Mutation in a Novel Connexin-like Gene (Gjf1) in the Mouse Affects Early Lens Development and Causes a Variable Small-Eye Phenotype

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PURPOSE. The purpose of the study was the characterization of the novel small-eye mutant Aey12 in the mouse.

METHODS. The eyes of the mutants were described morphologically and histologically and by in situ hybridization.

RESULTS. The homozygotes were viable and fully fertile, which identifies Aey12 as a new microphthalmia phenotype in the mouse, different from Maf or Pax6 mutants. Histologic analysis indicated the presence of the lens vesicle, however, the primary fiber cells did not elongate properly. Genome-wide linkage analysis mapped the mutation to the proximal region of chromosome 10 between the markers D10Mit206 and D10Mit189. Among the positional candidate genes, one EST (expressed sequence tag), D230044M03Rik, encodes a connexin-like protein. A G→T point mutation was identified at cDNA position 96, resulting in an R32Q amino acid exchange in a transmembrane domain. The mutation leads to a loss of an Sfi restriction site, which is present in five wild-type mouse strains (102, C3H, C57BL/6, DBA, and JF1). The gene is expressed in the posterior part of the lens vesicle, where the primary fiber elongation starts. In the mutants, the expression pattern of Pax6, Prox1, Six3, and Cryg1 are modified, but not the pattern of Pax6.

CONCLUSIONS. The mutated mouse gene belongs to the family of connexin-encoding genes (gene symbols Gja-Gje). Together with its rat and human homologues, it defines a new subgroup, referred to as Gjf1. The mouse mutant described herein offers a new functional candidate gene for microphthalmia-related disorders at the corresponding locus on human chromosome 6, area q24. (Invest Ophtalmol Vis Sci. 2008;49:1525–1532) DOI:10.1167/iovs.07-1035

Microphthalmia in humans and small eye in the mouse are frequent, but genetically diverse phenotypes caused by severe defects in early eye development (for a recent review, see Graw5). The major players resulting in small-eye phenotypes in the mouse are Mlf (encoding the microphthalmia-associated transcription factor2–5) or Pax6 (paired-box gene 66–8). In addition to these major players characterized by a high number of affected alleles, the Pitx3, Maf, Sox2, and Fox genes have been shown to lead to similar phenotypes; however, only a few alleles of these genes have been characterized up to now (for a recent review, see Graw5).

In humans, severe microphthalmia frequently shows some evidence of familial recurrence but usually no clear Mendelian transmission pattern.10 Nevertheless, the molecular basis of several congenital diseases, including microphthalmia, have been identified. The causative mutations affect Pax2, Pax6, Rax, Chx10, Maf, Shh, or Sox2 (for a recent overview, see Ragge et al.11).

We present a novel dominant mouse model for microphthalmia, which was identified in a currently running ENU (N-ethyl-N-nitrosourea) screen with C3H mice.12 In contrast to mice that carry mutations in most of the genes just mentioned, homozygous Aey12 mutants are viable and fully fertile. At the genetic level, the mutation is characterized by a point mutation in a novel gene that codes for a connexin-like protein. The phenotype is characterized by an arrest of the outgrowth of the primary lens fibers. This new mouse model, Aey12, points to the outgrowth of the primary lens fiber cells as a crucial step in lens and eye development. The phenotype is different from other mutants that carry connexin-encoding genes.

MATERIAL AND METHODS

Mice

Mice were kept in specific pathogen-free conditions at the National Research Center for Environment and Health (GSF) and monitored within the ENU mouse mutagenesis project.12,13 The use of animals was in accordance with the German Law on Animal Protection and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male C3HeB/FeJ mice were treated with ENU (3 ± 100 mg/kg body weight) at the age of 10 weeks according to Ehling et al.14 and mated to untreated female C3HeB/FeJ mice. The offspring of the ENU-treated mice were screened at the age of 3 weeks for abnormalities of the eye (Aey). Mice with phenotypic deviation were tested for a dominant mode of inheritance.

