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V76D mutation in a conserved γD-crystallin region leads to dominant cataracts in mice

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Abstract. During a large-scale ENU mutagenesis screen, a mouse mutant with a dominant cataract was detected and referred to as *Aey4*. Aim of this study was the morphological description of the mutant, the mapping of the mutation, and the characterization of the underlying molecular lesion. The slit-lamp examination revealed a strong nuclear cataract surrounded by a homogeneous milky opacity in the inner cortex. The histological analysis demonstrated remnants of cell nuclei throughout the entire lens. The mutation was mapped to Chromosome 1 by a genome-wide linkage making the six γ-crystallin encoding genes and the closely linked βA2-crystallin encoding gene to relevant candidate genes. Finally, a T→A exchange in exon 2 of the γD-crystallin encoding gene (symbol: *Crygd*) was demonstrated to be causative for the cataract phenotype; this particular mutation is, therefore, referred to *Crygo*^{Aey4}. The alteration in codon 76 leads to an amino acid exchange of Val→Asp. Val at this position is highly conserved; it is found in all mouse and rat γD/E/F-crystallins as well as in the human γA- and γD-crystallins. It may be replaced solely by Ile, which is present in all bovine γ-crystallins, in the rat and mouse γA/B/C-crystallins, as well as in the human γB/C-crystallins. It is predicted that the exchange of a hydrophobic side chain by a polar and acidic one might influence the microenvironment by a dramatic decrease of the isoelectric point by 1.5 pH units in the 10 amino acids surrounding position 76. The *Crygd*^{Aey4} additionally demonstrates the importance of the integrity of the *Cryg* gene cluster for lens transparency.

Introduction

Large-scale ENU mutagenesis screens have been demonstrated to be a source for mouse mutants with clinically important phenotypes (Ehling et al. 1985; Hrabé de Angelis and Balling 1998; Justice et al. 1999; Hrabé de Angelis et al. 2000; Nolan et al. 2000; Brown and Balling 2001). One of the phenotypes we are looking for are opacities of the ocular lens (cataracts). The method was demonstrated to be quick and sensitive (Kratochvilova and Ehling 1979), and about 200 different cataract mutants have been collected (Favor and Neuhäuser-Klaus 2000).

Among the proteins that are responsible for lens transparency, the superfamily of the β/γ-crystallins is most prominent. They were considered for a long time to be present mainly in the ocular lens. However, just recently, expression of the βB2-crystallin mRNA and protein was reported also in retina, brain, and testis (Magabo et al. 2000; Graw et al. 2001b). The common characteristic of all β- and γ-crystallins is the so-called Greek-Key motif. Crystallography has shown that each of the β- and γ-crystallins is composed of two domains, each built up by two Greek Key motifs (for reviews, see Slingsby and Clout 1999; Graw 1997; Wistow and Piatigorsky 1988). The γ-crystallin encoding *Crygs* genes are mainly located in a cluster of six highly related genes (*Cryga* → *Crygf*) on mouse Chromosome (Chr) 1; the 7th *Crygs* gene (*Crygs*) is mapped on mouse Chr 16. Several mutations in *Crygs* genes have been characterized and are causative for cataracts (for reviews see Graw 1999; Francis et al. 2000; He and Li. 2000).

The mutant *Aey4* was found as a dominant cataract by slit-lamp screening of mice after paternal treatment with ethylnitrosourea (ENU). We demonstrate here linkage of this mutation close to the *Crygs* gene cluster on mouse Chr 1. Molecular analysis revealed that the *Crygd* gene is affected in this particular mutant line.

Materials and methods

Animals. Mice were kept under specific pathogen-free conditions at the GSF-Research Center and monitored within the ENU-mouse mutagenesis screen project (Hrabé de Angelis et al. 2000; Hrabé de Angelis and Balling 1998). Male C3HeB/FeJ mice were treated with ethylnitrosourea (ENU; 160 mg/kg) at the age of 10 weeks, according to Ehling et al. (1985), and mated to untreated female C3HeB/FeJ mice. The offspring of the ENU-treated mice were screened at the age of 4–6 months with the aid of a slit lamp (SLM30, Zeiss, Oberkochen, Germany) for the presence of cataracts (Kratochvilova and Ehling 1979). Mice with lens opacities were tested for a dominant mode of inheritance. Homozygotes were obtained by brother × sister mating.

