Molecular Vision 3: 5, 1997 <http://www.emory.edu/molvis/v3/steele>
Received 24 Mar 1997 | Accepted 2 May 1997 | Published 7 May 1997

Identification of a mutation in the MP19 gene, *Lim2*, in the cataractous mouse mutant *To3*

Ernest C. Steele, Jr.,[1] Stefan Kerscher,[2] Mary F. Lyon,[2] Peter H. Glenister,[2] Jack Favor,[3] JianHua Wang,[1] and Robert L. Church[1]*

[1]Emory Eye Center, Emory University School of Medicine, Department of Ophthalmology, Atlanta, GA, USA, [2]MRC Mammalian Genetics Unit, Harwell, Didcot, Oxfordshire OX11 ORD, United Kingdom, and [3]Institute of Mammalian Genetics, GSF - National Research Center for the Environment and Health, D-85758 Neuherberg, Germany

Purpose: *Lim2*, the gene encoding the second most abundant lens specific integral membrane protein, MP19, has recently been proposed as an ideal candidate gene for the cataractous mouse mutant, *To3*. The aim of this study was to screen the *Lim2* gene in the *To3* mutant for a genetic lesion that was correlated and consistent with the mutant phenotype.

Methods: Genomic DNA was isolated from both normal mouse parental strains as well as the heterozygous and homozygous To3 cataract mutant. PCR was used to generate overlapping fragments of the entire *Lim2* gene from these DNAs. The coding regions, including splice junctions and the translational termination site, of these fragments were then sequenced.

Results: A single G -> T transversion was identified within the first coding exon of the *Lim2* gene in the *To3* mutant DNA. This DNA change results in the nonconservative substitution of a valine for the normally encoded glycine at amino acid 15 of the MP19 polypeptide.

Conclusions: The identified genetic lesion in the *Lim2* gene of the cataractous mouse mutant, *To3*, confirms *Lim2* as an ideal candidate gene. Future transgenic experiments should provide proof or disproof of a causative relationship between the identified mutation and the cataractous phenotype. These studies indicate that MP19 may play an important role in both normal lens development and cataractogenesis, and warrants more intense investigation of its role within the ocular lens.

MP19 (lens intrinsic **m**embrane **p**rotein, **19** kDa) is the second most abundant integral membrane protein within vertebrate ocular lens fiber cells (1,2). MP19 has been shown to be localized to tight junctional domains of differentiated lens fiber membranes (2-6). The protein has four predicted transmembrane domains and has been demonstrated to be phosphorylated (2, 7-9) and to bind calmodulin *in vitro* (2, 7, 10, 11). These features are characteristic of functional proteins that are regulated or modulated. To date, no clearly defined structural or functional role within the lens has been elucidated for MP19. However, there is now a preponderance of evidence that a genetic lesion in *Lim2* (**l**ens **i**ntrinsic **m**embrane protein **#2**), the gene encoding MP19, is the underlying molecular defect in the cataractous mouse mutant, *To3* (**t**otal **o**pacity of lens **#3**)(18). This new evidence suggests an important role for MP19 in both normal lens development and cataractogenesis.

To3, originally named ENU-288, was produced and characterized in a set of experiments testing the efficacy of ENU (ethylnitrosourea) as a mouse spermatogonial mutagen (12). This mutant was further characterized in extensive outcrossing experiments, and the phenotype was demonstrated to segregate in a simple Mendelian fashion with full penetrance, having no effect on viability or fertility (13). The *To3* mutant phenotype is both hereditary, being expressed in a semi-dominant fashion, and congenital. Mice heterozygous or homozygous for the *To3* mutation have total opacity of the lens with a dense cataract. In addition, the *To3*/*To3* homozygotes exhibit microphthalmia, abnormally small eyes. Histological sections of the eyes of homozygotes show that the lens is vacuolated and the fibers are grossly disorganized (Figure 1). In the central region, fibers lose their nuclei, but are not regularly arranged. Disorganized secondary fibers retaining nuclei are also present (Figure 1b). In homozygotes, but not heterozygotes, the lens capsule ruptures posteriorly with lens material leaking into the vitreous chamber (Figure 1c), and in some cases the lenses were vacuolated. The vitreous chamber was abnormally small, and the eyes appeared shrunken. The development of the abnormality has not yet been studied. Rupture of the lens is also found in various other mouse cataracts, including lens rupture, *lr*, (14), and rupture of lens cataract, *rlc*, (15). Similarly, microphthalmia accompanies cataract in other mouse mutants, including alleles of dominant cataract, *Cat* (16) and of dominant cataract 2, *Cat2* (17).

