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Establishment of Tight Junctions Between Cells from Different Animal Species and Different Sealing Capacities

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Summary. Epithelial cells establish tight junctions (TJs) that offer an ample range of transepithelial electrical resistances (TER), in adjustment to physiological requirements. In the present work, we demonstrate that cells from different animal origins, co-cultured in monolayers, can make sealed TJs, suggesting that this structure has a basic universal structure. TJs cannot be established, however, if one of the partners does not normally express TJs, indicating that each neighbor has to contribute its moiety. Furthermore, we observe that clones of the same cell line, with widely different values of TER, do not differ in the number and length of their junctional strands, suggesting that the difference is due to their ability to express ionic channels traversing their strands. The value of TER achieved in mixed monolayers of cells of the same or different lines is the one that may be expected by taking into account the proportion of each type in the mixture and adding in parallel the electrical resistance that they exhibit in pure monolayers. Therefore, epithelial TJs appear to behave as parallel resistances.

Key Words tight junctions · cell attachment · epithelia · cultured monolayers · freeze fracture · ruthenium red · paracellular route

Introduction

Tight junctions (TJs) located at the outermost end of the interspace between the cells enable epithelia to act as diffusion barriers (Farquhar & Palade, 1963). The degree of sealing of these TJs varies over a wide range. Thus, the transepithelial electrical resistance (TER) may be as low as 10 $\Omega \cdot cm^2$ in the proximal tubule of the kidney, or as high as several thousand ohms in the epithelium of the urinary bladder. This ability to make TJs is retained in some established cell lines, whose values of TER vary also over a wide range, not only among different cell lines, but in different clones of the same line as well (Cereijido et al., 1978*a,b;* Simmons 1981). This range as TERs is not due to variations in the conductance across the transcellular, but across the paracellular route limited by the TJ (Cereijido, Stefani & Martinez-Palomo, 1980; Stefani & Cereijido, 1983). According to current models of TJs, these variations may involve: (i) the length of the intercellular cleft around the cells; (ii) the number of strands opposed to the flux of current; and (iii) the probability that permeating channels across these strands would be in an open or in a closed state. Therefore, the first aim of this article is to analyze morphometric parameters of the TJ of two clones of MDCK cells with different values of TER, to establish which of these factors is responsible for this difference. The second aim is to assess whether the degree of sealing achieved by co-culturing two clones with different values of TER would be the one that may be expected from their proportion in the mixture and the sum in parallel of their individual TERs. The third aim relates to the specificity of TJs, i.e., given two cell lines derived from different animal species, each one with the capacity to make TJs, would they form sealed TJs when mixed in a monolayer and, in this case, would the value of TER of the monolayer depend on their porportions in the mixture and the sum of their individual TERs? The fourth aim is to study whether, in order to seal the intercellular space, it is sufficient that one cell can attach to the partner and confer on it the ability to make TJs. For this purpose we co-cultured epithelial and nonepithelial cells in monolayers.

Materials and Methods

Cell Culture

Starter MDCK cultures (Madin & Darby, 1958) derived from the kidney of a normal dog were obtained from the American Type Culture Collection (MDCK, CCL-34). In most experiments, cells were between 60–80th passage. Cells were grown at 36.5°C in disposable plastic bottles (Costar 3150, Cambridge, MA) with an

air (5% CO_2) atmosphere (VIP CO_2 incubator 417, Lab. Line Instruments, New Brunswick, NJ) and 20 ml of Dulbecco's modified Eagle's basal medium DMEM (Grand Island Biological Co., GIBCO, 430-1600, Grand Island, NY) with 100 U/ml of penicillin, 100 μg/ml of streptomycin (In Vitro S.A., Mexico D.F.), 0.08 U/ml of insulin (Eli Lilly, Mexico D.F.), and 10% fetal bovine serum (Flow Laboratories, McLean, VA); this complete medium is referred to in the following text as CDMEM. Cells were harvested with trypsin-EDTA (In Vitro S.A., Mexico D.F.) and plated on nitrocellulose filters (RAWP 013-00; Millipore Corporation, Bedford, MA) contained in a tissue culture multiwell plate (Falcon, Labware, Oxnard, CA), or on tissue culture plates (Lux 5221, Miles Laboratories, Naperville, IL). To eliminate floating cells, 1 hr after plating the medium was replaced with fresh CDMEM.

