

## Water Channel Properties of Major Intrinsic Protein of Lens\*

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The functions of major intrinsic protein (MIP) of lens are still unresolved; however the sequence homology with channel-forming integral membrane protein (CHIP) and other Aquaporins suggests that MIP is a water channel. Immunolocalizations confirmed that *Xenopus* oocytes injected with bovine MIP cRNA express the protein and target it to the plasma membrane. Control oocytes or oocytes expressing MIP or CHIP exhibited small, equivalent membrane currents that could be reversibly increased by osmotic swelling. When compared with water-injected control oocytes, the coefficient of osmotic water permeability ( $P_f$ ) of MIP oocytes was increased 4–5-fold with a low Arrhenius activation energy, while the  $P_f$  of CHIP oocytes increased > 30-fold. To identify structures responsible for these differences in  $P_f$ , recombinant MIP proteins were expressed. Analysis of MIP-CHIP chimeric proteins revealed that the 4-kDa cytoplasmic domain of MIP did not behave as a negative regulator. Individual residues in MIP were replaced by residues conserved among the Aquaporins, and introduction of a proline in the 5th transmembrane domain of MIP raised the  $P_f$  by 50%. Thus oocytes expressing MIP failed to exhibit ion channel activity and consistently exhibited water transport by a facilitated pathway that was qualitatively similar to the Aquaporins but of lesser magnitude. We conclude that MIP functions as an Aquaporin in lens, but the protein may also have other essential functions.

Major intrinsic protein (MIP)<sup>1</sup> is a 26-kDa protein expressed exclusively in lens fiber cells where it comprises over 60% of the membrane protein. The cDNA encoding MIP was isolated from a bovine lens cDNA library, and hydrophobicity plots predicted that MIP is an integral membrane protein with cytoplasmic amino and carboxyl termini and six bilayer-spanning domains (Gorin *et al.*, 1984). MIP reconstituted into liposomes exhibits voltage-dependent channels permeable to ions and small molecules that may be closed by  $\text{Ca}^{2+}$  and calmodulin (Nikaido and

Rosenberg, 1985; Girsch and Peracchia, 1985; Peracchia and Girsch, 1989; Shen *et al.*, 1991). In planar lipid bilayers, MIP forms single channels of various conductances (up to 3 nano-siemens) and a weak selectivity for anions (Ehring *et al.*, 1990); Nodulin-26, a homologous protein from soy bean root nodules, was recently shown to behave similarly (Weaver *et al.*, 1994). In spite of these studies with reconstituted MIP protein, studies of MIP in native membranes or expressed in oocytes have failed to confirm a physiologic function for MIP. For example, although MIP was initially considered to be the gap-junction protein, immunological, biochemical, and electrophysiological studies failed to identify electric coupling of lens fiber cells via MIP (Swenson *et al.*, 1989).

MIP was the first identified member of an ancient family of membrane proteins from diverse organisms that now includes more than 20 members. Several MIP family members from animal and plant tissues were shown to function as water-selective membrane channels and are now referred to as the "Aquaporins" (Chrispeels and Agre, 1994). The purification and cDNA cloning of the 28-kDa channel-forming integral membrane protein, CHIP (Denker *et al.*, 1988; Preston and Agre, 1991), a MIP homolog from red cells and renal proximal tubules, permitted the first demonstration of a molecular water channel (Preston *et al.*, 1992). CHIP is now designated Aquaporin-1 (AQP1), and cDNAs encoding four other Aquaporins have subsequently been isolated from diverse mammalian tissues (Fushimi *et al.*, 1993; Ishibashi *et al.*, 1994; Ma *et al.*, 1994; Echevarria *et al.*, 1994; Hasegawa *et al.*, 1994; Jung *et al.*, 1994b; Raina *et al.*, 1995).

The hourglass model was recently proposed to merge structural and functional features of the Aquaporins (Jung *et al.*, 1994a). The model describes two tandem repeats with the first and second half of the molecule oriented at 180° to each other, and each half contains the sequence asparagine-proline-alanine (NPA) in intracellular loop B and extracellular loop E. In the hourglass model, it was predicted that loop B and loop E fold back into the membrane, forming a single water-selective pore. The striking sequence homology with the Aquaporins suggests that MIP may also be involved in water movement through the lens fiber cell membranes. In this report, we investigated the water transporting capacities of MIP using the *Xenopus* oocyte expression system, and water channel properties qualitatively similar to the Aquaporins were observed.

### EXPERIMENTAL PROCEDURES

**Plasmid DNA Mutagenesis, DNA Sequencing, and in Vitro RNA Synthesis**—Standard molecular procedures were used (Sambrook *et al.*, 1989). The bovine MIP coding sequence (Gorin *et al.*, 1984) flanked 5' and 3' by *Xenopus*  $\beta$ -globin gene untranslated sequences (Swenson *et al.*, 1989) was transferred into pBluescript II KS (Stratagene) following digestion with *Hind*III and *Xba*I. The CHIP expression vector was constructed as described (Preston *et al.*, 1992). These constructs served as templates for site-directed mutagenesis reactions using the Muta-Gene phagemid *in vitro* mutagenesis kit (Bio-Rad). Table I lists the

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<sup>1</sup> The abbreviations used are: MIP, major intrinsic protein of lens;  $P_f$ , coefficient of osmotic water permeability; CHIP, channel forming integral protein of 28-kDa (CHIP28).

