Molecular Biology of the Cell
Vol. 4, 7–20, January 1993

Connexin40, a Component of Gap Junctions in Vascular Endothelium, is Restricted in Its Ability to Interact with Other Connexins

Roberto Bruzzone,* Jacques-Antoine Haefliger,*† Robert L. Gimlich,‡ and David L. Paul§

Department of Anatomy and Cellular Biology and §Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115

Submitted September 14, 1992; Accepted November 2, 1992

The cellular distribution of connexin40 (Cx40), a newly cloned gap junction structural protein, was examined by immunofluorescence microscopy using two different specific anti-peptide antibodies. Cx40 was detected in the endothelium of muscular as well as elastic arteries in a punctate pattern consistent with the known distribution of gap junctions. However, it was not detected in other cells of the vascular wall. By contrast, Cx43, another connexin present in the cardiovascular system, was not detected in endothelial cells of muscular arteries but was abundant in the myocardium and aortic smooth muscle. We have tested the ability of these connexins to interact functionally. Cx40 was functionally expressed in pairs of *Xenopus* oocytes and induced the formation of intercellular channels with unique voltage dependence. Unexpectedly, communication did not occur when oocytes expressing Cx40 were paired with those expressing Cx43, although each could interact with a different connexin, Cx37, to form gap junction channels in paired oocytes. These findings indicate that establishment of intercellular communication can be spatially regulated by the selective expression of different connexins and suggest a mechanism that may operate to control the extent of communication between cells.

INTRODUCTION

Cells communicate directly with their neighbors through specialized intercellular channels present in gap junctions. Genes encoding the structural components of these channels have been cloned and comprise a family of highly related proteins, the connexins, consisting of at least 11 members (Kumar and Gilula, 1986; Paul, 1986; Beyer *et al.*, 1987, 1992; Gimlich *et al.*, 1988, 1990; Ebihara *et al.*, 1989; Zhang and Nicholson, 1989; Beyer, 1990; Hoh *et al.*, 1991; Paul *et al.*, 1991; Willecke *et al.*, 1991; Haefliger *et al.*, 1992; Hennemann *et al.*, 1992; White *et al.*, 1992). Different connexins display distinct spatial and temporal patterns of expression (Paul, 1985; Beyer *et al.*, 1989; Dermietzel *et al.*, 1989; Beyer, 1990; Gimlich *et al.*, 1990; Nishi *et al.*, 1991; Risek and Gilula, 1991; Valdimarsson *et al.*, 1991). In addition, channels composed of different connexins exhibit different functional properties with regard to unitary conductance (Burt and Spray, 1988; Eghbali *et al.*, 1990; Fishman *et al.*, 1990) and gating by voltage and phosphorylation (Swenson *et al.*, 1990; Bennett *et al.*, 1991). A critical issue to be resolved is how differences in connexin distribution or channel properties contribute to the function of organs constructing gap junctions from these proteins.

One important consequence of connexin diversity is that cells expressing different connexins may come into contact and form gap junctions that are mixed in connexin content. This idea is supported by Northern analyses of connexin mRNAs that indicate that certain organs express multiple connexins (Haefliger *et al.*, 1992). Because intercellular channels span two plasma membranes, they require the contribution from each cell of a hemi-channel, or connexon. Two connexons interact in the extracellular space to form the complete intercellular channel. Thus, intercellular channels may be defined as homotypic, when both connexons are com-

* This work reflects equal contributions of these authors.
† Present address: Département de Médecine Interne B, CHUV, CH-1011 Lausanne, Switzerland.
‡ Present address: Genetics Institute, Cambridge, MA 02140.

posed of the same connexin, or heterotypic, when the connexons differ. Using in vitro functional expression systems, it has been shown that some connexins can form both homotypic and heterotypic channels (Dahl *et al.*, 1987; Swenson *et al.*, 1989; Werner *et al.*, 1989; Barrio *et al.*, 1991). These studies indicate that heterotypic intercellular channels display asymmetric properties with respect to voltage dependent closure and support the suggestion that such channels could account for the asymmetric voltage sensitivity observed in vivo at rectifying electrotonic synapses (Giaume *et al.*, 1987). However, the occurrence of heterotypic junctions in vivo has not been demonstrated conclusively.

The expression of different connexins in adjacent cells could also result in a failure to establish communication. For example, when connexins from very different organisms were expressed in paired *Xenopus* oocytes, the development of communication was dependent on the type of connexins expressed. Thus, rat connexin43 (Cx43) could establish heterotypic communication with *Xenopus* Cx38, whereas rat Cx32 could not (Swenson *et al.*, 1989). Clearly, if connexins expressed in the same organism had the ability to discriminate between one another, then it could provide a powerful mechanism for limiting or segregating communication.

To investigate the potential for interactions between connexins, it is necessary to establish the cellular location of specific connexins more precisely than possible by mRNA analysis and to describe their characteristic functional properties, such as voltage dependence. We selected Cx40 and Cx43 for this study because they exhibited broad mRNA distribution, making them likely candidates for interactions. Cx40 was abundant in vascular endothelium of aorta and of muscular arteries in lung and heart but not other elements of the vascular wall. Cx43, in contrast, was readily detected in myocardial cells and aortic smooth muscle but not in endothelium or the media of small muscular arteries. We also tested the ability of Cx40 and Cx43 to form homo- and heterotypic intercellular channels. Intercellular channels composed of Cx40 displayed a characteristic voltage dependence similar to that recently described for the mouse homolog (Hennemann *et al.*, 1992), whereas Cx43 channels, as previously shown, were not voltage sensitive in oocytes (Swenson *et al.*, 1989; Werner *et al.*, 1989; but see also Veenstra, 1990; Wang *et al.*, 1992; Lal and Arnsdorf, 1992, who found that cardiac gap junctions are modulated by voltage). Unexpectedly, it was found that oocytes expressing Cx40 could not form intercellular channels when paired with cells expressing Cx43. This restriction was not absolute, as both Cx40 and Cx43 could form heterotypic channels with another rat connexin, Cx37. This is the first demonstration, using connexins obtained from the same organism, that cells may limit their ability to communicate by the expression of incompatible connexins. These results suggest a general mechanism that may operate to regulate the extent of communication between cells.

MATERIALS AND METHODS

Production and Purification of Antisera

Peptides corresponding to amino acids 313–330 (40S; TQYGQKPEQPSGASAGHR) and 339–356 (40B; KRRLSKAS-SKARSDDLSV) of Cx40 (Haefliger *et al.*, 1992) and amino acids 252–271 of Cx43 (Beyer *et al.*, 1989) were synthesized commercially. The Cx43 peptide was conjugated to keyhole limpet hemocyanin, and the Cx40 peptide 40B was conjugated to bovine serum albumin (BSA) using glutaraldehyde (Harlow and Lane, 1988). The Cx40 peptide 40S was synthesized with a cysteine residue at the N-terminal end and coupled to BSA preactivated with sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate according to the manufacturer's recommendations (Pierce, Rockford, IL). Immunization and collection of sera were performed by the Pocono Rabbit Farm and Laboratory (Canadensis, PA). For affinity purification, peptides were coupled to Sepharose 4B preactivated with cyanogen bromide according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). Purification of crude sera was performed as described by Musil *et al.* (1990).

Immunocytochemistry

Adult CD rats were fixed using whole body perfusion through the left ventricle after cutting the inferior vena cava. To prevent vascular spasm and ensure complete perfusion, a prefix perfusion was performed using 10 ml of a solution containing 150 mM NaCl, 0.025% heparin, 0.6% polyvinylpyrrolidone, and 0.5% procaine-HCl, pH 7.4. This was followed by 20 ml of 4% formaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in phosphate-buffered saline (PBS; 135 mM NaCl, 3 mM NaH_2PO_4, 7 mM Na_2HPO_4, pH 7.4). Tissues were manually cut into 2- to 3-mm slices and fixed for 1 additional h at room temperature and then incubated in 0.5 M sucrose/PBS overnight at 4°C for cryoprotection before embedding in Tissue-Tek (Miles, Elkhart, IN) and freezing in liquid nitrogen. Rat aorta was surgically isolated, cleaned from most of the adventitial layer, and carefully rinsed in PBS. Five-millimeter pieces were embedded in Tissue-Tek and frozen in liquid nitrogen. Five- to 10-μm frozen sections were cut as described previously (Goodenough *et al.*, 1988). Sections were incubated with affinity purified antibodies at 1:250–1:1000 dilution for 1 h at room temperature, washed in PBS, and then stained with a 1:500 dilution of rhodamine-conjugated secondary antisera (Pierce, Rockford, IL). Fluorescence microscopy was performed with a Zeiss Axioskop (Thornwood, NY) recorded on Kodak (Rochester, NY) Tmax400 film.

