Ethynitrosourea-Induced Base Pair Substitution Affects Splicing of the Mouse γE-Crystallin Encoding Gene Leading to the Expression of a Hybrid Protein and to a Cataract

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ABSTRACT

A novel ENU-induced mutation in the mouse leading to a nuclear and cortical opacity of the eye lens (ENU418) was mapped to proximal chromosome 1 by a genome-wide mapping approach. It suggests that the cluster of γ-crystallin encoding genes (Cryg) and the βA2-crystallin encoding gene Cryba2 are excellent candidate genes. An A → G exchange in the middle of intron 1 of the Cryg gene was found as the only alteration cosegregating with the cataractous phenotype. The mutation was confirmed by the presence of a novel restriction site for Apol in the corresponding genomic DNA fragment. The mutation represses splicing of intron 1; the additional 92 bp in the corresponding cDNA leads to a frameshift and the expression of a novel hybrid protein containing 3 amino acids of the γE-crystallin at the N terminus, but 153 novel amino acids. The CrygENU418 protein has a calculated molecular mass of ~15.6 kD and an alkaline isoelectric point (pH 10.1) and is predicted to have two hydrophobic domains. Western blot analysis using a polyclonal antibody against the hydrophilic C-terminal part of the Cryg1993-specific protein demonstrated its stable expression in the cataractous lenses; it was not found in the wild types. Histological analysis of the cataractous lenses indicated that the expression of the new protein disrupts the cellular structure of the eye lens.

The β- and γ-crystallins were first characterized by Mörner (1893) more than 100 years ago. They are recognized now 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 also be found in the retina (Head et al. 1995; Jones et al. 1999), brain, and testes (Magabo et al. 2000; Graw et al. 2001c). The common characteristic of all β- and γ-crystallins is the Greek-key motif, which allows a dense packing of proteins in the ocular lens. The Cryg genes in all mammals consist of three exons: the first one codes 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 Piaticgorsky 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 or human chromosome 3. The Cryba2 gene encoding the βA2-crystallin is located ~8 cM distal to the mouse Cryg gene cluster; in humans, 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 ENU-436 affects the Cryga gene, the Nop mutation the Crygb gene (Klopp et al. 1998), the Chl3 mutation the Crygc gene (Graw et al. 2002b), and Lop12 (Smith et al. 2000) and Aey4 (Graw et al. 2002a) the Crygd gene. Five cataract-causing alleles of Cryge have been reported so far in the mouse: Elo (Cartier et al. 1992), Cat2 (Klopp et al. 1998), Cat2a (Graw 1999), Cat2b (Klopp et al. 2001), and Aey1 (Graw et al. 2001d). Also a mutation in the mouse Cryg gene was demonstrated recently to be causative for a dominant cataract (Sinha et al. 2001). Several hereditary cataracts in humans 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; Santhiya et al. 2002).

While analyzing mice obtained from a large-scale ethynitrosourea (ENU) mouse mutagenesis program (Ehling et al. 1985; Favor and Neuhäuser-Klaus 2000) we identified several mutants with dominant cataracts. Here we report the map position and the identification of the underlying mutation and its biochemical and histological consequences in the mutant ENU418.

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The mutant was recovered after paternal treatment with 250 mg ENU/kg body weight and previously characterized by a variable phenotype ranging from corneal and anterior polar opacity with corneal lens attachment to total lens opacity. The mutation has full penetrance and no effect on viability even in the homozygotes (Favor 1983, 1984).

