Mouse Cx50, a Functional Member of the Connexin Family of Gap Junction Proteins, is the Lens Fiber Protein MP70

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The crystalline lens is an attractive system to study the biology of intercellular communication; however, the identity of the structural components of gap junctions in the lens has been controversial. We have cloned a novel member of the connexin family of gap junction proteins, Cx50, and have shown that it is likely to correspond to the previously described lens fiber protein MP70. The N-terminal amino acid sequence of MP70 closely matches the sequence predicted by the clone. Cx50 mRNA is detected only in the lens, among the 12 organs tested, and this distribution is indistinguishable from that of MP70 protein. A monoclonal antibody directed against MP70 and an anti-Cx50 antibody produced against a synthetic peptide identify the same proteins on western blots and produce identical patterns of immunofluorescence on frozen sections of rodent lens. We also show that expression of Cx50 in paired *Xenopus* oocytes induces high levels of voltage-dependent conductance. This indicates that Cx50 is a functional member of the connexin family with unique physiological properties. With the cloning of Cx50, all known participants in gap junction formation between various cell types in the lens are available for study and reconstitution in experimental systems.

INTRODUCTION

The vertebrate eye lens provides an attractive system to study the establishment, regulation, and function of intercellular communication through gap junction channels. The lens is an organ containing two cell types, an anterior epithelium and specialized lens fibers, with a zone of differentiating cells at the equator. Intercellular communication occurs between epithelial cells, between fiber cells, as well as between the epithelium and the fibers. It has been proposed that intercellular communication allows the metabolically active epithelium to maintain precise intracellular ionic conditions for the fibers, necessary to prevent precipitation of fiber proteins and cataract formation (for review, see Rae, 1979).

There are structural, biochemical, and physiological differences in the gap junctions connecting the different cell types in the lens. Gap junctions between epithelial cells appear in conventional freeze-fracture as crystallized arrays (Peracchia, 1978; Goodenough *et al.*, 1980). Epithelial junctions contain connexin 43 (Cx43), a

member of the connexin family of gap junction proteins (Beyer *et al.*, 1987, 1989; Musil *et al.*, 1990), and communication in the epithelium is sensitive to cytoplasmic acidification (Schuetze and Goodenough, 1982). In contrast, the freeze-fracture appearance of lens fiber junctions is not ordered, these junctions do not contain detectable Cx43, and communication between the fibers is not sensitive to acidification in some species (Benedetti *et al.*, 1974, 1976; Goodenough, 1979; Schuetze and Goodenough, 1982; Musil *et al.*, 1990).

Although the structural components of the lens fiber junctions have not been completely characterized, they do contain Cx46, another member of the connexin family (Paul *et al.*, 1991). In addition, the fiber junctions contain the lens-specific protein MP70 (Kistler *et al.*, 1985), which was identified by immunocytochemical localization using a specific monoclonal antibody (Gruijters *et al.*, 1987). N-terminal sequence obtained from ovine MP70, although degenerate in three positions, indicated that it was related to other connexins (Kistler *et al.*, 1988). Although one possible ovine MP70

Table 1. Specific connexin PCR primers

Connexin	Primer sequences	Reference
Cx26	5'-CGGAAGTTCATGAAGGGAGAGAT-3' 3'-ACGAGTCCCTTCAGGTTTCTGG-5'	Zhang and Nicholson, 1989
Cx31.1	5'-TATACCCTCCCGTCTATGGTC-3' 3'-TTTGGTAAGACACT-5'	Haefliger <i>et al.</i> , 1992
Cx32	5'-CTGCTCTACCCGGGCTATGC-3' 3'-TTCTCGCTGGCTACGAGTCGGAC-5'	Paul, 1986
Cx33	5'-AAGCATATCAAGTGTTGGC-3' 3'-CTCTTGCTTGGTACA-5'	Haefliger <i>et al.</i> , 1992
Cx37	5'-GGCTGGACCATGGAGCCGGT-3' 3'-CGGGGGGTCCACCGGTCTT-5'	Haefliger <i>et al.</i> , 1992
Cx40	5'-CTGGCCAAGTCACGGCAGGG-3' 3'-GGAGTCCCGATGGTGTCACTGTT-5'	Haefliger <i>et al.</i> , 1992
Cx43	5'-TACCACGCCACCACCGGCCCA-3' 3'-AAGGGACTGCTGTCTGGTTTACGG-5'	Beyer <i>et al.</i> , 1987
Cx46	5'-GGAAAGGCCACAGGGTTCTCTGG-3' 3'-GGCAACCAGGAGGACCTGGG-5'	Paul <i>et al.</i> , 1991

sequence precisely matches rat Cx46, it is clear that Cx46 and MP70 are different proteins that can be distinguished with specific antibodies (Paul *et al.*, 1991).

