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Molecular Cloning and Functional Characterization of Chick Lens Fiber Connexin 45.6

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The avian lens is an ideal system to study gap junctional intercellular communication in development and homeostasis. The lens is experimentally more accessible in the developing chick embryo than in other organisms, and chick lens cells differentiate well in primary cultures. However, only two members of the connexin gene family have been identified in the avian lens, whereas three are known in the mammalian system. We report here the molecular cloning and characterization of the third lens connexin, chick connexin45.6 (ChCx45.6), a protein with a predicted molecular mass of 45.6 kDa. ChCx45.6 was encoded by a single copy gene and was expressed specifically in the lens. There were two mRNA species of 6.4 kilobase (kb) and 9.4 kb in length. ChCx45.6 was a functional connexin protein, because expression in *Xenopus* oocyte pairs resulted in the development of high levels of conductance with a characteristic voltage sensitivity. Antisera were raised against ChCx45.6 and chick connexin56 (ChCx56), another avian lens-specific connexin, permitting the examination of the distribution of both proteins. Immunofluorescence localization showed that both ChCx45.6 and ChCx56 were abundant in lens fibers. Treatment of lens membranes with alkaline phosphatase resulted in electrophoretic mobility shifts, demonstrating that both ChCx45.6 and ChCx56 were phosphoproteins in vivo.

INTRODUCTION

Gap junctions are clusters of transmembrane channels that connect the cytoplasms of adjacent cells. These channels permit small metabolites, second messengers, and ions to pass from cell to cell (Bennett and Goodenough, 1978; Goodenough *et al.*, 1980) and may play important roles in cellular signaling and growth regulation. These channels are formed by members of a family of proteins known as connexins, which contain highly conserved membrane spanning and extracellular regions, whereas cytoplasmic regions are unique (Zimmer *et al.*, 1987; Goodenough *et al.*, 1988; Hertzberg *et al.*, 1988; Milks *et al.*, 1988; Yancey *et al.*, 1989; Beyer *et al.*, 1990). The expression of connexins is cell type specific (Bennett *et al.*, 1991; Haefliger *et al.*, 1992), although the physiological significance of these different expression patterns is unknown.

The cells of the vertebrate eye lens are networked by an extensive system of gap junction–mediated cell-cell communication pathways subserving organ metabolic homeostasis. The lens is an avascular organ composed of two cell types: an anterior epithelium and highly differentiated lens fibers. The epithelial cells covering the anterior surface of the lens are continuous with the differentiating, highly elongated lens fibers at the equator. Posteriorly, the fibers in the center of the lens, because they have neither blood supply nor organelles, are uniquely dependent on communication with cells at the lens surface. Lens fibers have been shown to be joined into a functional syncytium (Duncan, 1969; Eisenberg and Rae, 1976; Rae, 1979; Mathias *et al.*, 1981; Mathias and Rae, 1985, 1989) mediated by gap junctions between adjacent fibers, which permit ions (Phillipson *et al.*, 1975; Rae, 1979; Rae and Stacey, 1979) and small transported metabolites (Goodenough *et al.*, 1980) as well as dyes (Schuetze and Goodenough, 1982; Miller and Goodenough, 1986) to diffuse between adjacent cells.

The lens has three different cellular interfaces: epithelium/epithelium, fiber/fiber, and epithelium/fiber.

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Lens epithelial gap junctions contain connexin43 (Cx43) (Musil *et al.*, 1990a), which joins cells in many tissues (Beyer *et al.*, 1987, 1989). Two lens fiber connexins have been cloned and characterized in rodent, connexin46 (Cx46) (Paul *et al.*, 1991; Jiang *et al.*, 1993) and connexin50 (Cx50) (White *et al.*, 1992). Ovine fiber junctions contain the lens-specific protein MP70 (Kistler *et al.*, 1985, 1988; Gruijters *et al.*, 1987), which is the sheep counterpart of rodent Cx50 (White *et al.*, 1992).

The avian lens offers several experimental advantages for the study of gap junctions compared to lenses from other organisms. First, avian embryos are very accessible for manipulation, permitting intervention and study at all stages of lens development (Beebe and Piatigorsky, 1981; Schuetze and Goodenough, 1982; Miller and Goodenough, 1986). Second, methods for the culture of embryonic avian lens cells are well developed, and the cultured cells differentiate into "lentoids", clusters of cells that express proteins unique to the differentiated lens fibers and that acquire some of the differentiated fiber morphology (Okada *et al.*, 1971; Piatigorsky *et al.*, 1973; Menko *et al.*, 1984, 1987). Moreover, these avian cultures are capable of assembling large numbers of fiber-fiber junctions, which is not true for cultures of rat lens cells (FitzGerald and Goodenough, 1986; Jiang *et al.*, 1993).

Although three connexins are known in the lens from other species, only two lens connexins have been identified in the chick. Chick connexin43 (ChCx43) has been cloned from lens epithelial cells by Musil *et al.* (1990a) and is highly homologous to rat and mouse Cx43. ChCx56 has been cloned and characterized by Rup *et al.* (1993), but it remained unclear which of the two rodent lens fiber connexins (Cx46 and Cx50) is the counterpart of ChCx56. This study reports the cloning and characterization of a new member of the avian connexin family, chick connexin 45.6. Analysis of sequence and physiological properties, together with the cellular distribution, suggest that ChCx45.6 is the avian counterpart of rodent Cx50 and ChCx56, the counterpart of rodent Cx46. Southern blotting suggests that ChCx45.6 is encoded by an intronless, single copy gene that is expressed in the lens. Voltage clamp analysis of pairs of *Xenopus* oocytes expressing ChCx45.6 showed that they formed gap junction channels with distinct voltage sensitivities.

MATERIALS AND METHODS

Reagents

An avian genomic library constructed in lambda phage EMBL-3 was purchased from Clontech (Palo Alto, CA). Fertilized avian eggs were obtained from SPAFAS (Norwich, CT) and were incubated for the desired times in a humidified 37°C incubator. Nylon transfer membrane-Hybond H^+ was from Amersham (Arlington Heights, IL). Glycogen and the random priming kit were from Boehringer Mannheim (Indianapolis, IN). ^{32}P-dCTP, ^{35}S-methionine (translational grade), and rabbit reticulocyte lysate were from New England Nuclear (Boston, MA). Alkaline phosphatase conjugated goat anti-rabbit IgG and Taq polymerase sequencing kit were from Promega (Madison, WI). Rhodamine-conjugated goat anti-rabbit IgG and BCA kit were from Pierce Chemical (Rockford, IL). Nitrocellulose membranes were from Millipore (Bedford, MA). Bluescript KS^+ was from Stratagene (LaJolla, CA). Tissue-Tek compound was from Miles Scientific (Naperville, IL). Formaldehyde (16% stock solution) was from Electron Microscopy Sciences (Ft. Washington, PA). RNA standards were from GIBCO (Grand Island, NY). CNBr-Sepharose was from Pharmacia (Piscataway, NJ). All other chemicals were obtained from either Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Polymerase Chain Reaction (PCR) Cloning with Degenerate Oligonucleotides and Screening of a Genomic Library

