THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 271, No. 26, Issue of June 28, pp. 15623–15628, 1996
Printed in U.S.A.

A Retinaldehyde Dehydrogenase as a Structural Protein in a Mammalian Eye Lens

GENE RECRUITMENT OF η-CRYSTALLIN*

(Received for publication, February 14, 1996, and in revised form, April 2, 1996)

Caroline Graham, Jason Hodin, and Graeme Wistow‡

From the Section on Molecular Structure and Function, Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda, Maryland 20892-2730

η-Crystallin is a taxon-specific crystallin, a major component of the eye lens in elephant shrews (Macroscelidea). Sequence analysis of η-crystallin from two genera of elephant shrews and expression of recombinant η-crystallin show that the protein is a cytoplasmic (class 1) aldehyde dehydrogenase (ALDH1, EC 1.2.1.3) with activity for the oxidation of retinaldehyde to retinoic acid. Unlike many other mammals, elephant shrews have two ALDH1 genes. One encodes ALDH1/η-crystallin which, in addition to its very high expression in lens, is also the predominant form of ALDH1 expressed in other parts of the eye. The second gene encodes a "non-lens" ALDH1 (ALDH1-nl) which is the predominant form expressed in liver. This pattern of tissue preference contrasts with other mammals which make use of the same major ALDH1 transcript in both ocular and non-ocular tissues. Thus the gene recruitment of ALDH1/η-crystallin as a structural protein in elephant shrew lenses is associated with its collateral recruitment as the major form of ALDH1 expressed in other parts of the eye.

During vertebrate evolution, the composition and properties of eye lenses have been modified by the direct gene recruitment of enzymes as crystallins, the abundant structural proteins of the lens (1–4). It has been suggested that the recruitment of these novel crystallins is an adaptive process in diurnal terrestrial species, replacing or diluting the specialized γ-crystallins which are particularly associated with the harder, myopic lenses typical of fish and burrowing nocturnal rodents (1, 2). Enzyme crystallins may also confer benefits such as protection from UV damage or other stresses. In most cases the result of gene recruitment is that a single gene codes for both enzyme and crystallin and a new protein function is acquired without gene duplication.

η-Crystallin is a major component (up to 25% total protein) of the lens in elephant shrews (Macroscelidea) (5), a group of active diurnal insectivores. Previously, protein microsequencing and immunochemistry suggested identity between η-crystallin of *Elephantulus rufescens* and cytoplasmic aldehyde dehydrogenase (ALDH1)[1] (EC 1.2.1.3) (5). An important and rather specific activity of ALDH1 is to act as a retinaldehyde (retinal) dehydrogenase, catalyzing the synthesis of retinoic acid (RA) (6–9). To clarify this relationship η-crystallin cDNA was cloned from lenses of two species of elephant shrews representing two genera (*Elephantulus edwardi* and *Macroscelides proboscideus*) and recombinant η-crystallin was analyzed for retinal dehydrogenase activity.

EXPERIMENTAL PROCEDURES

Cloning and PCR Analysis—Elephant shrew tissues were obtained as post-mortem samples from National and Philadelphia Zoos. Rats were from Taconic Farms, PA, and bovine tissues from a local slaughterhouse. RNA was extracted using RNAzol (Tel-Test, Inc., Friendswood, TX).

Sequences were compiled by several PCR strategies including RT-PCR (10), RACE (11), and inverse PCR (12). For RT-PCR 1 μg of total RNA was primed with oligo(dT), random primers, or sequence-specific primers. For 5′-RACE 100 ng of lens RNA was primed with a specific primer, the product was tailed with poly(dA) and amplified using oligo(dT) and a nested, specific primer. RNA was transcribed with SuperScript RT (Life Technologies, Gaithersburg, MD) followed by amplification with *Taq* DNA polymerase (Boehringer Mannheim) using 30 cycles of 1 min at 94° C, 1 min at 55° C, 2 min at 72°, followed by 10 min at 72° C. Products were cloned into pCRII (Invitrogen Corp., San Diego, CA) and multiple clones sequenced using Sequenase version 2.0 (U. S. Biochemical Corp.). Sequences of all primers are available on request.

