Connexin46, A Novel Lens Gap Junction Protein, Induces Voltage-gated Currents in Nonjunctional Plasma Membrane of *Xenopus* Oocytes

D. L. Paul, L. Ebihara,* L. J. Takemoto,‡ K. I. Swenson, and D. A. Goodenough

Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, Massachusetts 02115; *Department of Pharmacology, Columbia University, New York 10032; and ‡Department of Biology, Kansas State University, Manhattan, Kansas 66506

Abstract. Gap junctions are composed of a family of structural proteins called connexins, which oligomerize into intercellular channels and function to exchange low molecular weight metabolites and ions between adjacent cells. We have cloned a new member of the connexin family from lens cDNA, with a predicted molecular mass of 46 kD, called rat connexin46 (Cx46). Since a full-length cDNA corresponding to the 2.8-kb mRNA was not obtained, the stop codon and surrounding sequences were confirmed from rat genomic DNA. The RNA coding for this protein is abundant in lens fibers and detectable in both myocardium and kidney. Western analysis of both rat and bovine lens membrane proteins, using the anti-MP70 monoclonal antibody 6-4-B2-C6 and three anti-peptide antibodies against Cx46 demonstrates that Cx46 and MP70 are different proteins. Immunocytochemistry demonstrates that both proteins are localized in the same lens fiber junctional maculae. Synthesis of Cx46 in either reticulocyte lysate or *Xenopus* oocytes yields a 46-kD polypeptide; all anti-Cx46 antisera recognize a protein in rat lens membranes 5–10 kD larger, suggesting substantive lenticular posttranslational processing of the native translation product. Oocytes that have synthesized Cx46 depolarize and lyse within 24 h, a phenomenon never observed after expression of rat connexins 32 or 43 (Cx32 and Cx43). Lysis is prevented by osmotically buffering the oocytes with 5% Ficoll. Ficoll-buffered oocytes expressing Cx46 are permeable to Lucifer Yellow but not FITC-labeled BSA, indicating the presence of selective membrane permeabilities. Cx43-expressing oocytes are impermeable to Lucifer Yellow. Voltage-gated whole cell currents are measured in oocytes injected with dilute concentrations of Cx46 but not Cx43 mRNA. These currents are activated at potentials positive to −10 mV. Unlike other connexins expressed in *Xenopus* oocytes, these results suggest that unprocessed Cx46 induces nonselective channels in the oolemma that are voltage dependent and opened by large depolarizations.

Gap junctions are composed of a family of membrane structural proteins, called connexins, which oligomerize into hexamers (connexons), which in turn pair between cells to form intercellular channels permitting intercellular diffusion of small molecules and ions (Bennett and Goodenough, 1978). While connexins are detectable both within assembled gap junctions and in intracellular compartments by immunolocalization methods (Hertzberg and Skibbens, 1984; Paul, 1986; Beyer et al., 1989; Musil et al., 1990*b*), no data are available that demonstrate the presence of either monomeric or oligomeric connexins in the nonjunctional plasma membrane. Due to the substantive 50–160-pS conductances associated with the nonselective gap junctional channels (Neyton and Trautmann, 1985; Veenstra and DeHaan, 1986; Burt and Spray, 1988; Somogyi and Kolb, 1988; Brink and Fan, 1989; Fishman et al., 1990), open connexons assembled in the nonjunctional plasma membrane would be incompatible with the maintenance of cellular resting potentials. Thus, if connexons are present in the nonjunctional plasma membrane, they are likely to spend most of the time in a closed state.

The structure and physiology of the lens suggest that it may contain specialized mechanisms of intercellular communication (Goodenough, 1979). The fibers in the center of the lens are uniquely dependent on intercellular communication with cells at the lens surface because they have no blood supply. Lens fibers have been shown to be joined into a syncytium with respect to ions (Duncan, 1969; Eisenberg and Rae, 1976; Rae, 1979; Mathias and Rae, 1985, 1989). This syncytium may be achieved by gap junctions between adjacent fibers, which permit ions and fluorescent dyes (Rae, 1974; Phillipson et al., 1975; Rae and Stacey, 1979; Schuetze and Goodenough, 1982; Miller and Goodenough, 1986) as well as small transported metabolites (Goodenough et al., 1980) to diffuse from cytoplasm to cytoplasm between adjacent cells.

The anatomical relationships of epithelial cells and fibers allow three classes of junctional interactions: epithelial/epi-

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thelial, fiber/fiber, and epithelial/fiber. Epithelial/epithelial gap junctions were shown to contain connexin43 (Cx43),[1] a gap junction protein found between many cell types, prototypically myocardial cells (Beyer et al., 1987, 1989; Musil et al., 1990*a*). These junctions display a crystalline freeze-fracture morphology, and communication between these cells is inhibited by cytoplasmic acidification (Miller and Goodenough, 1985, 1986). Fiber/fiber junctions (Cohen, 1965; Leeson, 1971) in ungulates contain a 70-kD protein called MP70 (Kistler et al., 1985; Gruijters et al., 1987; see Zampighi et al., 1989, for a discussion of other potential junctional proteins). MP70 is defined by the monoclonal antibody 6-4-B2-C6, rather than by its molecular weight, since there are significant species differences in protein mobility on SDS polyacrylamide gels (data in this article). Published NH_2-terminal sequence analysis of ovine MP70 protein and its degradation products demonstrates that MP70 is a member of the connexin family (Kistler et al., 1988). In addition, the sequence analysis suggests that the sequenced material contains two different but related proteins. cDNA corresponding to MP70 has not been cloned. Fiber/fiber junctions display a characteristic noncrystalline morphology and are not sensitive to cytoplasmic acidification (Schuetze and Goodenough, 1982; Miller and Goodenough, 1986). While the molecular components of the epithelial/fiber junctions are not known, they resemble fiber/fiber junctions in structural characteristics and pH insensitivity (Miller and Goodenough, 1986).

Here, we report the cloning of a novel connexin cDNA from rat lens. The cDNA encodes a connexin with a calculated molecular mass of 46 kD. The predicted NH_2-terminal amino acid sequence of connexin46 (Cx46) exactly matches one of the 20 amino acid NH_2-terminal sequences obtained from the 38-kD fragment of MP70 by Kistler et al. (1988). The cDNA hybridizes with a ~2.8-kb mRNA from lens fibers, myocardium, and kidney. A partial sequence of Cx46 containing some sequencing errors was reported previously (Beyer et al., 1988). We have prepared antisera directed against three different regions of the predicted Cx46 sequence, using synthetic oligopeptides as antigens. All three antipeptide sera appear to recognize the same polypeptide, which is distinct from MP70. Light and electron microscope immunocytochemistry reveal that the two proteins coexist in the same fiber/fiber junctions, intermingled to the level of resolution of the gold-labeled secondary antisera.

The translation product of Cx46 mRNA, detected either in reticulocyte lysate or in *Xenopus* oocytes, migrates in SDS-polyacrylamide gels at 46,000 M_r. The native protein detected in rat lens fiber plasma membranes migrates 5–10 kD larger, and the bovine counterpart shows an even slower electrophoretic mobility. Expression of Cx46 mRNA in *Xenopus* oocytes suggests that the regulation of channel function may be different for Cx46 than for other connexins. Lysis occurs when oocytes expressing Cx46 are maintained in amphibian Ringer's, as compared with oocytes expressing either Cx32 or Cx43. Lysis is prevented when the cells are osmotically buffered with 5% Ficoll. The osmotically rescued oocytes are highly permeable to Lucifer Yellow CH, but not to FITC-labeled BSA, and display voltage-activated whole cell currents.

1. *Abbreviations used in this paper*: Cx, connexin; PCR, polymerase chain reaction.

Materials and Methods

Northern blotting was performed as described in Paul (1986). The sizes of RNA transcripts were determined by comparison with sized RNA markers purchased from Gibco/BRL (Bethesda, MD). Lens membrane isolation and Western blotting were performed as described in Paul and Goodenough (1983). In general, molecular biology techniques were as described by Maniatis et al. (1982) except where indicated.

cDNA Library Construction and Screening

A rat lens library was constructed from manually decapsulated adult rat lens. Double-stranded cDNA with EcoRI linkers was prepared according to Watson and Jackson (1985). This cDNA was ligated to EcoRI-cut and dephosphorylated λgt11 arms obtained from Promega Corp. (Madison, WI). Lambda packaging extract was obtained from Stratagene Inc. (La Jolla, CA).

The unamplified rat lens cDNA library was plated in Y1090 and probed at reduced stringency with rat Cx32 cDNA (Paul, 1986). To prepare the probe, the 1.5-kb Cx32 cDNA in pGEM-3 (Promega Corp.) was digested with HindIII and BglI, releasing an ~850-bp fragment containing most of the coding portion. This fragment was labeled by random priming (Feinberg and Vogelstein, 1983) in LMP agarose (SeaPlaque; FMC Corp., Rockland, ME) after separation from the plasmid by gel electrophoresis in TAE buffer. Plaque lifts were performed according to Maniatis et al. (1982). Hybridization was conducted in 5% SDS, 0.75 M $NaPO_4$, pH 7.2, at 50°C for 16 h, followed by three 20-min washes in 0.3 M $NaPO_4$, pH 7.2, 1% SDS at 50°C. The lifts were then exposed overnight at −70°C with intensifying screens. Initial screening of 250,000 plaques resulted in seven consistently positive clones after four rounds of plaque purification. Phage DNA was prepared according to Helms et al. (1985), and inserts were subcloned into pBS-KS (Bluescript; Stratagene Inc.) for sequencing. Single-stranded templates were rescued from pBS-KS according to supplied protocols, and DNA sequence was obtained using Sequenase (United States Biochemical Corp., Cleveland, OH) according to the manufacturer's instructions.