For co-culturing, besides the wild MDCK cells, the following renal epithelial lines were used: high resistance MDCK (dog); PtK_2 (Kangaroo rat); MDBK (bovine); MK_2 and Ma-104 (rhesus monkey); LLC-RK_1 (rabbit); LLC-PK_1 (pig). Ma-104 and high resistance MDCK cells were a generous gift from Dr. Pedro Salas. CPA_{52} (bovine pulmonary artery) and the fibroblastic VERO cells (green monkey kidney) were a generous gift from Dr. Fernando Vargas and Dr. Enrique Rodriguez-Boulan, respectively. Other lines were purchased from the American Type Culture Collection. All these cell lines were cultured in CDMEM with 0.1 mM nonessential amino acids (Sigma Chemical Co., MT145) and 2 mM glutamine (GIBCO, 320-5039).

To prepare mixed monolayers, cells were incubated with EDTA in PBS without Ca^{2+} for 30 min. This procedure allowed them to separate from each other while still attached to the substrate. Cells were then harvested with trypsin-EDTA (2 min), filtered through a nylon mesh with 20-μm windows to avoid possible cell clumps, counted (Coulter Electronics, Hialeah, FL), mixed in the desired proportion and plated on nitrocellulose filters.

Transepithelial Electrical Resistance (TER)

The degree of sealing of the TJs was assessed by measuring the TER (Cereijido et al., 1978*a*). The filter with the monolayer was mounted as a flat sheet between two Lucite chambers with an exposed area of 0.69 cm^2. Current was delivered via Ag/AgCl electrodes placed 2.0 cm from the monolayer; the voltage deflection elicited was measured with a second set of electrodes placed 1.0 mm from the membrane. Values of TER reported were obtained by subtracting the contribution of the filter and the bathing solution. A given monolayer was used only once and discarded.

Transmission Electron Microscopy (TEM)

Monolayers were fixed with 2.5% glutaraldehyde for 1 hr. After washing in 0.1-M cacodylate buffer, cells were postfixed in 1% OsO_4-cacodylate buffer with ruthenium red (0.5 mg/ml), then dehydrated in ethanol and embedded in Epon 812. Thin sections were cut with a diamond knife, then examined and photographed in a Zeiss EM 10 electron microscope.

Freeze Fracture Electron Microscopy

Freeze fracture replicas were obtained from monolayers fixed with 2.5% glutaraldehyde for 30 min, and gradually infiltrated with glycerol up to 20% concentration, in which they were left for 1 hr. The monolayers were then detached from the substrate and frozen in the liquid phase of Freon 22 cooled with liquid nitrogen. Freeze fracture was carried out at -120°C, and 2×10^{-6} mm Hg using a Balzers apparatus (FL 9496 Balzers, Leichenstein) equipped with a turbomolecular pump. After evaporation of platinum and carbon, replicas are shown with the shadowing direction from bottom to top.

To assess modifications in the pattern of TJ strands, we counted the number of strands intercepting lines drawn perpendicular to the main axis of the junction. These procedures were previously described in detail elsewhere (González-Mariscal et al., 1985).

Scanning Electron Microscopy

Cell monolayers were fixed with glutaraldehyde and dehydrated. Critical point drying from absolute ethanol into liquid CO_2 was carried out in a Technics (Springfield, IL) apparatus. Dried specimens affixed to aluminum stubs with double-sided tape were coated in an ion sputter (JFC-1100, JEOL, Japan) evaporator with gold. Specimens were examined in a JEOL JSM 35C scanning electron microscope at 15 kV.

[^{35}S]-Methionine Labeling

Two μCi of ^{35}S-methionine per ml of culture media were added to a confluent cell monolayer. They were thoroughly washed 24 hr later and left in media for 48 hrs with 2 mM cold methionine. Cells were harvested, mixed with unlabeled cells and plated on a multiwell plate. Each well was thoroughly washed 24 hr later with PBS, and the monolayers were digested for 1 hr with 0.5-M NaOH. Measurements were done on the ^{14}C channel of a Packard 2000B Counter.

Results are expressed as mean ± SE (number of observations).