Expression patterns of η-crystallin and ALDH1-nl (non-lens) was examined using RT-PCR with 100 ng of lens RNA and 500 ng of RNA from non-lens tissues. Conserved primers in the 3′ regions (Fig. 1) were designed such that the η-crystallin product was 13 bp shorter than ALDH1-nl. Products were confirmed by subcloning and sequencing.

Southern Blot Analysis—Southern blots (13) of *M. proboscideus* genomic DNA, extracted from a eviscerated carcass, were probed with cDNA fragments corresponding to positions 58–1603 of η-crystallin (Fig. 1) and the equivalent part of human ALDH1, or with a shorter η-crystallin probe, nucleotides 908-1092 (Fig. 1), equivalent to exon 9 of human ALDH1 (14). Probes were derived by PCR of η-crystallin cDNA, human genomic DNA, or human kidney RNA, subcloned and sequenced. Probes were labeled with [α-^{32}P]dCTP by random priming (Life Technologies). Hybridization followed standard methodology (13) with final stringency 0.1 × SSC, 0.1% SDS at 55 °C.

Expression of Recombinant η-Crystallin—The coding sequence (CDS) of *E. edwardi* η-crystallin, with a *Nde*I site engineered into the start codon and a *Bam*HI site 40-bp downstream from the stop codon, was prepared by PCR, sequenced, and subcloned into the pET17b expression vector (Novagen, Madison, WI) and designated pETη. This plasmid was transformed into pLysS cells and induced by isopropyl-1-thio-β-D-galactopyranoside at room temperature. Cells were lysed by sonication (15) and soluble extract analyzed by native or SDS-polyacrylamide gel electrophoresis. For activity assays (16) native gels were immersed in 0.1 M sodium pyrophosphate buffer, pH 9.0, containing 22.5 mg of nitro blue tetrazolium (Boehringer Mannheim), 25 mg of NAD$^+$, 1 mg of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U02483, U03906, and U40486.

‡ To whom correspondence should be addressed: Chief, Section on Molecular Structure and Function, LMDB, National Eye Institute, Bldg. 6, Rm. 222, National Institutes of Health, Bethesda, MD 20892-2730. Tel.: 301-402-3452; Fax: 301-496-0078; E-mail: graeme@mge2.nei.nih.gov.

[1] The abbreviations used are: ALDH, aldehyde dehydrogenase; RA, retinoic acid; RT-PCR, reverse transcriptase polymerase chain reaction; bp, base pair(s); CDS, coding sequence; RACE, rapid amplification of cDNA ends; nl, non-lens.