Preparation of cRNAs and Oligonucleotides

The coding sequence of either Cx43, Cx40, or Cx37 was subcloned into the *Bgl* II site of the RNA expression vector SP64T, in between the 5′ and 3′ noncoding regions of *Xenopus* β-globin (Krieg and Melton, 1984). Recombinant plasmids were linearized with either *Bam*H1 or *Xba* I (New England Biolabs, Beverly, MA), gel purified, and used as templates (Maniatis *et al.*, 1982). In vitro synthesis of cRNA with unmethylated cap was carried out with SP6 polymerase (Promega, Madison, WI), according to the manufacturer's instructions. Purified cRNAs were aliquoted and stored in aqueous solutions at −120°C until use.

Phosphothioate-modified DNA oligonucleotides (Baker *et al.*, 1990) were prepared by the Dana Farber Cancer Institute Molecular Biology Core Facility (Boston, MA). The sequence of the oligonucleotide antisense to a portion of mRNA coding for *Xenopus* Cx38 (nucleotides 204–227 within the protein coding region) (Ebihara *et al.*, 1989; Gimlich *et al.*, 1990) was 5′-CTGACTGCTCGTCTGTCCACACAG-3′. The control oligonucleotide was a permuted ("scramble") sequence of

identical nucleotide composition: 5'-CAGTCTGGTCCTCTGTCGA-GACAG-3'. Oligonucleotides were deprotected and purified on a 19% polyacrylamide gel containing 7 M urea. After elution from gel slices, they were further purified on Sep-Pak cartridges (Waters, Milford, MA) and ethanol precipitated (Maniatis *et al.*, 1982). Oligonucleotides were stored at −20°C in aqueous solution (66 μg/ml) until use.

Isolation, Microinjection, and Pairing of Oocytes

Stage V–VI oocytes were isolated from *Xenopus laevis* by collagenase digestion in modified Barth's (MB) medium (110 mM NaCl, 1.3 mM KCl, 3 mM $NaHCO_3$, 19 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 0.9 mM $MgSO_4$, pH 7.6), followed by manual defolliculation as previously described (Swenson *et al.*, 1989). Isolated oocytes were then placed in MB medium supplemented with 0.4 mM $CaNO_3$ and 0.5 mM $CaCl_2$, and injected with 40 nl of a 0.06 ng/nl solution of either an oligonucleotide antisense to *Xenopus* Cx38 mRNA or a "scrambled" version of it. Cells were incubated 24 h, injected with 40 nl of either water or a 0.01–0.1 ng/nl solution of cRNA coding for a rat connexin, and then manually paired in MB medium, after the removal of the vitelline membrane in hypertonic solution (200 mM potassium aspartate, 10 mM HEPES, 1 mM $MgCl_2$, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 20 mM KCl, pH 7.4) (Methfessel *et al.*, 1986). Oocytes were kept at 18°C throughout the experimental procedures.

Labeling of Oocytes and Immune Precipitation of Connexins

Oocyte proteins were metabolically labeled with ^{35}S-methionine (1140 Ci/mmol, New England Nuclear, Boston, MA) essentially as described by Swenson *et al.* (1989). Briefly, oocytes were coinjected with either cRNA or water along with the label (6 μCi in 40 nl/cell) and incubated for 6 h. Labeling was terminated by homogenizing each oocyte in 500 μl of lysis buffer (0.4% sodium dodecyl sulfate [SDS], 20 mM tris(hydroxymethyl)aminomethane [Tris]-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.02% N_aN_3, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of chymostatin, leupeptin, and pepstatin). A 50-μl aliquot was dried under vacuum, resuspended in an equal volume of gel sample buffer (25 mM Tris-HCl, pH 6.8, 0.5% SDS, 0.1% beta-mercaptoethanol, 17% glycerol, 0.01% bromophenol blue), and analyzed by SDS-polyacrylamide gel electrophoresis to assay incorporation of ^{35}S-methionine into total protein. The remaining homogenate was incubated at 100°C for 3 min, Triton X-100 was added to a final concentration of 2%, and samples were cleared by centrifugation for 5 min at room temperature in an Eppendorf (Brinkmann Instruments, Westbury, NY) microcentrifuge. The supernatant was divided into two aliquots and incubated overnight on a rotating plate at 4°C with either preimmune serum or primary antibodies, used at a final dilution of 1:40 to 1:100. After the addition of Protein A-Sepharose CL-4B (Sigma, St. Louis, MO), samples were incubated for 1–2 h at 4°C. Beads were pelleted by brief centrifugation, washed two times in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% N_aN_3) with 2% Triton X-100, two times in TBS, and eventually solubilized in 120 μl of gel sample buffer. Aliquots of oocyte lysate (1/50 to 1/100 of one oocyte) and immune precipitates (1/10 to 1/20 of one oocyte) were separated on an 11% SDS gel and autoradiographed.

Measurements of Junctional Conductance

The formation of junctional channels was assessed 24–48 h after pairing by a dual voltage clamp procedure that enables junctional conductance to be directly quantitated (Spray *et al.*, 1981; Swenson *et al.*, 1989). The two cells of a pair were voltage clamped, usually at −80 mV, and alternating symmetrical depolarizing pulses of various amplitudes were imposed. Under these conditions, the current supplied by the voltage clamp to the cell not stepped is equal in amplitude but opposite in sign to the junctional current. Junctional conductance is the ratio of junctional current to the transjunctional voltage step. G_j values were normalized to the maximal conductance measured at the lowest V_j (10 mV). Time resolution of initial junctional currents was ~10–20 ms. To establish steady-state G_{jss} values, 60-s depolarizing steps were applied to ensure that junctional currents had reached equilibrium.

RESULTS

Characterization of Anti-Connexin Antibodies

Two different antibodies against Cx40 were produced by immunization of rabbits with synthetic peptides. One peptide (40B) corresponded to the ultimate C-terminal 18 amino acids (residues 339–356). The other peptide (40S) corresponded to residues 313–330 that are located in the large C-terminal cytoplasmic region (Haefliger *et al.*, 1992). Antibodies against Cx43 were produced against a peptide corresponding to residues 252–271 (Beyer *et al.*, 1989). All antibodies were affinity purified using the cognate peptide coupled to sepharose activated by cyanogen bromide, as described under MATERIALS AND METHODS.

The specificities of the affinity purified antibodies were assessed by immune precipitation of metabolically labeled proteins synthesized by *Xenopus* oocytes (Figure 1). Oocytes were injected with ^{35}S-methionine and either water or connexin cRNAs. Oocytes injected with water produced a characteristic pattern of ^{35}S-labeled proteins (Figure 1, lane 2) (see also Swenson *et al.*, 1989). Injection of rat Cx43 cRNA resulted in the appearance of a novel band with an apparent mobility of 45 kDa (Figure 1, lane 3). Injection of cRNA encoding rat Cx40 produced a doublet migrating at ~40 kDa (Figure 1, lane 4). Affinity purified antibodies against rat Cx43 specif-

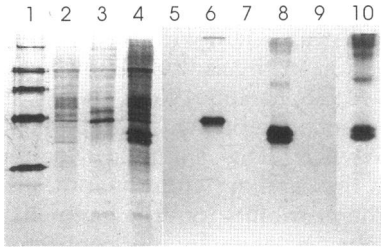


Figure 1. Production of connexin proteins in *Xenopus* oocytes and characterization of anti-connexin antibodies by immune precipitation. *Xenopus* oocytes were injected with 40 nl of either water (lanes 2, 5, 7, and 9) or cRNAs coding for Cx43 (lanes 3 and 6) or Cx40 (lanes 4, 8, and 10) and labeled with ^{35}S-methionine. Homogenates were made from each oocyte and aliquots were analyzed for incorporation of label into total protein (lanes 2, 3, and 4). Oocytes injected with cRNAs produced high levels of the encoded proteins, which were easily detected above the background of endogenously synthesized proteins. An anti-Cx43 antibody specifically precipitated one band of ~45 kD from an oocyte injected with rat Cx43 cRNA (lane 6), whereas no bands were detected in water injected oocytes using the same antibody (lane 5). Similarly, both anti-Cx40 antibodies precipitated a doublet migrating at ~40 kD from oocytes injected with cRNA for Cx40 but not from water-injected cells (40S; lanes 7 and 8; 40B, lanes 9 and 10). Lane 1 contains molecular weight markers: 200 kD, 97 kD, 69 kD, 46 kD, and 30 kD.

ically precipitated one band of ~45 kDa from a lysate of an oocyte injected with rat Cx43 cRNA (Figure 1, lane 6). No bands were detected when control oocyte lysates were precipitated with the same antibody (Figure 1, lane 5). Both antibodies against rat Cx40 precipitated the ~40 kDa doublet from oocytes injected with Cx40 cRNA (Figure 1, lanes 8 and 10) as well as some slower migrating bands. No bands were detected in precipitates of control oocyte lysates (Figure 1, lanes 7 and 9). Neither Cx40 antibody precipitated Cx43. We have not investigated the reason for the appearance of a doublet of ~40 kDa. Because it is recognized by both antibodies, it could be caused by either proteolytic cleavage at the N-terminus or abnormal initiation of protein synthesis. Although posttranslational modifications such as phosphorylation have been described for other connexins (Musil *et al.*, 1990), this possibility seems unlikely, because synthesis of rat Cx40 in reticulocyte lysate also resulted in the appearance of an ~40-kDa doublet.

