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Evolution

μ-Crystallin is a mammalian homologue of *Agrobacterium* ornithine cyclodeaminase and is expressed in human retina

(crystallin recruitment/molecular evolution/amino acid metabolism)

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ABSTRACT **μ-Crystallin is the major component of the eye lens in several Australian marsupials. The complete sequence of kangaroo μ-crystallin has now been obtained by cDNA cloning. The predicted amino acid sequence shows similarity with ornithine cyclodeaminases encoded by the tumor-inducing (Ti) plasmids of *Agrobacterium tumefaciens*. Until now, neither ornithine cyclodeaminase nor any structurally related enzymes have been observed in eukaryotes. RNA analysis of kangaroo tissues shows that μ-crystallin is expressed at high abundance in lens, but outside the lens μ-crystallin is preferentially expressed in neural tissues, retina, and brain. An almost full-length cDNA for μ-crystallin was cloned from human retina. In human tissues, μ-crystallin mRNA is present in neural tissue, muscle, and kidney. This pattern of expression and relationship to an enzyme involved in unusual amino acid metabolism suggests the interesting possibility that mammalian μ-crystallins could be enzymes participating in processes such as osmoregulation or the metabolism of excitatory amino acids.**

Crystallins, the major structural components of the lens, have arisen by the direct recruitment of enzymes and stress proteins (1–4). In many, perhaps all, cases the acquisition of another role occurred without prior gene duplication. Probably because of the evolutionary plasticity of the lens, many different taxon-specific crystallins with recent, independent recruitment events have been found in different lineages. It has been suggested that all the recruited proteins represent molecules expressed in the developing lens for other functional reasons, such as metabolic control or response to stresses such as the osmotic swelling of lens fiber cells (5, 6). The genes for such proteins are already active in the lens and may need only minor modification to increase expression to structurally useful levels. Recent recruitment events may have selected enzymes to dilute the lens-hardening effects of the more specialized γ-crystallins.

μ-Crystallin accounts for about a quarter of total lens protein in several Australian marsupials (5), although it is not detectable in the lenses of an American marsupial or of other vertebrates. Partial peptide sequences of μ-crystallins from a macropod and a dasyurid suggested distant similarities with certain dehydrogenases (5), but no obvious superfamily relationship was indicated. To obtain complete identification of μ-crystallin a gray kangaroo (*Macropus fuliginosus*) lens cDNA library has been constructed and screened with probes derived from known peptide sequences.§ Results suggest that μ-crystallin is a eukaryotic enzyme related only to bacterial ornithine cyclodeaminases (OCDs), enzymes of unusual amino acid metabolism.

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MATERIALS AND METHODS

RNA Extraction and cDNA Cloning. Total RNA was extracted from gray kangaroo lenses by the guanidinium thiocyanate method (7) with RNazol reagents (Tel-Test, Friendswood, TX). Five micrograms of poly(A)$^+$ RNA was then purified by using Dynabeads (Dynal, Great Neck, NY). cDNA was synthesized using the Lambda Zap kit from Stratagene, following manufacturer's specifications. A cDNA probe for μ-crystallin was produced by PCR of 1 μg of kangaroo lens total RNA with oligo primers GARGGGGTGGTGCARCC and GATCACATCCCCAGAYTC, designed from previously determined wallaby μ-crystallin peptides (5), with a Perkin–Elmer reverse transcription PCR kit. The probe was labeled by random-priming (United States Biochemical) and used to screen 200,000 plaques of the cDNA library. Approximately 5% of the plaques gave positive hybridization; plaques were purified by standard methods (8). A full-length clone was sequenced by using Sequenase reagents and protocols from United States Biochemical. This clone was used as a probe to screen a human fetal retina cDNA library (Stratagene).

Primer-Extension Analysis. Two different primers were used to extend on a template of kangaroo lens total RNA. A single initiation site was identified by both primers. Oligo primers (5936, CCGCCCGCTGTCCGCAGCCT; 5937, AAAGCCGGACTCCAACTCAT) were labeled with [γ-^{32}P]-dATP (Amersham). Labeled primer (10^6 cpm) was hybridized to 10 μg of lens total RNA and extended by using a cDNA synthesis kit from Boehringer Mannheim.