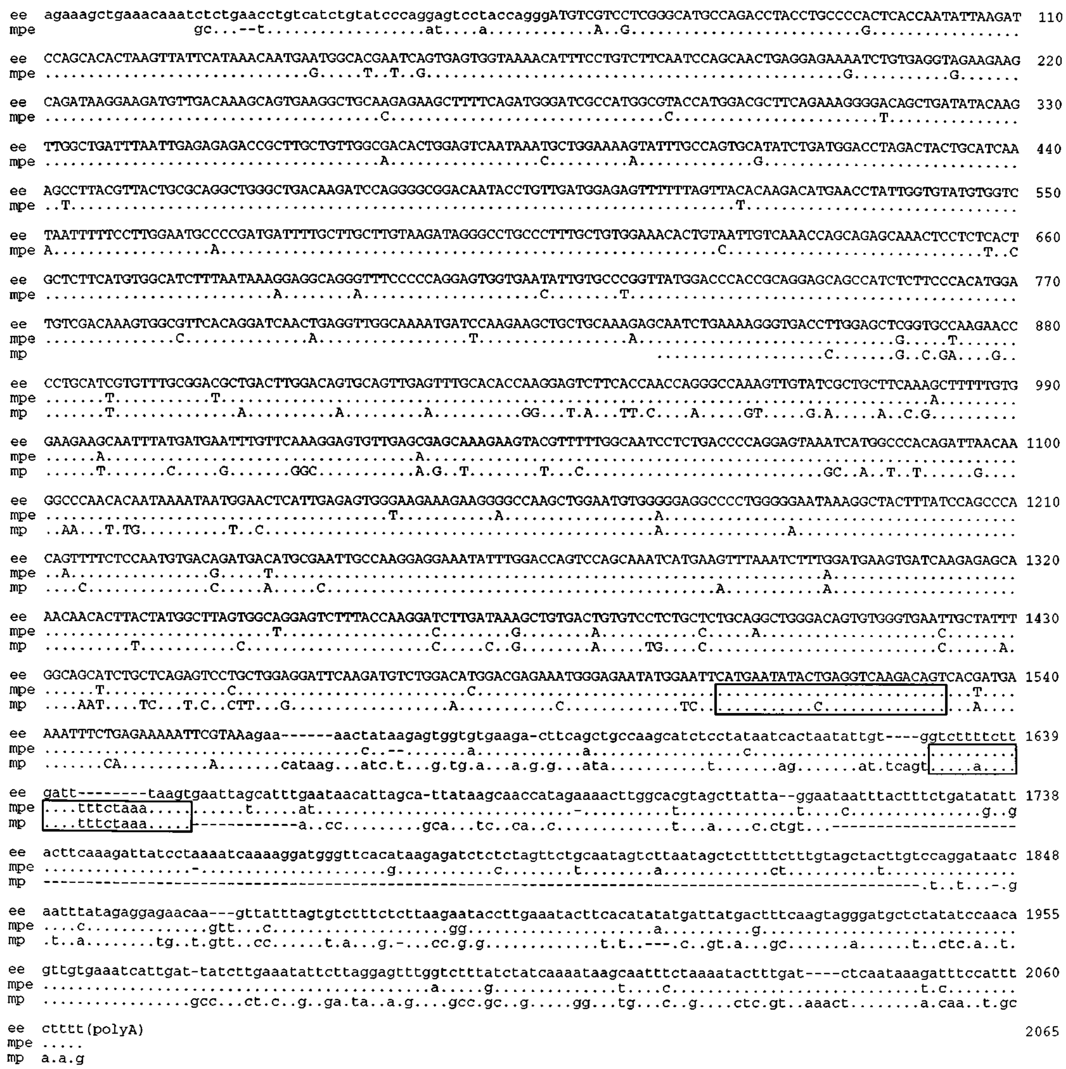

FIG. 1. **cDNA sequences of *E. edwardi* η-crystallin (*ee*), *M. proboscideus* η-crystallin (*mpe*), and (partial) *M. proboscideus* non-lens ALDH1 (*mp*).** Sequences numbered relative to ee. CDS in *upper case.* Identity to ee shown as *dots, hyphens* show gaps. A minor form of ee had an additional sequence, ACCT at the 3′ end and a similar minor form of mpe had ACATG. A possible allelic form of ee had six silent differences with the sequence shown: 1200/C; 1266/C; 1317/G; 1347/G; 1572/G; 1865/T. Primers used in Fig. 3 are *boxed.*

phenazine methosulfate (Sigma) in a light-proof container. All-*trans*-retinal (Sigma) was added to 0.1 mM and color developed at 37° C. Reaction was terminated with 7% acetic acid. Control yeast ALDH was from Boehringer Mannheim.

Sequence Analysis—Sequence analysis used the GCG package (17) implemented at the Frederick Cancer Research and Development Facility (Frederick, MD) and BLAST (18) at the National Library of Medicine. Cladistic analysis used MEGA (19). Approximately 500 bp of 3′ vector sequence in RATALDHA and a long 5′ region of chicken GGADHR were removed for comparison.

RESULTS

Cloning η-Crystallin from Elephant Shrew Lenses—Several procedures were used to assemble the complete cDNA sequences of *M. proboscideus* and *E. edwardi* η-crystallin (Fig. 1). An initial attempt to construct a cDNA library from *M. proboscideus* lens was unsuccessful but about 1400 bp of sequence data were rescued by PCR of uncloned cDNA using primers designed from *E. rufescens* η-crystallin peptides (5). Further sequence was obtained by inverse PCR of self-ligated cDNA (12). The *M. proboscideus* sequence was completed by RT-PCR (10) and 3′-RACE PCR (11) using RNA from two additional lenses. Lenses were also obtained from *E. edwardi* and full-length η-crystallin cDNA cloned by RACE PCR (11). No other ALDH sequences were detected in lens although similar procedures amplified a different transcript from liver.