Immunofluorescent Localization of Cx40 and Cx43 In Vivo

The antibodies were used for immunofluorescence on frozen sections of rat lung, heart, and aorta. The phase-contrast image of the section of lung (Figure 2A) displays a large artery that is mostly longitudinally sectioned. A small portion of the artery turns obliquely, resulting in a grazing section. This reveals a region of the intimal surface *en face*. The phase-dark lines that delineate the endothelial cells probably represent extensive tight junctional complexes. Labeling produced by the affinity purified 40B antibody consisted of numerous punctate or macular regions of fluorescence, which appear at the borders between endothelial cells (Figure 2B, arrows). This staining is consistent with the pattern of gap junction distribution in the endothelium of arteries (Simionescu *et al.*, 1975; Larson, 1988). There was also readily detectable punctate labeling in the parenchyma of the lung (arrowhead). This staining may represent gap junctions in the capillary endothelium of the alveolus. However, the possibility that the parenchymal staining arose from alveolar epithelium or connective tissue cells cannot be excluded. In corroborative studies, the pattern of labeling produced by 40S antibodies on similar sections was indistinguishable. Staining was abolished by preincubation of the antibody with 20 μg/ml of the cognate peptide.

The distribution of Cx40 in the heart and aorta and its relationship to Cx43 expression was determined by immunofluorescence on similar frozen sections (Figures 3 and 4). Sections of heart (Figure 3) contained cross-sectioned small muscular arteries surrounded by myocardium. The artery displayed in Figure 3, A and B, was perpendicularly sectioned at one end but turned obliquely at its other end. At the oblique end, an *en face* view similar to the section from lung was presented. The staining with anti-Cx40 (40S antibody; Figure 3B) was punctate and appeared to be confined to appositional areas between cells, consistent with the expected location of gap junctions. Nuclei of endothelial cells that protrude into the lumen are evident (arrowheads). Comparison of fluorescence and phase contrast images (Figure 3, A and B) indicated that the labeling was confined to the endothelial cells. The pattern of labeling observed with the anti-Cx43 antibody was dramatically different (Figure 3D). Cx43 staining was detected only between the myocardial cells. The arrangement of punctate staining into bands corresponding to the intercalated disks between myocardial cells is consistent with previous studies of the distribution of Cx43 in the heart (Beyer *et al.*, 1989). No specific staining above background was observed in the vessel wall.

The relationship between expression of Cx40 and Cx43 was also examined in the rat aorta (Figure 4). Similar to lung and heart, labeling with anti-Cx40 antibody (40S) produced macular or punctate fluorescence in the lumenal aspect of the vessel wall (Figure 4B). No labeling was detected in other parts of the vascular wall. In contrast, a high level of Cx43 expression was detected only in the smooth muscle of the tunica media (Figure 4D). In corroborative studies, we found that arterial endothelial cells from liver and kidney also express Cx40 but not Cx43.

Formation of Homotypic Junctions Between Xenopus Oocytes

We have used the paired *Xenopus* oocyte assay to 1) determine if rat Cx40 is capable of forming intercellular channels, 2) compare their voltage gating behavior to intercellular channels formed from other connexins, and 3) test the ability of rat Cx40 to functionally interact and form heterotypic intercellular channels with other connexins. The rat connexins Cx40, Cx43, and Cx37 were expressed by microinjection of their specific cRNAs into oocytes. After removal of the vitelline envelope and manual pairing of two oocytes, the development of junctional conductance was monitored by dual voltage clamp (Spray *et al.*, 1981; Swenson *et al.*, 1989).

It has been demonstrated that *Xenopus* oocytes contain an endogenous connexin, *Xenopus* Cx38 (Ebihara *et al.*, 1989; Gimlich *et al.*, 1990). Potentially, this endogenous connexin could both contribute to background conductance and interact with exogenous expressed connexins. In our laboratory and some others, the background level of communication between water-injected oocytes is generally low; 100–1000 times lower than communication produced by expression of exogenous connexins (Table 1, row 1) (Dahl *et al.*, 1987; Swenson *et al.*, 1989, 1990). However, some rat connexins can efficiently interact with the endogenous connexin (Swenson *et al.*, 1989). Therefore, the endogenous connexin must be eliminated in experiments involving

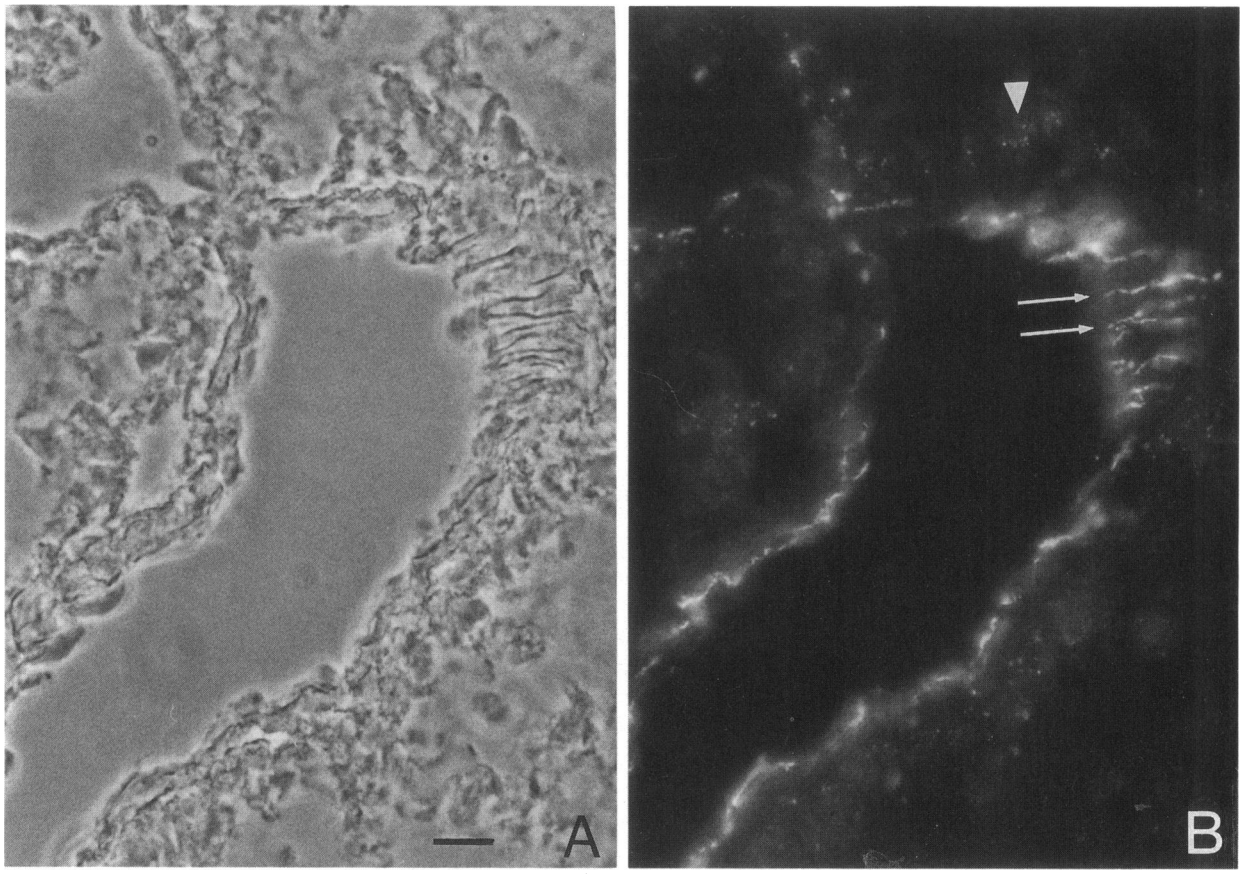
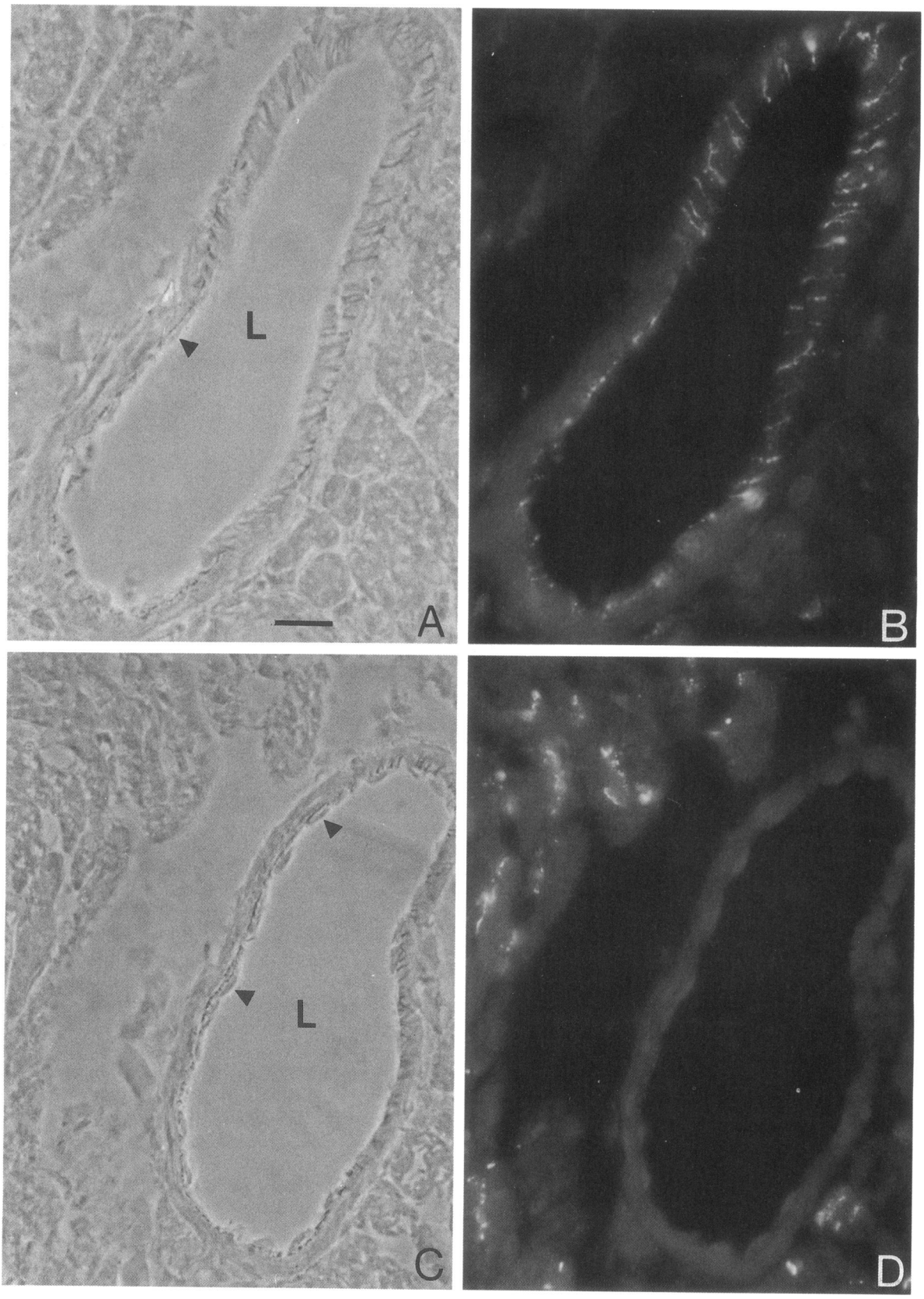