The structural components of lens gap junctions must be fully characterized to understand how their different functional properties contribute to organ homeostasis. To further characterize lens fiber junctions, we have searched for DNA clones encoding MP70 using Cx46 cDNA to probe a genomic library. A novel mouse connexin was cloned, Cx50, that appears to encode MP70. This relationship is established by five criteria. First, the N-terminal amino acid sequence of MP70 closely matches the sequence predicted by the clone. Second, the distribution of Cx50 mRNA is indistinguishable from that of MP70 protein. Third, antibodies raised against a Cx50 synthetic peptide identify the same proteins as the anti-MP70 monoclonal antibody on western blots of lens membranes. Fourth, the monoclonal antibody prepared against MP70 cross-reacts with Cx50 synthesized *in vitro*. Fifth, the anti-Cx50 and anti-MP70 antibodies produce identical patterns of immunofluorescence on frozen sections of rodent lens. We also show that expression of Cx50 in paired *Xenopus* oocytes induces high levels of characteristic voltage-dependent conductance. This indicates that Cx50 is a functional member of the connexin family with unique physiological properties.

MATERIALS AND METHODS

Screening of a Genomic Library

A mouse genomic library constructed in lambda phage EMBL-3 (Clontech, Palo Alto, CA) was screened at moderate stringency (Haefliger *et al.*, 1992) with a rat Cx46 probe containing the complete protein coding sequence (Paul *et al.*, 1991). Positive plaques were picked and lambda phage was eluted from each agar plug into 1 ml H₂O for 1 h at room temperature. Lambda phage DNA was isolated

for polymerase chain reaction (PCR) analysis by phenol extraction and ethanol precipitation. To eliminate clones containing previously identified connexins, phage DNA from positive plaques was subjected to PCR amplification using specific oligonucleotide primer sets corresponding to unique sequences of the rodent connexins (Table 1). Template DNA, which failed to yield a product with the specific primers, was then amplified using degenerate oligonucleotides corresponding to the highly conserved sequences encoding the extracellular domains of previously cloned rodent connexins (primers 2 and 3 of Haefliger *et al.*, 1992). To confirm the identification of novel connexins, the products of the second amplification were gel purified (GeneClean, BIO 101, Vista, CA), subcloned into the *EcoRV* site of pBluescript KS+, and sequenced on both strands (Sequenase, United States Biochemical, Cleveland, OH). Clones encoding novel connexins were purified through three additional rounds of replating. Phage DNA from the Cx50 clone was isolated (Helms *et al.*, 1985), and restriction analysis indicated that the connexin coding sequence was contained in a 3.8-kb *Bam*HI fragment that was subcloned into the *Bam*HI site of pBluescript KS+ and sequenced on both strands.

Northern and Southern Blotting

RNA was isolated by homogenization of different adult (8–10 wk) mouse organs in guanidine isothiocyanate followed by centrifugation through CsCl (Chirgwin *et al.*, 1979). RNA samples (10 µg) were electrophoresed on 1% agarose/formaldehyde/3-(N-morpholino)propanesulfonic acid gels and capillary blotted onto nylon transfer membranes (Hybond N+, Amersham, Arlington Heights, IL) in 2× SSC for 16 h (Maniatis *et al.*, 1982). Samples of mouse genomic DNA (5 µg, Clontech) were digested with various restriction enzymes, electrophoresed on 0.7% agarose/Tris-Acetate-EDTA gels, and blotted onto membranes in 0.4 M NaOH for 3 h. Nucleic acids were fixed to the membranes by incubation at 80°C for 1 h under vacuum. ³²P-labeled Cx46 or Cx50 probes were obtained by random priming (Boehringer Mannheim, Indianapolis, IN) in the presence of ³²P-dCTP (New England Nuclear, Boston, MA). Blots were hybridized in 0.7 M sodium phosphate, pH 7.2, 1 mM EDTA, 5% sodium dodecyl sulfate (SDS) at 65°C for 16 h and washed three times in 0.3 M sodium phosphate, pH 7.2, 1% SDS, for 30 min at 65°C (Paul, 1986). Southern blots were washed for an additional 30 min in 30 mM sodium phosphate, pH 7.2, 0.1% SDS at 65°C.

***In Vitro* Transcription and Translation of Cx50 mRNA**

The transcription vector SP64T (Krieg and Melton, 1984) was digested with *Bgl* II, filled with Klenow fragment, and dephosphorylated with calf intestinal alkaline phosphatase. A 1.4-kb *Bal* I/*Eco*RV fragment of the Cx50 genomic clone containing the entire open reading frame was gel purified using Gelase (Epicentre Technologies, Madison, WI) and subcloned into SP64T. Recombinant plasmid was linearized with *Bam*HI and used for the *in vitro* transcription of mRNA with SP6 RNA polymerase (Swenson *et al.*, 1989). *In vitro* synthesized Cx50 mRNA (50 ng) was translated in a rabbit reticulocyte lysate (New England Nuclear) in the presence of 80 mM potassium acetate, 0.65 mM magnesium acetate, and 25 μ Ci of 35 S-methionine (Musil *et al.*, 1990).

Production of Polyclonal Antibodies

A synthetic peptide corresponding to residues 124–136 of the predicted amino acid sequence of Cx50 (ERVPIAPDQASIR) was commercially synthesized (Multiple Peptide Systems, San Diego, CA) and coupled to bovine serum albumin (BSA) using glutaraldehyde as described by Goodenough *et al.* (1988). Antiserum was produced in rabbits and used without purification.