The η-crystallins of the two species were 95% identical in cDNA sequence and 98.8% identical in protein sequence. In both species there was evidence for two distinct populations of cDNAs distinguished by sets of silent changes in CDS or changes in the 3′-untranslated region (Fig. 1). This variation of about 0.3% may represent polymorphic alleles.

η-Crystallins are highly similar to ALDH1 sequences of other mammals. The *E. edwardi* cDNA shows 80% identity overall with human ALDH1, with 84% identity in the CDS. Similar high conservation is seen at the protein level and all residues required for ALDH activity (20, 21) are present in the deduced protein sequences (Fig. 2).

Recombinant η-Crystallin Has Retinal Dehydrogenase Activity—Recombinant *E. edwardi* η-crystallin (Fig. 3*A*) was examined for retinal dehydrogenase activity. Retinal is hydrophobic, light-sensitive, and has an absorption maximum close to that of NADH. To minimize practical problems an *in situ* colorimetric assay after native gel electrophoresis was chosen (Fig. 3*B*) (16). A specific positive color reaction, corresponding in mobility to the abundant recombinant protein was observed only in pETη extracts in repeated experiments. No positive reaction was seen in any control extracts lacking cloned insert. Highly active yeast ALDH served as a positive control (Fig. 3*B*). Thus recombinant η-crystallin has RA synthetic activity characteristic of