Phenotypic Characterization

The eyes of the mutants were examined by slit lamp at the age of 3 weeks. Histologic analysis of the eyes was performed during embryogenesis only. The embryos or their heads were fixed for 3 hours in

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Carnoy’s solution and embedded in JB-4 plastic medium (Polysciences Inc., Eppelheim, Germany), according to the manufacturer’s protocol. Sectioning was performed with an ultramicrotome (OMU3; Reichert-Jung, Walldorf, Germany). Serial transverse 3-μm sections were cut with a glass knife and stained with methylene blue and basic fuchsin.

**In Situ Hybridization**

In situ hybridization of sections from embryonic day (E)12.5 was performed, essentially as described by Grimm et al. Briefly, the embryos were fixed in paraformaldehyde and embedded (Jung Histowax; Cambridge Instruments, Nussloch, Germany). Sections (7-10 μm) were cut with a microtome (model RM-2065; Leica, Nussloch, Germany) and mounted on slides. The sections were evaluated with a light microscope (Axioplan; Carl Zeiss Meditec, Oberkochen, Germany). The images were acquired by means of a scanning camera (Progress 3008; Jenoptik, Jena, Germany) and imported into an image-processing program (Photoshop ver. 6.0, Adobe Illustrator. ver. 9.0; Adobe Systems, Unterschleissheim, Germany).

**Mapping**

Heterozygous carriers (first generation) were mated to wild-type C57BL/6j mice, and the offspring (second generation) were backcrossed to wild-type C57BL/6j mice. DNA was prepared from tail tips of affected offspring of the third generation (G3) according to standard procedures. DNA was adjusted to a concentration of 50 ng/μl. For a genome-wide linkage analysis of the Aey12 mutation, several microsatellite markers were used for each autosome (Graw et al. 15).

**PCR and Sequencing**

For the molecular analysis, RNA was isolated at E12.5 from the heads of the Aey12−/− or wild-type embryos (C3HeB/FeJ). The RNA samples were reverse transcribed to cDNA (Ready-To-Go First-Strand Kit; GE Healthcare, Freiburg, Germany), and genomic DNA was isolated from the tail tips or spleens of wild-type C3HeB/FeJ and C57BL/6j mice or homozygous mutants, according to standard procedures. For RT-PCR amplification, primers were selected from the EMBL-GenBank databases (smart.embl-heidelberg.de) provided in the public domain by the European Molecular Biology Laboratory, Heidelberg, Germany; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

Total RNA was prepared from the analyzed tissues (peqGOLD RNAPure extraction kit; Peqlab, Erlangen, Germany) in accordance with the protocols supplied by the manufacturer. Total RNA (5 μg) was transcribed to the first strand of cDNA (Superscript II RT kit; Invitrogen, Karlsruhe, Germany). In particular, RNA from the retina was isolated by placing the eye globe into ice-cold PBS. After the cornea was dissected and the lens removed, the retina was pulled out and immediately frozen on dry ice. RNA was isolated (RNA-Bee Kit; AMS Biotechnology, Abingdon, UK) and reverse transcribed (Ready-To-Go T-Primed First-Strand Kit; GE Healthcare, Freiburg, Germany).

PCR cycling after the RT step was performed with the primers D230044M03Rik-1 (5’-GCCAGGATAACACCTCCTGA-3’ and D230044M03Rik-2 (5’-TGACTGATAGAACCTATGGTATGG-3’), which were designed to flank both intron regions of D230044M03Rik to assure specific amplification of the cDNA. To verify that cDNA synthesis had been successful, we amplified a fragment of the housekeeping gene gapdh by use of the forward gapdh primer 5’-TGAAGTCCGGTGTGAACGGA-3’ and the reverse primer 5’-GATGGCATGGACGTGTTGCA-3’). The products were analyzed by electrophoresis on a 1.5% agarose gel.

PCR was performed with a thermocycler (BD-Clontech, Heidelberg, Germany, or ABL, Weiterstadt, Germany). PCR products were analyzed on a 1% agarose gel. Sequencing was performed commercially (SequiServe, Vaterstetten, Germany), after direct purification of the PCR products (Nucleospin Extract II; Machery-Nagel, Düren, Germany), after isolation of the DNA from the gel with an extraction kit (Qiagreen; Qiagen, Hilden, Germany), or after cloning into the pCR-2.1 cloning vector (Invitrogen).