Western Blotting

Mouse or rat lenses were collected and homogenized on ice in 5 mM tris(hydroxymethyl)aminomethane-HCl, pH 8.0, 1 mM EDTA, 1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, containing 20 μ M phenylmethylsulfonyl fluoride and 10 μ g/ml leupeptin (lysis buffer). The homogenate was centrifuged at $100\,000 \times g$ at 4°C for 30 min, the membrane pellet resuspended in fresh lysis buffer, and the protein concentration determined using the BCA assay (Pierce Chemical, Rockford, IL). *Xenopus* oocytes were transferred to Eppendorf tubes and homogenized in 1 ml of lysis buffer by passage through a 20-gauge needle. The homogenate was precleared by centrifugation at $3000 \times g$ at 4°C for 5 min to pellet yolk granules. The supernatant was then centrifuged at $100\,000 \times g$ at 4°C for 30 min and the membrane pellet resuspended in fresh lysis buffer. Gel electrophoresis and western blotting were carried out as previously described (Paul *et al.*, 1991). Western blots were probed with the monoclonal antibody 6-4-B2-C6 (1:500 dilution of ascites fluid), which recognizes MP70 (Kistler *et al.*, 1985), or anti-Cx50 antiserum (1:500 dilution of unfractionated serum). Primary antibodies were detected with alkaline phosphatase conjugated goat anti-mouse Ig (Boehringer Mannheim) or goat anti-rabbit IgG (Promega, Madison, WI).

Immunocytochemistry

Embryonic rat (E18) and adult mouse lenses were fixed in 1% formaldehyde, freshly prepared from paraformaldehyde, in phosphate-buffered saline (PBS) for 1 h at 4°C. Fixed lenses were rinsed in PBS, embedded in OCT (Miles, Elkhart, IN), and frozen in liquid nitrogen. Seven-micrometer frozen sections were prepared and processed according to Paul *et al.* (1991). Sections were incubated with anti-Cx50 antiserum diluted 1:100 and anti-MP70 ascites fluid diluted 1:500, with 1% normal goat serum and 2% BSA in PBS, for 1 h at room temperature. Sections were washed in PBS and then incubated with FITC conjugated goat anti-mouse (Sigma, St. Louis, MO) and rhodamine conjugated goat anti-rabbit (Pierce). Fluorescence microscopy was performed using a Zeiss Axioskop (Zeiss, Thornwood, NJ) and recorded on Tmax 400 film (Kodak, Rochester, NY).

Injection of Xenopus Oocytes and Electrophysiological Measurements

Oocytes were collected from *Xenopus laevis* females and processed for the paired *Xenopus* oocyte expression assay essentially as described

(Swenson *et al.*, 1989). To eliminate the possible contribution of endogenous intercellular channels to the measured conductances, manually defolliculated oocytes were injected with an antisense oligonucleotide (Barrio *et al.*, 1991) corresponding to *Xenopus* Cx38 (Ebihara *et al.*, 1989) (3 ng/oocyte, 5'-CTGACTGCTCGTCTGTCCACACAG-3'). After incubation overnight at 18°C, antisense-treated oocytes were then injected with 40 nl of Cx50 mRNA (10–100 pg) or H₂O and paired for quantitation of junctional communication by double voltage clamp (Spray *et al.*, 1981). Both cells were initially clamped at –80 mV to ensure zero transjunctional potential. Although cell 1 in the pair was depolarized in 10-mV steps, cell 2 was held at –80 mV. The current delivered by the clamp to cell 2 during the voltage step is equal in magnitude but opposite in sign to the current flowing through the junctional channels. To calculate the conductance, the junctional current was divided by the trans-junctional voltage step.

RESULTS

Identification of Cx50

The primary goal of this study was to identify a DNA clone encoding the lens gap junction protein MP70. Based on the assumption that Cx46 and MP70 would be highly related (Kistler *et al.*, 1988; Paul *et al.*, 1991), a mouse genomic library was screened at moderate stringency with a rat Cx46 probe. As it was likely that previously identified connexins would also be detected by this procedure, novel connexin sequences were distinguished using a two-step PCR analysis. In the first step, each isolated phage DNA was subjected to PCR amplification in the presence of oligonucleotide primer sets corresponding to unique sequences present in rat connexins 26, 31.1, 32, 33, 37, 40, 43, and 46 (Table 1). Clones whose DNA amplified with one of the specific primer sets were considered to contain known connexins and eliminated from further processing. In the second step, template DNA that failed to amplify with the specific primers was amplified in the presence of degenerate primers corresponding to highly conserved sequences shared between rat connexins 26, 32, 43, and 46 (Haeffliger *et al.*, 1992). Template DNA that amplified with the degenerate primers should produce a DNA fragment encoding the highly conserved second and third trans-membrane sequences and the unique central cytoplasmic domain. Each PCR product was first subcloned and sequenced to confirm the identification of a new connexin, followed by sequencing of the genomic clone.

Screening of 300 000 plaques resulted in the selection of 46 positive clones. PCR analysis revealed that 41 of the positive clones contained previously described connexins. Five new connexin clones were identified, two of which encoded a novel connexin sequence with a predicted molecular mass of 49 602 Da that has been designated mouse Cx50.

