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Sequence Characterization of γ-Crystallins from Lip Shark (*Chiloscyllium colax*): Existence of Two cDNAs Encoding γ-Crystallins of Mammalian and Teleostean Classes

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γ-Crystallin is a common lens protein of most vertebrate eye lenses and the major protein component in lenses of fishes and in many mammalian species during embryonic and neonatal stages. To facilitate the structural characterization of γ-crystallin possessing extensive charge heterogeneity, a cDNA mixture was constructed from the poly(A)$^+$ mRNA isolated from shark eye lenses, and amplification by polymerase chain reaction (PCR) was carried out to obtain cDNAs encoding multiple shark γ-crystallins. Sequencing analysis of multiple positive clones containing PCR-amplified inserts revealed the presence of a multiplicity of isoforms in the γ-crystallin class of this cartilaginous fish. It was of interest to find that two shark cDNA sequences coexist, one encoding γ-crystallin (γM_1) of high methionine content (15.5%) and the other encoding one (γM_2) of low methionine content (5.1%), each corresponding to the major teleostean and mammalian γ-crystallins, respectively. Comparison of protein sequences encoded by these two shark cDNAs with published sequences of γ-crystallins from mouse, bovine, human, frog, and carp lenses indicated that there is about 61–80% sequence homology between different species of the piscine class, whereas only 47–66% is found between mammals and shark. A phylogenetic tree constructed on the basis of sequence divergence among various γ-crystallin cDNAs revealed the close relatedness between shark γM_2-crystallin and mammalian γ-crystallins and that between shark γM_1 and teleostean γ-crystallins. The results pointed to the fact that ancestral precursors of γ-crystallins were present in the sharp lens long before the appearance of modern-day mammalian and teleostean γ-crystallins.

KEY WORDS: γ-Crystallins; shark lens; polymerase chain reaction (PCR); multigene family; phylogenetic tree.

1. INTRODUCTION

The abundant presence of various common and specific classes of structural proteins, e.g., lens crystallins, in different species of vertebrates constitutes a good model system to unravel the complex process of evolution in structurally homologous proteins (Chiou, 1986; de Jong and Hendriks, 1986). Extensive proteins and cDNA sequence data have been obtained from various species of vertebrates, allowing evolutionary relationships to be derived (de Jong *et al.*, 1985; de Jong and Hendriks, 1986; Chiou *et al.*, 1995; Lu *et al.*, 1995, 1996; Pan *et al.*, 1995*a*).

Fish represent the oldest and most diverse

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group of vertebrates (Keeton, 1972). The modern *Chondrichthyes* class of fishes, for example sharks and skates, are distinguished by their cartilaginous skeletons, in contrast to the bony skeletons of *Osteichthyes* (bony fishes). Sharks diverged from the Placodermi (one class of armored fishes) long before the appearance of modern bony fishes and amphibians (Keeton, 1972). The characterization of shark crystallins would be very important for the phylogenetic comparison in light of the recent elucidation of the complete sequences of γ-crystallins from several species of teleostean fishes in *Osteichthyes* (Chang *et al.*, 1988; Pan *et al.*, 1994, 1995*b*). The present study was performed in the endeavor to make a systematic characterization of lens crystallins from the lip shark (also called the brown-spotted catshark), with a characteristic and flabby lower lip. We have amplified cDNA constructed from the lenses of a single shark using PCR methodology to aid in the structural analysis of multiple isoforms of γ-crystallins. Several cDNAs encoding major γ-crystallins of shark lenses have been cloned and sequenced without the need for time-consuming library construction and screening. It was unexpected and interesting to find that two positive clones encode γ-crystallins with deduced protein sequences corresponding to two major γ-crystallins, each presumed to be uniquely expressed in the mammalian or piscine lenses alone (Chiou *et al.*, 1987, 1988; Chiou, 1989). This finding provides a crucial missing link in tracing evolutionary relationships among diverse γ-crystallins present in the lenses of the animal kingdom and may shed light on the molecular origin of this multigene γ-crystallin family.[4]

2. MATERIALS AND METHODS

2.1. Preparation of mRNA from Shark Lenses

A small shark (*Chiloscyllium colax*, brown-banded bamboo shark or brown-spotted catshark) less than 1-year-old was obtained from a local aquarium shop under special contract for scientific research. Shark lenses were removed and stored in liquid nitrogen container immediately after they were dissected and before the processing for mRNA isolation. Two deep-frozen lenses from one shark were homogenized and RNA was extracted according to the standard cloning manual of Maniatis *et al.* (1989). To obtain a full-length crystallin cDNA, poly(A)$^+$ RNA was purified using a QuickPrep mRNA preparation kit (Pharmacia, Uppsala, Sweden) and then subjected to the synthesis of cDNA mixture by a cDNA Synthesis System/Plus kit (Amersham, England).

