Characterization of a Mutation in the Lens-Specific CP49 in the 129 Strain of Mouse

Azita Alizadeh,1 John Clark,2 Teri Seeberger,2 John Hess,1 Tom Blankenship,1 and Paul G. FitzGerald1

PURPOSE. The 129 strain of mouse carries a mutation in the gene for CP49 (phakinin), an intermediate filament protein thus far demonstrated only in the lens fiber cell. As such, these mice represent naturally occurring mutants of interest in the study of the lens cytoskeleton. However, this strain of mouse is also widely used as a source of embryonic stem cells in gene-targeting studies. The presence of a mutation in a lens-specific gene can confound interpretation of studies in which lens genes have been knocked out. In the present study, both the genotype and phenotype of these mice were characterized, to permit an evaluation of the biological impact of this mutation and to facilitate the discrimination between wild-type and mutant animals that have been derived from this strain in gene-targeting studies.

METHODS. The CP49 cDNA and, when relevant, the genomic DNA sequences were determined for the 129/SvJ and C57BL/6J mice and from a commercially available 129/OLA P1 genomic clone. PCR primers were screened for their capacity to discriminate between the mutant and wild-type CP49 genes. Northern blot analysis was used to assess mRNA levels for CP49, filensin, and y8-crystallin (control). Western blot analysis was used to identify changes in protein size and abundance. The impact of the mutation on lens architecture was evaluated at the light-microscope level. Lens fiber cell ghosts from mutant and wild-type mice were examined in the electron microscope for the presence of beaded filaments. Lens clarity was assessed by slit lamp.

RESULTS. The 129 strain of mice exhibited a 6303-bp deletion from the end of intron B, and the beginning of exon 2. This deletion results in the loss of the exon 2 splice acceptor site, absence of exon 2 from the CP49 mRNA, and dramatically reduced levels of CP49 mRNA. The CP49 protein was undetectable by Western blot analysis. Messenger RNA levels for filensin, CP49’s assembly partner, were normal, but protein levels were sharply reduced. Light microscopy established that the initial differentiation and elongation of the fiber cells proceeded normally. Electron microscopy showed the absence of beaded filaments, whereas slit lamp microscopy showed a slowly emerging and progressive loss of optical clarity.

CONCLUSIONS. The 129/SvJ and 129/OLA strains of mice harbor a mutation that sharply reduces CP49 mRNA levels and essentially eliminates both CP49 and the beaded filament. These lenses exhibited a slow but progressive loss of optical clarity with age. Thus, the 129 strain of mouse behaves as a functional CP49 knockout. The loss of clarity in the lenses of these animals and the absence of beaded filaments (and any attendant interactions that may exist between beaded filaments and other lens proteins/structures) suggest that gene-targeting studies of lens proteins in which the 129 strain was used as a source of embryonic stem cells may need reevaluation.

Two members of the intermediate filament (IF) family of proteins, CP49 and filensin, have been demonstrated only in the differentiated lens fiber cell.1–6 These two proteins are localized to the beaded filament, a structure identified almost 30 years ago.7 Evidence has been presented that suggests that these proteins, and by inference the beaded filament, are essential for the maintenance of optical clarity: Point mutations in the human CP49 have been implicated as the cause of inherited autosomal dominant cataract in two separate families. Afflicted individuals are born with clear lenses, but suffer a loss of clarity some years after birth.8,9; and targeted genomic deletion of the CP49 and filensin in mouse results in animals that are born with clear lenses but exhibit a loss of clarity with age.10–12 Thus, the beaded filament is not essential to clarity, but is necessary to maintain it. The mechanism(s) by which the absence of CP49 produces the loss of clarity is undetermined and may in fact be different in the two reported models.

