Ethynitrosourea-Induced Mutation in Mice Leads to the Expression of a Novel Protein in the Eye and to Dominant Cataracts

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ABSTRACT

A novel ENU-induced mutation in the mouse leading to a nuclear and zonular opacity of the eye lens (Aey1) was mapped to chromosome 1 between the markers D1Mit303 and D1Mit332. On the basis of the chromosomal position, the γ-crystallin encoding gene cluster (Cryg) and the B2-crystallin encoding gene Cryba2 were tested as candidate genes. An A → T mutation destroys the start codon of the Cryg gene in the mutants; this mutation was confirmed by the absence of a restriction site for NolI in the corresponding genomic fragment of homozygous mutants. The next in-frame start codon is 129 bp downstream; this predicted truncated γ-crystallin consists of 131 amino acids, resulting in a molecular mass of 14 kD. However, another open reading frame was observed just 19 bp downstream of the regular Cryg start codon, resulting in a protein of 119 amino acids and a calculated molecular weight of 13 kD. Western blot analysis using polyclonal antibodies against γ-crystallins or the novel Aey1-specific protein demonstrated the specific expression of the Aey1 protein in the cataractous lenses only; the truncated form of the γ-crystallin could not be detected. Therefore, it is concluded that the novel protein destroys the sensitive cellular structure of the eye lens.

The β- and γ-crystallins were first characterized by Mörner (1893) more than 100 years ago. Nowadays, they are recognized as members of one β/γ-crystallin superfamily. The corresponding genes are expressed preferentially in the eye and mainly in the ocular lens; low expression can be found also in the retina (Head et al. 1995; Jones et al. 1999), brain, and testes (Mugabo et al. 2000). The common characteristic of all β- and γ-crystallins is the so-called Greek key motif, which allows a dense packing of proteins in the ocular lens. The Cryg genes in all mammals consist of 3 exons: the first one codes only for three amino acids, and the subsequent two are responsible for two Greek key motifs each. Biochemically, the γ-crystallins are characterized as monomers with a molecular mass of 21 kD (for reviews see Wistow and Piatigorsky 1988; Graw 1997; Slingsby and Clout 1999).

Six members of the Cryg family (Cryga → Crygf) are located in a cluster on mouse chromosome 1 or human chromosome 2q33–35, whereas the seventh Cryg gene (Crygs) maps on mouse chromosome 16 and human chromosome 3, respectively. The Cryba2 gene encoding the B2-crystallin is located ~8 cM distal to the mouse Cryg gene cluster; in human, the relative map positions of the CRYG gene cluster and the CRYBA2 gene are similar, with the CRYBA2 located at chromosome 2q34–36 (Hulsebos et al. 1995).

In mice, several mutations in the Cryg genes have been identified and demonstrated to lead to cataracts: the mutation ethynitrosourea (ENU)-436 affects the Cryga gene, the Nop mutation affects the Crygb gene (Klopp et al. 1998), and Lop12 affects the Crygd gene (Smith et al. 2000). Two cataract-causing alleles of Cryge have been reported so far in the mouse: Ebo (Cartier et al. 1992) and Cat2 (Klopp et al. 1998). Several hereditary cata-racts in man have also been shown to be caused by mutations in CRYG genes (Héon et al. 1999; Stephan et al. 1999; Knoch et al. 2000; Ren et al. 2000), while the human CRYGE and CRYGF genes are pseudogenes.

While analyzing mice obtained from a large-scale ENU mutagenesis program (Hrabé de Angelis and Balling 1998; Hrabé de Angelis et al. 2000; http://www.gsf.de/ieg/groups/enu-mouse.html), we identified several mutants with dominant cataracts. Here we report the map position and identification of the underlying mutation in Aey1; this mutation maps to mouse chromosome 1 and is a mutation affecting the start codon of the Cryg gene. Interestingly, instead of a (modified) γ-crystallin, a novel protein is formed, which does not exhibit any
Figure 1.—Morphology of cataract formation in the Aey1 mutant. Gross appearance of unfixed lenses from 3-week-old mice under the dissecting microscope. Right, a clear, wild-type lens; lower left, a slightly smaller lens of a heterozygous mutant with a nuclear cataract and a moderate zonular opacity; top left, a strong nuclear opacity from a homozygous Aey1 mutant.

remarkable similarity to another previously described protein.

