Evolution of Crystallins: Expression of Lens-Specific Proteins in the Blind Mammals Mole (Talpa europaea) and Mole Rat (Spalax ehrenbergi)

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The mole (Talpa europaea; Insectivora) and the mole rat (Spalax ehrenbergi; Rodentia) both have degenerated eyes as a convergent adaptation to subterranean life. The rudimentary eye lenses of these blind mammals no longer function in a visual process. The crystallin genes, which display a lens-specific expression pattern, were studied in these blind mammals and in related species with normal eyes by hybridizing their genomic DNAs with probes obtained from cDNA clones for αA-, αB-, and βBp-crystallins from calf and γ3-crystallin from the rat. For all crystallin genes examined, the hybridization signals of mole and mole rat genomic DNA were comparable, respectively, with those of shrew and of rat and mouse, normal-vision representatives of the orders Insectivora and Rodentia. The expression of the crystallins at the protein level was tested by using antiserum specific for α-crystallin in immunofluorescence reactions on lens sections of mole and mole rat eyes and by using antisera against the β- and γ-crystallins on sections of the mole eye. All antisera gave positive fluorescence reactions exclusively with lens tissue of these blind mammals, indicating that the crystallins are still normally expressed despite the fact that these lenses have had no function in a visual process in these mammals for at least many million years. These findings apparently imply that some unknown selective advantage has conserved the crystallin genes and their expression after the loss of normal function of the lenses.

Introduction

In the past 2 decades much information has become available on the evolutionary changes of genes and proteins. Most of this information has been obtained from comparative studies of functionally normal proteins and their genes. But what happens with genes and proteins that have lost their biological function in the course of evolution? What types of changes do they undergo—and at what rates—as compared with their functional counterparts? Such knowledge might complement our understanding of neutral and adaptive processes in molecular evolution.

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The analysis of pseudogenes, which are redundant and inactive copies of functional genes, has in fact already shown that these silenced genes, free from selective constraints, evolve at a much higher rate than do the coding genes from which they originate (e.g., Miyata and Yasunaga 1981; Lee et al. 1983). An entirely different and unique opportunity to study the evolutionary effects of the loss of functional constraints on genes and proteins is offered by the rudimentary eyes of many subterranean or cave-dwelling vertebrates. Because much is known about the structural and evolutionary characteristics of the lens-specific proteins, the crystallins, in vertebrates with normal visual capacities (Bloemendal 1981), we have now begun a comparative study of the crystallins and their genes in two blind mammals, the mole (Talpa europaea) and the mole rat (Spalax ehrenbergi). Both species are considered to be effectively blind, and this adaptation is an example of evolutionary convergence that is attributable to subterranean life (Nevo 1979). The eyes of these animals are degenerated, and their structures represent an unusual persistence into adult life of features classically associated with early prenatal development in the normal mammalian eye (Cei 1946; Quilliam 1966). The lenses, for example, lack the normally differentiated fiber cells, and all lens cells contain a nucleus.

In vertebrate lenses the crystallins are the principal structural proteins and account for almost 90% of the water-soluble protein (reviewed in Harding and Dilley 1976; Bloemendal 1981). There are four immunologically distinct major classes, called α-, β-, γ-, and δ-crystallins, of which the last occurs only in birds and reptiles. Comparative studies of the crystallin polypeptides have revealed a high degree of evolutionary conservation of these lenticular proteins. The four main classes of crystallins are each composed of multiple polypeptides that, except for δ-crystallins and β,-crystallin, form multimeric proteins. The polypeptides within a class of crystallins have related amino acid sequences and share antigenic determinants. Analysis of the amino acid sequences of the A chain of α-crystallin from 45 vertebrate species has provided a detailed picture of the evolutionary history of this protein and shown its usefulness in the study of mammalian phylogeny (de Jong 1981).

