

Connexin trafficking and the control of gap junction assembly in mouse preimplantation embryos

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SUMMARY

Gap junction assembly in the preimplantation mouse embryo is a temporally regulated event, beginning a few hours after the third cleavage during the morphogenetic event known as compaction. Recently, we demonstrated that both mRNA and protein corresponding to connexin43, a gap junction protein, accumulate through preimplantation development beginning at least as early as the 4-cell stage. Using an antibody raised against a synthetic C-terminal peptide of connexin43, this protein was shown to assemble into gap junction-like plaques beginning at compaction (G. Valdimarsson, P. A. De Sousa, E. C. Beyer, D. L. Paul and G. M. Kidder (1991). *Molec. Reprod. Dev.* 30, 18-26). The purpose of the present study was to follow the fate of nascent connexin43 during preimplantation development, from synthesis to plaque insertion, and to learn more about the control of gap junction assembly during compaction. Cell fractionation and reverse transcription-polymerase chain reaction were employed to show that connexin43 mRNA is in polyribosomes at the 4-cell stage, suggesting that synthesis of connexin43 begins at least one cell cycle in advance of when gap junctions first form. The fate of nascent connexin43 was then followed throughout preimplantation development by means of laser confocal microscopy, using two other peptide (C-terminal)-specific antibodies. As was reported previously, con-

nexin43 could first be detected in gap junction-like plaques beginning in the 8-cell stage, at which time considerable intracellular immunoreactivity could be seen as well. Later, connexin43 becomes differentially distributed in the apposed plasma membranes of morulae and blastocysts: a zonular distribution predominates between outside blastomeres and trophoctoderm cells whereas plaque-like localizations predominate between inside blastomeres and cells of the inner cell mass. The cytoplasmic immunoreactivity in morulae was deemed to be nascent connexin *en route* to the plasma membrane since it could be abolished by treatment with cycloheximide, and redistributed by treatment with monensin or brefeldin-A, known inhibitors of protein trafficking. Treatment of uncompact 8-cell embryos with either monensin or brefeldin-A inhibited the appearance of gap junction-like structures and the onset of gap junctional coupling in a reversible manner. These data demonstrate that the regulated step in the onset of gap junction assembly during compaction is downstream of transcription and translation and involves mobilization of connexin43 through trafficking organelles to plasma membranes.

Key words: connexin43, mRNA translation, protein trafficking, RT-PCR, laser confocal microscopy, dye coupling, mouse embryo

INTRODUCTION

Gap junctions are aggregations (plaques) of aqueous intramembranous channels that couple adjoining cells metabolically. Hemichannels in each cell are referred to individually as connexons, each of which is a hexamer of integral membrane proteins termed connexins (reviewed by Bennett et al., 1991). The connexins constitute a large family of related proteins, commonly identified according to their relative molecular masses as predicted from their cDNAs (e.g. connexin26 is 26×10^3 , connexin31 is 31×10^3 , etc; Haefliger et al., 1992).

Although much is now known about the expression of connexin genes in various adult tissues, comparatively little is known about the regulation of nascent connexin trafficking, membrane insertion, and assembly into plaques. Nor is there much precise information about the role of gap junctional coupling in embryonic development. The preimplantation mouse embryo provides a unique opportunity to explore all of these aspects. The assembly of gap junctions occurs *de novo* in this system and is a temporally regulated event, being initiated during the process of compaction, which begins a few hours after completion of the third cleavage (reviewed by Kidder, 1987). The establishment of

intercellular coupling is independent of cell flattening, one of the other components of compaction, but is required for maintenance of the compacted state, and hence for continued development (Buehr et al., 1987; Lee et al., 1987; Bevilacqua et al., 1989).

Connexin43 (Cx43) is one member of the connexin gene family whose zygotic expression supplies subunits for gap junction assembly during early development in the mouse. Cx43 mRNA has been detected as early as the 4-cell stage, and accumulates steadily thereafter (Valdimarsson et al., 1991; Nishi et al., 1991). Likewise, Cx43 itself was found by western blotting to accumulate from the 4-cell stage onward (Valdimarsson et al., 1991). These findings explain the relative insensitivity of gap junction formation to inhibition of transcription and protein synthesis from the 4-cell stage (McLachlin et al., 1983; McLachlin and Kidder, 1986). Cx43 can also be detected in the cytoplasm of 4-cell embryos, and in both cytoplasmic and gap junction-like structures beginning with compaction in the 8-cell stage, using the same antibody used for western blotting (Valdimarsson et al., 1991). The expression of three other connexin genes (Cx26, Cx32, and Cx46) has been examined in preimplantation embryos, and none has been found to be zygotically transcribed or to contribute subunits to gap junctional plaques (Barron et al., 1989; Valdimarsson et al., 1991; Nishi et al., 1991).

In the present study we have used two other antibodies specific for Cx43 to examine the distribution of this protein and its assembly into gap junctions during preimplantation development. Our goal was to learn more about the control of gap junction assembly during compaction and the contribution of Cx43 to gap junctional channels in later stages. Our evidence indicates that the regulated step in de novo gap junction assembly involves the transfer of nascent Cx43 from intracellular membranes to the plasma membrane, where it is quickly incorporated into plaques. Although Cx43 is inserted into the plasma membranes of all blastomeres, qualitative differences in its distribution become evident with cell polarization and the divergence of the inner cell mass from the trophectoderm.

MATERIALS AND METHODS

Embryo collection and culture

Embryos were flushed from the reproductive tracts of superovulated CF1 mice (Charles River Canada Ltd., St. Constant, Québec) mated with CB6F₁/J males (The Jackson Laboratory, Bar Harbor, ME), as described previously (Barron et al., 1989). Collections were carried out at the following times (hours post-hCG): 1- to 2-cell, 24 hours; uncompact 4- to 8-cell, 58-67 hours; 8- to 16-cell compacted morulae, 74-76 hours; late morulae (up to 32 cells), 80 hours; blastocysts, 90-92 hours. Embryos were cultured in standard egg culture medium (SECM; Spindle, 1980), at 37°C in 5% CO₂, in the presence or absence of cycloheximide, monensin, brefeldin-A or ethanol. Cycloheximide (Sigma Chemical Co., St. Louis, MO), was dissolved in distilled water to make a stock of 5 mg/ml, which was then diluted in culture medium to give a final concentration of 50 µg/ml (McLachlin et al., 1983). Monensin (Sigma) and brefeldin-A (Epicentre Technologies, Madison, WI) were dissolved in ethanol to make a stock of 10 mg/ml, and used in culture at a concentration of 1-5 µg/ml. Ethanol was added to control cultures at the same final concentrations (0.01-0.05%).

Ribonucleoprotein fractionation and RNA isolation

In order to investigate the utilization of Cx43 mRNA for protein synthesis we developed a procedure for microscale fractionation of embryos into subribosomal (i.e. less than 80S) and polyribosomal (i.e. 80S and greater) ribonucleoprotein fractions that could then be assayed for mRNA by reverse transcription coupled with the polymerase chain reaction (RT-PCR). Approximately 500 embryos were collected for each RNP fractionation. Embryos were washed 5× through ice cold Ca²⁺- Mg²⁺-free phosphate-buffered saline containing 0.3% polyvinylpyrrolidone (PBS-PVP), and deposited in 10-20 µl of PBS-PVP in the bottom of a mini-Dounce homogenizer on ice. They were homogenized using the 'A' pestle in 300 µl of a solution containing 1% NP-40, 0.4% sodium deoxycholate, 500 units/ml RNasin (Promega, Madison, WI), 10 µg/ml cycloheximide, and 20 µg *E. coli* rRNA in TSM/EGTA buffer (40 mM Tris-HCl, 150 mM NaCl, 20 mM magnesium acetate, 10 mM EGTA, 10 mM dithiothreitol, pH 7.5; Kidder and Conlon, 1985). The homogenate was centrifuged for 3 minutes at 24,000 revs/minute (22,000 g), at 4°C, in the TLA-100 rotor of a Beckman tabletop ultracentrifuge, to pellet nuclei and mitochondria. The post-mitochondrial supernatant was divided into two equal portions and each was layered over 50 µl of 40% sucrose in TSM/EGTA and centrifuged in the same rotor for 40 minutes at 50,000 revs/minute (100,000 g), at 4°C, forming a polyribosomal pellet and a subribosomal supernatant.