**Phylogenetic Analysis**

Degrees of similarities among the connexin proteins of Homo sapiens, Mus musculus, and Rattus norvegicus were calculated by an alignment program (DiAlign; Genomatix, Munich, Germany) based on the unweighted pair–group method with arithmetic mean (UPGMA). The unrooted phylogenetic tree diagram was plotted by the Drawtree software tool (developed by Joe Felsenstein, Departments of Genomic Science and Biology, University of Washington, Seattle, WA; available at www.phylodiversity.net/rree/drawtree).

**General**

Chemicals were from Merck (Darmstadt, Germany) or Sigma-Aldrich Chemicals (Deisenhofen, Germany). The enzymes used for cloning and reverse transcription were from Roche (Mannheim, Germany).

**RESULTS**

**Phenotype**

The Aey12 mutant was detected during an ongoing ENU mutagenesis screening because of its dominant small-eye phenotype; subsequently, it was established as a homozygous line. In addition to the small-eye phenotype, slit lamp analysis identified opacities in the cornea and the lens of variable intensity. Figure 1 demonstrates the reduced size of the lenses at the age of 6 months in hetero- and homozygous mutants compared with the wild-type control. Besides the size difference, the
heterozygotes have a polar lens opacity. In the homozygotes, the lens is empty and consists only of an envelope. It is grown together with several other ocular tissues.

Because the small eyes were obvious at weaning, we investigated eye development in the mutants (homozygous and heterozygous) during embryogenesis (Fig. 2) starting at E10.5. This stage represents the classic placode stage where the lens placode just begins to invaginate, forming the lens vesicle. At this particular stage, no major differences were obvious between the wild-types and the mutants. One day later, the lens vesicle formed and the primary lens fibers started to elongate in the wild-type embryos. Similarly, in the heterozygotes, the posterior part of the lens vesicle seemed to be thicker, but in the homozygotes no preparation for elongation was obvious. At E12.5, the primary lens fibers of the wild-type elongated from the posterior side of the lens vesicle and finally filled the entire lens vesicle. This process did not take place in homozygous mutants; the lens vesicle remained empty. In the heterozygotes, the primary fibers elongated but did not reach the anterior epithelium. In heterozygous mutants, the lens fiber elongation was not complete at E15.5 and E17.5. Similar to the situation in homozygotes, the lens was smaller than in the wild-type. In homozygotes, some further tissue was present in the small lens, and the cornea was thickened (which led later to the frequently observed cornea opacity). The retina was misfolded because of the smaller size of the lens and was poorly differentiated. The optic stalk was present.

For a first screening of potentially affected pathways, at E12.5 we investigated the expression of genes well-known to be essential for early eye development, including Pax6, Pax2, Prox1, Six3, and Crygd. These results are summarized in Figure 3. The spatial expression pattern of Pax6 was restricted to the central area of the central anterior epithelium of the lens vesicle, whereas Pax2 expression in the posterior part of the eye and in the developing optic nerve was not altered. In contrast, transcription factors involved in the regulation of Cryg genes (such as Prox1 and Six3) were more strongly expressed over a larger region. Consequently, Crygd was also strongly expressed, but, because of the missing lens fibers, it was present in the posterior part of the lens vesicle only.
Mapping of the Aey12 Mutation and Candidate Gene Analysis

For linkage analysis, homozygous Aey12 mutants on a C3Heb/Fej background (G1) were crossed to wild-type C57BL/6J mice; heterozygous mutants (G2) were backcrossed to C57BL/6J C3Heb/Fej mice. The analysis of 70 G3 offspring revealed a clear linkage of the small-eye phenotype to chromosome 10, close to the centromere (between the markers D10Mit123 and D10Mit168) spanning more than 8 Mb. This mapping result excluded other genes that lead to a similar phenotype (like Maf or Pparbp), because these genes are located on different chromosomes. Because a further fine mapping did not reveal conclusive results, a straightforward positional candidate gene approach was not possible. Therefore, we made a second linkage analysis and collected, in 230 G3 mice, much more than in the first approach. Four animals (0.02%) did not show any linkage to chromosome 10 and were therefore not considered in further analyses. (These four animals were not homogenous for sex, phenotype, or coat color.)

