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Normal development of preimplantation mouse embryos deficient in gap junctional coupling

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SUMMARY

The connexin multigene family (13 characterized members in rodents) encodes the subunits of gap junction channels. Gap junctional intercellular coupling, established during compaction of the preimplantation mouse embryo, is assumed to be necessary for development of the blastocyst. One member of the connexin family, connexin43, has been shown to contribute to the gap junctions that form during compaction, yet embryos homozygous for a connexin43 null mutation develop normally, at least until implantation. We show that this can be explained by contributions from one or more additional connexin genes that are normally expressed along with connexin43 in preimplantation development. Immunogold electron microscopy confirmed that roughly 30% of gap junctions in compacted morulae contain little or no connexin43 and therefore are likely to be composed of another connexin(s). Confocal immunofluorescence microscopy was then used to demonstrate that connexin45 is also assembled into membrane plaques, beginning at the time of compaction. Correspondingly, embryos homozygous for the connexin43 null mutation were found to retain the capacity for cell-to-cell transfer of fluorescent dye (dye coupling), but at a severely reduced level and with altered permeability characteristics. Whereas mutant morulae showed no evidence of dye coupling when tested with 6-carboxyfluorescein, dye coupling could be demonstrated using 2′,7′-dichlorofluorescein, revealing permeability characteristics previously established for connexin45 channels. We conclude that preimplantation development in the mouse can proceed normally even though both the extent and nature of gap junctional coupling have been perturbed. Despite the distinctive properties of connexin43 channels, their role in preimplantation development can be fulfilled by one or more other types of gap junction channels.

Key words: Gap junction, Connexin43, Connexin45, Preimplantation development, Mouse, Intercellular coupling, Immunogold labeling, Confocal microscopy, Gene targeting

INTRODUCTION

Gap junctional coupling is established very early in mouse development, during compaction in the 8-cell stage (reviewed by Kidder, 1987). Once gap junctions have formed, the embryo remains coupled throughout until ‘communication compartments’, areas of spatially restricted coupling, begin to appear around the time of implantation (Kalimi and Lo, 1988, 1989). Experimental approaches aimed at disrupting the synthesis of gap junction proteins or gap junctional coupling in the preimplantation embryo have provided evidence that coupling is essential for blastocyst development (Lee et al., 1987; Bevilacqua et al., 1989; Becker et al., 1995).

A gap junction is an array of paired hemichannels called connexons (reviewed by Bruzzone et al., 1996). The pairing of connexons from closely apposed plasma membranes in end-to-end alignment creates intercellular channels through which small molecules, including second messengers, can pass from cell to cell. Each connexon is a cylinder constructed of multiple subunits called connexins, encoded in mammals by a large multigene family. 13 connexins have been cloned and characterized thus far from rodent cDNA or genomic libraries (Bruzzone et al., 1996; Dahl et al., 1996). Because the permeability, biophysical and regulatory properties of gap junction channels composed of different connexins differ, it has been proposed that each type of channel may play a unique role in cellular regulation (Elfgang et al., 1995). We have begun to test this hypothesis using gene targeting technology.

Previous work from one of our laboratories had identified one member of the connexin family, connexin43 (Cx43), as contributing to the gap junctions that form during compaction in the mouse (Barron et al., 1989; Valdimarsson et al., 1991).

The Cx43 gene is active from the onset of embryonic transcription in the 2-cell stage, but nascent Cx43 remains in an intracellular location in a state of suspended trafficking until an as yet unknown signal, linked to the developmental 'clock', triggers its assembly into gap junctions (De Sousa et al., 1993; Valdimarsson and Kidder, 1995). The precise temporal control of this event, together with its association with a critical morphogenetic process such as compaction, imply that Cx43 plays an important role in preimplantation development. However, this assumption was challenged when it became clear that embryos deficient in Cx43 as a result of gene targeting develop normally through the preimplantation period and go on to establish full-term pregnancies (Reaume et al., 1995).

Our recent experiments have been directed towards understanding the lack of any preimplantation effects of the Cx43 null mutation. Using RT-PCR, we identified transcripts of five additional connexin genes (encoding Cx30.3, 31, 31.1, 40 and 45) in preimplantation embryos (Davies et al., 1996). This raised the possibility that embryos homozygous for the Cx43 null mutation might utilize these other connexins to retain a level of intercellular coupling sufficient to support development. In the present report, we show that Cx43-deficient embryos do indeed remain coupled, but at a severely reduced level. The channels remaining in the absence of Cx43 differ in their permeability properties from those of wild-type embryos. Thus preimplantation development can proceed normally even though both the extent and nature of gap junctional coupling have been perturbed.