One approach to addressing the question of beaded filament function and, indeed, many lens protein functions has been to create loss-of-function models. Targeted deletion of the gene of interest, followed by comparison of the mutant and wild-type animals, is a valuable method for highlighting functions associated with that gene product. However, this approach is predicated on the assumption that, for practical purposes, the difference between the wild-type and knockout animals is limited to the absence of the targeted gene product. Quinlan reported at the 2000 ICER meeting that the 129 strain of mouse used commonly as the source of embryonic stem (ES) cells in the preparation of knockout animals carries a mutation in CP49, an observation we also noted in preparation of CP49 knockouts.10 Thus, the 129 strain of mouse may offer an interesting naturally occurring mutant with the potential to yield insight into beaded filament biology. It may also, however, offer a confounding factor in the interpretation of other lens gene targeting studies. For these reasons we have characterized the genetic mutation carried by the 129 strain and assessed its impact on gene expression, protein levels, filament assembly, lens structure, and optical clarity. In addition, we describe PCR primer sets that permit discrimination between the wild-type and mutant CP49 alleles so that existing knockouts can be characterized as wild-type or mutant with respect to the CP49 gene.
**Materials and Methods**

**Animals and Clones**

129/SvJ and C57BL/6j mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animal use was conducted in adherence with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The 129/Ola P1 clone containing CP49 gene sequence was obtained from Genome Systems.

**PCR Primers**

RNA was isolated from decapsulated 129/SvJ and C57BL/6j mouse lenses and reverse transcribed using random hexamers or oligo-dT, to generate first-strand cDNA. Several PCR primer sets were developed to discriminate between wild-type and mutant CP49 alleles. PCR primer sets and primer locations are presented in Figure 1. Genomic DNA was isolated from mouse tail clips or from the P1 clone. PCR conditions used for each pair of primers was the same: 25 μL PCR reaction were prepared with final concentration of 1X PCR buffer, 2.5 mM MgCl2, 0.5 μM of each primer, 0.1 mM dNTP mix, 0.625 U Taq DNA polymerase (Invitrogen, Carlsbad, CA), and 2 μL genomic DNA containing 100 to 200 ng total DNA. PCR was conducted in a Peltier-effect thermal cycler (PTC-200; MJ Research, Watertown, MA). Initial denaturation was 95°C for 1 minute, then 35 cycles of 3 steps: 95°C for 30 seconds, 68°C for 30 seconds, and 72°C for 30 seconds and then a final step of 72°C for 10 minutes.

**Northern Blot Analysis**

Northern blot analysis was performed as described previously.10 Total RNA was isolated by single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture.13 Total RNA was quantified by spectroscopy. Approximately 10 μg of total RNA was electrophoresed in formaldehyde-agarose gels and transferred to nylon membrane (Imaging Quant; Amersham, San Francisco, CA). The values determined for the imager (Storm Phosphorimager) and accompanying software (ImageQuant; Amersham, San Francisco, CA) according to standard procedures. After UV cross-linking, the blots were probed with 32P-labeled cDNA for CP49 (full length cDNA probe), filensin (partial cDNA probe consisting of 500 bp from the filensin rod domain), or γs (537 bp representing the entire coding region), washed, exposed, and developed according to standard procedures. Each sample was run in triplicate. Signal density was determined using a phosphorescence imager (Storm Phosphorimager) and accompanying software (ImageQuant; Amersham, San Francisco, CA). The values determined for the γs signal in the wild-type and knockout lanes were used to normalize the signals for the filensin and CP49 blots.

**Western Blot Analysis**

Whole lenses were decapsulated and immersed in SDS-PAGE sample buffer containing 2% SDS, 5 mM β-mercaptoethanol and 5% glycerol in 50 mM Tris (pH 8.0). Samples were resolved on 12.5% polyacrylamide gels and either stained with Coomassie blue or transferred electrophoretically to a membrane (Immobilon-P; Millipore, Billerica, MA) for Western blot analysis. After blocking in 5% nonfat milk, 2% powdered milk, and 0.1% Tween 20 in Tris-buffered saline for 20 minutes, samples were probed with antisera raised against recombinant human filensin and recombinant mouse CP49, diluted 1:1000 in blocker. Antiserum to vimentin was generously provided by Roy Quinlan (University of Durham, Durham, UK). Visualization was achieved by second-stage labeling with goat anti-rabbit antibody conjugated to alkaline phosphatase, developed with 5bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium (BCIP/NBT).

