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Expression of the Gap Junction Protein Connexin43 in Embryonic Chick Lens: Molecular Cloning, Ultrastructural Localization, and Post-Translational Phosphorylation

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Summary. Lens epithelial cells are physiologically coupled to each other and to the lens fibers by an extensive network of intercellular gap junctions. In the rat, the epithelial-epithelial junctions appear to contain connexin43, a member of the connexin family of gap junction proteins. Limitations on the use of rodent lenses for the study of gap junction formation and regulation led us to examine the expression of connexin43 in embryonic chick lenses. We report here that chick connexin43 is remarkably similar to its rat counterpart in primary amino acid sequence and in several key structural features as deduced by molecular cDNA cloning. The cross-reactivity of an anti-rat connexin43 serum with chick connexin43 permitted definitive immunocytochemical localization of chick connexin43 to lens epithelial gap junctional plaques and examination of the biosynthesis of connexin43 by metabolic radiolabeling and immunoprecipitation. We show that chick lens cells synthesize connexin43 as a single, 42-kD species that is efficiently post-translationally converted to a 45-kD form. Metabolic labeling of connexin43 with ^{32}P-orthophosphate combined with dephosphorylation experiments reveals that this shift in apparent molecular weight is due solely to phosphorylation. These results indicate that embryonic chick lens is an appropriate system for the study of connexin43 biosynthesis and demonstrate for the first time that connexin43 is a phosphoprotein.

Key Words gap junctions · connexin43 · lens epithelium · molecular cloning · protein phosphorylation · intercellular communication

Introduction

In order for lens epithelial cells to maintain osmotic balance for the entire ocular lens, they must be joined by low resistance connections to each other and to the ATPase-poor, differentiated lens fibers (reviewed in Mathias & Rae, 1985). A combination of morphological and physiological studies has demonstrated that this is accomplished by an extensive network of epithelium-epithelium, fiber-fiber, and epithelium-fiber intercellular gap junctions which serve as nonselective channels for the passage of low molecular weight metabolites (Goodenough, Dick & Lyons, 1980). During differentiation of the epithelial cells into the lens fibers, the pH sensitivity and structure of the lens junctions changes such that the mature lens fiber junctions are less sensitive to acidification and no longer crystallize in response to chemical fixation (Schuetze & Goodenough, 1982; Miller & Goodenough, 1985, 1986). It has been proposed that these physiological and structural differences are due to a developmental change in expression from one class of gap junction protein, synthesized by epithelial cells, to another class expressed by the differentiating fibers. The ocular lens is thus an excellent system for the examination of two physiologically (and most likely biochemically) distinct gap junction types and also provides an opportunity to study the developmental transition from one junctional phenotype to another.

The composition of the gap junctions connecting mature lens fiber cells is controversial. Numerous investigators have provided evidence that the lens fiber junctional channels are composed of MP26, the principal polypeptide of the lens fiber membrane (reviewed in Zampighi et al., 1989). Although it is generally accepted that MP26 is found in abundance in the lens fiber nonjunctional plasma membrane, and asymmetrically disposed in one of the two membranes in thin, pentalaminar profiles of unknown function (Paul & Goodenough, 1983; Zampighi et al., 1989), there is disagreement as to whether MP26 forms intercellular channels in junctional membranes. A second lens membrane protein, called MP70, has been demonstrated to reside exclusively in cortical fiber junctions (Kistler, Kirkland & Bullivant, 1985; Gruijters et al., 1987); this protein, unlike MP26, is closely related to other

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known gap junction proteins in its N-terminal amino acid sequence (Kistler, Christie & Bullivant, 1988). At the present time there are no biochemical data describing the composition of the epithelium/fiber gap junctions.

In contrast to the uncertainty surrounding the composition of fiber cell junctions, the identity of the protein responsible for gap junctional communication between lens epithelial cells seems clear. In the rat, it has been shown that the lens epithelial cells, but not the lens fibers, are interconnected by gap junctions which are recognized by an antiserum raised against a peptide sequence specific to connexin43 (Beyer et al., 1989). Connexin43 is a member of the connexin family of gap junction proteins which share certain features of sequence and structure and have been demonstrated to form functional junctions in a *Xenopus* oocyte expression system (Beyer, Goodenough & Paul, 1988; Swenson et al., 1989). Although originally cloned from a rat myocardial cDNA library and localized to gap junctions of myocardial intercalated discs (Beyer, Paul & Goodenough, 1987), connexin43 is present in other cell types as well, such as ovarian granulosa cells, fibroblasts, uterine myometrial cells, and corneal epithelial cells (Beyer et al., 1989).

A major drawback to the use of rodent lenses for the study of lens gap junction biology is the lack of a mammalian in vitro culture system that faithfully recapitulates the developmental transition from the epithelial-epithelial to the fiber-fiber junctional phenotypes observed in vivo. FitzGerald and Goodenough (1986) demonstrated that, although rat lens epithelial cells in primary culture slowly differentiate to form small lentoids, the fiber-like cells comprising these structures have very few morphologically recognizable gap junctions and are consequently only poorly coupled as assayed by dye transfer. In contrast, a similar preparation of epithelial cells from 10-day embryonic chick lenses differentiates efficiently in primary culture, leading to the formation of extensive, well dye-coupled lentoids which contain large gap junctions similar to those seen between fiber cells in vivo (Menko, Klukas & Johnson, 1984; Menko et al., 1987). The advantages of these well-characterized chick cultures led us to clone the chick counterpart of rat connexin43 and to examine the expression of this protein in the embryonic chick lens using a variety of molecular biological, biochemical, and morphological approaches. The results presented here demonstrate that chick connexin43 is extremely homologous to its rat counterpart and that *m*RNA transcripts encoding this protein are present in whole chick lenses and in primary cell cultures derived from them. We also demonstrate that the anti-rat connexin43 oligopeptide antiserum cross-reacts with the chick lens protein. Immunofluorescence and electron microscopic immunocytochemistry of developing chick lenses employing this antiserum reveal that connexin43 is localized to junctional structures joining the epithelial cells and to larger, plaque-shaped structures in the differentiating bow region, which disappear as the cells take up positions as mature fibers. Finally, we demonstrate by metabolic labeling and immunoprecipitation that chick connexin43 is synthesized and efficiently posttranslationally phosphorylated in both the intact lens and in lens cell primary cultures. This latter observation is especially intriguing in light of the extensive literature suggesting a role for protein phosphorylation in the regulation of gap junctional communication (Wiener & Loewenstein, 1983; Loewenstein, 1985; Saez et al., 1986; Azarnia et al., 1988).

Materials and Methods

Reagents

Tissue culture reagents were purchased from Gibco (Grand Island, NY), except for phosphate-free MEM[1], which was prepared in the laboratory from tissue culture grade reagents and concentrated MEM vitamin and amino acid solutions. Alpha ^{32}P-dCTP (>3000 Ci/mmol) and ^{35}S-methionine (>800 Ci/mmol) were from New England Nuclear (Boston, MA); $H_3[^{32}P]O_4$ (285 Ci/mg) was from ICN (Irvine, CA); and ^{35}S-dATP (>600 Ci/mmol) was from Amersham (Arlington Heights, IL). All other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise specified.

*c*DNA Library Screening and Sequencing

The *c*DNA clone for rat connexin43 (G2) has been described in detail previously (Beyer et al., 1987; Genbank accession number M19317). This *c*DNA was isolated from a rat heart *c*DNA library and contains 1392 bp of DNA including sequences coding for a 43-kD gap junction protein, 202 bp of 5′-noncoding sequence, and 44 bp of 3′-noncoding sequences. Rat connexin43 *c*DNA probe was prepared by isolation of the *c*DNA insert by electrophoresis in low melting temperature agarose and was radiolabeled with ^{32}P-dCTP using the Klenow fragment of DNA polymerase I and hexanucleotide primers as described by Feinberg and Vogelstein (1983). A *c*DNA library in lambda gt11 prepared from *m*RNA from whole 10-day-old chick embryos was obtained commercially (Clontech, Palo Alto, CA). This library was screened by hydribization of nitrocellulose filter plaque lifts with the ^{32}P-labeled rat connexin43 *c*DNA in 0.75 M $NaPO_4$, 1% SDS, 100 μg/ml salmon sperm DNA at 55°C as described previously (Beyer et al., 1987).