Figure 2. Immunohistochemical localization of Cx40 in rat lung. Five-micron frozen sections of formaldehyde-fixed rat lung were examined by phase contrast (A) and immunofluorescence (B) microscopy. (A) The phase contrast displays a large artery that has been cut obliquely to reveal a region of the intimal surface *en face*. (B) Labeling produced by the affinity purified 40B antibody consists of numerous punctate or macular regions of fluorescence that outline endothelial cells (arrows). There is also smaller punctate staining in the parenchyma of the lung (arrowhead). Scale bar is 10 μm.

expression of some rodent connexins. Pairing oocytes expressing either rat Cx43 or Cx37 with control oocytes injected with water induced the development of large junctional conductances, as the result of heterotypic interaction with an endogenous connexin (Table 1, rows 2 and 3). By contrast, Cx40 failed to form junctional channels with water-injected oocytes (Table 1). Injection of antisense oligonucleotides against *Xenopus* Cx38 greatly inhibited the ability of both rat Cx43 and Cx37 to produce heterotypic channels with the endogenous connexin (Table 1, rows 6 and 7). This effect resulted from reduced Cx38 mRNA and protein levels (Gimlich and Goodenough, personal communication). Although the use of antisense oligonucleotides to suppress the endogenous conductance of water-injected oocyte pairs has been reported previously (Barrio *et al.*, 1991; Hennemann *et al.*, 1992), our data demonstrate for the first time the efficacy of this strategy in preventing the establishment of heterotypic coupling between mammalian and *Xenopus* connexins. The suppression of these high levels of conductance (much higher than those reported for endogenous coupling) is essential to analyze the formation of heterotypic channels between foreign connexins expressed by oocyte pairs.

All three connexins tested formed homotypic intercellular channels with high conductances (Table 2). Thus, expression of rat Cx40 was capable of inducing the formation of intercellular channels. Junctional currents between oocytes expressing rat Cx40 decreased in a time and voltage-dependent manner (Figure 5A). For comparison, junctional currents in oocytes expressing rat Cx37 are presented in Figure 5B. In both cases, the rate of channel closure increased with increasing transjunctional potentials (V_j). The junctional currents for Cx40 channels decayed exponentially and achieved equilibrium more quickly than for channels composed of Cx37. The decay in junctional currents from Cx37 channels did not fit simple exponentials and thus may reflect several independent processes. For both connexins, the rate of voltage-dependent closure was much slower than for other well-characterized voltage-gated ion channels. As previously reported, channels formed


L
A
B
L
C
D

from Cx43 did not display voltage dependence over the range of transjunctional potentials tested (Swenson *et al.*, 1989; Werner *et al.*, 1989). The characteristics of rat Cx40 and Cx37 channels are similar to those recently reported for their mouse homologs (Cx37, Willecke *et al.*, 1991; Cx40, Hennemann *et al.*, 1992).

The normalized initial junctional conductance (G_{j0}) and steady-state conductance (G_{jss}) of homotypic Cx40 intercellular channels are displayed in Figure 5C. Both cells were initially clamped at −80 mV to ensure zero transjunctional potential. Although one cell was held at a constant potential, depolarizing voltage steps (10–80 mV) were sequentially applied to the other cell, and the resulting junctional currents were recorded. G_j values were normalized to the maximal conductance measured at the lowest V_j (10 mV). Time resolution of initial junctional currents was ~10–20 ms. To establish steady-state values, 60-s depolarizing steps were applied. Initial conductance of Cx40 channels was insensitive to transjunctional potential. However, the steady-state conductances decreased significantly at $V_j > 30$ mV. Half-maximal response (V_0) occurred at $V_j = 38$ mV, and the normalized equilibrium value (G_{jmin}) was 0.18 at $V_j = 80$ mV. Results are the mean ± SEM of 18 pairs from eight experiments (G_{j0}) and 5 pairs from five experiments (G_{jss}). As expected for homotypic channels, the observed conductances were the same regardless of the polarity of transjunctional potential.

Unlike Cx40, Cx37 formed channels in which G_{j0} decreased substantially for increasing V_j (Figure 5D). G_{jss} of Cx37 was more sensitive to transjunctional voltage than that of Cx40 ($V_0 = 25$ mV). In addition, G_{jmin} was lower than for Cx40 (0.08 at $V_j = 80$ mV). Results are the mean ± SEM of 17 pairs from three experiments (G_{j0}) and 12 pairs from three experiments (G_{jss}). For channels composed of either Cx37 or Cx40, G_j decreased symmetrically for depolarizing and hyperpolarizing steps applied to either cell, indicating that conductance was not directly affected by membrane potential.

Formation of Heterotypic Gap Junctions Between Xenopus Oocytes

To examine heterotypic junction formation, we paired oocytes expressing different connexins (Table 2). Unexpectedly, when oocytes expressing Cx40 were paired with those expressing Cx43, no communication was established. In these pairs the conductance was below the background levels (compare Table 2, heterotypic, row 3, with Table 1, rows 4 and 6). Because the oocyte expression system can support the formation of homotypic and heterotypic channels, as described below, it is likely that the inability of Cx40 and Cx43 to functionally interact reflects an intrinsic property of these connexins.

In contrast, when oocytes expressing Cx40 or Cx43 were paired with those expressing Cx37, high levels of conductance were observed (Table 2). The voltage dependence of these heterotypic channels is displayed in Figure 5E, where depolarizing steps were applied to the cell expressing Cx40, and in Figure 5F, where the cell expressing Cx37 was depolarized. The voltage sensitivity of these heterotypic channels, unlike homotypic channels, depended on the polarity of the transjunctional potential (Figure 5, E and F). For each polarity, the voltage dependence was qualitatively similar to that of the corresponding homotypic channels, although a small increase in sensitivity was observed for steady-state Cx40 conductance in heterotypic pairs (compare Figure 5, E with C and F with D). Results are the mean ± SEM of 7 (G_{jss}) and 10 (G_{j0}) pairs. Similarly, asymmetric voltage-dependent conductance was observed with heterotypic channels composed of Cx43 and Cx37. The asymmetry of junctional currents and the relative conservation of each connexin's voltage sensitivity showed that each cell contributed a connexon composed of a different connexin and that a heterotypic channel had been established.

