62.0	58.0	33.0	2	Bullfrog rM1-2
3	15.8	16.2		59.0	50.0	49.0	64.0	61.0	35.0	3	R.temporaria r2
4	17.2	17.7	15.1		52.0	56.0	60.0	55.0	34.0	4	Xenopus r1
5	25.6	26.0	24.8	24.3		79.0	54.0	48.0	31.0	5	Shark rM1-1
6	24.1	24.6	22.0	22.0	10.3		51.0	48.0	31.0	6	Carp rM1
7	15.6	16.1	12.8	15.2	23.7	21.3		75.0	33.0	7	Bovine rII
8	18.7	18.6	16.0	19.1	24.1	23.7	9.2		32.0	8	Human r5
9	34.1	34.0	33.4	35.4	38.5	37.6	33.1	34.3		9	Bovine beta Bp
	1	2	3	4	5	6	7	8	9		

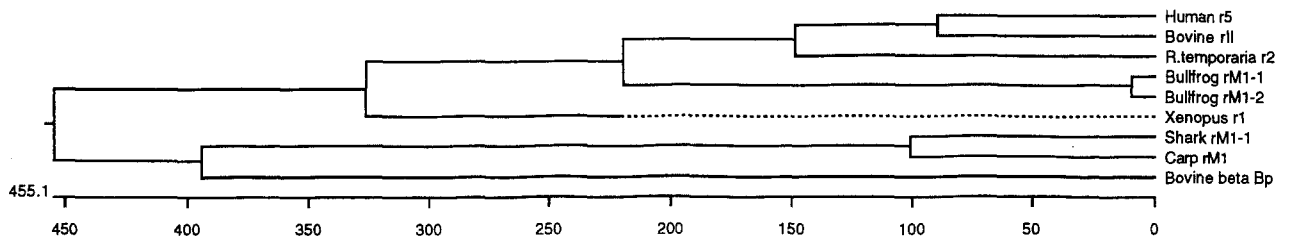

Fig. 6. Pairwise comparison of amino acid sequence similarity and divergence (top) and construction of phylogenetic tree (bottom) of nine crystallin sequences from various species of three major classes of vertebrates. Analysis of sequence data was carried out in a LaserGene software package for the Apple Macintosh computer (DNASTAR, Madison, WI). Percent divergence is calculated by comparing sequence pairs in relation to the relative positions in the phylogenetic tree. On the other hand, the percent similarity is estimated by comparing percent sequence identity directly without accounting for phylogenetic relationships. A phylogenetic tree was then constructed based on the percent divergence between protein sequences using a combination of distance matrix and approximate parsimony methods in the phylogeny generation program of Hein (1990). This algorithm carries out multiple alignment by imposing restrictions based on evolutionary relatedness of the aligned sequences. The length of each pair of branches represents the sequence distance between aligned pairs. The scale beneath the tree measures the distance between sequences (in millions of years). The dotted line shown for *Xenopus* γ1 indicates that the sequence distance is not proportional to the scale.

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