[1] *Abbreviations used*: MEM, minimal essential medium: PBS, phosphate-buffered saline; PMSF, Phenylmethylsulfonyl fluoride; and SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The cDNAs from positive lambda clones were purified from plate lysates by DEAE cellulose chromatography (Helms et al., 1985) and were subsequently subcloned into the EcoRI site of the plasmid Bluescript (Stratagene, San Diego, CA). These constructs were then used to transform *Escherichia coli* strain JM-101 cells, from which single-stranded DNA was isolated after culture with the helper phage R408 (Stratagene, San Diego, CA) as described by the supplier. All sequencing was performed by the chain termination method of Sanger, Nicklen and Coulsen (1977) using this single stranded template and Sequenase dideoxy sequencing reagents (United States Biochemical, Cleveland, OH). Three independent clones were sequenced. Most of the sequence was determined using clustered overlapping deletions as in Beyer et al. (1987). In addition, three oligonucleotides were synthesized on a Biotix (Danbury, CT) oligonucleotide synthesizer and used as sequencing primers: TCCTGCTATTGGGGAC (184-199), TTATGGGTTTAGCCTGA (611-627), and ATCTCCAGGTCATCAGG (antisense 1225-1209).

RNA Isolation and Northern Blots

RNA was isolated by homogenization of tissues or cultured cells in guanidine isothiocyanate followed by centrifugation through CsCl (Chirgwin et al., 1979). RNA samples (10 μg, as estimated by optical density measurements at 260 and 280 nm) were electrophoresed on 1% agarose/formaldehyde gels and capillary blotted onto nylon membranes (Hybond-N, Amersham, Arlington Heights, IL). RNA was cross-linked to the membrane by exposure to a medium length (300 nM) UV transilluminator for 5 min. Blots were hybridized at high stringency in 0.75 M Na_2HPO_4 (pH 7.2), 5% SDS, 100 μg/ml salmon sperm DNA at 65°C and processed as described previously (Beyer et al., 1987).

Anti-connexin43 Serum

A synthetic oligopeptide whose sequence corresponds to a unique region of the predicted cytoplasmic tail domain of rat connexin43 (amino acids 252-271; GPLSPSKDCGSPKYAYFNGC) was conjugated to keyhole limpet hemocyanin and used to generate a connexin43-specific antiserum. The preparation and characterization of this rabbit antiserum, referred to here as anti-connexin43 (252-271), have been described elsewhere (Beyer et al., 1989).

Immunohistochemistry and Immunocytochemistry

Day-10 chick lenses were dissected from the embryos and fixed 1–2 hr at room temperature in 1% formaldehyde (prepared fresh from paraformaldehyde) in phosphate buffered saline (PBS). For immunohistochemistry, lenses were then immersed in Tissue-Tek compound (Miles Scientific, Naperville, IL) in aluminum foil holders and frozen in Freon-22 slush. Ten micrometer frozen sections were collected on gelatin-coated slides and immersed in absolute acetone at −20°C for 3 min, then immersed in PBS (Goodenough, Paul & Jesaitis, 1988). Sections were incubated with a 1 : 500 dilution of anti-connexin43(252-271), nonspecific antiserum (anti-connexin32(98–124) (Goodenough et al., 1988), or preimmune antiserum overnight at 4°C. Following 3 × 10 min washes in PBS, sections were counterstained with 1 : 500 rhodamine-labeled goat anti-rabbit IgG antiserum in PBS (Boehringer Mannheim Biochemicals, Indianapolis, IN). Fluorescent microscopy was performed as described previously (Goodenough et al., 1988). For immunocytochemistry with horseradish peroxidase-labeled secondary antiserum, formaldehyde-fixed lenses were embedded in 3% agar in PBS and 50–100 μm vibratome sections cut with a razor. The sections were immersed for 1 week each in PBS + 0.2% saponin, 1 : 50 dilution of anti-connexin43(252–271) in PBS-saponin, PBS-saponin alone, 1 : 50 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG antiserum (Boehringer Mannheim, FRG) in PBS-saponin, and PBS-saponin alone. The sections were then reacted for peroxidase as described previously (Goodenough & Revel, 1971), fixed in tannic acid-glutaraldehyde, postfixed in OsO_4, stained, and embedded in epoxy resins as described previously (Paul & Goodenough, 1983). For immunocytochemistry with colloidal gold-labeled secondary antiserum, chick embryo lenses were fixed with the PLP fixative of McLean and Nakane (1974) containing 1% formaldehyde (prepared fresh from paraformaldehyde) for 1 hr at room temperature. The fixed lenses were washed in PBS, then equilibrated with 65% ethylene glycol in PBS for 1 hr. The lenses were then chilled to −20°C and dehydrated through 50, 95, and 100% ethanol at −20°C, after which they were equilibrated with a 1 : 1 mixture of ethanol : LR White (Electron Microscopy Sciences, Ft. Washington, PA) for 2 hr and then left in 100% LR White overnight at the same temperature. The lenses were embedded in BEEM capsules in fresh LR White and polymerized in UV light for 4 days at 4°C. Thin sections mounted on formvar/carbon-coated copper grids were stained sequentially by floating grids at room temperature on Tris-buffered saline with 0.2% fish skin gelatin (TBS-FSG) at pH 8.0 (10 min), 1 : 50 dilution of anti-connexin43(252–271) in TBS-FSG (2 hr), TBS—FSG (2 × 10 min), 1 : 3 dilution of 5 nm gold-conjugated goat anti-rabbit IgG antiserum (Janssen Life Sciences, Piscataway, NJ) in TBS-FSG (2 hr), TBS-FSG (2 × 10 min), distilled water (5 min), 1% aqueous uranyl acetate (20 min), and distilled water (1 min). All electron microscopy was performed on a JEOL 100CX operating at 60 kV.

Preparation of Lenses and Primary Lens Cell Cultures

Lenses were dissected from 10- to 11-day white leghorn chicken embryos, taking care to minimize the amount of contaminating extra-capsular tissue. The lenses were collected into TD buffer as formulated by Menko et al. (1984) (140 mM NaCl, 5 mM KCl, 0.7 mM Na_2PO_4, 5 mM glucose, 25 mM Tris base, pH 7.4) and were rinsed in this buffer three times prior to metabolic labeling of whole lenses (*see below*) or to establishment of primary lens cell cultures. Cultures were prepared using a modification of the procedure of Menko et al. (1984). Briefly, washed lenses were incubated without agitation for 30 min at 37°C in TD containing 0.1% trypsin. The intact lenses were then freed of digested extracapsular tissue by gentle repeated resuspension with a wide-bore pipette and were collected into trypsin-free TD. The washed lenses were subsequently resuspended in culture medium (Medium 199 with Earle's salts containing 10% fetal calf serum, penicillin G, and streptomycin) and ruptured by vigorous manual trituration, after which the dissociated cells were pelleted by low speed centrifugation (150 × g, 10 min), resuspended in culture medium, and plated onto 35-mm tissue culture dishes. The cultures were fed on day two and day four after preparation and were generally used on day seven, at which time they consisted of a monolayer of epithelioid cells interspersed with lentoid

structures indicative of the differentiation of epithelial cells into fiber-like cells (Menko et al., 1984, 1987).