To attempt isolation of full-length clones, this library, and a commercially obtained rat lens cDNA library (Clontech Laboratories, Inc., Palo Alto, CA), were screened conventionally under high stringency conditions using a 64-base synthetic oligonucleotide corresponding to bases 207–269 in the original cDNA sequence (EMBL X57970). No clones longer than the original cDNA or containing any additional sequences in the 3′ or 5′ directions were obtained.

Inverse PCR Amplification of Genomic Sequences

Inverse polymerase chain reaction (PCR) amplification of PstI cut and circularized rat genomic DNA was performed according to Ochman et al. (1988). To identify appropriate restriction enzymes, rat genomic DNA digested with various restriction enzymes was blotted and probed with a 3′ portion of the Cx46 cDNA. This 3′ portion was prepared by digesting the cDNA, appropriately oriented in pBS-KS, with PstI. PstI-digested genomic DNA contained a 1.1-kb fragment that hybridized with the 3′ portion of the Cx46 cDNA (data not shown). The template for PCR amplification was produced by ligating PstI-digested genomic DNA at 1 μg/ml to promote formation of closed circles. Ligation was performed at 15°C in 50 mM Tris, pH 7.5, 20 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, and 800 U/ml T4 DNA ligase (New England Biolabs, Beverly, MA) for 16 h. PCR reactions contained 1 μg of phenol-extracted, ethanol-precipitated template DNA, 200 ng of each primer, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) in 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 200 μM each dNTP. The sense primer corresponded to bases 789–812 in the Cx4 cDNA sequence; the antisense primer corresponded to the reverse complement of bases 756–799. 30 cycles of 94°C × 1′, 65°C × 1′, and 72°C × 3′ were performed. The 1.1-kb band was isolated from TAE/agarose gels with Gene-clean (Biol 101, Inc., Vista, CA) and sequenced directly using Sequenase after generating single-stranded template by asymmetric PCR according to Wilson et al. (1990).

Preparation and Injection of Oocytes

Oocytes were collected from gravid *Xenopus laevis* females (Nasco, Fort At-

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CX26   M-DWGTLQSILGGVNKHSTSIGKIWLTVLFIFRIMILVVAAKEVWGDEQADFVCNTLQPG   59
CX32   M-NWTGLYTLLSGVNRHSTAIGRVWLSVIFIFRIMVLVVAAESVWGDEKSSFICNTLQPG   59
CX43   MGDWSALGKLLDKVQAYSTAGGKVWLSVLFIFRILLLGTAVESAWGDEQSAFRCNTQQPG   60
CX46   MGDWSFLGRLLENAQEHSTVIGKVWLTVLFIFRILVLGAAAEEVWGDEQSDFTCNTQQPG   60
                RL   A
MP70   -GDWSFLG  LEN QEHSTVI
                NI   V

CX26   CKNVCYDHYFPISHIRLWALQLIMVSTPALLVAMHVAY--RRHEKKRKFMKGEIKNEFKD  117
CX32   CNSVCYDHFFPISHVRLWSLQLILVSTPALLVAMHVAHQQHI-EKKMLRLEGHGDPLHL-  117
CX43   CENVCYDKSFPISHVRFWVLQIIFVSVPTLLYLAHVFYVMRKEEKLNKKEEELKVAQTDG  120
CX46   CENVCYDRAFPISHIRFWALQIIFVSTPTLIYLGHVLHIVRMEEKKKEREEELLRRDNPQ  120

CX26   -----------------IEEIKTQKVRIEGSLWWTYTTSIFFRVIFEAVFMYVFYIMYNGF  161
CX32   ------------------EEVKRHKVHISGTLWWTYVISVVFRLLFEAVFMYVFYLLYPGY  160
CX43   VNVEMHLKQIEIKKFKYGIEEHGKVKMRGGLLRTYIISILFKSVFEVAFLLIQWYIY-GF  179
CX46   HGRGREPMRTGSPRDPPLRDDRGKVRIAGALLRTYVFNIIFKTLFEVGFIAGQYFLY-GF  179

CX26   FMQRLVKCNAWPCPNTVDCFISRPTEKTVFTVFMISVSGICILLNITELCYLFIRYCSGK  221
CX32   AMVRLVKCEAFPCPNTVDCFVSRPTEKTVFTVFMLAASGICIILNVAEVVYLIIRACARR  220
CX43   SLSAVYTCKRDPCPHQVDCFLSRPTEKTIFIIFMLVVSLVSLALNIIELFYVFFKGVKDR  239
CX46   QLQPLYRCDRWPCPNTVDCFISRPTEKTIFVIFMLAVACASLVLNMLEIYHLGWKKLKQG  239

CX26   SKRPV                                                          226
CX32   AQRRSNPPSRKGSGFGHRLSPEYKQNEINKLLSEQDGSLKDILRRSPGTGAGLAEKSDRC  280
CX43   VKGRSDPYHATTGPLSPSKDCGSPKYAYFNGCSSPTAPLSPMSPPGYKLVTGDRNN----  295
CX46   VTNHFNPDASEVRHKPLDPLSEAANSGPPSVSIGLPPYYTHPACPTVQGKATGFPGAPLL  299

CX32   SAC                                                            283
CX43   SSCRNYNKQAS------------------EQNWANYSAE-QNRMGQAGSTISNSHAQPFD  336
CX46   PADFTVVTLNDAQGRGHPVKHCNGHHLTTEQNWASLGAEPQTPASKPSSAASSPHGRKGL  359

CX43   FPDDNQNAKKVAAGHEL---QPLAIVDQ---RPSS----RASSRASS-RPRPDDLEI     382
CX46   TDSSGSSLEESALVVTPEGEQALATTVEMHSPPLVLLDPERSSKSSSGRARPGDLAI     416
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Figure 1. Comparison of the amino acid sequences of Cx26, 32, 43, and 46, and MP70. All these proteins show a high degree of similarity. Spaces introduced manually for maximum alignment are indicated by dashes. The exact matching residues are boxed and shaded. The NH_2-terminus of MP70 was determined by Kistler et al. (1988) by manual protein sequencing and exhibits two possible residues at positions 8, 9, and 13. One possible sequence combination for MP70 exactly matches the predicted sequence of Cx46. The underlined residues indicate the amino acid sequences used to produce anti-Cx46 antibodies.

kinson, WI) as described previously (Swenson et al., 1989), except that clumps of ovarian tissue were immersed directly into the protease inhibitor cocktail immediately after the 10–20-min incubation in 20 mg/ml collagenase B (Boehringer Mannheim Corp., Indianapolis, IN). Oocytes were then manually defolliculated and stored overnight at 18°C in modified Barth's medium containing 5 μg/ml gentamicin (Gibco Life Technologies, Grand Island, NY). Microelectrodes were pulled with a vertical electrode puller (model PE-2; Narishige, Tokyo, Japan) and carefully broken during observation with a light microscope to a tip diameter of 15 μm. The electrodes were calibrated by injection into Wesson® oil, and measurement of droplet diameter. The diameter of the droplet was adjusted with a Picospritzer II (General Valve Corp., Fairfield, NJ) to 4–5 nl. The electrode tip was then pressed against the vitelline envelope overlying the vegetal pole. This resulted in distortion of the surface inward until the pipette was observed to pop into the ooplasm. The depth of needle penetration was variable between oocytes, but the pipette was not pushed further into the cell. Oocytes were injected with either RNA (1 μg/μl) or autoclaved water using 10 4–5-nl pulses/oocyte. For metabolic labeling, oocytes were injected with 40 nl mRNA with 2 μCi/oocyte [^{35}S]methionine (800 Ci/mmol; New England Nuclear, Boston, MA) as described previously (Swenson et al., 1989). Injected oocytes were then incubated at 18°C either in modified Barth's made 5% with Ficoll (type 400-DL; Sigma Chemical Co., St. Louis, MO) or in 130 mM K-aspartate, 20 mM KCl, 1.5 mM $MgCl_2$, 10 mM $CaCl_2$, 1 mM EGTA, 10 mM HEPES, and 5% Ficoll, pH 7.4 (Ficoll buffer). For some experiments, oocytes were manually devitellinized according to Methfessel et al. (1986) and stored similarly. Resting potentials of oocytes were measured with intracellular microelectrodes as described by Swenson et al. (1989). For whole cell current measurements, oocytes were voltage clamped using a two-microelectrode voltage clamp (Axoclamp-2A; Axon Instruments, Inc., Foster City, CA). The current and voltage electrodes had resistances ranging between 0.5 and 2 MΩ and were filled with 0.5 M K-aspartate, 0.5 M KCl, 10 mM EGTA, and 10 mM Hepes, pH 7.4. The bathing solution consisted of modified Barth's solution without Ficoll. Immune precipitations of labeled oocytes were performed as described previously (Swenson et al., 1989).

Oocytes were incubated for 1 h in 1 mg/ml Lucifer Yellow CH (Polysciences Inc., Warrington, PA) or in 1 mg/ml FITC-BSA in modified Barth's with 5% Ficoll. Oocytes were rinsed twice for 5 min in buffer without Lucifer Yellow, then fixed in 2% formaldehyde (freshly made from paraformaldehyde) in PBS, pH 7.4. Fixed oocytes were then frozen and sectioned as described previously (Swenson et al., 1989) and viewed by epifluorescence.