MATERIALS AND METHODS

Embryo collection

Embryos to be used for immunocytochemistry were flushed from the reproductive tract of CF1 females that had been superovulated and mated with CB6F$_1$/J males as described previously (De Sousa et al., 1993). After rinsing with phosphate-buffered saline containing 0.3% polyvinylpyrrolidone (PBS-PVP), the embryos were transferred into the appropriate fixation solution. Embryos lacking Cx43 were generated by mating males and females (C57BL/129×CD1) that were heterozygous for a targeted mutation in the gap junction protein gene *Gja1* (ES24 line; Reaume et al., 1995). In this case, the females were not superovulated. Pregnant females were killed 2 days after detection of a vaginal plug and the embryos flushed from their reproductive tracts using flushing medium 1 (Spindle, 1980). Compacted morulae were selected and left in the flushing medium for the dye-coupling experiments. Afterwards, the embryos were individually rinsed five times in PBS-PVP prior to genotyping (see below).

Immunogold electron microscopy

Unless noted otherwise, all steps were performed at room temperature using filtered solutions, with constant rotation. Compacted morulae (74-76 hours post-human chorionic gonadotropin injection) and cumulus-oocyte-complexes (COCs), isolated from the antral follicles of superovulated females 34 hours post-hCG, were fixed for 1 hour in 1.6% glutaraldehyde in fix buffer (0.1 M phosphate buffer, pH 7.3, containing 2% w/v sucrose). Fix was removed with three 10 minute changes of fix buffer, after which the specimens were pre-embedded in molten 2% agar, which was then trimmed to a block size of 1-2 mm^3. The specimens were post-fixed for 15 minutes with 1% OsO_4 in fix buffer. Following passage through three more 10 minute changes of fix buffer, the specimens were washed through four changes of sterile double distilled water (ddH_2O) and stained with saturated aqueous (5%) uranyl acetate for 2.5 hours, which was then removed with three 20 minute changes of ddH_2O. The specimens were dehydrated by 10 minute exposures to an ascending series of 50%, 70% and 95% ethanol. They were infiltrated first for 1 hour in 50% LR White resin (Polysciences Inc., Warrington, PA) in absolute ethanol, and next for 14 hours in LR White. Infiltrations were performed in light-tight and air-tight glass vials. The specimens were embedded in blocks of fresh LR White resin polymerized at 60°C for 24 hours in gelatin capsules (size 00, Polysciences). Between 5 and 15 specimens (embryos or COCs) were embedded in each block. Data on morula gap junctions were collected from four different blocks and data on cumulus-cumulus gap junctions from three blocks, whereas two blocks were used for preimmune controls.

Silver and silver/gold sections (600-1,000 Å) were collected on hexagonal mesh nickel grids. Prior to immunostaining, sections were etched on one side to expose antigenic sites by treatment at 37°C for 1 hour with saturated aqueous sodium metaperiodate. Once etched, grids were washed with ddH_2O, blotted dry with filter paper, and blocked for 30 minutes with 3% bovine serum albumin in Ca^{2+}- and Mg^{2+}-free PBS (CMF-PBS) before treatment for 3 hours with primary antibody in CMF-PBS. Cx43 was immunolocalized using an affinity-purified rabbit polyclonal antibody raised against a synthetic peptide corresponding to the carboxy terminus of Cx43 (A.A. 360-382). This antibody (CT360) and the preimmune serum were generously provided by Dr Dale Laird (Department of Anatomy and Cell Biology, The University of Western Ontario), and were used at a dilution of 1:500. Specificity of the CT360 antibody had previously been confirmed by immunoblotting, immunoelectron microscopy and immunofluorescence (Laird and Revel, 1990; De Sousa et al., 1993).

After immunostaining, grids were washed by passage through at least four changes of CMF-PBS in microdrops in moisture chambers placed onto magnetic stirrers set at low speed. Primary antibody was recognised by a goat anti-rabbit IgG, conjugated to 10 nm gold (Cedarlane Laboratories Ltd., Hornby, Ontario), used at a dilution of 1:20 to 1:35. This secondary antibody treatment, also in CMF-PBS, was normally for 90 minutes and was followed by renewed washing as described following primary antibody treatment. Lastly, grids were rinsed in ddH_2O for up to 1 minute before being dried on filter paper. Grids were counterstained by treatment for 1 hour with saturated aqueous uranyl acetate at 37°C, followed by washing with ddH_2O, and 10 minutes with lead citrate in a CO_2-free environment created in a Petri dish by the placement of 2-3 potassium hydroxide pellets. Lead staining was removed by washing with freshly boiled (CO_2-free) ddH_2O. Lead citrate stain was prepared by dissolving approximately 35 mg of lead citrate in 10 ml of freshly boiled ddH_2O, to which 3 drops of 10 N NaOH were added to dissolve the lead. Undissolved lead was pelleted by centrifugation at 2,000 rpm before use. Sections were viewed with a Phillips CM10 transmission electron microscope at 60 KV.