2.2. cDNA Amplification by PCR and cDNA Sequencing of γ-Crystallins

We synthesized three oligonucleotide primers of sense and antisense orientations covering 5′- and 3′-nucleotide coding regions for N- and C-terminal 5- to 7-amino acid segments of two previously determined partial sequences for shark (Chiou, 1989) and carp γ-crystallins (Chang *et al.*, 1988), i.e., γ-M_1 and γ-M_2, with the forward sequence 5′-CATGGGCAAG(A/G)TCA(T/C)CTT(C/T)-3′ for γ-M_1 and γ-M_2, and the two reverse sequences 5′-(T/C)TAACA(G/C)ATATC(A/C)(G/A)TGA-(T/C)ACG-3′ for γ-M_1 and 5′-(T/C)TAGTACCA-(G/C)GA(G/A)TCCATGA(T/C)ACG-3′ for γ-M_2 (with slashes indicating use of degenerate condons in the primers). The conditions for PCR reactions were similar to the previous report for amplification of teleostean lenses, i.e., subjected to 40 cycles of heat denaturation at 94°C for 2.5 min, annealing the primers to the DNAs at 48°C for 1 min and 20 sec and running DNA chain extension with *Taq* polymerase at 72°C for 3 min, followed by a final extension at 72°C for 10 min. The PCR amplification cycle for γ-M_2 was repeated twice using the eluted band of first PCR products in order to get enough material for cloning. Products were treated with Klenow Fragment and T4 Polynucleotide kinase and separated on a 1.2% agarose gel and electroeluted according to standard procedures. The DNA fragments were subcloned into pUC18 previously digested with *Sma*I/BAP and then transformed into *Escherichia coli* strain JM 109. Plasmids purified from positive clones were prepared for nucleotide sequencing by dideoxynucleotide chain-termination method (Sanger *et al.*, 1977). The DNA sequence determined by a conventional isotope-labeled manual method was double-checked by automatic fluorescence-based sequencing of templates amplified by PCR using Model 373A DNA Sequencing System (Applied Biosystems, CA) with a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems).

[4] The sequence data of cDNAa for γ-crystallins in this study have been deposited in the EMBL Data Library under the accession numbers X79228 and X79229 for γM_1 and γM_2, respectively.

2.3. Sequence Comparison of Shark γ-Crystallins and Homology Search

A commercially available software package (DNASTAR Inc., Madison, WI) was used for the estimation of sequence homology based on percent sequence identity, similar to the previous report (Pan *et al.*, 1994).

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

A LaserGene software package for the Apple Macintosh computer from DNASTAR was used for the estimation of sequence homology based on percent similarity and divergence among different cDNA sequences of γ-crystallins. 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 relations. 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 to align highly evolved gene families that have clear evolutionary relationships such as γ-crystallins reported here.

3. RESULTS AND DISCUSSION

The modern fishes comprise two major classes of piscine species: (1) *Osteichthyes* or teleostean (bony) fishes and (2) *Chondrichthyes* or cartilaginous fishes (sharks and skates). The study of lens crystallins from the lowest piscine class, *i.e.*, sharks of the cartilaginous class, is of special interest from the evolutionary point of view because they constitute the early protein forms of vertebrates and are thought to have been ancestral to those of land vertebrates. There were sharks in the oceans long before the first animals had begun to colonize the land. Their history stretches back for at least 700 million years, a vast period of time as compared with the shorter evolutionary history of other vertebrates (Keeton, 1972). Once considered to be most primitive, sharks are now thought to be relatively specialized and, considering, their complex biology, to be ranked with birds and mammals as highly evolved creatures. In this report the structural characterization of γ-crystallin from shark lenses by PCR and its comparison with those of teleostean and mammalian lenses is of special import to unravel the complex evolutionary history of lens crystallins in the animal kingdom (Chiou, 1984, 1986, 1988).