**MATERIALS AND METHODS**

**Animals:** (102/Elx3C3H/El)F1 male mice were treated with ENU (250 mg/kg) at the age of 10–12 weeks. Treated mice were mated with untreated female T-stock mice (Favor 1983). Offspring were ophthalmologically examined for eye abnormalities at weaning using a slit lamp (SLM90, Zeiss, Oberkochen, Germany). Presumed mutations were genetically confirmed and further outcrossed to either strain 102/El or (102/Elx3C3H/El)F1 hybrid mice (Favor 1984). Homozygous mutant lines were established and have been maintained by brother × sister matings. An allelism test was performed on the Cat2 group of mutants using the Cat2<sup>+</sup> mutant allele as reference (Kratochvilova and Favor 1992). Before initiating the mapping experiments, the mutation ENU418 was crossed 32 generations to (102/Elx3C3H/El)F1 hybrids. A homozgyous line was established and kept for 12 generations by brother × sister matings. For fine mapping on chromosome 1, the microsatellite markers D1Mit156 and D1Mit181 were used in an outcross/backcross to C57BL/6 mice. Genomic DNA was prepared from liver according to standard procedures. All data concerning the linkage of genes or markers are taken from the MGI database (http://www.informatics.jax.org). Mice were kept under specific pathogen-free conditions at the GSF according to the German law on the protection of animals.

**Morphological analysis:** For gross documentation, lenses were enucleated under a dissecting microscope (MZ APO, Leica, Bensheim, Germany) and photographed. For detailed histological analysis, eye globes were fixed for 24 hr in Carnoy’s solution, dehydrated, and embedded in JB-4 plastic medium (Polysciences, Eppelheim, Germany) according to the manufacturer’s procedure. Sectioning was performed with an ultramicrotome (Ultratom OUM3; Reichert, Waldorf, 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. Alternatively, eye globes were fixed in 10% buffered formalin, embedded in paraffin, and sectioned with a Jung RM 205 microtome (Leica). Slides were stained with hematoxylin/eosin or propidium iodide. 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 imported into an image processing program (Photoshop V6.0, Adobe, Unterschleißheim, Germany). All wild-type controls were of the strain C3H/El.

**Isolation of RNA, DNA, and PCR conditions:** RNA was isolated from lenses (stored at −80°C) of newborn mice according to standard procedures. cDNA synthesis and PCR for mouse Cryga or Cryba2 genes using genomic DNA or cDNA as template were performed as described previously (Klopp et al. 1998; Graw et al. 2001d).

PCR products were sequenced commercially (SequiServe, Vaterstetten, Germany) 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 glycerogen.

**Biochemical analysis of the lens extracts:** Computer-assisted prediction of the biochemical properties of the mutated protein were performed using the Protocols tools of the ExPASy Molecular Biology server (http://www.expasy.ch). In particular, we used Kyte-Doolittle algorithms for hydrophobicity (Kyte and Doolittle 1982), the TMpred and TopPred2 programs to detect trans-membrane domains, GOR4 (Garnier et al. 1996) for secondary structures, and the PROSCAN program for additional biochemical features. Western blot analysis was performed according to standard procedures (Klopp et al. 1998). To detect the novel protein, a specific antibody was made commercially against the peptide RSTSERRTEAKWR corresponding to amino acids (aa) 123–136 shown in Figure 5 (Sequence Laboratories, Göttlingen, 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:** By slit lamp analysis of 28- to 42-day-old mice the ENU418 mutant was initially identified as a variable ocular phenotype ranging from corneal and anterior polar opacity with corneal lens attachment to total lens opacity (Favor 1983). After several generations of crossing to (102/Elx3C3H/El)F1 hybrids, the major phenotype is a nuclear and cortical opacity of the ocular lens as demonstrated by a gross morphological analysis of enucleated lenses. The anterior suture was often affected. In comparison to heterozygotes, the lenses of homozygous mutants are only slightly smaller and the nuclear opacity is more severe (Figure 1).