Recently, the *To3* cataract was mapped to a position approximately 7.3 cM proximal to the coat color marker, *p*, on mouse chromosome 7 and the name *To3* (for Total Opacity #3) was proposed for the genetic locus (18). Using a bovine *Lim2* (MP19) cDNA probe, that possesses greater than 90% nucleotide identity with the mouse sequence, the *Lim2* gene was recently mapped to the same region of mouse chromosome 7 as the *To3* cataract (approximately 8 cM proximal to *p*) (19). Although *To3* and *Lim2* have not been directly mapped against one another, the closeness of their apparent locations on mouse chromosome 7 makes *Lim2* a likely candidate gene in the *To3* mutant. This is further supported by the fact that the only other lens specific genes known to map to this region of mouse

*To whom correspondence should be addressed:
Robert L. Church, Emory Eye Center, Room B5601, 1365B Clifton Rd, NE, Atlanta, GA 30322 (USA), Phone: (404) 778-4101, FAX: (404) 778-2232, e-mail: rlchurc@emory.edu

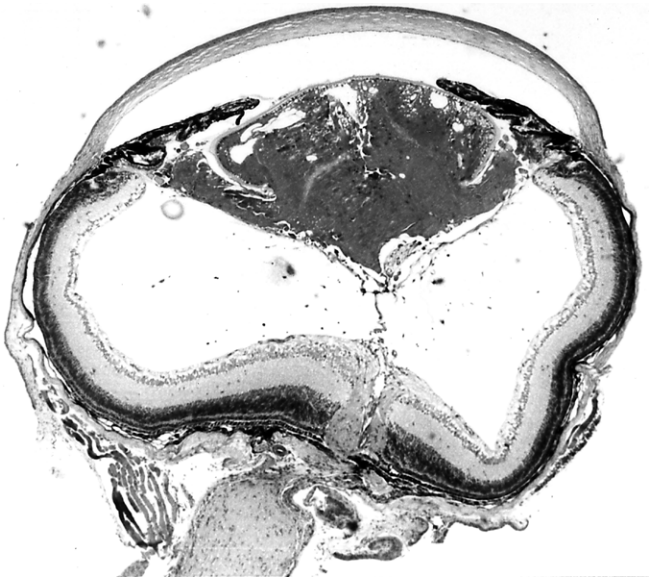

Figure 1a. Eye of To3/To3 homozygote, age 18 days. The lens is vacuolated, and the lens capsule has ruptured. The vitreous chamber is abnormally small.

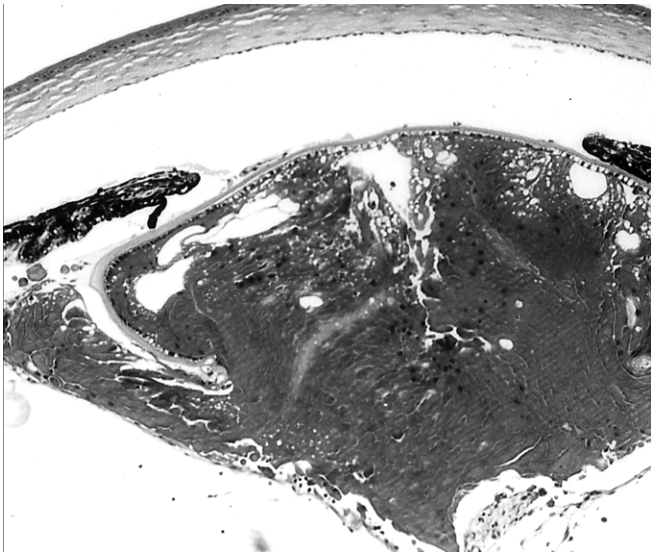

Figure 1b. Higher power view of lens in Fig. 1a. The lens fibers are disorganized and some retain the nuclei.

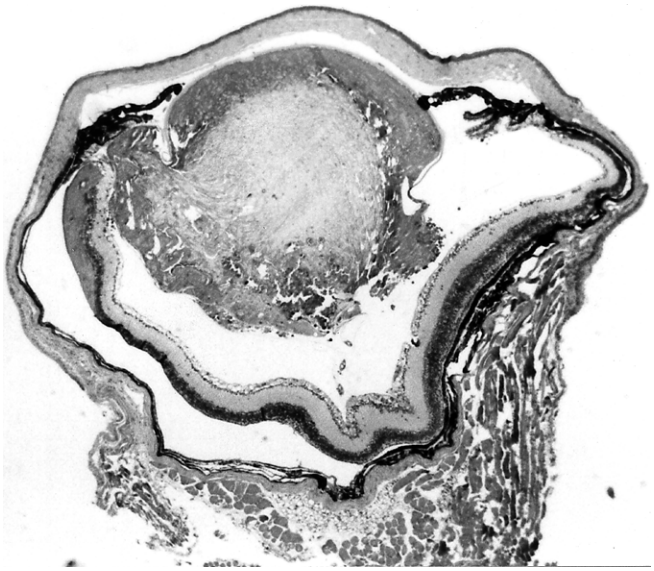

Figure 1c. Eye of To3/To3 homozygote, age 69 days. The lens capsule has ruptured, and lens material has leaked into the abnormally small vitreous chamber.