Metabolic Labeling of Cells and Lenses

Primary lens cell cultures and intact embryonic chick lenses, prepared as described above, were labeled with ^{35}S-methionine as follows. Monolayers or lenses were washed twice with warmed D-MEM without methionine prior to the addition of 1.5 ml of labeling medium (methionine-free D-MEM with 5% dialyzed fetal calf serum, 20 μM methionine, and 0.2 mCi of ^{35}S-methionine). The cells were incubated for the times indicated in the individual experiments. For pulse-chase analysis, the radioactive medium was removed after a 40-min pulse and the cultures or lenses rinsed three times with complete D-MEM supplemented with 0.5 mM methionine and 10% fetal calf serum. The cells were then incubated in the chase medium for up to 4 hr. Metabolic labeling with ^{32}P was accomplished by first incubating lenses or cultures for 15 min with phosphate-free MEM (containing 8 mM glutamine and 1X MEM nonessential amino acids) in order to reduce intracellular phosphate levels. The cells were then labeled for 4 hr in the same medium supplemented with 0.5 mCi/ml $H_3[^{32}P]O_4$ in 0.02 N HCl.

Preparation of Cell Lysates and Immunoprecipitation

In the case of whole lenses, the labeling or chase period was terminated by washing them three times in ice-cold TD buffer. The lenses were then incubated in TD containing 0.01% trypsin for 30 min at 4°C followed by a 7-min incubation in the same solution at 37°C. They were subsequently transferred to 4°C TD buffer containing an excess of trypsin inhibitors (0.5 mM diisopropylfluorophosphate, 0.1 mg/ml soybean trypsin inhibitor, and 200 μM leupeptin) and rinsed three times in this solution. This procedure removes any residual ciliary epithelial cells (which are rich in connexin43) from the lenses without affecting the cells protected by the collagenous lens capsule.

Intact lenses treated in this manner or metabolically labeled lens cell cultures were lysed by rinsing them three times on ice with PBS supplemented with 5 mM EDTA, 5 mM EGTA, 10 mM N-ethylmaleimide, and 2 mM PMSF, followed by a single wash with lysis buffer (5 mM each of Tris base, EDTA, and EGTA, containing 0.5 mM diisopropylfluorophosphate, 10 mM N-ethylmaleimide, and 2 mM PMSF, pH 8.0). The buffer was removed and the cells incubated for ~30 sec at room temperature with lysis buffer supplemented with 0.6% SDS and 200 μM leupeptin. For ^{32}P-labeled cells, 10 mM PO_4, 2 mM Na orthovanadate, and 100 mM Na fluoride were added to the lysis buffer to inhibit phosphatases. Whole lenses were disrupted by passing them several times through a 20-gauge needle, whereas an 18-gauge needle was used to lyse the lens cultures. The lysates were boiled for 3 min and passed three times through a 27-gauge needle to shear DNA released from lysed nuclei. Samples were then diluted with four volumes of "immunoprecipitation buffer" (0.1 M Nacl, 0.02 M Na borate, 15 mM EDTA, 15 mM EGTA, 0.02% Na azide, 10 mM N-ethylamleimide, 2 mM PMSF, pH 8.5) supplemented with 0.5% Triton X-100. Immunoprecipitation with the anti-connexin43(252–271) serum was conducted using the method described by Musil et al. (1988), except that Protein A-Sepharose CL-4B was substituted for Immunoprecipitin and the immune complexes were incubated with the immunoabsorbant for 2 hr rather than 20 min.

SDS Gel Electrophoresis and Fluorography

Immunoprecipitated samples were analyzed on 10% polyacrylamide gels according to the method of Laemmli (1970); 5.5 cm-long minigels were used unless otherwise specified. The gels were processed for fluorography by soaking them for 30 min in 1 M Na salicylic acid prior to drying.

In Vitro Transcription, Translation, and Immunoprecipitation of Chick Connexin43

The complete coding region of the 13A chick connexin43 *c*DNA clone was linearized and subcloned into the EcoR1 site of the Bluescript expression vector (Stratgene, San Diego, CA). Plasmid was purified, linearized by digestion with Cla 1, and transcribed with T7 RNA polymerase (Promega, Madison, WI) in the presence of 0.5 mM m7G(5′)ppp(5′)G and nucleoside triphosphates using a modification of the procedure of Melton et al. (1984) as described by the manufacturer. *m*RNA encoding rat connexin43 was synthesized as described by Swenson et al. (1989). The *m*RNA was purified and translated in a nuclease-treated rabbit reticulocyte system (New England Nuclear, Boston, MA) using 80 mM added potassium acetate, 0.65 mM added magnesium acetate, and 50 μCi of ^{35}S-methionine according to the supplier's protocol. Translation reactions were diluted 20-fold into immunoprecipitation buffer (*see above*) containing 0.6% SDS and boiled for 3 min prior to immunoprecipitation with the anti-connexin43(252–271) serum as described for lens cells.

Dephosphorylation of Connexin43

After metabolic labeling with either ^{35}S-methionine or $[^{32}P]O_4$, lenses were lysed and reacted with the anti-connexin43(252–271) serum and Protein A-Sepharose CL-4B as described above. The resulting immune complexes were washed three times with a modified immunoprecipitation buffer (0.1 M NaCl, 0.02 M Na borate, 0.02% Na azide, 0.5 M sucrose, 0.5% Triton X-100, 0.1% SDS, 2 mM PMSF, pH 8.5) containing 0.5% bovine serum albumin and then once with the same buffer containing only 0.2% bovine serum albumin. The samples were subsequently washed twice with phosphatase reaction buffer (50 mM Tris HCl, 10 mM $MgCl_2$, 150 mM NaCl, pH 8.0) supplemented with 0.1% Triton X-100, 0.05% SDS, and 2 mM PMSF. After aspiration to dryness, the pellets were each resuspended with 12.5 μl of phosphatase reaction buffer containing 1% SDS, 1% 2-mercaptoethanol, and 2 mM PMSF and heated to 60°C for 3 min to dissociate the immunoprecipitated connexin43 from the immunoabsorbant. The released connexin43 was then diluted with 50 μl of phosphatase reaction buffer and incubated for 4 hr at 37°C in the presence of 2 units of molecular biology grade calf intestinal alkaline phosphatase (Boehringer Mannheim, FRG). Prior to use, the enzyme was treated with 2 mM diisopropylfluorophosphate for 1 hr to inhibit any contaminating serine proteases. The dephosphorylation reaction was terminated by the addition of trichloroacetic acid (final concentration = 10%) and the resulting precipitates were washed three times with −20°C acetone prior to resolubilization in SDS-PAGE sample buffer and analysis of SDS-PAGE. Control reactions done in the presence of phosphatase inhibitors were conducted with the addition of 2 mg/ml Na orthovanadate, 10 mM EDTA, and 10 mM PO_4 to the phosphatase reaction buffer.

Results

cDNA Cloning

To facilitate the study of connexin43 in chick lens cells, we isolated *c*DNA clones encoding this protein from a lambda gt11 library constructed from 10-day-old chick embryos. The library was screened by hybridization using the G2 *c*DNA corresponding to the complete coding region of rat connexin43 (Beyer et al., 1987) as a probe. The hybridization temperature was reduced to 55°C to allow for possible mismatching due to the species difference. Several positive clones were isolated, three of which were sequenced and found to overlap without discrepencies. One of these clones, 13A, contained the complete coding sequence of the chick *c*DNA as shown in Fig. 1; the complete 3′-noncoding sequence has not been determined. This sequence contains an open reading frame of 1143 nucleotides which codes for a predicted protein containing 381 amino acids with a molecular mass of 43,177 daltons[2]. Based on its calculated molecular weight and the nomenclature system introduced previously (Beyer et al., 1987), we will refer to this protein as *chick connexin43*.