Southern Analysis. Ten micrograms of liver DNA was digested with *Bam*HI, *Eco*RI, and *Hin*dIII restriction enzymes (BRL). Gels were run according to standard methods (8) and blotted onto nylon membranes. A cDNA probe was generated by PCR of the μ-crystallin clone shown in Fig. 1 with T3 and T7 primers used to amplify the entire insert (9). Probe was labeled with ^{32}P by using the United States Biochemical random priming kit. Hybridization was in Nylohybe (Oncor, Gaithersburg, MD) at 42°C overnight. The blot was washed in 0.1× standard saline citrate (SSC)/1% SDS at 65°C. A commercial "zoo blot" (Evoblot, Bios, New Haven, CT) hybridized with the same probe. Hybridization was in Nylohybe at 42°C overnight, and subsequent washing was in 0.1× SSC/1% SDS at room temperature.

Northern (RNA) Analysis. Total RNA was extracted from kangaroo lens, cornea, retina, brain, liver, heart, and kidney as for Fig. 1. Five micrograms was loaded in each lane; an additional lens lane contained 0.5 μg. Northern blots were

Abbreviation: OCD, ornithine cyclodeaminase.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M90841).

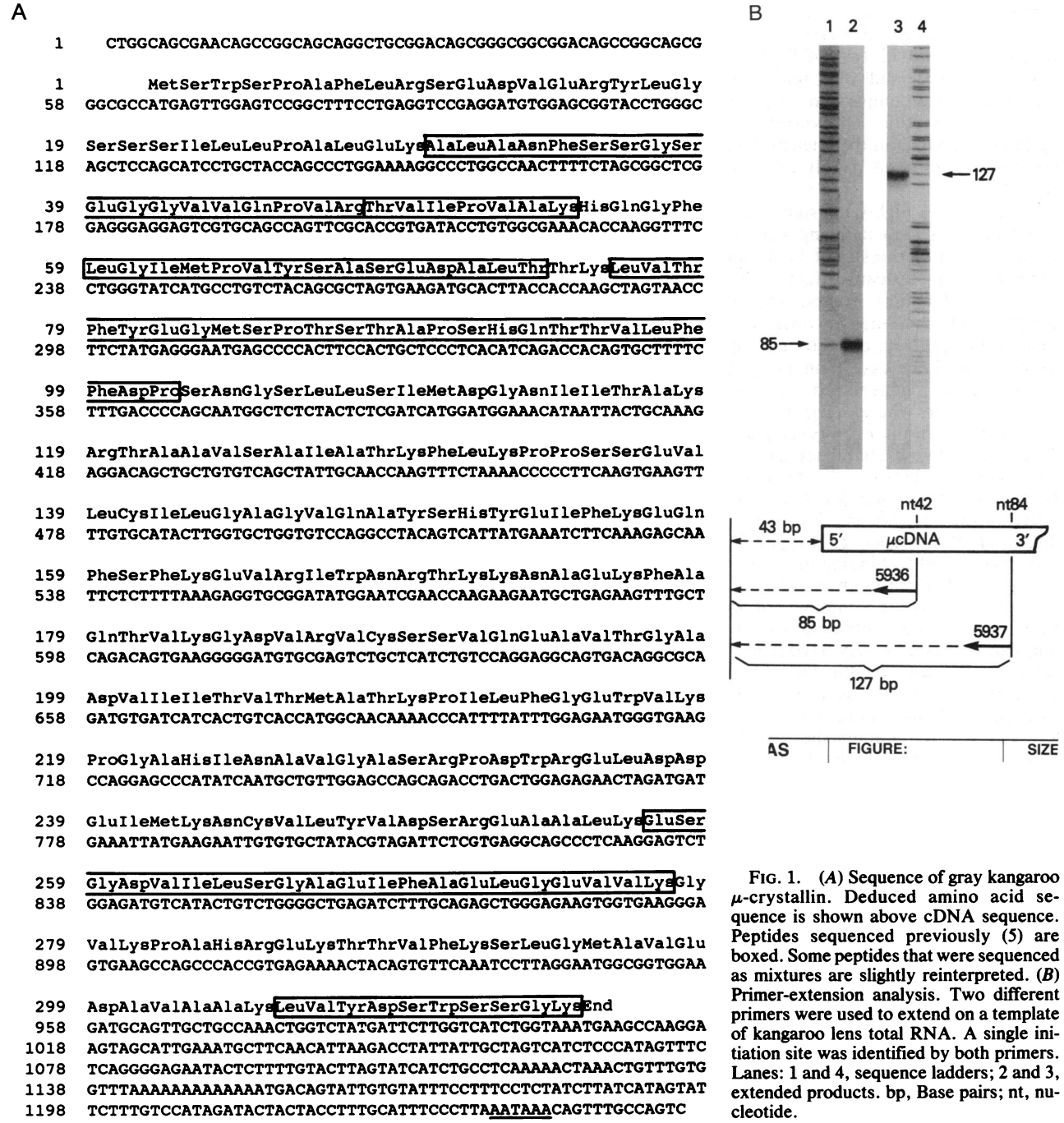

FIG. 1. (*A*) Sequence of gray kangaroo μ-crystallin. Deduced amino acid sequence is shown above cDNA sequence. Peptides sequenced previously (5) are boxed. Some peptides that were sequenced as mixtures are slightly reinterpreted. (*B*) Primer-extension analysis. Two different primers were used to extend on a template of kangaroo lens total RNA. A single initiation site was identified by both primers. Lanes: 1 and 4, sequence ladders; 2 and 3, extended products. bp, Base pairs; nt, nucleotide.

performed by standard methods (8) under the conditions described above for Southern analysis. For human tissues a Northern blot of mRNA (Clontech) was probed in a similar way with the human cDNA.