DISCUSSION

We have demonstrated that rat Cx40 was prominently expressed by vascular endothelial cells but not any other cells in the vascular wall. In contrast, Cx43 was detected in ventricular myocardium and aortic smooth muscle. The paired *Xenopus* oocyte system was used to characterize the behavior of rat Cx40, Cx43, and Cx37. Although each connexin formed homotypic gap junction channels, we found that rat Cx43 and Cx40 were incompatible and did not form heterotypic channels. This is the first indication that, with connexins of the same organism, two cells may limit their ability to communicate by expressing different gap junction proteins. In addition, we have established that incompatible connexins are expressed in adjacent cells in the vascular wall.

Connexin Expression in the Cardiovascular System

Our observations of connexin expression are consistent with physiological demonstrations of extensive dye and

Figure 3. Immunohistochemical localization of Cx43 and Cx40 in rat heart. Five-micron frozen sections of formaldehyde-fixed rat heart were examined by phase contrast (A and C) and immunofluorescence (B and D) microscopy. (A and C) Myocardial muscle and a transverse section of a small muscular artery are visible in the phase contrast micrograph. The arterial lumen (L) and nuclei of endothelial cells that protrude into the lumen are apparent (arrowheads). (B) Cx40 strongly labels the arterial endothelium. (D) In contrast, Cx43 is detected in the intercalated discs between cardiac myocytes, consistent with previous reports and the known location of gap junctions in these cells. No staining of the arterial wall above background can be observed. Scale bar is 10 μm.

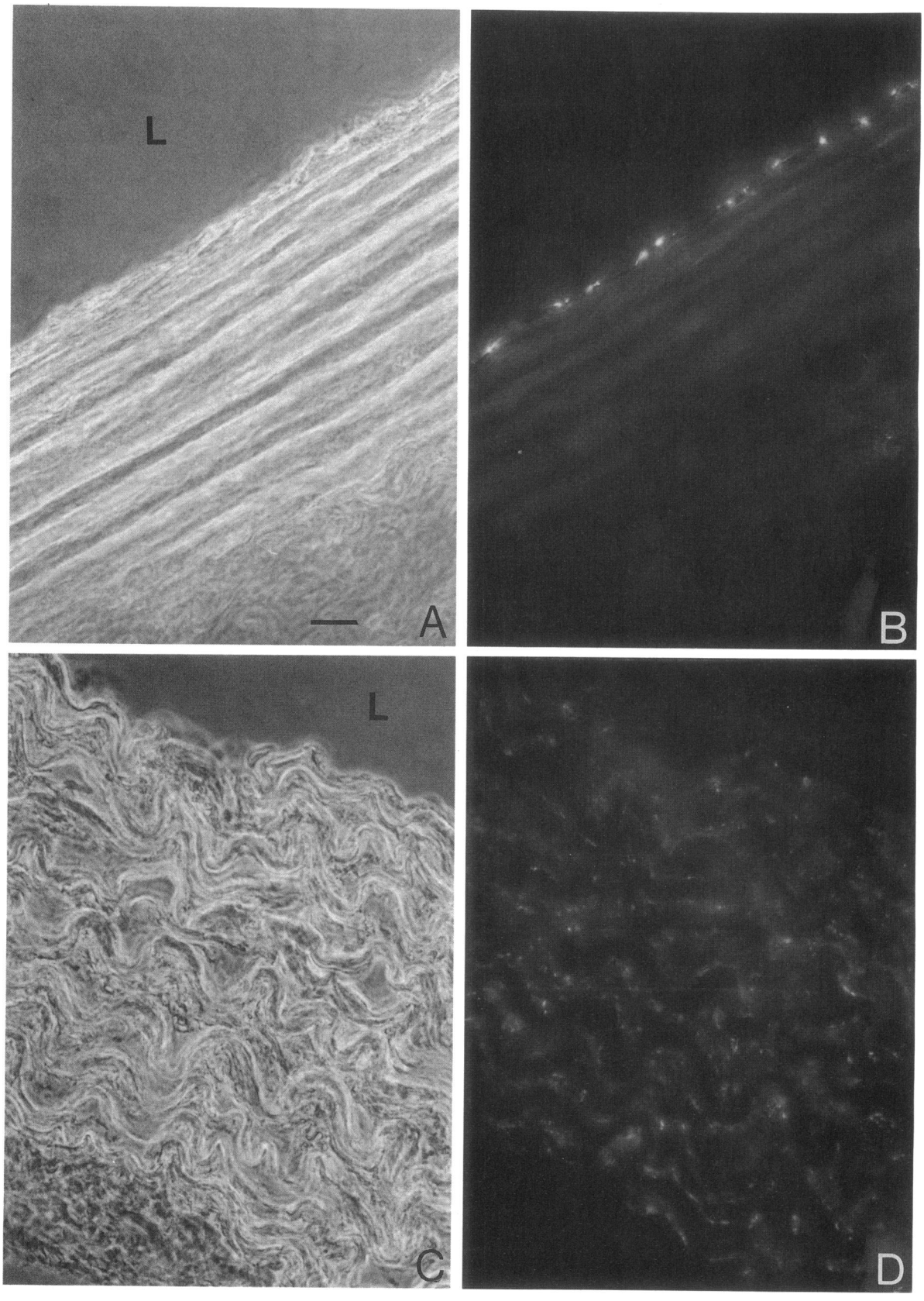

L
A
B
L
C
D

Table 1. Injection of oligonucleotides antisense to mRNA encoding *Xenopus* Cx38 prevents the formation of heterotypic channels between either rat Cx43 or rat Cx37 and *Xenopus* Cx38

Oocyte injection cell 1/cell 2	Conductance (μS)[a]	No. of experiments (no. of pairs)
Water + scramble/water + scramble	0	6 (13)
Cx43 + antisense/water + scramble	8.2 ± 2.3	7 (23)
Cx37 + antisense/water + scramble	19.5 ± 0.8	9 (30)
Cx40 + antisense/water + scramble	0.06 ± 0.07	3 (10)
Water + antisense/water + antisense	0	4 (9)
Cx43 + antisense/water + antisense	0.4 ± 0.2	7 (23)
Cx37 + antisense/water + antisense	1.2 ± 0.2	9 (29)

[a] Values are means ± SEM.

Oocytes were injected with either an oligonucleotide antisense to a sequence within the coding region of *Xenopus* Cx38 or a scrambled version of it. After an overnight incubation, oocytes were injected a second time with either cRNAs for the rat connexins studied or with water and incubated another 24–48 h before measurements of junctional conductance were performed.

electrical coupling between endothelial cells in vivo (Beny and Gribi, 1989; Beny, 1990). Although other connexins may have been present in endothelial cells (see below), the abundance and selective expression of Cx40 suggests a significant role for Cx40 in the establishment of junctions between arterial endothelium.

We do not detect Cx43 in the endothelium of small or large arteries. However, the ability of cultured bovine endothelial cells to express Cx43 in vitro has been documented (Larson *et al.*, 1990; Beyer *et al.*, 1992; Pepper *et al.*, 1992). Because these reports were based on studies of primary cultures or cell lines, the apparent discrepancy with our results may reflect changes in both types and levels of connexin expression under culture conditions. This hypothesis is supported by a recent report (Stutenkemper *et al.*, 1992) that the types of connexins expressed by cells in vivo can change in culture. It is also possible that Cx43 is present in endothelium in vivo in amounts too small to detect. Further experiments are needed to resolve this issue.

Cx43 was readily detected in the smooth muscle of aorta. The fact that we did not detect Cx43 in the tunica media of small muscular arteries may reflect the small size and low frequency of gap junctions between these cells. Supporting this idea, electron microscopic examination of smooth muscle in coronary arteries failed to reveal the presence of gap junctions, even though these cells are demonstrably coupled (Beny and Connat, 1992). Although Cx43 was found in A7r5 cells, an aortic smooth muscle cell line (Moore *et al.*, 1991), it is also possible, as discussed above for endothelial cells, that either Cx43 expression in smooth muscle cells is heterogeneous or changes under culture conditions.

In contrast to our results, Cx40 mRNA has been reported in cultures of vascular smooth muscle (Beyer *et al.*, 1992). It is difficult to directly compare their results with ours because we demonstrate connexin protein in vivo, whereas they demonstrate only connexin mRNA in cultured cells. It is possible that smooth muscle expresses Cx40 mRNA in vivo but does not synthesize or accumulate Cx40 protein. However, it seems more likely that the types of connexins expressed by endothelial and smooth muscle cells in vivo can change in culture as recently shown for liver cells (Stutenkemper *et al.*, 1992).

Finally, our results do not confirm the finding of Kanter *et al.* (1992), who reported immunoreactivity in isolated canine ventricular myocytes using an anti-peptide antibody directed against chicken Cx42. Rodent and canine Cx40 are the closest connexin homologs to chicken Cx42, although significant differences are present. For example, the chicken sequence used to generate the anti-peptide antibody was 55% conserved in canine Cx40 (Kanter *et al.*, 1992). Our failure to observe Cx40 immunoreactivity in rat myocardial cells was consistent with both of our two distinct anti-rat Cx40 antibodies. It is possible that canine and rodent myocardium express different connexins. However, we are not able to clearly reconcile these results at this time.