The development of recombinant DNA technology allows direct examination of the crystallin genes. Recently crystallin-specific cDNA clones have become available (Dodemont et al. 1981; Quax-Jeuken et al. 1984) that can be used as probes for the detection of crystallin sequences in the genomes of blind animals. Here we report the demonstration of crystallin DNA sequences in the genomes of mole and mole rat. The expression of the crystallins at the protein level has been examined by immunofluorescence studies on sections of the rudimentary eyes of mole and mole rat using antisera prepared against bovine crystallins.

Material and Methods

Animals

Livers and heads were obtained immediately after death from adult specimens of two species of “blind” mammals: the European mole, Talpa europaea, and the Palestine mole rat, Spalax ehrenbergi. Livers were stored in liquid nitrogen or, for periods of less than a few days, at -20 C before further use. Eyes, with some surrounding tissue, were immediately dissected and put into fixation fluid. The mole rat was of the chromosomal species 2n = 52 (Nevo 1982). For comparative purposes four other species, with normally developed eyes, were used: rat (Rattus norvegicus) and mouse (Mus musculus), which are rodents like the mole rat; shrew
(Sorex araneus), which is an insectivore like the mole; and calf (Bos taurus). The latter was included in our experiments because most of the cDNA clones used in the hybridization experiments were made from bovine mRNA.

Blot Analysis

Chromosomal DNA was isolated from liver tissue of the different species using standard methods (van der Putten et al. 1979). Equal amounts of the genomic DNAs were digested to completion with the restriction enzymes EcoRI, BamHI, and HindIII and electrophoresed on 0.6% agarose gels. Transfer of DNA to nitrocellulose filters was performed according to the methods of Thomas (1980). Prehybridization and hybridization were conducted under standard conditions with 50% formamide; 5 × SSC (0.75 M NaCl; 0.075 M sodium citrate, pH 7.0); 5 mM EDTA; 100 μg/ml herring sperm single-stranded DNA; 20 mM sodium phosphate, pH 6.8; 0.1% SDS (sodium dodecyl sulfate); and 1 × Denhardt's medium (0.02% BSA, 0.02% Ficoll 400, and 0.02% polyvinylpyrrolidone) at 42 C (Wahl et al. 1979) and with nick-translated plasmid probe (1–5 × 10⁸ cpm/μg), respectively. After hybridization, filters were washed twice with the hybridization solution (without the probe) at 42 C for 1 h, twice with 2 × SSC and 0.1% SDS at 55 C for 15 min, and, in some more stringent cases, at 60 C with 0.1 × SSC and 0.1% SDS for another 15 min. cDNA clones specific for the bovine lens crystallins βBp (pBLβBp), αA (pBLαA), and αB (pBLαB) were used as probes in the hybridization assay. The construction of these clones is detailed in Quax-Jeuk (1984). The γ-crystallin probe was a mixture of three PstI fragments of the insert of the rat γ-crystallin cDNA clone pRLγ3 (Dodemont et al. 1981).

Preparation of Lens Sections

Dissected eyes of mole and mole rat were fixed in Carnoy's and Bouin's fluid, respectively, and embedded in paraffin. Serial sections of 4 μm were cut in parallel orientation to the axis of the eye. Sections that contained lens tissue were used for immunofluorescence reactions or stained with hematoxylin and azofloxine (Romeis 1968).

Preparations of Antibodies

Rabbits (2.5–3.0 kg) were injected subcutaneously with 0.6 ml of a 1:1 (v/v) mixture of complete Freund’s adjuvant and a solution (1 mg/ml) of bovine α-, β-, or γ-crystallins, purified by repeated gel filtration over Ultrogel AcA34. After 6 wk the rabbits were bled and the γ-globulin fraction of the serum was obtained by affinity chromatography on a Protein A–Sepharose column. We also used an antiserum prepared against the bovine βB1 chain that was purified by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (J. Mulders, unpublished data). Immunoelectrophoresis and immunodiffusion analysis established the specificity of the antisera for each of the crystallin fractions (results not shown).