OCD Assay. OCD enzyme assays were done as described elsewhere (10, 11). Extracts of adult gray kangaroo lenses were prepared by standard methods (12). *Escherichia coli* expressing *Agrobacterium tumefaciens* OCD were prepared as described (10).

Computer Methods. The IDEAS (13) and GCG (14) packages were used for data base searches and sequence analysis.

RESULTS AND DISCUSSION

A cDNA library was constructed from poly(A)$^+$ RNA extracted from Western gray kangaroo (*M. fuliginosus*) lenses. A probe for μ-crystallin was produced by PCR of kangaroo lens RNA with primers designed from tryptic peptide sequences obtained previously for the tammar wallaby (*Macropus eugeni*). The PCR fragment was then labeled and used to screen the cDNA library, yielding abundant positive clones. Several clones were isolated and partially sequenced. Synthetic probes for the 5′ sequences of the longest clone were used to rescreen the library, and a clone (Fig. 1*A*) was obtained, which, as determined by primer-extension analysis, is only 43 bases short of the single transcription-initiation site in lens (Fig. 1*B*). This clone has a long open reading frame corresponding to a protein of 314-amino acid residues and molecular mass 33,931, in good agreement with the size of μ-crystallin estimated by SDS/PAGE (5). This open reading frame accounts for all the known tryptic peptides previously obtained for tammar wallaby (*M. eugeni*) μ-crystallin (5), although two peptides that had been sequenced as a mixture were slightly reinterpreted in light of the cDNA sequence.

The complete predicted amino acid sequence was compared with translated GenBank version 70 (13), and a striking similarity was seen with two OCDs encoded on different Ti (tumor-inducing) plasmids of *A. tumefaciens* (11, 15) (Fig. 2). Individual pairwise comparisons show 31–33% identity between the prokaryotic and vertebrate proteins, placing μ-crystallin and OCD in the ancient category of proteins, a group that is composed mainly of common metabolic enzymes (16).

