Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 5320–5324, August 1987
Evolution

The lens protein αA-crystallin of the blind mole rat, *Spalax ehrenbergi*: Evolutionary change and functional constraints

(protein evolution/rodent phylogeny/substitution rate/retina/photoperiodicity)

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Communicated by Francisco J. Ayala, April 13, 1987 (received for review January 5, 1987)

ABSTRACT **The complete structure of the single-copy αA-crystallin gene of the blind mole rat (*Spalax ehrenbergi*) has been determined in order to elucidate the evolutionary effects of the loss of vision on a lens-specific protein and its gene. The αA-crystallin gene appears to have all the necessary transcriptional and translational signal sequences to be expressed in the rudimentary lens of the mole rat and gives rise to probably two protein products by means of alternative splicing, as in rodents with normal vision. Comparisons of the blind mole rat αA-crystallin sequence with αA sequences from other rodents reveal a considerable acceleration of the substitution rate at nonsynonymous positions in the mole rat lineage, which reflects a relaxation of selective constraints, but the acceleration is not to the extent that might be expected if the gene were now without any function. The remaining evolutionary constraints still imposed upon the mole rat αA-crystallin gene may possibly reflect the need for α-crystallin expression as an indispensable component in the developmental program of the atrophied eye.**

Functional constraints working at the protein level are recognized as a major determinant of the rate of molecular evolution (1–3). Although several well-known examples clearly seem to illustrate this principle, such as the fast-evolving fibrinopeptides versus the conserved histones (4), the structure–function relationships of most proteins are, in fact, not understood in sufficient detail to reliably correlate functional constraint with rate of evolution. This difficulty may also hamper the clear distinction of other types of constraints, such as amino acid composition (5), transcriptional and translational requirements (6), and developmental programs (7, 8). The existence and importance of these additional selective forces might be revealed by studying genes whose protein products have lost their normal principal function in the course of evolution. The substitution rate of such a gene would be expected to increase and might eventually become as fast as in pseudogenes unless other constraints interfere. A unique opportunity for such a study is offered by the rudimentary eyes of blind vertebrate species. The eye-specific genes of these animals are no longer subject to the selective constraints associated with the maintenance of vision.

The eye lens protein α-crystallin is very suitable for this purpose because much comparative and evolutionary data are available (9–11). α-Crystallin is a slowly evolving protein (10), and, thus, relaxation of constraints should be readily detectable. An important advantage, too, is the fact that the αA and αB subunits, of which α-crystallin is composed, are both encoded by single-copy genes located on different chromosomes (12). The αA and αB chains have ≈57% sequence homology, due to an ancient gene duplication (10), and conspicuous sequence similarity with the small heat shock proteins indicates that the ancestral α-crystallin gene originated from this protein family (13, 14). αA-Crystallin DNA sequences are mainly available from rodents (14–17), which makes the αA gene of the completely blind mole rat, *Spalax ehrenbergi* (18), an excellent target for a study of evolutionary constraints. The eyes of this rodent are highly degenerated (19), as an adaptation to a subterranean way of life probably more than 25 million years (myr) ago (20).

Previous studies have shown that crystallin cDNA clones hybridize with genomic DNA of the mole rat (21). The hybridization signals were comparable to those in rodent species with normal vision. In addition, immunofluorescence reactions on sections of the mole rat eye revealed that crystallins are still expressed in the atrophied lens cells. In the present study, we have determined the nucleotide sequence of the mole rat αA-crystallin gene and have reconstructed the evolutionary changes in this gene and its product in the mole rat lineage.

MATERIALS AND METHODS

Protein Sequence Analysis. Beaver lenses (*Castor canadensis*) were supplied by J. E. Storer and D. Baron (Regina, Canada) and were sent to Nijmegen at ambient temperature in a saturated solution of guanidine hydrochloride, from which the crystallins were reconstituted by controlled dialysis. Gundi lenses (*Ctenodactylus gundi*) were collected in Tunisia by J. J. Beintema and were stored frozen until used. Frozen squirrel lenses (*Sciurus vulgaris*) were provided by J. Bouten (Venlo, The Netherlands). All subsequent procedures have been described in detail elsewhere (refs. 9 and 11 and references therein).