Production of Antisera

Peptides corresponding to amino acids 121–141, 372–382, and 411–416 were synthesized, conjugated to keyhole limpet hemocyanin, and injected into rabbits as described previously (Takemoto et al., 1985). Serum was used without further purification.

Immunocytochemistry

Bovine lenses were dissected from eyes obtained from a local abattoir and manually cut into 2–3-mm slices in 1% formaldehyde made from paraformaldehyde in PBS, pH 7.4. After fixation for 30 min at room temperature, the tissue slices were washed in PBS, then cut into frozen sections as described previously (Goodenough et al., 1988). Sections were stained with polyclonal antisera at 1:500 dilution or with undiluted hybridoma supernatant overnight at 4°C, washed in PBS, and then stained with 1:500 dilutions of fluorescein- or rhodamine-conjugated secondary antisera (Boehringer Mannheim Corp.). Fluorescence microscopy was performed with a Zeiss Axioskop (Carl Zeiss, Inc., Thornwood, NY) recorded on Kodak (Rochester, NY) Tmax400 film.

Results

cDNA Sequence Analysis

To enrich for mRNAs from developing fibers, libraries were constructed using mRNA from manually decapsulated lenses. This procedure removed both the lens and ciliary epithelia, which are known to express Cx43 (Beyer et al., 1989). Screens of two lens cDNA libraries prepared from mRNA from decapsulated lens produced positive clones of two size classes containing inserts of ~1.3 and ~1.6 kb. The sequences of these cDNAs were identical in the region of overlap. The difference between these two classes was in the length of the 3′ sequence. The 5′ aspects of the cDNAs were very similar, containing ~200 bases of noncoding DNA ahead of the presumptive start codon (data not shown). Northern analysis, to be described below, demonstrated that neither size class

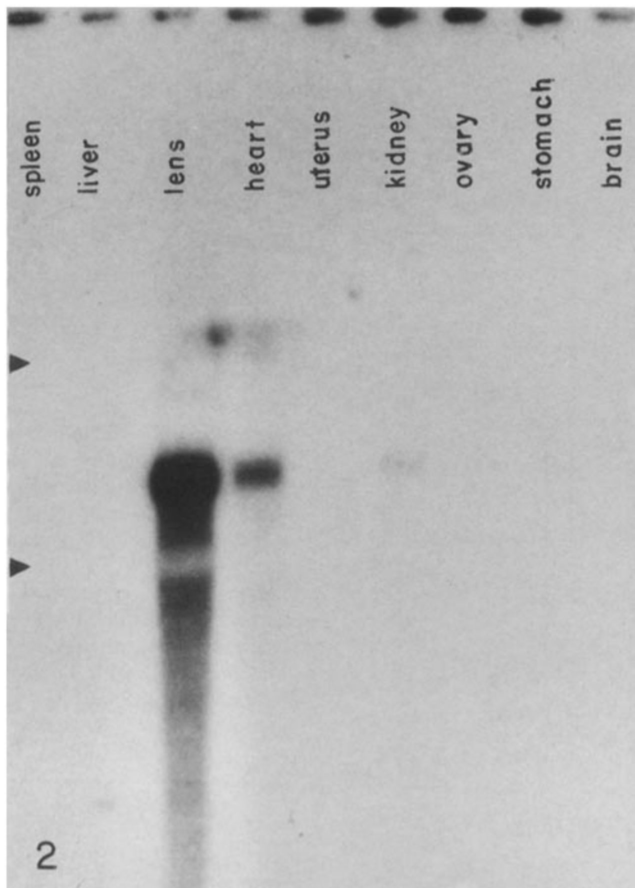


Figure 2. Northern blot of RNA from a variety of organs hybridized with Cx46 cDNA. Each lane consists of 10 μg of total RNA isolated from spleen, liver, lens, heart, uterus, kidney, ovary, stomach, and brain. A single band ~2.8 kb in size is detected in lens, heart, and kidney RNAs. The autoradiograph was exposed for 48 h to reveal the weak signal in kidney RNA. Positions of the 28S and 18S ribosomal RNAs are indicated by arrowheads.

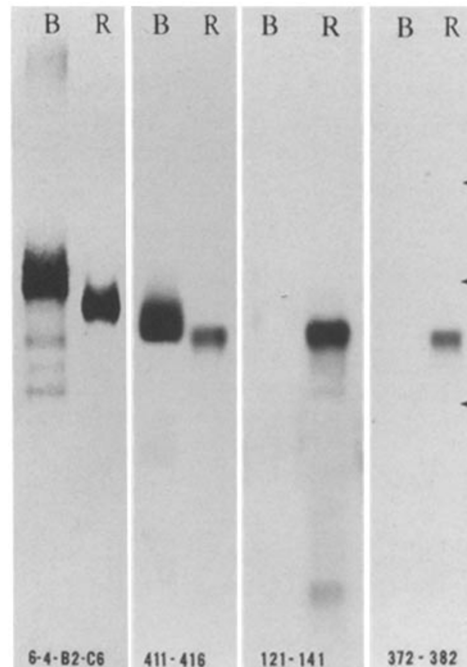


Figure 3. Western blot of bovine and rat lens membranes indicates that MP70 and Cx46 are unique proteins. Bovine (*B*) and rat (*R*) lens membranes were separated by SDS-PAGE and transferred to nitrocellulose filters. Identically prepared filters were stained with anti-MP70 (6-4-B2-C6) and the three anti-Cx46 antibodies (411–416, 121–141, 372–382). For the rat specimens, the three anti-Cx46 antibodies recognize a protein with the same mobility, while anti-MP70 recognizes a distinctly different band. For the bovine samples, one of the anti-Cx46 antibodies (411–416) cross-reacts. The bovine component recognized by anti-Cx46(411–416) is again a different protein than that recognized by anti-MP70. The other anti-Cx46 antibodies do not cross-react with bovine samples. Lower molecular weight bands recognized by anti-Cx46(121–141) are presumed proteolysis products of Cx46; this staining was blocked with immunizing peptide. These bands do not migrate at the expected position (faster than the ovalbumin standard) of Cx43. Arrows indicate the position of methylated protein standards (top to bottom): rabbit muscle phosphorylase B = 97 kD, BSA = 69 kD, and ovalbumin = 46 kD.

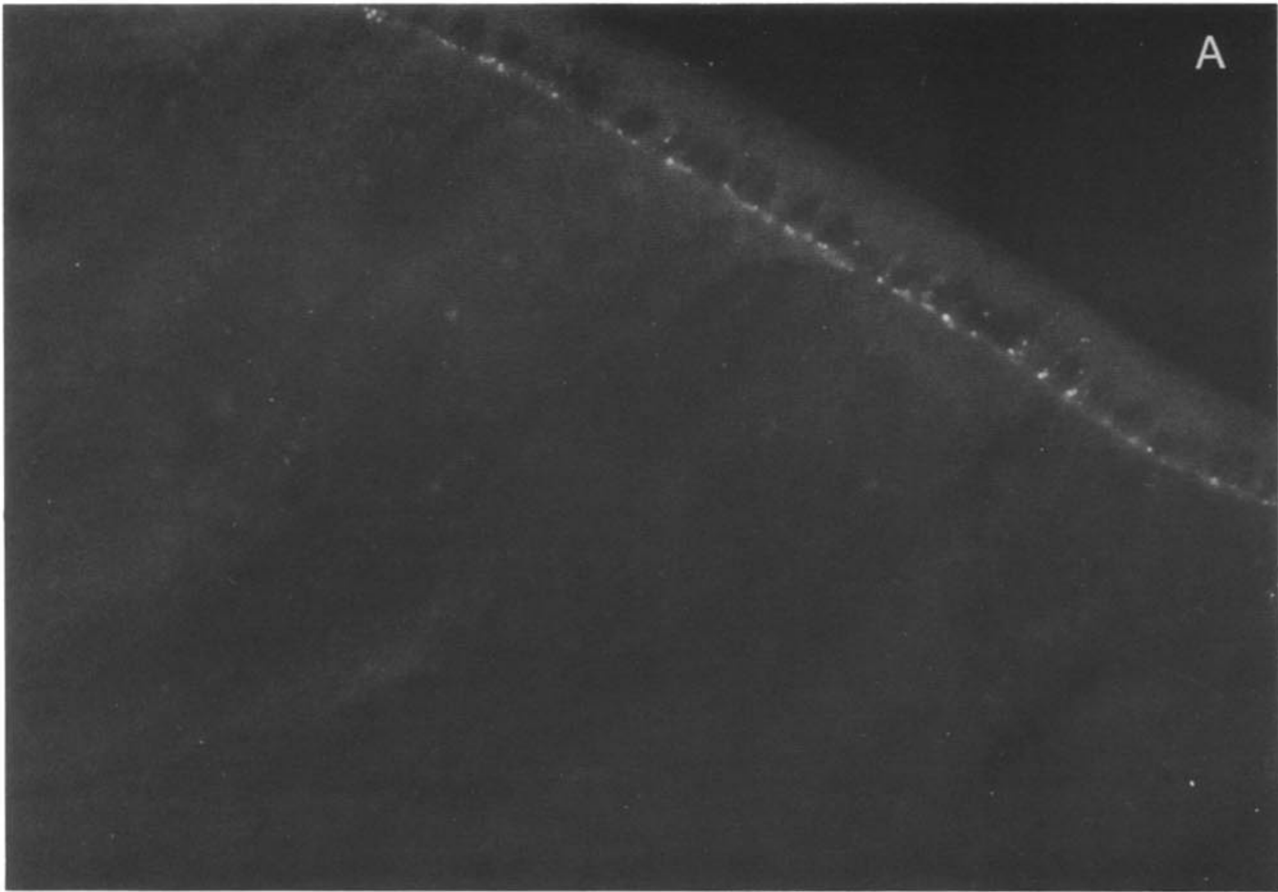
of cDNA was full length. To attempt isolation of full-length clones, these libraries, and a commercially obtained rat lens cDNA library (Clontech) were screened conventionally under high stringency conditions using a 64-base synthetic oligonucleotide corresponding to bases 207–270 in the original cDNA sequence (EMBL #X57970). No clones longer than the original cDNA or containing any additional sequences in the 3′ or 5′ directions were obtained.