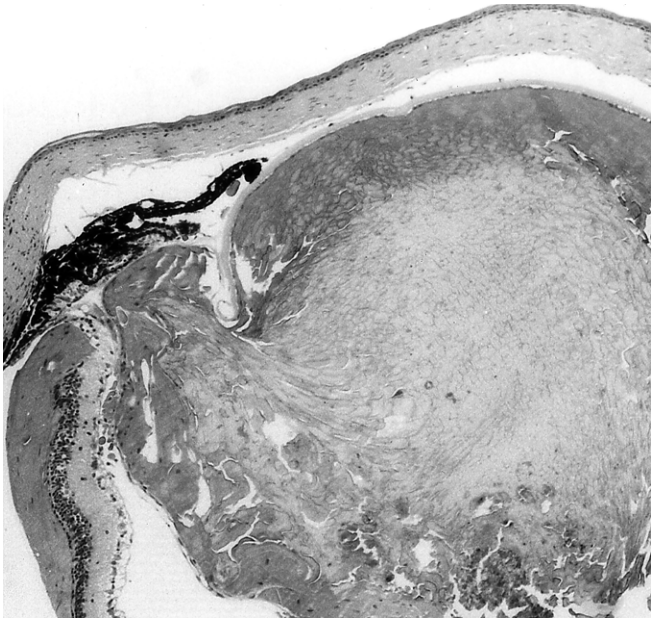

Figure 1d. Higher power view of lens in Fig. 1c. The lens has a nucleus with non-nucleated fibers, but these are disorganized.

chromosome 7, *Zfp61* and *Zfp63* (both of which are transcription factors), map too proximally to be candidates for *To3* (20).

Based upon the above evidence, we have screened the *Lim2* genes of both the *To3* mutant and normal parental strains in an attempt to detect an alteration in the *Lim2* gene of the *To3* mutant that was associated and consistent with the observed phenotype. In this communication we report the identification of a single base change within the first coding exon of the *To3* mutant *Lim2* gene that results in a single amino acid substitution within the MP19 protein. This new evidence indicates that MP19 may play an important role in both lens development and cataractogenesis and warrants further investigation into the precise nature of its role within the ocular lens.

MATERIALS AND METHODS

Isolation of murine genomic DNAs—High molecular weight genomic DNA was isolated from 200 mg of frozen mouse spleens from normal parental mouse strains and heterozygous and homozygous *To3* mice according to standard methods using Proteinase K digestion of cells followed by organic extraction and precipitation of DNA (21). The DNA's were quantitated using a UV spectrophotometer and were analyzed on agarose gels to confirm high molecular weight and lack of degradation.

PCR amplification of murine Lim2 gene fragments— An approximately 3.8 kb fragment spanning from about 130 bases upstream of the transcription start site through the end of exon 3 (Fragment 1 in Figure 2) was amplified using r*Tth* DNA

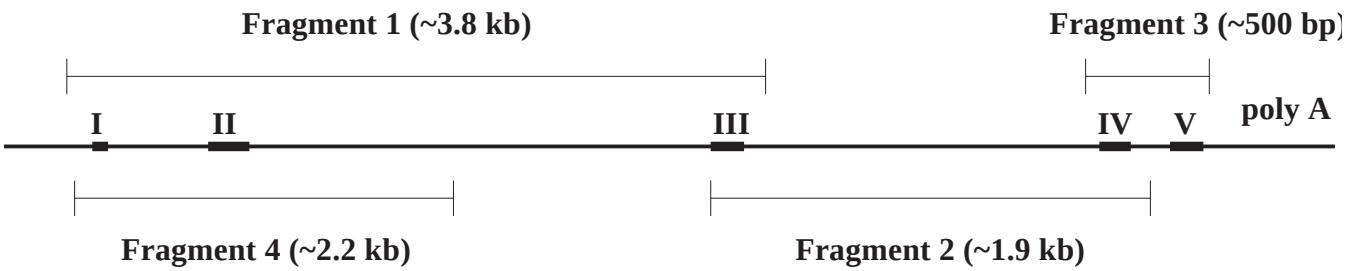

Figure 2. Strategy for PCR amplification of murine Lim2 gene fragments. Four overlapping fragments that encompassed the entire coding region were amplified as shown using the oligonucleotides indicated in Table 1.

polymerase (Perkin-Elmer) and the H19P-132/MP19-386 primer pair (See Table 1 for sequences) in a PTC-200 DNA Engine (MJ Research, Watertown, MA). Briefly, PCR was carried out using 100 ng genomic DNA as template, the supplied buffer, 200 µM each dNTP and 25 pmoles of each primer using a "hot start" technique. The following cycling conditions were used: 120s initial denaturation at 94°C followed by 40 cycles of 60s at 93°C, 60s at 58°C, and 360s at 72°C followed by 10 min at 72°C.