Morphological analysis. For gross documentation, lenses were enucleated under a dissecting microscope (Leica MZ APO, Bensheim, Germany) and photographed. For histological analysis, eye globes were fixed for 3 h in Carnoy's solution and embedded in JB-4 plastic medium (Polysciences Inc., Eppelheim, Germany). Serial transversal 2-μm sections were cut with a dry glass knife (Ultratom OMU3, Reichert, Walldorf, Germany), stained with methylene blue and basic fuchsin, and evaluated with a light microscope (Axioplan, Zeiss). Images were processed with the Adobe software packages Illustrator and Photoshop (Adobe, Unterschleissheim, Germany).

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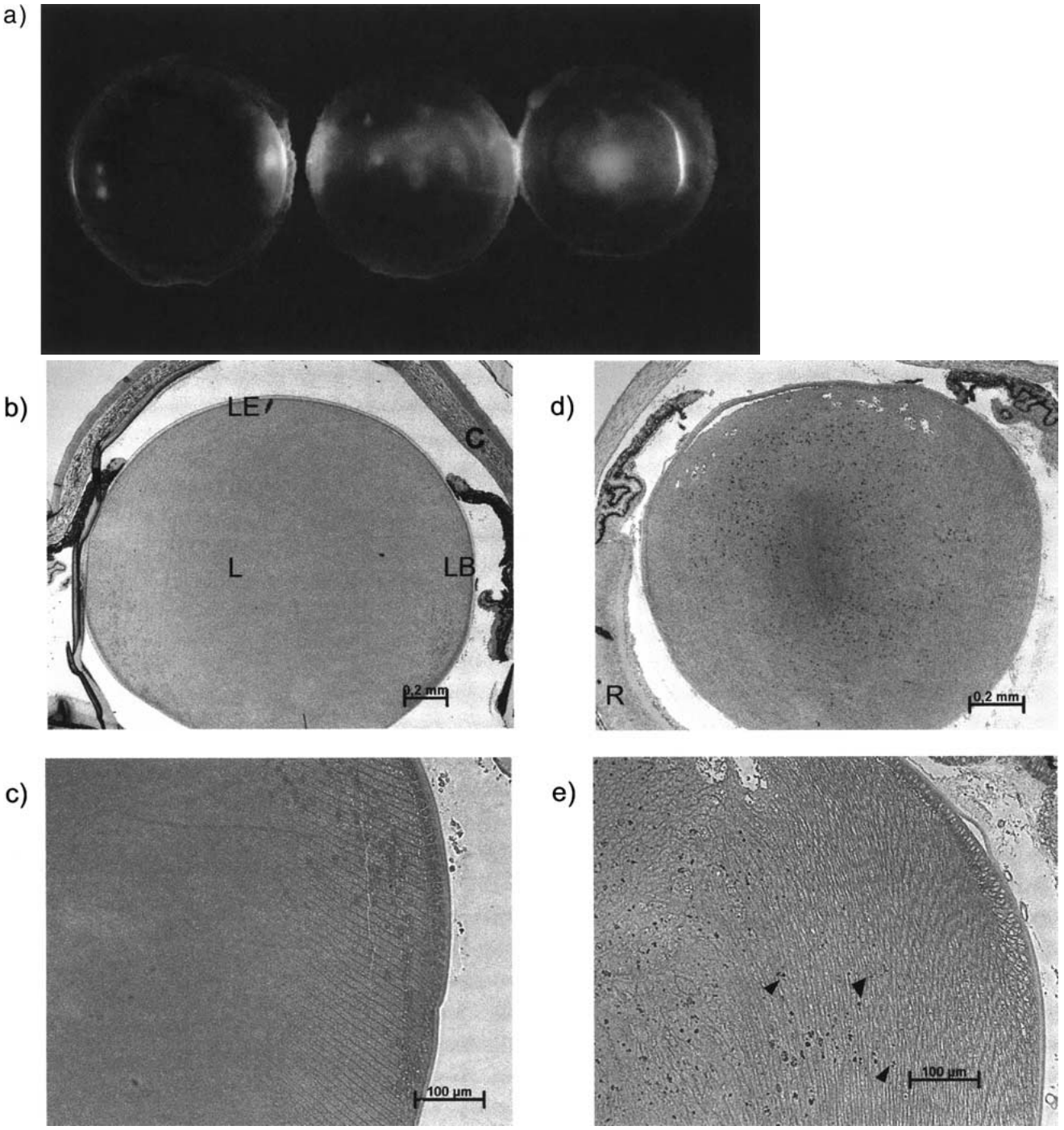