The subribosomal supernatants were carefully withdrawn and transferred to microfuge tubes. 20 µl of 3 M sodium acetate and 440 µl of absolute ethanol were added to each and the RNPs allowed to precipitate overnight at -20°C. Each polyribosomal pellet was dissolved in 8 µl of solubilization solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 100 units/ml RNasin, 10 mM dithiothreitol) containing 10 µg *E. coli* rRNA, and then the two pellet fractions were combined. 85 µl of solution D were added [solution D is 4 M guanidinium thiocyanate (GIBCO/BRL, Burlington, Ont.), 25 mM sodium citrate, pH 7.0, containing 0.5% Sarkosyl and 100 mM β-mercaptoethanol; Chomczynski and Sacchi, 1987] and the sample was stored at -20°C overnight. After removal from ethanol by centrifugation, the subribosomal precipitates were likewise each dissolved in 8 µl of solubilization solution, combined, and then diluted with 85 µl of solution D. The two fractions in solution D were then layered over 100 µl of 5.7 M CsCl in 0.1 M EDTA, pH 7.5 and centrifuged in a Beckman TLA-100 rotor at 80,000 revs/minute (250,000 g) for 4 hours at 20°C to pellet RNA. The pellets were dissolved at room temperature in 100 µl of 2.5 M ammonium acetate and ethanol precipitated overnight at -20°C.

Reverse transcription/polymerase chain reaction

After centrifugation to remove the ethanol, the RNA pellets were rinsed twice with cold 70% ethanol before air drying. Reverse transcription (RT) and amplification of cDNA by polymerase chain reaction (PCR) was based on the method of Rappolee et al. (1989). RNA from each RNP fraction was reverse transcribed into cDNA by incubation at 42°C for 90 minutes with a mixture of 30 units of AMV-RT (Boehringer Mannheim, Laval, Québec) and the following reagents: 0.4 µg oligo-dT primer, 10 mM MgCl₂, 100 mM Tris-HCl buffer, pH 8.3, 10 mM dithiothreitol, 1 mM each dNTP, and 40 units of RNasin (Promega) in a 20 µl volume. Reactions were stopped by boiling for 10 minutes followed by flash cooling on ice, and divided into suitable aliquots (20-25 embryo equivalents/µl) which were stored at -20°C or used immediately for PCR. Reverse transcriptions were also performed on 2 µg of mouse brain and liver RNA.

PCR was performed on 50-75 embryo equivalents of cDNA using 2.5 units of Taq DNA polymerase (GIBCO/BRL) in a solu-

tion consisting of 1 μ M oligonucleotide 5' and 3' sequence-specific primers, 200 μ M dNTP, 10 mM Tris-HCl, pH 8.8, 3.5 mM MgCl₂, 50 mM KCl, and 0.1% Triton X-100, in a 50 μ l volume. Amplification reactions were carried out for 30 cycles using a Perkin Elmer Cetus DNA Thermal Cycler (1 minute at 94°C, 1 minute at 65°C, and 2 minutes at 72°C). Oligonucleotide primers for mouse Cx43 amplified a 332 bp segment encoding a peptide corresponding to a.a. 270-379 of rat Cx43 (Beyer et al., 1987). This segment contains a diagnostic restriction site for *Hpa*II producing 162 and 170 bp fragments. The mouse Cx43 primers were (5'-primer) 5'-TGGCTGCTCCTACCAACGGC-3', and (3'-primer) 3'-CGTCTGGAGCCGGACTACTGG-5'. Primers for β -actin were also used; these amplify a 243 bp segment of the cDNA spanning an intron (87 bp) in the genomic sequence. The mouse β -actin primers were (5'-primer) 5'-CGTGGGCCGCCCTAG-GCAACCA-3', and (3'-primer) 3'-GGGGGACTTGGGATTC-CGGTT-5' (Tokunaga et al., 1986). Restriction enzyme digestion of the 332 bp Cx43 amplified fragment was performed on PCR products which had been precipitated overnight at -20°C with 3 M ammonium acetate and ethanol, pelleted, washed with 70% ethanol, and air dried. Reactions were performed with 10 units of *Hpa*II (BRL, Burlington, Ont.) for 1 hour at 37°C.

PCR products were separated by electrophoresis in an 80 V constant voltage field in a 4% agarose gel (NuSieve 3:1, FMC BioProducts, Rockland, ME) containing 40 mM Tris-acetate, 1 mM EDTA and 0.75 μ g/ml ethidium bromide. After denaturation, PCR products were transferred to Hybond-N (Amersham, Oakville, Ont.) by capillary blotting overnight in 10 \times SSC. Blots were probed with cDNAs labeled by the random primer method (Feinberg and Vogelstein, 1983) to a specific activity of 1-2 \times 10⁹ counts/minute per μ g. Probe hybridizations were as described in Valdimarsson et al. (1991) except that they were carried out overnight at 42°C in a Robbins Hybridization Incubator (model 310). Cx43-specific PCR amplification products were detected by hybridization with a previously characterized 1.4 kb Cx43 cDNA (Beyer et al., 1987; Barron et al., 1989).

Protein solubilization and western blotting

Four-cell embryos (approx. 670) and late morulae (approx. 600), frozen separately in approximately 5 μ l of PBS-PVP as described previously (Valdimarsson et al., 1991), were thawed by the addition of an equal volume of lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 1% NP-40, 2 mM N-ethylmaleimide, 2 mM phenylmethylsulfonylfluoride, 2 mM ortho-phenanthroline) containing 1% SDS. To dephosphorylate total protein, 1/10 volume of 10 \times dephosphorylating buffer (0.5 M Tris-HCl, 1 mM EDTA, pH 8.5) was added followed by 2 units of calf intestinal alkaline phosphatase (Boehringer-Mannheim) and the sample was incubated at 37°C for 8 hours. Dephosphorylated total ovarian protein was prepared as described for embryos. To obtain total native phosphorylated ovarian protein, ovaries were homogenized in lysis buffer containing phosphatase inhibitors (100 μ M Na₃VO₄, 50 mM NaF). Lysed embryos and ovary homogenates were mixed with an equal volume of 2 \times Laemmli buffer, vortexed, boiled for 10 minutes, sonicated, and centrifuged at 10,000 g for 10 minutes at room temperature. Supernatants were subjected to electrophoresis on minigels (12.6% resolving gel) according to Laemmli (1970), and separated proteins were transferred to Immobilon-P (Millipore, Bedford, MA; Towbin et al., 1979). Western blotting was performed according to Valdimarsson et al. (1991), except that bound primary antibody was detected with the use of IBI High Sensitivity Enzygraphic Webs (Intersciences, Markham, Ontario).

Immunofluorescence

Prior to fixation, embryos were washed by passage through at least

five drops of PBS-PVP, followed by one drop of PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 1 mM MgCl₂, pH 6.9). Embryos up to the late morula stage were fixed and processed for immunofluorescence as described in Valdimarsson et al. (1991). Immunofluorescence on blastocysts required the substitution of 0.2%-0.5% Triton X-100 for 0.1% Tween-20 in the protocol. Immunostained embryos were wholemounted in FITC-Guard (Testog Inc., Chicago, IL) and viewed either with a Zeiss Photomicroscope I equipped with epifluorescence optics or a Bio-Rad MRC 600 confocal laser scanning microscope.

Antibodies

Cx43 was detected on western blots and by immunofluorescence using an affinity-purified rabbit antiserum raised against a synthetic peptide corresponding to a carboxy-terminal cytoplasmic domain of Cx43 (a.a. 302-319; Garcia, White, Willecke, and Nicholson, unpublished data; Naus et al., 1992). The specificity of the immunostaining patterns obtained with this antibody was tested using preimmune serum and crude serum absorbed three times with peptide. For immunofluorescence analysis, affinity-purified, peptide absorbed, and preimmune sera for the 302-319 peptide were all used at a concentration of 8-9 μ g/ml IgG. A second affinity-purified rabbit antiserum raised against another synthetic peptide corresponding to a different carboxy-terminal cytoplasmic domain of Cx43 (a.a. 360-382) was provided by Dr D. Laird (Laird and Revel, 1990). It was used at a dilution of 1:250. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (ICN Biomedicals Canada Ltd., St. Laurent, Québec) was used at a dilution of 1:50. For western blot analysis, anti-302-319 was used at a concentration of 0.25 μ g/ml IgG. Primary antibody bound to the blot was detected using goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories Ltd., Mississauga, Ontario).

Dye coupling

To test for intercellular coupling via gap junctions, a single blastomere was injected with the fluorescent dye 6-carboxyfluorescein (10 mM in distilled water; Kodak, Rochester, NY) using a continuous train of hyperpolarizing current pulses of 17 nA (200 millisecond duration, one pulse per second). Dye delivery was terminated 1 minute after impalement, and the electrode was maintained in the injected cell for the duration of each observation. Microelectrodes were prepared as described in McLachlin et al. (1983). Injections were performed in flushing medium-1 (Spindle, 1980). The spread of fluorescent dye between blastomeres was observed intermittently over a 30 minute period through a Zeiss IM35 inverted microscope fitted with phase-epifluorescence optics.