Results

Since this study involves the use of different cell types, the first step was to select them. Table 1 shows the value of TER in monolayers of the epithelial and nonepithelial cell lines chosen, prepared by plating them at confluence. TER was determined the day after, but in some cases it was also followed up to seven days after plating. The value of TER was of course much higher in epithelial than in nonepithelial cells, but it may vary by 15-fold among epithelial cells, e.g., MDCK (HR) *vs.* MK_2. TER remained remarkably constant in monolayers of some cell types (e.g., MDCK, MDBK), but it rose by 200% in some others, e.g. MDCK (HR), PTK_2. To avoid these variations in the evolution of TER when working with mixed populations of cells, comparisons were made one day after plating.

The second step was to compare the relationship between TER and TJ structure in two different clones of the same type of cells. Table 2 shows the values of TER obtained in MDCK, 185 ± 17 (23) Ω ·

Table 1. Electrical resistance across monolayers of cultured cells

Cell line	Origin	Days after plating			
		1	2	3	7
		($\Omega \cdot cm^2$)[a]			
MDCK	Dog kidney (epithelial)	217 ± 7 (152)	236 ± 12 (8)	206 ± 14 (31)	186 ± 9 (5)
MDCK (HR)	Dog kidney (epithelial)	316 ± 28 (5)		625 ± 32 (22)	789 ± 47 (5)
PTK_2	Kangaroo rat kidney (epithelial)	87 ± 11 (9)		168 ± 39 (8)	
MDBK	Bovine kidney (epithelial)	16 ± 1 (4)		15 ± 3 (4)	
MK_2	Rhesus monkey kidney (epithelial)	18 ± 4 (16)		11 ± 2 (6)	
$LLCRK_1$	Rabbit kidney (epithelial)	33 ± 9 (5)		40 ± 14 (5)	
MA-104	Rhesus monkey kidney (epithelial)	42 ± 4 (25)		50 ± 6 (5)	
$LLC\text{-}PK_1$	Pig kidney (epithelial)	164 ± 12 (23)			
CPA_{52}	Bovine Pulmonary artery (endothelial)	8 ± 2 (6)		7 ± 1 (6)	
VERO	Green monkey kidney (fibroblast)	5 ± 1 (17)			

Cells were plated at confluence on Millipore filters (pore diameter 1.2 μm).

[a] Transepithelial resistance was measured through the voltage deflection elicited by a 100 $\mu A/cm^2$ current pulse. The contribution of media and support was subtracted.

Table 2. Comparison of theoretical and experimental transepithelial electrical resistance (TER) in wild and high resistance MDCK cells

Cell type	Specific junctional resistance	Linear amount of TJ per unit area	n	TER	
				theor.	exper. ($\Omega \cdot cm^2$)[a]
	($\Omega \cdot$ cm)	(cm/cm^2)			
MDCK	2.64×10^4	765 ± 23	111	35	185 ± 17 (23)
MDCK (HR)	2.64×10^4	792 ± 41	135	29	625 ± 32 (22)

[a] Mean ± SE (number of observations).

cm^2, and in MDCK (HR) cells, 625 ± 32 (22) $\Omega \cdot cm^2$, three days after plating. This difference in TER is accounted for by differences in the paracellular permeation route limited by the TJ (*see* Appendix 1). Routine inspection of freeze fracture replicas of TJs of both types of monolayers as those shown in Fig. 1, revealed no major differences in their number and arrangements of the strands. However, a valid comparison requires a quantitative morphological analysis of the strands, and an evaluation of the electrical resistance that such pattern of strands would offer. Morphometric analysis is based on counting the number of strands every 133 nm of TJ and determining the fraction of the junctional belt having a given number of strands. Figure 2 shows the result of the analysis performed in monolayers of both clones. Each column represents the fraction (f_i) of the total TJ composed of a given number (i) of strands (e.g., wild MDCK cells have $f_3 = 21$; and high resistance MDCK ones have an $f_3 = 23$). No major differences in the distribution of strands is observed.