In summary, we have established that rat Cx40 is prominently expressed in rat arterial endothelium,

Figure 4. Immunohistochemical localization of Cx43 and Cx40 in rat aorta. Five-micron frozen sections of unfixed rat aorta were examined by phase contrast (A and C) and immunofluorescence (B and D) microscopy. Luminal aspect of the vessel is indicated by L. (B) Labeling with anti-Cx40 antibodies (40S) produced punctate fluorescence detected only in the endothelial layer. (D) In contrast, signal for Cx43 was confined to the smooth muscle cells in the tunica media. Scale bar is 10 μm.

Table 2. Conductances developed by homotypic and heterotypic gap junction channels in oocyte pairs

Oocyte injection cell 1/cell 2	Conductance (μS)[a]	No. of experiments (no. of pairs)
	Homotypic	
Cx43/Cx43	16.1 ± 2.9	7 (19)
Cx37/Cx37	16.9 ± 3.8	9 (31)
Cx40/Cx40	14.1 ± 5.7	6 (25)
	Heterotypic	
Cx37/Cx40	23.4 ± 5.9	5 (20)
Cx37/Cx43	6.0 ± 1.1	4 (17)
Cx40/Cx43	0.2 ± 0.07	3 (14)

[a] Values are means ± SEM.

All oocytes received antisense oligonucleotides as detailed in MATERIALS AND METHODS. Measurements of junctional conductance were performed 24–48 h after the injection of connexin cRNAs and pairing of the oocytes.

whereas Cx43 is abundant in the tunica media of large elastic arteries.

Voltage Dependence of Connexins

The influence of transjunctional voltage on the permeability of gap junction channels has been the subject of numerous studies (see Bennett *et al.*, 1991, for a review). Functional expression of connexins in communication-deficient cells (Eghbali *et al.*, 1990; Fishman *et al.*, 1990) and *Xenopus* oocytes (Swenson *et al.*, 1989; Werner *et al.*, 1989; Barrio *et al.*, 1991; Willecke *et al.*, 1991; Rubin *et al.*, 1992; White *et al.*, 1992) has demonstrated that voltage dependence of individual connexins is markedly different. Some connexins display a relatively high sensitivity to voltage (e.g., Cx37, Cx40); others show different behaviors depending on the expression system (e.g., Cx32 and Cx43). Interpretation of these data is complicated by the possibility that unique physiological properties of connexins may not depend only on differences in the primary sequence lining the channel pore but also on cell specific posttranslational modifications. For example, phosphorylation of Cx43 may occur in a tissue specific fashion (Kadle *et al.*, 1991) and may be associated with a shift in unitary conductance and a modification of voltage dependent channel kinetics (Moreno *et al.*, 1992). From this perspective, it will be interesting to determine whether Cx40 is phosphorylated by vascular endothelial cells in vivo. The marked differences in voltage sensitivity between Cx40 and Cx37 reported here and the lack of voltage-induced closure of channels made of Cx43 (Swenson *et al.*, 1989) must be related, however, to the connexins themselves and not to the cytoplasmic environment, because measurements were made in the same type of cell.

Why are connexins expressed in nonexcitable tissues sensitive to transjunctional voltage? One possibility is that closure of gap junction channels may serve as a mechanism to prevent communication between injured cells, which have become depolarized, and the surrounding population, thereby protecting functionally intact cells next to dying ones. By contrast, the presence of intercellular channels relatively insensitive to voltage in electrically excitable tissues, as for Cx43 in myocardium and smooth muscle, would maintain communication between cells that become depolarized during the normal propagation of electrical signals, thereby playing a role in the coordination of contraction. In this context, it is interesting to note that Cx43 is the principal connexin expressed by pancreatic β-cells (Meda *et al.*, 1991). Because their secretory response to glucose is triggered by depolarization (Prentki and Matschinsky, 1987), this may reflect a role for junctional coupling in the coordinated regulation of insulin release (Meda *et al.*, 1984). Further studies are clearly needed to evaluate the role of voltage-induced closure of gap junction channels in physiological situations.

Significance of Selectivity Among Connexins

Both homo- and heterotypic gap junction channels have been demonstrated in functional expression systems (Dahl *et al.*, 1987; Swenson *et al.*, 1989; Barrio *et al.*, 1991), thus reinforcing previous suggestions that intercellular communication is a promiscuous phenomenon that is likely to occur between contacting cells (Michalke and Loewenstein, 1971; Epstein and Gilula, 1977). Earlier, we reported that connexins from markedly different species failed to form heterotypic channels (Swenson *et al.*, 1989). In this study, we demonstrate that there also can be incompatibility among connexins from the same organism. Thus, the establishment of intercellular communication is not a uniformly permissive process but one that may be controlled by the expression of alternative connexins.

How connexons composed of particular connexins recognize and discriminate between alternative partners in adjacent cells remains to be elucidated. Cx40, Cx43, and Cx37 all belong to the α (Risek *et al.*, 1990) or Type II (Bennett *et al.*, 1991) subgroup of connexins and are very highly related. Although it is reasonable to speculate that the extracellular regions of the proteins, which must physically interact, participate in this process of recognition, these regions are the most highly conserved among all connexins. The ability of connexins to discriminate between each other could be influenced by posttranslational modifications. Because *Xenopus* oocytes may not accurately carry out these modifications, it will be of interest to explore the in vivo physiology of cells expressing incompatible types.