To understand the process of cataract formation in this particular mutant, histological analysis with different staining techniques was performed on eyes from animals at different ages. In lenses of newborn wild-type mice (Figure 2a), the lens fiber cells are ordered regularly, and their cell nuclei are present only in the cortical region. In the cortical region of the mutant lenses (Figure 2b), the cell nuclei of the lens cortex are also visible and they are arranged in a wave-like manner as in the wild types. In contrast, in the core of the mutant lenses, the structure of the fiber cells is completely disorganized; clefts, vacuoles, and swollen cells are visible. At the transition zone the cell nuclei appear to be swollen. At the transition zone, individual larger spots can be recognized. It indicates that DNA persists in the cataractous region, which is usually completely degraded from the agarose gel using kits from QIAGEN (Hilden, Germany) or Bio-Rad (Munich, Germany). Mice were kept under specific pathogen-free conditions.
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excluding a distance between Cat2\textsuperscript{\textprime\prime} and ENU18 > 2.6 cM (95% confidence interval). The fine mapping revealed no recombination with the marker D1Mit156 (position 32.8 cM from the centromere) and just one recombination with the marker D1Mit181 (position 42.0 from the centromere) among 104 backcross offspring tested. These different linkage tests suggest that the critical region for the ENU18 mutation is < 1 cM between the markers D1Mit156 and D1Mit181, including the Cryg gene cluster. Assuming 1 cM to correspond to 750 kb and an average gene density of 12–15 genes per Mio base pair, we can estimate that the ENU18 critical region might contain 9–12 genes. The chromosomal location and the lens phenotype suggest that the Cryg gene cluster (position 32.0 cM from the centromere) and the closely linked Cryba2 gene (position 40.8 cM from the centromere) are excellent candidate genes.

**Mutation analysis:** At first, the Cryba2 gene was tested as a candidate for the ENU18 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 ENU18.

Therefore, all six Cryg genes from the chromosome 1 gene cluster were amplified specifically by PCR using genomic DNA or lens cDNA as a template and sequenced. Several polymorphic sites were observed in the Cryga, Crygd, and Crygf genes, which were not associated with the cataractous phenotype. No sequence differences were observed for the Crygb and Crygc genes.

**Biochemical analysis:** The CrygeENU18 phenotype is caused by a mutation in intron 1 leading to a less efficient splicing of this intron. Therefore, two Cryg mRNA products are present: a correctly spliced mRNA and an abnormal mRNA containing intron 1. The deduced amino acid sequence of the abnormal CrygeENU18 cDNA suggests that the corresponding protein will consist of the three N-terminal γ-crystallin amino acids followed by 153 amino acids, where the degradation of the cell nuclei takes place in the wild type. Other ocular tissues besides the lens are not affected.

Histological analysis of the cataractous ENU18 lenses in 8-day-old homozygous mutants (Figure 3, a and b) demonstrated the same major phenotype of the central nuclear cataract as in the lenses of newborn mice. Additionally, alterations in the anterior suture become visible, indicating a reduced attachment of the fiber cells. The nuclei of the lens fiber cells remain visible in the lens central region. The phenotype is more severe at 3 weeks of age, including both the central cataractous region and clefts at the anterior suture (Figure 3, c and d). It is obvious from the analysis of serial sections that the extension of the suture defects is smaller than that of the nuclear cataractous region.

**Mapping:** An allelism test with the Cat2\textsuperscript{\textprime\prime} mutant revealed no wild-type mice among 115 offspring tested,

![Figure 1](#)

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**Figure 1.—Morphology of cataract in the ENU18 mutant.** Gross appearance of unfixed lenses from 3-week-old mice. (A) The wild-type lens is clear. (B) On the left, a lens of a heterozygous mutant with a nuclear cataract and a moderate cortical opacity is demonstrated; on the right, a strong nuclear opacity from a homozygous mutant. (C) As in B, but a lateral view of the homozygous lens (right). The nuclear opacity extends toward the anterior polar region, and the cortical opacity is evident.
Inhibition of degradation of cell nuclei in cataractous lenses of newborn mice. Sections from 1-day-old wild-type (a and c) and homozygous ENU418 mice (b and d) are shown. The sections were stained with either hematoxylin/eosin (a and b) or propidium iodide (c and d). The sections were made through the center of the lens as indicated by the presence of the optic nerve. The bars indicate magnification. In the wild-type lenses (a and c), the cell nuclei are present in a wave-like orientation in the lens cortex, but absent in the center of the lens. In the homozygous mutant lenses (b and d), they are present in the cortical area as in wild type. The cataractous region in the anterior core of the lens corresponds to the zone where degradation of the cell nuclei usually takes place. Instead of a complete degradation of the nuclei in this area, the cataractous lenses demonstrate the presence of chromatin as indicated by the diffuse propidium iodide staining (d). C, cornea; LB, lens bow; LE, lens epithelium; R, retina.