The relationship between the total resistance (R_T) offered by a TJ and the distribution of its strands is given by Eq. (6) (Appendix 2). Inserting in this equation the values of f_i for wild and high resistance MDCK cells, and taking the values of specific resistances R_i for fragments with 1, 2 . . . n strands found by Claude (1978), one obtains the specific junctional resistance for the strands of wild ($2.64 \times 10^4 \Omega \cdot$ cm) and high resistance MDCK cells ($2.32 \times 10^4 \cdot$ cm) (Table 2). However, the resistance of the TJs in a given epithelium depends also on the linear amount of TJ, i.e., the length of the intercellular space surrounding the cells per cm^2 of monolayer. This was in turn evaluated by measuring directly with a planimeter the length of the intercellular cleft in scanning electron micrograph of monolayers of each clone (Fig. 3), as described by Madara and coworkers (Marcial, Carlson & Madara, 1984; Madara & Dharmsathaporn, 1985). The linear amount of TJ found in monolayers of each type was 765 ± 23 (111) for the wild, and 792 ± 41 (135) cm/cm^2 for the high resistance MDCK cells,

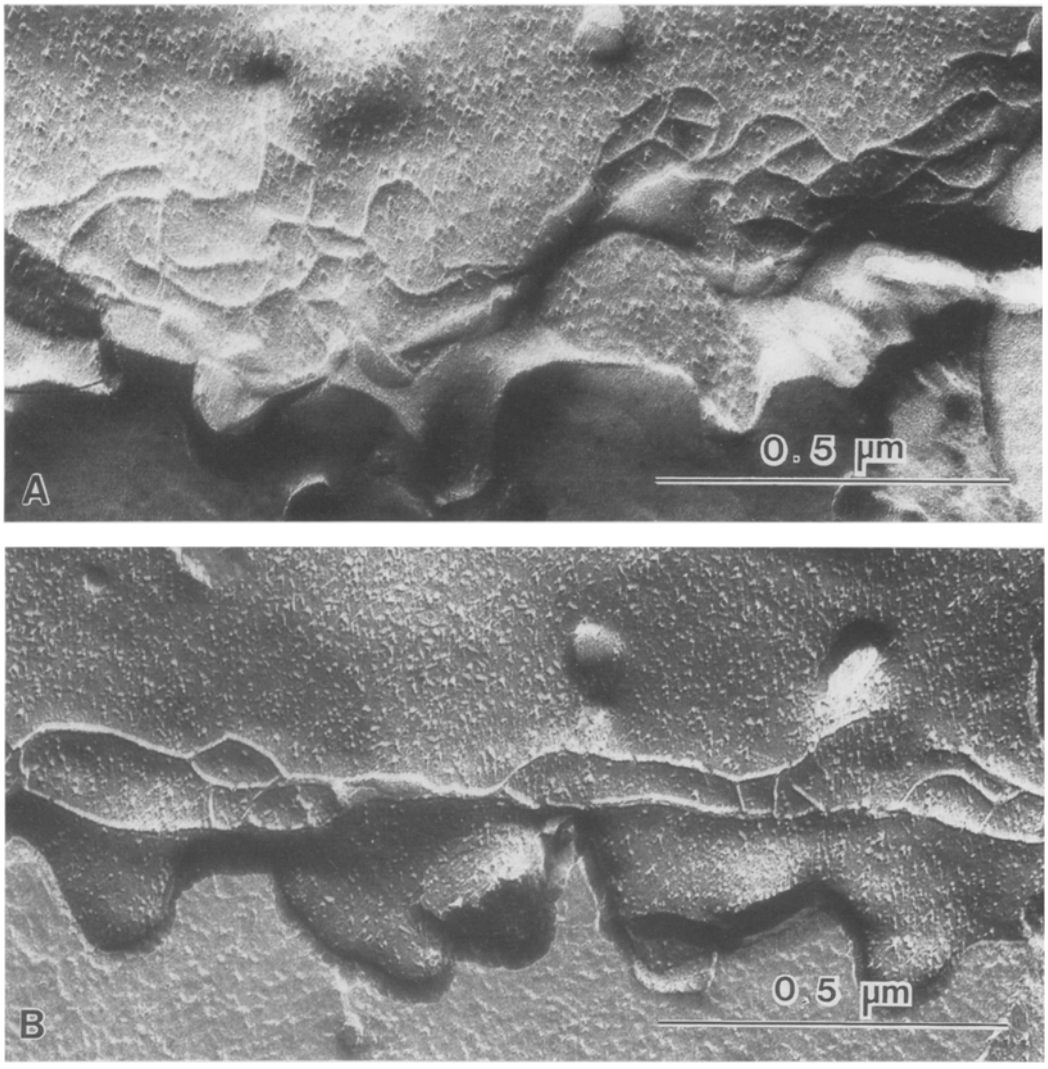

Fig. 1. Freeze fracture replicas of monolayers of wild (*A*) and high resistance MDCK cells (*B*). The two cell types were obtained by cloning a starter cell batch of MDCK obtained from the American Type Culture Collection. Cells were grown on Millipore filters, fixed with 2.5% glutaraldehyde, infiltrated with glycerol and processed as described in Materials and Methods. The horizontal bar corresponds to 0.5 μm

respectively (Table 2). Dividing the specific junctional resistance (in $\Omega \cdot$ cm) by the linear amount of TJ per unit area (in cm/cm^2) for each type of monolayer, one obtains theoretical values of 35 and 29 $\Omega \cdot$ cm^2 for monolayers of wild and high resistance cells, respectively. Therefore, this analysis indicates (i) that the amount of junction; (ii) the specific resistance of