OCD catalyzes a highly unusual reaction, known only in certain bacteria. Tumor-inducing *Agrobacteria* subvert host plant metabolism by transfer of Ti plasmid genes to the host genome. This transfer causes formation of crown galls and the synthesis of opines, compounds metabolized only by means of a Ti plasmid-encoded operon (17). OCD is the final enzyme in the pathway, converting ornithine to proline in a single step with the release of ammonia. The reaction mechanism is not well understood but, surprisingly, requires NAD^+ as cofactor, whereas arginine may have a regulatory role (11, 15). Given the evolutionary distance involved and the fact that the two bacterial sequences are <70% identical (15), the similarity between μ-crystallin and the two known OCD sequences is striking. Most of the identical residues are clustered in regions that are those best-conserved between the bacterial OCD sequences (Fig. 2). It is likely that these regions correspond to binding or active sites. Indeed, residues 193–202 of μ-crystallin are similar to a sequence previously proposed as an ornithine- or arginine-binding site common to OCD and ornithine transcarbamylases (11), suggesting that μ-crystallin may have the same substrate as the bacterial OCDs. μ-Crystallin may also share the NAD^+-binding activity of OCD because, in preliminary experiments, it has been shown to bind blue-Sepharose (P. V. Rao, J. S. Zigler, and G.J.W., unpublished work), although neither OCD nor μ-crystallin contain classical NAD(H)-binding sequences. The major difference between μ-crystallin and the OCD sequences is the smaller size of the mammalian protein, primarily due to the lack of a C-terminal region, which, at least in part, is well-conserved between the bacterial proteins.

Southern blots of kangaroo genomic DNA give a very simple pattern, consistent with the presence of a single μ-crystallin gene (Fig. 3*A*). Similar blots for human and mouse DNA suggest that related genes are conserved in other vertebrates (Fig. 3*B*). Given the similarities between OCD and μ-crystallin and the conservation of related sequences in other vertebrates in which μ-crystallin is not a major lens structural protein, it seems likely that μ-crystallin represents another example of the over-expression of a functional eukaryotic enzyme (1–4).

Bacterial OCD enzyme assays were performed on extracts of adult kangaroo lenses and *E. coli* expressing *A. tumefaciens* OCD (10). No activity was observed in kangaroo lens under conditions optimal for activity of *Agrobacterium* OCDs (11). It is possible that, although μ-crystallin belongs to the same functional enzymatic superfamily as OCD, it may require different conditions for activity. The lack of the bacterial OCD C-terminal extension may have an effect on activity or specificity. Another likely possibility is that age-related or postmortem loss of activity may have occurred. Posttranslational modification in lens occurs in lactic dehydrogenase-B/ε-crystallin (1, 18) and is suspected as a cause for low activity in turtle lens α-enolase/τ-crystallin (1, 19) and for the lack of aldehyde dehydrogenase activity in elephant shrew lens η-crystallin (5). In the latter case, aldehyde dehydrogenase enzyme activity depends on a cysteine residue sensitive to oxidation (20). With this in mind, it is interesting that Cys-187 of μ-crystallin is conserved in both OCD enzymes (Fig. 2) and might, therefore, be expected to have functional importance.

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            *   *         **        *    *  *       *
 C58     1  MPALANLNIVPFISVENMM..DLAVSTGIENFLVQLAGYIEEDFRRWESF
Ach5     1  MPIDPKLNVVPFISVDHMM..KLVLKVGIDTFLTELAAEIEKDFRRWPIF
 Mu      1  MSWSPAF..LRSEDVERYLGSSSILLPALEKALANFSSGSEGGV....VQ
 Hum        _______  _____________________________GPEGGV....MQ

                 *      *    **    *           *    *  *    **
 C58    48  DKIPRIASHSRDGVIELMPTSDGTLYGFK......YVNGHPKNTKSGRQT
Ach5    48  DKKPRVGSHSQDGVIELMPTSDGSLYGFK......YVNGHPKNTHQGRQT
 Mu     44  PVRTVIPVAKHQGFLGIMPVYSASEDALTTKLVTFYEGMSPTSTAPSHQT
 Hum        PVRTVVPVTKHRGYLGVMPAYSAAEDALTTKLVTFYEDRGITSVVPSHQA

                 **  *  **   *** *   * ** **** **** * *  * *
 C58    92  VTAFGVLS.DVDSGYPLLLSEM..TILTALRTAATSAIAAKYLARKDSRT
Ach5    92  VTAFGVLS.DVGNGYPLLLSEM..TILTALRTAATSALAAKYLARPNSKT
 Mu     94  T....VLFFDPSNGS..LLSIMDGNIITAKRTAAVSAIATKFLKPPSSEV
 Hum        T....VLLFEPSNGT..LLAVMDGNVITAKRTAAVSAIATKFLKPPSSEV