3.1. Characterization of cDNAs Encoding γ-Crystallins from Shark Lenses

Previous study has indicated the unusual structural characteristics of shark γ-crystallin as compared with those associated with the lenses of teleostean fishes such as carp (Choiu, 1989; Siezen *et al.*, 1988; Chiou *et al.*, 1990). Especially noteworthy is the finding that the amino acid composition of shark γ-crystallins seems to lack the unique characteristic of high methionine content (>10%) commonly observed for that of teleostean fishes. In contrast, shark γ-crystallin is closer to those γ-crystallins characterized from mammalian species regarding amino acid composition, N-terminal partial sequence, plus some conformational properties as revealed by circular dichroism (Chiou, 1989; Chiou *et al.*, 1990). Shark γ-crystallin showed complex multiple bands in isoelectric focusing (Chiou, 1989; Siezen *et al.*, 1988), revealing that it also consists of various isoforms and probably forms a multigene family. Due to the complexity of γ-crystallin, we have resorted to recent rapid and improved protocols of cloning and sequencing by means of the PCR technique for the determination of protein sequences of these multiple isoforms in order to circumvent the difficulties encountered by protein sequencing.

PCR amplification of total lens cDNA mixtures prepared from two lenses of a single shark with the designed primers based on DNA coding sequences of γ-M_1 and γ-M_2 of carp γ-crystallins (Chang *et al.*, 1988; Pan *et al.*, 1994) achieved the isolation of two PCR fragments corresponding to complete reading frames encoding two γ-crystallin isoforms from this novel species, i.e., γM_1 and γM_2 in this report. Figure 1 shows the size determination of PCR-amplified cDNAs coding for γM_1 and γM_2. The DNA bands were estimated to be about 530 base pairs, in agreement with a protein of about 170–180 amino acid residues. It is noteworthy that

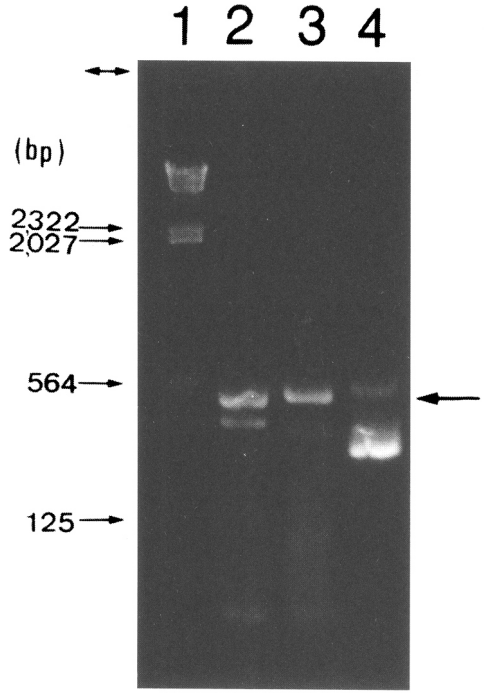

Fig. 1. Identification and size determination of PCR-amplified shark γ-crystallin cDNAs. Electrophoresis was carried out in 1.2% agarose gel. Lane 1, DNA molecular size markers of 0.125–2.322 kb (Lambda *Hind*III markers, Promega); lane 2, PCR reaction product coding for γs-crystallin; lane 3, PCR reaction product of about 530 bp coding for γM_1-crystallin; lane 4, PCR reaction product of about 530 bp (arrow) coding for γM_2-crystallin. The band below the indicated product showed a nonspecific PCR product of lower size.

at the annealing temperature of 48°C it was very easy to get PCR product for γM_1, whereas minute amounts of γM_2 product did not form until the annealing was lowered to 42°C. Therefore a second PCR-cycle reaction was carried out using the eluted band of the first PCR product in order to get enough PCR-amplified fragments for cDNA cloning. The PCR-amplified DNA fragments were then subcloned into PUC18 previously digested with *Sma*I/BAP and then transformed into *E. coli* strain JM 109. Plasmids purified from positive clones were then prepared for nucleotide sequencing.

3.2. cDNA Sequence Analysis

It is noteworthy that more than five positive clones have been identified, with their 5′ and 3′ nucleotide sequences being determined to be essentially identical to those of the designed primers. In order to avoid the sequencing errors introduced in the manual sequencing of isotope-labeled cDNA, sequence accuracy was doubly checked and confirmed by an automatic fluorescence-based sequencing technique. The deduced protein sequences together with their genetic coding sequences of two clones, designated as γM_1 and γM_2, are shown in Figs. 2A and 2B. The cDNA sequences encoding γM_1 and γM_2 were found to consist of 525 and 528 nucleotides, respectively, each covering a full-length protein of 175 and 176 amino acid residues including the initiating methionine. We have taken these two as exemplars to illustrate the general sequence characteristics of γM_1 and γM_2 subclasses of the shark γ-crystallin family.