Another fragment, spanning from the beginning of exon 3 through the end of exon 4 (Fragment 2 in Figure 2, approximately 1.9 kb) was amplified as above using the MP19-239/MP19-524 primer pair (See Table 1 for the sequences). PCR was carried out as for Fragment 1 above using the following cycling conditions: 120s initial denaturation at 94°C followed by 40 cycles of 60s at 93°C, 60s at 60°C, 120s at 72°C followed by 10 min at 72°C.

A third fragment, spanning from the beginning of exon 4 through to about 100 bp past the translational stop site (Fragment 3 in Figure 2, approximately 500 bp) was amplified using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and the 129X5S2/129X5A2 primer pair (See Table 1 for sequences). PCR was carried out using 100 ng genomic DNA as template, the supplied buffer, 200 µM each dNTP, and 100 pmoles of each primer with a "hot start" technique. The following cycling conditions were used: 120s at 94°C initial denaturation followed by 40 cycles of 60s at 94°C, 60s at 58°C, 60s at 72°C followed by 10 min at 72°C.

Table 1. Sequences of DNA oligonucleotides used in PCR amplification of murine Lim2 gene fragments

Fragment	Primer name	(direction)	Sequence
1	H19P-132	(sense)	5'-CCTGCTTCAGCGAATTCCA-T-3'
	MP19-386	(antisense)	5'-GAGGCGAAAAACATGATG-CCT-3'
2	MP19-239	(sense)	5'-CATACTGGAATGCCACCCG-GG-3'
	MP19-524	(antisense)	5'-CCTGCAAAGAAGGTCATGA-GC-3'
3	129X5S2	(sense)	5'-TGGAGTCACCGTCAGTTTC-C-3'
	129X5A2	(antisense)	5'-CTATCTAGAATCCAGGACT-GA-3'
4	M19TG-S2	(sense)	5'-GTAGAGCTCGATCCTACCG-TACAAAGAACCC-3'
	M19TG-A3	(antisense)	5'-GACTTTGAAGCTTCAAAAG-GCCC-3'

PCR of Lim2 exon 2 fragments for confirmation—An approximately 2.2 kb fragment spanning exon 2 (Fragment 4 in Figure 2) was amplified using r*Tth* DNA polymerase and the M19TG-S2/M19TG-A3 primer pair (See Table 1 for sequences). PCR was carried out as for Fragments 1 and 2 above using the following cycling conditions: 120s at 94°C initial denaturation followed by 39 cycles of 60s at 93°C, 60s at 60°C, and 240s at 72°C followed by 10 min at 72°C. Fragment 4 PCR products were digested with *BstEII* restriction endonuclease (Life Technologies, Gaithersburg, MD) for 30 min at 37°C, then electrophoresed on a 2% agarose gel stained with ethidium bromide, and visualized using the Gel Doc 1000 apparatus and Molecular Analyst Software (Bio-Rad Laboratories, Hercules, CA).

DNA sequencing— Fragments 1 and 2 described above were subcloned into the pCRII vector (Invitrogen, Carlsbad, CA). These constructs were then used as template in cycle sequencing reactions using Cy5-labeled oligonucleotide primers and the Cy5 AutoCycle Sequencing Kit (Pharmacia Biotech, Piscataway, NJ). These were then electrophoresed and analyzed using an ALFexpress automated DNA Sequencer (Pharmacia Biotech, Piscataway, NJ). Fragments 3 and 4 were purified on a preparative agarose gel and then sequenced directly without subcloning using the same reagents and methods.

Nucleotide sequence and predicted amino acid sequence analysis was performed using DNASIS and PROSIS software (Hitachi America, Ltd.). Secondary structure analyses were performed using Macimdad (Molecular Applications Group) and PROSIS software.

RESULTS AND DISCUSSION

Before undertaking the screening of the *Lim2* gene in *To3*, the type of mutagenesis used to create this mutant, the alkylating agent N-ethyl-N-nitrosourea (ENU), was considered. ENU is a highly effective mutagen for mouse spermatogonial stem cells (22) that was predicted from studies in *Drosophila* (23) to induce single-base-pair changes in mammalian DNA, resulting in the recovery of intragenic mutations. The available data on the genetic and molecular nature of ENU-induced specific locus mutations in the mouse are consistent with this notion (24-26).

Therefore, it was sound to hypothesize that the mutation in the *To3* mutant would be a single-base-pair mutation.