The nucleotide and deduced amino acid sequences of chick connexin43 are very similar to those reported for rat connexin43 (Beyer et al., 1987). In the coding region 82% of the nucleotides are identical, with many of the mismatches occurring in the third positions of codons. Noncoding regions also show some homology but are much less highly conserved. Ninety-two percent of the predicted amino acids are identical in the derived rat and chick sequences, with the majority of the substitutions being conservative with respect to charge (Fig. 1). Substitutions occur in all regions of the molecule, but appear to be especially concentrated in two portions of the predicted cytoplasmic tail sequence (amino acids 243–253 and 337–348). Other differences between the chick and rat connexin43 sequences are the lack of a chick counterpart to rat histidine 248 and the lower calculated isoelectric point of chick as compared to rat connexin43 (pI = 8.68 *vs.* 10.19, respectively). Hydropathicity analysis of the chick connexin43 sequence using the method of Kyte and Doolittle (1982) suggests, however, that the overall topology of the protein with respect to the membrane is likely to be very similar to that previously proposed for rat connexin43 (Beyer et al., 1987) (*not shown*). In this context, it is interesting that the six cysteine residues perfectly conserved in the predicted extracellular domains of all connexin sequences published to date (connexin43 (Beyer et al., 1987), connexin32 (Paul, 1986), connexin46 (Beyer et al., 1988), and connexin26 (Nicholson & Zhang, 1988) from rat; human connexin32 (Kumar & Gilula, 1986); connexin38 (Ebihara et al., 1989) and XE11a (Gimlich, Kumar & Gilula, 1988) from *Xenopus*) are also present in analogous positions in the chick connexin43 sequence (residues 54, 61, 65, 187, 192, and 198). A peptide corresponding to amino acids 252–271 of rat connexin43 was used in a previous study to generate a rabbit antiserum, anti-connexin43(252–271). This peptide shares 17 out of 20 of the amino acids in the corresponding region of chick connexin43, providing the basis for the cross-reactivity of the antiserum between chick and rat connexin43 (*see below*).

[2] Sequence data submitted to Genbank and assigned the accession number M29003.

Northern Blot Analysis

Total RNAs were isolated from chick embryo heart and lens and from cultured chick embryo lens cells. Approximately 10 μg of each RNA sample (as estimated from optical density measurements at 260 and 280 nm) was run on a formaldehyde/agarose gel prior to transfer to a nylon membrane. Equivalence of loading was verified by visualization of the *r*RNA with ethidium bromide before and after blotting. The blots were examined for *m*RNAs that hybridized to the 13A chick connexin43 *c*DNA by Northern blot analysis (Fig. 2). A single band of 3.0 kb was seen in all of these RNAs at high stringency, and while no effort was made to quantitate the *m*RNA levels, the band appeared most intense in the RNA derived from the lens (lane *B*). Hybridization at reduced stringency has no effect on the relative intensities of these bands. A band of similar mobility was observed when a rat connexin43 probe was hybridized to adult rat RNA under similar conditions (Beyer et al., 1987), although in that system connexin43 *m*RNA appeared to be much more abundant in heart than in lens. This apparent discrepancy with the results presented here most likely reflects a higher proportion of connexin43-synthesizing cells in embryonic, relative to adult, lens (*see* Discussion).

In Vitro Translation and Immunoprecipitation of Chick Connexin43

Properties of the protein encoded by the 13A chick connexin43 clone were examined by expression in a cell-free system. The 13A *c*DNA was transcribed in vitro and translated in a reticulocyte lysate devoid of endogenous translatable *m*RNA. A single labeled

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       GCTCTTCCGTTTTCCCTTAACCCTCCAGAGAAGTACCCCGAGCCACTCGCACTGCTCATC      60
       AGCAGCGCCAATATCCAGTGAAGATGGGTGATTGGAGTGCCCTGGGAAAACTTCTTGACA     120
CHICK                      m  g  d  w  s  a  l  g  k  l  l  d  k      13
       AGGTTCAAGCTTATTCTACTGCAGGAGGAAAAGTGTGGCTGTCTGTCCTCTTTATTTTCC     180
CHICK   v  q  a  y  s  t  a  g  g  k  v  w  l  s  v  l  f  i  f  r      33
       GAATCCTGCTATTGGGGACAGCAGTCGAGTCTGCTTGGGGAGATGAACATGTCGCTTTTC     240
CHICK   i  l  l  l  g  t  a  v  e  s  a  w  g  d  e  h  v  a  f  r      53
RAT                                                 q  s
       GGTGCAATACTCAACAGCCTGGTTGTGAGAACGTCTGCTATGACAAGTCCTTCCCCATCT     300
CHICK   c  n  t  q  q  p  g  c  e  n  v  c  y  d  k  s  f  p  i  s      73
       CCCACGTGCGTTTCTGGGTTCTGCAGATCATATTTGTGTCTGTACCAACCCTCTTGTACC     360
CHICK   h  v  r  f  w  v  l  q  i  i  f  v  s  v  p  t  l  l  y  l      93
       TGGCNCACGTGTTCTACGTGATGAGGAAAGAAGAGAAGCTGAACAAAAGAGAGGAAGAGC     420
CHICK   a  h  v  f  y  v  m  r  k  e  e  k  l  n  k  r  e  e  e  l     113
RAT                                                 k
       TCAAGGTGGTCCAGAATGATGGTGTGAATGTGGATATGCACCTCAAACAAATAGAAATTA     480
CHICK   k  v  v  q  n  d  g  v  n  v  d  m  h  l  k  q  i  e  i  k     133
RAT        a     t                 e
       AGAAATTCAAGTATGGAATTGAAGAGCACGGCAAGGTAAAGATGCGTGGAGGACTGCTTC     540
CHICK   k  f  k  y  g  i  e  e  h  g  k  v  k  m  r  g  g  l  l  r     153
       GTACTTACATCATCAGCATCCTGTTTAAGTCTGTCTTCGAGGTGGCTTTCCTGCTGATAC     600
CHICK   t  y  i  i  s  i  l  f  k  s  v  f  e  v  a  f  l  l  i  q     173
       AGTGGTACATTTATGGGTTTAGCCTGAGTGCCATCTACACCTGTGAGCGAGACCCATGCC     660
CHICK   w  y  i  y  g  f  s  l  s  a  i  y  t  c  e  r  d  p  c  p     193
RAT                                    v           k
       CTCACAGGGTGGACTGTTTCCTGTCCCGTCCAACTGAGAAAACCATTTTCATCGTCTTCA     720
CHICK   h  r  v  d  c  f  l  s  r  p  t  e  k  t  i  f  i  v  f  m     213
RAT      q                                                 i
       TGCTGGTAGTGTCTTTGGTGTCTCTTGCCTTGAACATCATTGAGCTTTTCTACGTGTTCT     780
CHICK   l  v  v  s  l  v  s  l  a  l  n  i  i  e  l  f  y  v  f  f     233
       TCAAGGGTGTCAAGGATCGTGTGAAAGGAAAAACCGACCCCTAC---TCCCACAGTGGCACGA  840
CHICK   k  g  v  k  d  r  v  k  g  k  t  d  p  y     s  h  s  g  t  m  253
RAT                                  r  s        h  a  t  t     p  l
       TGAGCCCTTCCAAGGACTGTGGTTCCCCCAAATATGCGTATTACAATGGCTGTTCCTCAC     900
CHICK   s  p  s  k  d  c  g  s  p  k  y  a  y  y  n  g  c  s  s  p  273
RAT                                        f
       CGACCGCCCCCCTTGTCTCCCATGTCTCCCCCGGGGTACAAGCTTGTTACCGGAGACAGGA    960
CHICK   t  a  p  l  s  p  m  s  p  p  g  y  k  l  v  t  g  d  r  n  293
       ACAATTCCTCCTGTCGTAACTACAATAAGCAAGCCAGCGAGCAAAACTGGGCCAACTACA    1020
CHICK   n  s  s  c  r  n  y  n  k  q  a  s  e  q  n  w  a  n  y  s  313
       GTGCGGAGCAGAATAGGATGGGGCAGGCTGGCAGCACCATCTCCAACTCTCATGCCCAGC    1080
CHICK   a  e  q  n  r  m  g  q  a  g  s  t  i  s  n  s  h  a  q  p  333
       CCTTCGACTTCGCCGATGAGCACCAGAACACGAAAAAACTGGCGTCGGGACACGAGCTGC    1140
CHICK   f  d  f  a  d  e  h  q  n  t  k  k  l  a  s  g  h  e  l  q  353
RAT           p     d  n        a        v     a
       AACCCCTCACCATTGTGGACCAGAGGCCTCCCAGCAGAGCCAGCAGCCGAGCCAGCAGCA    1200
CHICK   p  l  t  i  v  d  q  r  p  p  s  r  a  s  s  r  a  s  s  r  373
RAT      a
       GGCCTCGACCTGATGACCTGGAGATCTAAGCTTCTCACTGCAGTACTTACTCAACTATGA    1260
CHICK   p  r  p  d  d  l  e  i  .                                     381
       AACAACGTGCATTGGAAGATGCAGTCAGTGCTGATCTTGTTCCAGTGGAGGTGGTACTTA    1320
       ACAGTCTCAAGCAGGAGATTTTAACAATCGTAAAAACAAACAGGCAAACAAACTTTTAAA    1380
       ATACTTGCTGTTTTTTGGGGAGGATATGGGGCTGGTGTGGGGTGGTACAATACACATTGG    1440
       TATTTAATGTAGCTGGTTTAAA                                          1462
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Fig. 1. Sequence of chick embryo connexin43 *c*DNA. The complete coding sequence of nucleotides is shown in light type with residues numbered on the right. Two independently derived clones were completely sequenced in both directions: a ~1-kb *c*DNA spanning nucleotides 405–1327 and a ~1.5-kb *c*DNA (nucleotides 1–1462; designated clone 13A) containing the entire coding region of chick connexin43. Partial sequence from a third, 1.8-kb clone was also obtained and overlapped with that of the other *c*DNAs without discrepencies. The derived amino acid sequence of chick connexin43 is shown in lower case bold face. The positions of amino acids in rat connexin43 which differ from the chick connexin43 sequence are also shown. The sequence corresponding to the region used for the anti-rat connexin43(252–271) oligopeptide antiserum is indicated by underlining