Degenerate oligonucleotide primers corresponding to portions of the first and second extracellular domains were synthesized on an ABI model 391 (Applied Biosystems, Foster City, CA). Avian lens cDNA was prepared according to Sambrook *et al.* (1989) by reverse transcription of total RNA from chick lens. PCR reactions contained 0.5 μg of avian lens cDNA, 200 ng of sense and antisense primers, 200 μM dNTP, 50 mM tris(hydroxymethyl)aminomethane (Tris) pH 8.3, 50 mM KCl, and 2.5 mM $MgCl_2$ in 100 μl. Addition of Taq polymerase (2.5 U) was performed after the reaction reached 94°C to minimize undesired priming during the initial cycle. Thirty cycles were performed as follows: 94°C for 1 min, 40°C for 1 min, and 72°C for 1 min. This was followed by a final extension for 10 min at 72°C. Reaction products were separated on 3% Nu-Sieve gels, excised, and electroeluted in dialysis bags as described by Sambrook *et al.* (1989). Eluted DNA was phenol-extracted and ethanol-precipitated with 10 μg of glycogen as a carrier. PCR products were phosphorylated and subcloned into Bluescript KS^+ as described by Haefliger *et al.* (1992). Restriction analysis with *Hin*fI and *Hin*pI was used to distinguish potentially novel connexins from ChCx43 and ChCx56. Plasmid DNA from unique clones was isolated and sequenced using Taq polymerase sequencing kit according to the manufacturer's recommendations. A partial sequence for a new connexin was obtained. This sequence was used as a probe to screen an avian genomic DNA library constructed in λ phage EMBL-3. Plaques (200 000) were plated on six (15 cm) plates. Plaques were lifted according to Sambrook *et al.* (1989) onto nitrocellulose, and hybridization was performed as described by Beyer *et al.* (1987). Three 30-min washes were performed at 65°C in 0.3 M Na_2HPO_4 and 1% sodium dodecyl sulfate (SDS). Four consistently positive plaques were carried through three rounds of replating to purity, and all of them were hybridized under high stringency conditions by washing at 65°C in 0.03 M Na_2HPO_4 and 1% SDS. λ DNA was isolated on a DEAE column according to Helms *et al.* (1985). For all four lambda clones, *Sal* I and *Spe* I digestion of λ DNA yielded five fragments of ~9, 4, 1.4, 0.6 and 0.2 kilobase (kb) in size. The fragments were separated on 1% agarose gels, isolated by electroelution of gel strips in dialysis bags, subcloned into Bluescript KS^+, and sequenced.

Northern and Southern Blotting

For Northern blotting, RNA was isolated by homogenization of 11 d and 19 d embryonic avian tissues in guanidine isothiocyanate followed by centrifugation through CsCl (Chirgwin *et al.*, 1979). Ten-microgram samples were electrophoresed on 1% agarose/formaldehyde gels and capillary blotted onto nylon membrane (Hybond N^+) in 2× SSC for 14 h (Sambrook *et al.*, 1989). For Southern blotting, 5-μg aliquots of avian genomic DNA (Clontech) were digested with various restriction enzymes, electrophoresed on 0.7% agarose/Tris-acetate-EDTA gels, and blotted onto nylon membranes in 0.4 M NaOH for 3 h. Nucleic acids were fixed to the membranes by incubation at 85°C for 1 h under vacuum. ^{32}P-labeled ChCx45.6 probe was prepared from a 996 basepair (bp) *Bsp* mI fragment of the ChCx45.6 clone in SP64T (see below) by random-prime labeling. Blots were prehybrided in 0.7 M

Na phosphate pH 7.2, 1 mM EDTA, 5% SDS, and 100 μg/ml salmon sperm DNA at 65°C for 2 h and then hybridized with the addition of the labeled probes for 16 h. The blots were washed 3 × 30 min in 0.3 M Na phosphate pH 7.2 and 1% SDS at 65°C (Paul, 1986) and were further washed 2 × 30 min in 6.7 mM Na phosphate at 65°C.

Preparation and Immunoaffinity Purification of Anti-ChCx45.6 and Anti-ChCx56 Sera

Bacterial fusion proteins containing glutathione-S-transferase plus C-terminal portions of ChCx45.6 (nucleotides [nt] 1029–1274) and ChCx56 (nt 1081–1467) were produced using the vector pGEX-1 (Smith and Johnson, 1988). Overnight cultures were diluted 1/100 in fresh LB medium and grown for 2 h before inducing synthesis of the fusion protein by the addition of a final concentration of 0.5 mM isopropyl β-D-thiogalactopyranoside. After 4 h of induction, cells were pelleted by centrifugation at 2100 × *g* for 10 min. The pellet was resuspended in cold phosphate-buffered saline (PBS), sonicated with 1% Triton X-100 for 1 min on ice, and centrifuged at 5600 *g* at 4°C. The supernatant was passed through an affinity column of glutathione immobilized on Sepharose. The bound fusion protein was released with 5 mM glutathione. To separate full length fusion proteins from degradation products, the eluted fusion proteins were separated on a 10% SDS-polyacrylamide gel, which was then stained with copper chloride as described in Harlow and Lane (1988), and the full length fusion protein band was excised from the gel. The gel strips were electroeluted in dialysis bags, and the eluted proteins were concentrated in ULTRAFREE-MC 30 000 NMWL Filter Unit (Millipore, Bedford, MA). The purified full length fusion proteins were used to raise polyclonal antibodies in rabbits (Pocono Rabbit Farm, Canadensis, PA).

Antibodies were affinity purified according to Harlow and Lane (1988). First, antibodies directed against glutathione S-transferase were removed by passing 2 ml of immune serum over a Sepharose column containing 1 mg of glutathione S-transferase. Next, the flow-through was applied to a second Sepharose column containing 1 mg of purified fusion protein. The bound ChCx45.6- and ChCx56-specific antibodies were eluted with 0.1 M glycine pH 2.5 and immediately neutralized. Columns were produced using preactivated CNBr-Sepharose 4B according to the manufacturer's directions.

Dephosphorylation Analysis and Western Blotting

A crude membrane fraction of avian lenses was prepared as described in White *et al.* (1992). The dephosphorylation assay was done according to Musil *et al.* (1990a). Briefly, membrane preparations were incubated for 3 h, at 37°C in the absence or presence of 2 U of alkaline phosphatase. Control reactions were done with incubation of 2 U of alkaline phosphatase plus alkaline phosphatase inhibitors (2 mg/ml Na orthovandate, 10 mM EDTA, and 10 mM PO_4). The protein concentration was determined using the BCA assay; 0.5 μg of total protein was loaded on each lane of 10% SDS-polyacrylamide gel and transferred to nitrocellulose (White *et al.*, 1992). Western blots were probed with a 1:500 dilution of preimmune serum and anti-ChCx45.6 or anti-ChCx56 serum. Primary antibodies were detected with a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG.