Immunofluorescence on Lens Sections

The indirect technique of Coons (1956) was used throughout, with some modifications. Deparaffinized sections were hydrated and treated with rabbit preimmune serum for 30 min to avoid unspecific binding. After rinsing in Veronal buffer (pH 7.1) for 10 min. the sections were treated with the rabbit antisera against α-, β-, or γ-crystallin for 30 min. After rinsing again in Veronal buffer, the slides were
treated with goat γ-globulins, conjugated with fluorescein isothiocyanate (Roboz, Washington, D.C.), and directed against rabbit γ-globulins. After 30 min the sections were rinsed again for 10 min in Veronal buffer and mounted with 50% (v/v) glycerol.

Results
Detection of Crystallin Sequences in the Genomes of Mole and Mole Rat

To test the genomic DNA of mole and mole rat for the presence of crystallin sequences, we used cDNA clones of bovine and rat crystallins as probes in hybridization assays. We constructed cDNA clones of bovine lens mRNAs that were identified by means of hybridization-selection translation followed by one- and two-dimensional gel electrophoresis of the translational products. We obtained clones specific for αA (pBLαA) and for αB (pBLαB) (to be published elsewhere) and for five different β-crystallin subunits, among which was a βBp cDNA clone, pBLβBp (Quax-Jeuken et al. 1984). The insert of the rat γ-crystallin cDNA clone (pRLγ3) was used as the γ-crystallin probe (Dodemont et al. 1981).

The genomic DNA from mole (insectivore) and mole rat (rodent) was digested with the restriction enzymes EcoRI, HindIII, and BamHI. For comparison with these hybridization signals, we also digested the genomic DNA of normal-vision representatives of the different orders to which these blind animals belong, a shrew (insectivore) and mouse and rat (rodents). Furthermore, bovine genomic DNA was included in our experiments, since most of the probes in the hybridization assays were cDNA clones from bovine mRNAs. BamHI and HindIII digests of DNAs from rat, mole rat, mole, shrew, and calf were hybridized with the clone pBLαA (fig. 1A). An EcoRI and a BamHI digest of the DNAs of the same species plus the mouse were hybridized with pBLαB (fig. 1B) and with pRLγ3 (fig. 1D), and another EcoRI digest was hybridized with clone pBLβBp (fig. 1C). All species showed clear bands under the rather stringent conditions used (see Material and Methods), with only one exception: for the shrew we could not detect any hybridization signal with the αA probe, either in the BamHI or in the HindIII digest (fig. 1A, lane d). The meaning of this finding is not clear. It seems unlikely that the αA gene is absent, since it is present in all investigated mammals, including the insectivores, hedgehog (de Jong 1981), and mole. Perhaps αA sequences of the shrew are located on DNA fragments that are too large to be electrophoresed into the gel or too small to be recovered with the blotting and hybridization procedures.