the TJ; and (iii) the theoretical resistance calculated on the number, distribution and frequency of segments with a given number of strands, is similar for the two cell types. However, the experimental TER measured simultaneously in both types of monolayers at the third day after plating is significantly different ($P \leq 0.001$). It may be noticed that experimental values of TER are remarkably higher than the theoretical ones that were derived from observations made in natural transporting epithelia. This may reflect the fact that cell lines achieve a comparatively low degree of differentiation, and may express a small number of channels across their TJs (*see below*). Number and length of strands are two of the most important variables in the resistance offered by a TJ, but the other basic factor is constituted by permeating channels traversing them (Claude, 1978). Therefore, results in Table 2 suggest that, at a given time, the strands in high resistance MDCK cells may contain a smaller number of channels in the open state.

Current models of junctional structure consider that a strand is not the protrusion of a cell mem-

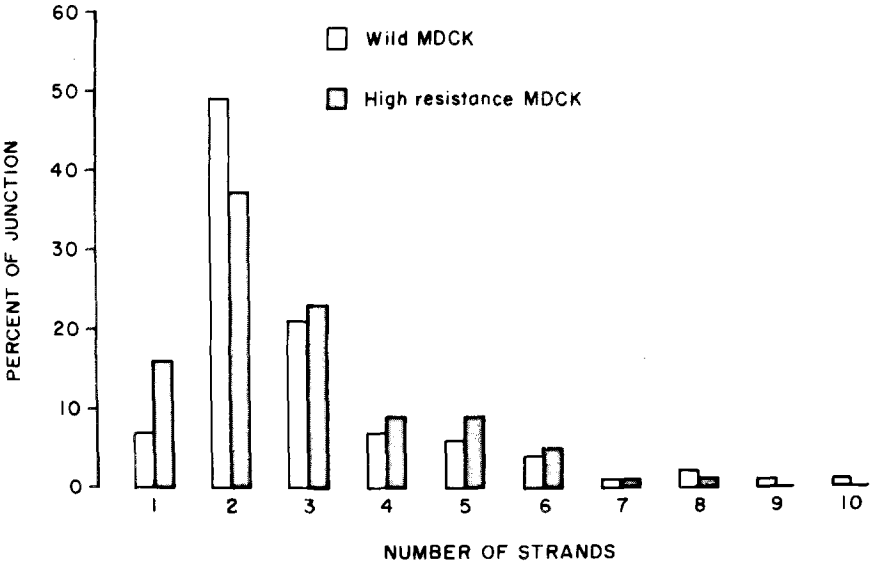

Fig. 2. Percent of the (f_i) TJ constituted by a given number of strands ($i = 1 \ldots n$), in monolayers of wild and high resistance MDCK cells, three days after plating at confluency. Pictures of freeze fracture replicas like the one shown in Fig. 1 were studied morphometrically, as described by González-Mariscal et al. (1984). A main line was drawn along the axis of the TJ, and secondary lines perpendicular to the first one were drawn every 133 nm. The number of strands intersecting each secondary line was recorded. f_i represents the probability of finding segments of the TJ with 1, 2 . . . n strands. It may be observed that the general pattern is similar in the two clones; segments with 2 strands being the ones most frequently found in both of them followed by segments with 3, and 1, etc.