Confocal immunofluorescence microscopy

Embryos were processed for wholemount immunofluorescence microscopy according to the procedure described by Valdimarsson et al. (1991) and De Sousa et al. (1993), except that the embryos were blocked with 150 mM glycine in PBS-Tween-20 for 40 minutes after the permeabilization step. The affinity-purified rabbit primary antibody was raised against a synthetic peptide corresponding to amino acids 285-298 of the C-terminal segment of Cx45. The primary antibody and corresponding preimmune serum were supplied by Dr Eric Beyer (Division of Pediatric Hematology, Washington University) and used at a dilution of 1:500. Specificity of the antibody for mammalian Cx45 has been demonstrated by immunofluorescence, immunoprecipitation and immmunoblotting (Kanter et al., 1992; Laing et al., 1994). The secondary antibody was fluorescein-conjugated goat anti-rabbit IgG (ICN Pharmaceuticals Canada Ltd., Montreal), used at a dilution of 1:50.

Dye coupling assays

10 mM solutions of 6-carboxyfluorescein (molecular mass 376 Da; Eastman Kodak, Rochester, NY) and 2′,7′-dichlorofluorescein (molecular mass 401 Da; Sigma Chemical Co., St Louis, MO) were made up in distilled water, adjusted to pH 7 with 1 N NaOH, and filtered through 0.45 µm filters. Glass micropipettes (Kwikfill capillary tubing 1B100F; W-P Instruments, Sarasota, FL) were pulled on a vertical electrode puller (Narashige Scientific, Tokyo), back-filled with dye by capillary action and mounted on a deFonbrune micromanipulator (CH Beaudouin, Paris) as described in Safranyos and Caveney (1985). Embryos were placed singly into a plastic bath chamber constructed with a glass coverslip base, grounded through a Ag/AgCl electrode and a KCL/agar bridge immersed in the medium. Electrode tip resistance was typically less than 500 MΩ. Dye was iontophoretically injected into a single blastomere in each embryo for 1-2 minutes using a continuous train of hyperpolarizing current (1-6 nA; 200 milliseconds duration at 1 Hz). The spread of dye fluorescence from the injected blastomere to its neighbours was detected using a Zeiss Axiovert 35 inverted phase-fluorescence microscope equipped with a mercury vapor lamp (HBO 50) and a standard fluorescein fluorescence filter set (excitation filter BP485, dichroic mirror FT510 and barrier filter BP520). Video fluorescence images were recorded with a silicon-intensified target (SIT 66) camera (Dage-MTI, Michigan City, IN) and a Sony (TVO-9000) 3/4 second time-lapse videocassette recorder. Dye bleaching and cytotoxicity during the 3-4 minute period of dye spread was reduced both by exciting the injected dye and viewing its spread in the embryo for less than 5 seconds during each 30 second interval following the start of dye injection, and by placing a 5% transmittance neutral density (ND) filter in the excitation light path. 3 minutes after dye injection began, the ND filter was removed from the excitation light path and the full extent of coupling between the blastomeres of the embryo was scored. In order to avoid mistaking dye transfer via intercellular bridges for gap junctional coupling (Kidder et al., 1988), only those embryos in which dye was passed to more than one blastomere were scored as coupled.

Embryo genotyping

After being tested for dye coupling, morulae were genotyped by PCR. We used a strategy based on the fact that the Cx43 allele mutated by gene targeting retains a portion of the C-terminal coding region (Reaume et al., 1995). To detect the normal allele, the upstream Cx43 primer was 5′-CCCCACTCTCACCTATGTCTCC-3′ and the downstream Cx43 primer was 5′-ACTTTTGCCGCCTAGCTATCCC-3′; the resulting amplicon is approximately 0.5 kb in length. To detect the targeted allele we used the same downstream primer in conjunction with an upstream primer (5′-GCTTGCCGAATATCATGGTGGA-3′), specific for the neo^R sequence in the targeting vector, to produce an amplicon that is approximately 1 kb in length. The two PCR reactions were performed separately.

Each individual morula was digested at 56°C overnight in 10 µl of a solution containing proteinase K (GIBCO-BRL). This solution was prepared by adding 4 µl of proteinase K stock solution (20 mg/ml) to 96 µl proteinase K buffer (10 mM Tris at pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, 0.45% Tween-20). 2 µl of digested embryo solution served as template for each PCR reaction; this was combined with 5.1 µl Milli-Q water, placed in a PCR reaction tube, heated to 95°C for 15 minutes (to inactivate the proteinase K) and then held at 85°C while the remaining reaction ingredients were added (‘hot start’). The final reaction mixture contained the heat-treated embryo digest plus 0.2 µl of 10 mM dNTPs (Pharmacia), 1 µl of 15 mM $MgCl_2$, 0.3 µl of each primer stock solution (10 µM), 0.1 µl of *Taq* polymerase (5 U/µl; GIBCO-BRL) and 1 µl of 10×PCR buffer (GIBCO-BRL) for a total reaction volume of 10 µl. PCR was carried out for a total of 40 cycles using a ‘touch down’ protocol. Each cycle began with 1 minute denaturation at 94°C followed by 1 minute primer annealing; for the first 10 cycles the annealing temperature was 65°C, but this was decreased by 1°C every other cycle thereafter until the annealing temperature had been reduced to 58°C, which was maintained for the remaining 18 cycles. Primer extension at 72°C was for 4 minutes initially but was increased by 3 seconds in each succeeding cycle. The samples were held at 72°C for 10 minutes at the end of the PCR program. The amplicons were visualized on 1.5% agarose gels.