Based on the 226 G3 mice, the genetic order was calculated (genetic distance ± SD in parenthesis): D10Mit206 (0.9 ± 0.6 cM), Aey12; D10Mit188–D10Mit189 (0.9 ± 0.6 cM). The critical interval can be calculated to be 4.3 Mb, including 17 genes and a pseudogene. Several genes in the critical interval are predicted only because of the existence of ESTs, but are not yet fully annotated. Among the genes in the critical interval, none is known to be responsible for ocular disorders.

Because the marker D10Mit188 did not show any recombination among the 226 mutant offspring, it indicates a very close linkage to the Aey12 mutation. Since the EST D230044M03Rik encoding a connexin-like protein is very close to this marker and because mutations in the genes Gja3 and Gja8 (coding for connexin46 or connexin50, respectively) have been shown frequently to cause cataracts in mouse and human (for a review, see Graw), we considered this EST to be a good candidate to be involved in this particular mutation. Indeed, RT-PCR demonstrated that it is strongly expressed in the embryonic lens, and sequence analysis detected a G→A exchange at position 95 of the cDNA (counting the starting ATG as position 1; Figs. 4a, b). Moreover, it leads to a loss of an Ssil restriction site, which was confirmed in the genomic DNA of all tested homozygous Aey12 mutants (n = 5); all wild-type strains tested (C3H, C57BL/6J, JF1, 102, and DBA/2) kept this restriction site, indicating that it is not a polymorphic site (Fig. 4c).

**Figure 3.** In situ hybridization of genes known to be involved in eye development. In situ hybridizations were performed at E12.5 with probes specific for Pax2, Pax6, Prox1, Six3, and Crygd. It is obvious that the expression pattern of Pax6 was not altered, but the pattern of Pax2 was restricted to very central areas. In contrast, the expression areas of Prox1 and Six3 were broader than in the wild-type, and Crygd was present in the posterior part of the empty lens vesicle. C, cornea; L, lens; OS, optic stalk; R, retina.

**Figure 4.** Sequence analysis and restriction digests. (a, b) Sequence analysis detected a G→A mutation in homozygous Aey12 mutants. (c) Restriction digest in five different wild-type strains and five homozygous Aey12 mutants. M, marker; −, without digestion; +, digested with Ssil; RS, PCR product after cleavage by Ssil.
The 95G→A exchange is predicted to cause a alteration of Arg to Gln at amino acid position 32. According to secondary structure prediction programs, it is suggested that this amino acid exchange is located within the first transmembrane domain of the new connexin-like gene. (This new protein has four transmembrane domains like other connexins.) Comparison of the sequence surrounding the Aey12 mutation with other connexins (data not shown) indicated that the exchanged Arg residue at position 32 is highly conserved among the family of connexins and is present in all members described so far.

Phenotypic Heterogeneity of Aey12 Mutants in G3 Offspring

Surprisingly, the phenotype of the G3 offspring in both sets of experiments was quite heterogeneous. In the first mapping approach, 41 mutants were characterized by small eyes with cataracts, 25 had small eyes with cornea opacities, and 4 had small eyes with small lenses. In the second mapping approach 4 years later, the phenotypic heterogeneity was similar. To exclude the possibility that other genes on the C3H background are responsible for the variation of the phenotype, we tried to establish independent lines from the heterogenous G3 offspring. However, this approach failed, and subsequent breeding of these heterogenous phenotypes resulted again (G4) in a rather homogenous phenotype, mainly with cornea opacities. Therefore, a genetic modifier may act on the C57BL/6 background and interfere somehow with the connexin-like gene, most likely with the regulation of its spatial and temporal expression pattern. Additional experiments are currently undertaken to characterize this phenotypic heterogeneity in more detail.

Expression Pattern of D230044M03Rik

To confirm that the new connexin-like gene is expressed in the developing eye, we performed in situ hybridization analysis during early embryonic stages. As demonstrated in Figure 5a, **D230044M03Rik** was expressed at E11.5 in the posterior lens vesicle in a very restricted central area. It fits the region exactly, where the primary fiber cells start. Therefore, it can be concluded from this study, that the mutation in **D230044M03Rik** is very likely to be causative of the phenotype observed in the **Aey12** mutants.