MATERIALS AND METHODS

Animals: C3Heb/FeJ male mice were treated with ENU (160 mg/kg) at the age of 10 wk according to Ehling et al. (1985). Treated mice were mated with untreated female C3Heb/FeJ mice and kept under specific pathogen-free conditions at the GSF Research Center according to the German law on the protection of animals. The resulting offspring were monitored for cataract phenotypes within the ENU mouse mutagenesis screen project (Hrabe de Angelis and Balling 1998; Hrabe de Angelis et al. 2000).

Cataracts were identified at weaning using a slit lamp (SLM30; Zeiss, Oberkochen, Germany). Homozygous mutant lines were then established and have been maintained by brother × sister matings.

The mutation was mapped by mating heterozygous carriers (first generation) to wild-type C57BL/6J mice; offspring (second generation) with cataracts were backcrossed to wild-type C57BL/6J mice. DNA was prepared from tail tips of 42 cataractous offspring of the third generation (G3) according to standard procedures. For the genome-wide linkage analysis, several markers were used for each chromosome (Graw et al. 1999). All data concerning the linkage of genes or markers are taken from the Mouse Genome Informatics database (http://www.informatics.jax.org).

Morphological analysis: For gross documentation, lenses were enucleated under a dissecting microscope (Leica MZ APO) and photographed. For detailed histological analysis, eye globes were fixed for 3 hr in Carnoy’s solution and embedded in JB-4 plastic medium (Polysciences, Eppelheim, Germany) according to the manufacturer’s procedure. Sectioning was performed with an Ultratom OMU3 (Reichert, Walldorf, Germany). Serial 2-μm transverse sections were cut with a dry glass knife, collected in water drops on glass slides, and, after drying, the sections were stained with Methylene blue and basic Fuchsin. The sections were evaluated using a light microscope (Axioplan; Zeiss). Images were acquired by means of a scanning camera (Progress 3008; Jenoptik, Jena, Germany) equipped with a screen-capture program (KS100; Carl Zeiss Vision, Hallbergmoos, Germany) and were imported into an image processing program (Photoshop V5.0; Adobe, Unterschleißheim, Germany). All wild-type controls were of the strain C3H/El.

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 for mouse Cryg genes using genomic DNA or cDNA as tem-
Figure 3.—Haplotype analysis of the Aey1 mutant. Forty-two heterozygous Aey1 mutants from G3 of an (Aey1 × C57BL/6J) backcross have been genotyped with respect to the markers D1Mit211, D1Mit216, D1Mit303, and D1Mit332. The analysis demonstrated that Aey1 is located between the markers D1Mit303 and D1Mit332. Seven of the 42 G3 offspring showed double recombinations; 5 of them were observed between the most distant markers D1Mit211 and D1Mit216.

PCR for Cryba2 used the primer pair 5'-AGCGAACAC CAGGGTGTGAGC-3' (for the left side) and 5'-GAGCTTTTAT TGAAATCTTCTGTGATGAC-3' (for the right side). Using an annealing temperature of 55°C, a 690-bp fragment was amplified.

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 using kits from QIAGEN (Hilden, Germany) or Bio-Rad (Munich, Germany), and subsequent precipitation by ethanol and glycogen.

Biochemical analysis of the lens extracts: Computer-assisted prediction of the biochemical properties of the mutated protein were performed using the Proteomics tools of the ExPASy Molecular Biology server (http://www.expasy.ch). Western blot analysis was performed according to standard procedures using a γ-crystallin antibody was a gift from Dr. Roy Quinlan (Dundee, Scotland, United Kingdom). To detect the novel protein, a specific antibody was made commercially against the peptide STSERTTEAKWWRSQ corresponding to amino acids 86–100 shown in Figure 5 (Sequence Laboratories, Göttingen, Germany).