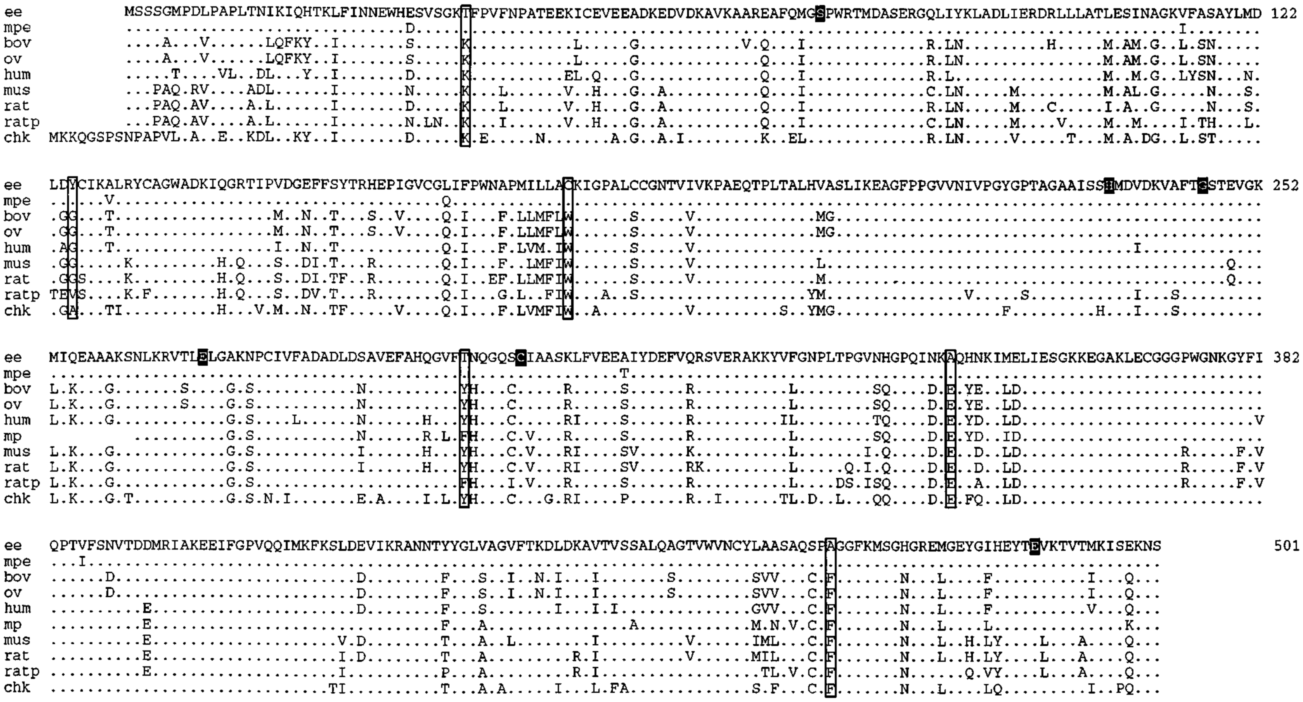

FIG. 2. **Alignment of ALDH1 protein sequences.** ee, mpe, and mp are as described in the legend to Fig. 1; *bov*, bovine retina ALDH1; *ov*, sheep liver ALDH1; *hum*, human liver ALDH1; *mus*, mouse liver ALDH1; *rat*, rat kidney ALDH1; *ratp*, phenobarbital-inducible ALDH1; *chk*, chicken liver ALDH1. Sequences compared to ee as in Fig. 1. Positions of η-crystallin specific non-conservative changes are *boxed*. Residues essential for catalytic activity (20, 21) are in *reverse print*. Sequences were from GenBank.

ALDH1 (6–9).

Southern Blot Analysis and the Existence of Processed Pseudogenes—M. proboscideus genomic DNA was probed with equivalent 1500-bp cDNA fragments of *M. proboscideus* η-crystallin and human ALDH1. Both probes gave very similar complex patterns of strongly hybridizing bands. In an attempt at simplification, a η-crystallin probe equivalent to exon 9 of human ALDH1 (14) was used with the object of hybridizing one band for each gene present. At moderate stringency the same complex pattern produced by the cDNA probes was seen. At higher stringency the complexity decreased to 1 or 2 bands which were not among those strongly hybridizing to the long cDNA probes. A representative pattern for one restriction enzyme is shown in Fig. 4.

Processed pseudogenes, being uninterrupted sequences, may hybridize more efficiently to cDNA probes than do the isolated exons of functional genes. These results thus suggested the presence of multiple pseudogenes in addition to one or more functional ALDH1 gene. Indeed, PCR, cloning, and partial sequencing of *M. proboscideus* genomic DNA confirmed the presence of intronless ALDH1-related sequences (not shown).

A Second ALDH1 Gene Expressed in Elephant Shrew Liver—Multifunctional enzyme crystallins are also expressed outside the lens in their pre-recruitment role. Non-lens expression of η-crystallin was examined by RT-PCR of RNA from *M. proboscideus* liver. After subcloning and sequencing, two ALDH1 sequences were observed, one identical to η-crystallin, the other corresponding to a new ALDH1. This was confirmed by RACE PCR which yielded the 3′-untranslated region and 700 bp of CDS of a transcript designated ALDH1-nl (non-lens) (Fig. 1).

Tissue-preferred Expression of η-Crystallin in Lens and Other Parts of the Eye—The relative abundance of η-crystallin and ALDH1-nl transcripts in different parts of the eye and in liver of *M. proboscideus* was estimated by RT-PCR using common primers (Fig. 5). Reflecting the high abundance of η-crystallin transcripts even in an adult, slowly growing lens, five times more RNA was used for non-lens tissues. Only η-crystallin was detected in lens. In the anterior and posterior parts of the eye η-crystallin was predominant although a minor product corresponding in size to ALDH1-nl was detectable. Liver, in contrast, yielded mainly ALDH1-nl, with only a minor component of η-crystallin.

To determine whether other mammals also exhibit tissue preference in ALDH1, bovine and rat tissues were examined. Primers were made from conserved regions of ALDH1 sequences. RT-PCR of bovine liver RNA yielded a single product identical to the retinal dehydrogenase (ALDH1) from retina (8), suggesting that the same ALDH1 gene product predominates in both eye and liver. Similarly, ALDH1 transcripts of rat were amplified and sequenced from lens, the remainder of the eye and liver. Of six clones sequenced from liver one matched rat phenobarbital-inducible ALDH1 (22). All other clones from liver, lens, and the remainder of the eye corresponded to a different sequence 99.5% identical (out of 640 bp) with that determined for the rat kidney enzyme of RA synthesis (GenBank: RATALDHA) (23, 24). This suggests that even though there is more than one gene for ALDH1 in rat there is no tissue preference in expression of the major transcript between liver and eye.