3.3. Sequence Comparison of Shark, Teleostean, and Mammalian γ-Crystallins

In the pairwise sequence comparison (Table I) of deduced amino acid sequences using a commercial software package (DNASTAR program), it is found that γM_1 and γM_2 subclasses of this shark γ-crystallin show 51.2% and 66.5% sequence identity to bovine γII crystallins, respectively. On the other hand, they show 79.1% and 61.9% sequence identity to carp γM_1, and 64.9% and 61.1% to carp γM_2 crystallins, respectively (Chang *et al.*, 1988). This certainly underlines the distinct differences between shark γM_1 and γM_2, sharing a sequence identity of only about 58.5% (compare two sequences in Fig. 2). Close scrutiny of their sequences indicates that γM_1 possesses a high methionine content of 15.5% and γM_2 a low methionine content of 5.1%. It is well known that the salient and characteristic difference between γ-crystallins of teleosteans and mammals lies in their methionine contents, and shark γ-crystallin belongs to the class of low-methionine crystallin (Chiou, 1988; Siezen *et al.*, 1988). Therefore, in contrast to previous studies, which indicated a lack of typical γ-crystallin with high-methionine content in shark lenses, cDNA analysis did detect and indicate the presence of two types of γ-crystallins, encompassing crystallins from the teleostean and mammalian lenses. The high-methionine γ-crystallin encoded by shark γM_1 probably exists in such a low quantity as to escape detection by conventional protein analysis (Chiou, 1989).