The 1.6-kb cDNA contained an in-frame stop codon at base 1,455, immediately preceding an unusually A-rich region (see EMBL #X57970). Since a full-length cDNA could not be obtained, inverse PCR of genomic DNA was used to obtain genomic sequence corresponding to this area which exactly matched the stop codon and neighboring A-rich region (data not shown).

Protein Sequence Analysis

Fig. 1 compares the amino acid sequence predicted for rat Cx46 by the open reading frame in the 1.6-kb cDNA (EMBL #X57970) with rat Cx26, 32, and 43. Included also are 20 amino acids from the NH_2 terminus of the 38-kD fragment of ovine MP70 (Kistler et al., 1988). Residues 8, 9, and 13 of MP70 were reported to contain equimolar amounts of two amino acids each. There is an exact amino acid match between Cx46 and one of the possible sequences for MP70. Inspection of the Cx46 sequence reveals that the homologous regions with other connexins (*shaded*) correspond principally to transmembrane and extracellular domains (see general model in Beyer et al., 1990). However, the cytoplasmic NH_2-terminal regions of all connexins, and the ultimate COOH-terminal regions of Cx46 and 43, show obvious similarities. Other putative cytoplasmic amino acid sequences are unique. The three underlined, unique sections of Cx46

Figure 4. Cx46 is expressed by lens fibers. Adjacent frozen sections of bovine lens were stained with anti-Cx43 (*A*) and anti-Cx46(411–416) (*B*). Cx43 staining is confined to the lens epithelium while Cx46 staining is abundant between lens fibers. It is not possible to determine if either Cx43 or Cx46 is present in junctions between epithelial cells and fibers. Bar = 20 μm.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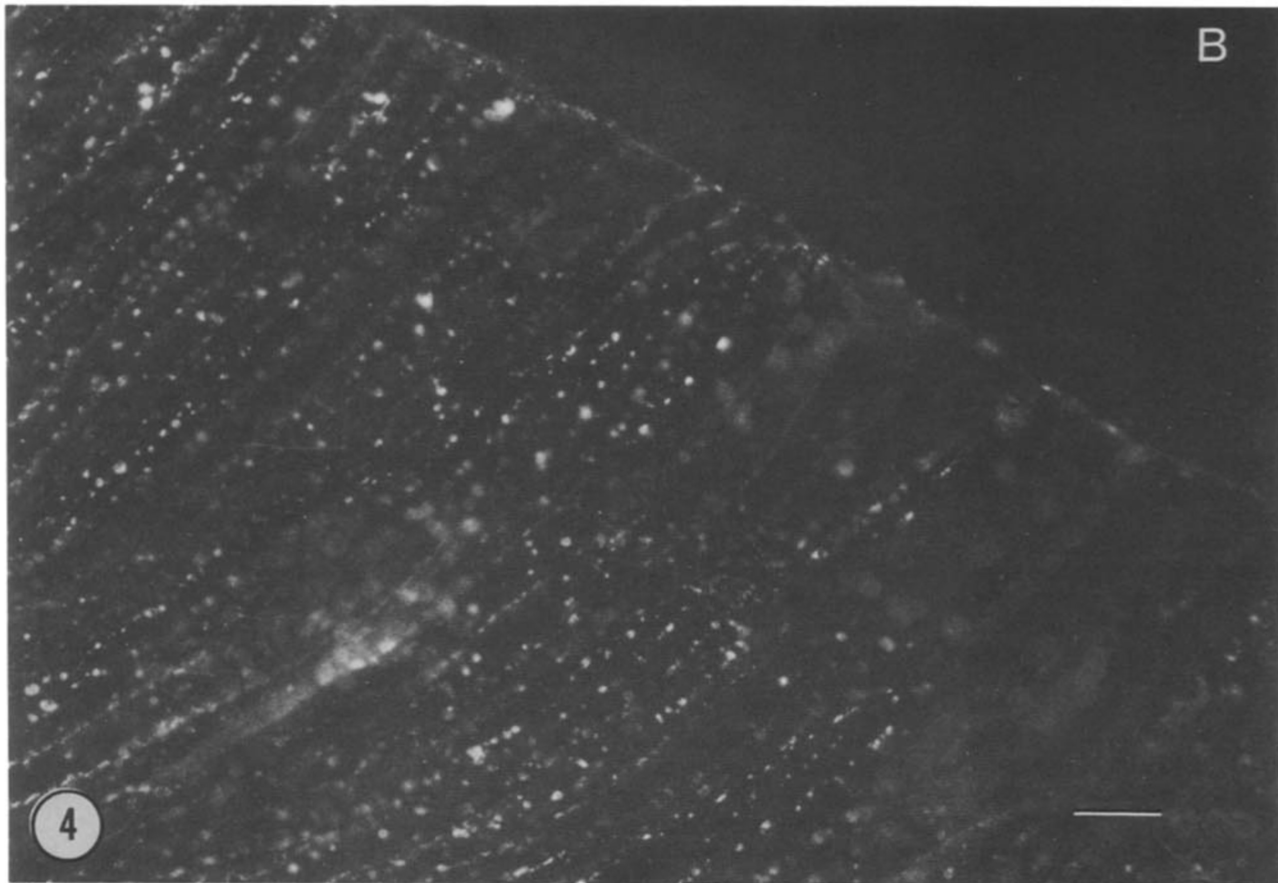
B
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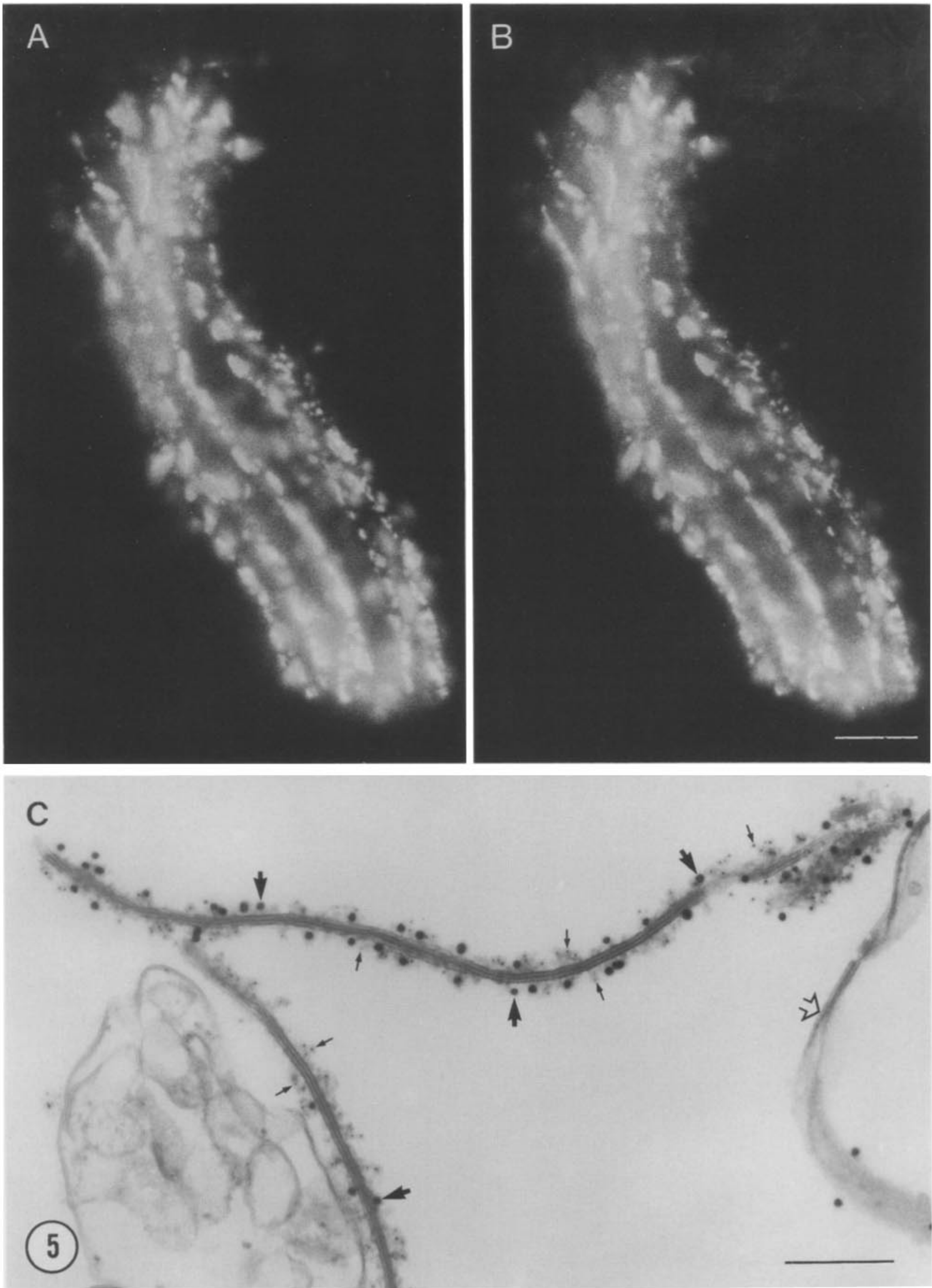
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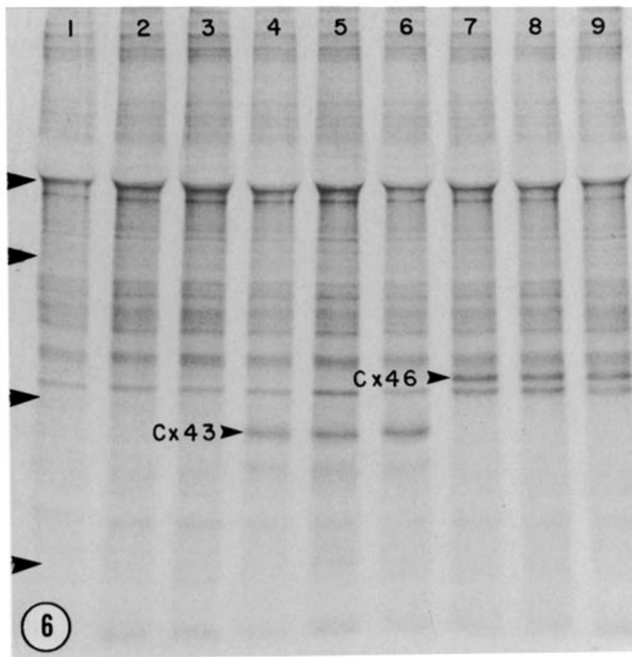