Immunohistochemistry

Eleven- and 19-d embryonic chick lenses were fixed in 1% formaldehyde (diluted from 16% stock) in PBS for 30 min at room temperature. Lenses were then immersed in Tissue-Tek compound wrapped by aluminum foil and frozen in nitrogen-cooled Freon-22 slush. Seven micron sections were collected and processed according to Paul *et al.* (1991). Sections were first incubated in blocking solution containing 2% normal goat serum, 2% fish skin gelatin, and 1% BSA in PBS for 30 min and then incubated with either an affinity-purified anti-ChCx45.6 or anti-ChCx56 antibody diluted 1:500 in blocking solution for 1 h at room temperature. Sections were washed 3 times, 5 min each in PBS and then incubated with rhodamine-conjugated goat anti-rabbit IgG (Sigma) diluted 1:500 in blocking solution for 1 h. Fluorescence microscopy was performed using a Zeiss Axioskop (Thornwood, NJ) and recorded on Tmax400 film (Kodak, Rochester, NY).

Injections of Xenopus Oocytes and Electrophysiological Measurements

Oocytes were collected from *Xenopus laevis* females and processed for the paired *Xenopus* oocyte expression assay as described (Swenson *et al.*, 1989). To eliminate the possible contribution of endogenous intercellular channels to the measured conductance, manually defolliculated oocytes were injected with an antisense oligonucleotide corresponding to a portion of the coding sequence of *Xenopus* Connexin38 (Cx38) (Bruzzone *et al.*, 1993) (3 ng/oocyte, 5′-CTGACTGCTCG-TCTGTCCACACAG-3′). After overnight incubation at 18°C, each antisense-treated oocyte was then injected with 40 nl of ChCx45.6 mRNA (10–100 pg) and paired for quantitation of junctional communication by double voltage clamp (Spray *et al.*, 1981). Both cells were initially clamped at −40 mV to ensure zero transjunctional potential. Although cell 1 of the pair was hyperpolarized or depolarized in 5 or 10 mV steps, cell 2 was held at −40 mV. ChCx45.6 pairs exhibiting conductance < 5 μS were selected for analysis of voltage sensitivity. Initial currents were resolved at 5–10 ms, and steady state currents were measured at 30 s. Normalized steady-state conductance was fitted to a Boltzmann equation of the form

$$G_j = \{(G_{jmax} - G_{jmin})/[1 + e^{(A(V_j - V_o)}]\} + G_{jmin},$$

where G_{jmax} is the normalized maximal conductance (=1), G_{jmin}, is the normalized minimal conductance at the largest V_j, V_o is the voltage at which half-maximal decrease of the steady-state conductance (G_{jss}) occurs, and A is a parameter reflecting the slope of the curve (Spray *et al.*, 1981).

In Vitro Transcription and Translation ChCx45.6

A DNA fragment containing the ChCx45.6 coding sequence, from the start to stop codon, was produced by PCR amplification of DNA from one of the lambda genomic clones. Primers contained *Bgl* II sites to facilitate subcloning. The PCR product was gel isolated and subcloned into the *Bgl* II site of the transcription vector SP64T (Krieg and Melton, 1984). Recombinant plasmid was linearized with *Bam*HI and used as a template for the in vitro transcription with SP6 RNA polymerase (Swenson *et al.*, 1989). In vitro transcribed ChCx45.6 mRNA (50 ng) was translated in a rabbit reticulocyte lysate in the presence of 80 mM potassium acetate, 0.65 mM Mg acetate, and 25 μCi of ^{35}S-methionine (Musil *et al.*, 1990a). Immunoprecipitation of in vitro translation product was conducted using the methods described by Musil *et al.* (1990a). For metabolic labeling of *Xenopus* oocytes, each oocyte was injected with 10–100 pg of mRNA and 2 μCi of ^{35}S-methionine in a volume of 40 nl, and then incubated at 18°C for 6 h (Swenson *et al.*, 1989). The oocytes were lysed and immunoprecipitated as described by Swenson *et al.* (1989).

RESULTS

Molecular Cloning and Sequence Analysis of ChCx45.6

The strategy we used to identify additional connexins in avian lens relied on PCR amplification of lens cDNA and restriction digestion analysis of clones to eliminate those corresponding to already known connexins. Degenerate oligonucleotide primers were constructed based on the similarity between previously identified chick connexins (ChCx): ChCx45 (Beyer, 1990), ChCx42 (Beyer, 1990), ChCx56 (Rup *et al.*, 1993), and ChCx43 (Musil *et al.*, 1990a). The two consensus degenerate oli-

gonucleotides are shown in Table 1. To minimize probe degeneracy, not all of the possible sequence variations in the alignment were present in the primer sequences. The amplified regions started within the first extracellular loop and ended in the second extracellular loop. The two major PCR products visible by electrophoresis were excised, and the DNA was isolated, subcloned, analyzed by restriction digestion for novel genes (see MATERIALS AND METHODS), and sequenced. A clone that contained novel connexin sequences was identified and used to screen a chick genomic library.

Figure 1 shows the sequence of portions of 4 kb of *Sal* I/*Spe* I and 0.2 kb of *Spe* I fragments from a positive clone of chick genomic library. There was an open reading frame starting at nt 108 and ending at nt 1310. As with all other connexins, there appeared to be no introns in the coding region (Miller *et al.*, 1988; Zhang and Nicolson, 1989; Paul *et al.*, 1991; Haefliger *et al.*, 1992; White *et al.*, 1992; Rup *et al.*, 1993). The DNA sequence predicts a protein of 400 amino acids with a molecular mass of 45619 Da that, by current convention, we have designated chick connexin45.6. ChCx45.6 is structurally similar to other connexins in that the deduced amino acid sequence predicts four transmembrane domains, two extracellular loops, and one cytoplasmic loop. In addition, the similarity of ChCx45.6 to other connexins is highest in the characteristically conserved transmembrane domains and extracellular loops. Therefore, ChCx45.6 is a member of the avian connexin family. Comparison of ChCx45.6 sequence to other known lens connexins (Figure 2A) demonstrates that the amino acid sequence of ChCx45.6 is 75% identical to that of mouse Cx50 (White *et al.*, 1992), 58% identical to ChCx56 (Rup *et al.*, 1993), and 46% identical to rat Cx46 (Paul *et al.*, 1991), demonstrating that ChCx45.6 is most homologous to mouse Cx50. Comparison of amino terminal sequences with the degenerate sequences of ovine MP70 (Kistler *et al.*, 1988) (Figure 2B) shows that ChCx45.6 and mouse Cx50 are identical except for two amino acid residues and that they match one of the MP70 degenerate sequences; ChCx56 and rat Cx46 are exactly identical, and they match the other MP70 degenerate sequence.

Southern blot analysis was performed by digestion of chick genomic DNA with various restriction enzymes (Figure 3). Digestion with *Ban* I and *Bam* HI produced two hybridizing bands, consistent with predicted restriction sites within the probe region (see MATERIALS AND METHODS). Digestion with *Eco*RI, *Hin*dIII, *Pst* I, and *Xba* I/*Xho* I, which have no predicted sites in the probe region, resulted in only one hybridizing band. These data suggest that the coding region of ChCx45.6 is contained in a single exon of a single copy gene, consistent with other connexins.