Immunofluorescence Studies on Lens Sections

The expression of the different crystallin genes in the lenses of mole and mole rat was examined by immunofluorescence experiments with antisera specific for α-, β-, and γ-crystallins. Dissected eyes of mole and mole rat were fixed and embedded in paraffin. Serial sections were stained and examined under the microscope (figs. 2A–B and 3A–B). Especially the morphology of the eye of the mole rat (fig. 3A) deviates greatly from the general bauplan of the mammalian eye, showing several unusual structures, including a rudimentary lens, undifferentiated sclerochoroidea, poorly developed retina, indistinct iris and ciliary body developed into an enormous anterior pigmented and folded mass, an embryonic-like vitreous body, absence of eye muscles, and an extremely hypertrophic Harderian gland (Cei 1946). The lenses of both mole and mole rat (figs. 2B and 3B) are composed entirely of
Fig. 1.—Detection of crystallin sequences in the mole (Talpa europaea) and mole rat (Spalax ehrenbergi). Ten-microgram amounts of DNA from mole and mole rat and several related species were digested with restriction enzymes, run on 0.6% agarose gels, blotted onto nitrocellulose, and hybridized with nick-translated cDNA clones of αA-, αB-, βBp-, and γ3-crystallin. Hybridization was under rather stringent conditions, as described in Material and Methods. HindIII digests of λ DNA were used as molecular weight markers. A, DNA blot hybridization with the αA-crystallin probe. DNA from rat (a), mole rat (b), mole (c), shrew (d), and calf (e) were digested with BamHI (I) and HindIII (II) and hybridized with the bovine cDNA clone pBlαA. λ/HindIII fragment sizes are indicated. B, DNA blot hybridization with the αB-crystallin probe. An EcoRI digest (I) of DNA from rat (a), mouse (b), mole rat (c), mole (d), and calf (e) and a BamHI digest (II) of DNA from mole (a), shrew (b), and calf (c) were hybridized with the bovine αB-crystallin probe pBlαB. C, Blot hybridization with the βBp-crystallin probe. DNA from rat (a), mouse (b), mole rat (c), mole (d), shrew (e), and calf (f) were digested with EcoRI and hybridized with the bovine βBp cDNA clone pBlβBp. Sizes of λ/HindIII fragments are indicated. D, Blot hybridization with the γ3-crystallin probe. An EcoRI digest (I) of rat (a), mouse (b), and mole rat (c) and a BamHI digest (II) of rat (a), mole rat (b), mole (c), shrew (d), and calf (e) were hybridized with the nick-translated γ3 probe of the rat (pRLγ3).
nucleated cells and in this respect resemble the lens of the normal mammalian eye in the early stages of prenatal development. The mole lens still retained the characteristic lens shape, whereas some differentiation from the anterior epithelial cell layer into the irregularly elongated or enlarged posterior cells can be observed. By contrast, the lens of the mole rat consists of an irregularly shaped mass of apparently undifferentiated cells. In fact, these cells display degenerative features, for example, cytoplasmic clearing and vacuolization, which have been likened to certain pathological conditions in the human cataractous lens (Cei 1946). Comprehensive anatomical and histological descriptions of the degenerated eyes of mole rat and mole have been given by Cei (1946) and Quilliam (1966), respectively.

Sections of the mole eye that contained lens tissue were treated with the antisera against α-, β-, and γ-crystallins (fig. 2C–E). Sections of the mole rat eye were treated only with antiserum against α-crystallin (fig. 3C). The immunofluorescence reactions with all antisera were positive in the lens of the mole eye, and the same holds true for the α-crystallin antiserum in the lens of the mole rat eye. Immunofluorescence was not seen in other ocular tissues than the lens. Replacement...
of the antiserum by normal rabbit serum abolished the reaction. This led us to conclude that mole and mole rat display a lens-specific crystallin expression.

Discussion

The mole and mole rat are considered to be "blind," an adaptation to subterranean life (Nevo 1979). While the mole may still be capable of some form of light/dark discrimination (Lund and Lund 1965), the mole rat does not respond to light stimuli at all (Haim et al. 1983). Whether the atrophied eyes of these species, after the loss of their visual function, may have retained or developed other
functions, e.g., the perception of photoperiodicity (Haim et al. 1983; Pévet et al. 1984), is a matter for further investigation.

Because eyes do not fossilize, it is obviously difficult to establish precisely the evolutionary time since the beginning of the degeneration of the eyes in the ancestors of moles and mole rats. However, the fossil evidence shows that the earliest known representatives of the family Spalacidae, from the Lower Miocene (approximately 25 Myr), had already adapted to a burrowing, subterranean way of life (de Bruijn 1985). Similarly, the oldest fossil remnants of typical moles, family Talpidae, are from the Upper Eocene (~45 Myr) (Siège et al. 1977). The degeneration of the eyes of these mammals is likely to have started shortly after the animals had adopted fossorial lives.