              * * * *       **        *      *   * *   **   *
 C58   140  MALIGNGAQSEFQALAFKALIGVDRIRLYDIDPEATARCSRNLQRFGFQI
Ach5   140  MAIIGNGAQSEFQARAFRAILGIQKLRLFDIDTSATRKCARNLTGPGFDI
 Mu    138  LCILGAGVQAYSHYEIFKEQFSFKEVRIWNRTKKNAEKFAQTVKG...DV
 Hum        LCILGAGVQAYSHYEIFTEQFSFKEVRIWNRTKENAEKFADTVQG...EV

              * ** *** ***** ***       **    * ** ******   *
 C58   190  EACTSAEQAVEGADIITTATADEHNATILSDNMIGPGVHINGVGGDCPGK
Ach5   190  VECGSVAEAVEGADVITTVTADKQFATILSDNHVGPGVHINAVGGDCPGK
 Mu    185  RVCSSVQEAVTGADVIITVTMATK..PILFGEWVKPGAHINAVGASRPDW
 Hum        RVCSSVQEAVAGADVIITVTLATE..PILFGEWVKPGAHINAVGASRPDW

             *   **       *        *     *       **  *  * *
 C58   240  TEMHRDILLRSDIFVEFPPQTRIEGEIQQLAPDHPVTELWRVMTGQDVGR
Ach5   240  TEISMEVLLRSDIFVEYPPQTWIEGDIQQLPRTHPVTELWQVMTGEKTGR
 Mu    233  RELDDEIMKNCVLYVDSREAALKESGDVILSGAEIFAELGEVVKGVKPAH
 Hum        RELDDELMKEAVLYVDSQEAALKESGDVLLSGAEIFAELGEVIKGVKPAH

                  * * * * * **  *   * *     * **
 C58   290  KSDKQITIFDSVGFAIEDFSALRYVRDRVEGSSHSSPLDLLADPDEPRDL
Ach5   290  VGDRQITMFDSVGFAIEDFSALRYVRAKITDFEMFTELDLLADPDEPRDL
 Mu    283  R..EKTTVFKSLGMAVEDAVAAKLVYD.....SWSSGK
 Hum        C..EKTTVFKSLGMAVEDTVAAKLIYD.....SWSSGK

 C58   340  FGMLLRRQAF RRLGG
Ach5   340  YGMLLRCEKK LEPTAVG
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FIG. 2. Kangaroo lens and human retina μ-crystallins are related to OCDs. C58, OCD from Ti plasmid pTiC58 (11); Ach5, OCD from Ti plasmid Ach5 (15); Mu, kangaroo lens μ-crystallin; Hum, human retina μ-crystallin. Residues identical between either μ-crystallin and either of the OCD sequences are indicated by stars.

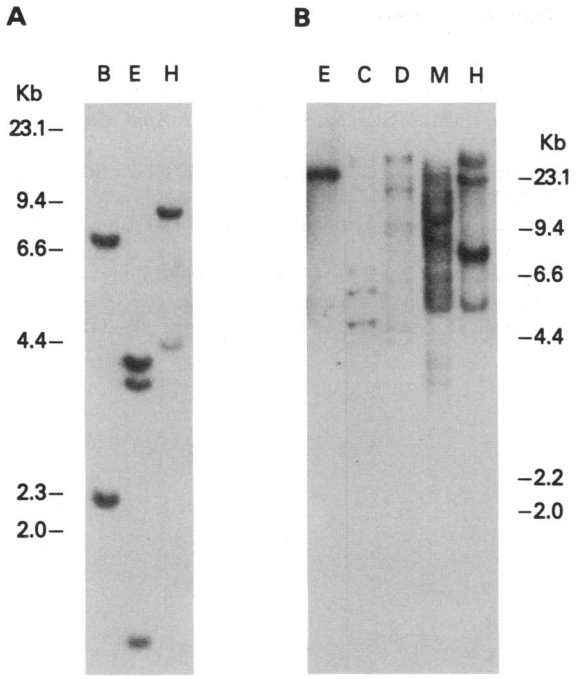

FIG. 3. Genomic Southern blots probed with gray kangaroo μ-crystallin cDNA. (*A*) Gray kangaroo genomic DNA. Ten micrograms of liver DNA was digested with *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H). (*B*) A commercial "zoo blot." Species are *E. coli* (E), *Caenorhabditis elegans* (C), *Drosophila* (D), mouse (M), and human (H). Kb, kilobases.