TABLE I  
Site-specific mutations, insertional mutations, and chimeric constructs of MIP and CHIP

MIP constructs	Wild-type amino acid/codon	Mutant amino acid/codon
MIP (V112Bam)	Val-112/GTC	Val-Asp-Pro/GTggatccC
MIP(C14V)	Cys-14/TGT	Val-14/GTT
MIP(V160P)	Val-160/GTG	Pro-160/CCA
MIP(A181C)	Ala-181/GCA	Cys-181/TGT
MIP(S243V/S245E)	Ser-Glu-Ser-245/AGTgagTCC	Val-Glu-Glu/GTTgagGAA
CHIP constructs	Wild-type amino acid/codon	Mutant amino acid/codon
CHIP(T120Bam)	Thr-120/ACT	Thr-Asp-Pro/ACggatccT
CHIP(D131P)	Asp-131/GAT	Pro-131/CCT
CHIP(P169A)	Pro-169/CCC	Ala-169/GCC
Chimeric constructs	Amino acids	Total amino acids
MIP-CHIP (loop A)	MIP(1-32)-CHIP(34-51)-MIP(44-263)	270
MIP-CHIP-1	MIP(1-112)-CHIP(121-269)	263
MIP-CHIP-2	MIP(1-226)-CHIP(235-269)	261
CHIP-MIP-1	CHIP(1-120)-MIP(113-263)	273
CHIP-MIP-2	CHIP(1-234)-MIP(227-263)	271

mutants used in this study. The locations of mutations within the MIP polypeptide are illustrated in Fig. 1.

The chimeric protein vectors CHIP-MIP-1 and MIP-CHIP-1 were constructed by inserting a *Bam*HI restriction site at Val-112 of MIP and Thr-120 of CHIP, resulting in the insertion of the amino acids aspartic acid and proline. MIP(V112Bam) and CHIP(T120Bam) were digested with *Bam*HI, the 5'-half of CHIP was ligated to the 3'-half of MIP (CHIP-MIP-1), and the 5'-half of MIP was ligated to the 3'-half of CHIP (MIP-CHIP-1). The chimeric protein vectors MIP-CHIP-2 and CHIP-MIP-2 were constructed using the megaprimer polymerase chain reaction method (Barik and Galinski, 1991) exchanging at Arg-226 of MIP and Arg-234 of CHIP, using the following antisense primers: MIP-CHIP-2, 5'-GTCTGTGAGGTCACCTGCTG-CGAGGGAAGAGGAGA-AAG-3'; CHIP-MIP-2, 5'-CTCAGAAACACTCTTGAGC-CGTGGGGC-CAGGATGAAG-3'.

The chimeric protein, MIP-CHIP (loop A), contains the first exofacial loop (A) of CHIP (amino acids 34-51) in place of the first exofacial loop of MIP (amino acids 33-43) and was constructed with an 82-base pair insertional-substitutional oligonucleotide primer (not shown) in a site-directed mutagenesis reaction. All mutations were confirmed by enzymatic nucleotide sequencing (U. S. Biochemical Corp.).

Capped RNA transcripts were synthesized *in vitro* using T3 RNA polymerase with *Xba*I-digested MIP, CHIP, or mutant expression vector DNA, and the RNA was purified as described (Yisraeli and Melton, 1989).

**Preparation of Oocytes and Measurement of *P<sub>f</sub>*—Female *Xenopus laevis*** were anesthetized on ice, and stage V and VI oocytes were removed and prepared (Lu *et al.*, 1990). The day after isolation, oocytes were injected with either 50 nl of water or 0.5–25 ng of cRNA in 50 nl of water. Injected oocytes were maintained for 2–3 days at 18 °C prior to osmotic swelling, membrane isolation, or voltage clamp experiments. Oocyte swelling was performed at 22 °C following transfer from 200 mosM (*osm<sub>in</sub>*) to 70 mosM (*osm<sub>out</sub>*) CHIP) or either 70 or 20 mosM (*osm<sub>out</sub>*) MIP) modified Barth's solution diluted with water. Sequential oocyte images were digitized at 5-s intervals for a total of 1 min, and the volumes of the sequential images were calculated as described (Preston *et al.*, 1993). The change in relative volume with time,  $d(V/V_0)/dt$ , was fitted by computer to a quadratic polynomial, and the initial rates of swelling were calculated. The osmotic water permeability ( $P_f$ ,  $\mu\text{m/s}$ ) was calculated from osmotic swelling data between 5 and 10 s, initial oocyte volume ( $V_0 = 9 \times 10^{-4} \text{ cm}^3$ ), initial surface area ( $S = 0.045 \text{ cm}^2$ ), and the molar ratio of water ( $V_w = 18 \text{ cm}^3/\text{mol}$ ) (Zhang *et al.*, 1990) using the formula

$$P_f = [V_0 \times d(V/V_0)/dt] / [S \times V_w \times (\text{osm}_{\text{in}} - \text{osm}_{\text{out}})] \quad (\text{Eq. 1})$$

**Oocyte Membrane Isolation and Immunoblot Analysis**—Total oocyte membranes (Preston *et al.*, 1993) and plasma membranes (Wall and Patel, 1989) were isolated from groups of 4–30 oocytes, solubilized in 1.25% (w/v) SDS at 60 °C for 10 min, electrophoresed into 12% SDS-polyacrylamide gels (Laemmli, 1970), transferred to nitrocellulose (Davis and Bennett, 1984), incubated with a 1:10,000 dilution of anti-MIP antibody, or a 1:1000 dilution of anti-CHIP antibody (Smith and Agre, 1991), and visualized using the ECL Western blotting detection system (Amersham Corp.). Molecular weights were determined relative to the mobility of prestained SDS-PAGE standards (Bio-Rad).

**Anti-MIP Antibody**—A synthetic peptide corresponding to the 15

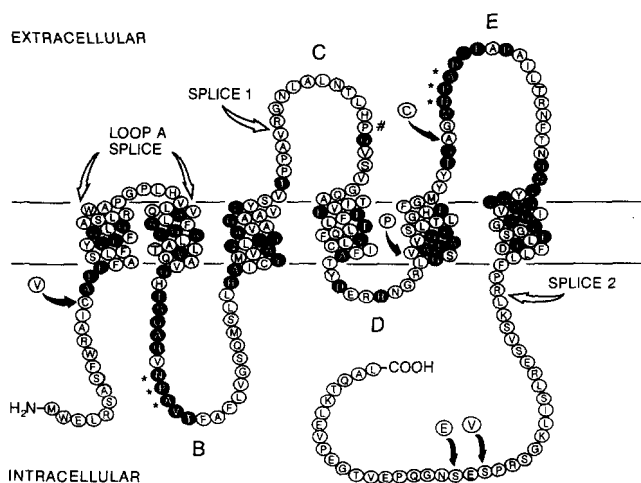


FIG. 1. Membrane topology of MIP showing sites targeted for mutagenesis and domain exchanges in this study. *SPLICE 1* is the splice site for MIP-CHIP-1 and CHIP-MIP-1; *SPLICE 2* is the splice site for MIP-CHIP-2 and CHIP-MIP-2. The proline introduced in CHIP at position Asp-131 is indicated with #. NPA motifs are indicated with asterisks. Filled symbols indicate amino acids that are conserved in more than half of the members of the MIP family from bacteria, yeast, plants and animals (Reizer *et al.*, 1993).