species of M_r = 42 kD was obtained after SDS-PAGE (Fig. 3, lane *2*), which comigrated with rat connexin43 translated similarly (lane *1*). The anti-rat connexin43(252–271) serum immunoprecipitated the 42-kD protein (lane *3*), whereas preimmune serum or normal rabbit serum did not. Furthermore, the ability of the anti-connexin43(252–271) serum to recognize this species was blocked by the presence of an excess of the synthetic peptide against which it was raised (lane *6*). Taken together, these results demonstrated that the antiserum raised against rat connexin43 cross-reacts with the analogous protein in the chick, as expected from comparison of the deduced chick and rat connexin43 sequences.

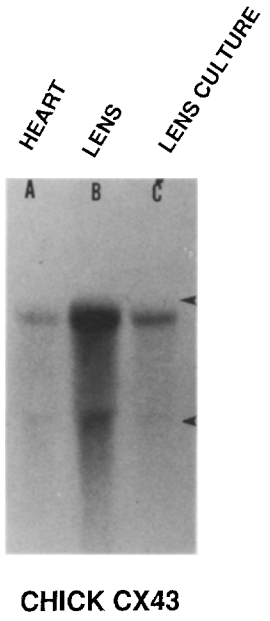

Fig. 2. Northern blot analysis. Total RNA was prepared from 10 to 12-day-old chick embryonic heart (lane *A*) and lens (lane *B*) and from cultured lens cells (lane *C*). Ten micrograms of RNA was loaded in each lane and probed by hybridization at high stringency with ^{32}P-labeled chick connexin43 *c*DNA (clone 13A) as described in Materials and Methods. A band of ~3 kb was detected in all samples. Arrowheads on the right-hand side indicate the positions of the 28S and 18S *r*RNA subunits

Biosynthesis of Connexin43 in Lens Cells

To examine the synthesis of connexin43 in lens cells, intact lenses dissected from 11-day-old chick embryos were metabolically labeled with ^{35}S-methionine for 4 hr. Whole cell lysates made from these lenses were then incubated with the anti-connexin43(252–271) serum and the resulting immune complexes washed extensively in detergent-containing buffers prior to analysis by SDS-PAGE. Two immunoprecipitated species were obtained: a 42-kD band which comigrated with chick connexin43 translated in vitro and a more heterogeneous M_r = ~45 kD species which occasionally migrated as an incompletely resolved doublet (Fig. 3, lane *4*). Neither band was immunoprecipitated by preimmune serum or by anti-connexin43(252–271) serum preabsorbed with the corresponding peptide (lane *7*). Results identical to these were obtained when primary monolayer cultures of embryonic chick lens epithelial cells were labeled and immunoprecipitated similarly (lanes 5 and 8). These results raised the possibility that the 42-kD protein represented the primary translation product of connexin43 and that the 45-kD species were derived from it by post-translational modification.

The precursor-product relationship between the 42-kD and the 45-kD forms of connexin43 suggested by these data was confirmed by a pulse-chase experiment (Fig. 4). Whole embryonic chick lenses were metabolically labeled with ^{35}S-methionine for

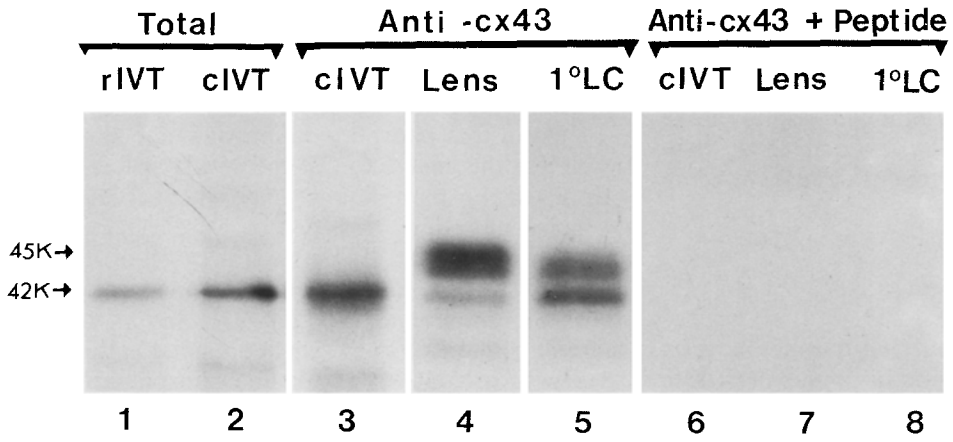

Fig. 3. Immunoprecipitation with anti-connexin43(252–271) serum of connexin43 translated in vitro or in chick lens cells. *m*RNA encoding chick connexin43 was synthesized in vitro from a clone 13A chick connexin 43 *c*DNA template and translated in a nuclease-treated rabbit reticulocyte system supplemented with ^{35}S-methionine. The translation reaction was immunoprecipitated with the anti-rat connexin43(252–271) oligopeptide serum in the absence (lane *3*) or presence (lane *6*) of 100 μg/ml of the immunizing peptide. The total products (no immunoprecipitation) of in vitro translation of rat and chick connexin43 are shown in lanes *1* and *2*, respectively. In lanes *4*, *5*, *7*, and *8*, whole 10-day-old embryonic chick lenses (lanes *4* and *7*) or primary monolayer cultures of chick lens cells (lanes *5* and *8*) were metabolically labeled for 4 hr with ^{35}S-methionine, lysed, and immunoprecipitated with the anti-connexin43(252–271) serum either without (lanes *4* and *5*) or with (lanes *7* and *8*) 100 μg/ml of the competing peptide