**Light Microscopy**

Whole mouse lenses were processed for light microscopy, as described. Briefly, whole globes were removed, opened, and immersed in a formaldehyde-glutaraldehyde fixative. Lenses were then dehydrated through graded alcohols and embedded in glycol methacrylate. One-micrometer sections were stained with toluidine blue.

**Electron Microscopy**

The preparation of lens ghosts has been described. Briefly, whole eyes were submerged in optimal cutting temperature (OCT) compound and immediately frozen on dry ice, then stored at −80°C. Lens sections were generated at −15°C on a cryostat (Leica, Deerfield, IL) in a trim mode at 150 μm. Sections were floated through two changes of PBS-EDTA, with protease inhibitors, then fixed and processed for thin-section electron microscopy.

**Slit Lamp Microscopy**

Nonanesthetized mice were examined with a slit lamp ophthalmoscope (model FS-2; Nikon, Tokyo, Japan), as described elsewhere.10 Mouse eyes were diluted with a 1:1 mixture of 1% tropicamide (Alcon, Fort Worth, TX) and 10% phenylephrine hydrochloride (Akorn, Abita Springs, LA). The angle of the slit lamp was approximately 40°, and the slit width was approximately 0.2 mm. Examinations were recorded by digital video (Canon Optura Pi, Tokyo, Japan). Still images were captured (Premiere; Adobe, San Diego, CA) and processed (Photoshop; Adobe).

Slit lamp images were converted to grayscale and opened in Image J (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). A graph of pixel brightness was plotted along a single horizontal line of pixels through the center of the eye, and values were pasted into a spreadsheet (Excel; Microsoft, Redmond, WA). Values were normalized to the cornea to compare plots between images. The intensity at the cornea was adjusted to 100%, assuming that all images had the same corneal clarity. The minimum value relative to the cornea corresponded to the aqueous chamber and was adjusted to 0%. All other values were adjusted accordingly, using the equation (intensity value − minimum of aqueous chamber)/maximum of cornea) × 100%. These adjusted values were plotted to obtain the graphs in Figure 8.

**Results**

**Gene Structure**

The structure of the relevant part of the wild-type CP49 gene locus is shown in Figure 1a. The 6-kb region of the wild-type gene that is deleted from the 129 strains occurs at the end of intron B and includes the first 8 amino acids of exon 2. This 6-kb region is bolded in Figure 1a, and the mutant locus of the 129/SvJ mouse, lacking this 6-kb region, is shown in Figure 1b. Sequences of the primer sets developed for the purposes of discriminating between wild-type and mutant animals are listed in Figure 1e, and their location is shown schematically in Figure 1a. PCR was also conducted on the P1 clone containing 129/Ola CP49 genomic DNA, which was used for the generation of the CP49 knockout animals.

The genomic sequence flanking the beginning and end of the 6-kb fragment is presented in Figure 2. The remainder of the sequence can be accessed online (http://www.ncbi.nlm.nih.gov, accession number 28546163). Sequence absent from the 129 strain is shown in lowercase letters. Italicized letters are from exon 2. A schematic which compares the wild-type and mutant mRNAs is presented in Figures 1c and 1d, and depicts the absence of the entire exon 2 in the mutant animal.

**Amino Acid Sequence**

To confirm that the 6kb deletion resulted in the absence of exon 2 from 129 mouse mRNA, we sequenced cDNA from the 129 mouse lens. This sequence was then compared with that of the Swiss Webster mouse, as well as CP49 sequence reported for both human and cow. The results are shown in Figure 3. This alignment shows strong conservation of sequence between the CP49s, with the exception of the absence of amino acids 165-192 in the 129 strain. These 28 residues...
represent the entirety of exon 2. PCR amplification of the relevant region of the C57Bl/6j CP49 cDNA, and subsequent sequencing, showed that the C57Bl/6j strain also contains exon 2. Thus, the absence of these 28 residues in the 129 strain is unique among the CP49s sequenced to date, indicating that the 129 sequence is abnormal.