Cx50 is a Member of the Connexin Family

Figure 1 shows a partial nucleotide sequence of a 3.8-kb *Bam*HI fragment encoding mouse Cx50. A single uninterrupted open reading frame begins at nucleotide 267 and ends at nucleotide 1589. The absence of introns in

GGATCCTTTCAACAACATGTGAGGTGAATGAGGACAGATGTTGTTAGTA 50
 ACCCTACTTTATAGATTAGTATTAACACACTTTAAGAGCCCTTTTCATCT 100
 CTTAGCAGTAGTCGGACAGATGAGGCATTTGATAGAAGCTGTTGGATAC 150
 TATGATTGTTCCATCAGTTCCAAAAGGAAAGTCACTCCAAGAGCTAGGAA 200
 AGAGATCATCTCAGAGTTGCACTGTGGCCAAATAGATTGCTTCTGCT 250
 TCCTTGGTAGTGACCAATGGGCGACTGGAGTTTCTGGGAAACATCTTGG 300
 AAGAGGTGAATGAGCAGTCCACTGTCACTCGGAGAGTCTGGCTCAGAGT 350
 CTCCTCATCTTCCGCATCTCATCTCGGACAGCAGCGAGTTGTGTG 400
 GGGCGATGAGCAATCTGATTTGTATGCAACACCCAGCAGCCAGGCTGTG 450
 AGAATGCTGTGATGAGGCTTTCCCATCTCACACATCCGCTCTG 500
 GTGCTGAGATCATCTTCTGCTCCACTCCATCGTGTACGTGGGGCA 550
 CGCGGTACACACGTTCCGATGGAGGAGAAGCGAAAGACCGTGAAGCTG 600
 AGGAGCTGTGACAGCAGTCGCGCAGCAACGGGGTGAGAGGTACCAATC 650
 GCCCCAGACAGGCGAGCATCGGAAGAGCAGCAGCAGTAGCAAAGGCAC 700
 CAAGAAGTTCGGCTGGAGGCGACATGCTAAGGACCTATGCTGCCACA 750
 TCATCTTCAAGACCTCTTTGAGGTGGGCTTCATCGTGGGCACTTACTCT 800
 CTGTATGGTTTCCGCATCTGCCCCCTCTATCGTGCAGCCGTTGGCCCTG 850
 CCCCAATGTGGTAGACTGCTTTGTATCCCGGCTACTGAGAAGACCATCT 900
 TCACTCTCTCATGTTATCATCGCTTTTGTGCTACTCTTCTCAACATCT 950
 ATGGAGATGAGCCACCTGGGATGAAAGAAATCCGCTGCTGCTTCAAGAG 1000
 GCCTGTAGAGCAACCACTGGGGGAGATTGCTGAGAAGTCCCTCCACTCCA 1050
 TTGCAGTTTCTCCATCCAGAAAGCCAAAGGCTACCACTTCTAGAAGAA 1100
 GAGAAGATCGTATCACACTATTTCCCTTTGACAGAGGTGGAATGTTGGA 1150
 GACCAGCCCTCTTTCCGCCAAGCCTTTTAGTCAGTTTGGAGAGAAGATCG 1200
 GCACAGACCCCTGGCAGATATGTCACGGAGTTACCAAGAAACCTGCTCT 1250
 TCTTATGCTCAGTGGGGTCCAGGAAGTGGAGCGGGAAGAGCCGCTAT 1300
 AGAAGAGGCTGTGGAACCGGAAGTGGGAGAGAAGCAAGAAGCAGAGA 1350
 AGGTGGCCCCAGAAGGGCAGGAGACAGTTGCACTGCCAGACAGGAGAGA 1400
 GTAGAGACCCCTGGAGTGGGAAGGAGGATGAGAAAGAAGAGCTGCAAGC 1450
 TGAAAAGGTAACCAAGCAAGGCTGTCTGCTGAGAAGCACCTCACTCT 1500
 GTCCGAGCTGACACCCGATGACAAATCGGCCCTTGAGCAGGCTGAGTAAA 1550
 GCCAGCAGCAGGCGAGGTGAGATGATCTCACCATAAGACCTGGTGCAGA 1600
 AGAAGATATCCCACTCCAGCCAATACAGACCAAGATAAACCAGCTGG 1650
 TGCCAATATACTTTGAGTCTTATGTGTCCTTGGTCCACCCCAACCCCTG 1700

Figure 1. Sequence of the genomic Cx50 clone. A portion of the 3.8-kb *Bam*HI fragment of the genomic clone was sequenced on both strands. A single uninterrupted open reading frame starting at nucleotide 267 and ending at nucleotide 1589 (bold, double underline) encoded a protein with a predicted molecular mass of 49 602 Da, which was named Cx50. This sequence is available in the GenBank database under the accession number M91243.

the coding sequence is a common feature of all connexins for which genomic DNA have been analyzed (Miller *et al.*, 1988; Zhang and Nicholson, 1989; Paul *et al.*, 1991; Haefliger *et al.*, 1992). Hydropathy analysis predicted four transmembrane domains, with cytoplasmic amino and carboxy termini. The transmembrane domains are highly homologous to those of other connexins, as are the two predicted extracellular domains. Each extracellular domain contains the characteristically spaced three cysteine residues found in all connexins. In contrast, the central and carboxy terminal cytoplasmic domains are unique. This pattern of conserved and unique structure is found in all connexins (for reviews, see Beyer *et al.*, 1990; Bennett *et al.*, 1991), and Cx50 represents a new member of the connexin family of gap junction proteins.