carboxyl-terminal amino acids of bovine MIP (PEVTGEPVELKTQAL) (Gorin *et al.*, 1984) was coupled to keyhole limpet hemocyanin. Rabbits were injected with 400  $\mu\text{g}$  of conjugated synthetic peptide mixed with Freund's complete adjuvant. After 4 weeks, and every 3 weeks thereafter, the rabbits were boosted with 200  $\mu\text{g}$  of conjugated synthetic peptide mixed with incomplete Freund's adjuvant. The produced anti-MIP antiserum was tested for specificity and cross-reactivity by an enzyme-linked immunosorbent assay.

**Immunolocalization of MIP in *Xenopus* Oocytes**—Oocytes were frozen for immunofluorescence microscopy in Tissue-Tek mounting medium (Miles Inc.). Sections of 5  $\mu\text{m}$  were cut by cryostat, collected on gelatin-coated slides, and fixed at room temperature for 5 min in 1% (w/v) periodate-lysine-paraformaldehyde fixative (McLean and Nakane, 1974). Sections were washed 3 times with TBS (0.9% NaCl, 25 mM Tris, pH 7.4) and incubated overnight at 4 °C with anti-MIP antibody at a dilution of 1:1,000 in TBS containing 10% (v/v) goat serum. After 3 washes with TBS, the sections were incubated for 1 h at 37 °C with affinity-purified fluorescein isothiocyanate-labeled goat-anti-rabbit IgG (Sigma Immuno Chemicals) at a dilution 1:50 in TBS containing 10% (v/v) goat serum. After 3 more washes in TBS, the sections were embedded in Mounting Medium (Sigma) and analyzed by immunofluorescence microscopy.

**Electrophysiology**—Studies were carried out as described (Preston *et al.*, 1992) using a two-microelectrode voltage clamp with Clampex software. Water- and cRNA-injected oocytes were voltage-clamped at a holding membrane potential of  $-70 \text{ mV}$ , repolarized to  $-90 \text{ mV}$ , and

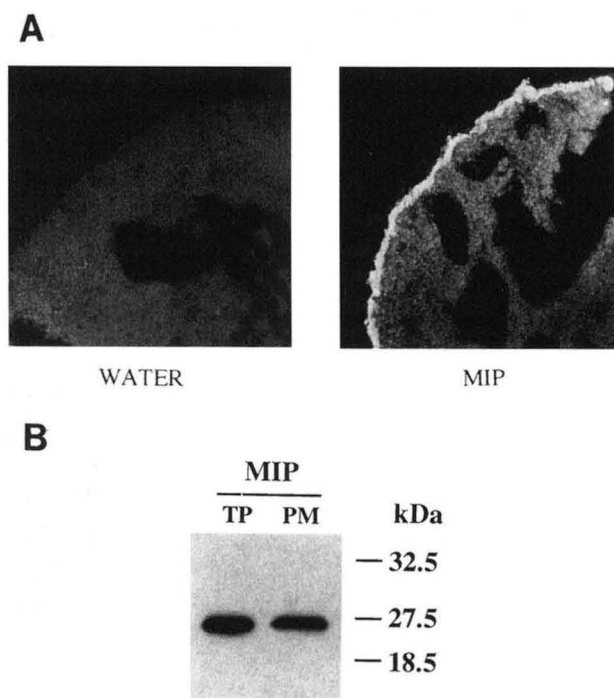


FIG. 2. A, immunofluorescence microscopy of control oocytes and oocytes expressing MIP. Frozen sections were prepared from *Xenopus* oocytes injected with 50 nl of water or 50 nl of water containing 10 ng of MIP cRNA and probed with anti-MIP antibody (see "Experimental Procedures"). Note strong labeling of plasma membrane and negligible labeling of cytoplasm of the oocyte expressing MIP. B, immunoblot of total protein (TP, equivalent of 0.2 oocytes) and plasma membranes (PM, equivalent of 15 oocytes) from oocytes expressing MIP. SDS-polyacrylamide gel electrophoresis immunoblot was incubated with a polyclonal antibody directed against the carboxyl terminus of MIP.

depolarized to +50 mV in 20 mV step intervals. The membrane potential was returned to the holding potential between each step.

## RESULTS

**Immunolocalization**—Oocytes from *X. laevis* have provided the standard system for expression of water channels and measurement of their functional activity by a swelling assay (see "Experimental Procedures"). Expression of MIP and insertion of the protein into the oocyte plasma membrane was studied by immunofluorescence microscopy of frozen sections. Oocytes injected with MIP cRNA showed a strong anti-MIP immunolabeling of the plasma membrane, whereas the cytoplasm showed only weak immunostaining (Fig. 2A). Immunoblot analysis of isolated oocyte plasma membranes also showed a single strong signal at 27 kDa, confirming that MIP is targeted to the plasma membrane (Fig. 2B).

**Conductance**—It was reported that MIP reconstituted into planar lipid bilayers forms channels with high conductance (Ehring *et al.*, 1990). The hypothesis that MIP is an ion channel was tested by measuring the conductance of oocyte membranes expressing MIP with a two-microelectrode voltage clamp. In the absence of osmotic swelling, no differences in conductance were measured in oocytes injected with water, MIP cRNA, or CHIP cRNA (Fig. 3). The oocytes were placed in hypotonic medium, and, after being osmotically swollen to a similar size, membrane currents were again measured. Oocytes injected with water, MIP cRNA, or CHIP cRNA exhibited a similar increase in conductance. After replacing oocytes in isotonic buffer to cause shrinkage to the initial volume, the conductance decreased again to the initial values, although the shrinking process for water-injected oocytes was too slow for analysis (not shown).