Moreover, the expression pattern of **D230044M03Rik** suggests that it is involved in the transition of the cells during their terminal differentiation process. In the elongating primary fiber cells, it is expressed at the anterior tip of the cells, but not at the posterior end. When the differentiation of the secondary fibers begins, it is present in the lens epithelial cells and in the elongating epithelial cells at the lens equator (but no longer in the primary lens fiber cells). Since the identity of the lens cells in the **Aey12** mutant is different, it is not surprising that **D230044M03Rik** is expressed mainly in the cells surrounding the empty lens vesicle. However, in addition to the lens, it is also present in the developing retina—at weak intensities in the wild-type, but strongly expressed in the homozygous mutants. Since the retina in the homozygous mutants is not well differentiated and is also heavily folded, it is tempting to speculate whether **D230044M03Rik** in the tip of the retina interferes with the proper differentiation of the retina. However, further analysis demonstrated that **D230044M03Rik** mRNA cannot be detected in the retinas of 6-week-old wild-type mice. RT-PCR with cDNA from other organs of these mice demonstrated the specific expression of **D230044M03Rik** in the lens; no expression was found in brain, lung, heart, liver, or kidney (Fig. 5b).

**DISCUSSION**

We have described a novel dominant mouse mutant, **Aey12**, that is characterized by small eyes, homozygous viability, full penetrance, and fertility. The phenotypic features include cornea opacities and small lenses with cataracts and vacuoles. Analysis of eye development during embryogene-
sis showed a characteristic feature of the Aey12 mutant to be retarded lens fiber elongation in addition to partly disrupted fiber–epithelium connections (heterozygotes) and the arrest of eye development at the lens vesicle stage (homozygotes). Irregular cell-to-cell appositions between epithelial cells and fibers (however, in a weaker form) have been described previously in lenses of mice lacking the activity of the gap junction protein connexin 43. Concerning the major observations for Aey12/−/− (the empty lens vesicle in the homozygous mutants), two similar phenotypes have been reported previously. First of all, in the Maf knockout18 and in the Maf mutant mouse Of1,19 the primary lens fiber cells do not grow out, and therefore the lens vesicle remains empty. The second phenotype similar to our Aey12 mutant is caused by the knockout of the Pparbp gene (coding for the peroxisome proliferator activator receptor [PPAR] binding protein20). However, linkage analysis of the Aey12 mutation to the proximal part of mouse chromosome 10 excludes these genes as candidates, since Gja1 (connexin43) is located on a significantly different region of mouse chromosome 10, whereas Maf and Pparbp are located on mouse chromosomes 8 and 11, respectively. Moreover, another dominant cataract mutation, Cat5, has been mapped to the same region (5 cM distal to the centromere).21 This mutant is not yet characterized.

Fine mapping placed the Aey12 mutation within a 4.3-Mb interval containing 17 coding genes (some of them are not yet fully characterized in the database). Even if none of the genes in the candidate region has been reported to be involved in eye development or in (congenital) ocular disorders, we demonstrated in the current study that the EST D230044M03Rik is expressed in the developing lens and a mutation affecting the first (predicted) transmembrane domain of the connexin-like protein is very likely to be causative of the mutation. The phenotypic heterogeneity in the heterozygous Aey12 mutants on the C57BL/6 background could suggest an additional modifier gene that may be involved in the spatial and temporal expression of D230044M03Rik.

The EST D230044M03Rik was identified as a member of the connexin-encoding genes a few years ago (referred to as PC17005) and was characterized to be expressed in the eye22; however, no functional data have been reported up to now. The gene was found within a representative transcript and protein set (RTPS; ftp://fantom2.gsc.riken.go.jp/RTPS/); the encoded connexin was designated as Cx23. Because of its difference from the other connexin-encoding genes, it was grouped into the δ group of divergent connexins and therefore referred to as Gjd5.23-24 However, to characterize the relationship of the EST D230044M03Rik to the other connexin-encoding genes, we compared the entire family of the mouse genes to those from rat and human, where also all the sequences are available. The result (Fig. 6) demonstrates clearly that D230044M03Rik is significantly different from the members of the Gjd gene group. The EST D230044M04Rik forms together with the also unknown gene LOC684664 (rat) and ENSG000000203733 (human) a group of its own within the connexin-encoding family. Based on this relationship, we propose Gjf1 as the corresponding new gene symbol (gap junction protein f1; Gjf1 is the next one in the alphabetical series: the mouse Gje1 gene encodes Cx2925; see Appendix).