Fig. 1. Morphological description of *Aey4* lenses. Lenses of 12-week-old *Aey4* mice demonstrate a strong nuclear and milky cortical cataract **(a)**. Both mutant genotypes show smaller lenses than the wild type (left); the homozygotes (right) are more severely affected than the heterozygotes (middle). Histological comparison of eyes of 8-day-old wild types **(b, c)** and homozygous *Aey4* mice **(d, e)** indicate the opaque cataractous core of the lens and persistence of remnants of cell nuclei in the *Aey4* mutants **(d)**. The clefts and vacuoles in the anterior part of the lens might be due to sectioning; however, since it is never observed in the wild types, it reflects a weaker adherence of the cells in this region of the cataractous lenses. The magnification of the lens bow region **(e)** indicates that the nuclei are fragmented at the outer cortex and not fully degraded in the deeper zones. Obviously, other parts of the eye are not different between wild-type and mutants. C, cornea; L, lens; LB, lens bow; LE, lens epithelium; R, retina; the bars represent the given distance.

Mapping. Homozygous carriers (1st generation) were mated to wild-type C57BL/6J mice; the offspring (2nd generation) were backcrossed to the wild-type C57BL/6J mice. DNA was prepared from tail tips of 46 cataractous offspring of the 3rd generation (G3) according to standard procedures; DNA was adjusted to a concentration of 50 ng/µl. Genome-wide linkage analysis was done as described previously (Graw et al. 1999).

Isolation of RNA, DNA, and PCR conditions. Genomic DNA was prepared from spleen or tail tips of 3-week-old mice according to standard procedures. RNA was isolated from lenses (stored at −80°C) of newborn mice. cDNA synthesis and PCR with genomic DNA or cDNA as template were performed essentially as reported previously (Klopp et al. 1998, 2001). In addition, for amplification of the *Crygd* exons 1 and 2 including their flanking regions, we used

the primer pair 5′-CGCGGGCCCCTTTTGTGCC-3′ (L) and 5′-CGTTCTTATGCCACAAATACGTAGGGGCC-3′ (R; according to GenBank/EMBL acc. nos, M16512 and AJ224342); the annealing temperature was 65°C. PCR products were sequenced commercially (SequiServe, Vaterstetten, Germany) either after cloning into the pCR2.1 vector (Invitrogen, Leek, The Netherlands) or directly after elution from the agarose gel with kits from Qiagen (Hilden, Germany) or BIORAD (Munich, Germany) and subsequent precipitation by ethanol and glycogen. PCR fragments spanning the genomic region of the exons 1 and 2 of the *Crygd* gene have been digested by *Psy*I and subsequently analyzed on a 10% polyacrylamide gel.

General. Chemicals were from Merck (Darmstadt, Germany) or Sigma Chemicals (Deisenhofen, Germany). The enzymes used for cloning and reverse transcription were from Roche (Mannheim, Germany), and restriction enzymes were from MBI Fermentas (St. Leon-Rot, Germany), if not otherwise mentioned.

Results and discussion

Phenotype and lens morphology. The *Aey4* mutant lenses were examined biomicroscopically by a slit lamp and, after extraction, under a dissecting microscope. They exhibit a strong nuclear cataract and a homogeneous milky opacity of the surrounding area. At the age of 12 weeks, the opacities are more pronounced in the homozygous mutants than in the heterozygotes; the lenses of the homozygotes are also smaller, indicating a semi-dominant effect of the mutation (Fig. 1a). The histological sections of homozygous mutants at the age of 8 days (Fig. 1d, e) revealed some clefts and vacuoles in the anterior part of the lens. They may be caused by a reduced adhesion of the fiber cells. Remnants of cell nuclei are present throughout the whole lens. Other parts of the eye are obviously not affected.