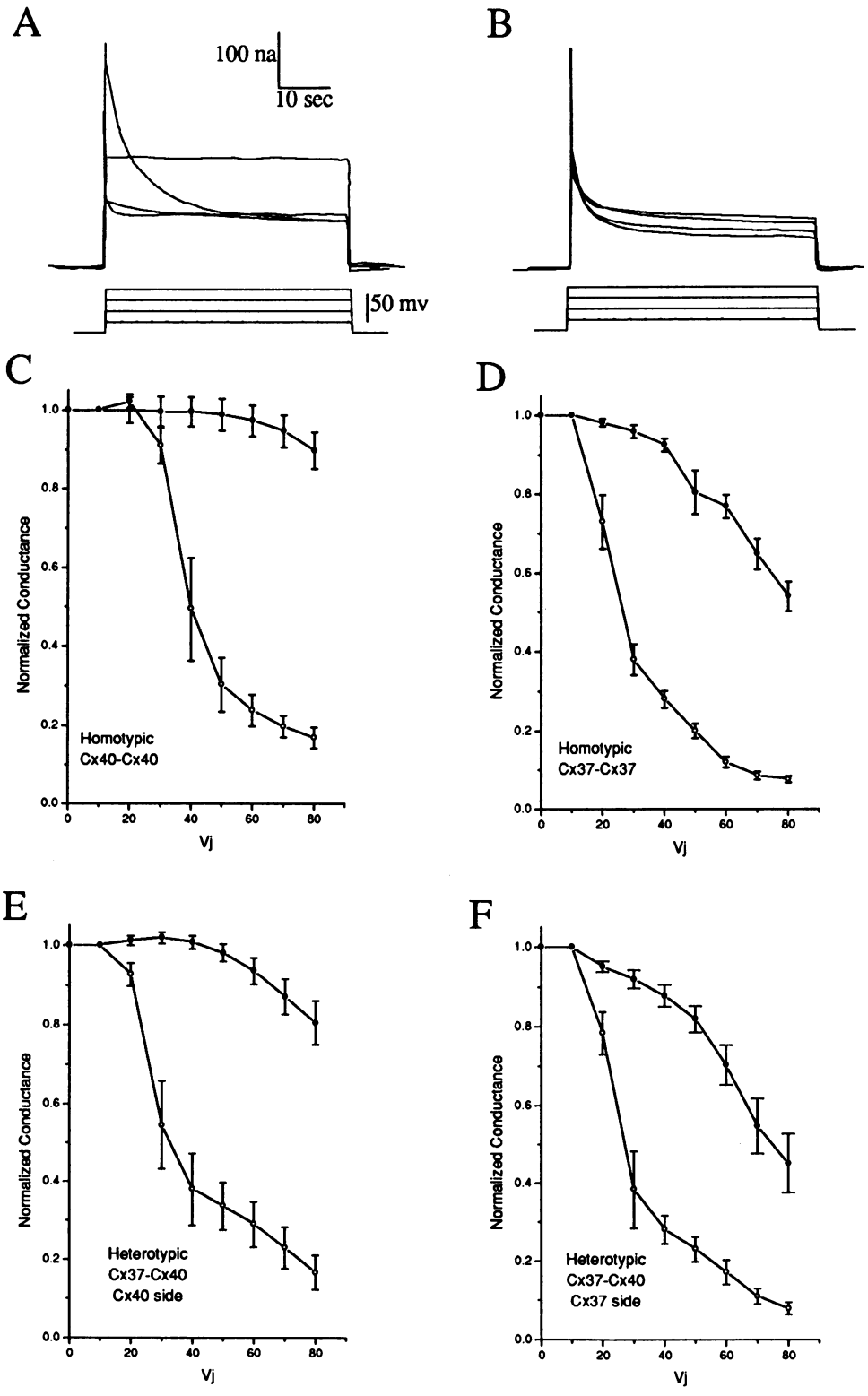


Figure 5. Voltage dependence of homotypic (A–D) and heterotypic (E and F) gap junction channels composed of Cx40 or Cx37 in *Xenopus* oocyte pairs. (A and B) Junctional currents (I_j) developed by pairs of oocytes injected with synthetic cRNA coding for Cx40 (A) and Cx37 (B). Both cells were initially clamped at −80 mV to ensure zero transjunctional potential. Although one cell was held at a constant potential, depolarizing voltage steps (20–80 mV) were sequentially applied to the other cell and the resulting junctional currents were recorded. The currents reflect voltage-induced closure occurring with a relatively slow time course, obtaining equilibrium within 10–50 s. (C and D) The relationship of transjunctional voltage (V_j) to initial (G_{j0}, filled circles) and steady-state (G_{jss}, open circles) junctional conductances is displayed for homotypic channels composed of Cx40 (C) and Cx37 (D). For Cx40, G_{j0} was not affected at V_j < 70 mv. G_{jss} decreased for transjunctional potentials larger than 30 mv. The decreases were faster for greater transjunctional potentials. For Cx37, G_{j0} decreased substantially for increasing V_j and G_{jss} was more sensitive to voltage than Cx40. (E and F) The relationship of V_j to G_{j0} and G_{jss} in heterotypic gap junctions when depolarizing steps were applied to the cell expressing Cx40 (E) and Cx37 (F). Voltage dependence of heterotypic junctional channels was asymmetric as the properties of each connexin were qualitatively maintained. A small decrease in V_0 (~10 mv) was observed for steady-state Cx40 conductance in heterotypic pairs (compare E with C and F with D).

Selectivity among connexins may represent the molecular mechanism underlying the establishment of "communication compartments," where cells communicate within, but not between, groups. Such compartments, which have been observed in a variety of developing and adult organisms (Lo and Gilula, 1979; Warner and Lawrence, 1982; Weir and Lo, 1982; Blennerhasset and Caveney, 1984; Kalimi and Lo, 1988; Serras *et al.*, 1990; Kam and Hodgins, 1992), could result from the inability of different connexins to interact functionally. For example, in the pancreas, acinar cells are not coupled to adjacent ductal cells (Findlay and Petersen, 1983; Chanson *et al.*, 1991). It remains to be established whether other connexins are incompatible and whether they may be present in physically adjacent cells in developing and/or adult organisms.

In the vascular wall, because incompatible connexins are expressed in physically adjacent endothelial and smooth muscle cells, the purpose of alternative connexin expression might be to reduce or regulate the frequency of communication between those cells. In support of this notion, no physiological demonstration of coupling between these cell types in vivo has been reported (Beny, 1990; Segal and Beny, 1992).

ACKNOWLEDGMENTS

We thank Katherine Swenson and Dan Goodenough for help with oocyte handling and Linda Musil and Jeff Goliger for their careful reading of the manuscript and extensive and thoughtful comments. This work was supported by National Institutes of Health grants GM-37751 to D.L.P. and GM-18974 to D.A. Goodenough, by Swiss National Science Foundation grants 823A-28370 to J.-A.H. and 83.627.0.88 to R.B., and by National Science Foundation grant DCB-9096202 to R.L.G.

REFERENCES

Baker, C., Holland, D., Edge, M., and Colman, A. (1990). Effects of oligo sequence and chemistry on the efficiency of oligodeoxyribonucleotide-mediated mRNA cleavage. Nucleic Acids Res. *18*, 3537–3543.

Barrio, L.C., Suchyna, T., Bargiello, T., Xu, L.X., Roginsky, R., Bennett, M.V.L., and Nicholson, B.J. (1991). Gap junctions formed by connexins 26 and 32 alone and in combination are differently affected by voltage. Proc. Natl. Acad. Sci. USA *88*, 8410–8414.

Bennett, M.V.L., Barrio, L.C., Bargiello, T.A., Spray, D.C., Hertzberg, E.L., and Sáez, J.C. (1991). Gap junctions: new tools, new answers, new questions. Neuron *6*, 305–320.

Bény, J.-L. (1990). Endothelial cells and smooth muscle cells hyperpolarized by bradikinin are not dye coupled. Am. J. Physiol. *258*, H836–H841.

Bény, J.-L., and Connat, J.-L. (1992). An electron-microscopic study of smooth muscle cell dye coupling in the pig coronary arteries. Circ. Res. *70*, 49–55.

Bény, J.-L., and Gribi, F. (1989). Dye and electrical coupling of endothelial cells in situ. Tissue & Cell *21*, 797–802.

Beyer, E.C. (1990). Molecular cloning and developmental expression of two chick embryo gap junction proteins. J. Biol. Chem. *265*, 1439–1443.

Beyer, E. C., Kistler, J., Paul, D.L., and Goodenough, D.A. (1989). Antisera directed against connexin43 peptides react with a 43-kD protein localized to gap junctions in myocardium and other tissues. J. Cell Biol. *108*, 595–605.

Beyer, E.C., Paul, D.L., and Goodenough, D.A. (1987). Connexin43, a protein from rat heart homologous to a gap junction protein from liver. J. Cell Biol. *105*, 2621–2629.

Beyer, E.C., Reed, K.E., Westphale, E.M., Kanter, H.L., and Larson, D.M. (1992). Molecular cloning and expression of rat connexin40, a gap junction protein expressed in vascular smooth muscle. J. Membr. Biol. *127*, 69–75.

Blennerhasset, M.G., and Caveney, S. (1984). Separation of developmental compartments by a cell type with reduced junctional permeability. Nature *309*, 361–364.

Burt, J.M., and Spray, D.C. (1988). Single-channel events and gating behavior of the cardiac gap junction channel. Proc. Natl. Acad. Sci. USA *86*, 3431–3434.

Chanson, M., Orci, L., and Meda, P. (1991). Extent and modulation of junctional communication between pancreatic acinar cells in vivo. Am. J. Physiol. *261*, G28–G36.

Dahl, G., Miller, T., Paul, D., Voellmy, R., and Werner, R. (1987). Expression of functional cell-cell channels from cloned rat liver gap junction complementary DNA. Science *236*, 1290–1292.

Dermietzel, R., Traub, O., Hwang, T.K., Beyer, E., Bennett, M.V.L., Spray, D.C., and Willecke, K. (1989). Differential expression of three gap junction proteins in developing and mature brain tissues. Proc. Natl. Acad. Sci. USA *86*, 10148–10152.

Ebihara, L., Beyer, E.C., Swenson, K.I., Paul, D.L., and Goodenough, D.A. (1989). Cloning and expression of a *Xenopus* embryonic gap junction protein. Science *243*, 1194–1195.

Eghbali, B., Kessler, J.A., and Spray, D.C. (1990). Expression of gap junction channels in communication-incompetent cells after stable transfection with cDNA encoding connexin32. Proc. Natl. Acad. Sci. USA *87*, 1328–1331.

Epstein, M.L., and Gilula, N.B. (1977). A study of communication specificity between cells in culture. J. Cell Biol. *75*, 769–787.

Findlay, I., and Petersen, O.H. (1983). The extent of dye-coupling between exocrine acinar cells of the mouse pancreas. Cell Tissue Res. *232*, 121–127.

Fishman, G.I., Spray, D.C., and Leinwand, L.A. (1990). Molecular characterization and functional expression of the human cardiac gap junction channel. J. Cell Biol. *111*, 589–598.

Giaume, C., Kado, R.T., and Korn, H. (1987). Voltage clamp analysis of a crayfish rectifying synapse. J. Physiol. *386*, 91–112.

Gimlich, R.L., Kumar, N.M., and Gilula, N.B. (1988). Sequence and developmental expression of mRNA coding for a gap junction protein in Xenopus. J. Cell Biol. *107*, 1065–1073.

Gimlich, R.L., Kumar, N.M., and Gilula, N.B. (1990). Differential regulation of the levels of three gap junction mRNAs in *Xenopus* embryos. J. Cell Biol. *110*, 597–605.

Goodenough, D.A., Paul, D.L., and Jesaitis, L. (1988). Topological distribution of two connexin32 antigenic sites in intact and split rodent hepatocyte gap junctions. J. Cell Biol. *107*, 1817–1824.

Haefliger, J.-A., Bruzzone, R., Jenkins, N.A., Gilbert, D.J., Copeland, N.G., and Paul, D.L. (1992). Four novel members of the connexin family of gap junction proteins. Molecular cloning, expression, and chromosome mapping. J. Biol. Chem. *267*, 2057–2064.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Hennemann, H., Suchyna, T., Lichtenberg-Frate, H., Jungbluth, L., Dahl, E., Schwarz, J., Nicholson, B.J., and Willecke, K. (1992). Mo-

lecular cloning and functional expression of mouse connexin40, a second gap junction gene preferentially expressed in lung. J. Cell Biol. *117*, 1299–1310.