RESULTS

Cx43 mRNA is recruited into polyribosomes by the 4-cell stage

Given that Cx43 mRNA and the protein itself are both detectable at least as early as the 4-cell stage (Valdimarsson et al., 1991; Nishi et al., 1991), we began by asking whether some of the protein might be newly synthesized, i.e. whether Cx43 mRNA is recruited into polyribosomes for translation as soon as it becomes available. The procedure for fractionating embryo RNPs into subribosomal supernatant (S) and polyribosomal pellet (P) fractions was developed using 10 mg samples of mouse liver and then tested with cultured rat glioma cells exhibiting a high level

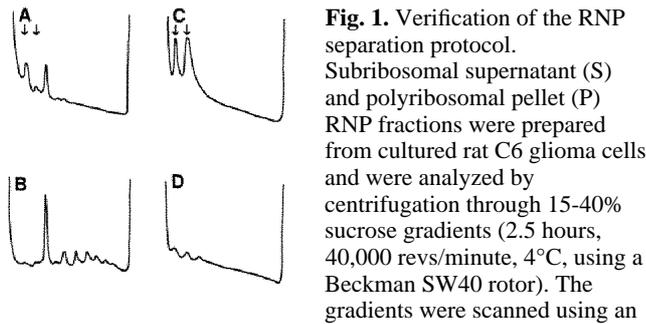


Fig. 1. Verification of the RNP separation protocol. Subribosomal supernatant (S) and polyribosomal pellet (P) RNP fractions were prepared from cultured rat C6 glioma cells and were analyzed by centrifugation through 15-40% sucrose gradients (2.5 hours, 40,000 revs/minute, 4°C, using a Beckman SW40 rotor). The gradients were scanned using an

ISCO density gradient fractionation system. Traces A (S fraction) and B (P fraction) were obtained from RNP fractions prepared as described for embryos in Materials and Methods. Traces C and D (S and P respectively) were obtained from post-mitochondrial supernatants that had been treated with 50 mM EDTA for 15 minutes at room temperature to dissociate polyribosomes into ribosomal subunits prior to the RNP fractionation step. The 40S and 60S ribosomal subunits are indicated by arrows.

of Cx43 expression, so that the size distributions of the RNPs in the two fractions, and the distribution of Cx43 mRNA between them, could be analyzed. As shown in Fig. 1A,B, this procedure resulted in an almost complete sepa-

ration of subribosomal and polyribosomal RNPs, with 80S monosomes being split between the two fractions. Whereas the supernatant was nearly devoid of polyribosomes, the pellet did contain a small proportion of the ribosomal subunits; attempts to eliminate this by shortening the 100,000 g separation step resulted in significant contamination of the S fraction with polyribosomes (not shown). The samples in Fig. 1C,D, in which the fractionation step was performed after the post-mitochondrial supernatant had been treated with EDTA to dissociate monosomes and polysomes into 40S and 60S subunits, show that the proportion of ribosomal subunits pelleting with the polyribosomes is very small. Northern blot analyses of the S and P fractions from exponentially growing cells had revealed that all of the Cx43 mRNA is in polyribosomes, and EDTA treatment caused most of it (0.87 ± 0.09 , $n=3$) to partition into the S fraction (Naus et al., 1992). Thus, only about 13% of any subribosomal Cx43 mRNA could be expected to contaminate the P fraction.

RNA isolated from 4-cell stage S and P RNP fractions prepared in the same way was reverse transcribed to cDNA and then subjected to DNA amplification using oligonucleotide primers designed to amplify a 332 bp segment of mouse Cx43. Amplification products were separated on an

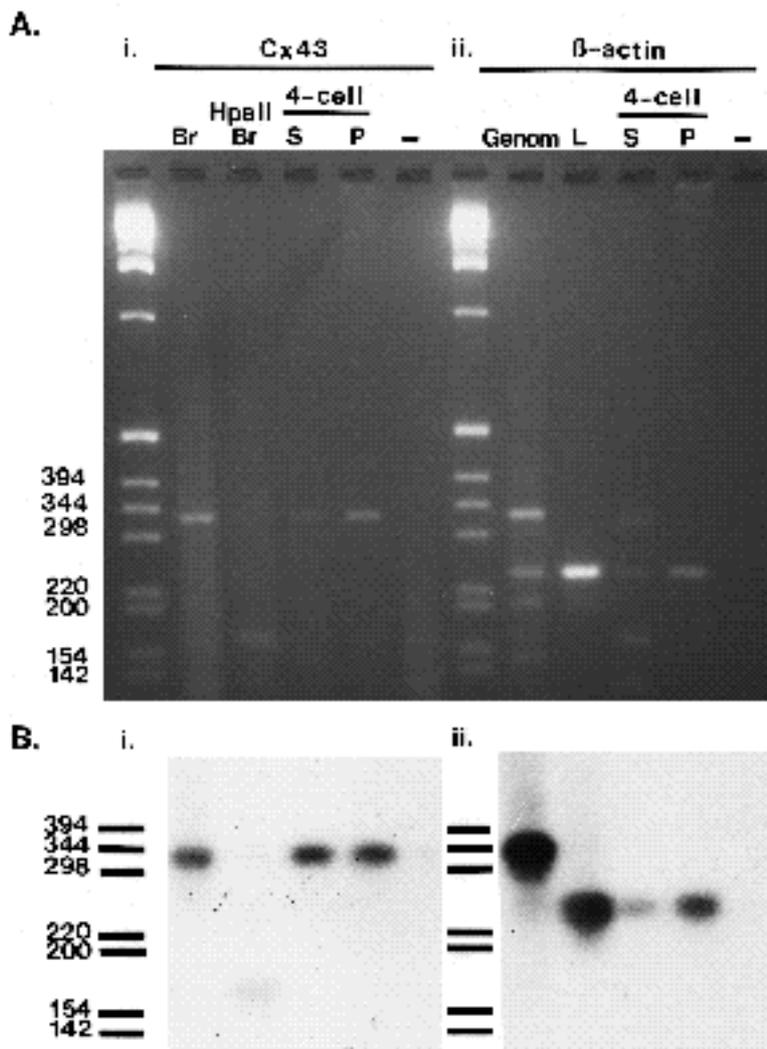


Fig. 2. Cx43 mRNA is recruited into polysomes in the 4-cell stage. (A) Ethidium bromide-stained agarose gel containing PCR products obtained with primer sets that amplify a 332 bp segment of Cx43 cDNA (i), or a 243 bp segment of β -actin cDNA (ii); the 330 bp β -actin segment derived from amplification of genomic DNA is also shown (Genom). (B) Southern blot of the gel in (A) probed with 32 P-labeled cDNA for Cx43 (i) or β -actin (ii). The 332 bp Cx43 fragment from mouse brain cDNA (Br), included as a positive control, was digested with *Hpa*II to reveal the presence of a diagnostic restriction site indicated by the doublet at 162-170 bp. Liver cDNA (L) served as positive control for the β -actin amplification. Absence of the 330 bp intron-containing β -actin amplification product in the 4-cell embryo subribosomal (S) and polysomal (P) RNP fraction cDNAs demonstrates that these are free of genomic DNA contamination. These lanes are derived from 25 embryo equivalents. In three separate experiments Cx43 mRNA was found predominately in the P fraction. No amplification products were obtained from *E. coli* rRNA (-), the carrier RNA used during RNA purification, using either of the two primer sets.

ethidium bromide-containing agarose gel and probed with radiolabeled cDNA after Southern blotting. The Cx43 primers selectively amplified only the expected 332 bp fragment from both the S and P RNP fractions of 4-cell embryos, with most of the amplification product being contributed by the P fraction (Fig. 2Ai). A similar fragment was always amplified from brain cDNA, the positive control. *Hpa*II digestion of this fragment generated a poorly resolved doublet at the approximate location expected for the diagnostic restriction fragments (162 and 170 bp). In addition, hybridization with the 1.4 kb Cx43 cDNA detected only the single 332 bp band in brain and embryo fractions (Fig. 2Bi). Although faint in this figure, longer autoradiographic exposures also detected the faster migrating restriction fragments produced by *Hpa*II digestion.

One problem with the use of RT-PCR to detect Cx43 mRNA is the absence of introns from the genomic coding sequence (Willecke et al., 1991). Since the amplified segment obtained from genomic DNA is the same size as that from cDNA, precautions were taken to ensure the absence of contaminating DNA, including the isolation of embryo RNA by pelleting through CsCl. To check that the 332 bp Cx43 fragment obtained from the S and P fraction cDNA did not arise from contamination, an oligonucleotide primer set for β -actin was used with the same RT preparations. This primer set amplifies a 243 bp segment from cDNA but, because the primers bracket an 87 bp intron, amplifies a 330 bp segment from genomic DNA. When this primer set was used for PCR with genomic DNA both 243 and 330 bp fragments could be detected on ethidium bromide-stained gels, although using the more stringent criterion of hybridization with the cloned cDNA, only the larger (intron containing) fragment could be confirmed as β -actin (Fig. 2Aii,Bii). This larger fragment could not be detected in the embryo RNP fractions on either the gel or the Southern blot, even upon longer autoradiographic exposures, confirming the absence of genomic DNA from these preparations. No amplification products were evident when either Cx43 or β -actin primers were used to amplify cDNA prepared from *E. coli* rRNA, the carrier RNA used during embryo fractionation and RNA purification.