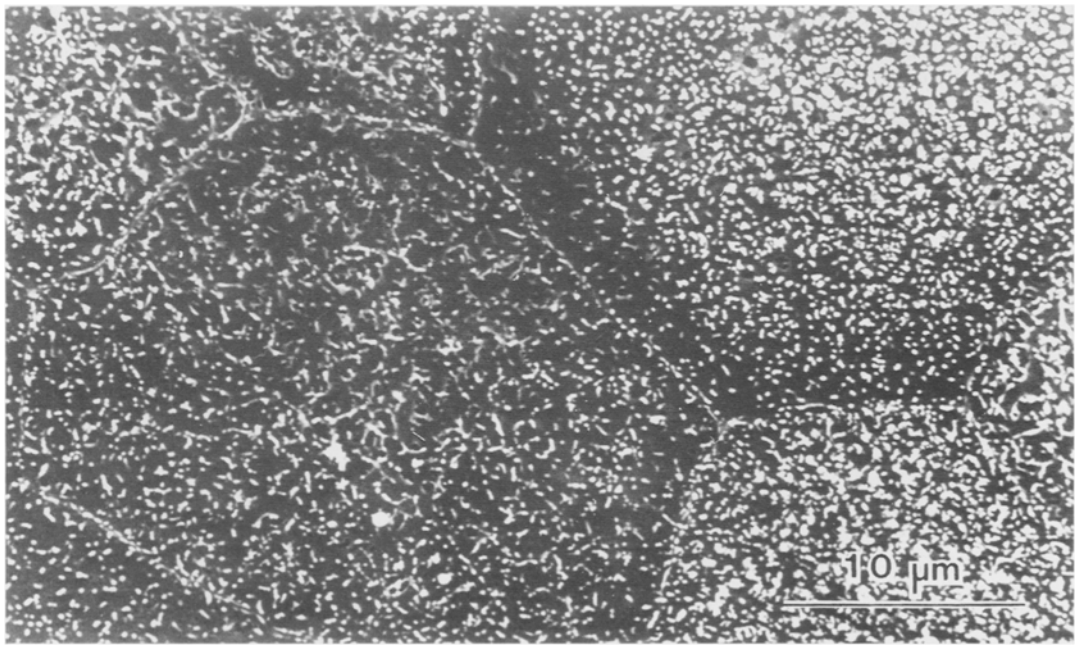

Fig. 3. Scanning electron micrograph of a confluent monolayer of MDCK cells showing the boundaries between neighboring cells. To measure the linear amount of TJ per unit area, cell borders are first marked on similar photographs with a pen, and then measured with a planimeter

brane that reaches and attaches to a neighboring one, but that each strand is formed by the two neighboring cells (for a review, *see* Cereijido et al., 1988). Therefore, the second question was: in monolayers prepared by mixing two cell types, would the value of TER reflect the parallel arrangement of elements afforded by each neighbor, as expected from current models? Would it be dominated

Table 3. Theoretical *vs.* experimental TER in monolayers of mixed[a] types of MDCK cells

Cell type	TER ($\Omega \cdot cm^2$)
Low resistance	236 ± 12 (8)
High resistance	625 ± 32 (22)
Mixed (theoretical)	306 ± 44
Mixed (experimental)	332 ± 18 (5)

[a] Mixed in a 50 : 50 ratio

by the cell type that makes more channels (the lower resistance one) or would it rather exhibit the ability to make the more resistant strands of the other clone? To answer this question we prepared monolayers by mixing equal numbers of cells from two clones of MDCK in a suspension, and plating them at confluence.

Table 3 shows the TER values of MDCK monolayers formed with individual clones and a mixture of both of them. The theoretical value (R_{theor}) of the mixture was calculated as follows:

$$\frac{1}{R_{Theor}} = \frac{F_1}{R_1} + \frac{F_2}{R_2} \quad (1)$$

where F_1 and F_2 are the fractions (0.5) of each cell type in the mixture, and R_1 and R_2 are their experimental values of TER. The fact that the experimental value found in the mixed monolayers is so close to the theoretical one, suggests that strands reflect the participation of wild and high resistance cells working in parallel and, therefore, supports current junctional models elaborated on the basis of freeze fracture information (Madara & Dharmsathaporn, 1985; Marcial et al., 1984).