PCR Discrimination of Wild-Type from Mutant CP49 Alleles

PCR primer sets were developed to permit discrimination between the wild-type and mutant loci. Figure 4 shows PCR results using these primer sets. In Figure 4a, lanes 2 to 4,

Figure 1. (a) A map of the wild-type CP49 locus, as determined from the C57Bl/6j mouse. The region that is absent from the 129 strains is in **bold** and encompasses the end of intron B and the beginning of exon 2. PCR primers used in this study are indicated by lowercase letters. Letters indicate downstream primers, whereas letters with a ('') notation indicate upstream primers. Their approximate location and orientation are indicated. (b) Map of the 129/SvJ mutant CP49 gene, lacking the 6kb fragment. (c) Schematic of the primary transcript from the C57Bl/6j mouse and its splicing to a final transcript that includes exon 2. (d) The primary transcript derived from the mutant CP49 allele, showing how the loss of the exon 2 splice site yields a final transcript lacking exon 2. (e) Primer sets used in this study. Each primer site is given a lowercase letter designation. The location of the primer relative to the wild-type gene is shown in (a).

DNA sequence flanking the 6kb deletion. **Uppercase letter:** sequence present in both wild-type and mutant animals. *Italicized uppercase letters:** exon 2 sequence, which is absent from the mRNA of the 129 strains because of alternative splicing caused by the deletion. **Lowercase letters:** sequence deleted from the 129 strains. Lowercase bold letters: intron sequence; lowercase lightface letters: exonic sequence. The complete sequence of the intron is not shown, with the point of interruption indicated by an **ellipsis.** This missing sequence can be found at http://www.ncbi.nlm.nih.gov (accession number 28546163). Primers c and e', which flank the deleted sequence, are underscored.

Figure 2. DNA sequence flanking the 6kb deletion.
primer pair a-a\textsuperscript{H11032} was used as a positive control. This primer set amplifies a region within exon 1 that should be present in both wild-type and mutant animals and that is predicted to amplify an approximately 450-bp fragment. The reaction yields the expected result, amplifying the appropriate-sized product in the C57BL/6J wild-type animal (lane 2), the P1 clone derived from the 129/OLA animal (lane 3), and the 129/SvJ mouse (lane 4). In lanes 5 to 7 primer set c-e\textsuperscript{H11032}, which flanks the entire 6-kb deletion, was used. In the wild-type locus (lane 5) this primer pair should amplify a product in excess of 6 kb, but under these conditions yielded no product. In the DNA derived from the 129 strains, the 6-kb region is missing, and the PCR reaction yielded the predicted 400-bp fragment, confirming the absence of the 6-kb region. In lanes 8 to 10, primer set e-c\textsuperscript{H11032} was used, which should amplify an approximately 450-bp fragment found within the 6-kb deleted region. The wild-type locus yielded the expected product, whereas no product was amplified in the DNA derived from the 129 strains, again confirming the presence of this region in the wild type and its absence in the 129 strains. In Figure 4b, lanes 2 to 4, primer set d-b\textsuperscript{H11032}.

Figure 3. Alignment of CP49 amino acid sequences from cow (BoCP49), human (HuCP49), Swiss Webster mouse (MoSWCP49), and 129/SvJ mouse (Mo129/SVJCP49). The absence of exon 2 from the 129/SvJ mouse CP49 is evident in the absence of 28 amino acids (165-193).
Northern Blot Analysis

To explore whether the 129 mutation affected lens CP49 and filensin mRNA levels, we conducted Northern blot analysis on cDNA generated from lenses of 129/SvJ and C57BL/6J mice. The results are presented in Figure 5, and show a sharp reduction in the levels of CP49 mRNA (top panel), but no significant change in filensin mRNA levels. Why the message level for CP49 is reduced is not clear, but raises the possibility that the deleted region plays a role in transcriptional regulation and/or message stability. As a positive control for RNA loading, these same blots were probed for S-crystallin (not shown) as an additional control. Gel densitometry was conducted on the Northern blot analysis, and normalized against the γS signal to adjust for differences in gel loading. This established that CP49 levels in the 129 strain are reduced to approximately 15% of the wild-type, whereas filensin levels are comparable to the wild-type.