Cx43 is distributed in both cytoplasmic and plasma membrane arrays

We next examined the subcellular distribution of Cx43 throughout preimplantation development. Using an antibody raised against a synthetic peptide unique to the C-terminal cytoplasmic domain of Cx43 (a.a. 252-271), we had previously demonstrated that Cx43 can be identified in the cytoplasm of uncompact embryos, although plaque-like structures resembling gap junctions could only be detected in the 8-cell stage after the onset of compaction (Valdimarsson et al., 1991). In the present study, our ability to resolve structural detail was substantially improved by using confocal laser scanning microscopy. In addition, we used a new antibody against a different synthetic peptide, also unique to the C-terminal domain of Cx43 (a.a. 302-319). Anti-302-319 has been shown to label specifically cardiac intercalated discs, and produces punctate surface labeling in Cx43-expressing fibroblasts and glioma cells. It also specifically recognizes protein with an approximate M_r of 43×10^3 on

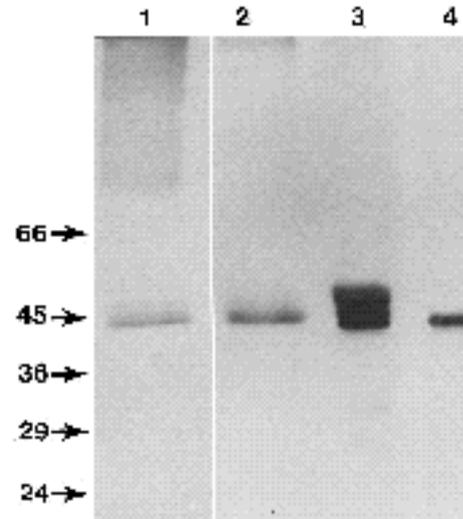


Fig. 3. An antibody against a.a. 302-319 of Cx43 recognizes a $43 \times 10^3 M_r$ protein in 4-cell embryos and late morulae. A western blot containing dephosphorylated protein preparations of 4-cell embryos (1), late morulae (2), ovary (4), and ovarian lysate prepared in the presence of phosphatase inhibitors (3), was probed with anti-302-319. A single band with $M_r 43 \times 10^3$ was detected in dephosphorylated samples of both embryos and ovary. Four prominent bands with M_r approximately 42, 45, 47 and 49×10^3 were evident in ovarian lysate prepared in the presence of phosphatase inhibitors.

western blots of brain, heart, and Cx43-expressing glioma cells, but does not bind to homologous connexins in liver (Cx32 and 26; Garcia, White, Willecke and Nicholson, unpublished data; Naus et al., 1992). To confirm that this antibody specifically recognizes Cx43 in embryos, western analysis was performed on total protein solubilized from 4-cell embryos and late morulae. Since our previous western blot analyses had revealed multiple, variable Cx43 bands in embryo lysates, possibly derived from posttranslational phosphorylation, we sought to eliminate this variability by treating the sample with alkaline phosphatase before electrophoresis. Anti-302-319 recognized protein with a M_r of approximately 43×10^3 in dephosphorylated preparations from both embryonic stages and ovary (Fig. 3). In contrast, four prominent bands with M_r of approximately 42, 45, 47, and 49×10^3 were evident in ovarian lysate prepared in the presence of phosphatase inhibitors.

Having confirmed the specificity of anti-302-319, this antibody was used for immunofluorescence experiments. As before, Cx43 was first detected in plasma membranes as plaque-like intercellular foci suggestive of gap junctions beginning with the first signs of cell flattening in the 8-cell stage (Fig. 4C). Compacting and postcompaction embryos were also rich in cytoplasmic immunoreactivity, often seen as small, diffuse foci (Fig. 4C,D). Both the membrane and the cytoplasmic staining patterns seen in postcompaction embryos with anti-302-319 strongly resembled those seen previously with the anti-252-271 antibody (Valdimarsson et al., 1991). However, despite the ability of anti-302-319 to detect Cx43 at the 4-cell stage by western blotting, we were unable to detect Cx43 in the cytoplasm of precompaction embryos by immunofluorescence (Fig. 4A,B).

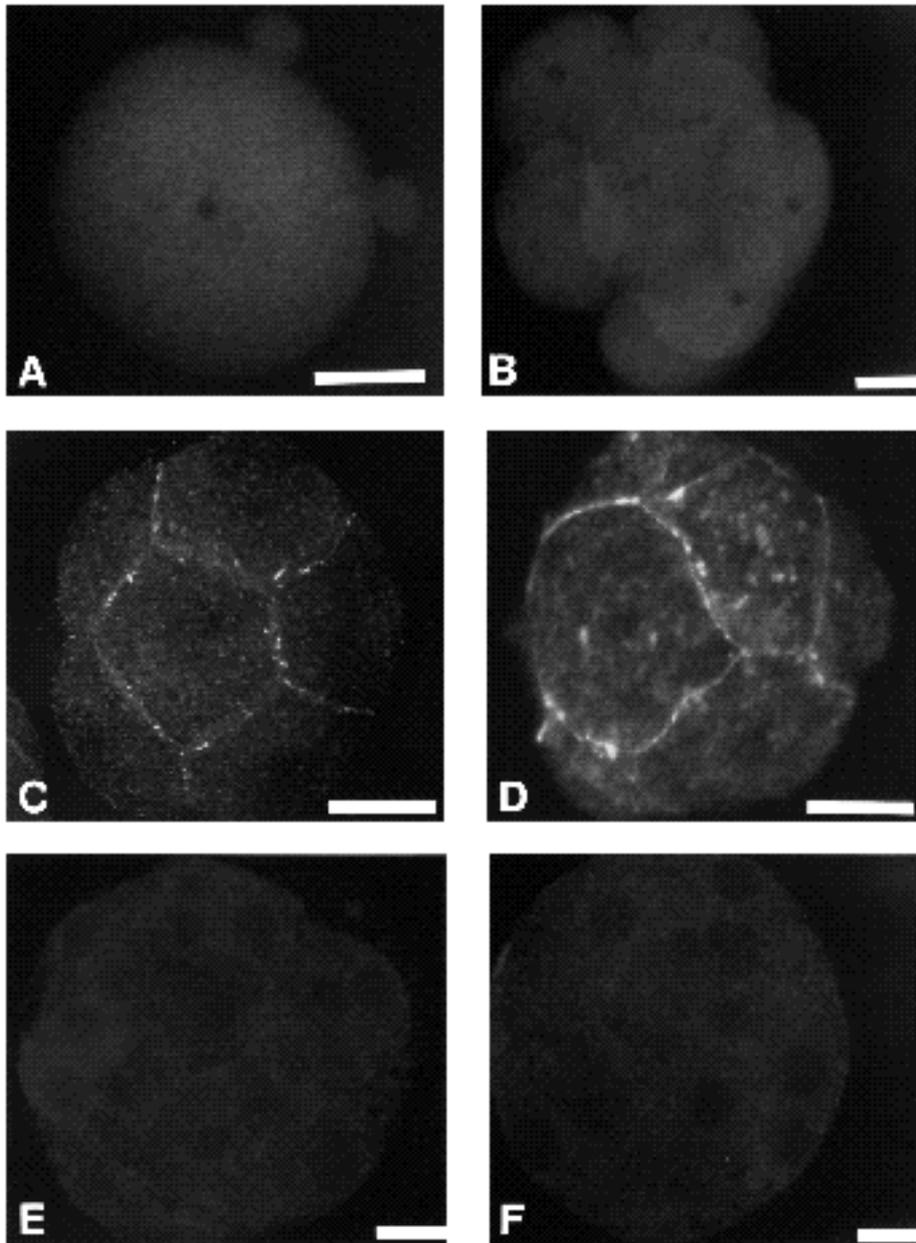


Fig. 4. Pattern and specificity of Cx43 staining with anti-302-319. No immunoreactivity was detected with this antibody in 1-cell zygotes (A) or uncompact 8-cell embryos (B). Plaque-like foci at apposed membrane regions were first detected beginning with signs of cell flattening in the 8-cell stage, at which time cytoplasmic immunoreactivity also became evident (C). With increasing cell number, morulae develop a zonular pattern of intercellular staining at apposed membrane regions while still retaining plaque-like localizations (D). Cytoplasmic and membrane staining patterns were absent from morulae stained with peptide absorbed (E) or preimmune serum (F). The images are confocal z-series projections (2–2.5 μm thick). Bars, 25 μm .

Therefore, we sought to determine if the cytoplasmic staining in precompaction embryos observed previously (Valdimarsson et al., 1991) could be reproduced with other antibodies. Using an antibody directed against a different C-terminal domain (a.a. 360–382), Cx43 was detected in the cytoplasm of both pre- and postcompaction embryos, as well as in plaque-like structures in the latter (Fig. 5). The cytoplasmic immunoreactivity in precompaction embryos was reminiscent of that which we had seen with anti-252-271 (Valdimarsson et al., 1991), and differed qualitatively from that seen in postcompaction embryos by being organized into larger, more irregular foci.