We have also included mouse and human

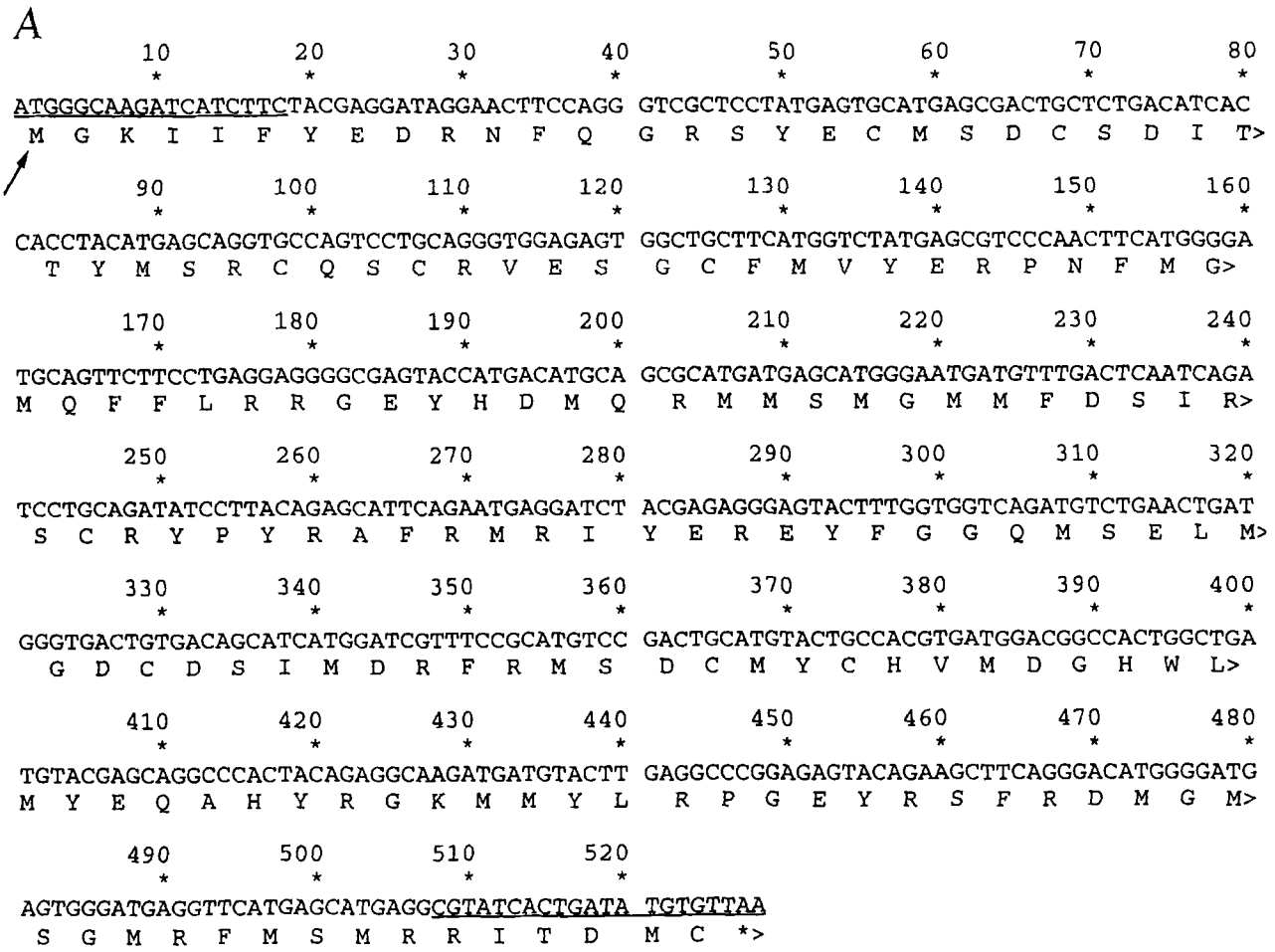

Fig. 2. Nucleotide and deduced protein sequences of shark γM_1(A) and γM_2(B) crystallin cDNAs. (A) The nucleotide sequence of 528 base pairs is shown above the amino acid sequence of 174 residues excluding translation initiation methionine (arrow) and termination codon; (B) the nucleotide sequence of 531 base pairs encoding a protein sequence of 175 amino acids. Asterisks (*) are indicated in every 10-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.

γ-crystallins in the pairwise comparison with shark γM_1 and γM_2 (Fig. 3A). The results clearly indicate that shark γM_2 is closer to these two mammalian crystallins than is shark γM_1. This seems to imply that in the evolution of γ-crystallin from the piscine γ-crystallins to modern mammalian γ-crystallins, two independent pathways developed, one leading to the teleostean and the other to the mammalian γ-crystallin class, with shark γ-crystallins probably as the ancestral precursors for both. All these determined sequences have laid a firm molecular basis for the construction of a molecular phylogenetic tree among these apparently quite heterogeneous and complex families of structurally homologous crystallins.

In Fig. 3 we align five sequences encompassing the major shark γM_2-crystallin with low methionine (5.1%) and carp γM_2-crystallin with high methionine (14%) plus γ-crystallins from three mammalian species, which have all been deduced from nucleotide sequences coding for these γ-crystallins. It is noteworthy that the extent of protein sequence similarity among these sequences is about 57–67%. One salient feature is that some of the key residues (such as Tyr-6, Glu-7, Phe-11, Gly-13, and Ser-34 based on bovine γII sequence numbering) for the maintenance of stability in γ-crystallins (Blundell *et al.*, 1981; Wistow *et al.*, 1983; Liaw *et al.*, 1992) are mostly retained and conserved in all sequence pairs including shark/bovine and shark/carp pairs. This