Genomic analysis. The *Aey4* mutation has a complete penetrance, and the litter size in the matings of heterozygotes as well as of homozygotes showed normal fertility and viability of the mutants. Genome-wide mapping indicated linkage with Chr 1; a detailed haplotype analysis is given in Fig. 2a, and the partial map of mouse Chr 1 in Fig. 2b. On the basis of this mapping information, the *Cryg* gene cluster (32 cM from the centromere), as well as the *Cryba2* gene at position 40.8 cM from the centromere, were tested as candidates. In the *Cryba2* cDNA, no difference was found between the wild types and the *Aey4* mutants.

In the *Cryg* genes, some polymorphic sites were discovered in this mutant as described previously (Klopp et al. 2001; Smith et al. 2000). The only difference between wild-type C3H and mutant *Aey4* cDNA, which leads to an alteration of the amino acid sequence, could be identified in the second exon of the *Crygd* gene (acc. # NM_007775: exchange of T→A at pos. 227 of the cDNA). The mutation was confirmed by the absence of the restriction site for *Psy*I in the genomic DNA of four different mutants (one heterozygous, three homozygous); it was always present in four wild-type mice from different strains (C3H/El, C57BL/6, T-stock, JF-1). The heterozygous mutant tested clearly showed both alleles. Therefore, we conclude that this point mutation in the *Crygd* gene is causative for the cataractous phenotype; the new allele symbol is suggested as $Crygd^{Aey4}$.

Predicted biochemical alterations. The deduced amino acid sequence demonstrates a replacement of Val at pos. 76 by Asp. At this position, Val is highly conserved and found in all mouse and rat γD/E/F-crystallins as well as in the human γA- and γD-crystallin. It may be substituted solely by Ile, which is present in all bovine γ-crystallins, in the rat and mouse γA/B/C-crystallins, as well as in the human γB/C-crystallins. This position belongs to the structurally conserved region within the second Greek Key motif and is usually inaccessible for solvents (Zarina et al. 1994).