An alternative protocol was used to detect the targeted allele for genotyping embryos after immunofluorescence assessment of Cx45 expression. After proteinase K digestion, 6.5 µl of each embryo lysate was added to 3.5 µl of master mixture consisting of 2.0 µl water, 0.5 µl of 10 mM dNTPs and 0.5 µl of each primer stock solution (10 µM). After inactivation of the proteinase K, this reaction mixture was combined at 80°C with 2.5 µl each of buffers A and B supplied with the ELONGASE™ enzyme kit (GIBCO-BRL), 9.5 µl water and 0.5 µl ELONGASE™ enzyme. PCR was carried out as described above, except that amplification was for 50 cycles for both alleles.

RESULTS

Cx43 null homozygotes develop normally through the preimplantation period

Visual inspection of embryos from heterozygote crosses developing in vitro failed to give any indication of developmental abnormalities (results not shown). To confirm the presence of homozygotes in those populations, we genotyped 85 morulae and 44 blastocysts. As expected from our earlier genotypic analysis of late gestation fetuses (Reaume et al., 1995), we found a 1:2:1 ratio of genotypes, indicating that there is no loss of homozygotes between the morula and blastocyst stages when gap junctional coupling becomes established. We therefore sought to determine whether additional members of the connexin family might also contribute to the gap junctions that form during compaction, thus explaining the lack of any effects of the Cx43 null mutation.

Some morula gap junctions contain little or no Cx43

As a test of the ability of the CT360 antibody to label Cx43-containing gap junctions at the electron microscope level, we prepared cumulus-oocyte complexes (COCs) for immunogold labeling (Cx43 is highly expressed in cumulus cells and localizes to gap junctional plaques connecting them; Beyer et al., 1989; Risek et al., 1990; Valdimarsson et al., 1993). So as not to bias interpretation of the specificity of anti-Cx43 labeling as opposed to that seen with preimmune serum, unequivocal gap junctions were classified as labeled if they possessed at least one 10 nm gold particle within 10 nm of their structure. Only about 10% of cumulus-cumulus (C-C) or morula gap junctions treated with preimmune serum were ‘labeled’, and this labeling tended to consist of a single gold particle. The CT360 antibody, however, labeled 100% of C-C gap junctions, and this labeling was much more extensive (Fig. 1, Table 1). In contrast, only 70% of gap junctions seen in morulae were labeled with this antibody using identical methods of fixation and preparation (Table 1). Unlabeled and labeled gap junctions were frequently found together in the same embryo (Fig. 1C,D), as well as side by side along the same apposed membrane region.

Although C-C gap junctions labeled with CT360 appeared to have more gold particles associated with them than did morula gap junctions, this difference could be attributed to the