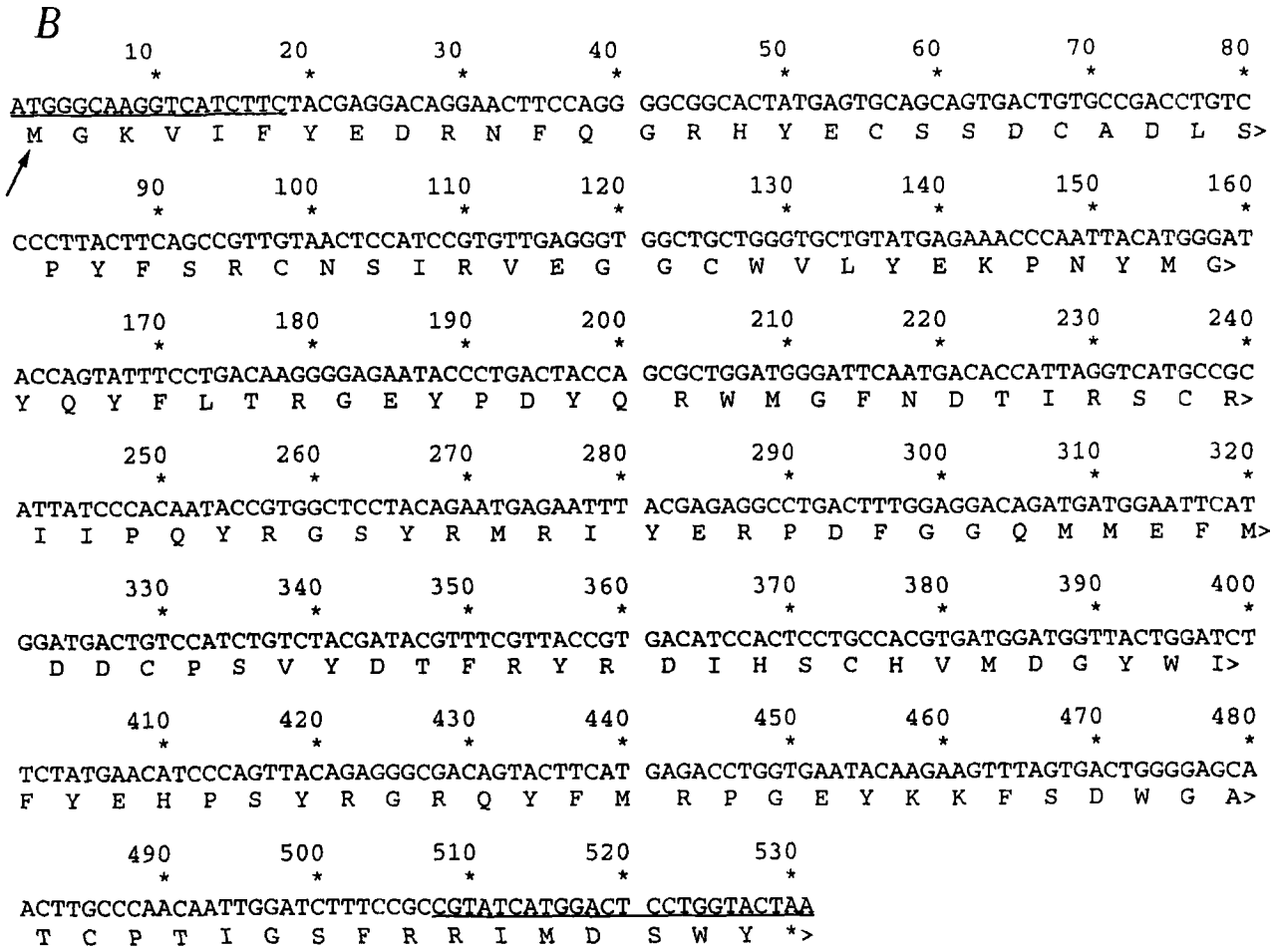

Fig. 2. Continued.

would indicate that the evolutionary rate of shark γM_2-crystallin change is probably slow when compared with that of carp γM_1 or γM_2-crystallins with high methionine content, resulting in the conservation of protein structure (shark $\gamma M_2 \rightarrow$ mammalian γ-crystallins) during the long period of evolution.

3.4. Construction of a Phylogenetic Tree Based on cDNA Sequences

In our systematic pairwise sequence comparison of crystallin genes from various species of the animal kingdom, higher sequence homology is generally found between cDNA sequences than

Table I. Pairwise Comparison of Amino Acid Sequence Homology Between Shark and Carp γ-Crystallins Together with Various Mammalian γ-Crystallins[a]

Percent homology	Bovine γII	Carp γM_1	Carp γM_2	Mouse γ_1	Mouse γ_2	Human γ_5
Shark γM_1	51.2	79.1	64.9	49.7	54.6	46.6
Shark γM_2	66.5	61.9	61.1	59.0	64.2	57.2

[a] Analysis of sequence homology was carried out using a software package from DNASTAR (Madison, WI) using the published sequences for carp (Liaw *et al.*, 1992), bovine (Bhat and Spector, 1984), mouse (Goring *et al.*, 1992), and human (Meakin *et al.*, 1987) γ-crystallins.