Computer Programs. DNA sequence gel readings were recorded, compared, edited, and assembled by the programs of Staden (22). Evolutionary trees were constructed using the programs FITCH, DNAPARS, and DNACOMP, as supplied in the phylogeny inference package PHYLIP, distributed by J. Felsenstein. FITCH uses distance matrices to construct trees, without allowing negative branch lengths; DNAPARS infers a phylogeny by minimizing the number of substitutions needed to convert one sequence into another; and DNACOMP constructs trees by maximizing the number of compatible sites in each tree topology. To calculate the number of synonymous and nonsynonymous substitutions per site from homologous sequences (23), corrected for multiple events (24), a program was written in C and run on a VAX 11/780 computer.

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Abbreviations: Myr, million years; kb, kilobase(s).

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RESULTS

The Structure of the Mole Rat αA-Crystallin Gene. A mole rat genomic library (25) was screened using a rat αA-crystallin cDNA clone (pRLαA-1; ref. 15). Four cosmid clones bearing sequences homologous to αA-crystallin were isolated. Restriction enzyme digestion followed by gel electrophoresis, blotting, and screening with the rat αA cDNA probe demonstrated that all hybridizing bands in the mole rat genomic DNA that were observed in earlier studies (21) were also present in the cloned fragments. The above results confirm that all genomic αA-crystallin sequences were obtained. Restriction enzyme mapping revealed that the inserts represent a region of ≈55 kilobases (kb), containing a 7.3-kb *Eco*RI–*Hin*dIII fragment responsible for the hybridization signal with the rat cDNA probe. This *Eco*RI–*Hin*dIII fragment was subcloned; clone pSCr-1, which was used for sequence determination, was generated.

By using the shotgun strategy of Deininger (26) and the dideoxy sequencing method (27), the nucleotide sequence of the major part of pSCr-1 was established (Figs. 1 and 2). This clone contains the complete single-copy αA-crystallin gene, flanked by 2.3 kb of upstream and 0.8 kb of downstream sequences. As in mouse (17) and hamster (14), the mole rat αA-crystallin gene consists of four exons and three introns and appears to have all the necessary transcriptional and translational signal sequences. In mouse and hamster, the single-copy αA-crystallin gene encodes two primary gene products, αA- and αA^{Ins}-crystallin (14, 17). The minor αA^{Ins} chain has hitherto only been found in muroid rodents. It contains an insertion of 23 amino acid residues as compared with normal αA. This insertion peptide is encoded by the optional exon II, which is flanked at its 3′ end by the dinucleotide GC instead of the consensus splice donor sequence GT. This deviation has been proposed to explain the alternative splicing of the αA transcript (17). The fact that in the mole rat αA gene the 3′ splice junction sequence of exon II is also GC (Fig. 2) strongly suggests that the transcripts of this gene also use alternative splicing pathways, which leads to the synthesis of both αA and αA^{Ins} chains.

The mole rat insert peptide has three or four amino acid replacements as compared with the insertion sequence of hamster, rat, and mouse αA^{Ins}, whereas the latter three differ from each other at only a single position (Fig. 2). This reflects a considerable change in this part of the mole rat protein. Because of the limited number of known αA^{Ins} sequences, we further focused our attention on the αA-crystallin sequence as deduced from exons I, III, and IV.

Amino Acid Replacements in Mole Rat αA-Crystallin. In order to evaluate the amino acid replacements in the mole rat αA chain in the context of evolutionary change of αA-crystallin in rodents with normal vision, we increased the number of six known rodent αA sequences (9, 10, 14–17) by analyzing the primary structure of αA-crystallin for three additional species: squirrel, beaver, and gundi (see the legend to Fig. 3). Together these nine αA sequences represent the major groups among the ≈32 rodent families (28). From Fig. 3, it is immediately obvious that mole rat αA deviates from the general pattern of αA evolution in rodents. In *Spalax* αA, there are no less than nine amino acid replacements, which is in sharp contrast with the few replacements in other rodent αA chains. Not even a single replacement has occurred in the investigated muroid rodents mouse, rat, gerbil, and hamster (to which superfamily the mole rat also belongs) or in squirrel.