**Water Permeability of MIP**—Osmotic water permeability ( $P_f$ )

of *Xenopus* oocytes was measured in hypotonic medium 3 days after injection with 50 nl of water or 10 ng of MIP or CHIP cRNA. Water-injected control oocytes swelled minimally ( $P_f = 10 \pm 3.2 \mu\text{m/s}$ ). Oocytes injected with MIP cRNA displayed a 4–5-fold increase in water permeability above the basal level of control oocytes ( $P_f = 45 \pm 8.2 \mu\text{m/s}$ ). A more than 30-fold increase in  $P_f$  was exhibited by oocytes injected with the same amount of CHIP cRNA ( $P_f = 351 \pm 43 \mu\text{m/s}$ , Fig. 4, A and B). Although the  $P_f$  of MIP-injected oocytes was only ~15% of CHIP-injected oocytes, the MIP-injected oocytes had significantly greater  $P_f$  than the control oocytes ( $p < 0.05$ , Fig. 4A), a difference that was consistently reproduced in each of 14 independent experiments.

Water transport through aqueous channels is characterized by a low Arrhenius activation energy, which is equivalent to diffusion of water in bulk solution ( $E_A \sim 4 \text{ kcal/mol}$ ), whereas diffusion of water through lipid membranes is characterized by a high value ( $E_A > 10 \text{ kcal/mol}$ ) (Finkelstein, 1987). Water permeability of oocytes injected with 25 ng of MIP cRNA was measured at multiple temperatures; consistent with facilitated water transport, the activation energy was low ( $E_A = 3.9 \text{ kcal/mol} \pm 1.4 \text{ kcal/mol}$ , average of 4 experiments, Fig. 5). Indicative of diffusional water movement, the activation energies of water-injected oocytes were measured in two experiments ( $E_A = 10$  and 18 kcal/mol).

**Chimeric MIP-CHIP Proteins**—Domains of MIP and CHIP that might be of importance for the specific functional characteristics of the two proteins were exchanged, and the functional consequences were measured (Table I, Fig. 1). The MIP-CHIP (loop A) chimera represents a MIP molecule with residues 33–43 in loop A replaced by the residues 34–51 from the loop A of CHIP. The MIP-CHIP-1 and CHIP-MIP-1 chimeras contain the amino-terminal half of one molecule and the carboxyl-terminal half of the other spliced at a site in loop C where the tandem repeats are normally joined. The MIP-CHIP-2 and CHIP-MIP-2 chimeras are spliced at a site in the carboxyl-terminal cytoplasmic domains of the molecules. When membranes of oocytes expressing each of these constructs were analyzed by immunoblot, polypeptides of the anticipated size were identified, and the levels of expression were comparable with oocytes expressing native MIP and CHIP proteins (not shown).

Unlike all of the known Aquaporins, MIP does not contain a potential *N*-glycosylation site in either loop A or loop C, and no glycosylation of MIP was detected when the polypeptide was expressed in oocytes (Fig. 2B). The MIP-CHIP (loop A) construct contains the CHIP glycosylation site at residue asparagine 42 in loop A. Despite the presence of a potential glycosylation site, oocytes expressing this construct still did not exhibit an increase in  $P_f$ , and immunoblots revealed a 27-kDa polypeptide but not higher molecular mass bands, indicating that *N*-glycosylation had still not occurred (not shown).

Oocytes expressing MIP-CHIP-1 and CHIP-MIP-1 chimeras did not exhibit a significant increase in  $P_f$  above water-injected controls, suggesting that these chimeric proteins cannot form functional water channels. Oocytes expressing MIP-CHIP-2 also exhibited  $P_f$ s similar to control oocytes. In contrast, oocytes expressing CHIP-MIP-2 were found to have  $P_f$ s similar to oocytes expressing wild-type CHIP (Fig. 6), indicating that the carboxyl-terminal cytoplasmic domain of MIP does not interfere with the water-transporting domains of CHIP.

**Single Amino Acid Substitutions**—Unlike CHIP, the increase in water permeability induced by MIP expression is not blocked by  $\text{HgCl}_2$ . A putative mercury-sensitive cysteine residue was introduced into MIP at position 181, which conforms to the mercury-sensitive cysteine 189 in CHIP, but the substitu-