Using anti-302-319, plaque-like foci of Cx43 were distributed throughout apposed membranes in compacted morulae (Fig. 6Ai–iv). However, beginning in late morulae and persisting through to the blastocyst stage, Cx43 becomes differentially distributed in inner and outer blas-

tomeres. In late morulae, the outer blastomeres exhibit a zonular array of Cx43 in apposed membranes, punctuated by plaque-like foci (Figs 4D, 6Ai, 6Aiv). As the plane of focus passes deeper into the embryo, the zonular array is lost (Fig. 6Aii–iii). This zonular array was also detected in morulae stained with anti-252-271 (Valdimarsson et al., 1991). In blastocysts, plaque-like Cx43 staining is prominent in the inner cell mass (ICM) whereas a zonular distribution of immunoreactivity punctuated by intermittent, small, plaque-like foci is evident between trophectoderm cells (Fig. 6Bi–iv). Visualization of Cx43 in the ICM necessitated the substitution of Triton X-100 for Tween-20 during the permeabilization step. This substitution persistently resulted in the staining of nuclei with anti-302-319, but did not affect the specificity of staining with anti-360-382.

To assess the specificity of the immunostaining patterns

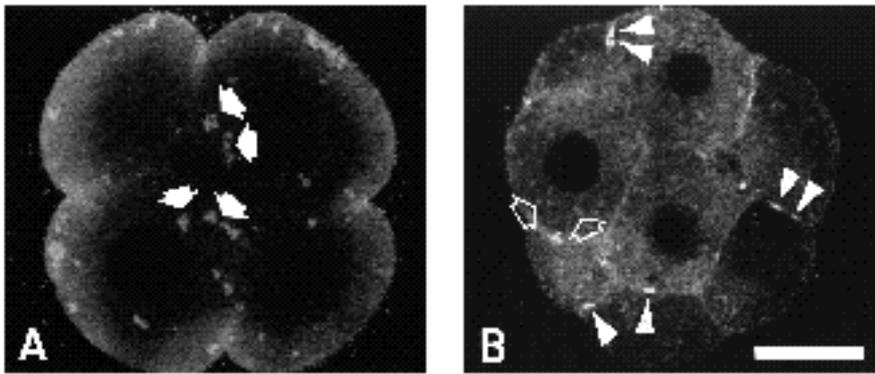


Fig. 5. Cx43 can be detected in the cytoplasm of 4-cell embryos and membrane and cytoplasm of morulae with a second Cx43 peptide-specific antibody. Affinity-purified antibody against a different C-terminal peptide unique to Cx43 (a.a. 360-382) detects antigen in the cytoplasm of both 4-cell embryos (A) and morulae (B), as well as in gap-junction-like structures (arrowheads) in the latter. Large irregular foci detected in the

cytoplasm of 4-cell embryos (solid arrows) are qualitatively similar to previously published findings with anti-252-271 (Valdimarsson et al., 1991), and are different from the diffuse foci seen in the cytoplasm of morulae (open arrows). Bar, 25 μ m.

provided by the anti-302-319 antibody, 8- to 16-cell morulae were scored blindly, i.e. their identities were revealed to the experimenter only after scoring had been completed. Diffuse cytoplasmic foci, punctate plaque-like foci, and zonular staining of apposed membrane regions were detected in 82%, 93%, and 61% of embryos ($n=28$), respectively. None of these staining patterns was ever observed using preimmune serum ($n=28$) or secondary antibody alone ($n=29$), or if the anti-302-319 was preabsorbed with the peptide prior to use ($n=28$; Fig. 4E,F).

Cytoplasmic immunoreactivity represents nascent Cx43 en route to the plasma membrane

To determine if the cytoplasmic staining detected in post-compaction embryos represents nascent Cx43, 8- to 16-cell morulae (74-76 hours post-hCG) were cultured for 4 hours in the presence of 50 μ g/ml cycloheximide (CHX), and scored blindly. This treatment effectively inhibits protein synthesis, causing greater than 97% inhibition of [35 S]methionine incorporation (McLachlin et al., 1983). Cycloheximide reduced the incidence of small diffuse foci to approximately one quarter of that seen in control embryos without affecting the incidence of plaque-like foci (Table 1). Affected embryos were completely devoid of any cytoplasmic staining pattern above a uniform, diffuse background (Fig. 7).

Given the likelihood that most of the Cx43-specific cytoplasmic immunoreactivity represents nascent connexin, we next considered how the distribution of this protein might

be altered by treatment with inhibitors of protein trafficking. Post-compaction 8- to 16-cell embryos were treated for 4 hours with 5 μ g/ml of either monensin or brefeldin-A (BFA) and scored blindly. Monensin is a well-known inhibitor of flow through the trans-Golgi network (TGN), while BFA blocks translocation of proteins from the endoplasmic reticulum to the Golgi apparatus (Mollenhauer et al., 1990; Klausner et al., 1992). Compacted 8-cell embryos and morulae treated with monensin acquired a new cytoplasmic staining pattern consisting of large, juxtannuclear clouds of immunoreactivity (Fig. 8C,D), whereas the frequency of the small diffuse foci remained unaltered compared to control embryos (Fig. 8A,B; Table 1). In contrast, treatment with BFA abolished the small diffuse foci characteristic of controls, replacing them instead with a reticulated network of immunoreactivity (Fig. 8E,F). BFA treatment over this period had the added effects of inducing decompaction and inhibiting cytokinesis, the latter evidenced by the presence of binucleate blastomeres (not shown). Treatment for 4 hours with either monensin or BFA did not diminish the incidence of plaque-like foci, or the zonular staining pattern.

Monensin and BFA interfere with the de novo assembly of gap junctions and the acquisition of dye coupling

Although we could not visualize Cx43 in precompaction embryos with the anti-302-319 antibody, its detection in the cytoplasm but not membranes of such embryos using both

Table 1. Anti-302-319 immunoreactivity in morulae is sensitive to inhibitors of protein synthesis and intracellular trafficking

Treatment*	Number scored	Morulae (%)				
		Cytoplasmic staining			Intercellular staining	
		Small diffuse foci	Large juxta-nuclear	Reticulated	Punctate foci	Zonular
Control	87	99	0	0	99	66
50 μ g/ml CHX	92	26	0	0	98	55
5 μ g/ml Monensin	73	100	97	0	100	69
5 μ g/ml BFA	52	0	0	93	98	87

*Embryos were cultured for 4 hours in SECM containing either 0.05% ethanol (control) or inhibitor, prior to being fixed and processed for immunofluorescence. Culturing in the presence of 0.05% ethanol did not effect the frequency or nature of Anti-302-319 immunoreactivity. For the BFA treatment group the data are from two experiments whereas the other treatment data are from three experiments.