Figure 6. Expression of Cx46 and Cx43 in mRNA-injected *Xenopus* oocytes. 40 nl of mRNA and water solutions were injected with 2 μCi [^{35}S]methionine and incubated for 8 h at 18°C. Aliquots of total ^{35}S-labeled proteins from oocytes injected with water (lanes *1–3*), Cx43 mRNA (lanes *4–6*), and Cx46 (lanes *7–9*) are shown. Samples from separate oocytes were loaded in each lane, indicating the reproducibility of the protein levels. The labeled arrowheads indicate the unique polypeptides resulting from mRNA injection. Arrowheads along the left edge of the gel indicate the position of nonmethylated protein standards, from top to bottom: phosphorylase B (97 kD), BSA (66 kD), hen ovalbumin (43 kD), and carbonic anhydrase (31 kD).

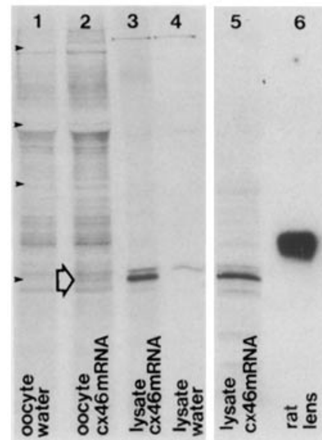


Figure 7. SDS-PAGE mobility of in vitro synthesized and native Cx46 are different. Cx46 synthesized in *Xenopus* oocytes (lane *2*, *open arrow*) and in reticulocyte lysate and labeled with [^{35}S]methionine (lanes *3* and *5*) display an indistinguishable mobility. Cx46 present in rat lens membranes (lane *6*), detected by immunoblot with anti-Cx46(411–416), migrates more slowly with an apparent relative molecular mass ~5–10 kD higher than the in vitro synthesized products. Arrowheads indicate migration of protein standards (myosin = 200 kD; phosphorylase B = 97 kD; BSA = 69 kD; ovalbumin = 46 kD).

were synthesized as oligopeptides and used to prepare antisera as described in Materials and Methods. Corresponding to the amino acid numbers, the antisera recognizing these peptides will be referred to as anti-Cx46(121–141), anti-Cx46(372–382), and anti-Cx46(411–416).

Cx46 mRNA Is Not Restricted to Lens

Cx46 cDNA was used to probe a Northern blot consisting of total RNA from spleen, liver, lens, heart, uterus, kidney, ovary, stomach, and brain (Fig. 2). Under the stringency conditions used here, we reliably observed a positive signal in three organs: lens, heart, and kidney. Hybridization to a single major band of ~2.8 kb was readily detected in lens RNA, to a lesser degree in myocardial RNA, and at low levels in kidney RNA. The Cx46 mRNA was distinctly different in size from Cx43 mRNA, which migrated at ~3.0 kb on the same blot after reprobing (data not shown). Under the conditions of hybridization used in these blots, no cross-hybridization between Cx46 and Cx43 was observed. This blot was significantly overexposed to demonstrate the weak Cx46 signal in the kidney lane. The smear of lower molecular weight RNA in the lens sample is presumably degradation products of the Cx46 mRNA.

Antibodies against MP70 and Cx46 Recognize Different Proteins

All three anti-Cx46-peptide antisera and anti-MP70 monoclonal 6-4-B2-C6 were used in Western blots of both rat and bovine lens plasma membranes. Fig. 3 shows a composite blot with all four reagents aligned using identical standards, indicated by the arrows at the right. 6-4-B2-C6, which defines MP70, stained a broad doublet in the bovine lens membrane lane (*B*) and a prominent, broad band in the rat lens membrane lane (*R*). The bovine and rat immunoreactive bands migrate at significantly different sizes, reflecting a species difference. Using this gel system, it was not possible to resolve these bands further. All three anti-Cx46 reagents stained single, comigrating bands in the rat lanes, which ran significantly faster than the band recognized by 6-4-B2-C6 in the rat. Of the three, only anti-Cx46(411–416) cross-reacted strongly with the bovine membranes, staining the presumed bovine counterpart of rat Cx46: a broad band that migrated faster than the 6-4-B2-C6-stained bovine MP70. All staining with the antipeptide antisera could be completely inhibited with the specific immunizing peptides (data not shown). These data demonstrate that MP70 and Cx46 are different proteins.

Anti-Cx43 antisera detect Cx43 in Western blots of whole lens homogenates which comigrates with Cx43 from a variety of different tissues (Beyer et al., 1989). None of the anti-Cx46 antisera used in this study detect antigen in the region of the gel corresponding to Cx43, which would run ahead of the ovalbumin standard (*lowest arrow*) in Fig. 3. Together

Figure 5. Cx46 and MP70 colocalize in lens fiber junctions. Fragments of bovine lens fibers are double stained with anti-MP70 (*A*) and anti-Cx46(411–416) (*b*). Staining is coincident to the level of light microscopic resolution. Bar = 20 μm. In the electron microscope, individual junctional plaques show double staining with anti-MP70 (5-nm gold particles, *small arrows*) and anti-Cx46 (15-nm gold particles, *large arrows*). Some junctional profiles stain with neither antibody (*open arrow*). Nonjunctional membrane does not stain above background. Bar = 200 nm.

with the immunohistochemical data presented below, this demonstrates that none of the anti-Cx46 antisera cross-react with Cx43.

Cx46 Is Found in Lens Fibers but Not Lens Epithelium and Colocalizes with MP70

Fig. 4 shows a pair of fluorescent photomicrographs of adjacent frozen sections of bovine lens, stained with anti-Cx43(252–271) and anti-Cx46(411–416). Anti-Cx43(252–271) localized exclusively to the lens epithelium (Fig. 4 *A*). Anti-Cx46(411–416) stained only macular structures between the lens fibers (Fig. 4 *B*). Since it is not possible to tell whether Cx43 and Cx46 are found at the epithelium-lens fiber contacts, it is not possible to state that either connexin is expressed solely by one cell type.

The antisera were used to study the relative distribution of MP70 and Cx46 on the surfaces of lens fibers and in lens membrane fractions. Frozen sectioning of lenses resulted in occasional fragments of lens fibers which permitted high resolution immunohistochemistry. Double staining of fragments of lens fibers with the anti-MP70 monoclonal 6-4-B2-C6 (Fig. 5 *A*) and anti-Cx46(411–416) (Fig. 5 *B*) revealed exact one-to-one staining with the two reagents. These results were also seen in intact areas of sectioned fibers. Coincident staining was further resolved by EM immunocytochemistry of subfractionated bovine lens fiber plasma membranes using these same two reagents. MP70 and Cx46 were visualized with 5- and 15-nm gold-conjugated secondary antisera, respectively (Fig. 5 *C*). The 5-nm (*small arrows*) and 15-nm (*large arrows*) gold particles were intermingled on both sides within the same lens fiber junction with no obvious clustered domains. It was not possible to resolve whether Cx46 and MP70 were contained within the same connexon, or if they existed in microdomains of tens of connexons. No staining was observed on the nonjunctional lens fiber plasma membranes. Some lens fiber junctions stained with neither antibody (*open arrow*). Some lens fiber junctions were seen stained only with anti-Cx46(411–416), while none were seen with only 6-4-B2-C6 staining (data not shown), probably related to proteolytic removal of the 6-4-B2-C6 epitope (Kistler et al., 1985; Gruijters et al., 1987). Neither reagent stained the "thin" pentalaminar profiles (Paul and Goodenough, 1983; Zampighi et al., 1989; data not shown). Identical ultrastructural observations colocalizing both Cx46 and MP70 within the same junctional maculae were also seen with isolated rat lens fiber membranes (data not shown).