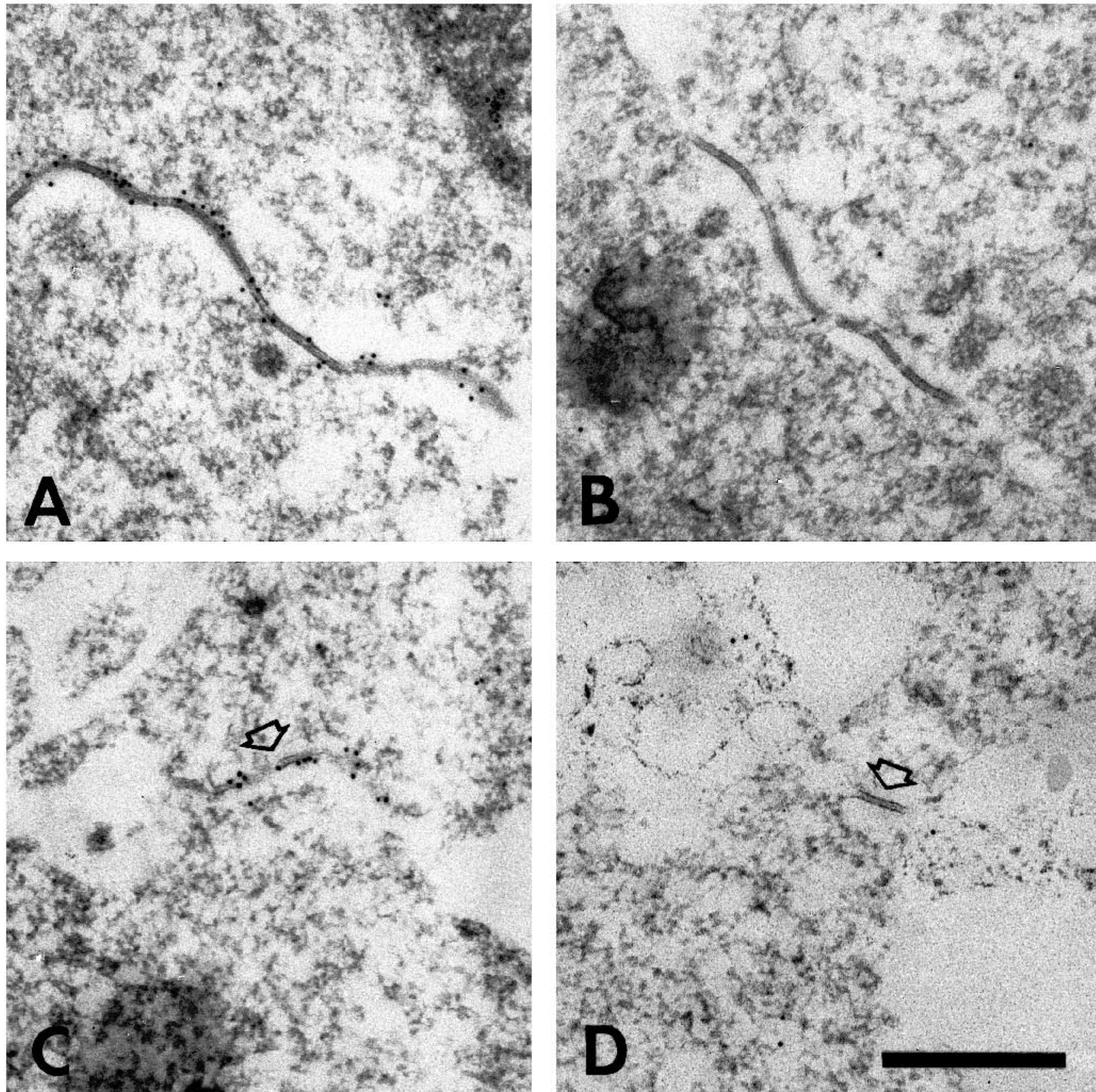

Fig. 1. Distribution of Cx43 in gap junctions between cumulus cells and in morulae. Immunostaining of sections with CT360 antibody resulted in labeling of 100% of gap junctions between cumulus cells (A), whereas both labeled (C) and unlabeled (D) gap junctions (arrows) were evident in morulae. Little, if any, labeling of gap junctions between cumulus cells (B) and in morulae (not shown) was observed with preimmune serum. Bar, 400 nm.

larger size of C-C gap junctions. There was no significant difference in the labeling density of junctions, defined as the number of gold particles per µm of labeled gap junction (Table 1). Thus, although there is a lower proportion of Cx43-containing gap junctions in morulae than in cumulus cells, there appears to be no difference in the density of Cx43 in those gap junctions that contain this protein. We conclude that roughly one third of morula gap junctions contain little if any Cx43 and must incorporate one or more of the other co-expressed connexins.

Cx45 is assembled into membrane plaques during compaction

Wholemount immunofluorescence microscopy was used to explore the possibility that Cx45, which is coexpressed with Cx43 in other cell types (Kanter et al., 1992), participates in gap junction assembly during compaction. Small foci of Cx45 immunoreactivity were seen with the confocal microscope in embryos that were undergoing or had completed compaction (Fig. 2A,B), but not in precompaction embryos (Fig. 2C). These foci were infrequent (very few were visible in any one optical section), restricted to regions of blastomere apposition, and variable in number. No such immunoreactivity was evident with the preimmune serum (Fig. 2D). We conclude that at least a few gap junctions in compacted morulae normally contain Cx45. To determine whether this conclusion is also valid for embryos lacking Cx43, 36 morulae derived from a heterozygote cross were immunolabeled with the Cx45 antibody,

Table 1. Immunogold labeling of Cx43 in gap junctions of mouse cumulus cells and compacted morulae

Gap junction type	Treatment	% Gap junctions labeled	No. gold particles per labeled junction*	No. gold particles per micron of junction*,†
Cumulus	CT360	100 (47)‡	31.3±17.5	32.4±8.9 (12)‡
	Preimmune	12 (57)	1.6±1.5	
Morula	CT360	70 (20)	4.5±2.1	34.5±13.3 (13)
	Preimmune	11 (9)	1.0	

*Mean ± standard deviation

†As determined from micrographs of labeled gap junctions, where the number of gold particles within 10 nm of a junction was determined and divided by the length of the junction. The difference between gold particle densities in cumulus and morula gap junctions is not significant (P>0.05, Student's t-test).

‡Numbers in parentheses indicate the number of gap junctions examined.

examined for the presence of Cx45, and then genotyped (Fig. 3). Ten of these embryos were homozygous for the targeted allele, seven were wild type and the remainder were heterozygotes. Whereas the number of Cx45-containing gap junction-like foci in this population also varied from one embryo to the next, this number was not correlated with Cx43 genotype.