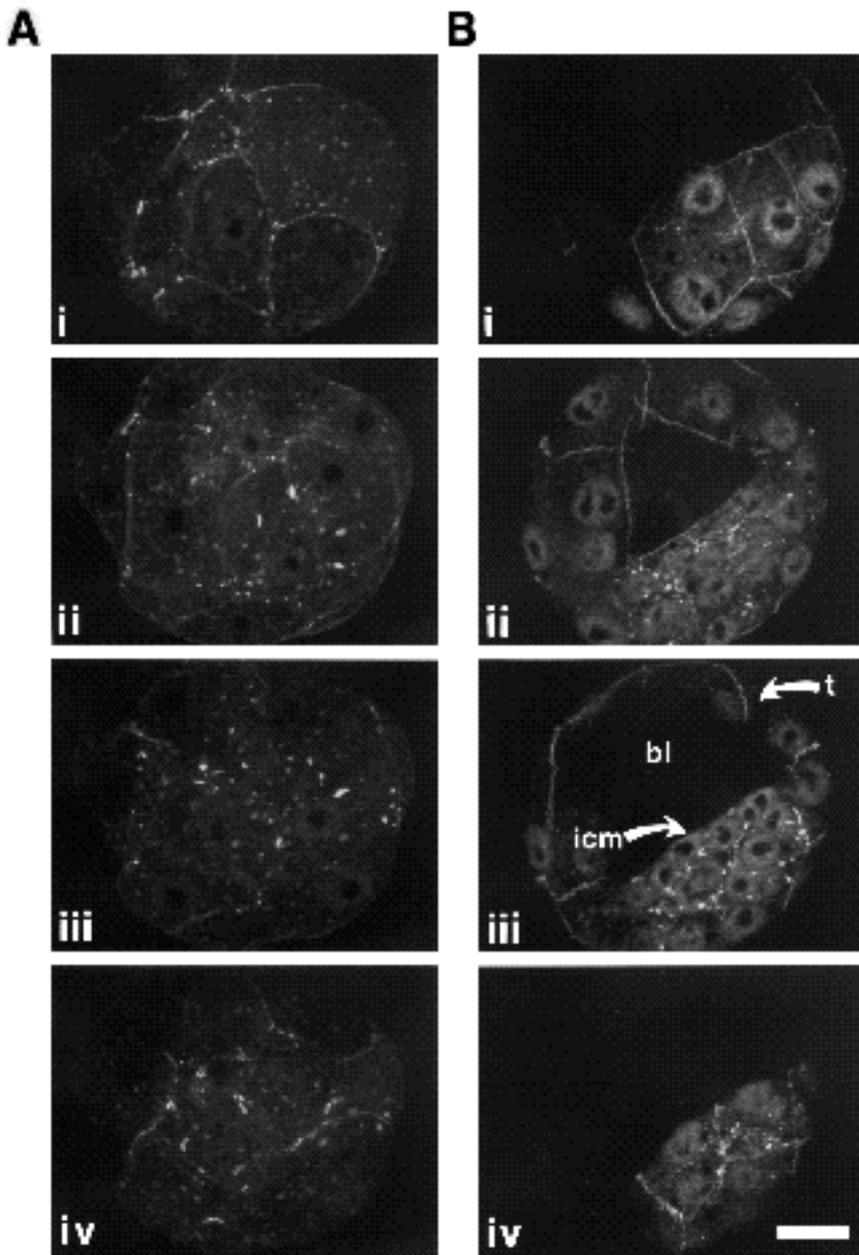


Fig. 6. The zonular Cx43 staining pattern at apposed membrane regions is confined to the outside cells of morulae and trophectoderm of blastocysts. Each column is a through focus series of successive z-series projections (2-3 μm thick) of a morula (Ai-iv) or blastocyst (Bi-iv) stained with anti-302-319. The top and bottom images in each column are superficial optical sections whereas the middle images are deeper sections. Large, plaque-like foci typical of embryonic gap junctions predominate in the icm (Bii, iii) whereas the zonular pattern, punctuated by small, bright foci that may also represent gap junctional plaques, characterizes the trophectoderm (t) which surrounds the blastocoel (bl). Bar, 25 μm .

the anti-252-271 (Valdimarsson et al., 1991) and anti-360-382 antibodies, along with our finding that Cx43 mRNA is translated in the 4-cell stage, imply that a cytoplasmic pool exists from which nascent Cx43 is recruited into plasma membranes. We therefore tested whether inhibition of protein trafficking with either monensin or BFA could prevent the *de novo* assembly of gap junctions and delay the onset of intercellular coupling at compaction. Intercellular communication via gap junctions was assayed by dye coupling, wherein the intercellular transfer of microinjected carboxyfluorescein was monitored. Uncompacted 8-cell embryos were cultured in the continued presence of either 5 $\mu\text{g}/\text{ml}$ monensin or 1-5 $\mu\text{g}/\text{ml}$ BFA until control embryos treated with carrier solvent (0.01-0.05% ethanol) began exhibiting rapid dye transfer to all blastomeres. To guard against the inactivation of inhibitors during the culture period, embryos

(control and treatment groups) were transferred to fresh culture drops every 4-5 hours. Embryos exhibiting no dye transfer or transfer to only a single blastomere were designated as not coupled, the latter situation presumably arising from transfer of dye through remnant cytoplasmic bridges connecting sister blastomeres (Kidder et al., 1988). Conversely, embryos exhibiting transfer to more than one blastomere were considered coupled. The extent of coupling was further categorized based on the rate of dye transfer, defined as the time necessary for dye to enter all the cells in the focal plane of the injected cell, up to 30 minutes. Dye transfer times of 10 minutes, 10-20 minutes, or 20-30 minutes were considered rapid, moderate, and slow, respectively. Coupling in embryos with incomplete dye transfer after 30 minutes was considered very slow.

Both monensin and BFA markedly reduced both the inci-

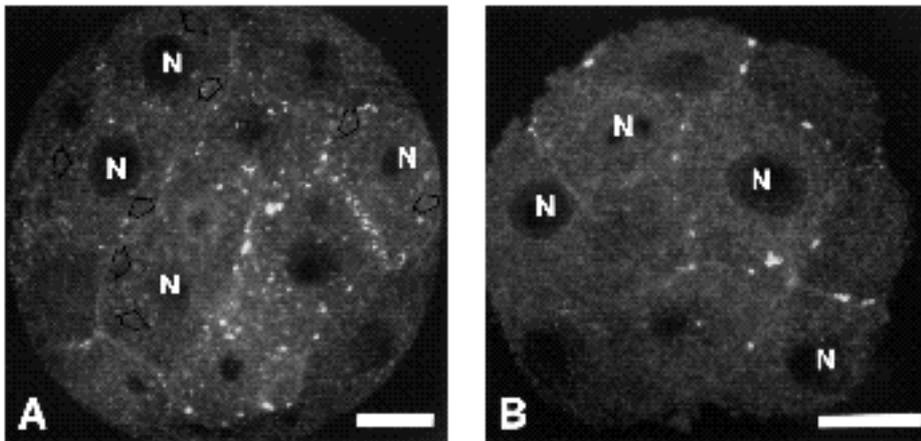


Fig. 7. Cycloheximide abolishes the Cx43-specific, diffuse cytoplasmic foci (arrows). Each image is a confocal z-series projection (1.5 μm thick) of a 16-cell morula stained with anti-302-319 following 4 hours of culture in SECM with (B) or without (A) 50 $\mu\text{g}/\text{ml}$ cycloheximide. Nuclei are indicated (N). Bars, 25 μm .

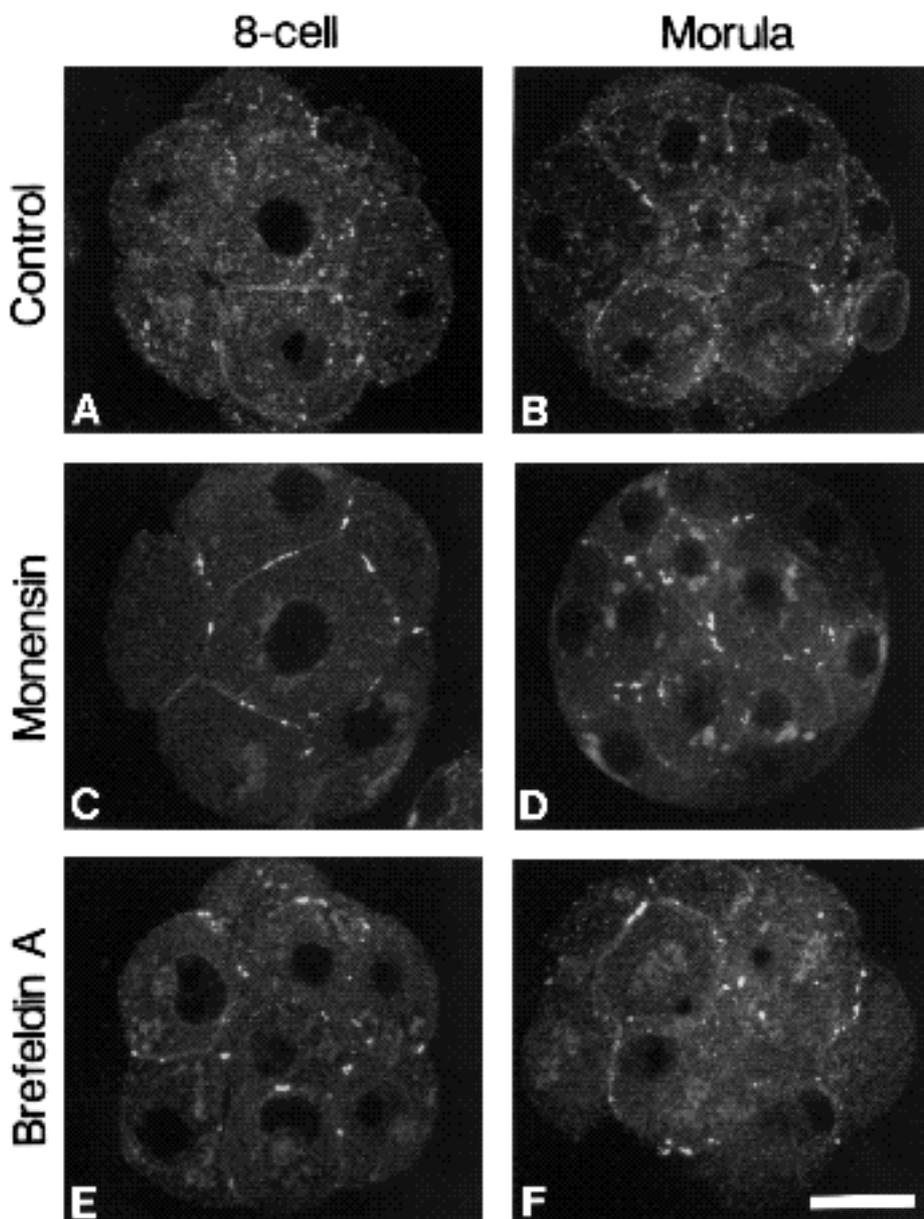


Fig. 8. Treatment for 4 hours with monensin or brefeldin-A causes redistribution of cytoplasmic Cx43 immunoreactivity in morulae. Each image is a confocal z-series projection (2 μm thick) of early (A,C,E) or late (B,D,E) morulae stained with anti-302-319 following 4 hours culture in SECM containing 0.05% ethanol (A,B), 5 $\mu\text{g}/\text{ml}$ monensin (C,D), or 5 $\mu\text{g}/\text{ml}$ BFA (E,F). Cytoplasmic immunoreactivity in ethanol-treated embryos did not differ qualitatively from untreated embryos (see Fig. 6), and consisted mainly of small diffuse foci. Monensin induced the formation of large juxtannuclear clouds while BFA caused the replacement of small diffuse foci with larger, reticulated structures. Bar, 32 μm .