Cx46 Expressed in Xenopus Oocytes and in Reticulocyte Lysate Migrates Differently in SDS-PAGE Than Native Lens Cx46

For expression studies, Cx46 cDNA was subcloned into the BglII site of the transcription vector SP64T (Krieg and Melton, 1984; Swenson et al., 1989). In vitro transcribed RNA was injected into three *Xenopus* oocytes and compared with oocytes similarly injected with Cx43 mRNA and water. All oocytes were coinjected with [^{35}S]methionine. Fig. 6 shows an autoradiogram of an SDS polyacrylamide gel loaded with an aliquot of total protein from one oocyte/lane. Lanes *1–3* were injected with water; lanes *4–6* were injected with Cx43 mRNA; lanes *7–9* were injected with Cx46 mRNA. Unique bands corresponding to the connexin RNAs are indicated directly on the figure. These bands were immunoprecipitable with specific antisera (data not shown).

Cx46 was also translated in the reticulocyte lysate system and compared with Cx46 expressed in *Xenopus* oocytes. The Cx46 synthesized in the reticulocyte lysate and in the oocyte were compared with Cx46 identified in whole rat lens by immunoblot (Fig. 7). Comparison of lanes *1* and *2* demonstrates the synthesis of a 46-kD peptide in the oocyte injected with synthetic Cx46 mRNA (*open arrow,* lane *2*) which comigrated with the major polypeptide synthesized in Cx46 mRNA-directed reticulocyte lysate (lane *3*) not seen in the water-directed lysate (lane *4*). In a separate experiment, the same lysate sample shown in lane *3* was reelectrophoresed in lane *5* adjacent to a rat lens homogenate (lane *6*). After transfer to nitrocellulose, both the native lens Cx46 immunolabeled with anti-Cx46(411–416) and ^{125}I-protein A, and the [^{35}S]methionine-labeled in vitro synthesized product were detected by autoradiography. The relative mobility of the native Cx46 in rat lens varied with the gel system used, but consistently showed an additional relative molecular mass of 5–10 kD compared with either the in vitro or oocyte-synthesized molecules.

Expression of Cx46 in Xenopus Oocytes Caused Cell Lysis

Xenopus oocytes were injected with Cx46, Cx43, and Cx32 mRNA and then devitellinized and followed for 24 h. All oocytes injected with Cx46 mRNA became visibly altered by 8 h and had lysed by 16–24 h. Oocytes that were not devitellinized were protected from lysis by the vitelline envelope, but the oocytes nonetheless lost resting potentials and experienced a disorganization of pigment distribution. Control oocytes injected with Cx32 or Cx43 mRNA remained unlysed, with 60–80-mV resting potentials for up to 2 d. Pairing the Cx46-injected oocytes failed to save them from lysis. Fig. 8 shows photographs of oocyte pairs constructed following the injection of either water or Cx46 mRNA. Fig. 8 *A* shows the pairs at 12 h incubation. The three Cx46-injected pairs have begun to show disorganization of the pigment in the animal hemispheres. This process is more pronounced at 16 h (Fig. 8 *B*) and some oocytes have noticeably flattened, increasing their apparent diameter. By 24 h (Fig. 8 *C*), all but one of the Cx46-injected oocytes have completely lysed ("white death"), while the water-injected oocyte pair shows no visible alterations. Pairs were also constructed joining Cx46 mRNA-injected with either Cx32 or Cx43 mRNA-injected oocytes. While these heterologously injected cells initially formed tightly adherent pairs, by 16–24 h the oocyte containing Cx46 lysed, leaving those containing Cx32 or Cx43 intact.

Single oocytes were prepared for immunohistochemistry as described in Swenson et al. (1989). Anti-Cx46(372–382) and anti-Cx46(411–416) were used to stain 10-μm sections of oocytes injected with amounts of Cx46 mRNA identical to that shown in Fig. 7, lane *2*. Immunofluorescence revealed a cytoplasmic and cortical distribution for Cx46 (data not shown), similar to the distribution of Cx32 and Cx43 reported in single oocytes (Swenson et al., 1989). The distribution of Cx46 in oocyte pairs was not studied.

Cx46 mRNA-injected oocytes could be prevented from lysing by incubation in modified Barth's buffer with 5% Fi-