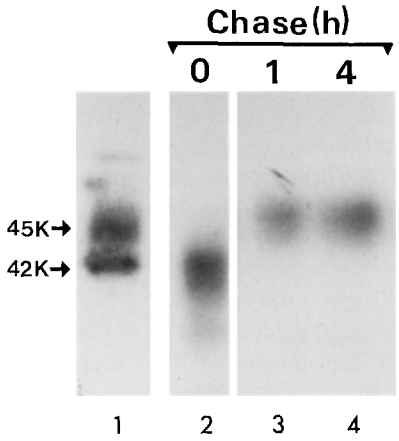

Fig. 4. Pulse-chase analysis of the synthesis and post-translational processing of connexin43 in embryonic chick lenses. Intact lenses from 10-day embryonic chicks were metabolically labeled with ^{35}S-methionine for 40 min and chased for up to 4 hr in the presence of an excess of unlabeled methionine. The lenses were then lysed, immunoprecipitated with the anti-connexin43(252–271) serum, and analyzed by SDS-PAGE and fluorography. Electrophoresis was conducted on a 12.5-cm rather than a 5.5-cm gel in order to achieve greater resolution between the 42-kD and the 45-kD connexin43 species. Also shown is an anti-connexin43 (252–271) immunoprecipitate from lenses continously labeled with ^{35}S-methionine for 3 hr (lane *1*)

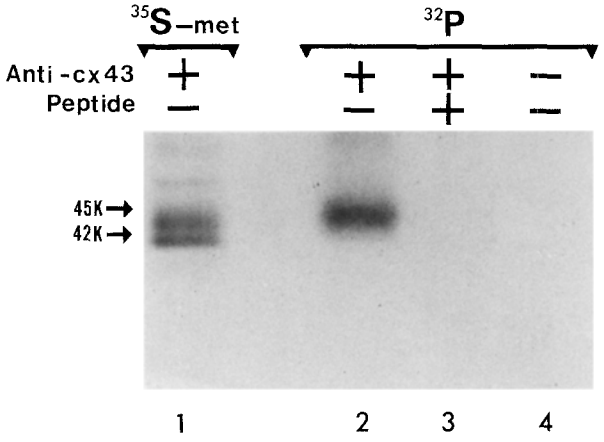

Fig. 5. Phosphorylation of connexin43 in primary cultures of embryonic chick lens cells. Lens cell monolayers were labeled for 4 hr with either $[^{32}P]O_4$ (lanes *2–4*) or, for comparison, ^{35}S-methionine (lane *1*). Cell lysates were prepared and immunoprecipitated with pre-immune serum (lane *4*) or with the anti-connexin43(252–271) serum in the absence (lanes *1* and *2*) or presence (lane *3*) of 100 μg/ml of the immunizing peptide

40 min and were then chased in the presence of an excess of unlabeled methionine for up to 4 hr. Immunoprecipitation of connexin43 after the various chase periods revealed that connexin43 was initially synthesized as the 42-kD protein (lane *2*) but was completely converted to the 45-kD form within 1 hr of chase (lane *3*). An identical experiment conducted with primary cultures of lens cells yielded qualitatively similar results, although a faster rate of degradation of connexin43 in these cells made visualization of the processing event somewhat more difficult (*not shown*).

Phosphorylation of Connexin43

We were interested in identifying the modification responsible for the conversion of connexin43 from the 42-kD to the 45-kD form. Saez et al. (1986) and Traub et al. (1987) have reported that connexin32 in primary cultures of rodent hepatocytes is phosphorylated, a modification that decreases the electrophoretic mobility of some proteins although apparently not that of connexin32. To see if connexin43 was phosphorylated, lens cell cultures were metabolically labeled for 4 hr with ^{32}-P-orthophosphate prior to immunoprecipitation and SDS-PAGE. As shown in Fig. 5, the anti-connexin43(252–271) serum (lane *2*), but not preimmune serum (lane *4*) or anti-connexin43(252–271) serum preabsorbed with the corresponding peptide (lane *3*), immunoprecipitated a single ^{32}P-labeled species from these cells. Significantly, this protein comigrated with the 45-kD rather than with the 42-kD form of connexin43 obtained after metabolic labeling of a parallel culture of cells with ^{35}S-methionine (lane *1*). Thus, only the mature form of connexin43 was metabolically labeled with $[^{32}P]O_4$, raising the possibility that phosphorylation itself accounted for the shift from the 42-kD to the 45-kD species.

Proof that phosphorylation was indeed responsible for the generation of the 45-kD species came from dephosphorylation experiments (Fig. 6). Whole embryonic chick lenses were metabolically labeled with either ^{35}S-methionine or ^{32}P-orthophosphate for 3 hr followed by immunoprecipitation with the anti-connexin43(252–271) serum. Incubation of ^{32}P-labeled connexin43 obtained in this manner with calf intestinal alkaline phosphatase completely removed the radioactive signal (lane *5*). When a parallel sample of connexin43 labeled with ^{35}S-methionine was treated identically, the 42–45 kD connexin43 doublet was quantitatively converted to the lower molecular weight species (lane *2*). Control experiments showed that incubating ^{32}P- or ^{35}S methionine-labeled connexin43 without alkaline phosphatase (lanes *1* and *4*) or in the presence of alkaline phosphatase plus phosphatase inhibitors (lanes *3* and *6*) had no effect on either the electro-

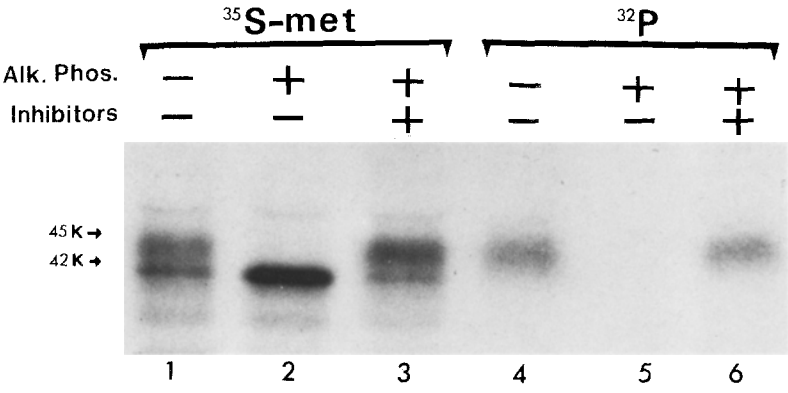

Fig. 6. Dephosphorylation of connexin43 with alkaline phosphatase. Embryonic chick lenses were metabolically labeled for 3 hr with either ^{35}S-methionine (lanes *1–3*) or [^{32}P]-orthophosphate (lanes *4–6*), lysed, and immunoprecipitated with the anti-connexin43(252–271) serum. The immunoprecipitates were then incubated for 4 hr at 37°C in the presence of either alkaline phosphatase (lanes *2* and *5*), alkaline phosphatase plus an excess of phosphatase inhibitors (lanes *3* and *6*), or digestion buffer alone (lanes *1* and *4*)