As for the other connexins, the same general feature of four transmembrane domains is predicted for the new member Gjf1. In addition, the cytosolic N- and C-terminal parts are also present. In contrast to many other connexins, the C-terminal region is very short, leading to a calculated molecular mass of 23.8 kDa and an isoelectric point at pH 8.7. Moreover, the protein has only two, rather than three, highly conserved cysteine residues in each of the presumed extracellular loops,25 which also explains the relative large evolutionary difference from the other GJ genes.

The new connexin-encoding gene Gjf1 is expressed in the posterior part of the lens vesicle, later in the anterior part of the primary lens fibers, and finally in the lens epithelium and the lens equator, suggesting that it has an important function during the process of lens fiber cell differentiation. It is not expressed in the cornea; therefore, the observed cornea opacities have to be considered to be secondary effects. In the mutant lenses, Gjf1 expression in the early embryonic stages remains restricted to the posterior area of the lens vesicle, but is strongly expressed in the anterior part of the developing retina at later stages as well. However, RT-PCR data in 6-week-old wild-type mice demonstrated clearly that the expression in the retina is only transient (Fig. 5). This finding points to a crucial role of Gjf1...
during early development and differentiation of the retina, but shows that it is not needed at later stages.

In accordance with the consequences of the loss of the Gf1 function in early lens development, important lens-related transcription factors are also changed in their expression patterns. While Pax6 is restricted to a more central area, Prox1 (in the lateral parts of the lens vesicle) and Six3 (in the anterior part of the lens vesicle and in the prospective retina) are markedly enhanced; Crygd (as a marker for lens fiber cells) is expressed in the very posterior part of the lens vesicle. This aspect is of particular surprise, since Crygd expression in the wild-type lens at this age is found at the anterior part of the primary lens fibers—the same position in which Gf1 is expressed in the wild-type.

Besides Gja3 and Gja8, the newly identified Gf1 gene is the third connexin-encoding gene that affects early eye and lens development. For Gja3 and Gja8, several knockout and knockin mutations have been described in the mouse. These investigations led to the concept that Gja3 is important for the functional integrity of the lens (its knockout leads to a dense nuclear cataract in a lens of regular size), whereas Gja8 is responsible for the size of the lens (Gja8+/− are smaller, but have only a mild nuclear cataract; for recent reviews, see Gong et al.26 or Gerido and White75). In addition, in mice, two-point mutations in Gja8 have been described to be involved in congenital cataracts26,29; there is no further mutation known in the mouse Gja3 gene. In contrast, several point mutations in both genes are reported in humans (for a review, see Graw3). To this list Gf1 must now be added, and it would be interesting to see the clinical phenotype of mutations in the corresponding human gene. Up to now, there is no entry in the OMM database for an uncharacterized eye disorder at the homologous position on human chromosome 6, area q24.

**CONCLUSION**

We identified a new mouse mutant that has arrested early lens development. Molecular analysis characterized a causative mutation in a novel gene, which is now referred to as Gf1. The corresponding protein obviously has an important role in the initiation of various steps of lens fiber cell differentiation, but also in the early development and differentiation of the anterior retina.

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**APPENDIX**

Because of a change in the connexin nomenclature system by the connexin nomenclature committee, the D230044M03Rik gene has recently been referred to as Gje1. The new nomenclature was based on that suggested by Sohl and Willecke23 and the phylogenetic tree from Cruciani and Michalens.50 As is obvious from the comparison of the old and the new system (see Supplementary Table S1 online at http://www.iovs.org/cgi/content/full/49/4/1525/DC1), two genes have the same symbol in the old and new system, however, with different meanings (Gje1old is Cx30.2 and Gje1new is Cx45; Gje1old is Cx29 and Gje1new is Cx23). To avoid confusion, at least for Gje1, we still prefer Gf1 as the appropriate gene symbol for D230044M03Rik (Cx23).

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