Despite the loss of normal structure and function of the lens in mole and mole rat, our hybridization experiments show that the genes for lens-specific proteins, the crystallins, are conserved during the evolution of these species. The apparent conservation of the crystallin genes in blind animals seems remarkable, since one might imagine that acceptance of mutations in these genes was no longer constrained by selective forces at the protein level after the loss of functional eyes. Such supposedly functionless genes could thus be expected to be freer than coding genes to accumulate base substitutions, deletions, and insertions, in a manner analogous to the evolution of pseudogenes. Sequence analysis of an α-globin pseudo gene (Miyata and Yasunaga 1981) and three β-tubulin pseudogenes (Lee et al. 1983) indeed revealed that the rate of evolutionary change of these silenced genes can be nearly twice the rate of silent site substitutions in active genes, which is approximately 1%/Myr (Jeffreys 1982). The rate of substitutions in fully functionless and unconstrained “junk” DNA has also been predicted to be 1%–2%/Myr (Jeffreys 1982). After 25–45 Myr of unconstrained evolution one would expect that the similarity of the crystallin genes in mole and mole rat might fall below the detection limit in DNA-hybridization experiments. Nevertheless, the intensity of the hybridization bands is roughly comparable to that in their rodent and insectivore relatives with normal eyes, giving no evidence that nucleotide substitutions have occurred at greatly accelerated rates in the crystallin genes of the blind mammals.

The hybridization patterns of all species examined are rather similar for a particular crystallin class (fig. 1A–D). αA-crystallin, a single-copy gene (King and Piatigorsky 1983; Dodemont 1984), gives only one or two bands in the genomes of all species except the bovine BamHI digest, which shows three bands. The same holds true for the hybridization patterns with the αB- and the βBp-probe: only two or three bands can be detected in each lane. For the γ-crystallins we see a more complicated pattern. The γ-crystallins in the rat are encoded by six linked genes that show more than 90% sequence identity in the coding regions (Moormann 1984). This great similarity results in cross hybridization even under the stringent washing conditions we used. After hybridization with the rat γ-crystallin probe, we see several bands in the lanes of rat, mouse, mole rat, and calf. But the insectivores, i.e., mole and shrew (fig. 1D II, lanes c and d), each reveal only one band, which is 15 kb and 30 kb, respectively. This simple pattern for γ-crystallin sequences is perhaps characteristic for the insectivores. It may indicate either that the insectivores have fewer γ-crystallin genes than do the other mammals we examined or that these genes have no BamHI sites between them.

The immunofluorescence experiments on sections of the eyes of the mole demonstrate that the crystallin genes are still effectively expressed, since all crystallin
antisera used gave positive reactions. Similarly, α-crystallin was shown to be present in the rudimentary eye lens of the mole rat.

If one considers that base substitutions in unconstrained DNA sequences are estimated to occur at rates of up to 2%/Myr (Jeffreys 1982) and also that deletions and insertions are hardly less frequent evolutionary events (De Jong and Ryden 1981), one might expect that, after the loss of selective constraint on a protein, the corresponding gene would certainly be silenced by nonsense and frame-shift mutations within a few million years. The fact that the crystallin genes are still expressed in the degenerated lenses of mole and mole rat can, according to current opinions about molecular evolution (e.g., Wilson et al. 1977), only be explained by assuming that the ability to synthesize crystallins bestows some as yet unknown selective advantage on these animals.

It will be interesting to see whether this presumed selective pressure has conserved the crystallin amino acid sequences in mole and mole rat as rigidly as it has in other mammals. This can be determined by deriving these amino acid sequences from the nucleotide sequences of the crystallin genes in mole and mole rat. Studies at the sequence level are important for understanding some of the basic rules that govern DNA evolution. The relationship between biochemical and organismal evolution can only be understood by integration of knowledge from such fields as paleontology, physiology, and morphology with that from biochemistry and molecular genetics.

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LITERATURE CITED


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