Protein Levels

To explore how or whether the production of low levels of mutant CP49 mRNA affected beaded filament protein levels, we probed both C57BL/6J and 129/SvJ lenses by Western blot analysis (Fig. 6). Figure 6a shows a Coomassie-blue-stained 12.5% polyacrylamide gel that did not reveal any obvious differences in the total protein profiles between the wild-type and mutant lenses. Figure 6b is a Western blot probed with antisera to CP49, and shows that CP49 in the mutant animals is either missing or is present at levels that were not detected under these conditions. Thus, CP49 is at least dramatically reduced in the mutant animal, if not absent entirely. Figure 6c shows a Western blot probed with anti-filensin antisera and establishes that filensin levels are sharply reduced in the mutant animal, mirroring the results obtained in the CP49 knockout animals. The absence of the CP49 causes no major changes in filament protein levels, but sharply reduces protein levels. The many lower-molecular-mass immunoreactive products in the blots of CP49 and filensin have been shown to be naturally occurring breakdown products of CP49 and filensin, which are found in older lens fiber cells. They are reactive with monoclonal antibodies to both proteins and are absent in knockouts of both animals.1,10

Vimentin is a type III IF protein which is also expressed in lens. To determine whether the CP49 impact on filensin was specific to filensin, or more generalizable to lens IF proteins,
we probed a Western blot with antisera to vimentin (Fig. 6d). No apparent differences were seen in the vimentin levels of the wild-type and mutant animals.

**Microscopy**

Light microscopic examination of 129/SvJ and C57BL/6J mice showed no apparent differences in the initial elongation of fiber cells, nor in the initial assumption of long-range order in the differentiating fiber cells (not shown). Thus, the histologic organization of the newly formed fiber cells appears unaltered in the younger (3 week) mutant animals, a finding similar to that reported in the CP49 knockout. Sandilands et al., however, report a loss of order and cell shape in the deeper regions of the older lens (5 month) of the CP49 knockout.

**Electron Microscopy**

Ghosts of C57BL/6J and 129/SvJ lens fiber cells were prepared to explore the impact of the CP49 mutation on the beaded filaments. The ghosting process permits crystallins to diffuse away, thus allowing visualization of the underlying cytoskeleton. Thick sections are initially prepared from blocks containing the ghosts and examined by light microscopy so that regions that center on the mature nucleated fiber cells can be identified for further study. Figure 7 shows images taken from comparable cortical regions of both C57BL/6J and 129/SvJ animals. Beaded filaments were very abundant in the wild-type lens (Fig. 7a), but undetectable in the mutant animal (Fig. 7b), suggesting that they were absent, or at extremely low levels. Residual crystallins cross-link to the plasma membrane during fixation, giving the plasma membrane a granular or beaded appearance. Note that the profiles of the fiber cells in the mutant animal (Fig. 7b) are more regular and geometric than in the wild-type animal (Fig. 7a). These differences are not intrinsic to the genotype and probably result from distortion to the lens slice during tissue processing, because the geometric/nongeometric appearance can be found in both animals.

**Slit Lamp Examination**

Age-matched C57BL/6J and 129x1/SvJ mice were examined by slit lamp microscopy starting at 17 days after birth. Lenses of the C57 mice were mostly clear, although a very small, faint nuclear opacity began to appear at 4 to 5 months. By 1 month, lenses of the 129 mice showed faint opacity in the nucleus, with a clear ring around the nucleus and faint opacity in the cortex of the lens (Fig. 8). Opacification in all regions became more pronounced as the animals aged, although lenses did not progress to a mature cataract. Light scattering was no obvious and was only observed using a slit lamp view of the lens. Some animals exhibited anterior subcapsular regions of opacity in addition to the deep rings of opacity. Lenses of the 129 mice appeared similar to those of CP49-null mice in age of onset, progression, and regions of opacity.

**Discussion**

The 129/SvJ strain of mouse harbors a mutation in the CP49 gene that consists of an approximately 6-kb deletion spanning the end of intron B and the first eight amino acids of exon 2. Because the deletion eliminates the splice site at the 5’ end of exon 2, the entire exon is omitted from the mRNA. Little is known of the intermolecular interactions that occur in the beaded filament, so it is difficult to speculate about the impact of the absence of exon 2.