Furthermore, the dominant nature of the mutant phenotype implied that the mutation would probably result in a protein with an altered structure and/or function. There were three possible locations for a mutation that would be expected to alter a protein's structure and/or function:

1. Within a coding exon resulting either in an amino acid substitution or a premature translational stop within the polypeptide;
2. At a splice junction resulting in an altered polypeptide length;
3. In the translation termination signal resulting in an elongated polypeptide.

As there was no method of predicting the odds of any of these types of mutations being more likely than the other, all three were screened for.

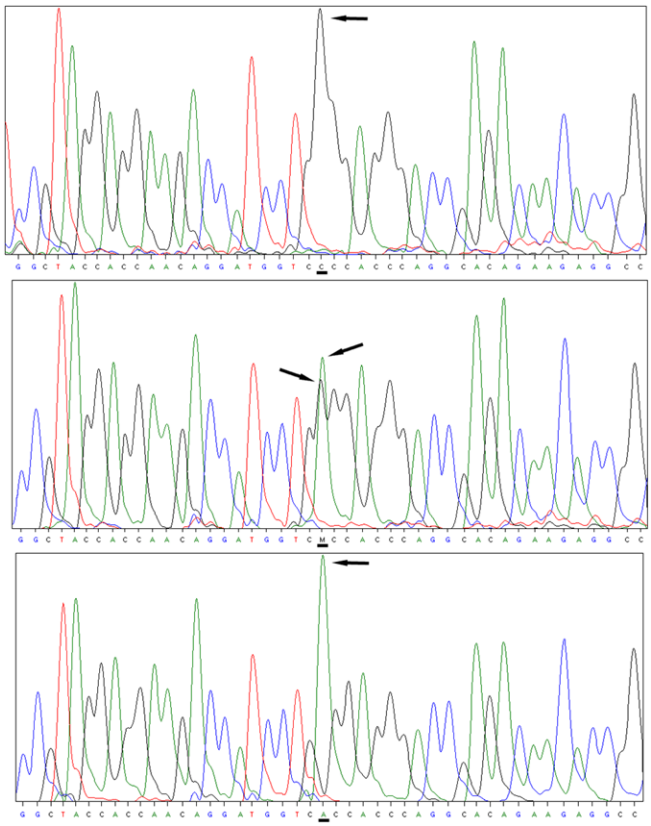

Figure 3a. Sequence of normal (top), heterozygous mutant (middle), and homozygousmutant (bottom) Lim2 exon 2 coding region. Sequence data shown is antisense sequence. The shown C -> A transversion corresponds to a G -> T transversion in the coding strand, which results in the nonconservative substitution of Val (GTG) for Gly (GGG) at amino acid position 15 in the mutant polypeptide. (Transversion peaks at arrows and transversion positions underlined.)

```
+/+    5' GCC TGG GTG GGG ACC ATC CTG 3'
          ALA TRP VAL GLY THR ILE LEU

                     BstEII
To3/To3   GCC TGG GTG GTG ACC ATC CTG
          ALA TRP VAL VAL THR ILE LEU
```

Figure 3b. The G ->T transversion in the mutant coding strand creates a unique BstEII restriction endonuclease recognition site and substitutes Val for Gly at amino acid 15 in the encoded polypeptide.

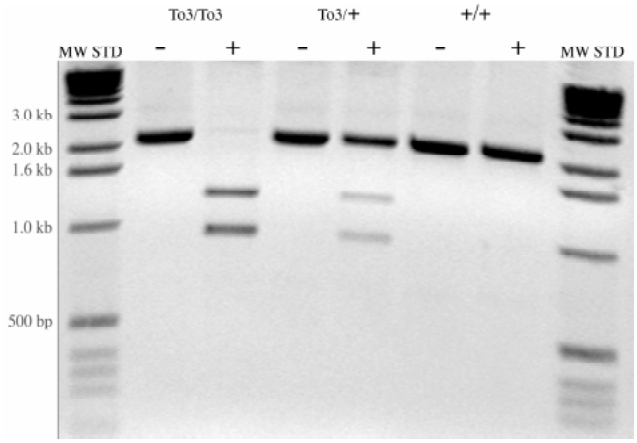

Figure 4. Bst EII restriction analysis of murine Lim2 Fragment 4 PCR products. Fragment 4 PCR products from To3/To3, To3/+, and +/+. DNA's were incubated without (-) or with (+) Bst EII restriction endonuclease and then electrophoresed on an agarose gel stained with ethidium bromide. Presence of the sequence is clearly linked to the mutant phenotype and corroborates the sequence data.