To examine the relationship of Cx50 to Cx46, their complete predicted amino acid sequences were aligned (Figure 2A). Cx50 shares 43% sequence identity with Cx46 over the entire amino acid sequence. When the sequence comparison is restricted to the first 230 amino acids of Cx50, eliminating the carboxy terminus that is highly divergent among all connexins (Haefliger *et al.*, 1992), the proteins share 68% identity. In contrast, this

region of Cx50 shows only 42–60% identity with all other cloned rat connexins, demonstrating that Cx50 is most related to Cx46.

Comparison of the amino terminal protein sequences predicted for Cx46 and Cx50 with the amino terminal sequence obtained from ovine MP70 (Kistler *et al.*, 1988) reveals the relationships between these different proteins. Cx50 and Cx46 both have predicted amino termini that are very similar to the MP70 sequence (Figure 2B). Residues 8, 9, and 13 of the ovine MP70 sequence are degenerate, each containing equimolar amounts of two amino acids. Mouse Cx50 is 90% identical to one of the six possible MP70 sequences, matching the three amino acids not present in the Cx46 sequence at each degenerate position. It clearly has been shown that Cx46 and MP70 are two discrete lens fiber proteins, distinguishable with specific antibodies in both rat and bovine lenses (Paul *et al.*, 1991). The amino terminal identity between Cx50 and MP70 is significantly higher than

A

Cx50	MGDWSFLGNILEEVNEHSTVIGRVMLTVLFIFRILILGTAEEFVWGDEQSD	51
Cx46	MGDWSFLGRLLLENAQEHSTVIGRVMLTVLFIFRILVLGAAAEVWGDEQSD	51
Cx50	FVCNTQQPGCENVCYDEAFPIHSHIRLMVLQIIFVSTFSLMYVGHVHHVRM	102
Cx46	FTCNTQQPGCENVCYDRAFFISHIRFMALQIIFVSTPTLIYLGHVHLIHRM	102
Cx50	BEKKRDREBELCQQSRNNGGERVPIAPDQASIRKSSSSSGTKKFRLEG	153
Cx46	BEKKKEREELLRDNPPQHGRGREPM---RTGSPDRPPLRDRGKVRIG	150
Cx50	LLRTYVCHIIFKTLFEVGFIVGHYFLYGRIFLPLRYCSRWPCPNVDFVS	204
Cx46	LLRTYVFNIIFKTLFEVGFIVGHYFLYGRIFLPLRYCDRWPCNTVDFIS	201
Cx50	RPTEKTFIFLFLMSVAFVSLFLNIMEMSHLGMKIRSAFVRPVEQPLGEIA	255
Cx46	RPTEKTFIVIFMLAVACASLVNLMLEIVHLGKWLKQGVNTHNFPDASEVR	252
Cx50	EKSLHSIAVSSIQKAGYQLLEEKIVSHYFPLTEVGMVETSPLSAKPFQS	306
Cx46	HKPLDPLSEANSGPPSVS-----IGLPPYTHPACPTVQKATGFPFGAPL	298
Cx50	FEKIGTGPLADMSRSYQETLPSYAVQGVQEVERBEPPIEEAVEPEVGEKK	357
Cx46	LPADFTVTVLND---AQGRGHVPKHCNGHHLTTQGNWASLGAEPTQPAK	345
Cx50	QEAKEVAPEQGETVAVPDRERVETPGVKGDEKBEELQAEKVTKQGLSAEKA	408
Cx46	PSSAASSPHGRKGLTDSGSSSLRESALVVTPGEQALATTV-----EMHS	390
Cx50	PSLCPFLTDDNRPLSRLSKASSRARSDDLTI	440
Cx46	PPL---VLLDPEPS---SKSSSGRARPGDLAI	416

B

Cx46	MGDWSFLGRLLLENAQEHSTVI..
MP70	-GDWSFLG^{RL}_{NI}LEN^A_VQEHSTVI..
Cx50	MGDWSFLGNILEEVNEHSTVI..

Figure 2. Comparison of amino acid sequence of lens connexins. Identical amino acids are indicated in bold type. (A) Alignment of the entire predicted amino acid sequences for Cx50 and Cx46 demonstrates that they are highly related. The two lens connexins show 43% identity over the entire amino acid sequence, and Cx50 exhibits all the characteristic structural features of connexin family members. Extracellular domains (double underline) are highly conserved, each containing the three conserved cysteine residues found in all connexins. Transmembrane domains and amino termini (single underline) are also well conserved, but the major cytoplasmic domains (no underline) are unique. (B) Alignment of the predicted amino termini of Cx46 and Cx50 with the amino terminal sequence of MP70. The MP70 sequence is degenerate at three positions. Cx50 is 90% identical to one possible MP70 sequence, whereas Cx46 exactly matches a second possible MP70 sequence.

the level of amino terminal identity between connexins in general.