Acids completely different in their sequence from γE-crystallin (Figure 4b). The calculated molecular weight is 15.6 kD and its isoelectric point at pH 10.1. A search in the nonredundant SwissProt database using the entire 156-aa protein did not reveal any significant similarity to other proteins.

Analysis for hydrophobicity revealed two hydrophobic regions (amino acids 57–78 and 80–104), which might be interpreted as membrane spanning segments. In a part of the first hydrophobic domain and in the regions between amino acids 38–49 and 118–125, α-helical regions are suggested (in total, 17% of the protein). All other regions are predicted to be randomly coiled (66%) or extended β-strands (17%; GOR4). As outlined by the PROSCAN program, additional putative biochemical features of the novel protein are three N-myristoylation sites and four phosphorylation sites (one for casein kinase II and three for protein kinase C).

Using a polyclonal antibody against the most hydrophilic region (amino acids 123–136), we could demonstrate that this particular protein is present in the water-soluble extract from cataractous lenses at the expected size (and a somewhat smaller form in a lesser amount) but not in the lenses of wild-type mice (Figure 6). In addition, the antibody recognizes a 20-kD protein in both the wild-type and mutant lens extracts. However, since the corresponding preimmune serum of this particular antibody did not show any reaction against either the wild-type or the mutant lens extract, it is concluded that the antibody cross-reacts with other (unknown)
Figure 3.—Histology of juvenile lenses. Sections through lenses of 8-day- (a and b) and 3-week-old (c and d) homozygous ENU418 mice are shown and compared to an age-matched control (3 weeks old; e and f); staining was done with methylene blue and basic fuchsin. The bars indicate magnification. The mutants showed dense material in the lens core as well as swollen fiber cells in the central cortex and the anterior suture. In the outer cortex, the fiber cells seem well organized. The major observation is the presence of pycnotic cell nuclei in the anterior part of the central cortex at both age stages investigated (b and d). The other ocular tissues are without defects. The age-matched control did not show abnormalities (e and f). C, cornea; LB, lens bow; LE, lens epithelium; R, retina.
proteins in both the wild-type and the cataractous lenses. Further experiments might be necessary to identify the corresponding protein(s) and to elaborate whether the antibody recognizes identical proteins in the wild type and in the mutant.

**DISCUSSION**

In this article, we describe the molecular characterization of an ENU-induced mouse cataract mutation, *ENU418*, due to a base pair substitution in intron 1 changing its splicing characteristics. Restriction analysis demonstrated that the mutation segregates with the phenotype; therefore, it is strongly suggested that the mutation in the *Cryg* gene is responsible for the cataractous phenotype.

The novel *Cryg* allele, *Cryg<sup>ENU418</sup>*, leads to a nuclear and cortical cataract. At the histological level it is obvious that the lens nucleus and the inner cortex are affected by the presence of pycnotic fiber cell nuclei. Comparing the histological observations at early postnatal stages, *Cryg<sup>ENU418</sup>* is phenotypically very similar to other cataract mutations in a variety of genes like *Gja8* (encoding connexin50; Graw et al. 2001b), *Cryaa* (encoding αA-crystallin; Graw et al. 2001a), or other *Cryg* genes (Graw et al. 1990, 2001d; Klopp et al. 2001). Besides *Cryg<sup>ENU418</sup>* several other mouse mutations have been reported to affect the *Cryg* gene cluster and proteins in both the wild-type and the cataractous lenses.