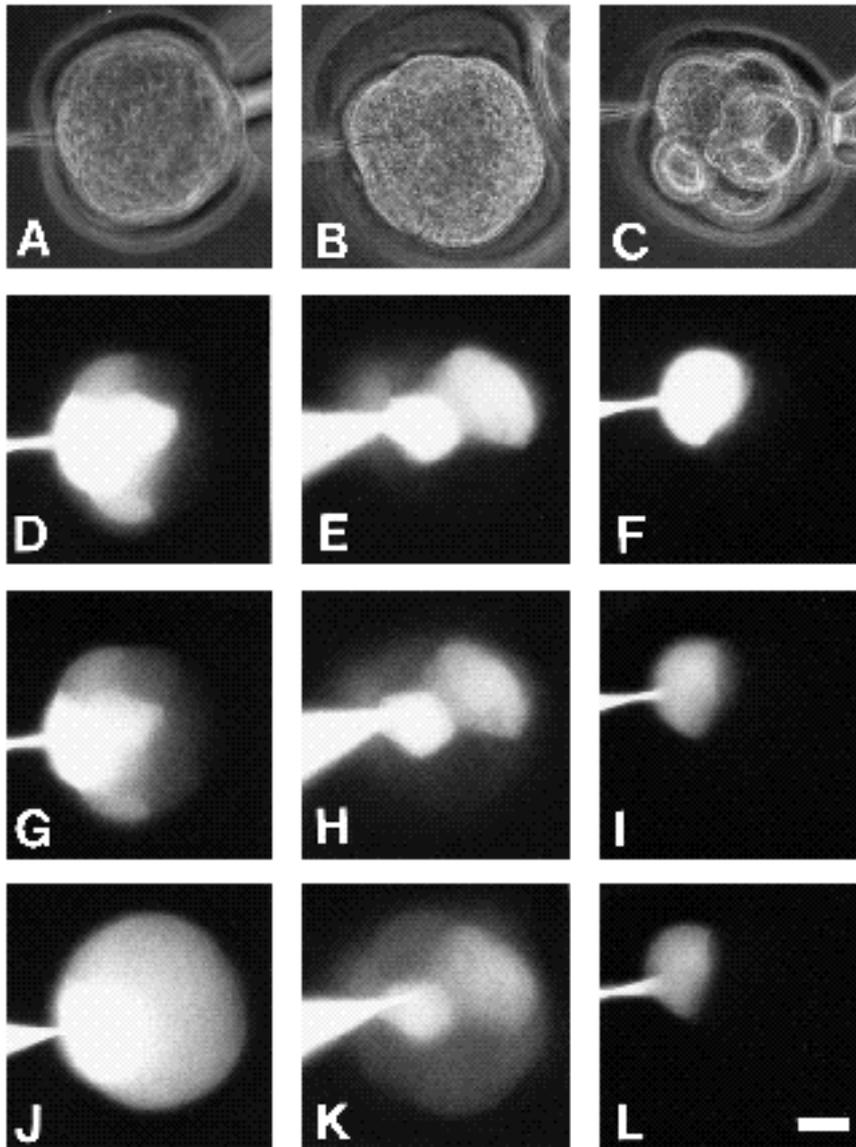


Fig. 9. Monensin or brefeldin-A interfere with the acquisition of dye coupling during compaction. Precompaction 8-cell embryos were cultured in SECM containing 0.01–0.05% ethanol (control; A,D,G,J), 5 $\mu\text{g/ml}$ monensin (B,E,H,K), or 1–5 $\mu\text{g/ml}$ BFA (C,F,I,L) for 10–18 hours before being injected with 6-carboxyfluorescein and monitored for dye transfer shown here after 1 minute (D,E,F), 5 minutes (G,H,I) and 30 minutes (J,K,L). Virtually all control embryos exhibited rapid dye transfer to all cells within 30 minutes (A,D,G,J). Greater than 50% of both monensin- and BFA-treated embryos showed either no dye transfer (C,F,I,L), or transfer to a single, adjacent blastomere. Remaining embryos exhibited either a reduced rate of dye transfer (B,E,H,K), or transfer to only a subset of cells (Table 2). Although the embryos in all treatment groups initially underwent cell flattening during culture, BFA-treated embryos later showed decompaction of one or more blastomeres. There was no correlation between the incidence of decompaction and the extent of dye coupling in these embryos. Bar, 20 μm .

dence of dye coupling and extent of coupling where it occurred (Fig. 9). Virtually all of the control embryos were coupled, with close to 75% of these embryos showing rapid dye transfer. In contrast, no dye coupling was detected in over 50% of embryos treated with either monensin or BFA. Similarly, coupling in over half of inhibitor-treated embryos classified as coupled was very slow (i.e. incomplete after 30 minutes; Table 2). Although the effects of monensin and BFA on dye coupling were virtually identical, BFA appeared to be slightly less effective with approximately twice as many coupled embryos exhibiting rapid dye transfer. Despite BFA's ability to induce decompaction of one or more blastomeres, there was no correlation between the compaction state of a treated embryo and its ability to transfer dye (data not shown). Most importantly, the inhibitory effect of monensin or BFA on the establishment of dye coupling was reversible. Non-injected embryos treated for up to 18 hours with either drug were washed free of inhibitor by passage through several drops of control culture medium and then observed for their ability to form blastocysts. Gap

junctional communication between embryonic blastomeres is an essential requirement for blastocyst development (Buehr et al., 1987). After 10 hours of recovery, only 40% of either monensin ($n=63$) or BFA ($n=25$) treated embryos had formed a blastocoel cavity, compared to 80% of controls ($n=64$). By 34 hours, however, there was virtually no difference between these groups with 96% of controls ($n=82$), 97% of monensin-treated ($n=63$), and 90% of BFA-treated embryos ($n=38$) having cavitated. Thus, although blastocyst formation was delayed, neither monensin nor BFA irreversibly damaged the ability of embryos to resume normal development.

Finally, to confirm that monensin and BFA delayed the acquisition of gap junctional communication by interfering with the *de novo* assembly of gap junctions, embryos treated with inhibitors from prior to compaction (68 hours post-hCG) until the acquisition of rapid dye coupling by controls 12 hours later, were stained for immunofluorescence with the anti-302-319 antibody. In three separate, blind experiments both monensin and BFA consistently reduced

Table 2. Treatment of uncompact 8-cell embryos with monensin or brefeldin-A reduces the frequency and limits the extent of dye coupling

Treatment†	Number Scored	% Embryos Coupled‡	% of coupled embryos			
			Rapid 10	Dye transfer time (minutes)* Moderate 10-20	Slow 20-30	Very slow §
Control	28	96	74	15	7	4
5 µg/ml Monensin	23	48	9	18	18	55
5 µg/ml BFA	22	41	22	0	22	56

*Defined as the time taken for dye to enter all the cells in the focal plane of injection, up to 30 minutes.

†Embryos were cultured from 10 to 18 hours in SECM containing either 0.01-0.05% ethanol (control) or inhibitor, prior to being injected with 6-carboxyfluorescein.

‡Defined as embryos exhibiting transfer of dye from an injected blastomere to more than one adjacent blastomere. Transfer to a single blastomere was assumed to be due to cytoplasmic bridges connecting mitotic sisters.

§Although embryos were coupled, dye had not entered all cells in the injection focal plane by the end of the 30 minute observation period.

the frequency and size of gap junction-like structures within treated embryos (Fig. 10). This effect was especially pronounced in BFA-treated embryos, which during the treatment period first compacted and then decompact as observed with morulae. Anti-302-319 did recognize cytoplasmic structures in both monensin- and BFA-treated embryos, and the staining patterns were very similar to those exhibited by compacted morulae treated with the same inhibitors (Fig. 8).