In order to screen the entire coding region, including the splice sites and the translation termination signal, the PCR primers shown in Table 1 were designed to amplify three large overlapping fragments encompassing different portions of the coding region as indicated in Figure 2. PCR was carried out on genomic DNA isolated from both normal parental strains as well as heterozygous and homozygous mutants. Both strands of the coding regions were then sequenced using oligonucleotides corresponding to sequences internal to these amplified fragments.

The only reproducible sequence change discovered was a single G -> T transversion within the first coding exon (exon 2) of the *Lim2* gene. The sequence comparison for this region is shown in Figure 3. The sequence shown in Figure 3a is the antisense strand and, therefore, shows a C -> A transversion. This DNA sequence alteration results in the creation of a *Bst EII* restriction endonuclease cleavage site within the DNA as well as the substitution of the normally encoded glycine by a valine at amino acid 15 within the polypeptide sequence as shown in Figure 3b. The *Bst EII* site was used as shown in Figure 4 to confirm the presence of the DNA sequence alteration in the *To3*/*To3* and *To3*/+ DNA, and its absence in the +/+ DNA.

Using predictive secondary structure algorithms (27,28), the glycine -> valine substitution was observed to result in a turn within the alpha helix of the first transmembrane spanning domain of the MP19 polypeptide. A graphical representation of this predicted change in the protein conformation was produced using Macimdad Software and is shown in Figure 5. Though speculative, such a disruption of the protein's normal folding pattern could interfere with normal insertion of the protein into fiber cell membranes and thus account for the defects observed in the mutant lenses. However, no physical structural analyses of normal and mutant MP19 protein have been conducted to confirm this theoretical model.

As Figures 3 and 4 illustrate, the identified mutation is linked to the observed cataractous phenotype of the *To3* mutant. The mutation is also consistent with the observed phenotype for the following reasons:

1.ENU predominantly produces single base substitutions in the mouse (22,29);
2.The mutation results in an amino acid substitution which may alter the structure and/or the function of the encoded polypeptide; and
3.The normal glycine residue at amino acid position 15 is conserved in all members of vertebrate phylogeny examined to date (3) as well as in 10 independent inbred strains of mice (data not shown).

It is of note that our results with the *To3* mutant bear a great deal of similarity to that of another murine mutant, *Lop* (for lens opacity). *Lop* is another autosomal semi-dominant, congenital, bilateral cataract. The *Lop* cataract has recently been shown to be the result of a single base pair transversion in the coding region of *Mip*, the gene encoding the most abundant lens specific integral membrane protein MP26. This nucleotide change results in the nonconservative substitution of proline for alanine at amino acid 51 (30). The evidence supports the hypothesis that the mutation alters the proper folding of the protein, resulting in its improper trafficking. Instead of being inserted into the membranes of the fiber cells, the mutant MP26 protein appears to accumulate in the endoplasmic reticulum. It is tempting to hypothesize that the same phenomenon might be occurring with the MP19 protein in the *To3* mutant. It is equally tempting to hypothesize that MP19 may play a role similar to, or perhaps even interactive with, MP26, as both proteins colocalize to the junction regions of the lens membranes and appear to result in similar phenotypes when one or the other is mutated.

Previously, immunofluorescent studies of MP19 in lens fiber cells colocalized MP19 with the gap junction protein Cx46 in a restricted area 0.5 mm to 1.0 mm into the lens(4). It is, therefore, another possibility that MP19 may serve a role similar to or interactive with this protein. These data provide evidence of a possible role for MP19 in mammalian lens fiber junctional formation or organization.

While the above correlations add strong support to the hypothesis that a genetic lesion in the *Lim2* gene of the *To3* mutant is the causative factor for the cataractous phenotype, they are by no means proof. It remains a possibility, albeit highly unlikely, that there exists an unidentified lens specific gene adjacent to *Lim2* which also bears a genetic lesion and is actually the cause of the phenotype in the *To3* mutant. In order to prove a causative relationship between the identified mutation and the cataractous phenotype, a transgene would have to be generated, by engineering this same mutation into a normal murine *Lim2* gene, and used in classical transgenic experiments.

In conclusion, these data support the hypothesis that a genetic lesion in the *Lim2* gene is the causative factor for the cataractous phenotype of the *To3* mouse mutant. The identified G -> T transversion in exon 2 results in a nonconservative substitution of valine for glycine at amino acid position 15 in the first transmembrane domain of the encoded MP19 polypeptide. This amino acid substitution may alter the normal conformation, and thereby any associated function, of the