As compared with the average rate of change for αA-crystallin in vertebrates (three amino acid replacements per 100 residues in 100 Myr; ref. 10), it can be calculated from Fig. 3 that in the mole rat lineage this rate increases to 13% per 100 Myr. Moreover, four of the inferred replacements in the mole rat lineage (at positions 12, 29, 60, and 163) occur among those 80 residues that are unchanged in all other 72 known αA sequences, ranging from dogfish to man (refs. 10, 11, and 32 and this paper). Three replacements occur at variable positions (51, 172, and 173) in the αA sequence, but they involve residues that have not been observed in other species. Only two of the replacements have been found earlier: phenylalanine-53→leucine in *Rana temporaria* (32) and asparagine-123→serine in hedgehog and alligator (10, 11). Several of the replacements involve radically different residues. These findings all clearly indicate an increased tolerance for change in the primary structure of αA in this blind animal.

Increased Rate of Change at Nonsynonymous Sites. The availability of αA nucleotide sequences for rat (15), mouse (16, 17), hamster (14), chicken (M. Thompson, J. Hawkins, and J. Piatigorsky, personal communication), and frog (32) made it possible to study in more detail the possible effects of the loss of vision on the evolution of the αA-crystallin gene in the mole rat. An analysis of the codons in the mole rat sequence as compared with those in other αA-crystallin genes did not reveal any change in the biased codon usage (data not shown). Using the coding sequences, the corrected number of nonsynonymous substitutions per nonsynonymous site (K_A^c) was calculated for each pairwise comparison of rodent and chicken sequences (Table 1). From this table it

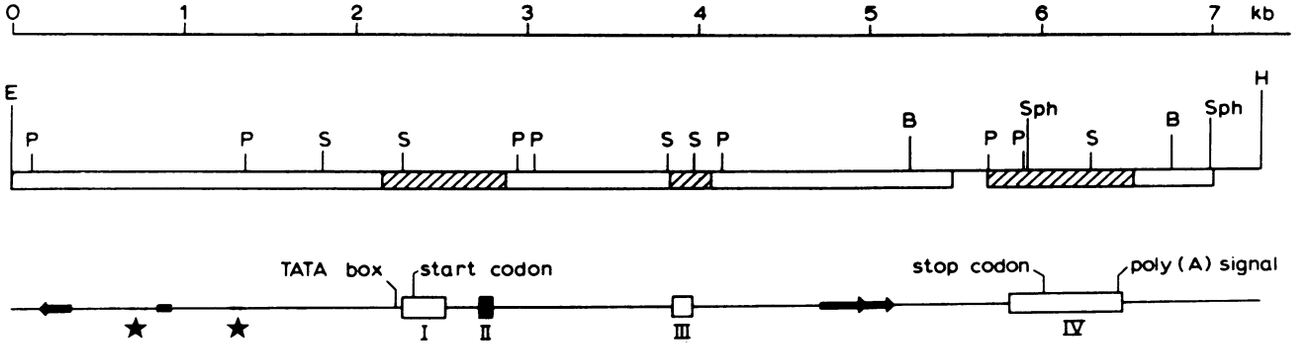

FIG. 1. Physical map of the mole rat αA-crystallin gene. The second line gives the restriction enzyme cleavage sites that were predicted from the nucleotide sequence and were checked by restriction enzyme analysis. E, *Eco*RI; H, *Hin*dIII; S, *Sac* I; P, *Pst* I; B, *Bam*HI; Sph, *Sph* I. The boxes in the second line represent the two regions for which the nucleotide sequence was established, and the hatched parts indicate the sequence regions that are shown in Fig. 2. On the third line the lengths and positions of the αA exons are represented by boxes (the optional exon is in black), and the positions of transcription and translation signals are given. The asterisks indicate two stretches of alternating purine and pyrimidine bases, $(GT)_{23}$ and $(GT)_{21}$, respectively. Three repeated sequences (black arrows) are present that are homologous to the rodent *Alu* type 2 sequences.