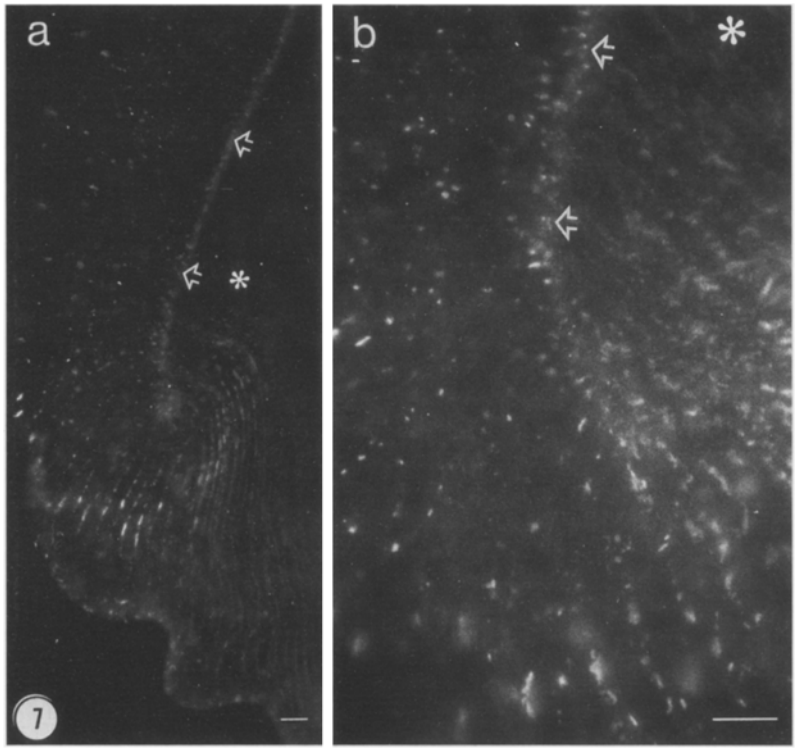

Fig. 7. Immunohistochemical localization of connexin43 in embryonic chick lens. (*a*) Frozen section of fixed 10-day embryonic chick lens stained with anti-connexin43(252–271) serum followed by rhodamine-labeled goat anti-rabbit IgG. Connexin43(252–271) localizes to bright spots between lens epithelial cells and is especially concentrated at the epithelium/fiber interface (arrows). This punctate pattern is replaced by much larger, positively staining maculae in the lens bow region, which gradually disappear in a fiberward gradient (asterisk). Bar = 10 μm. (*b*) Higher magnification micrograph of lens bow region shown in *a*. Bar = 10 μm

phoretic mobility or the intensity of labeling of connexin43. Thus, enzymatically active alkaline phosphatase was both necessary and sufficient for the conversion of the 45-kD form of connexin43 to the 42-kD form, demonstrating that the difference in the apparent molecular mass between the two species was due solely to phosphorylation.

Morphological Localization of Connexin43 in Embryonic Chick Lenses

Ten-day chick embryo lenses were studied morphologically with the anti-connexin43(252–271) serum at both the histological and ultrastructural levels. Figure 7*a* is a low-power immunofluorescence photomicrograph of a frozen section of the bow region of an embryonic chick lens. The letter *a* marks the approximate position of the capsule at the anterior lens surface, and the arrows delineate the epithelium/fiber interface. The region of transition between epithelium and fiber in the bow region is shown at higher magnification in an adjacent section in Fig. 7*b*. Within the stratified lens epithelium, numerous sites of macular and punctate anti-connexin43(252–271) staining were seen, particularly concentrated adjacent to the epithelium/fiber interface (arrows). Within the bow itself, this staining pattern was replaced by much larger, positively-

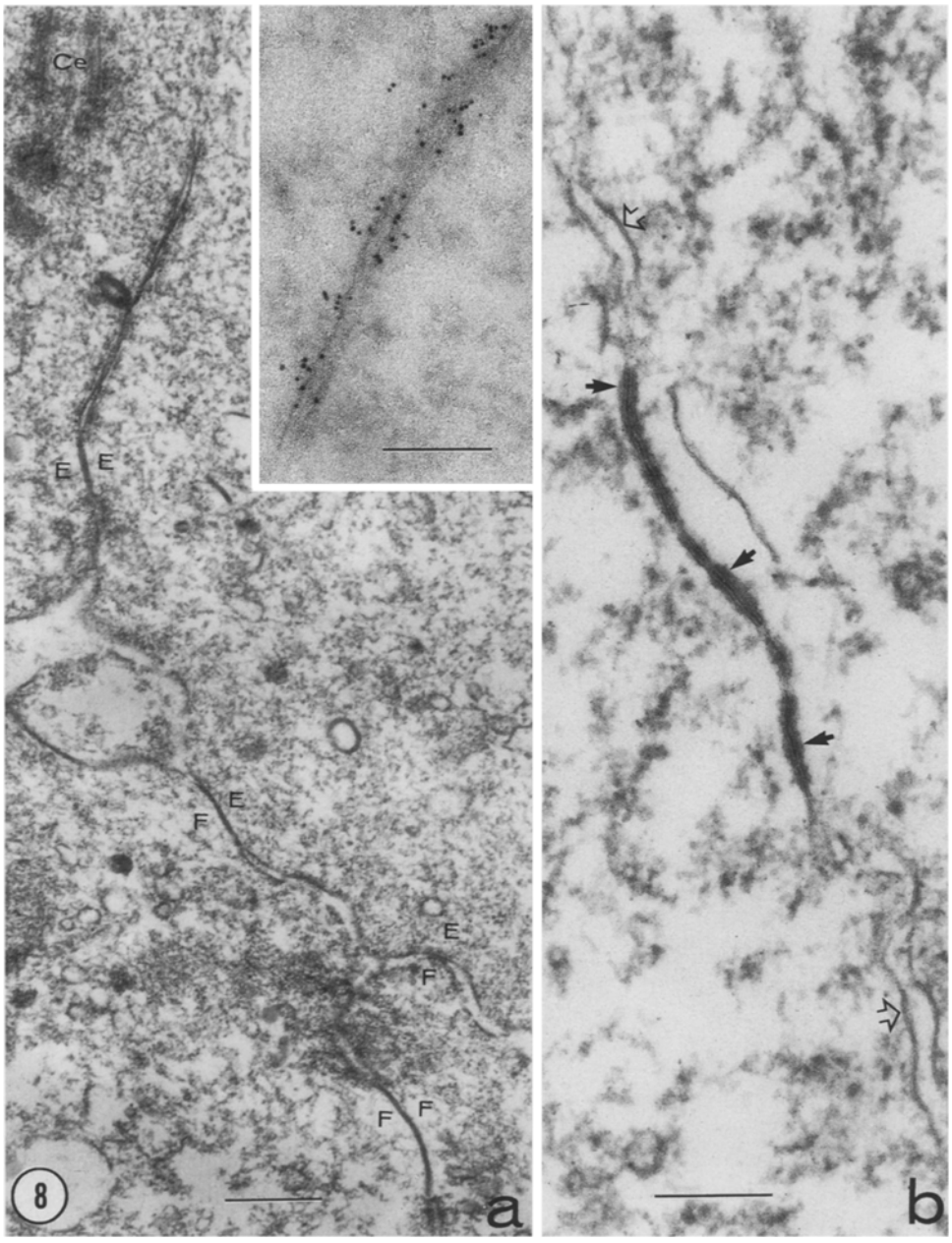

Fig. 8. Electron microscopic immunocytochemical localization of connexin43 in embryonic chick lens. (*a*) Low magnification photomicrograph of a section of 10-day embryonic chick lens epithelium stained with anti-connexin43(252–271) serum followed by horseradish peroxidase-labeled goat anti-rabbit IgG. The epithelial cells (*E*) are clearly distinguishable from the lens fibers (*F*). *Ce* = centriole. Bar = 0.2 μm. (*b*) At higher magnification, the cytoplasmic surfaces of an epithelium/epithelium gap junction in cells treated as in *a* can be seen to be labeled with the enzymatic reaction product (solid arrows) which is distinctly lacking on the nonjunctional plasma membranes (open arrows). Bar = 200 nm. (*a*) (*Inset*): Micrograph of an LR White section of 10-day chick lens stained with anti-connexin43(252–271) followed by gold-labeled goat anti-rabbit secondary reagent. The gold particles cluster only at sites of cell-cell interaction, but the method does not permit clear visualization of gap junction fine structure. Bar = 100 nm

staining maculae, which decreased in intensity and finally disappeared as the differentiating lens fibers came to occupy deeper positions in the lens cortex (asterisks, Fig. 7*a* and *b*).