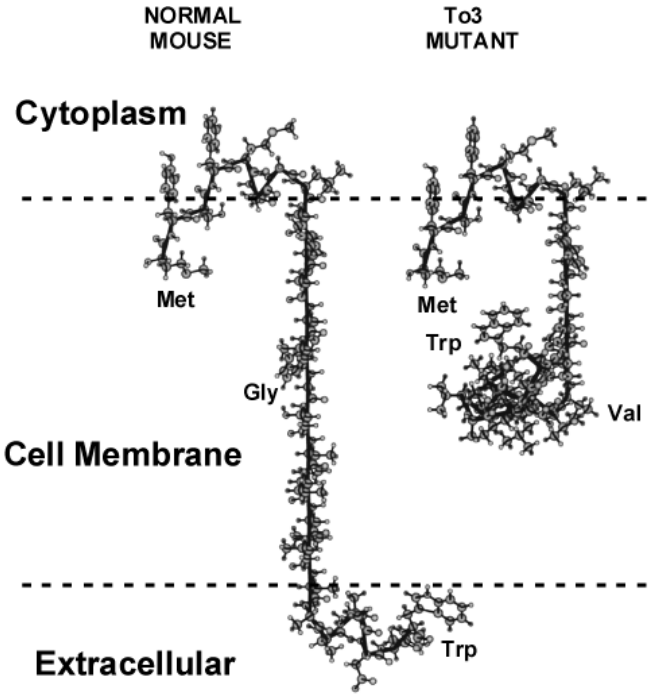

Figure 5. Molecular modeling of first 28 amino acids of predicted normal and mutant MP19 polypeptides. The Gly-->Val substitution at amino acid 15 may dramatically alter the folding pattern of the first transmembrane spanning domain of the polypeptide by introducing a turn in an alpha helix.

encoded MP19 polypeptide. As the role of MP19 in the ocular lens remains undetermined and no immunohistochemical analyses of the *To3* lenses have been conducted, it is impossible to predict whether the mutant MP19 is a gain of function or a loss of function allele. One hypothesis is that *To3* represents a gain-of-function mutation involving protein conformation defect or even cytotoxic effects in the fiber cells. An alternative hypothesis is that *To3* represents a loss-of-function allele such that a reduced concentration below a threshold concentration results in cataract formation. Transgenic experiments and immunohistochemical analyses should provide final proof or disproof of these hypotheses. If the mutation in the *Lim2* gene proves to be the underlying molecular defect in the *To3* mutant, the *Lim2* gene becomes another candidate for certain types of hereditary cataract in the human population.

ACKNOWLEDGEMENTS

This work was supported in part by National Institutes of Health grants R01 EY11516, T32 EY07092, P30 EY06360, C06 EY06307, RO1 EY10321, the Knights Templar Educational Foundation of Georgia, Inc., and a Departmental Grant from Research to Prevent Blindness, Inc.

REFERENCES

1. Gutekunst, KA, Rao, GN, and Church, RL, Molecular cloning and complete nucleotide sequence of the cDNA encoding a bovine lens intrinsic membrane protein (MP19), Curr. Eye Res. 9 (1990) 955-961.

2. Mulders, JWM, Voorter, CEM, Lamers, C, de Haard-Hoekman, WA, Montecucco, C, van de Ven, WJM, Bloemendal, H, and de Jong, WW, MP17, a fiber-specific intrinsic membrane protein from mammalian eye

lens, Curr. Eye Res. 7 (1988) 207-219.

3. Louis, CF, Hur, KC, Galvan, AC, TenBroek, EM, Jarvis, LJ, Eccleston, ED, Howard, JB, Identification of an 18,000-dalton protein in mammalian lens fiber cell membranes, J. Biol. Chem. 264 (1989) 19967-19973.

4. TenBroek, E, Arneson, M, Jarvis, L, and Louis, C, The distribution of the fiber cell intrinsic membrane proteins MP20 and connexin 46 in the bovine lens, J. Cell Science 103 (1992) 245-257.

5. TenBroek, EM, Johnson, R, and Louis, CF, Cell-to-cell communication in a differentiating ovine lens culture system, Invest. Ophthalmol. Vis. Sci. 35 (1994) 215-228.

6. Voorter, CEM, Kistler, J, Gruijters, WTM, Mulders, JWM, Christie, D, and de Jong, WW, Distribution of MP17 in isolated lens fiber membranes, Curr. Eye Res. 8 (1989) 697-706.

7. Galvan, A, Lampe, PD, Hur, KC, Howard, JB, Eccleston, ED, Arneson,m and Louis CF, Structural organization of the lens fiber cell plasma membrane protein MP18, J. Biol. Chem. 264 (1989) 19974-19978.

8. Garland, D and Russell, P, Phosphorylation of lens fiber cell membrane proteins, Proc. Natl. Acad. Sci. USA 82 (1985) 653-657.

9. Louis, CF, Johnson, R, Johnson, K, and Turnquist, J, Characterization of the bovine lens plasma membrane substrates for cAMP-dependent protein kinase, Eur. J. Biochem. 150 (1985) 279-286.