Sequence analysis of most IF proteins predicts that the initial stage of assembly is the formation of a coiled-coil dimer. Cross-linking data have supported this, but more recently electron paramagnetic resonance studies of spin-labeled vimentin has mapped the specific residue interactions in vimentin dimer formation, leaving little doubt about the coiled-coil nature of the vimentin dimer. Because the general plan of IF proteins structure is conserved, it is reasonable to extrapolate these observations to most IF proteins.

Thus, one might expect that CP49 and filensin similarly form a coiled-coil dimer. If true, then loss of exon 2, which is 28 residues of the central rod domain, would be predicted to be catastrophic to CP49-filensin assembly. However, CP49 and filensin primary sequence are the most divergent of the IF family, exhibiting variations that predict secondary structural divergence from the rest of the IF family. Filensin for example is unique in having a shortened central rod domain. Analysis of CP49 rod domain shows an absence of, or a weaker propensity for, coiled-coil structure in the beginning of the rod domain, and it is in this domain that the 28 residues of exon 2 are found. Reports have also appeared that suggest that the molar ratio of CP49:filensin is 2:1 or 3:1, observations that...
suggest something other than a simple heterodimer as the initial stage of assembly.\textsuperscript{23–25} Finally, CP49 and filensins are localized to beaded filaments and not 10-nm IFs. Thus, although the absence of a part of the central rod domain would most likely be catastrophic to most members of the IF family, the impact on the beaded filament remains unknown.

It is of interest to note that the resultant CP49 mRNA levels were much lower than that of the wild-type control. The reason for the reduced levels is unknown, but it raises the possibility that the 6 kb of intronic DNA contains regulatory sites that contribute to the control of CP49 transcription, and/or that exon 2 contributes to message stability. Though CP49 message is detectable at low levels in the 129/SvJ lenses, CP49 protein was either absent, or at least reduced to the point that protein was not evident by Western blot analysis of whole-lens homogenates. This apparent absence may reflect the sensitivity of the approach, or may suggest that CP49 without exon 2 does not undergo proper folding and is therefore targeted for destruction.

Additional phenotyping included northern and Western blot analysis of filensin, CP49’s assembly partner, as well as slit lamp examination, and both light and electron microscopy. The resultant data mirrored the results that have been reported for the CP49 knockout. Filensin mRNA levels are comparable to that of the wild-type, whereas filensin protein levels are sharply reduced. The initial elongation of the fiber cell appears to proceed normally, and the precision of fiber cell packing appears unperturbed.\textsuperscript{10}

It is important to note that the loss of clarity in the lenses of the 129/SvJ strain and in the CP49 knockouts is very subtle in the younger animals and is not readily demonstrated except by low angle slit lamp examination. Thus, an absence of an obvious cataract by direct examination should not be construed as an absence of the mutation.\textsuperscript{5,6} The cause of the loss of clarity has not been definitively established, but a recent report from Sandilands et al.\textsuperscript{11} suggests that older fiber cells may undergo structural changes that could account for the changes in optical properties. Alternatively, the loss of clarity could be due to the accumulation of the insoluble assembly partner filensin. This possibility is being directly tested by the creation of a CP49-filensin double knockout.

Although we describe herein the results of phenotyping the 129/SvJ mouse, we initially detected the mutation in a P1 clone derived from the 129/OLA strain that we used in the preparation of a targeting vector for the CP49 knockout. Thus, at least two substrains of the 129 animals are affected, both of which are a commonly used source of embryonic stem cells for gene-targeting studies.\textsuperscript{26–32} The presence of a naturally occurring mutation in a lens-specific gene in these strains thus has the potential to confound interpretation of other lens gene-targeting studies. Loss of clarity is one established issue that must be considered. However, the absence of CP49, the near absence of filensin, and the resultant absence of the beaded filament may well have specific molecular effects on any proteins or structures that bind to or are bound by these proteins. Thus, the true ramifications of the CP49 mutation on past lens gene-targeting studies may be difficult to assess fully and may require that the targeted gene be crossdated out to a wild-type background to eliminate the possibility that the CP49 mutation will affect the results. The wild-type/mutant status of the CP49 gene in existing knockouts, as well other mouse strains may be established with the primer sets described herein.

References