Cx50 is a Single Copy Gene Whose mRNA is Only Detected in the Lens

Southern blot analysis was performed on mouse genomic DNA digested with various restriction enzymes. The blot was hybridized with a 965-bp probe corresponding to the first two-thirds of the Cx50 coding region and washed under conditions of high stringency (Figure 3). Sequence analysis predicted a *Kpn* I site at base 477 of the probe sequence, and digestion with this enzyme resulted in two hybridizing bands. Digestion with all other enzymes tested yielded single bands. These data are consistent with Cx50 being present as a single copy gene in the mouse genome and containing the complete coding region in a single exon.

To determine the tissue distribution of Cx50 expression, a northern blot containing total RNA isolated from adult mouse brain, heart, kidney, lens, liver, lung, ovary, pancreas, skin, spleen, stomach, and testis was prepared. The blot was hybridized with a probe prepared identically to that used for Southern analysis. A single major band with a mobility of ~8.5 kb was detected in lens RNA, but not in RNA from any of the other organs tested (Figure 4). MP70 has been immunologically shown to be restricted to lens fiber cells, with no detectable signal in brain, heart, or liver (Kistler *et al.*, 1985). Therefore, the expression pattern of Cx50 is indistinguishable from the immunolocalization of MP70.

Figure 3. Southern blot analysis suggests Cx50 is encoded by single copy gene with no introns in the coding region. Ten-microgram aliquots of mouse genomic DNA, digested with various restriction enzymes, were probed with a portion of the Cx50 genomic clone corresponding to base 170 to base 1135 of the sequence in Figure 1. Sequence analysis predicted a *Kpn* I site within the region covered by the probe (base 647), and digestion with this enzyme yielded two hybridizing bands. Digestion with all other enzymes tested yielded single hybridizing bands. The migrations of DNA standards are indicated on the left in kilobases.

Figure 4. Northern blot analysis. Ten micrograms of total RNA, isolated from different adult (8–10 wk) mouse organs, was loaded in each lane. The blot was probed with a 965-bp fragment of Cx50 identical to that used for Southern analysis. An ~8.5-kb transcript was detected in mouse lens RNA, whereas no signal was observed in any of the other organs tested. The migrations of RNA standards (GIBCO, Grand Island, NY) are indicated on the left in kilobases.

Anti-Cx50 and Anti-MP70 Antibodies Recognize the Same Protein on Western Blots

To determine if Cx50 and MP70 are the same protein, a polyclonal antiserum raised against Cx50 and a monoclonal antibody directed against MP70 were used for western blot analysis of rodent lens membranes. The monoclonal anti-MP70 (6-4-B2-C6) (Kistler *et al.*, 1985) was produced against sheep lens MP70. The epitope recognized by this antibody has been determined to be located near the C-terminal of the protein (Kistler and Bullivant, 1987). To ensure that antibodies against Cx50 would recognize a different epitope, a synthetic peptide corresponding to the central cytoplasmic domain between the putative second and third transmembrane segments (Goodenough *et al.*, 1988) was prepared and used to immunize rabbits.

In rat lens membranes, the anti-MP70 monoclonal antibody recognizes three bands with apparent molecular masses of ~63–65 kDa (Figure 5, "anti-MP70"). The middle band is the most intensely labeled. The anti-Cx50 antibody recognizes the same set of bands (Figure 5, "anti-Cx50") and also labels the middle band most intensely. In addition, the anti-Cx50 antibody shows a weak reaction with several bands that migrate much faster, presumably proteolytic products that are not labeled by the anti-MP70 antibody because of the location of the epitope it recognizes.

Figure 5. Antibodies directed against MP70 and Cx50 recognize the same proteins in rat lens membranes. Total membrane proteins from adult rat lenses were separated by SDS-PAGE and transferred to nitrocellulose. Companion blots were probed with either the anti-MP70 monoclonal antibody 6-4-B2-C6 or a rabbit anti-Cx50 antibody produced against a synthetic peptide corresponding to amino acids 124–136. Three bands in the range of ~63–65 kDa are recognized by both antibodies. Molecular mass markers are indicated in kilodaltons.

To ascertain if the protein encoded by the Cx50 clone could be recognized by the anti-MP70 antibody, Cx50 was expressed in *Xenopus* oocytes by injection of synthetic mRNA. A western blot (Figure 6) of membranes isolated from rat lens, mouse lens, and oocytes injected with either Cx50 mRNA or water was probed with the 6-4-B2-C6 antibody. The profile of immunoreactive bands is slightly different in mouse and rat lens membranes; the antibody recognizes a doublet in mouse and a triplet in rat. A single band of ~63 kDa was detected in Cx50 mRNA-injected oocytes. This band clearly migrates faster than the major band detected in native lens membranes of either species. The relative electrophoretic mobilities of the native and oocyte-synthesized protein were verified by loading in vitro synthesized, ³⁵S-methionine-labeled Cx50 in the same lane as the native membranes. Subsequent autoradiography of the western blot confirmed the relationships outlined above (Figure 6, "In Vitro Cx50"). No signal was detected in water-injected oocytes.