Morulae that are homozygous for the Cx43 null mutation retain a severely reduced level of dye coupling

The existence of morula gap junctions containing Cx45 raises the possibility that intercellular coupling is maintained in Cx43 null homozygotes. We tested this by assaying for dye coupling in individual morulae from heterozygote crosses; after scoring for passage of the dye from the injected blastomere to more than one additional blastomere, each morula was processed for PCR genotyping (Fig. 3). Using 6-carboxyfluorescein as the permeant molecule, it was very clear that null homozygotes are, in fact, deficient in gap junctional coupling (Table 2). Of the 37 morulae tested, 10 failed to show any evidence of intercellular dye transfer and all of these were determined retrospectively to be homozygotes. There was variation in the strength of coupling (i.e. the rate of dye transfer) among the positives, but we could not distinguish between wild-type morulae and heterozygotes on that basis. Thus gap junctional coupling is severely impaired in morulae lacking Cx43.

Functional expression studies of individual connexin genes have revealed that gap junction channels are selectively and differentially permeable to various dyes (Elfgang et al., 1995).

Table 2. Dye coupling among morulae from heterozygote crosses tested with 6-carboxyfluorescein or 2′,7′-dichlorofluorescein

Morula genotype	Carboxyfluorescein		Dichlorofluorescein	
	Coupled	Not coupled	Coupled	Not coupled
+/+	13	0	4	0
+/–	14	0	18	2
–/–	0	10	5	6

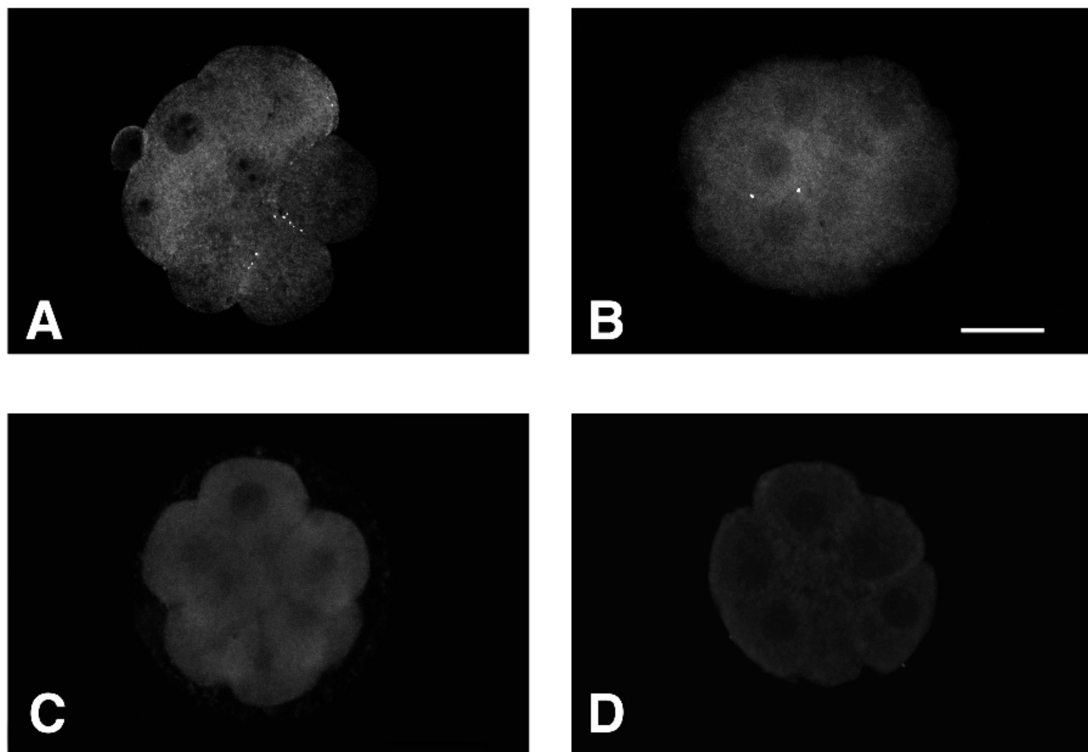

Fig. 2. Demonstration of Cx45-containing membrane plaques in morulae by confocal immunofluorescence microscopy. The affinity-purified anti-peptide antibody revealed small, infrequent foci of immunoreactivity in apposed membrane regions of compacted 8-cell embryos (A,B). Such immunoreactive foci were never seen in precompaction embryos (C). The preimmune serum did not reveal any specific immunoreactivity (D). The images are z-series projections of thickness: A=2 μm, B=3 μm, C=3 μm and D=6 μm. The image in D is overexposed relative to the others in order to make it more visible. Bar, 25 μm.