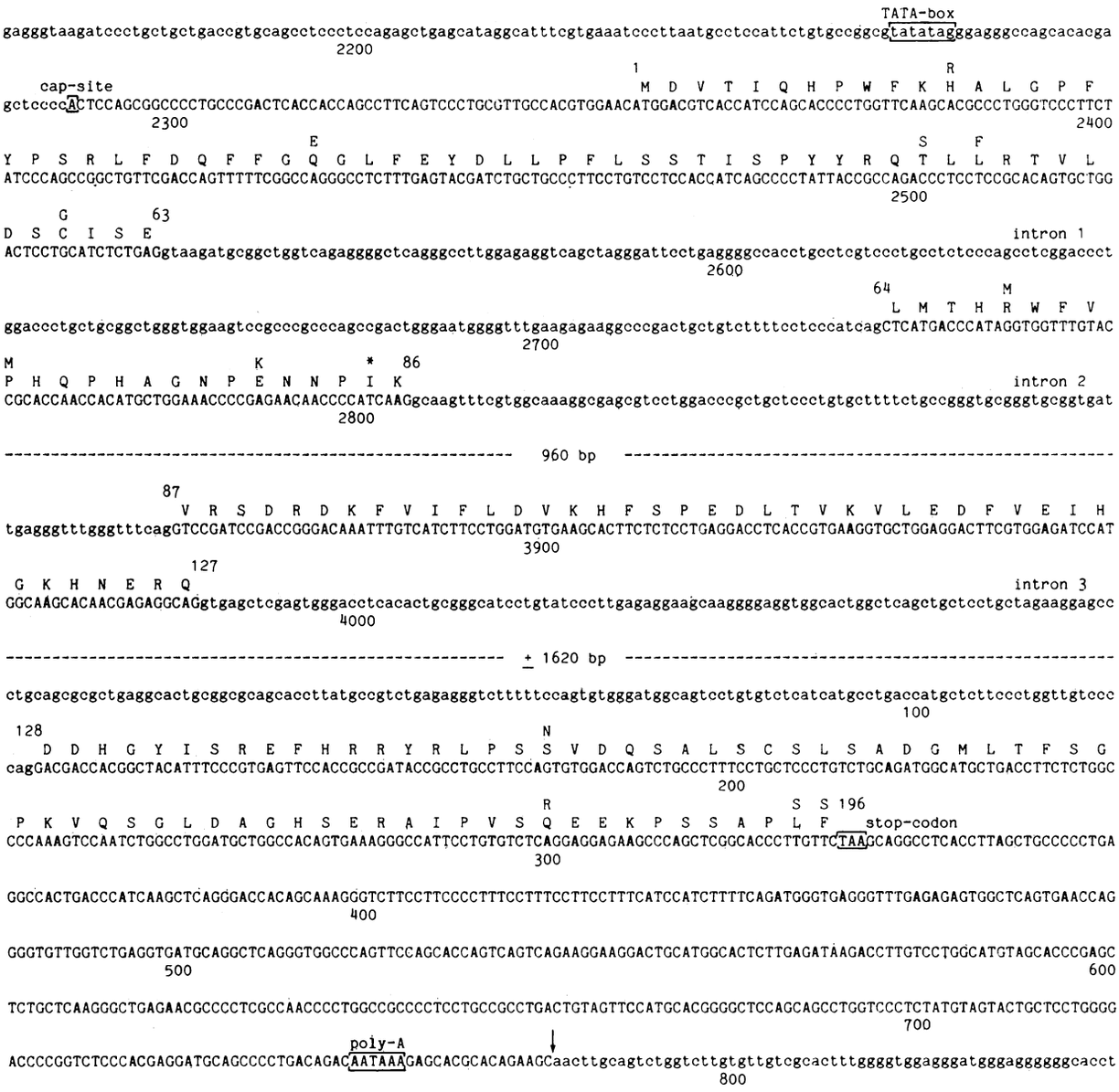

FIG. 2. Sequence of the mole rat αA-crystallin gene. Exon sequences are depicted in capital letters. The deduced amino acid sequence is given in single letter notation above the corresponding triplets. Above the mole rat amino acid sequence are the residues that are different in the hamster sequence, which is identical to that of mouse and rat, except for the presence of valine in mouse and glycine in rat (14) at position 85 in the insert peptide (*). The numbering of nucleotides is according to their position in the two sequenced regions of pSCr-1 (Fig. 1). The predicted cap site and predicted poly(A) addition site (↓) are indicated. The numbering of amino acid residues in this figure includes the optional insert of 23 amino acids (exon II). This gives a discrepancy of 23 in the numbering beyond residue 63 as compared with that in the rest of the paper, where the numbering according to the positions in the normal 173-residue αA chain is adhered to.

appears that in the mole rat lineage 0.023 substitutions per nonsynonymous site have occurred in the 40 Myr since the divergence from the other rodents; this gives an average rate of 0.58×10^{-9} substitution per site per year. This is almost four times the rate in the branches to chicken and the other rodents: 0.15×10^{-9} substitution per site per year (taking the rodent divergence times as given in Fig. 3 and 300 Myr for the divergence of birds and mammals; ref. 33).

The increased rate in the mole rat lineage is a conservative estimate because it is the average over the last 40 Myr of evolution of αA. Although it obviously is uncertain when the effective loss of vision occurred, it seems reasonable to assume that the accelerated evolution of αA only began after the mole rats had fully adapted to a burrowing, subterranean way of life, probably 25 Myr ago according to fossil evidence (20). In this case, the inferred average rate would increase to approximately 0.9×10^{-9} substitution per site per year. This still is only 20% of the rate of change observed in pseudogenes (34), which is assumed to be the neutrality standard for inferring the stringency of selective constraints in sequence regions. Thus, it appears that the αA-crystallin gene in the mole rat has still been under considerable selective constraint, although again it is uncertain whether this constraint has been uniform or discontinuous during the past 25 Myr.

Evolution at Synonymous Sites and in Noncoding Regions. The corrected number of synonymous substitutions per synonymous site (K_S^c) obtained from the comparison of mole rat and chicken αA coding sequences does not significantly differ from the mean value (1.298 with a standard deviation of 0.126) obtained for the other rodent sequences when compared with chicken (Table 1). However, a possible change in the synonymous substitution rate in the mole rat