The tissue distribution of ChCx45.6 was examined using a Northern blot containing total RNA from lens, heart, lung, liver, kidney, and brain. Bands (6.4 and 9.4 kb) were detected in lens RNA (Figure 4) but not in RNA from other organs tested. The ratio of 6.4 to 9.4 kb mRNA was higher in the 19-d than in the 11-d embryonic avian lens.

Both ChCx45.6 and ChCx56 Are Phosphorylated in Vivo

Membrane fractions prepared from 11-d embryonic chick lenses were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting using anti-ChCx45.6 serum. Three immunoreactive bands were detected at M_r = 53, 56, and 58 kDa, respectively (Figure 5, lane 1). No signals were detected by preimmune serum. Posttranslational phosphorylation has been shown to alter the electrophoretic mobility of other rodent lens connexins (Tenbroek *et al.*, 1992; Jiang *et al.*,

Table 1. Degenerate oligonucleotide primers derived from known chick connexins

ChCx43	GGTTGTGAGAACGTCTGCTATGAC . . . . .
ChCx56	GGTTGCGAAAATGTTTGCTATGAC . . . . .
ChCx42	GGCTGTGAAAACGTCTGCTACGAC . . . . .
ChCx45	GGCTGTGAGAACGTTTGTTACGAC . . . . .

5′ GGTTGTGAGAACGTCTGCTATGAC 3′
C C A T T T C

Sense Primer →

ChCx43	ACAAAGGACAGGGCAGGTTGACTC . . . . .
ChCx56	ACGAAGTAGAGTGCCGGGTGACTC . . . . .
ChCx42	ACGATGCAAAGGGCAGGGTGTCTC . . . . .
ChCx45	ACGAAGTAAAGTTCCGGCTGGCTT . . . . .

3′ ACAAAGGACAGGGCAGGGTGACTT 5′
G T T G T C
C A

← Anti-Sense Primer

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ACTAGTTGAA AGTAACAACA GGCATCAGTG GGAAAACATG AGATCTTTTA AATTTAGCAT        60
AACTTGTGGT CATTCTCTTT CTTTTTTCTT TCCTTAGACA GTAAGAA ATG GGT GAC TGG AGT 122
                                    M   G   D   W   S                    5

TTC TTG GGG AAC ATT TTA GAG CAG GTG AAC GAG CAG TCC ACT GTC ATC GGG     173
F   L   G   N   I   L   E   Q   V   N   E   Q   S   T   V   I   G      22

AGA GTT TGG CTC ACG GTG CTC TTC ATT TTC CGC ATC CTG ATC CTG GGA ACA     224
R   V   W   L   T   V   L   F   I   F   R   I   L   I   L   G   T      39

GCT GCT GAA CTA GTA TGG GGA GAT GAA CAG TCA GAC TTT GTG TGC AAC ACC     275
A   A   E   L   V   W   G   D   E   Q   S   D   F   V   C   N   T      56

CAG CAA CCT GGT TGT GAG AAC GTC TGC TAT GAT GAG GCC TTC CCC ATC TCC     326
Q   Q   P   G   C   E   N   V   C   Y   D   E   A   F   P   I   S      73

CAC ATC CGG CTC TGG GTC CTA CAG ATC ATT TTT GTA TCC ACG CCT TCG CTA     377
H   I   R   L   W   V   L   Q   I   I   F   V   S   T   P   S   L      90

GTG TAC TTT GGG CAT GCG GTG CAC CAT GTC CGC ATG GAG GAG AAG AGG AAA     428
V   Y   F   G   H   A   V   H   H   V   R   M   E   E   K   R   K     107

GAG AGG GAG GAA GCT GAG AGG CGT CAG CAA GCT GAG GTG GAT GAA GAG AAG     479
E   R   E   E   A   E   R   R   Q   Q   A   E   V   D   E   E   K     124

CTG CCC CTA GCT CCA AAT CAA AAC AAG GGC AAC AAC CCA GAT GGG ACC AAG     530
L   P   L   A   P   N   Q   N   K   G   N   N   P   D   G   T   K     141

AAG TTT CGC CTG GAG GGG ACC CTC CTG AGA ACC TAC ATC CTC CAC ATC ATT     581
K   F   R   L   E   G   T   L   L   R   T   Y   I   L   H   I   I     158

TTC AAA ACT CTC TTT GAA GTG GGA TTC ATT GTT GGC CAG TAT TTC CTG TAT     632
F   K   T   L   F   E   V   G   F   I   V   G   Q   Y   F   L   Y     175

GGC TTC CGC ATT CTC CCC CTT TAC CGC TGT GGG CGG TGG CCC TGT CCC AAC     683
G   F   R   I   L   P   L   Y   R   C   G   R   W   P   C   P   N     192

CTA GTG GAC TGT TTT GTC TCC AGG CCC ACA GAG AAG ACC ATC TTT ATT ATG     734
L   V   D   C   F   V   S   R   P   T   E   K   T   I   F   I   M     209

TTC ATG CTC GTG GTG GCT GCT GTG TCC CTC TTC CTC AAC CTG GTG GAG ATC     785
F   M   L   V   V   A   A   V   S   L   F   L   N   L   V   E   I     226

AGC CAC TTG ATC CTG AAA AGG ATC CGG AGG GCT CTG AGA AGA CCA GCA GAG     836
S   H   L   I   L   K   R   I   R   R   A   L   R   R   P   A   E     243

GAG CAG ATG GGG GAG GTG CCA GAG AAG CCC CTC CAT GCT ATT GCA GTG TCC     887
E   Q   M   G   E   V   P   E   K   P   L   H   A   I   A   V   S     260

TCC ATC CCG AAG GCC AAA GGC TAC AAG CTG CTA GAA GAA GAA AAG CCA GTG     938
S   I   P   K   A   K   G   Y   K   L   L   E   E   K   P   V     277

TCC CAC TAT TTC CCT CTC ACG GAA GTA GGG GTT GAG CCC AGT CCC CTT CCA     989
S   H   Y   F   P   L   T   E   V   G   V   E   P   S   P   L   P     294

TCA GCC TTC AAT GAG TTT GAG GAG AAG ATT GGG ATG GGG CCA CTG GAA GAT    1040
S   A   F   N   E   F   E   E   K   I   G   M   G   P   L   E   D     311

CTC TCC AGG GCA TTT GAT GAG AGG TTA CCA TCG TAT GCA CAA GCG AAG GAA    1091
L   S   R   A   F   D   E   R   L   P   S   Y   A   Q   A   K   E     328

CCG GAA GAG GAG AAG GTA AAA GCA GAG GAG GAA GAG GAA CAA GAA GAG GAG    1142
P   E   E   E   K   V   K   A   E   E   E   E   E   Q   E   E   E     345