The next question was whether these mixed strands could be made by cells from different animal species, and an associated question was: would junctions be made even if one of the mixed cell types had no ability to make them? Table 3 shows that the cells of the different types used exhibit a measurable value of TER. However, this does not necessarily indicate that they make TJs. Smulders, Tormey and Wright (1972) have shown that part of the electrical resistance offered by the paracellular route is accounted for by the narrow and tortuous intercellular space. This resistance is usually low but, when the total value of TER is also low, the intercellular space may account for a large fraction of this TER. When cells form sealed TJs, extracellular markers added to one side of the epithelium may be stopped at this level (Cereijido et al., 1978*b*). Therefore, they afford a useful method to assert whether an epithelium with a low value of TER would have permeable or impermeable TJs. Figures 4 to 7 illustrate the permeability (e.g. MDBK, Fig. 5*B*) or impermeability (e.g., MDCK, Fig. 4) to ruthenium red in monolayers prepared with different cell types. Whenever the penetration of marker was blocked, this blockade was made at the level of the TJ. To make a quantitative estimate we counted the number of intercellular spaces penetrated by the marker in the different monolayers (Table 4). As expected, we found an almost all-or-none pattern: all TJs of a given epithelium are permeable, or all of them are impermeable to ruthenium red.

Table 4. Penetration of ruthenium red beyond the occluding junction

Cell line	*n*	ICS Permeable to RR	%
MDCK	29	0	0
PTK_2	14	1	7
MDBK	23	23	100
MK_2	12	12	100
LLC-RK_1	17	17	100
MA-104	11	9	81
LLC-PK_1	27	0	0
CPA_{52}	8	8	100
VERO	30	30	100

ICS, intercellular space; RR, ruthenium red

Figure 8 compares the permeability to ruthenium red to the value of TER of the different monolayers studied. It seems clear that monolayers below some 40 $\Omega \cdot cm^2$ do not block the penetration of the marker, suggesting that they make no TJs and that their TER is due mainly to the resistance offered by the long, narrow, tortuous and in some cases interdigitated intercellular space. Figure 8 also includes data from nonepithelial cells that serve as a control of the criteria used: fibroblasts (VERO, triangle) and endothelial (CPA_{52}, filled circle). As expected, these have negligible values of TER and 100% of spaces penetrated by ruthenium red. We may now return to the question of whether these variety of cells will make sealed TJs when cocultured with MDCK cells.

Table 5 shows the theoretical *vs.* the experimental value of TER found in mixed monolayers. The theoretical value was obtained by inserting the experimental values of TERs from monolayers of single-cell types prepared and measured simultaneously, in Eq. (2). In general, we explored mixtures in a 50 : 50 proportion, but in some cases 75 : 25 and 25 : 75 MDCK cells were also studied. However, if the two cell types attach at a different