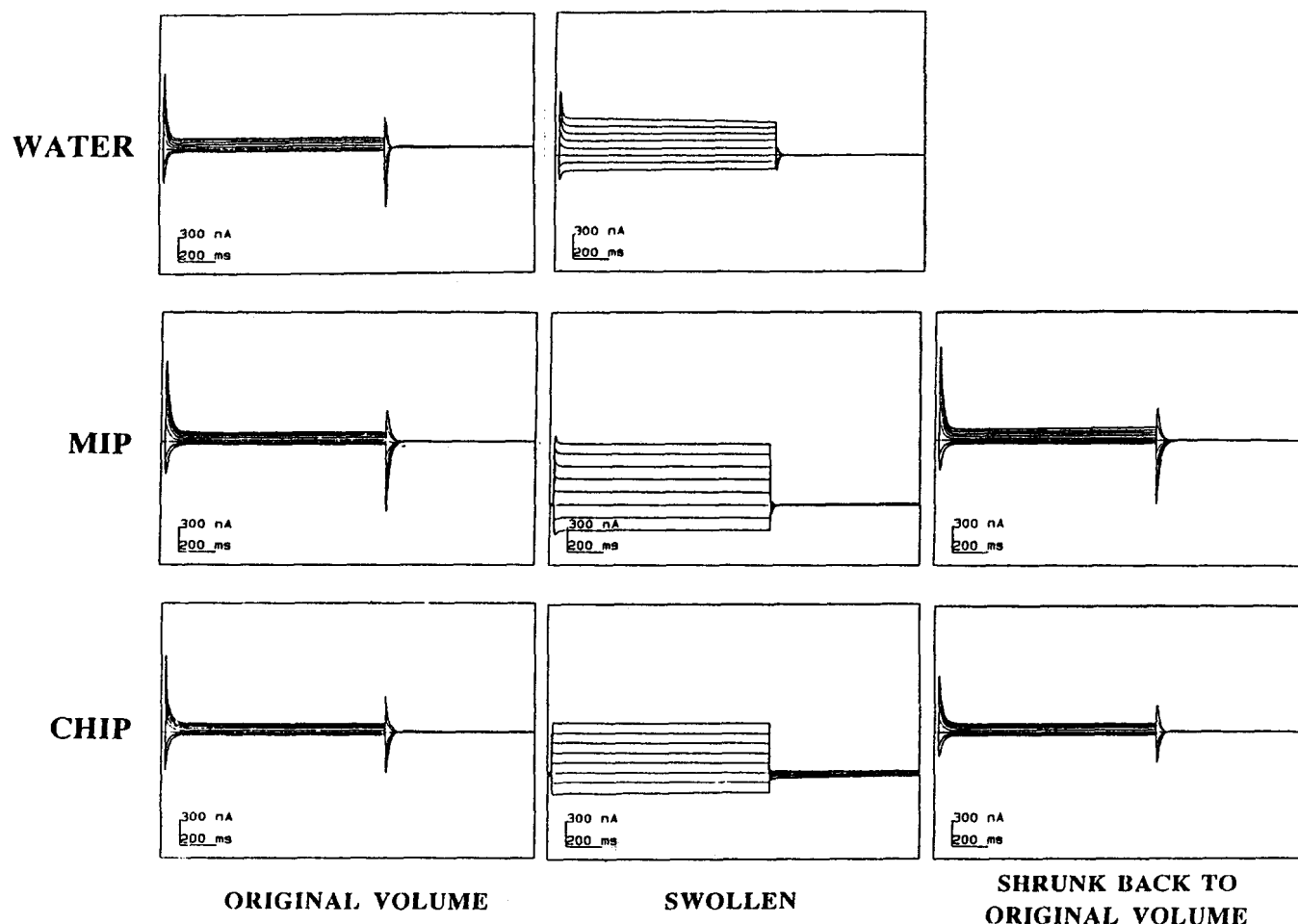


FIG. 3. **Membrane ion conductance.** Voltage clamp was used to measure currents at voltages from  $-90$  mV to  $+50$  mV of water-injected control oocytes, oocytes injected with 25 ng of MIP cRNA, or oocytes injected with 0.5 ng of CHIP cRNA. The currents were determined in 200 mosM modified Barth's solution. The oocytes were swollen by replacing modified Barth's solution with a hypotonic medium, and current measurements were repeated. Control oocytes were placed in  $H_2O$ , and the current was measured after 30 min. Oocytes expressing MIP and CHIP were placed in modified Barth's solution diluted from 200 to 70 mosM, and currents were measured after 20 and 30 s. Oocytes were shrunk back to their normal volume by replacing the hypotonic medium with isotonic modified Barth's solution, and currents in oocytes expressing MIP and CHIP were measured after 8 min.

tion MIP(A181C) did not confer mercury sensitivity to MIP (Fig. 7). The cysteine at position 14 in the amino-terminal domain of MIP was replaced by a valine, since other Aquaporins do not contain a cysteine at this position, but the  $P_f$  of C14V mutant MIP was not different from wild-type MIP (Fig. 8A). MIP contains a proline in the second extracellular loop (residue 123 in loop C), a site where other members of the MIP family do not contain this residue. Introducing a proline at this position in CHIP (D131P) also did not change the  $P_f$  (Fig. 8A).

All known Aquaporins except AQP4 contain a proline in the 5th transmembrane segment; no proline exists in the 5th transmembrane domain of MIP. When a proline was introduced in MIP (V160P), the substitution reproducibly enhanced the  $P_f$  by  $50 \pm 20\%$  relative to the  $P_f$  of oocytes expressing wild-type MIP (Fig. 8A). Notably, the amount of MIP protein expressed in oocytes was not different after injection of equal amounts of MIP cRNA and MIP(V160P) cRNA (Fig. 8B). Although not large, this increase in  $P_f$  was confirmed in each of five different experiments. A "gain of function" mutation suggests that the  $P_f$  of MIP can be increased by introducing subtle conformational changes in the molecule. Nevertheless, replacing the proline in CHIP at this position (P169A) to the corresponding residue of MIP had no measurable influence on the  $P_f$  of CHIP (Fig. 8A).

These results show that MIP exhibits a low  $P_f$  value that can be increased by introducing subtle changes in the molecule.

One explanation may be that MIP needs to undergo an activation or structural rearrangement to function as a water channel, whereas CHIP is constitutively in the activated state. Two sites in the carboxyl-terminal cytoplasmic domain of MIP are phosphorylated *in vivo* (Lampe and Johnson, 1990). Since these sites are not conserved in other members of the MIP family, the sites may play a role in regulation of MIP function. Mutation of the two putative phosphorylation sites to the corresponding residues in CHIP (S243V, S245E) slightly decreased the  $P_f$  (Fig. 8A), but the amount of protein expressed was comparably reduced as compared with wild-type MIP by immunoblot (Fig. 8B). The possibility of stretch-activation was also considered, but if it exists, it was not reproduced by simple increase in volume, since the rate of osmotic swelling did not increase with time (Fig. 9). Thus, if an activation step confers CHIP-level water permeability on MIP, the identity of this step remains unknown.