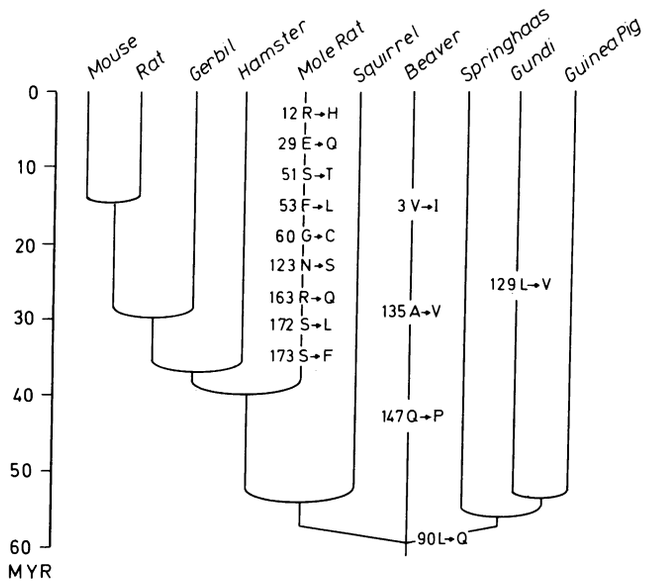

FIG. 3. Evolutionary tree showing the inferred distribution and types of replacements in the evolution of αA-crystallin in rodents. The αA sequences of mole rat, squirrel, beaver, and gundi were added to the already available rodent αA sequences (9, 10, 14–17). The squirrel, beaver, and gundi sequences were analyzed by comparing the amino acid compositions of tryptic peptides. All peptides were found to have the same compositions as those from rat αA-crystallin (9), except for gundi T11 and T17a and also beaver T1, T17b, and T18. Subsequent thermolytic digestion of these peptides revealed that the differences were due to the replacements leucine-90→glutamine and leucine-129→valine in gundi as well as valine-3→isoleucine, alanine-135→valine, and glutamine-147→proline in beaver αA-crystallin (data not shown). The branching pattern of this tree is based on a current consensus about rodent relationships, as inferred from the most recent multidisciplinary evidence (28), apart from the joining of springhaas to the gundi/guinea pig branch, which is solely based on the shared replacement leucine-90→glutamine. This is, however, a weak indicator for common ancestry because this replacement and vice versa occur repeatedly in several mammalian orders (10). The divergence time of rat and mouse is averaged from fossil (8–14 Myr; ref. 29), immunological (22–24 Myr; ref. 30), and DNA–DNA hybridization data (7–11 Myr; F. M. Catzeflis, E.N., J. Ahlquist, and G. C. Sibley, unpublished data; 17–25 Myr; ref. 31). The separation time of Gerbillidae from other rodents is according to fossil, immunological, and hybridization evidence estimated at 19 Myr (29), 35–40 Myr (30), and 36–55 Myr (31), respectively. For the divergence time of hamster from the other Muroidea, these estimates are 35 Myr (28), 35–40 Myr (30), and 38–58 Myr (31), respectively. Because the Spalacidae most probably are the sister group of all other Muroidea (28), their branch should be older than that of the Cricetidae and is placed at 40 Myr, in agreement with immunological data (43 Myr; ref. 30) but earlier than the fossil (25 Myr; refs. 20 and 28) and hybridization (18–21 Myr; F. M. Catzeflis, E.N., J. Ahlquist, and G. C. Sibley, unpublished data) evidence indicate. Immunological data place the origin of squirrels at 55 Myr (30), and the earliest fossil evidence for gundis dates back to 55 Myr (28).

lineage would be hard to detect in this test because the use of the distantly related chicken as an outgroup necessarily results in high and, thus, less reliable K_S^c values (34). Attempts to deduce such information from comparisons of K_S^c among the rodent αA coding sequences themselves (Table 1) also failed because of the gross uncertainties about rodent divergence times (see legend to Fig. 3). Comparisons of the various noncoding regions of the αA-crystallin genes of the different species, in a search for possible evolutionary effects of the change in selective constraints in the mole rat lineage, were not successful for the very same reasons. In fact, considerable variation is observed in substitution rates of untranslated regions of mammalian genes, whereas lack of

Table 1. Number of substitutions per site in αA-crystallin coding regions

	Mouse	Rat	Hamster	Mole rat	Chicken
Mouse		0.000	0.000	0.023	0.084
Rat	0.227		0.000	0.022	0.095
Hamster	0.293	0.344		0.023	0.085
Mole rat	0.534	0.403	0.410		0.112
Chicken	1.407	1.327	1.160	0.983	

K_A^c (upper right part) and K_S^c (lower left part) values are the numbers of nonsynonymous and synonymous substitutions per site, respectively, in the αA coding regions, corrected for multiple events (23, 24).

data have not yet allowed reliable estimates for flanking and intronic regions (34).

Elements that are involved in the expression of the αA-crystallin gene have been identified in the 5′ flanking sequences of the murine and chicken αA gene (35). Alignment of the first 320 base pairs upstream of the cap site of the mole rat αA gene with the other 5′ flanking sequences available (mouse, hamster, and chicken) revealed that these parts are at least 55% homologous and that the promoter elements are situated in the areas with the best overall homology.