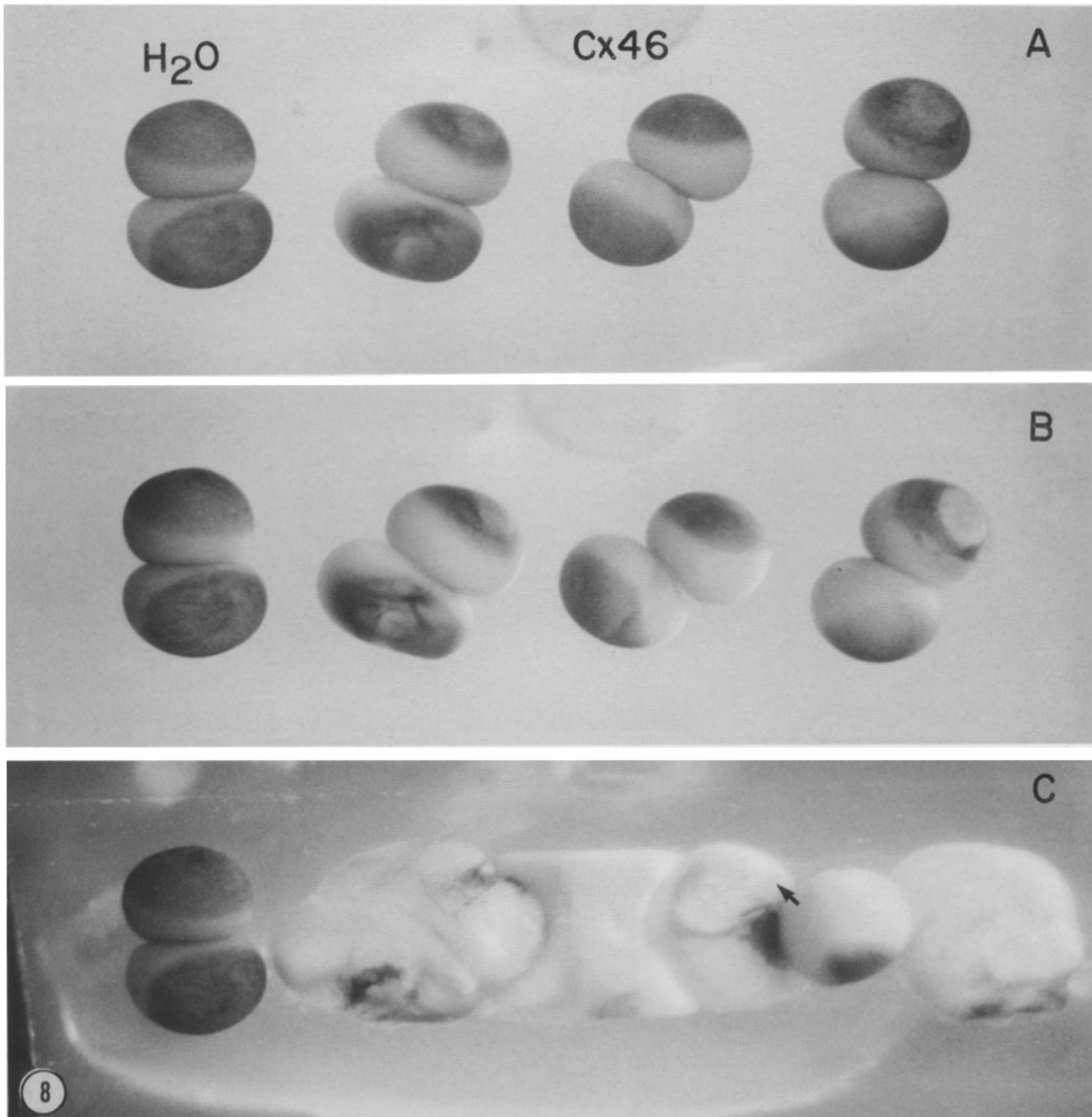
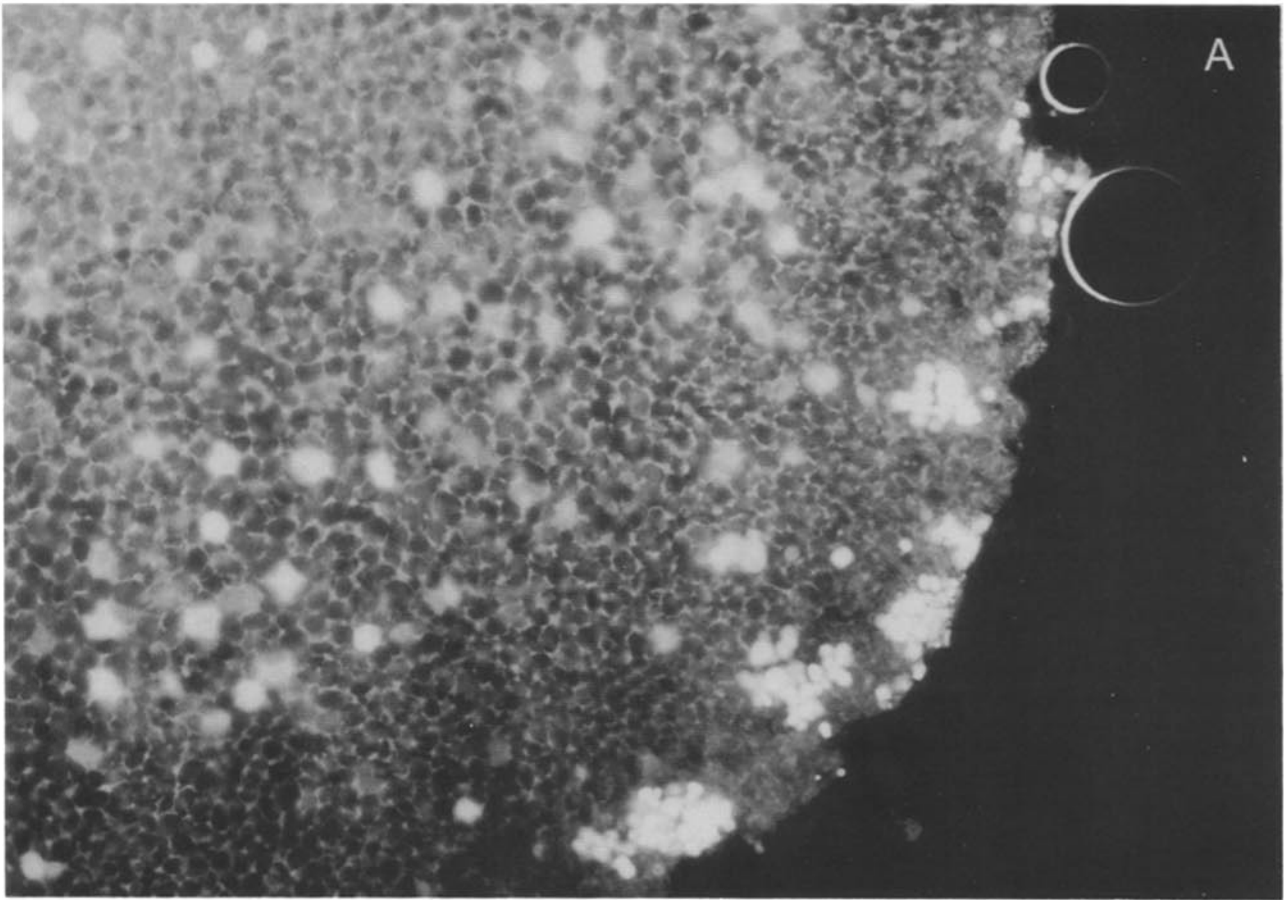
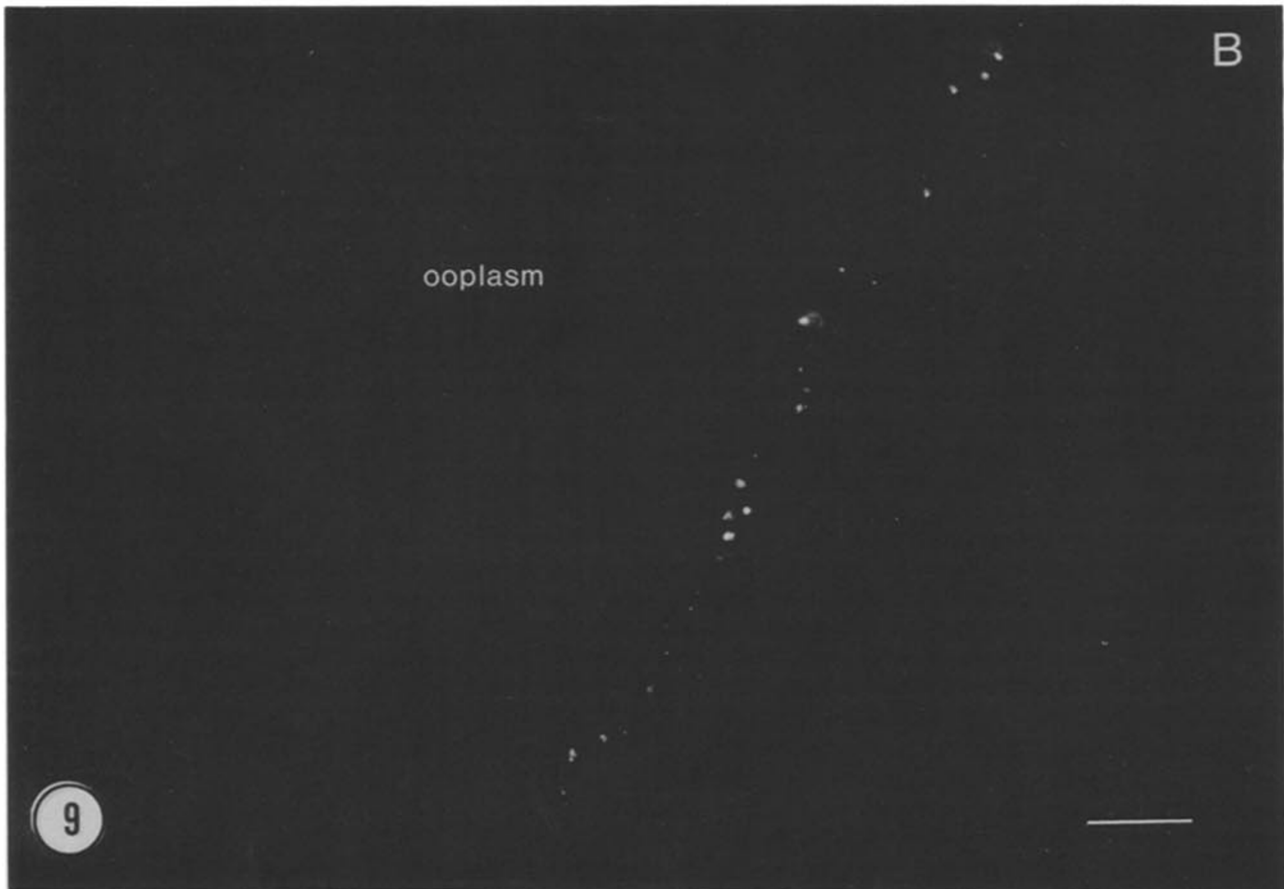


Figure 8. Xenopus oocytes were injected with either water or Cx46 mRNA, devitellinized, and paired on Teflon supports. One oocyte pair on the left-hand side was injected with water, the rest with Cx46 mRNA. The pairs were followed sequentially in time and photographed using a dissecting microscope at 4-h intervals. (*A*) Pairs at 12 h after injection of the Cx46 mRNA show disorganized pigment patterns in their animal hemispheres. (*B*) 16 h after injection the cells have flattened, increasing their apparent diameter. The pigment disorganization is more evident. (*C*) 24 h after injection all but one of the oocytes have completely lysed; the remaining cell is releasing yolk platelets in a broad stream (*arrow*). The water-injected controls show no apparent changes at any of the time points. Oocytes are ~1 mm in diameter.

coll, or in Ficoll buffer (see Materials and Methods). 0.25 M sucrose in Barth's was also effective in preventing lysis. Rescued cells showed no alteration in pigment distribution up to 48 h. Efforts to measure junctional conductances between oocyte pairs injected with Cx46 mRNA and incubated in 5% Ficoll were inconclusive; cells exhibited no resting potentials and such low input resistances that cell–cell coupling could not be measured. In the Barth's/Ficoll buffer system, most (70–90%, depending on the bath of oocytes) oocytes were seen (Fig. 9 *A*) to be permeable to the fluorescent dye Lucifer Yellow CH (443 mol wt), while control oocytes showed only limited vesicular uptake of Lucifer, detectable mostly at the surface of the vegetal hemisphere (Fig. 9 *B*). Fig. 9 *A* shows an example of a highly permeable oocyte;


A


B
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9

within each batch of oocytes there was a range of permeabilities as judged by the amount of cytoplasmic fluorescence detectable above background. A subset of yolk granules was also permeable to Lucifer, but it was not known if this was due to Cx46 mRNA injection, or if this indicated two classes of naturally occurring permeabilities. Control experiments included both Cx43 and Cx32 mRNA as well as water-injected oocytes. Since the Lucifer Yellow permeabilities of all control specimens were similar, only the water-injected control is presented in Fig. 9 *B*. Neither Cx46 nor control mRNA-injected oocytes were permeable to FITC-labeled BSA in the osmotic protection buffer (data not shown). Vesicular uptake of FITC-BSA was not noted in either experimental or control conditions.

Transmembrane conductance changes induced by expression of Cx46 in single oocytes could be observed under voltage clamp. In these experiments, oocytes were injected with Cx46 mRNA diluted to 0.02 $\mu g/\mu l$ and incubated in modified Barth's solution without Ficoll at 17°C overnight. Electrophysiological measurements were performed 6–15 h after injection of mRNA. Oocytes injected with Cx46 mRNA had reduced resting potentials relative to control oocytes injected with Cx43 mRNA (data not shown). These resting potentials were highly variable between experiments, but averaged around −30 mV. Large voltage-dependent currents were observed in Cx46 mRNA-injected oocytes which activated at potentials positive to −10 mV. Fig. 10 *A* shows current–voltage relations measured in a Cx46 mRNA-injected oocyte (*triangles*) compared with an oocyte injected with Cx43 (*squares*). Currents were measured 7 s after the imposition of each depolarizing or hyperpolarizing voltage step from a holding potential of −30 mV. The magnitude of the currents varied between oocytes and was sensitive to the amount of Cx46 mRNA injected. However, the −10-mV threshold for channel opening was relatively constant. Of 258 Cx46 mRNA-injected oocytes, 250 exhibited these voltage-gated currents; in 50 oocytes injected with either Cx32 or Cx43 mRNA, similar currents were never observed. The current–voltage relationship over time for Cx46-induced currents is illustrated in Fig. 10 *B*. Depolarizing voltage clamp steps were applied from a holding potential of −40 mV to potentials between −40 mV (baseline) and +20 mV in 10-mV increments. The slowly activating outward current was observed clearly in the 0-, +10-, and +20-mV traces. The inward currents seen upon repolarization back to −40 mV indicate more rapid channel closing kinetics.