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

Phenotype and lens morphology: The Aey1 mutant was initially identified as a nuclear and zonular cataract by slit lamp analysis of 3-week-old mice. This phenotype is also demonstrated by a gross morphological analysis of enucleated lenses. The lenses of homozygous mutants are smaller than those of heterozygotes and the nuclear opacity is more severe. Associated with the cataract formation is a slight microphthalmia (Figure 1). Histological analysis of the cataractous Aey1 lenses in juvenile homozygous mutants demonstrates the presence of pycnotic cell nuclei in deep cortical lens areas (Figure 2).

The pycnotic nature of these nuclei is characterized by a polar distribution of chromatin. Moreover, it should be noted that the cell nuclei in the more peripheral region have been degraded as usual. Therefore, there may be a developmental difference in fiber cell maturation: fiber cells produced in early stages of development may not lose their nuclei completely, while those produced at later stages may develop normally. This temporal difference in fiber cell nuclei degradation might be related to the temporal expression pattern of Cryge expression. Since the cataract is stable from the onset of observation (3 weeks of age), this observation supports the idea that only early fiber cells are formed abnormally. The lens bow and the anterior lens epithelium as well as other ocular tissues besides the lens, in particular the cornea and the retina, are formed regularly.

Mapping: Heterozygous carriers from the second generation were mated to wild-type C57BL/6J mice; among 419 offspring of the third generation were 219 cataractous mice and 200 wild types, indicating a dominant mode of inheritance with complete penetrance of the Aey1 mutation. The first 42 cataractous mice were used for the genome-wide mapping. The result indicates linkage to chromosome 1 of the mouse; the detailed haplo-
type analysis for this chromosome and the four markers used is given in Figure 3. It turned out that Aey1 maps between the markers D1Mit303 and D1Mit332.

The mapping of Aey1 between the markers D1Mit303 and D1Mit332 makes the Cryba2 gene an interesting candidate gene; however, since this cluster is only 8 cM apart from Cryba2 (Figure 4). Because of the small number of G3 animals genotyped and the resulting large standard deviation, a mutation in the Cry cluster could not be ruled out.

Genomic analysis: At first, the Cryba2 gene was tested as a candidate for the Aey1 phenotype. However, sequencing of PCR products derived from cDNA from the lenses of homozygous cataractous mice revealed no sequence alterations as compared to the database (accession no. AJ272228). Moreover, there was no apparent alteration in the amount of amplified Cryba2 cDNA, making a promoter mutation unlikely. Thus, the Cryba2 gene was eliminated as a candidate for Aey1.

Therefore, all six Cry genes were amplified specifically by PCR using genomic DNA or lens cDNA as a template and sequenced. Several polymorphic sites were observed in the Cryga and Crygd genes, which were not associated with the cataractous phenotype. The only difference between wild-type C3H and mutant Aey1 cDNA, which could be correlated to the cataractous phenotype, was identified in the Cryge gene (acc. no. NM_007777) as a transversion of an A at position 1 of the cDNA to a T (Figure 5). The mutation in Cryge was confirmed by sequencing exon 1 from genomic DNA.
of homozygous mutants. It destroys a restriction site for Nol, which was demonstrated in five homozygous mutants. This site is still present in five wild-type mice from different strains (Figure 6). Therefore, we conclude that this point mutation in the Cryge gene is responsible for the cataractous phenotype; the new allele symbol is suggested as Cryge<sup>Aey1</sup>.