#### DISCUSSION

Even though the cDNA encoding MIP was cloned more than a decade ago (Gorin *et al.*, 1984), the physiological roles of the protein are still not understood. Detailed studies of the homologous proteins MIP (Ehring *et al.*, 1990), Nodulin-26 (Weaver *et*

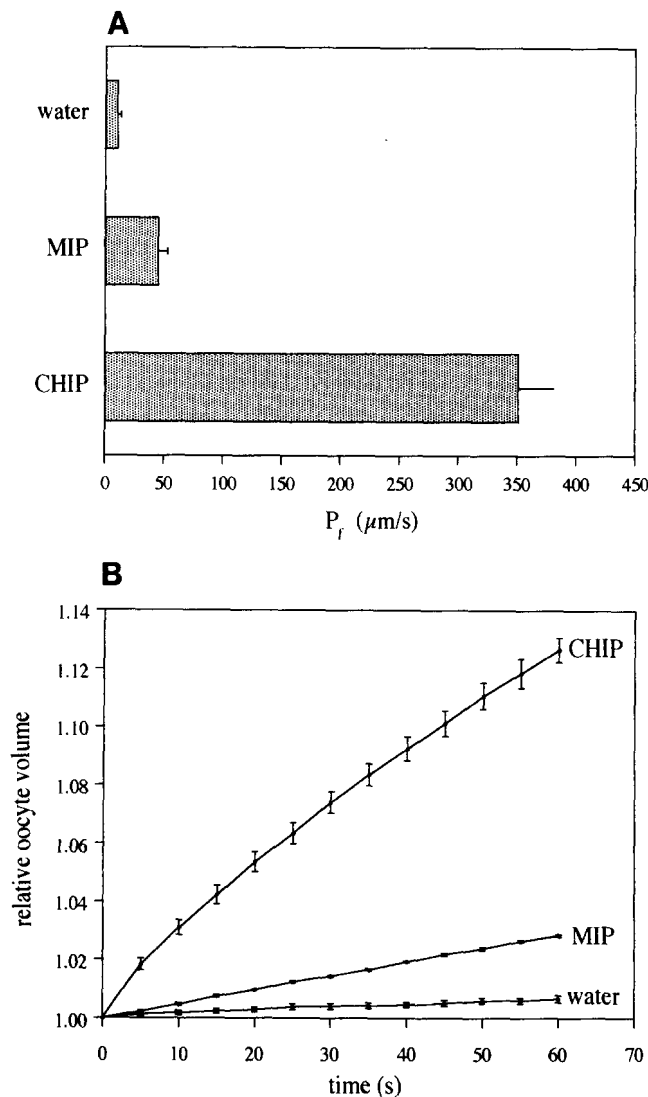


FIG. 4. A, increased osmotic water permeability of *Xenopus* oocytes expressing MIP and CHIP. Oocytes were injected with water or 10 ng of *in vitro* transcribed cRNA encoding MIP or CHIP. After 72 h,  $P_f$ s were determined by video microscopic measurement of the rate of swelling after transfer to hypotonic medium. Shown are the means  $\pm$  S.D. for 8 oocytes. B, time-dependent osmotic swelling of oocytes injected with water or 10 ng of cRNA encoding MIP or CHIP. Shown are the means from five traces  $\pm$  S.E.

*al.*, 1994), and preliminary studies with CHIP<sup>2</sup> revealed large voltage-dependent conductances when reconstituted into planar lipid bilayers. Nevertheless, the magnitude of the currents in bilayers containing MIP was far above the conductances measured in normal lens (Mathias *et al.*, 1979, 1991). Therefore, while the planar lipid bilayer studies are highly reproducible, their relevance to normal lens physiology remains uncertain. Moreover, the studies reported here (Fig. 3) and preliminary studies of other investigators (Kushmerick *et al.*, 1994) failed to confirm ion conductance by MIP expressed in oocytes.

Soon after the discovery that CHIP is a molecular water channel (Preston *et al.*, 1992), several labs evaluated MIP and other homologous proteins for osmotic water permeability. One group reported that MIP is not a water channel (Verbavatz *et al.*, 1994), but apparently these investigators used the same techniques with which they failed to detect osmotic water per-

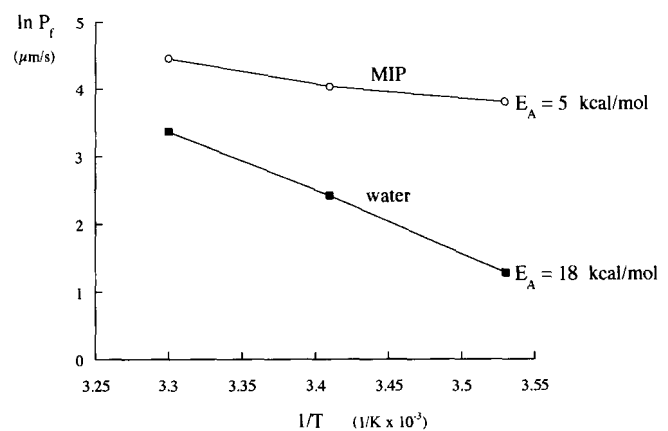


FIG. 5. Arrhenius activation energy ( $E_A$ ) of osmotic water permeabilities of control oocytes and oocytes expressing MIP. The  $E_A$  was determined by measuring the  $P_f$  at 10, 20, and 30 °C. Control oocytes were injected with water and MIP oocytes were injected with 25 ng of MIP cRNA. In this experiment, each point represents 7–10 control oocytes or 11–15 MIP oocytes.

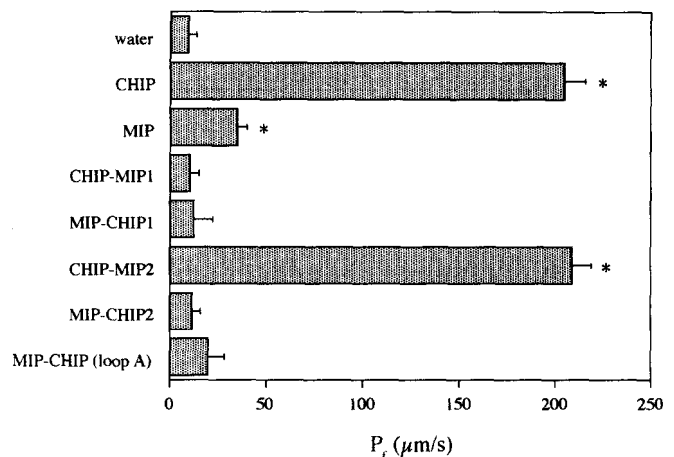


FIG. 6. Osmotic water permeability of oocytes expressing chimeric MIP-CHIP proteins.  $P_f$  was determined from oocytes injected with water or 10 ng of cRNA encoding MIP, CHIP, or the indicated MIP-CHIP chimeric proteins. Shown are the means  $\pm$  S.D. of five oocytes. \*,  $p < 0.05$  compared with water-injected control oocytes.