10. Louis, CF, Hogan, P, Visco, L, and Strasburg, G, Identity of the calmodulin-binding proteins in bovine lens plasma membranes, Exp. Eye Res. 50 (1990) 495-503.

11. Louis, CF, Johnson R, and Turnquist, J, Identification of the calmodulin-binding components in bovine lens plasma membranes, Eur. J. Biochem. 15 (1985) 271-278.

12. Favor, J, A comparison of the dominant cataract and recessive specific-locus mutation rates induced by treatment of male mice with ethylnitrosourea, Mutat. Res. 110 (1983) 367-382.

13. Favor, J, Characterization of dominant cataract mutations in mice: penetrance, fertility and homozygous viability of mutations recovered after 250 mg/kg ethylnitrosourea paternal treatment, Genet. Res., Camb. 44 (1984) 183-197.

14. Fraser, FC and Herer, ML, The inheritance and expression of the "lens rupture" gene in the mouse, J. Hered. 41 (1950) 3-7.

15. Matsushima, Y, Kamoto, T, Iida, F, Abujang, P, Honda, Y, and Hiai,H, Mapping of rupture lens cataract (rlc) on mouse chromosome 14, Genomics 36 (1996) 553-554.

16. Lyon, MF, Jarvis, SE, Sayers, I, and Holmes, RS, Lens opacity: a new gene for congenital cataract on chromosome 10 of the mouse. Genet. Res. 38 (1981) 337-341.

17. Graw, J, Bors, W, Gopinath, PM, Markle, S, Michel, C, Reitmeir, P, Schaffer, E, Summer, KH, and Wulff, A, Characterization of Cat-2t, a radiation-induced dominant cataract mutation in mice. Invest. Ophthalmol. Vis. Sci. 31 (1990) 1353-1361.

18. Kerscher, S, Glenister, PH, Favor, J, and Lyon, MF, Two new cataract loci, Ccw and To3, and further mapping of the Npp and Opj cataracts in the mouse, Genomics 36 (1996) 17-21.

19. Kerscher, S, Church, RL, Boyd, Y, and Lyon, MF, Mapping of four mouse genes encoding eye lens-specific structural gap junction, and integral membrane proteins: Cryba1 (Crystallin beta-A3/A1), Crybb2 (Crystallin beta-B2), Gja8 (MP70), and Lim2 (MP19), Genomics 29 (1995) 445-450.

20. Danciger, M, Brady, J, Adamson, MC, Farber, DB, Piatigorsky, J, and Kozak, CA, Chromosomal localization of the genes for five zinc finger proteins expressed in mouse lens, Genomics 28 (1995) 39-43.

21. Ausubel, FM, Brent, R, Kingston, RE, Moore, DD, Seidman, JG, Smith, JA, and Struhl K (eds.), Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Interscience, New York (1987) pp. 2.2.1-2.2.3.

22. Russell, WL, Kelley, EM, Hunsicker, PR, Bangham, JW, Maddux, SC, Phipps, EL, Specific-locus test shows ethylnitrosourea to be most potent mutagen in the mouse, Proc. Natl. Acad. Sci. USA 76 (1979) 5818-5819.

23. Vogel, E, and Natarajan, AT, The relation between reaction kinetics and mutagenic action of mono-functional alkylating agents in higher eukaryotic systems: recessive lethal mutations and translocations in Drosophila, Mutat. Res. 62 (1979) 51-100.

24. Popp, RA, Bailiff, EG, Skow, LC, Johnson, FM, and Lewis, SE, Analysis of a mouse alpha-globin gene mutation induced by ethylnitrosourea, Genetics 105 (1983) 157-167.

25. Russell, LB, and Rinchik, EM, In Moore, MM, Demarini, DM, de Sevres, FJ, and Tindall, KR (eds.), Mammalian Cell Mutagenesis, Cold Spring Harbor Laboratory Press (1987) pp. 109-121.

26. Zdarsky, E, Favor, J, and Jackson, IJ, The molecular basis of brown, an old mouse mutation, and of an induced revertant to wild type, Genetics 126 (1990) 443-449.

27. Levitt, M and Lifson, S, Refinement of protein conformations using a macromolecular energy minimization procedure, J. Mol. Biol. 46 (1969) 269-279.

28. Garnier, J, Osguthorpe, DJ, and Robson, B, Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins, J. Mol. Biol. 120 (1978) 97-120.

29. Provost, GS and Short, JM. Characterization of mutations induced by ethlnitrosourea in seminiferous tubule germ cells of B6C 3F1 mice. Proc. Natl. Acad. Sci. USA 91 (1994) 6564-6568.

30. Shiels, A, and Bassnett, S, Mutations in the founder of the MIP gene family underlie cataract development in the mouse, Nat. Genet. 12 (1996) 212-215.