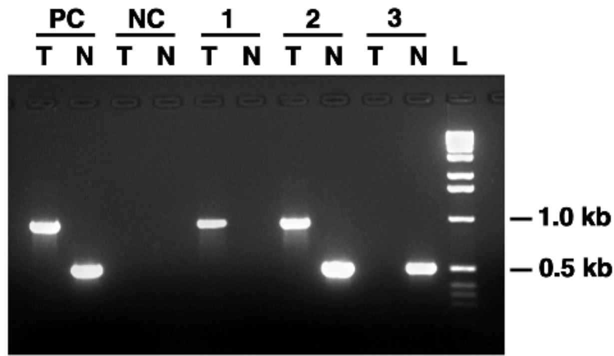

Fig. 3. Determination of individual embryo genotypes by PCR. DNA in embryo lysates was amplified in separate reactions using primer pairs that generate either a 1 kb amplicon from the targeted allele (T) or a 0.5 kb amplicon from the normal allele (N). Embryos 1, 2 and 3 represent –/–, +/– and +/+ genotypes, respectively. Lanes marked PC are the positive control (PCR using DNA from a known heterozygote); lanes marked NC are the negative control (PCR in the absence of embryo lysate). L, 1 kb DNA ladder.

In particular, rodent and avian Cx45 channels transmit anionic dyes such as Lucifer yellow or 6-carboxyfluorescein less well than they transmit the less polar dye, 2′,7′-dichlorofluorescein (Steinberg et al., 1994; Veenstra et al., 1994, 1995). We took advantage of this 'permeability phenotype' to assess the contribution of Cx45 channels to intercellular coupling in compacted morulae lacking Cx43. When morulae were injected with 2′,7′-dichlorofluorescein, five of the 11 homozygotes tested were dye-coupled, as were all but two of the 24 morulae that carried at least one normal Cx43 allele (Fig. 4 and Table 2). The failure of roughly half of the homozygotes to transmit a detectable amount of 2′,7′-dichlorofluorescein likely reflects a reduction in the total number of gap junctions due to the absence of Cx43. These results demonstrate that preimplantation development in the mouse can proceed normally despite a severe reduction in gap junctional coupling, and indicate that other channels, including those with permeability properties resembling Cx45, contribute to morula gap junctions in the absence of Cx43.

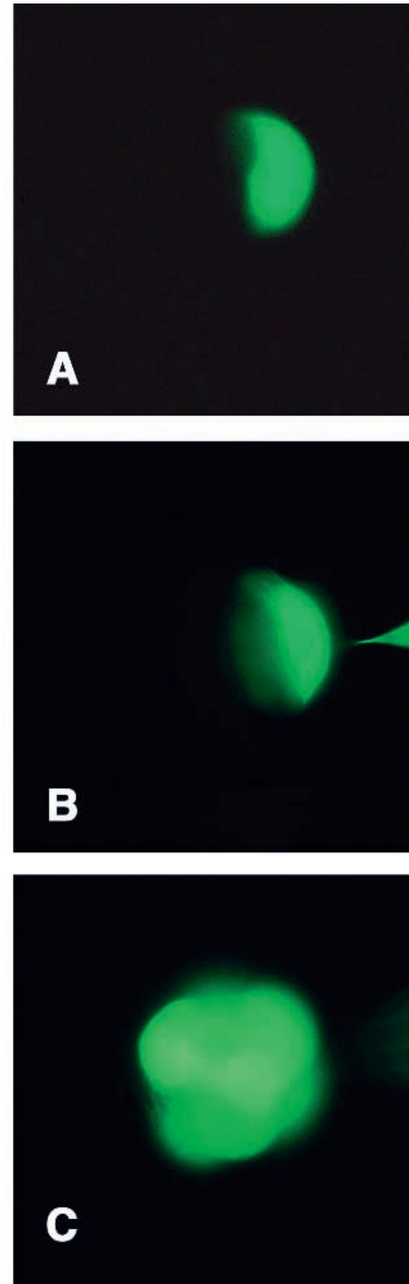

Fig. 4. Test for dye coupling in morulae using 2′,7′-dichlorofluorescein. A compacted embryo was scored as not coupled if the dye was retained within the injected blastomere (A) or passed to a single adjacent blastomere (not shown). An embryo was scored as coupled if the injected dye was transferred to more than one additional blastomere (B) or to all blastomeres (C). Embryos A and B are homozygous for the Cx43 null allele; embryo C is a heterozygote. The scale is the same as that for Fig. 2.

DISCUSSION

The great diversity of biophysical, permeability and regulatory properties displayed by gap junction channels composed of different connexins has led to speculation that the various members of this gene family may play functionally diverse roles in development or cellular regulation (Elfgang et al., 1995). The cardiac dysmorphogenesis that accompanies a Cx43 null mutation (Reaume et al., 1995) reflects an indispensable and perhaps unique role in that context. In the case of preimplantation development, however, Cx43 is clearly not indispensable, since its loss by mutation did not result in the absence of intercellular coupling or in developmental abnormalities.