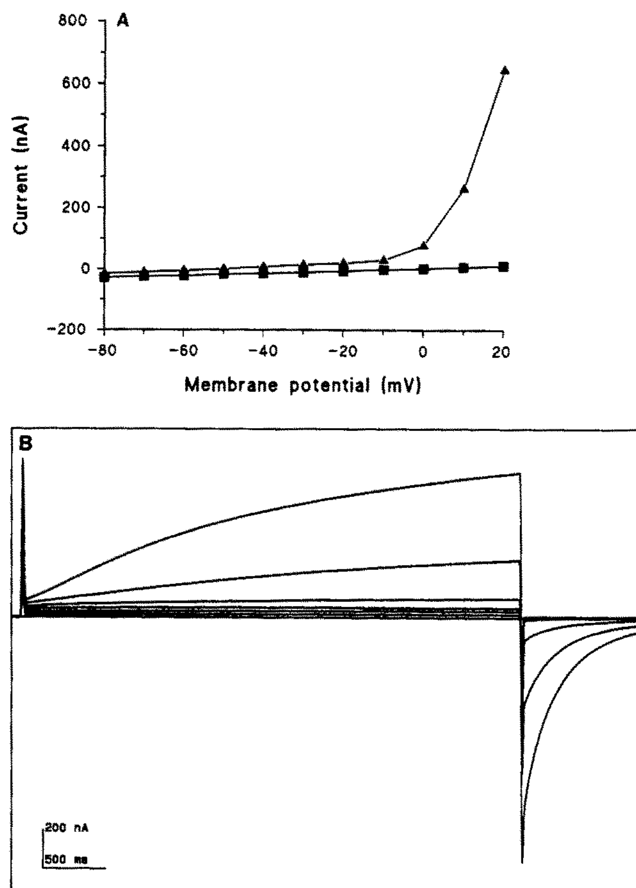

Figure 10. (*A*) Current–voltage relationships in Cx46 (*triangles*) and Cx43 (*squares*) mRNA-injected oocytes. Current was measured 7 s after the onset of a voltage-clamp pulse applied from a holding potential of −30 mV. Potentials positive to −10 mV activate whole cell currents in the Cx46-injected oocyte not observed in the Cx43-injected cell. (*B*) Time course of Cx46-induced currents. The oocyte was voltage clamped to −40 mV, then stepped in 10-mV increments to +20 mV. Slowly activating outward currents are observed positive to −10 mV in the 0 mV, +10 mV, and +20 mV traces. Release of the voltage clamp back to −40 mV results in an inward tail current with different kinetics.

Discussion

Cx46 Is a Member of the Connexin Family of Gap Junction Proteins

The amino acid sequence of Cx46 predicted by the cDNA reveals that this protein is a member of the connexin family of gap junction proteins (Beyer et al., 1988, 1990), with conserved regions corresponding to the predicted transmembrane and extracellular portions of the molecule, and completely unique sequences corresponding to those portions of the polypeptide predicted to face the cytoplasm. Since the anti-Cx46(411–416) antiserum is made to a predicted peptide at the COOH terminus of Cx46, the predicted cytoplasmic orientation of the COOH terminus is confirmed by the immunocytochemical localization of anti-Cx46(411–416) on the cytoplasmic surfaces of the junctional profiles (Fig. 5). Cx46 mRNA is most abundant in the lens, but is also clearly detectable in total RNA from heart and kidney.

Figure 9. Xenopus oocytes expressing Cx46 are permeable to Lucifer Yellow. Fluorescence micrographs of frozen sections of *Xenopus* oocytes injected with either Cx46 mRNA (*A*) or water (*B*) and incubated for 1 h in modified Barth's supplemented with 5% Ficoll and 0.1% Lucifer Yellow. In the Cx46-injected oocyte, dye is detected in the cytoplasm between yolk platelets and in a subset of the platelets. In contrast, in the water-injected oocyte, only small amounts of dye are seen which have a punctate, cortical distribution. Bar = 40 μm.

The Relationship of MP70 and Cx46

MP70 is a lens fiber plasma membrane protein recognized by the monoclonal antibody 6-4-B2-C6 (Kistler et al., 1985). This protein has been localized to fiber intercellular junctions in the cortex of ovine, rodent, and bovine lenses. It is not demonstrable in the deeper regions of the lens, presumably because the recognized epitope is removed by specific proteolysis as fibers age (Gruijters et al., 1987; Kistler and Bullivant, 1989). Amino acid analysis of the NH_2 termini of MP70 and its proteolytic fragments have suggested that this protein is a member of the connexin family.

The exact match between one possible ovine NH_2-terminal sequence of MP70 and the amino acid sequence predicted by the rat Cx46 cDNA suggested that the proteins were identical, although the significant difference in their molecular masses was not explained (Kistler et al., 1988; Beyer et al., 1988). In this report we have shown that Cx46 and MP70 cannot be the same protein because anti-Cx46 antibodies react with a protein of different size than that recognized by the 6-4-B2-C6 anti-MP70 monoclonal in both bovine and rat lens membranes.

Electron microscopic localization demonstrated that some fiber gap junctions contain both MP70 and Cx46. Other connexins, Cx32 and Cx26, have also been reported to be present in single gap junctional maculae in liver (Nicholson et al., 1987; Traub et al., 1989). Due to the technical limitations of resolution of immunocytochemical localization, it was not possible to determine if the two proteins can be localized to either the same connexon or to the same intercellular channel.

Posttranslational Processing of Cx46

Synthesis of Cx46 in either reticulocyte lysate or *Xenopus* oocytes results in a 46-kD polypeptide. The size of the translation product contrasts markedly with the size of the polypeptide recognized by the antisera in immunoblots of whole rat lens, where the protein appears 5-10 kD larger. This difference in size could be due to either a posttranslational modification of the protein, or to a cloning artifact. The latter possibility is unlikely since the sequence of the 3′ region surrounding the stop codon was confirmed from genomic DNA. The reason for the large shift in molecular weight of Cx46 is not known. However, posttranslational phosphorylation of Cx43 has been shown to result in a 3-4-kD increase in protein mobility (Crow et al., 1990; Musil et al., 1990*a*,*b*; Swenson et al., 1990; Laird et al., 1991). Theoretically, similar modifications could influence the mobility of Cx46. Oocytes are capable of serine phosphorylation of Cx43 (Swenson et al., 1990), a modification that is tightly correlated with gap junction assembly between cells in culture (Musil et al., 1990*b*), indicating that the oocyte has the capability to posttranslationally process at lest this connexin.

Expression of Cx46 in Xenopus Oocytes

The expression of Cx46 in the oocytes results in cell lysis unless the oocytes are osmotically buffered with Ficoll. Osmotically buffered oocytes injected with Cx46 mRNA are uniquely permeable to Lucifer Yellow CH but not to FITC-BSA. In addition, Cx46 mRNA-injected oocytes display voltage-activated ionic conductance. This unusual behavior is not shown by any of the other connexins that have thus far been expressed in oocytes (Dahl et al., 1987; Ebihara et al., 1989; Swenson et al., 1989; Werner et al., 1989). These data suggest that the Cx46 is forming water-permeable channels in the oolemma and that the mechanism of cell death is osmotic lysis. Therefore, Cx46 may form open connexons (hemichannels) in the membrane of the oocyte. Since the channel is closed at hyperpolarized potentials, why do the oocytes lyse? As Cx46 synthesis proceeds, the amount of protein potentially available for channel assembly becomes very large, enough for $\sim 10^8$ channels 24 h after mRNA injection (data not shown). With such large numbers of channels, the stochastic opening of even a small percentage of the total will result in a significant depression of input resistance and subsequent reduction of resting potential. This process of positive feedback would tend to open increasingly more channels over time. Thus, over the initial 6-8 h after mRNA injection, we observed a progressive loss of resting potentials in the oocytes, which eventually reaches zero before oocyte lysis.

Evidence for the presence of open gap junction hemichannels in nonjunctional membrane has been found in three systems. Lucifer-permeable channels which are modulated by dopamine have been observed by DeVries and Schwartz (1991) in teleost horizontal cells. Beyer and Steinberg (1991) have presented evidence that the ATP^{4-}-activated channel in a macrophage cell line may be related to Cx43. Finally, conductances with properties similar to those ascribed to gap junctions have been observed after incorporation of Cx32 into lipid bilayer systems (Young et al., 1987).

In the lens, where Cx46 is processed to a 54,000-M_r species, the formation of physiologically significant numbers of open hemichannels composed of Cx46 is unlikely to occur, since Mathias et al. (1981) have demonstrated that the lens fiber membranes are of unusually high resistance. However, Cx46 mRNA has been detected in heart and kidney, and may be present at lower levels in other tissues. Currently, no information is available about the synthesis or molecular mass of Cx46 in these organs. Whether Cx46 is used to form physiologically functional water channels in these organs must await further experimentation.

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