CAG CAA GCA CCT CAG GAA GAG CCA GGG GTG AAG AAA GCA GAG GAG GAG GTG    1193
Q   Q   A   P   Q   E   E   P   G   V   K   K   A   E   E   E   V     362

GTG AGC GAT GAA GTG GAA GGG CCT TCA GCA CCT GCT GAA CTT GCC ACC GAT    1244
V   S   D   E   V   E   G   P   S   A   P   A   E   L   A   T   D     379

GTG AGA TCC CTC AGC AGG CTA AGT AAA GCC AGC AGC CGG GCC AGG TCA GAC    1295
V   R   S   L   S   R   L   S   K   A   S   S   R   A   R   S   D     396

GAT CTG ACT GTA TGA GGATGCAGGA TATGAGGAGC ATATGAAAAG GAAAAGAGGA       1350
D   L   T   V                                                         400

AAAGGAAAAG GAGAGAGAAA GAATCAGAAG AATTTTAAGC AAAGTGCTAA AATGATCATT     1410
TAAATATTAT TTCATCTTGA GATTCTCACT G                                    1441
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Figure 1. Sequence of genomic ChCx45.6 clone. Portions of a *Sal* I/*Spe* I fragment (4 kb) and a *Spe* I fragment (0.2 kb) were sequenced on both strands. A single uninterrupted open reading frame starting at nt 108 and ending at nt 1310 (bold, underline) encoded a protein with a predicted molecular mass of 45 619 Da. The derived amino acid sequence of ChCx45.6 is shown in lower line. This sequence is available in the EMBL database under the accession number L24799.

1993). Therefore, the avian lens membranes were treated with alkaline phosphatase before Western blotting. The three bands were converted predominantly into a M_r = 58 kDa band (Figure 5, lane 2). Control experiments showed that phosphatase inhibitors blocked the effect of alkaline phosphatase on the electrophoretic mobility (Figure 5, lane 3). By these criteria, ChCx45.6 is a phosphoprotein whose electrophoretic mobility is increased in response to phosphorylation.

Neither the phosphorylation nor distribution of another cloned chick lens connexin, ChCx56 (Rup *et al.*, 1993), have been reported; therefore, anti-ChCx56 serum was raised and ChCx56 was analyzed as described above for ChCx45.6. Multiple bands were detected around 81 kDa (Figure 5, lane 4), and alkaline phosphatase digestion increased the mobility of at least some of the bands (Figure 5, lane 5). Control experiments (Figure 5, lane 6) with alkaline phosphatase inhibitors showed no mobility shift. Therefore, both ChCx45.6 and ChCx56 are phosphorylated proteins in the avian lens.

Both ChCx45.6 and ChCx56 Are Abundant in Embryonic Lens Fibers

The distribution of ChCx45.6 in 11-d avian embryonic lenses was determined by immunohistochemistry using affinity purified anti-ChCx45.6 antibody. Figure 6A is a phase contrast image of a frozen section of the lens epithelium and lens fibers. Immunofluorescence mi-

A:

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                                    30v                                 60v
CHCX45.6 MGDWSFLGNILEQVNEQSTVIGRVWLTVLFIFRILILGTAAELVWGDEQSDFVCNTQQPGC
         MGDWSFLGNILE VNE STVIGRVWLTVLFIFRILILGTAAE VWGDEQSDFVCNTQQPGC
CX50     MGDWSFLGNILEEVNEHSTVIGRVWLTVLFIFRILILGTAAEFVWGDEQSDFVCNTQQPGC
                                    30^                                 60^
                                   90v                                 120v
CHCX45.6 ENVCYDEAFPISHIRLWVLQIIFVSTPSLVYFGHAVHHVRMEEKRKEREEAERRQQAEVD-
         ENVCYDEAFPISHIRLWVLQIIFVSTPSL Y GHAVHHVRMEEKRK RE  E  QQ
CX50     ENVCYDEAFPISHIRLWVLQIIFVSTPSLMYVGHAVHHVRMEEKRKDREAEELCQQSRSNG
                                    90^                                120^
                                      150v                170v
CHCX45.6 EEKLPLAPNQ----NKGNNPDGTKKFRLEGTLLRTYILHIIFKTLFEVGFIVGQYFLYGFR
          E  P AP Q          GTKKFRLEGTLLRTY  HIIFKTLFEVGFIVG YFLYGFR
CX50     GERVPIAPDQASIRKSSSSSKGTKKFRLEGTLLRTYVCHIIFKTLFEVGFIVGHYFLYGFR
                                   150^                                180^
                                     210v
CHCX45.6 ILPLYRCGRWPCPNLVDCFVSRPTEKTIFIMFMLVVAAVSLFLNLVEISHLILKRIRRALR
         ILPLYRC RWPCPN VDCFVSRPTEKTIFI FML VA VSLFLN  E SHL  K IR A
CX50     ILPLYRCSRWPCPNVVDCFVSRPTEKTIFILFMLSVAFVSLFLNIMEMSHLGMKGIRSAFK
                                   210^                                240^
              250v                          280v
CHCX45.6 RPAEEQMGEVPEKPLHAIAVSSIPKAKGYKLLEEEKPVSHYFPLTEVG-VEPSPLPS-AFN
         RP E   GE  EK LH IAVSSI KAKGY LLEEEK VSHYFPLTEVG VE SPL    F
CX50     RPVEQPLGEIAEKSLHSIAVSSIQKAKGYQLLEEEKIVSHYFPLTEVGMVETSPLSAKPFS
                              270^                                300^
              310v                          330v
CHCX45.6 EFEEKIGMGPLEDLSRAFDERLPSYAQ--AKEPEEEKVKAEEEEEQEEEQQAPQEEPGVKK
          FEEKIG GPL D SR   E LPSYAQ    E E E    EE  E E        E
CX50     QFEEKIGTGPLADMSRSYQETLPSYAQVGVQEVEREEPPIEEAVEPEVGEKKQEAEKVAPE
                              330^                                360^
                                        370v
CHCX45.6 AEEEVVS-------------DEVE----------GPSAPA------ELATDVRS-LSRLS
           E V                DE E          G SA         EL TD    LSRLS
CX50     GQETVAVPDRERVETPGVGKEDEKEELQAEKVTKQGLSAEKAPSLCPELTTDDNRPLSRLS
                              390^                                420^
        390v          400
CHCX45.6 KASSRARSDDLTV
         KASSRARSDDLT
CX50     KASSRARSDDLTI
                    440
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B:

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Cx46                MGDWSFLGRLLENAQEHSTVI..
ChCx56              MGDWSFLGRLLENAQEHSTVI..
MP70                -GDWSFLG(RL/NI)LEN(A/V)QEHSTVI..
ChCx45.6            MGDWSFLGNILEEVNEHSTVI..
Cx50                MGDWSFLGNILEQVNEQSTVI..
```

Figure 2. Alignment of predicted amino acid sequences of lens connexins. (A) Alignment of the entire amino acid sequences for ChCx45.6 and rodent Cx50. Identical amino acids are written in bold type between sequences of ChCx45.6 and rodent Cx50. (B) Alignment of the predicted amino termini of ChCx45.6, ChCx56, rodent Cx50, and Cx46 with that of ovine MP70.

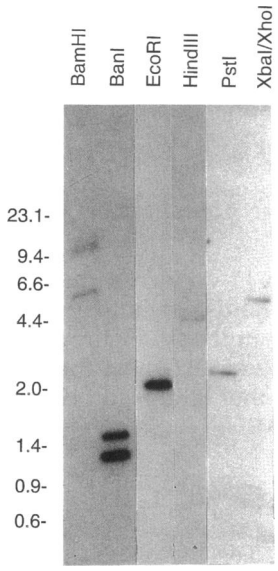

Figure 3. Southern blot analysis suggests ChCx45.6 is encoded by single copy gene with no introns in the coding region. Five microgram aliquots of avian genomic DNA were digested with various restriction enzymes and then were probed with a partial sequence of ChCx45.6. Sequence analysis predicted a *Ban* I and a *Bam* HI sites within the probe region, and digestion with these two enzymes yielded two hybridizing bands. Digestion with all other enzymes tested yielded a single hybridizing band. The DNA standards are indicated on the left in kilobases.

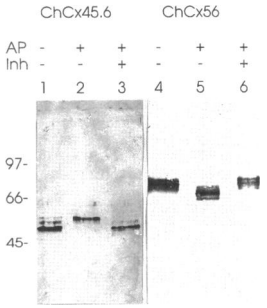

Figure 5. Dephosphorylation and Western blot analysis show that both ChCx45.6 and ChCx56 are phosphorylated proteins. Total membrane proteins from 11-d embryonic avian lenses were incubated in the absence (lane 1 and 3) or presence of alkaline phosphatase (lane 2 and 5), or alkaline phosphatase plus an excess of phosphatase inhibitor (lane 3 and 6). For ChCx45.6, triplet bands, 53, 56, and 58 kDa, respectively are recognized by anti-ChCx45.6 serum (lane 1 and 3) and mobility shifts to 58 kDa (lane 2) in the presence of alkaline phosphatase. For ChCx56, multiple bands around 81 kDa are recognized by anti-ChCx56 serum (lane 4 and 6), and mobility shifts to around 74 kDa (lane 5) in the presence of alkaline phosphatase.

croscopy showed that affinity-purified anti-ChCx45.6 antibody produced punctate staining between lens fibers (Figure 6B).

The localization of ChCx56 in embryonic lenses was also examined. A staining pattern similar to that of ChCx45.6 was produced by affinity-purified anti-ChCx56 antibody (Figure 6D). The corresponding phase contrast image is shown in Figure 6C. Taken together, these results indicate that both ChCx45.6 and ChCx56 are localized in lens fibers. At this developmental stage, the pseudostratified epithelium showed low numbers of ChCx45.6- and ChCx56-containing junctional maculae, although it was not known if these are colocalized either in the same junctional plaque or even in the same cells.

Immunohistochemical examination of lenses from older embryos (day 19) revealed that both ChCx45.6 and ChCx56 stainings are lost with lens fiber aging. Frozen sections of 19-d embryonic chick lenses were examined with either an affinity purified anti-ChCx45.6 antibody (Figure 6, E and F) or anti-ChCx56 antibody (Figure 6, G and H). Figure 6, F and H are immunofluorescent images of day 19 lenses taken at the center of the lens, where companion phase-contrast images (Figure 6, E and G) reveal the transition from lens fibers that contain oval nuclei through a zone of fibers that have pyknotic nuclei to those fibers that have no detectable nuclei. In this transition region, there was a spotty loss of immunofluorescent staining of the junctional maculae with both anti-connexin antibodies, indicating that there may be proteolytic processing of these connexins resulting in the removal of their antigenic sites, as has been documented for lens fiber connexins in other species (Gruijters *et al.*, 1987; Evans *et al.*, 1993).

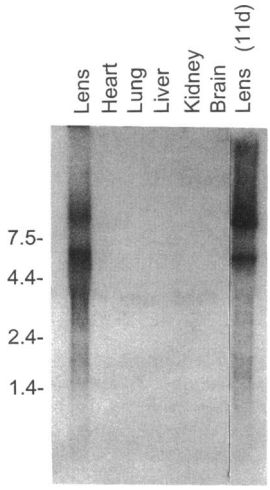

Figure 4. Northern blot analysis suggests that ChCx45.6 is a lens connexin. Ten micrograms of total RNA isolated from various 19-d embryonic avian organs and 11-d embryonic avian lenses were loaded in individual lanes. The blot was probed with the same probe as used for Southern analysis. Two hybridizing bands of 6.4 and 9.6 kb were detected in avian lens RNA, whereas no signal was observed in any other organs tested. The RNA standards are indicated on the left in kilobases.

Functional Expression of ChCx45.6 in Paired Xenopus Oocytes

The functional expression of ChCx45.6 was analyzed in the paired *Xenopus* oocyte system (Dahl *et al.*, 1987; Swenson *et al.*, 1989). Before injection of mRNA or water, an antisense oligonucleotide to *Xenopus* Cx38 was injected into the oocytes to eliminate the possible contribution of this endogenous connexin to the observed conductance (Bruzzone *et al.*, 1993). After ChCx45.6 mRNA injection, oocytes were then paired for measurements of junctional conductance. Table 2 shows a summary of conductance data for ChCx45.6. Although oocytes injected with water were not electrically coupled (<0.05 μS, n = 8), oocyte pairs injected with ChCx45.6 were highly coupled (33.5 ± 5.5 μS, n = 27), demonstrating that expression of ChCx45.6 results in the formation of functional intercellular channels in oocytes. The current voltage relationship for these channels is illustrated in Figure 7A. Depolarizing voltage steps ≥ 20 mV applied to either cell evoked junctional currents that decreased relatively slowly with time approaching