meability of AQP3 (Ma *et al.*, 1994), a protein found by two other groups to exhibit high  $P_f$  comparable with the other Aquaporins (Ishibashi *et al.*, 1994; Echevarria *et al.*, 1994). The studies reported here document that oocytes expressing MIP exhibit osmotic water permeabilities 4–5-fold above control oocytes (Fig. 4) with activation energies identical to the Aquaporins (Fig. 5). These observations are supported by preliminary studies of other investigators who also found an increase in  $P_f$  of oocytes expressing frog MIP (Kushmerick *et al.*, 1994) or bovine MIP (Chandy *et al.*, 1995). MIP may contribute to the maintenance of lens transparency by enhancing uptake of intercellular water by adjacent lens fiber cells. The narrow geographic separation of lens fiber cells (which contain MIP) and lens epithelial cells (which contain CHIP) suggests functional cooperativity. Osmotic gradients provide the driving force for Aquaporin-mediated water transport in kidney and most other tissues (Nielsen *et al.*, 1993a, 1993b). It is likely that hydrostatic forces move water through CHIP in endothelium of the proximal capillary bed (Nielsen *et al.*, 1993b), and a related process may occur through MIP when the lens shape is rapidly altered by contraction of muscles in the ciliary body to provide fine focus of the corneal image upon the retina.

A molecular explanation for why MIP is a weaker water

<sup>2</sup> J. Hall and P. Agre, unpublished observations.



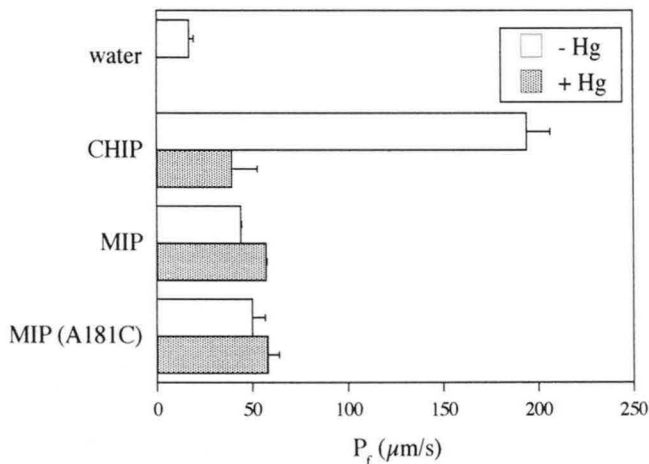


FIG. 7. Osmotic water permeability and  $\text{Hg}^{2+}$  inhibition of oocytes expressing CHIP, MIP, or MIP (A181C). Oocytes were injected with 1 ng of CHIP cRNA or 10 ng of MIP cRNA or 10 ng of MIP(A181C) cRNA.  $P_f$  was determined without pretreatment (open bars) or after 5 min in buffer containing 3 mM  $\text{HgCl}_2$ , followed by swelling in the presence of  $\text{HgCl}_2$  (solid bars). Shown are the means  $\pm$  S.D. of five oocytes.

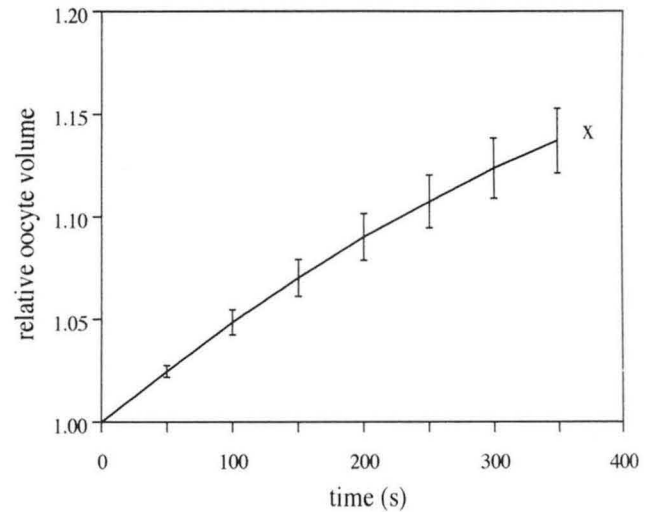


FIG. 9. Time-dependent osmotic swelling of oocytes expressing MIP. 3 days after injection of 25 ng of MIP cRNA, oocytes were transferred to 20 mosM modified Barth's solution, and swelling was recorded until the oocyte burst (X). Shown are the means from 7 traces  $\pm$  S.E.

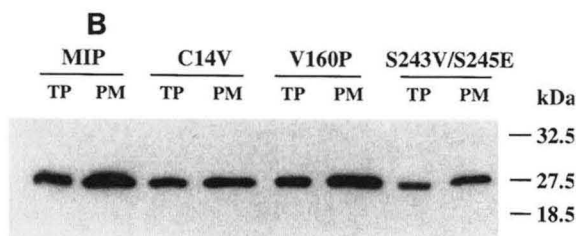
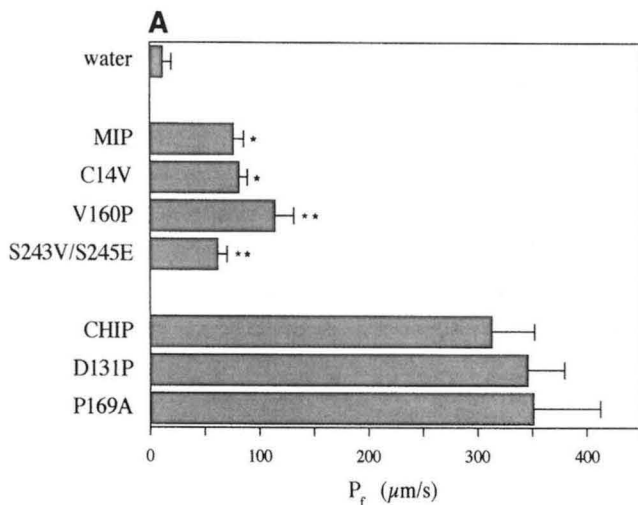