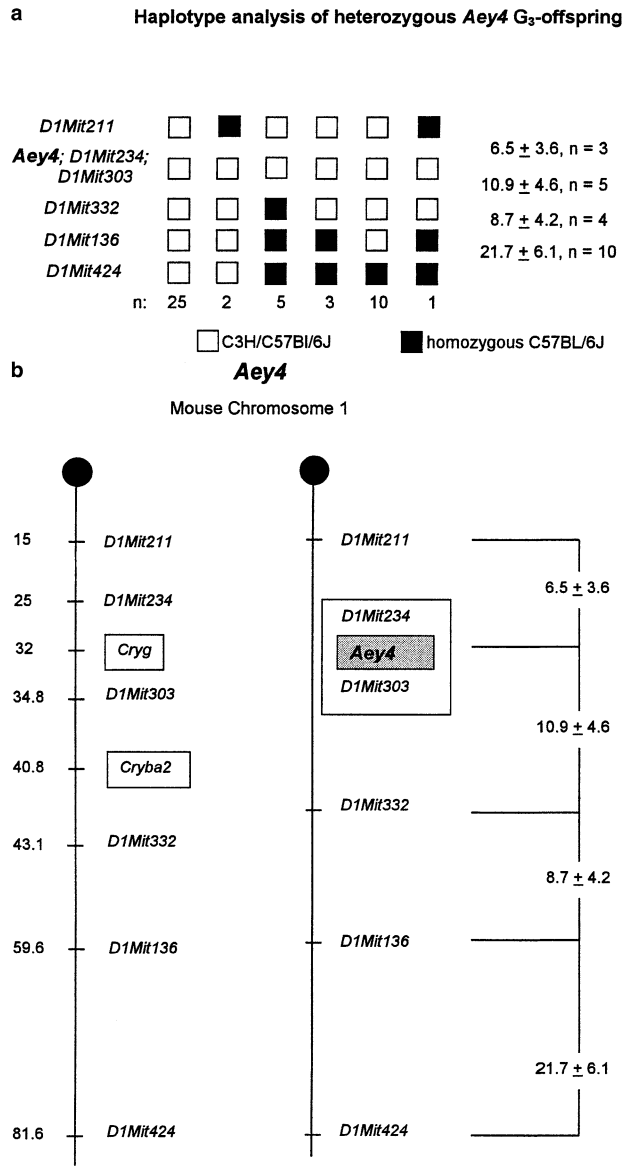

Fig. 2. Genetic analysis of the *Aey4* mutant. **a)** Haplotype analysis. Forty six heterozygous *Aey4* mutants from G3 of an (*Aey4* × C57BL/6J) backcross were genotyped with respect to the markers *D1Mit211*, *D1Mit234*, *D1Mit303*, *D1Mit332*, *D1Mit136*, and *D1Mit424*. The analysis demonstrated that *Aey4* is located between the markers *D1Mit211* and *D1Mit332*. One of the 46 G3 offspring showed a double recombination between the most distant markers *D1Mit211 and D1Mit424*. **b)** Partial map of mouse Chr 1. The partial map of mouse Chr 1 shows the location of the *Aey4* mutation in relation to the markers tested and to the candidate genes *Cryba2* and the *Crygcluster* (right). On the left side, the sequence of markers and genes is depicted as outlined by the 2000 Chromosome 1 Committee Report, representing a composite map derived by combining data from various sources. Numbers to the left of the chromosome indicate the genetic distance, in cM, from the centromere.

However, analyzing the folding properties of the affected region, GOR IV (Garnier et al. 1996), suggests alterations in the random coiled structure for the mutant protein. In addition, the Val→Asp changes a hydrophobic side chain to a polar and acidic one. This might influence the microenvironment of the corresponding area, as indicated by a dramatic

decrease of the isoelectric point from pH 7.96 in the wild-type decapeptide SDSVRSCRLI to pH 5.68 in the mutant peptide SDSDRSCRLI (changed position underlined; http://www.expasy.ch).

Further mutations affecting Cryg *genes*. The *Crygd*^{Aey4} mutation is the second mutation in the mouse *Crygd* gene, but several further mutations have been reported to affect other genes in the *Cryg* gene cluster: the *Crygе*^{elo} (Cartier et al. 1992) and the *Crygd*^{LopT2} (Smith et al., 2000) have been reported by other groups; we have characterized the *Cryga*^{1Neu}, *Crygb*^{nop}, *Crygc*^{Chl3}; *Crygе*^{t} the *Crygе*^{ns}, the *Crygе*^{nz}, and the *crygе*^{Aey1} (Graw 1999; Graw et al., 1999, 2001a, 2002; Klopp et al. 1998, 2001). Currently, in humans an increasing number of mutations in the *CRYG* genes has also been shown to be associated with cataract formation (Héon et al. 1999; Stephan et al. 1999; Kmoch et al. 2000; Ren et al. 2000). Just recently, Santhiya et al. (2002) reported three novel mutations in *CRYGC* and *CRYGD* genes; interestingly, one mutation is identical to that observed in the *Crygd*^{LopT2} mouse mutant.

Conclusions. This iterative finding of cataracts associated with mutations affecting a *Cryg* gene makes this gene cluster a very interesting locus. As a common feature so far, they all appear to lead to structural alterations of the proteins. In contrast, the evolutionary knockout of two of the six γ-crystallin-encoding genes as pseudogenes in human (ψ*CRYGE*, ψ*CRYGF*) suggests that the loss of function of only a few γ-crystallins might be without pathological consequences. Additionally, there are also several reports both for mouse and human for polymorphic sites within the *Cryg* genes. They do not lead to obvious effects concerning the functions of the proteins; however, they might be due to age-related cataract formation. Since such polymorphic sites have not been observed in the close *Cryba2* gene, it might reflect specific evolutionary properties of the *Cryg* genes.

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