**Biochemical analysis:** The deduced amino acid sequence of Cryge suggests that the Aey1 phenotype is caused by the destruction of the start codon of the Cryge gene. Since a second in-frame ATG is present 129 bp downstream, it is possible that a truncated γE-crystallin of 14 kD is expressed. To investigate whether this N-terminally truncated γE-crystallin is present in the eye lens, a polyclonal antibody against γ-crystallins was used for Western blot analysis of the water-soluble lens proteins. However, no immunoreactivity for γ-crystallin was apparent at 14 kD in either wild-type or Aey1 homozygous lenses (data not shown). Thus, it appears unlikely that a truncated γE-crystallin is present and responsible for cataract formation.

However, sequence analysis suggested that another long open reading frame (ORF) starts only 19 bp downstream of the destroyed ATG. The phase of this ORF is shifted by +1 as compared to the Cryge ORF and a protein consisting of 119 amino acids with a calculated molecular weight of 13 kD was predicted. Analysis for hydrophobicity using the Kyte-Doolittle algorithms (Kyte and Doolittle 1982) revealed two hydrophobic regions (Figure 7), which might be interpreted as membrane spanning segments. The TMpred program—and similarly also the TopPred2 program—strongly suggest the model of N terminus inside and the first transmembrane domain (amino acids 20–41) from inside to outside; correspondingly, the second transmembrane region (amino acids 43–67) runs from outside to inside. In the first putative transmembrane domain and in the regions between amino acids 8–12 and 91–98, α-helical regions are suggested. All other regions are suggested to be randomly coiled (65%) or extended β-strands (21%; GOR4, Garnier et al. 1996). As outlined by the PROSCAN program, additional putative biochemical features of the novel protein are three N-myristoylation sites and three phosphorylation sites (one for Casein kinase II and two for protein kinase C).

Using a specific antibody against the most hydrophilic region (−1.5 to −2.0 according to the hydrophilicity plot; Figure 7), we could demonstrate that this particular protein and a somewhat smaller degradation product are present only in the cataractous lenses but not in the lenses of wild-type mice (Figure 8). It might be proposed that the novel protein destroys the highly organized cellular structure of the lens fiber cells.

**DISCUSSION**

In this article, we describe the molecular characterization of an ENU-induced mouse cataract mutation, Aey1. The ENU-induced point mutation changes the start codon of the Cryge gene. Since this mutation segregates with the phenotype, this strongly suggests that the mutation in the Cryge gene is responsible for the cataractous phenotype. Since the 2000 Chromosome Committee Report places the Cry gene cluster proximal to the marker D1Mit303, this position of the Cry gene cluster should be changed on the basis of our mapping data to between the markers D1Mit303 and D1Mit332.

The novel Cryge allele Cryge<sup>Aey1</sup> leads to a nuclear and zonular cataract. At the histological level it is obvious that only the lens nucleus is affected by the presence of pycnotic fiber cell nuclei and the cortical regions of the lens remain intact. Comparing the histological observations at this stage, Cryge<sup>Aey1</sup> is very similar phenotypically to the Cryge<sup>gbop</sup> mutants described previously. Particularly, the nuclear fibers do not denucleate in either Cryge<sup>gbop</sup> or Cryge<sup>gbop</sup> (Graw et al. 1984, 1990b).

Cryge<sup>Aey1</sup> is the seventh mouse mutation reported to affect a gene of the Cry gene cluster and to lead to cataracts; these include the Cryge<sup>gbop</sup> (Cartier et al. 1992), the Cryge<sup>gbop</sup> (Smith et al. 2000), as well as the Cryge<sup>gbop</sup>, Cryge<sup>gbn</sup>, and the Cryge<sup>gb</sup> genes (Klopp et al. 1998). The deletion in the Cryge<sup>gb</sup> mutant is ≥2 kb and is not yet
Figure 7.—Hydrophobicity of the predicted Aey1 protein was calculated using the ProtScan program (http://expasy.cbr.nrc.ca). Positive scores indicate increasing hydrophobicity; negative scores indicate increasing hydrophilic character. The most hydrophilic region lies between the amino acid residues 86–100, which were used for the antibody production.

characterized in detail (Graw 1999). The novel Crygbr mutation, however, causes a less severe phenotype than observed in other Cryg alleles like Cryg′ or the Crygs. Also, the recently described cataract mutation Lop12, which was characterized as a mutation in Crygd (Smith et al. 2000), had a more severe phenotype and was similar to that of the Cryg′ (Graw et al. 1990a). All these Cryg mutants are characterized by an amino acid exchange at an important region of the corresponding γ-crystallin or contain a truncated form of the γ-crystallin with or without a few new amino acids. The Crygbr mutation is the only one that leads to the formation of an entirely novel protein in the eye lens.