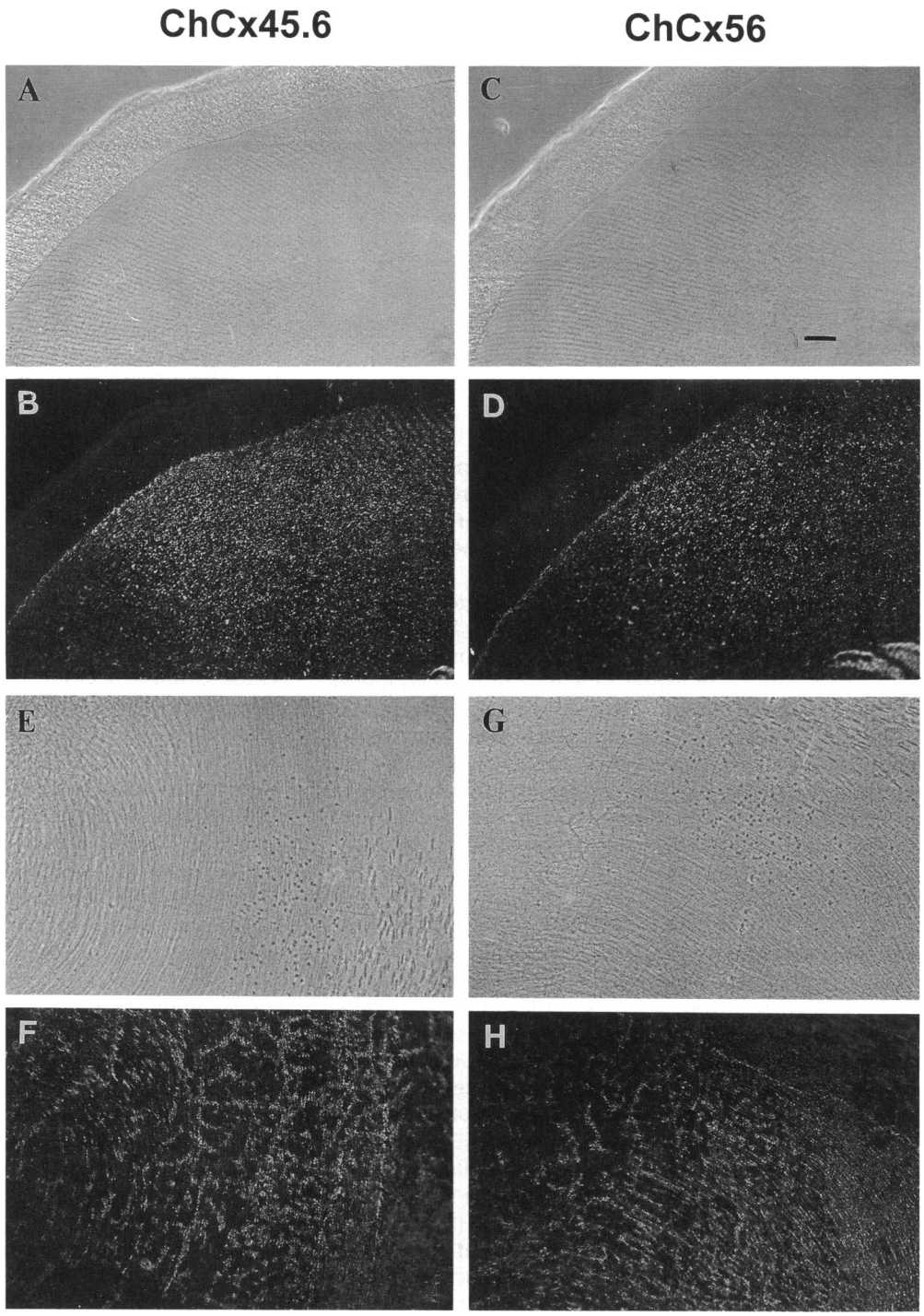

Figure 6. Immunohistochemical localization of ChCx45.6 and ChCx56 in embryonic avian lens. Seven micron frozen sections of 11-d embryonic chick lens and twenty-five micron frozen sections of 19-d embryonic chick lens were examined with either an affinity-purified anti-ChCx45.6 antibody (A, B, E, and F) or anti-ChCx56 antibody (C, D, G, and H) and followed by rhodamine-labeled goat anti-rabbit IgG. Staining patterns of 11-d embryonic chick lens produced by these antibodies are punctuate in the lens fiber (B and D). Phase contrast images are shown in A and C. F and H are immunofluroescent images of day 19 lenses taken at the center of the lens. Corresponding phase-contrast images (E and G) reveal in these sections that lens fibers are seen that contain oval nuclei, which have pyknotic nuclei, and that have no detectable nuclei. In this region of lens fiber differentiation, there is a spotty loss of immunoflurorescent staining of the junctional maculae with both anti-connexin antibodies, indicating that there may be proteolytic processing of these connexins resulting in the removal of their antigenic sites. Bar, 300 μm.

steady state. The rate of this decay increased with increasing transjunctional voltage. The current voltage relationship for ChCx45.6 is very similar to that of mouse Cx50 (White *et al.*, 1992).

The voltage dependence of these channels was further analyzed by plotting junctional conductance (G_j) as a function of transjunctional potential (V_j) (Figure 7B). G_j values for both initial (G_{jo}) and steady-state (G_{jss}) junctional conductance were normalized to the maximal conductance measured at the lowest V_j (=10 mV). No fast gating effects (<5 ms) of voltage on these channels were observed (Figure 7B, open squares). In contrast, G_{jss} was sharply dependent on voltage (Figure 7B, filled circles). This plot was fitted to a Boltzmann relation

Table 2. Summary of ChCX45.6-induced communication

Oocyte injection (cell1/cell2)	Conductance[a] [μS]	Number of pairs
ChCX45.6/ChCX45.6	33.5 ± 5.5	27
ChCX45.6/H_2O	<0.05	8
H_2O/H_2O	<0.05	4

[a] Values are means ± SE.

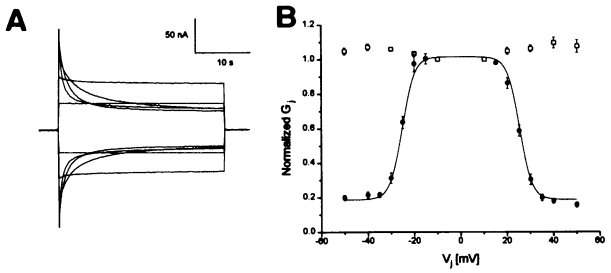

Figure 7. Electrophysiological properties of the junctional channels developed by paired oocytes injected with ChCx45.6 mRNA. Effect of transjunctional voltage (V_j) on the junctional current (I_j) is shown in A. Depolarizing or hyperpolarizing voltage steps of 10, 20, 30, 40, and 50 mV were applied to a cell pair initially clamped at −40 mV. At transjunctional potentials greater than 20 mV, junctional currents showed a symmetrical decline over the time of the voltage step. A plot of junctional conductance (G_j) vs. transjunctional potential (V_j) is shown in B. Initial (□) and steady state (●) conductance values are plotted against V_j. Steady-state conductance was fit by a Boltzmann equation with the following parameters: A = 0.38, V_o = 25 mV, G_{jmin} = 0.18.

(Spray *et al.*, 1981) whose parameters are given in the legend to Figure 7. From this analysis, ChCx45.6 channels displayed voltage sensitivity significantly different from rat Cx46 (calculated from Ebihara and Steiner, 1993).

The ability of *Xenopus* oocytes to support phosphorylation required to induce the SDS-PAGE mobility shifts observed in vivo was tested. Oocytes were coinjected with ChCx45.6 cRNA and ^{35}S-methionine then lysed and analyzed by immunoprecipitation using anti-ChCx45.6 serum (Figure 8). The immunoprecipate from oocytes (lane 2) displayed a single band at M_r = 58 kDa that comigrated with the in vitro translation product of ChCx45.6 mRNA in rabbit reticulocyte lysate (lane 1). No bands were seen with preimmune serum (lane 3) or in oocytes injected with water. For comparison, lane 4 shows a Western blot of isolated chick lens plasma membranes using anti-ChCx45.6 serum. Both the in vitro–and oocyte-synthesized products comigrated with the M_r = 58 kDa band recognized by the antiserum.

DISCUSSION

ChCx45.6 Is the Avian Counterpart of Murine Cx50 and Ovine MP70

We have cloned a DNA fragment encoding ChCx45.6 from a chicken genomic library. ChCx45.6 is a member of the connexin family of gap junction proteins because it exhibits primary sequence similarity to other connexins, and it induces communicating channels when expressed in paired *Xenopus* oocytes. ChCx45.6 is more homologous to mouse Cx50 than is ChCx56, the other cloned chick lens fiber connexin. The partial amino terminal sequences of ChCx45.6 and mouse Cx50 match one of the degenerate sequences of MP70 (Kistler *et al.*, 1988), whereas those of ChCx56 and rodent Cx46 match the other. In addition, the electrophysiological properties (A, V_o, and G_{jmin}) of the channels formed by ChCx45.6 (see Figure 7 legend) were more similar to those formed by mouse Cx50 (White *et al.*, 1992) than to those formed by rat Cx46 (Ebihara and Steiner, 1993). Taken together, these data suggest that ChCx45.6 is the homologue of rodent Cx50 and ovine MP70.

Are There Multiple ChCx45.6 mRNAs?