Cladistic and Phylogenetic Analyses—In cladistic analyses of ALDH1 cDNA sequences (Fig. 6), elephant shrew η-crystallin and ALDH-nl sequences group together. Similarly the two rat sequences and that from mouse form a distinct rodent clade. Whether any other mammals also have two ALDH1 genes is not known, but a search of the expressed sequence tag data bases reveals only one ALDH1 transcript in humans. It seems likely that ALDH1 gene duplications in rodents and elephant shrews were independent events not shared by other lineages.

Elephant shrews have been classified in various phylogenetic groups such as Insectivora, which would include true shrews, or Glires, which includes rodents and lagomorphs (25). Other classifications have placed them in their own order, Macroscelidea (26). Cladograms with ALDH1 sequences show

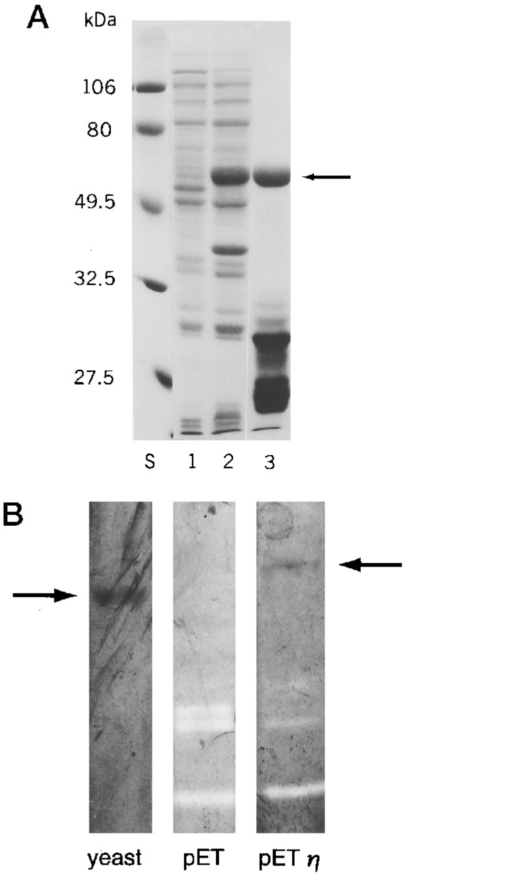

FIG. 3. *A,* SDS-polyacrylamide gel electrophoresis of recombinant *E. edwardi* η-crystallin. *Lane S,* markers; *1,* soluble extract of pET transformed bacteria; *2,* soluble extract pETη transformed bacteria; *3,* soluble extract of *E. edwardi* lens (5). *Arrow* indicates η-crystallin. *B,* native gel *in situ* colorimetric assay for retinal dehydrogenase activity. *Yeast,* positive control of purified yeast ALDH; *pET,* control lysate with no recombinant expression; *pETη,* lysate containing recombinant η-crystallin. *Arrows* indicate the positions of positive bands. Pale bands correspond to endogenous enzymes capable of oxidizing NADH.

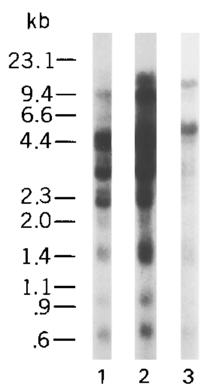

FIG. 4. **Southern analysis of ALDH1-like sequences in the *M. proboscideus* genome.** Representative lanes showing hybridization to *Pst*I digested genomic DNA. Probes: *1,* human ALDH1 cDNA; *2,* η-crystallin cDNA; *3,* single exon η-crystallin.

that elephant shrew sequences do not cluster with rodents, as would be expected for a Glires connection. Instead these data support an ancient branching of the elephant shrew line from those of ungulates, rodents and primates, consistent with data for αA-crystallin of *E. rufescens* which group elephant shrews with the paenungulates, an early offshoot of the placental family tree including hyrax and elephant (5, 27).