Immunogold electron microscopy revealed that approximately one third of the gap junctions in communication-competent morulae do not contain immunologically detectable Cx43. While it is possible that the C-terminal epitope of Cx43 that is recognized by the CT-360 antibody may be masked or absent in some connexons, it is more likely that failure of Cx43 immunostaining results from its being replaced by another connexin in those junctions. If this is the case, then it is not surprising that embryos deficient in Cx43 remain coupled, albeit at a greatly reduced level. That reduced level is sufficient for preimplantation development to proceed normally, indicat-

ing that the embryo maintains excess capacity for the intercellular transfer of metabolites and second messengers. Furthermore, the permeability properties of the gap junctions that remain after removal of Cx43 are very different from those of normal morulae, as indicated by their failure to transmit 6-carboxyfluorescein. The relationship between the dye permeability properties of a particular type of gap junction channel and its capacity to transmit biologically important molecules is not clear at present. What is clear is that, in the context of compaction and blastocyst development, any distinctive permeability properties provided by Cx43 junctions are unimportant.

At least five additional members of the connexin gene family are expressed along with Cx43 in preimplantation mouse development (Davies et al., 1996). Among these, connexins 30.3, 31, 40 and 45 could potentially contribute to gap junctional coupling in Cx43-deficient morulae because all have been shown to form functional intercellular channels when expressed in HeLa cells and/or paired *Xenopus* oocytes (Elfgang et al., 1995; White et al., 1995). Cx31.1, on the other hand, does not form intercellular channels in the oocyte expression system (White et al., 1995). Cx40 is also in doubt as a contributor because we have not been able to detect it in morula gap junctions using three different antibodies (Davies et al., 1996). Furthermore, Cx40 channels are only slightly permeable to 6-carboxyfluorescein and even less so to 2′,7′-dichlorofluorescein (Beblo et al., 1995). Both Cx31 (Dahl et al., 1996; Grümmer et al., 1996) and Cx45 (this report) have been localized in putative gap junction plaques in postcompaction embryos. In blastocysts, Cx31 and Cx43 co-localize in the same membrane plaques (Grümmer et al., 1996), raising the possibility that the absence of Cx43 in null mutant embryos might affect the assembly of Cx31 into functional gap junctions, thus reducing even further the diversity of channel types. The fact that Cx43-deficient blastomeres can transmit 2′,7′-dichlorofluorescein but not 6-carboxyfluorescein does imply a preponderance of functional Cx45 channels. Another explanation for the dye selectivity of the channels remaining in null mutant embryos might be that Cx45 co-assembles with other connexins present to form heterotypic or heteromeric gap junctions, whose permeability properties resemble those of Cx45 channels (the ability of Cx45 to alter the permeability of Cx43 channels has been demonstrated using transfected cells; Koval et al., 1995). On the other hand, it is not yet certain that the ‘dye coupling phenotype’ characteristic of Cx45-expressing cells is unique to this connexin. For the present, therefore, we must leave open the possibility that several different types of gap junction channels (Cx30.3 and Cx31 in addition to Cx45) contribute to the reduced level of coupling in Cx43 null homozygotes and are collectively able to compensate for the absence of Cx43.

Previously, other investigators had attempted to disrupt gap junctional coupling in preimplantation mouse embryos by means of connexin antibodies (Lee et al., 1987; Becker et al., 1995) or antisense RNA (Bevilacqua et al., 1989). In each of these experiments, the injected agent blocked intercellular dye (Lucifer yellow) transfer, with the result that affected blastomeres underwent ‘decompaction’. Thus it has been concluded that the maintenance of compaction and, as a consequence, blastocyst development depends on gap junctional coupling. Yet when dye coupling was severely reduced in our experiments by targeted disruption of the Cx43 gene, we could observe no effects on either compaction or blastocyst formation. One explanation for this discrepancy might be that in two of the above-cited reports, the agent used for disrupting gap junctional coupling was able to interfere with the full range of connexins present and thus produce a more complete blockade. Bevilacqua et al. (1989) injected Cx32 antisense RNA which, because of the extensive sequence similarity among the connexin genes, might be expected to base-pair with any connexin mRNA to an extent sufficient to interfere with its function. Lee et al. (1987) used antibodies raised against the intact major rat liver gap junction protein (i.e. Cx32); these are known to recognize at least one conserved epitope (Milks et al., 1988), and again could have interfered with several different connexins. On the other hand, the peptide-specific antibodies used by Becker et al. (1995) were designed to be highly specific for Cx43. Thus we are left with the implication that when Cx43 function is blocked by a specific antibody, compaction is affected, but when synthesis of Cx43 is prevented by mutational inactivation of the gene, there is no effect. As suggested by Becker et al. (1995), this may reflect up-regulation of one or more additional connexin genes in the absence of Cx43, a phenomenon that might not occur when Cx43 channels are blocked directly by an antibody. Our data indicate, however, that this up-regulation, if it occurs, does not involve Cx45. Perhaps a better explanation is suggested by the recent demonstration that different connexins can co-assemble to form heteromeric connexons (Konig and Zampighi, 1995; Stauffer, 1995; Jiang and Goodenough, 1996). If the multiple connexins expressed during preimplantation development normally co-assemble, then an antibody against Cx43 might have a much more pervasive effect on gap junction formation than a Cx43 null mutation. Our findings emphasize the complexity of intercellular coupling in development, given the multiplicity of connexins and the potentially great diversity of gap junction channel types.

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