Cx50 is Present in Lens Fibers, but not Epithelium, and is Coincident with MP70

If MP70 and Cx50 are the same gene product, then they should occupy the same cellular location. Double-label immunofluorescence microscopy was used to examine the distribution of Cx50 and MP70 in the same sections of rodent lens. An example of embryonic rat lens containing parts of the iris, lens epithelium, and cross-sectioned fibers is shown in Figure 7, A–C. Both anti-MP70 (7A) and anti-Cx50 (7B) antibodies produce strong punctate, or macular staining between lens fibers, consistent with the known distribution of gap junctions in

these cells. No specific signal is detected in the iris and epithelium. Longitudinally sectioned fiber cells from an adult mouse lens are displayed in Figure 7, D–F. The punctate staining patterns produced by the two antibodies are completely coincident (anti-MP70, 7D; anti-Cx50, 7E).

Cx50 Induces Intercellular Communication in Paired *Xenopus* Oocytes

The ability of Cx50 to form intercellular channels was tested in the paired *Xenopus* oocyte expression system (Dahl *et al.*, 1987; Swenson *et al.*, 1989). After injection of an antisense oligonucleotide to *Xenopus* Cx38 to eliminate the possible contribution of this endogenous connexin to observed conductances (Barrio *et al.*, 1991), oocytes were then injected with either Cx50 mRNA or water and paired for electrophysiological analysis of junctional conductance. Table 2 presents a summary of conductance data for Cx50. Antisense-treated oocyte pairs that were injected with water were not electrically coupled (<0.01-μS conductance) when tested 24–48 h

Figure 6. Cx50 expressed in oocytes is recognized by the anti-MP70 monoclonal antibody. Membranes were isolated from rat lens, mouse lens, Cx50 mRNA-injected oocytes, and H₂O-injected oocytes. The proteins were separated by SDS-PAGE and transferred to nitrocellulose. The western blot was probed with the anti-MP70 monoclonal antibody 6-4-B2-C6. The antibody recognized multiple bands of ~63–65 kDa in mouse and rat lens membranes. A single band was detected in Cx50-mRNA injected oocytes. No immunoreactivity was observed in H₂O-injected oocytes. Cx50 mRNA was translated in a reticulocyte lysate (In Vitro Cx50) in the presence of ³⁵S-methionine, separated by SDS-PAGE, and transferred to nitrocellulose and detected by autoradiography. In vitro synthesized Cx50 has an electrophoretic mobility identical to Cx50 expressed in oocytes but faster than the most intensely labeled band in lens membranes. Molecular mass markers are indicated in kilodaltons.

Figure 7. Patterns of immunofluorescence labeling produced by anti-MP70 and anti-Cx50 antibodies are indistinguishable. (A–C) Seven micron fixed-frozen section of embryonic (E18) rat lens displaying cross-sectioned fibers, lens epithelium, and iris. (D–F) Adult mouse lens displaying longitudinally sectioned fibers. Staining patterns produced by anti-MP70 antibodies (A and D) and anti-Cx50 antibodies (B and E) are punctate and coincident. For both antibodies, labeling is only detected in the lens fibers. Phase contrast images are shown in C and F. Bar, 20 μm .

later ($n = 12$). In contrast, antisense treated oocyte pairs that were injected with Cx50 mRNA were highly coupled after 24–48 h of incubation ($19.4 \pm 5 \mu\text{S}$, $n = 12$). Cx50 appears therefore to be a functional member of the connexin family of gap junction proteins because it

can induce junctional conductance between paired oocytes.

It previously has been shown that some mammalian connexins can interact with endogenous connexins present in *Xenopus* oocytes to form intercellular channels

Table 2. Summary of Cx50-induced communication

Oocyte injection cell1/cell2	Conductance ^a (μ S)	No. experiments ^b
Cx50/Cx50	19.4 ± 5	3 (12)
H ₂ O/H ₂ O	0.0	3 (12)
Cx50/H ₂ O	0.0	1 (4)
Cx43/H ₂ O	8.5 ± 2	1 (3)

^a Values are means $\pm$ SE.^b Values in parentheses are number of pairs.

(Swenson *et al.*, 1989). To determine if Cx50 has this property as well, Cx50 or Cx43 injected oocytes were paired with water-injected oocytes that had not received the antisense oligonucleotide. Table 2 summarizes that Cx50 was unable to induce cellular communication when expressed in only one cell of the pair ($n = 4$), unlike Cx43, which interacted with *Xenopus* connexins to form large conductances in oocyte pairs ($8.5 \pm 2 \mu$ S, $n = 3$).

The relationship between trans-junctional voltage (V_j) and junctional current (I_j) illustrated that Cx50 forms voltage-dependent intercellular channels (Figure 8). At trans-junctional potentials ≥ 30 mV, a time-dependent decrease in I_j to equilibrium values > 0 was observed. Complete closure of intercellular channels was not observed even at the highest V_j tested (80 mV).