Crystallin-related Sequence Changes—Overall, η-crystallins and *M. proboscideus* ALDH1-nl show similar relatedness to

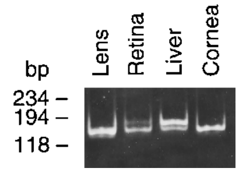

FIG. 5. **Tissue-preferred expression patterns of η-crystallin and ALDH1-nl in *M. proboscideus* analyzed by RT-PCR.** Primer positions are shown in Fig. 1. *Upper band* corresponds to ALDH1-nl and the lower band to η-crystallin. Lanes show results from 100 ng of lens RNA; 500 ng of retina (including sclera and pigment epithelium) RNA; 500 ng of liver RNA; 500 ng of cornea (including iris and ciliary body) RNA.

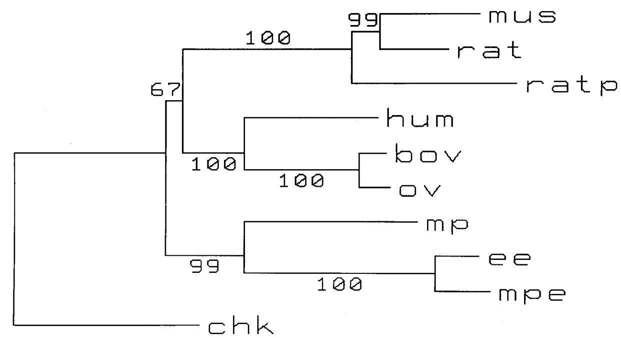

FIG. 6. **Cladogram derived from cDNA sequences of vertebrate ALDH1 genes.** *E. edwardi* η-crystallin (*ee*), *M. proboscideus* η-crystallin (*mpe*), and ALDH1 of cattle (*bov*), sheep (*ov*), human (*hum*), (*partial*) *M. proboscideus* non-lens ALDH1 (*mp*), mouse (*mus*), rat, rat phenobarbital-inducible (*ratp*), and chicken (*chk*), as outgroup. Tree was derived using neighbor-joining and Jukes-Cantor options in MEGA (19). Gaps were treated by pairwise deletion. Bootstrap values from 1000 replications are indicated. Sequences from present study or GenBank.

ALDH1 sequences of other vertebrates (Fig. 6). However, close comparison of protein sequences reveals some interesting differences specific to the η-crystallins (Fig. 2). For example, at positions 166, 178, and 297 in η-crystallin bulky aromatic residues (F, W, Y) are replaced by residues with smaller, less hydrophobic side chains (A, C, T). Since these non-conservative changes are associated only with η-crystallins, they may reflect modifications with no benefit for the ancient role as an enzyme which were instead selected by the new structural role in lens. Similar changes occur in lactate dehydrogenase B in species in which it serves as ϵ-crystallin (28–30).

DISCUSSION

In both *E. rufescens* and *E. edwardi*, η-crystallin accounts for almost a quarter of total soluble lens protein (5). As such it is probably the single most abundant gene product in the lens and in this genus may have largely supplanted γ-crystallins (5). In *M. proboscideus*, η-crystallin is somewhat less abundant, but at about 10% of total protein it is still a major component of the lens (5).

Like other taxon-specific crystallins (31), η-crystallin appears to have arisen by gene recruitment of an enzyme, in this case ALDH1. ALDH1 is the major cytosolic form of ALDH in most vertebrate tissues and is highly conserved among species suggesting an important function (32, 33). Indeed, even though ALDH1 has low activity against a broad range of aldehydes, it has been shown to play a major role in oxidation of retinal to RA (6–9), a potent morphogen and activator of gene expression (34, 35).

Analyses of sequence and enzyme activity (and also immunoreactivity (5)), show that η-crystallin is an ALDH1. However, it is not the only ALDH1 in elephant shrews. The existence of a second gene was revealed by cloning ALDH1-nl from elephant shrew liver. The two ALDH1 genes in *M. proboscideus* have different patterns of expression. In addition to its predominance in lens, η-crystallin is also the major ALDH1 transcript in other parts of the eye while it is detectable only at low levels in liver. ALDH1-nl shows a complementary pattern of expression. It is the major transcript in liver but is absent or at very low levels throughout the eye. There is no evidence for a similar pattern of tissue-preferred expression in other mammals, even when more than one ALDH1 gene is present. Thus the recruitment of an ALDH1 as a crystallin in the lens in elephant shrews is associated with its collateral recruitment as a tissue-preferred form in the rest of the eye.