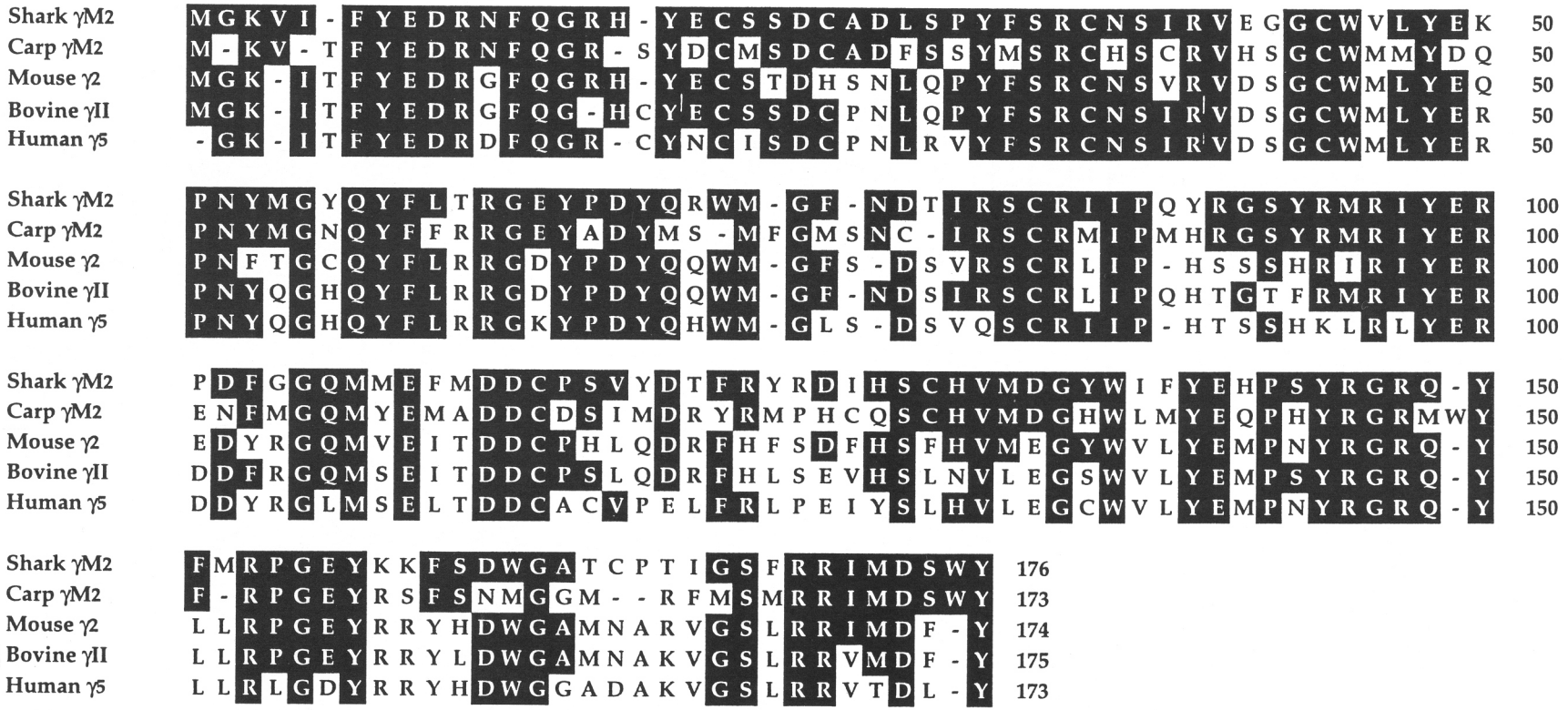

Fig. 3. Multiple sequence alignment and comparison of five γ-crystallin sequences from piscine and mammalian species. The identical amino acid residues among various sequences based on the first one (shark γM_2) are expressed as white letters against black background. The gaps were introduced for optimal alignment and maximum homology for the sequences. Note that shark γM_2 shows a high homologous sequence with those of mammalian γ-crystallins in some of the key amino acid residues (bold regions). Shark γM_2 exhibits an amino acid composition of low methionine content, which is similar to mammalian γ-crystallins, in contrast to that of high methionine for carp γM_2-crystallin. Amino acid residues are denoted by one-letter symbols.

Percent Similarity

	1	2	3	4	5	6	7	8		
1		61.0	54.4	31.2	42.2	42.4	58.5	26.3	1	Shark rM1
2	38.4		42.9	28.4	47.8	46.5	70.6	26.0	2	Shark rM2
3	21.7	33.6		29.8	41.1	38.6	42.4	29.7	3	Carp rM1
4	25.0	32.7	22.3		28.5	26.8	29.0	27.6	4	Carp rM2
5	44.3	33.3	39.9	40.2		42.7	45.8	27.2	5	Carp rS
6	44.6	29.8	41.5	39.0	40.7		44.3	24.5	6	Bullfrog rM1-1
7	40.6	29.0	36.5	36.3	34.0	33.5		27.3	7	Bovine rII
8	61.8	64.6	61.1	60.3	61.3	70.0	66.9		8	Bovine beta B2
	1	2	3	4	5	6	7	8		