Hoh, J.H., John, S.A., and Revel, J.-P. (1991). Molecular cloning and characterization of a new member of the gap junction gene family, connexin-31. J. Biol. Chem. *266*, 6524–6531.

Kadle, R., Zhang, J.T., and Nicholson, B.J. (1991). Tissue-specific distribution of differentially phosphorylated forms of connexin43. Mol. Cell Biol. *11*, 363–369.

Kalimi, G.H., and Lo, C.W. (1988). Communication compartments in the gastrulating mouse embryo. J. Cell Biol. *107*, 241–255.

Kam, E., and Hodgins, M.B. (1992). Communication compartments in hair follicles and their implication in differentiative control. Development *114*, 389–393.

Kanter, H.L., Saffitz, J.E., and Beyer, E.C. (1992). Canine cardiac myocytes express multiple gap junction proteins. Circ. Res. *70*, 438–444.

Krieg, P.A., and Melton, D.A. (1984). Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. Nucleic Acids Res. *12*, 7057–7070.

Kumar, N.M., and Gilula, N.B. (1986). Cloning and characterization of human and rat liver cDNAs coding for a gap junction protein. J. Cell Biol. *103*, 767–776.

Lal, R., and Arnsdorf, M.F. (1992). Voltage-dependent gating and single-channel conductance of adult mammalian atrial gap junctions. Circ. Res. *71*, 737–743.

Larson, D.M. (1988). Intercellular junctions and junctional transfer in the blood vessel wall. In: Endothelial Cells, ed. U.S. Ryan, Boca Raton, FL: CRC Press, 75–88.

Larson, D.M., Haudenschild, C.C., and Beyer, E.C. (1990). Gap junction messenger RNA expression by vascular wall cells. Circ. Res. *66*, 1074–1080.

Lo, C.W., and Gilula, N.B. (1979). Gap junctional communication in the postimplantation mouse embryo. Cell *18*, 411–422.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Meda, P., Chanson, M., Pepper, M., Giordano, E., Bosco, D., Traub, O., Willecke, K., El Aoumari, A., Gros, D., Beyer, E.C., Orci, L., and Spray, D.C. (1991). In vivo modulation of connexin43 gene expression and junctional coupling of pancreatic B-cells. Exp. Cell Res. *192*, 469–480.

Meda, P., Perrelet, A., and Orci, L. (1984). Gap junctions and cell-to-cell coupling in endocrine glands. Mod. Cell Biol. *3*, 131–196.

Methfessel, C., Witzemann, V., Takahashi, T., Mishina, M., Numa, S., and Sakmann, B. (1986). Patch clamp measurements on *Xenopus laevis* oocytes: currents through endogenous channels and implanted acetylcholine receptor and sodium channels. Pfluegers Arch. *407*, 577–588.

Michalke, W., and Loewenstein, W.R. (1971). Communication between cells of different type. Nature *232*, 121–123.

Moore, L.K., Beyer, E.C., and Burt, J.M. (1991). Characterization of gap junction channels in A7r5 vascular smooth muscle cells. Am. J. Physiol. *260*, C975–C981.

Moreno, A.P., Fishman, G.I., and Spray, D.C. (1992). Phosphorylation shifts unitary conductance and modifies voltage dependent kinetics of human gap junction channels. Biophys. J. *62*, 51–53.

Musil, L.S., Cunningham, B.A., Edelman, G.M., and Goodenough, D.A. (1990). Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. J. Cell Biol. *111*, 2077–2088.

Nishi, M., Kumar, N.M., and Gilula, N.B. (1991). Developmental regulation of gap junction gene expression during mouse embryonic development. Dev. Biol. *146*, 117–130.

Paul, D.L. (1985). Antibody against liver gap junction 27-kD protein is tissue specific and cross-reacts with a 54-kD protein. In: Gap Junctions, ed. M.V.L. Bennett, and D.C. Spray, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 107–122.

Paul, D.L. (1986). Molecular cloning of cDNA for rat liver gap junction protein. J. Cell Biol. *103*, 123–134.

Paul, D.L., Ebihara, L., Swenson, K.I., Takemoto, L.J., and Goodenough, D.A. (1991). Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of *Xenopus* oocytes. J. Cell Biol. *115*, 1077–1089.

Pepper, M.S., Montesano, R., El Aoumari, A., Gros, D., Orci, L., and Meda, P. (1992). Coupling and connexin43 expression in microvascular and large vessel endothelial cells. Am. J. Physiol. *262*, C1246–C1257.

Prentki, M., and Matschinsky, F.M. (1987). Ca^{2+}, cAMP and phospholipid-derived messengers in coupling mechanisms of insulin secretion. Physiol. Rev. *67*, 1185–1248.

Risek, B., and Gilula, N.B. (1991). Spatiotemporal expression of three gap junction gene products involved in fetomaternal communication during rat pregnancy. Development *113*, 165–181.

Risek, B., Guthrie, S., Kumar, N.M., and Gilula, N.B. (1990). Modulation of gap junction transcript and protein expression during pregnancy in the rat. J. Cell Biol. *110*, 269–282.

Rubin, J.B., Verselis, V.K., Bennett, M.V.L., and Bargiello, T.A. (1992). Molecular analysis of voltage dependence of heterotypic gap junctions formed by connexins 26 and 32. Biophys. J. *62*, 183–195.

Segal, S.S., and Bény, J.-L. (1992). Intracellular recording and dye transfer in arterioles during blood flow control. Am. J. Physiol. *263*, H1–H7.

Serras, F., Noteboom, R.G., and Van den Biggelaar, J.A. (1990). Communication compartments in the post-trochal ectoderm of the mollusc *Lymnaea stagnalis*. Exp. Cell Res. *187*, 284–291.

Simionescu, M., Simionescu, N., and Palade, G. (1975). Segmental differentiations of cell junctions in the vascular endothelium. The microvasculature. J. Cell Biol. *67*, 863–886.

Spray, D.C., Harris, A.L., and Bennett, M.V.L. (1981). Equilibrium properties of a voltage-dependent junctional conductance. J. Gen. Physiol. *77*, 75–93.

Stutenkemper, R., Geisse, S., Schwartz, H.J., Look, J., Traub, O., Nicholson, B.J., and Willecke, K. (1992). The hepatocyte-specific phenotype of murine liver cells correlates with high expression of connexin32 and connexin26 but very low expression of connexin43. Exp. Cell Res. *201*, 43–54.

Swenson, K.I., Jordan, J.R., Beyer, E.C., and Paul, D.L. (1989). Formation of gap junctions by expression of connexins in *Xenopus* oocyte pairs. Cell *57*, 145–155.

Swenson, K.I., Piwnica-Worms, H., McNamee, H., and Paul, D.L. (1990). Tyrosine phosphorylation of the gap junction protein connexin43 is required for the $pp60^{v\text{-}src}$-induced inhibition of communication. Cell Regul. *1*, 989–1002.

Valdimarsson, G., De Sousa, P.A., Beyer, E.C., Paul, D.L., and Kidder, G.M. (1991). Zygotic expression of the connexin43 gene supplies subunits for gap junction assembly during mouse preimplantation development. Mol. Reprod. Dev. *30*, 18–26.

Veenstra, R.D. (1990). Voltage-dependent gating of gap junction channels in embryonic chick ventricular cell pairs. Am. J. Physiol. *258*, C662–C672.

Wang, H.-Z., Li, J., Lemanski, L.F., and Veenstra, R.D. (1992). Gating of mammalian cardiac gap junction channels by transjunctional voltage. Biophys. J. *63*, 139–151.

Warner, A.E., and Lawrence, P.A. (1982). Permeability of gap junctions at the segmental border in insect epidermis. Cell *28*, 243–252.

Weir, M.P., and Lo, C.W. (1982). Gap junctional communication compartments in the *Drosophila* wing disk. Proc. Natl. Acad. Sci. USA *79*, 3232–3235.

Werner, R., Levine, E., Rabadan-Diehl, C., and Dahl, G. (1989). Formation of hybrid cell-cell channels. Proc. Natl. Acad. Sci. USA *86*, 5380–5384.

White, T.W., Bruzzone, R., Goodenough, D.A., and Paul, D.L. (1992). Mouse Cx50, a functional member of the connexin family of gap junction proteins, is the lens fiber protein MP70. Mol. Biol. Cell *3*, 711–720.

Willecke, K., Heykes, R., Dahl, E., Stutenkemper, R., Hennemann, H., Jungbluth, S., Suchyna, T., and Nicholson, B.J. (1991). Mouse connexin37: cloning and functional expression of a gap junction gene highly expressed in lung. J. Cell Biol. *114*, 1049–1057.

Zhang, J.-T., and Nicholson, B.J. (1989). Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. J. Cell Biol. *109*, 3391–3401.