For most taxon-specific crystallins a single gene encodes a multifunctional protein which serves as both enzyme and crystallin. However, in the case of δ-crystallins (1–4) gene duplication and specialization has occurred, possibly resolving an adaptive conflict between the separate roles of structural protein and enzyme (1, 2). In a similar way, a single ALDH1 gene may have first been recruited as a crystallin in an ancestor of elephant shrews. Adaptive conflict between dual roles may have been resolved through gene duplication and specialization. The opposing selective pressures involved in such adaptive conflict may be illustrated by the non-conservative amino acid changes in η-crystallin which are reminiscent of similar lens-associated changes in lactate dehydrogenase B/ε-crystallin (28–30).

The predominance of η-crystallin throughout the eye provides a rationale for the retention of enzymatic activity by this protein whose role in lens is primarily structural (1). In the developing mouse eye, ALDH1 is expressed at a very early stage in dorsal retina and in lens (7, 36) while ALDH1 is prominent in adult lenses of many mammals (5). RA receptors have been implicated in expression of γ-crystallin genes (37–39) and overexpression of retinoid-binding proteins caused defects in lenses of transgenic mice (40, 41). Since active, multimeric ALDH1 is probably important for normal eye development there must have been strong selective pressures to retain the catalytic activity of η-crystallin to avoid the possibility of "squelching" ALDH1 activity in eye.

Clearly the amount of ALDH1/η-crystallin present in an elephant shrew lens vastly exceeds the requirements of any enzymatic role. However, even if substrate is limiting, such abundance could have serious consequences for RA metabolism by sequestration of retinal or RA itself. Indeed, other enzyme crystallins seem to sequester cofactors in the lens, leading to a general increase in NAD(P)(H) levels. Transgenic mice are now being used in an effort to model this system.[2] Previous experiments have shown that transgenic mouse lens can tolerate a large increase in concentration of α-enolase (42), however, this is a relatively innocuous enzyme with no cofactors.

As has been suggested for other cases (1, 2), the primary selective pressure for this gene recruitment may have been the modification of the optical properties of the lens in an ancestor of elephant shrews, "diluting" the specialized γ-crystallins, to help make a softer accommodating lens. Other benefits may have been protection against the toxic effects of aldehydes resulting from light-induced oxidation of lens components, or generalized anti-oxidant and UV-filtering effects though sequestration of NAD(H) or retinoids in the lens (1, 28, 43, 44).

While there may have been selective advantages for the recruitment of ALDH1/η-crystallin in lens, there is no clear advantage to its collateral recruitment as the major ALDH1 expressed in other parts of the eye. This may instead have occurred as a side effect, through overlap in the transcriptional machineries of related tissues. For example, a key event in gene recruitment of guinea pig ζ-crystallin was the acquisition of an essential binding site for Pax-6 (45). However, since Pax-6 is not lens-specific (46–49) an early stage in such recruitment might have seen elevated in several sites in eye and brain. While additional promoter modifications seem to have fine-tuned tissue-specificity in ζ-crystallin (45, 50) this may not have occurred in η-crystallin. Instead, the enzyme may have maintained a state of high expression in lens coupled with increased expression throughout the eye.

Thus, possibly as a non-adaptive collateral effect of an adaptive process in the lens, elephant shrews have acquired a generally eye-preferred form of ALDH1. This presumably neutral difference between elephant shrews and other mammals recalls the non-adaptive consequences of evolutionary (and architectural) processes, famously discussed by Gould and Lewontin (51).

Acknowledgments—We thank the Pathology Department, National Zoo, Washington, D. C. and the Curator of Mammals of the Philadelphia Zoo for elephant shrew samples. We thank Dr. Cynthia Jaworski for cladistic analysis.

[2] C. Graham and G. Wistow, unpublished data.

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