**Figure 4.**—Sequence analysis of the *ENU418* mutant. (a) Alignment of genomic DNA. (b) Alignment of deduced amino acid sequences. Two types of cDNA from homozygous *ENU418* mutant lenses are compared to the genomic *Cryg* DNA sequence from two wild-type strains of mice [(102/ElxC3H/El)F<sub>1</sub>; X57-855 and T-stock] in exon 1, intron 1, and exon 2 (a). For evolutionary considerations, the corresponding rat sequence (GenBank accession no. X00271) also is given. *ENU418* variant 1 contains intron 1, whereas the second form corresponds to the correctly spliced cDNA. The exons are given in green. The branchpoint consensus sequence is boxed; the conserved part is shaded in yellow, the G/C polymorphism between mouse and rat at its last position in yellow-green, and the transition of A to G at the lariat-forming position is shown in red; the new *Apa*I restriction site is underlined. The difference in the intronic repeat region between mice and rats is highlighted in blue as well as one additional new polymorphic site in the T-stock sequence (exon 2). All other polymorphic sites between rat and mice were reported earlier (Graw et al. 1991). The γE-crystallin amino acid sequence is demonstrated in b. The small peptide used for specific antibody production is underlined and shown in boldface type. The polymorphic site in exon 2 is marked in blue.
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**Figure 5.** —ApaI digest of the 5’-end of the Cryge gene. (a) Restriction map of the 664-bp PCR fragment from Cryge, exon 1, intron 1, and exon 2. In wild types, one restriction site for ApaI exists, and endonuclease digestion yields two fragments of 171 and 483 bp. The additional ApaI site in the ENU418 mutants results in three instead of two fragments. (b) The PCR fragment was analyzed by agarose electrophoresis with (+) or without (−) digestion by ApaI. The genomic DNA from all wild types (derived from the strains C3H/El, C57BL/6J, T-stock, JF1, DBA/2, and BALB/c) can be digested into two fragments, but the DNA from six homozygous CrygeENU418 mutants revealed three fragments as expected from the restriction map.

to lead to cataracts: these include the Cryge "(Cartier et al. 1992), the Cryge " (Klopp et al. 2001), the Cryge " (Graw et al. 2001d), the Cryge " (Graw et al. 2002b), the Crygd " (Smith et al. 2000), and the Crygd " (Graw et al. 2002a), as well as the Cryga " and the Cryge " genes (Klopp et al. 1998). The deletion in the Cryge " mutant is >2 kb and not yet characterized in detail (Graw 1999). Most of these Cryg mutants are characterized by an amino acid exchange at an important region of the corresponding γ-crystallin or they express a truncated form of the γ-crystallin with or without a few new amino acids. The CrygeENU418 is the first mutation, which leads to a splicing variation within a Cryg gene.

The A → G mutation in intron 1 is 25 bp upstream of the 3’-end of the intron, where the consensus sequence that is suggested to be responsible for U2snRNA binding and lariat formation usually occurs. Indeed, the mutation destroys the adenosine residue exactly at the branch point. The surrounding sequence in (102/ElxC3H/El)F1 hybrid and T-stock mice (the genotypes in the mating that produced the founder mutant) matches in 6 out of 7 bp to the corresponding consensus sequence [YNCTGAC; Y, pyrimidine (T/C); N, any base; Reed and Maniatis 1988]. Obviously, the change of the C in the wild-type rat (perfectly matching the consensus sequence) to G in the wild-type mouse has no effect on the splicing efficiency. An additional polymorphism was observed in comparison to the rat. A 5-bp repeat element (GCCCTT) is present three times in this intron between the branch point and the 3’-splice site in both strains of mice, but only twice in the rat.

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 (Héon et al. 1999) and the variable zonular pulverulent cataract (Ren et al. 2000) with the CRYGC gene; the aculeiform cataract (Héon et al. 1999), a punctate cataract (Ste-

**Figure 6.** —Western blot of lens extracts. Water-soluble lens proteins from wild-type C3H/El or homozygous CrygeENU418 lenses were separated by polyacrylamide gel electrophoresis and analyzed for the presence or absence of the novel CrygeENU418-specific protein using a corresponding antibody. The arrows point to two specific bands with the appropriate sizes of 16 and 18 kD, respectively; these bands are visible only in extracts from mutant lenses. The upper band (*) is present in both wild-type and mutant lens extracts and indicates an interaction of the antibody with an as-yet-unidentified protein. (Left) Coomassie staining of the lens extracts. (Right) Western blot of lens proteins employing the CrygeENU418-specific antibody. C3H, C3H/El; ENU, CrygeENU418.
LITERATURE CITED


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