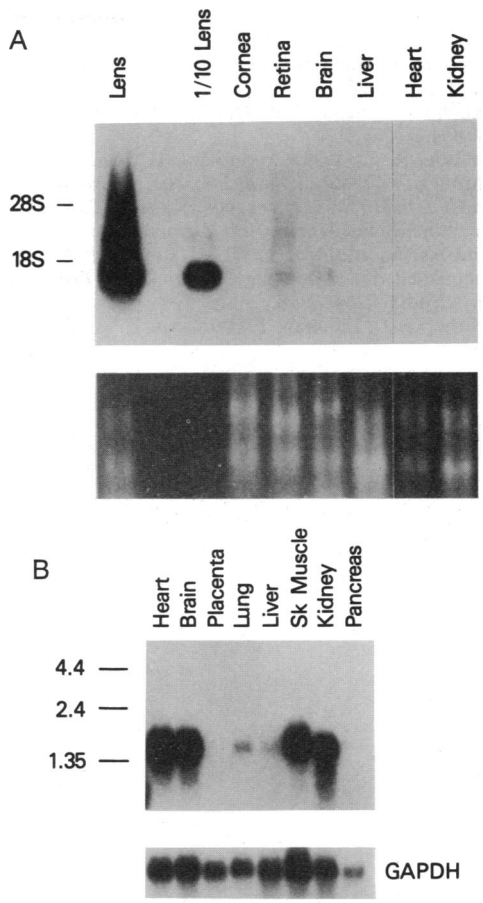

FIG. 4. Lens and nonlens expression of μ-crystallin in mammals. (*A*) μ-Crystallin is highly expressed in kangaroo lens and expressed at lower levels in neural tissues. (*Upper*) Northern blots of total RNA from kangaroo lens, cornea, retina, brain, liver, heart, and kidney. Positions of ribosomal bands are indicated. (*Lower*) Ethidium bromide staining pattern of RNA gel. (*B*) In human tissues μ-crystallin is preferentially expressed in neural tissue, muscle tissue, and kidney. RNA sizes are indicated in kilobases at left. At bottom is hybridization with a control glyceraldehyde 3-phosphate dehydrogenase probe.

If μ-crystallin is an enzyme, it should be expressed in other tissues. As expected, Northern analysis of RNA from gray kangaroo tissues reveals extremely high levels of μ-crystallin mRNA in lens (Fig. 4*A*). Outside the lens, however, kangaroo μ-crystallin is expressed preferentially in retina and brain, a pattern unique among known enzyme crystallins (1–4). With the kangaroo cDNA clone as probe, an almost full-length clone 86% identical to kangaroo μ-crystallin in predicted amino acid sequence was obtained from a human retinal cDNA library (Fig. 2). Northern analysis of human tissues confirmed the presence of μ-crystallin mRNA in neural tissue. In contrast to the kangaroo, however, μ-crystallin is also abundant in muscle tissues and in kidney (Fig. 4*B*). Weak hybridization in other tissues could come from associated nerves.

Because μ-crystallin is related to an enzyme of amino acid metabolism and because amino acids, notably glutamate, may serve as neurotransmitters, the neural expression of μ-crystallin raises the possibility of its involvement in the synthesis or breakdown of excitatory amino acids. Indeed, the familiar pathway of ornithine to proline involves at least one intermediate common to glutamate biosynthesis, 1-pyrroline-5-carboxylate; although this intermediate has not been detected in the reaction catalyzed by OCD (10). However, there seems to be a general rule that enzymes recruited as crystallins have a preexisting function in most lenses (5). If μ-crystallin fits this pattern, an alternative or additional role might be in the control of osmolytes essential to maintain lens transparency. Such a role could also be relevant to expression in human kidney. Proline, the product of OCDs, happens to be an important osmolyte and component of transport systems in various prokaryotes and eukaryotes (21–24); conceivably proline could have the same function in lenses. A common origin in osmotic stress and osmoregulation pathways may connect many of the proteins that now serve as crystallins (5, 6).

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