FIG. 8. Osmotic water permeability of oocytes expressing MIP and CHIP mutants. A, oocytes were injected with water or 25 ng of cRNA encoding MIP, MIP(C14V), MIP(V160P), MIP(S243V, S245E), or 5 ng of cRNA encoding CHIP, CHIP(D131P), or CHIP(P169A). The  $P_f$  was measured 3 days after injection. Shown are the means  $\pm$  S.D. \*,  $p < 0.05$  compared to water-injected oocytes, \*\*,  $p < 0.05$  compared with wild-type MIP. B, immunoblot comparison of total protein (TP, equivalent of 0.2 oocytes) or plasma membranes (PM, equivalent of 20 oocytes) from oocytes expressing MIP or the indicated MIP mutants. SDS-PAGE immunoblot was incubated with a polyclonal antibody specific for the carboxyl terminus of MIP.

transporter than the other Aquaporins was sought since the amino acid sequences of MIP and the Aquaporins are strikingly similar (Preston and Agre, 1991; Reizer *et al.*, 1993) (Fig. 10).

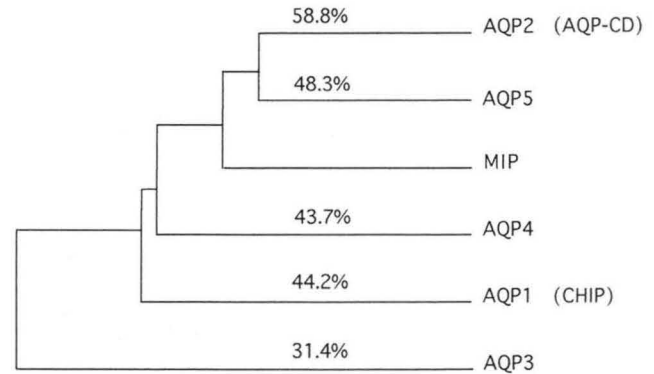


FIG. 10. Evolutionary tree of MIP and the mammalian Aquaporins. The percentage of amino acid identity between MIP and the five mammalian Aquaporins is indicated.

MIP was expressed well in oocytes and was targeted to the plasma membrane (Fig. 2). Neither the phosphorylation sites nor the other residues in the carboxyl-terminal cytoplasmic domain of MIP appeared to function as restraints (Figs. 6 and 8), and several different residues and domains from CHIP were spliced into MIP without increasing the  $P_f$  (Figs. 6–8). Despite introduction of an *N*-glycosylation site, MIP-CHIP (loop A) was still not glycosylated, and introduction of the potential mercurial inhibition site in MIP (A181C) failed to confer sensitivity (Fig. 7). Likewise, when a cysteine was introduced into the mercury-insensitive AQP4, the recombinant protein also failed to demonstrate mercury-inhibitable  $P_f$  (Jung *et al.*, 1994b), implying that MIP and AQP4 are structurally different from CHIP near the extracellular side of the aqueous pore. Nevertheless, substitution of proline in the 5th bilayer-spanning domain yielded a 50% increase in  $P_f$  (Fig. 8). This intriguing gain of function suggests that simple alterations in the bilayer-spanning domains can alter the conformation of MIP to more closely resemble CHIP. No loss of function was detected in the corresponding substitution (P169A) in CHIP; however, a small decrease in a high  $P_f$  should be more difficult to measure than a comparable increase in a low value. An alternative explanation may be that MIP requires a specific membrane organization, such as formation of orthogonal arrays, or a particular membrane environment that may be poorly reproduced in the oocyte expression system. For example, oocytes do not have

plasma membranes with wavy junctions similar to lens fiber cells (Zampighi *et al.*, 1989), and if this is important to the function of MIP, the water permeability studies may yield spuriously low values in oocytes.

Similar to other bilayer-spanning proteins, MIP may have multiple physiological functions, and it is possible that the primary role of MIP is not water transport. The red cell band 3 protein is the membrane anion exchanger (AE1), a cytosolic regulator of glycolytic enzyme activity, and the structurally important attachment site for ankyrin on the membrane (for review, see Low (1986)). MIP may also have a structural function, since the protein has been shown to enhance adhesion with membranes containing negatively charged phospholipids (Michea *et al.*, 1994). Also, it is known that some proteins are expressed in lens where their function is unrelated to their functions in other tissues (*e.g.* crystallins, for review, see Piatigorsky and Wistow (1989) and De Jong *et al.* (1994)). Thus the extremely high expression of MIP in lens may be far above the level needed for water permeability, since the abundance may be needed for an unrelated function.

A mutation has been identified in mice that may provide insight into other potential functions of MIP, since these mice develop cataracts prior to birth (Mugleton-Harris *et al.*, 1987). The *cat* mouse mutation results in lower abundance of MIP mRNA with the major transcript being truncated, and MIP was not detectable in lens by immunocytochemistry (Shiels and Griffin, 1993). The mutation is expressed as a dominant trait and has been mapped to the distal end of chromosome 10 (Mugleton-Harris *et al.*, 1987), coincident with the MIP locus (Griffin and Shiels, 1992). Mutations in genes encoding structural proteins usually produce dominantly inherited disorders, whereas mutations in transporters such as the CFTR are recessively inherited. Consistent with this, mutations in the AQP2 gene were recently identified in homozygotes and a compound heterozygote with severe nephrogenic diabetes insipidus, while the heterozygous relatives were unaffected (Deen *et al.*, 1994; van Lieburg *et al.*, 1994). Careful histological analysis of the early stages of disease in the *cat* mouse may provide clues to the critical function of MIP, which is the first defect leading to the development of cataracts in this animal model.

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