Percent Divergence

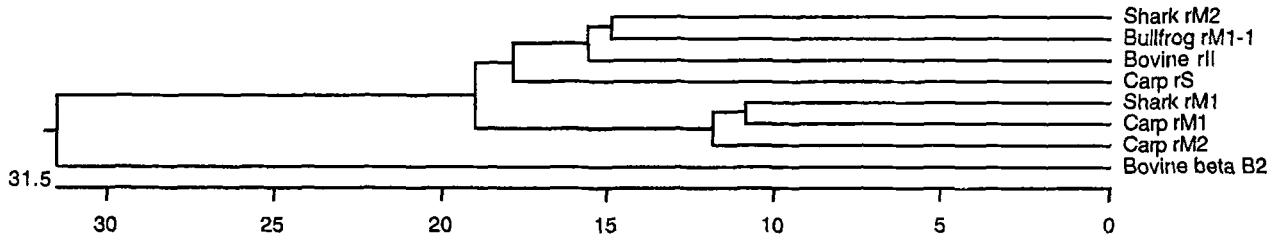

Fig. 4. Pairwise comparison of nucleotide sequence similarity and divergence (top) and construction of phylogenetic tree (Bottom) of eight crystallin sequences from various species of three major classes of vertebrates. Analysis of sequence data was carried out using a LaserGene software package for 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; 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 betwen cDNA sequences using a combination of distance matrix and approximate parsimony methods in the phylogeny generation program of Hein (1990). This algorithm carries out multiple alginment by imposing restrictions based on evolutionary relatedness of the aligned sequence pairs. The tree was built using clustal method and weighted residue-weight table. 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). Bovine βB2 crystallin is included to indicate the fact that β- and γ-crystallins form a β/γ superfamily.

between protein sequences (Chiou, 1988; Chiou *et al.*, 1995). We have therefore constructed a phylogenetic tree based on eight γ-crystallin and one bovine β-crystallin cDNA sequences (Fig. 4) using the algorithm of Hein (1990), which is a multiple-sequence alignment program that builds trees as it aligns DNA sequences using a combination of distance matrix and approximate parsimony methods. It has been shown to be useful in aligning highly evolved gene families and their corresponding protein sequences that have clear evolutionary relationships as in the case of the varied crystallin family (Chiou *et al.*, 1995; Lu *et al.*, 1995, 1996; Pan *et al.*, 1995*a*). It is noteworthy that the phylogenetic tree based on the sequence divergence among these crystallin cDNA sequences indeed exemplifies the close relatedness between shark γM_2-crystallin (low methionine content) and γ-crystallins from bullfrog and bovine lenses (both are also low-methionine crystallins). On the other hand, shark γM_1-crystallin (high methionine content) was grouped with carp γM1 and γM_2, in agreement with the classification based on methionine contents in these crystallins. Especially interesting is the observation that two shark γ-crystallin cDNAs characterized here indeed are located at different branching points of the tree, indicative of two distinct evolutionary pathways leading to these two types of γ-crystallins.

The results thus lead to the supposition that these two shark γ-crystallins diverged from the common ancestral γ-crystallin and may represent two origins for the modern γ-crystallins with high and low methionine contents in teleostean and mammalian species, respectively. There is only a slight discrepancy between trees constructed based on DNA and protein (data not shown), attesting to the general applicability of the tree construction

based on either DNA or protein to shed some light on the phylogenetic relationship among homologous proteins.

4. CONCLUSION

γ-Crystallin is the major and most abundant lens protein present in the eye lens of lower vertebrates such as amphibian and piscine species. Previous studies have revealed that γ-crystallins purified and characterized from lenses of different teleostean species 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 (Harding and Dilley, 1976; Chiou *et al.*, 1987, 1988; Chiou, 1989). Therefore it is reasonable to assume that there are two subclasses of γ-crystallin in this major γ-crystallin family of vertebrates.

Important insights into the evolution of different protein families are being discovered through the analysis of molecular sequences. Extensive cDNA and gene sequence data for various lens crystallins have now been obtained from rat, mouse, human, frog, and teleostean fishes, allowing evolutionary relationships to be derived. As the gene sequences for the shark γ-crystallin family become available (this report and unpublished data), our ability to reconstruct various phylogenetic relationships based on protein or nucleotide sequence information is limited only by the available analysis program to give a more meaningful elucidation of the interrelationship between various crystallin classes in the animal kingdom. The extensive characterization from the evolutionarily ancient and unique animal species such as the cartilaginous shark shown in this report may eventually provide some insight into the phenomenon of species diversification and the accompanying molecular origin of crystallins. The structural analysis of shark cDNAs encoding γ-crystallins by means of the PCR technique has revealed two cDNAs encoding two γ-crystallins hitherto supposed to be uniquely expressed only in teleostean or mammalian classes. Further genomic analysis of shark γ-crystallin genes based on information obtained from the characterization of the cDNA sequences should shed some light on the organization and evolution of this multigene γ-crystallin family in lower vertebrates.

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