Southern analysis suggested that ChCx45.6 has no introns in the coding region and is most likely a single copy gene. Northern analysis, however, revealed the existence of two distinct mRNA species, 6.4 and 9.4 kb. These could reflect either multiple transcription starts sites, alternatively spliced forms, or the existence of a relatively stable processing intermediate. Because the ratio of 6.4 to 9.4 kb mRNAs increased between day 11 and day 19 of embryonic development, it is possible that the 9.4-kb species is more abundant in the earlier embryo. A developmental change in the ratio of two mRNAs encoding another lens fiber membrane protein, MP26, has been observed (Gorin *et al.*, 1984), but the functional meaning of this phenomenon is also unclear. Both 6.4- and 9.4-kb mRNAs are much larger than would be required for encoding a protein of 45 619 Da, suggesting the presence of long 5′- and/or 3′-untranslated regions. Unusually large mRNAs have also been observed for two other connexins, both expressed in lens fibers, rodent Cx50 (White *et al.*, 1992) and ChCx56 (Rup *et al.*, 1993).

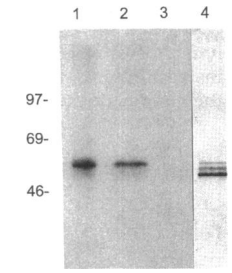

Figure 8. Translation of ChCx45.6 cRNA. Oocytes were microinjected with ChCx45.6 mRNA and ^{35}S-methionine. Lysates were prepared and immunoprecipitated with preimmune (lane 3) or with the anti-ChCx45.6 serum (lane 2). ChCx45.6 mRNA was translated in a reticulocyte lysate in the presence of ^{35}S-methionine and immunoprecipitated (lane 1). All immunoprecipitates were separated by SDS electrophoresis, transferred to nitrocellulose, and detected by autoradiography. Membrane preparation of 11-d embryonic chick lenses was separated on the same SDS gel as above immunoprecipitates, transferred to nitrocellulose, and blotted with anti-ChCx45.6 serum (lane 4).

Phosphorylation of Chick Lens Fiber Connexins, ChCx45.6 and ChCx56, and SDS-PAGE Mobility

The 45.6-kDa mass predicted by the DNA sequence is considerably smaller than the M_r = 58 kDa observed by SDS-PAGE for in vitro–synthesized ChCx45.6. This is also true for ChCx56, which migrates around M_r = 74 kDa on SDS gels. Differences between the molecular mass measured by electrophoretic mobility and the one calculated from the DNA sequence have been reported for rat connexin32 (Cx32), rat Cx26, and mouse Cx50 (Paul, 1986; Zhang and Nicholson, 1989; White *et al.*, 1992). The discrepancy between predicted and observed mass for both ChCx45.6 and ChCx56 can be attributed to anomalous migration of the protein in SDS polyacrylamide gels (Green *et al.*, 1988).

Anti-ChCx45.6 serum labeled a triad of bands in Western blots at M_r = 53, 56, and 58 kDa. After treatment of lens membranes with alkaline phosphatase, a single but more abundant band at M_r = 58 kDa was observed, suggesting that the three bands represent forms of ChCx45.6 differing in the extent of phosphorylation. The M_r = 58 kDa band comigrated with both in vitro–and oocyte-synthesized ChCx45.6. A second connexin from chick lens fibers, ChCx56, is also a phosphorylated protein. Phosphorylation of connexins has been observed in the lens fibers of other species (rat Cx46 [Jiang *et al.*, 1993], ovine MP70 and Cx46 [Tenbroek *et al.*, 1992] as well as other organs (rodent Cx32 [Saez *et al.*, 1986], rat Cx43 [Musil and Goodenough, 1991]) that may modulate connexin behavior. $pp60^{v\text{-}src}$-induced tyrosine phosphorylation of rodent Cx43 blocks the development of conductance between paired oocytes (Swenson *et al.*, 1990). Similarly, serine phosphorylation of rat Cx43 has been shown to correlate with the assembly of intercellular channels (Musil *et al.*, 1990b; Musil and Goodenough, 1991). Finally, Moreno *et al.* (1992) have suggested that phosphorylation may change single-channel conductance. The significance of lens connexin phosphorylation is not yet clear, although phosphorylation of rat Cx46 is developmentally regulated and appears to occur on a slow time scale (Jiang *et al.*, 1993).

Because the lens fiber connexins ChCx45.6, ChCx56, mouse Cx50, and rat Cx46 all display multiple forms of SDS-PAGE mobility, it is likely that they are phosphorylated at multiple sites. ChCx45.6 is unlike other connexins in that phosphorylation produces a mobility increase instead of a decrease, similar to what is observed for cyclin-dependent kinase-2 (Gu *et al.*, 1992).

Which Lens Gap Junctions Contain ChCx45.6?

We have now identified a third avian connexin, ChCx45.6, that can form functional intercellular channels. We have demonstrated that both ChCx45.6 and ChCx56 are present in fiber-fiber junctions. However, at present, we are unable to determine whether ChCx45.6 and ChCx56 are colocalized or in the same junctional plaques because both anti-ChCx45.6 and anti-ChCx56 are polyclonal sera from rabbits. The physiological properties of the gap junctional channels that join the lens epithelial cells to the underlying fibers in the chick have been shown to be similar to those joining the fibers themselves (Miller and Goodenough, 1986). Therefore, it is reasonable to suggest that the gap junctions joining epithelia to fibers may contain either ChCx45.6 or ChCx56. It is known that gap junctions joining epithelial cells contain ChCx43 (Beyer *et al.*, 1989; Paul *et al.*, 1991), which is localized on the lateral cell surfaces (Musil *et al.*, 1990b). It remains to be determined whether ChCx45.6 or ChCx56 are synthesized by the epithelial cells and specifically targeted to the epithelial/fiber junctions.

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