DISCUSSION

We have cloned DNA encoding a novel mouse connexin likely to correspond to the protein MP70, a previously identified component of lens fiber gap junctions. The five criteria that establish this relationship are 1) that the N-terminal amino acid sequence of MP70 closely matches the sequence predicted by the clone; 2) the distribution of Cx50 mRNA is indistinguishable from that of MP70 protein; 3) antibodies raised against a Cx50 synthetic peptide identify the same proteins as an anti-MP70 monoclonal antibody on western blots of lens membranes; 4) a monoclonal antibody prepared against MP70 cross-reacts with Cx50 synthesized in *Xenopus* oocytes; and 5) double-label immunofluorescence demonstrates that Cx50 and MP70 are coincident in lens fibers.

There are discrepancies between the size of Cx50 predicted by the DNA sequence, the size synthesized in oocytes, and the size observed in native lenses. Differences between the electrophoretic mobility and molecular size predicted by DNA analysis have been reported for Cx32 and Cx26 and are due to anomalous migration in SDS gels (Paul, 1986; Green *et al.*, 1988; Zhang and Nicholson, 1989). This appeared to be the

case for Cx50 as well, because Cx50 synthesized in a reticulocyte lysate migrated as an ~ 63 -kDa, rather than a 50-kDa, protein. However, the mobility of Cx50 synthesized in vitro or in *Xenopus* oocytes is slightly faster than the major band detected in rodent lenses. Therefore, anomalous SDS-polyacrylamide gel electrophoresis migration does not explain all of the discrepancy between predicted size and mass observed in native lens membranes. This difference may reflect posttranslational processing of Cx50 in the lens fiber, such as phosphorylation, not supported in the oocyte or reticulocyte lysate. Such processing could also explain the presence of multiple bands in native membranes. Post-translational modifications resulting in shifts to slower electrophoretic mobilities are seen with other connexins. For example, the mobilities of Cx43 and Cx46 are thought to be dependent on the state of phosphorylation (Musil *et al.*, 1990; Laird *et al.*, 1991; Paul *et al.*, 1991).

Paired *Xenopus* oocytes injected with Cx50 develop high conductances, demonstrating that this connexin can form intercellular channels. In paired oocytes, intercellular channels composed of Cx50 have physiological properties distinct from those composed of Cx43 and Cx46, the other connexins present in the lens. Cx50 induces voltage-dependent intercellular channels, whereas intercellular channels formed from Cx43 show little or no voltage dependence (Swenson *et al.*, 1989). Voltage dependence of Cx46 has not been examined, however, in contrast to connexins 50 and 43. Cx46

Figure 8. Effect of transjunctional voltage (V_j) on the junctional current (I_j) developed by oocyte pairs injected with Cx50 mRNA. Voltage steps of 10, 30, 50, and 70 mV are shown for a cell pair that initially displayed 13.8μ S of conductance. At transjunctional voltages > 20 mV, the junctional current decreased over the time of the voltage step (50 s). As the voltage steps increased in magnitude, the decrease in junctional current occurred more rapidly.

expression in oocytes leads to the formation of channels in the nonjunctional plasma membrane that cause cell depolarization and eventual lysis (Paul *et al.*, 1991). Voltage dependence of gap junctions in the lens has not been characterized *in vivo*.

There are multiple pathways of communication between cells in the lens. Cell-to-cell communication in the epithelium involves Cx43, whereas the fiber-to-fiber intercellular channels contain both Cx46 and Cx50. However, it is not known which connexins are involved in the epithelium to fiber communication pathways (Goodenough *et al.*, 1980; Rae and Kuszak, 1983; Miller and Goodenough, 1986). It remains to be determined if epithelium-fiber junctions contain Cx43 in the epithelial cells and Cx46 or Cx50 in the lens fibers. Alternatively, small amounts of Cx46 or Cx50 could be synthesized by the epithelium, or Cx43 could be present in the fibers. Immunohistochemical studies in the chick lens have demonstrated that Cx43 is expressed by differentiating lens fibers, but this connexin ceases to be detected in the mature fibers (Musil *et al.*, 1990). Although it is not known if there are additional connexins expressed in the lens, the availability of DNAs encoding these connexins which join the lens cells *in situ* offers the opportunity to reconstitute and study lenticular communication pathways.

There is a difference in the pH sensitivity of the gap junctions which join the epithelial cells, the superficial fibers, and the mature lens fibers. Schuetze and Goodenough (1982) demonstrated in the chick embryo that as lens fibers differentiate and acquire the noncrystalline morphology of the mature lens fiber junctions, intercellular communication becomes insensitive to acidification of cells with CO₂. Similarly, Miller and Goodenough (1986) demonstrated that dye transfer could be blocked in stage 24 chick lens epithelial cells by superfusion with 100% CO₂, whereas dye transfer between the underlying lens fibers and between the fibers and the epithelium was not detectably affected. These observations show some variability between species (Bassnett and Duncan, 1988; Emptage *et al.*, 1992) and age of the lens fibers (Mathias *et al.*, 1991). Communication between oocytes expressing Cx43 has been shown to be pH sensitive (Swenson *et al.*, 1989), in agreement with lens epithelial physiology *in situ*. With the availability of reagents specific for connexins joining the lens cells, the pH dependence of intercellular channels composed of various combinations of connexins 43, 46, and 50 in the *Xenopus* oocyte system are directly available for study.

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