DISCUSSION

Cx43 distribution in the preimplantation embryo

In the present study, Cx43 was detected in pre- and post-compaction embryos by both western blot analysis and by laser confocal microscopy. In postcompaction embryos, Cx43 could be distinguished in apposed membranes and in the cytoplasm, the latter pattern being sensitive to cycloheximide. To our knowledge this is the first experimental evidence identifying cytoplasmic immunoreactivity for Cx43 as nascent protein. This identification is based on results with three different antibodies, each raised against a different cytoplasmic, C-terminal domain unique to Cx43: a.a. 252-271 (Valdimarsson et al., 1991), a.a. 302-319, and a.a. 360-382 (this study). In cardiac cells as well as several fibroblast cell lines, no cytoplasmic staining with anti-302-319 has been seen (B.J. Nicholson, unpublished observa-

tions) indicating that this is not a consistent property of either that antibody or most types of cell.

Our ability to detect Cx43 in 4-cell embryos by western blotting with the anti-302-319 antibody, and by immuno-

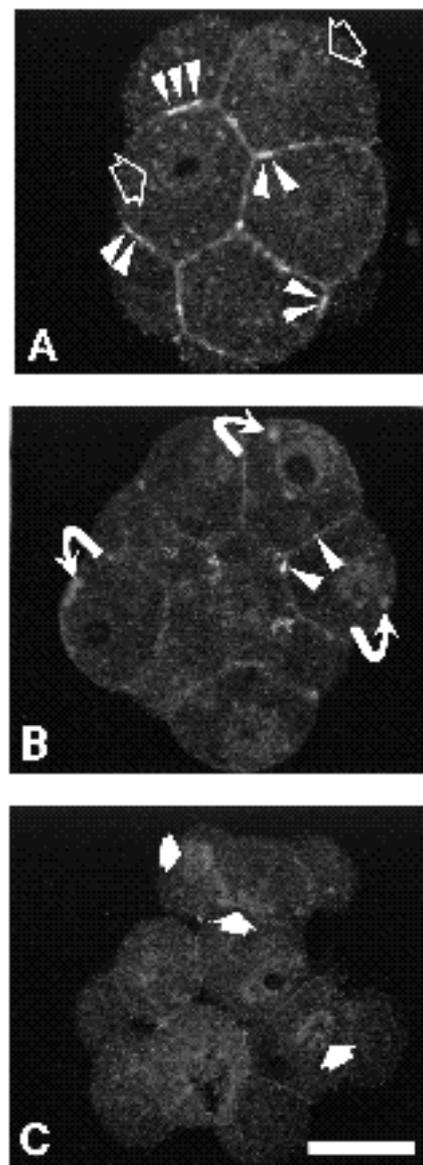


Fig. 10. Treatment with monensin or brefeldin-A beginning prior to compaction reduces and size and frequency of gap junction-like structures. Uncompact 8-cell embryos were cultured in the presence of either 0.05% ethanol (A), 5 µg/ml monensin (B), or 1 µg/ml BFA (C), as described for the dye coupling experiments. In three separate, blind experiments, monensin and BFA consistently reduced the size and frequency of gap junction-like structures (arrowheads) within most treated embryos. Effects ranged from complete abolishment of identifiable gap junctions, mostly seen in BFA-treated embryos (C), to partial abolishment wherein embryos exhibited either a uniform or regional reduction in plaque size and frequency (B). These treatments induced the same cytoplasmic staining patterns seen in postcompaction embryos treated for 4 hours. Small diffuse foci (open arrows) seen in controls (A) were augmented by large juxtannuclear clouds (curved arrows) in the presence of monensin (B), and replaced by a reticulated network of immunoreactivity (solid arrows) with BFA (C). Bar, 32 µm.

fluorescence with the anti-360-382 antibody, confirms our previous detection of Cx43 at this stage with anti-252-271 (Valdimarsson et al., 1991). It is likely that at least some of this protein is nascent as well, since translation of Cx43 mRNA begins as soon as it becomes available, at least as early as the 4-cell stage. The most reasonable explanation for our inability to detect Cx43 in precompaction embryos with the anti-302-319 antibody is that prior to compaction, this region of the molecule in its native form is not accessible to the antibody. This may reflect an incomplete state of maturation of the nascent protein. Cx43 may therefore undergo a conformational change, perhaps due to post-translational modification, correlated with the assembly of functional gap junctions in the 8-cell stage which makes the 302-319 epitope accessible. Since we were able to detect cytoplasmic Cx43 in embryos in which translocation to the plasma membrane had been blocked by monensin or brefeldin-A treatment starting in the uncompact 8-cell stage, it follows that the maturation step that renders the nascent protein accessible to anti-302-319 must occur prior to plasma membrane insertion. Preliminary results in our laboratory suggest that one such modification may involve phosphorylation. It is interesting to note that the reticulated cytoplasmic staining for Cx43 in precompaction embryos, observed with anti-252-271 and anti-360-382, is qualitatively different from the diffuse cytoplasmic foci seen after compaction using any of the three antibodies. This may reflect a difference in intracellular localization between these stages of development.

Cx43 in zonular arrays was confined to the apposed membrane regions of outer blastomeres and their descendants, the trophectoderm. This distribution is likely related to the organization of the trophectoderm as an epithelium. In the trophectoderm, gap junction plaques and adherens-type-junctions lie adjacent to and interspersed along apical *zonula occludens* (Ducibella et al., 1975; Magnuson et al., 1977). Due to the intermediate intensity of the zonular staining between the brightly staining plaque-like structures and the diffuse cytoplasmic foci, it seems likely that this pattern represents an intermediate distribution of either connexins or connexons in apposed membrane regions but outside of plaque-like aggregations. Although the ICM has also been reported to contain both adherens and occludens-type junctions, zonular arrays of the latter are confined to the trophectoderm (Magnuson et al., 1977). Connexins or connexons outside of plaques may serve as a reservoir of subunits for an expansion of gap junctions later in development.

Cx43 trafficking and the onset of gap junction formation

In morulae, treatment with BFA or monensin was found to redistribute nascent Cx43 in the cytoplasm. These treatments had little effect on surface labeling (either zonular or punctate), presumably because the half-life of Cx43 in plasma membranes is longer than the 4 hours treatment period used. Although both agents have been shown to have multiple species-specific effects on various aspects of intracellular protein trafficking, they are well-accepted inhibitors of Golgi apparatus function. Monensin is a Na⁺ ionophore capable of collapsing Na⁺ and H⁺ gradients. One conse-

quence of this is the neutralization of acidic intracellular compartments, such as the cisternae of the TGN and its associated elements (lysosomes and acidic endosomes). This neutralization is accompanied by loss of function, and in the case of the TGN, swelling of its cisternae (reviewed by Mollenhauer et al., 1990). Although BFA has also recently been shown to effect transcytosis of endosomes and the TGN (Wood et al., 1991; Hunziker et al., 1991; Lippincott-Schwartz et al., 1991), it is most noted for its ability to inhibit anterograde membrane traffic out of the endoplasmic reticulum (ER) and enhance retrograde movement of Golgi membrane back to the ER, resulting in collapse of the Golgi apparatus (reviewed by Klausner et al., 1992). In postcompaction embryos, the two agents had qualitatively different effects on the distribution of nascent Cx43 in the cytoplasm, suggesting each has a unique site of action. BFA, but not monensin, abolished the small, diffuse cytoplasmic foci, possibly representative of Cx43 in trafficking vesicles or in Golgi. In their place appeared reticular staining, possibly indicative of Cx43 being concentrated in the ER. Although monensin may have had multiple sites of action, the large juxtannuclear clouds observed following treatment with this inhibitor are consistent with accumulation of Cx43 downstream of the Golgi, such as within swollen TGN cisternae. The localization of Cx43 in intracellular trafficking organelles was recently substantiated by Hendrix et al. (1992), who demonstrated its colocalization with a Golgi-specific antigen in rat myometrium during labor.

Treatment of precompaction embryos with monensin or BFA interfered with the onset of dye coupling and the formation of gap junction-like structures during compaction. These results suggest that gap junction channels assembled at that time are dependent on one or more intracellular protein trafficking steps. Although translation of Cx43 mRNA begins by the 4-cell stage, at least 12 hours in advance of when gap junctions first form, it has not been possible to detect Cx43 in plasma membranes prior to compaction using antibodies raised against four different C-terminal domains (Valdimarsson et al., 1991; Nishi et al., 1991; this study). An additional piece of evidence that points to the absence of connexins from the plasma membranes of precompaction embryos is the finding that 4-cell embryos or their isolated blastomeres are unable to form gap junctions when paired with each other or with communication-competent 8-cell embryos (Goodall and Johnson, 1982; McLachlin et al., 1983). Thus, it is likely that treatment of embryos with either monensin or BFA prior to compaction interfered with the establishment of coupling by interrupting the delivery of nascent Cx43 to the cell surface. We conclude that the regulated step in the onset of gap junction assembly is downstream of transcription and translation, and involves mobilization of connexins into plasma membranes immediately prior to or during compaction. This is in contrast to the situation in cultured somatic cells, where the regulated step in gap junction assembly appears to be the formation of plaques after nascent connexin has arrived at the plasma membrane (Musil and Goodenough, 1991). Current investigation is directed at using immunoelectron microscopy to define more precisely the subcellular location of nascent Cx43 prior to compaction.

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