Corresponding to the increasing number of characterized cataract mutants in mice, mutations in human CRYG genes have been shown to be associated with cataract formation: the Coppock-like cataract (Heon et al. 1999) and the variable zonular pulverulent cataract (Ren et al. 2000) are associated with the CRYGC gene, whereas the aculeiform cataract (Heon et al. 1999), a punctate cataract (Stephan et al. 1999), and a crystal-deposition cataract (Kmoch et al. 2000) are associated with mutations in the CRYGD gene. Finally, a polymorphic congenital cataract was mapped very close to the CRYGB gene (Rogaev et al. 1996).

A common feature of all these mutations is changes in the amino acid sequence, which are considered to lead to altered folding properties. None of the altered proteins can behave like the intact γ-crystallin with the four Greek key motifs. The cataract mutation, which is reported here, is the only one that leads to the formation of a completely novel protein in the eye lens, because of the destruction of the regular start codon and the use of another ORF immediately downstream. The comparison of the deduced Aey1 amino acid sequence to the SwissProt database revealed as the best match just
45% identity to the chick octamer-binding transcription factor 1 (Petryniak et al. 1990) within a short stretch between amino acids 44 and 76. The same region of the Aey1 protein (between amino acids 37 and 77) shows just 39% identity to the Drosophila POU-domain protein dPOU-19 (termed POU for its presence in the Pit-1, Oct-1/Oct-2, and Unc-86 genes) (Dick et al. 1991); these weak homologies are not considered to allow speculations on functional similarities. Moreover, this short region of similarity is outside the conserved sequences of the POU-specific domain and the more C-terminal POU homeodomain, but is part of one of the predicted transmembrane domains. Further biochemical characterization of the new protein will be necessary to prove such similarities in detail.

The numerous mutations affecting Crya genes that cause cataracts makes this cluster very interesting. On one hand, this high number of pathological phenotypes supports the importance of these genes for lens transparency. On the other hand, the evolutionary “knockout” of two of these six genes in man (ψCRYGE and ψCRYGF) demonstrated that the loss of at least a few of them seems to be without any biological relevance. Additionally, there are several reports for polymorphic sites within these genes in both mouse and human. At these sites, sequences are allowed to be changed without any effect on the function of the proteins to keep the lens transparent (Klopp et al. 1998; Héon et al. 1999; Smith et al. 2000). On the basis of these studies and additional pathological and nonpathological alleles of the Crya genes, we will be able to characterize the domains of the corresponding γ-crystallins that are important for their biochemical function.

In summary, we demonstrated here that an ENU-induced mutation in the start codon of the Cryga gene leads to the use of an alternative start codon in another ORF and to the subsequent translation of a novel, but pathological, protein in the eye lens. To our knowledge, it is the first report of such consequences of a mutation in cataracts. The Aey1 mutant was kindly provided by Ingenium Pharmaceuticals AG (Martinsried, Germany). The expert technical assistance of Erika Bürkle, Gerlinde Berger, Andreas Mayer, Nicole Hirsch, Sabine Mandl, Jelina Prettin, Dagmar Reindl, and Monika Stadler is gratefully acknowledged. The authors also thank Dr. Melinda K. Duncan (University of Delaware, Newark, Delaware) for critical comments on the manuscript. Oligonucleotides were obtained from Utz Linzner (GSF-AG BIODV). Part of this work was supported by a grant from the German Human Genome Project (DHGP) to R.B., E.W., and M.H.d.A. (01KW9610/1).

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