In order to demonstrate that the fluorescent maculae were gap junctions, 10-day chick lenses were examined using the anti-connexin43(252–271) serum and either pre-embedding immunocytochemistry with horseradish peroxidase-labeled secondary antibody (Fig. 8*a* and *b*) or post-embedding staining of LR White thin sections with 5 nm colloidal gold-labeled secondary antibody (Fig. 8*a*, inset).

Peroxidase reaction product was found only on the cytoplasmic surfaces of the epithelial/epithelial gap junctions within the lens epithelium (arrowheads, Fig. 8*b*), where the epitope for anti-connexin43(252–271) has been shown to reside in rat myocardial gap junctions (Beyer et al., 1989). Similarly, colloidal gold particles were observed above background only at epithelial/epithelial intercellular interactions on LR White thin sections of chick embryo lenses (Fig. 8*a*, inset). The LR White embedding medium did not permit visualization of the 2 nm "gap." In the pre-embedded specimens, penetration of reagents into the tissue sections was limited, such that reaction product was seen only near the cut surfaces of cells. Thus, while positive anti-connexin43(252–271) staining was observed on epithelium/epithelium gap junctions, not all junctions were labeled, leaving open the possibility that other connexin types may contribute to epithelium/epithelium intercellular communication. No staining of either epithelium/fiber or mature fiber/fiber junctions was observed, subject to the same ambiguities regarding depth of penetration as mentioned above.

Discussion

Intercellular communication is critical for the maintenance of metabolic and ionic balance in the avascular lens. Correspondingly, the lens is rich in gap junctions, linking epithelial cell to epithelial cell, fiber to fiber, and epithelial cell to fiber (Goodenough et al., 1980; Rae & Kuszak, 1983). In this report we have focused on the class of gap junctions connecting epithelial cells, which a previous histochemical study in the rat had indicated contained connexin43 (Beyer et al., 1989). Using a combination of *c*DNA cloning and morphological and biochemical approaches, we have demonstrated that embryonic chick epithelial cells synthesize, and incorporate into gap junctions, the chick counterpart of rat connexin43. In addition, we have shown that connexin43 is post-translationally phosphorylated. Taken together, our results indicate that embryonic chick lenses and primary cultures derived from them are suitable systems for the study of connexin43 biosynthesis and its regulation during lens differentiation.

Chick and rat connexin43 are extremely homologous, showing 82 and 92% identity at the nucleotide and the amino acid levels, respectively. The structure and physiological properties of chick connexin43 are therefore likely to be very similar to those recently described for rat connexin43 (Beyer et al., 1987; Burt & Spray, 1988*a*,*b*). A high degree of evolutionary conservation has also been observed for connexin32, whose deduced amino acid sequence differs by only 4 out of 283 amino acids between rat and human (Kumar & Gilula, 1986; Paul, 1986). Rat connexin43 contains sequences located in the predicted cytoplasmic regions which are not shared with other known types of gap junction proteins (Kumar & Gilula, 1986; Paul, 1986; Beyer et al., 1987; Gimlich et al., 1988; Ebihara et al., 1989). The preservation of these sequences in chick connexin43 suggests that they may be involved in conferring physiological regulatory behavior specific to this connexin. Although such putative regulatory sequences have yet to be identified, it has been suggested that phosphorylation of connexins may be involved in modulating gap junctional communication (Saez et al., 1986). In light of our finding that connexin43 is a phosphoprotein, it is particularly intriguing that the unique regions of rat and chick connexin43 contain many serine and threonine residues and a single consensus tyrosine (Patchinsky et al., 1982) that might serve as phosphorylation sites.

Northern blots showed that connexin43 *m*RNA was much more abundant in chick embryo lens than in heart; one of our previous studies (Beyer et al., 1987) showed that the opposite was true in the adult rat. We believe that this most likely reflects development differences in the ratio of epithelial cells to fiber cells in the lens, with the higher proportion of epithelial and differentiating cells in embryonic lenses leading to correspondingly higher connexin43 expression. An alternative explanation would hold that our chick connexin43 clone encodes a protein not directly analogous to the rat connexin43 but instead a form particular to the lens. We feel this to be less likely because (i) Northern blots performed at lower stringency showed no relative increase in the heart signal and (ii) Southern blots demonstrated that connexin43 was a single copy gene (*data not shown*).

Morphological examination of chick lenses revealed that the lens epithelial cells assemble numerous intra-epithelial gap junctions containing connexin43 (Figs. 7 and 8). Junctions rich in connexin43 continued to be evident during the initial phases of lens fiber differentiation in the bow region, where they appeared larger than those in the nondifferentiating epithelium. The connexin43 signal in immunofluorescent images of the bow region diminished in a fiberward gradient. This distribution of connexin43-positive staining is similar to the distribution of gap junctions which contain crystallized connexons in freeze-fracture replicas (Benedetti, Dunia & Bloemendal, 1974; Peracchia, 1978; Miller & Goodenough, 1986), with the gradient of staining in the bow region consistent with the increased intermixing of noncrystallizing with crystallizing connexons in differentiating fibers (Schuetze & Goode-

nough, 1982; Menko et al., 1987). It is thus very likely that connexin43 is a component of the crystallized intra-epithelial gap junctions of the lens. The composition of the fiber/fiber and epithelium/fiber junctional interactions remains, however, to be clarified.

The immunoprecipitation data from whole lenses and from lens primary monolayer cultures provided further proof that connexin43 is synthesized by chick lens cells. Our results also demonstrated that connexin43 was translated in these cells as a single species with an apparent molecular weight of 42 kD which was subsequently converted to a M_r = ~45 kD form. This post-translational shift in apparent mass was due entirely to phosphorylation, a modification known to decrease the electrophoretic mobility of certain other proteins as well (Ralston & Bishop, 1985; Meijer et al., 1989; Moria et al., 1989). Pulse-chase analysis of connexin43 processing in whole lenses revealed that all of the connexin43 metabolically labeled during a 40-min incubation with ^{35}S-methionine was of the 42-kD form immediately after the pulse but was recovered exclusively as the 45-kD species after an hour of chase. Phosphorylation of connexin43 in lens cells thus appears to be a very efficient process, with all of the connexin43 surviving a 1-hr chase having undergone this modification. This is in contrast to connexin32, the only other connexin reported to be phosphorylated, for which no post-translational shift in apparent molecular weight has been observed and the extent of whose phosphorylation in intact cells is unknown (Saez et al., 1986; Traub et al., 1987). Immunoprecipitation of connexin43 from other junction-competent cell types (fibroblastic hamster BHK cells, rat NRK cells) revealed a similar post-translational decrease in the electrophoretic mobility of the protein due to phosphorylation (L.S. Musil, *unpublished observations*), demonstrating that this phenomenon is not confined to connexin43 from avian or lens sources. Studies of intercellular communication in NRK cells (Atkinson et al., 1981) and fibroblasts (Flagg-Newton, Dahl & Loewenstein, 1981) indicate that their gap junctional activity can be modulated by factors that effect cellular phosphorylation. Our finding that connexin43 is a phosphoprotein raises the possibility that these agents act, at least in part, by altering the phosphorylation of connexin43 itself. Future research will be directed towards examining this issue and towards characterizing gap junctional proteins in chick lens fiber cells.

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