Confirmation of Rodent Relationships. No mole rat or hamster DNA sequences have yet been used in studies of rodent relationships. We therefore used the K_S^c values of Table 1 in a distance matrix method (FITCH) for phylogenetic tree construction, and the results showed the mouse and rat αA sequences to be most related, followed by hamster and then mole rat. The same branching order was consistently obtained when the different αA coding sequences were used in other tree construction methods (DNAPARS and DNACOMP). The phylogenetic information contained in the αA-crystallin DNA sequences thus agrees with earlier parsimony analyses including *Spalax* myoglobin and hemoglobin sequences (36) and further strengthens the current opinion about the relationships of Muridae, Cricetidae, and Spalacidae, as depicted in Fig. 3.

DISCUSSION

The study described in this paper deals with the fate of the single-copy lens-specific αA-crystallin gene after loss of its most apparent function—the production of functionally and structurally normal αA-crystallin in the transparent lens fiber cells. Previous immunofluorescence studies had already indicated that α-crystallin is still present in the rudimentary lens of the mole rat (21). The present results show that the mole rat αA gene indeed has all prerequisites for normal expression and gives rise to probably two primary gene products, αA and $αA^{Ins}$, by alternative splicing like in other rodents (14, 17). The nonsynonymous substitution rate in the αA-crystallin gene has considerably increased in the mole rat lineage but has not reached the value observed in pseudogenes. The presence of unexpected, more subtle and complex constraints has apparently maintained the gene and its product in the mole rat lineage and kept its average rate of change at a level comparable with, for instance, that of the globin genes (34).

The fact that the αA gene is still expressed indicates that it is not the gene *per se* that bestows some selective advantage onto the organism, but rather its product. One could imagine that αA-crystallin fulfills some as yet unknown role as a minor but essential protein in cells outside the lens. Crystallins and their mRNAs have indeed been detected in trace amounts in nonlens tissues (37). In this connection, the relation of α-crystallin with the small heat shock proteins may also be recalled, although there is no indication that α-

crystallin itself behaves like a heat shock protein or that its gene has a heat shock-inducible promoter.

More likely, perhaps, is the possibility that the expression of the αA gene is an indispensable link in the developmental program of the atrophied eye of the mole rat. There is good evidence that this rudimentary eye, though not able to detect light anymore (38), is still of vital importance for photoperiod perception (39), which is required for the physiological adaptations of the animal to seasonal changes. It is becoming increasingly clear that in mammals not only the pineal gland is responsible for the induction of such physiological responses (through the synthesis of melatonin directed by photic information received from the retina) but also the retina itself is capable of melatonin synthesis (40). A retinal layer, in which melatonin is synthesized (41), can still clearly be recognized in the mole rat eye (19). If, indeed, this retina is functionally important, it can only be formed through the normal embryological stages of eye development, which necessarily involves the induction of a lens vesicle. Crystallin synthesis normally starts after this induction but before the appearance of specific morphological structures (42). It is conceivable that the expression of α-crystallin is an essential step at this stage of eye development. It might even be that α-crystallin is more directly involved in the development or function of the retina, considering the finding that Müller glia cells in the neural retina are immunostained by an antiserum to lens antigens enriched for α-crystallin (43).

Our present observations on the evolution of αA-crystallin in the blind mole rat thus lend support to the growing awareness that the concept of "constraints," determining the rate and mode of evolution of a gene, should not be confined to the most obvious functional properties of a gene and its products but should be considered in the much wider context of the complex molecular and cellular interrelationships imposed upon the organism by developmental programs (e.g., refs. 7 and 8).

We thank M. Globulic, Dr. F. Figueroa, and Prof. J. Klein for the mole rat genomic library; Dr. J. E. Storer, Dr. D. Baron, Prof. J. J. Beintema, S. Chouiref, Ministry of Agriculture (Tunisia), and Jac. Bouten BV for rodent lenses; the Israel Discount Bank Chair of Evolutionary Biology and the Ancell-Teicher Research Foundation for financial support; M. Versteeg for amino acid analyses; Dr. T. C. W. Luijben for statistical advice; and Dr. R. van den Heuvel for never-ending inspiration. The present investigation was carried out under the auspices of The Netherlands Foundation for Chemical Research and with financial aid from The Netherlands Organization for the Advancement of Pure Research.

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