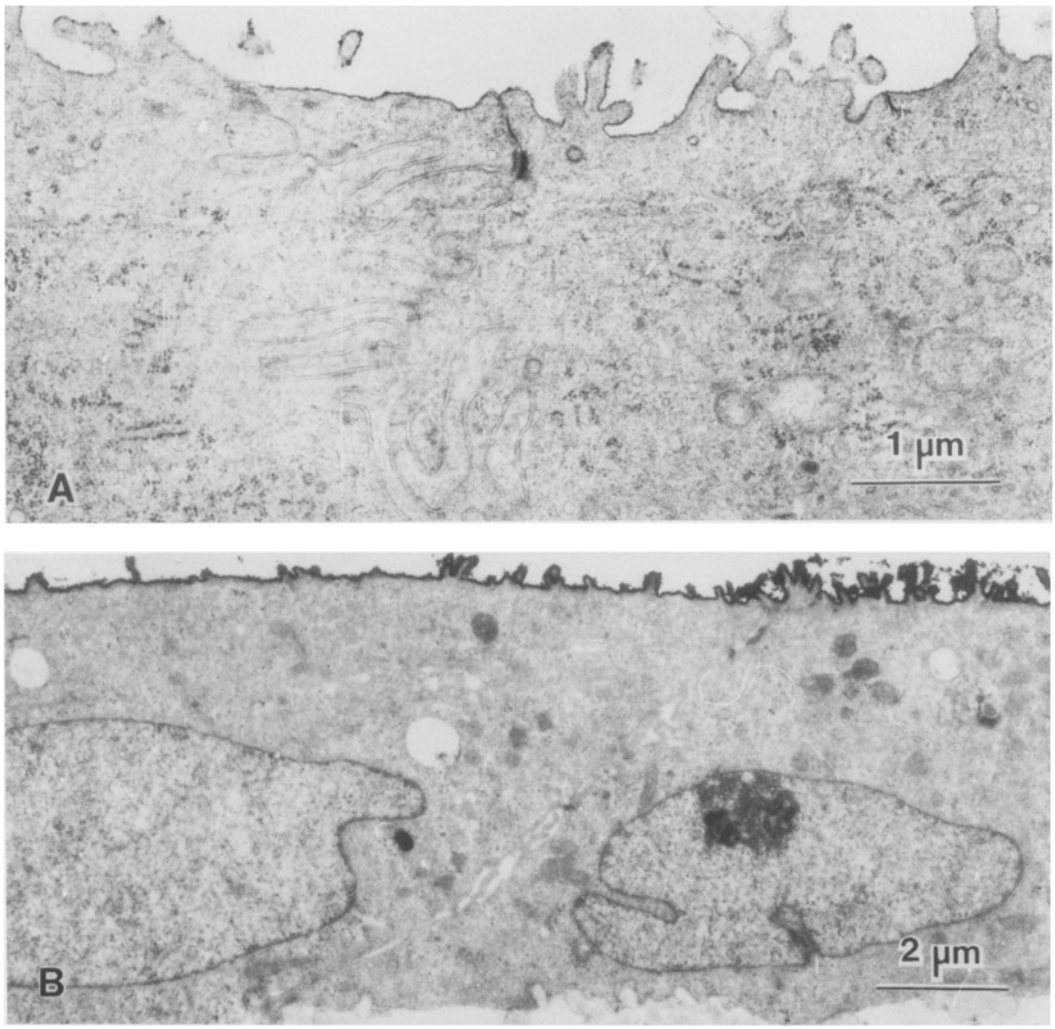

Fig. 4. Transmission electron micrographs of confluent monolayers of wild (*A*) and high resistance MDCK cells (*B*) treated on the apical side with ruthenium red. After fixing the monolayer with 2.5% glutaraldehyde, they were washed with cacodylate buffer and post-fixed in 1% OSO_4-cacodylate buffer with ruthenium red (0.5 mg/ml), then dehydrated in ethanol and embedded in EPON 812. It may be observed that the marker clearly stains the apical (upper) border, but does not penetrate into the intercellular space

speed, the resulting monolayer will not have the same proportion of cells as the suspension. To explore this possibility, we labeled the MDCK cells with [^{35}S]-methionine, mixed them with another unlabeled cell type in a given proportion, and prepared monolayers of pure labeled MDCK cells, and mixed monolayers containing labeled MDCK. Then, by counting the amount of ^{35}S in both sets of monolayers, it was possible to assert the proportion of each cell type. The first two columns in Table 5 show that the proportion experimentally found to attach follows closely the one in the plated mixture. Since comparisons involve theoretical and experimental values recorded in mixtures of different cell types and in different proportions, we found it convenient to represent discrepancies between theoretical and experimental values of TER as a function of the second one (Fig. 9). It may be observed that (i) regardless of the cell type involved, the higher the value of TER (better sealed TJs), the smaller the discrepancy, suggesting that the making of a TJ may be achieved by cells from different animal species, like PTK_2/MDCK (kangaroo rat/dog) and LLC-PK_1/MDCK (pig/dog; (ii) mixtures of MDCK and fibroblastic cells (VERO, triangles in Fig. 10) present the largest discrepancies; (iii) points below 40 $\Omega \cdot cm^2$ in the abscissa correspond to mixtures with epithelial cells that make no TJs, as revealed by the fact that in pure monolayers these allow the passage of ruthenium red. This indicates that the ability of a single neighbor (the MDCK cell) to make TJs, is not sufficient to establish them.

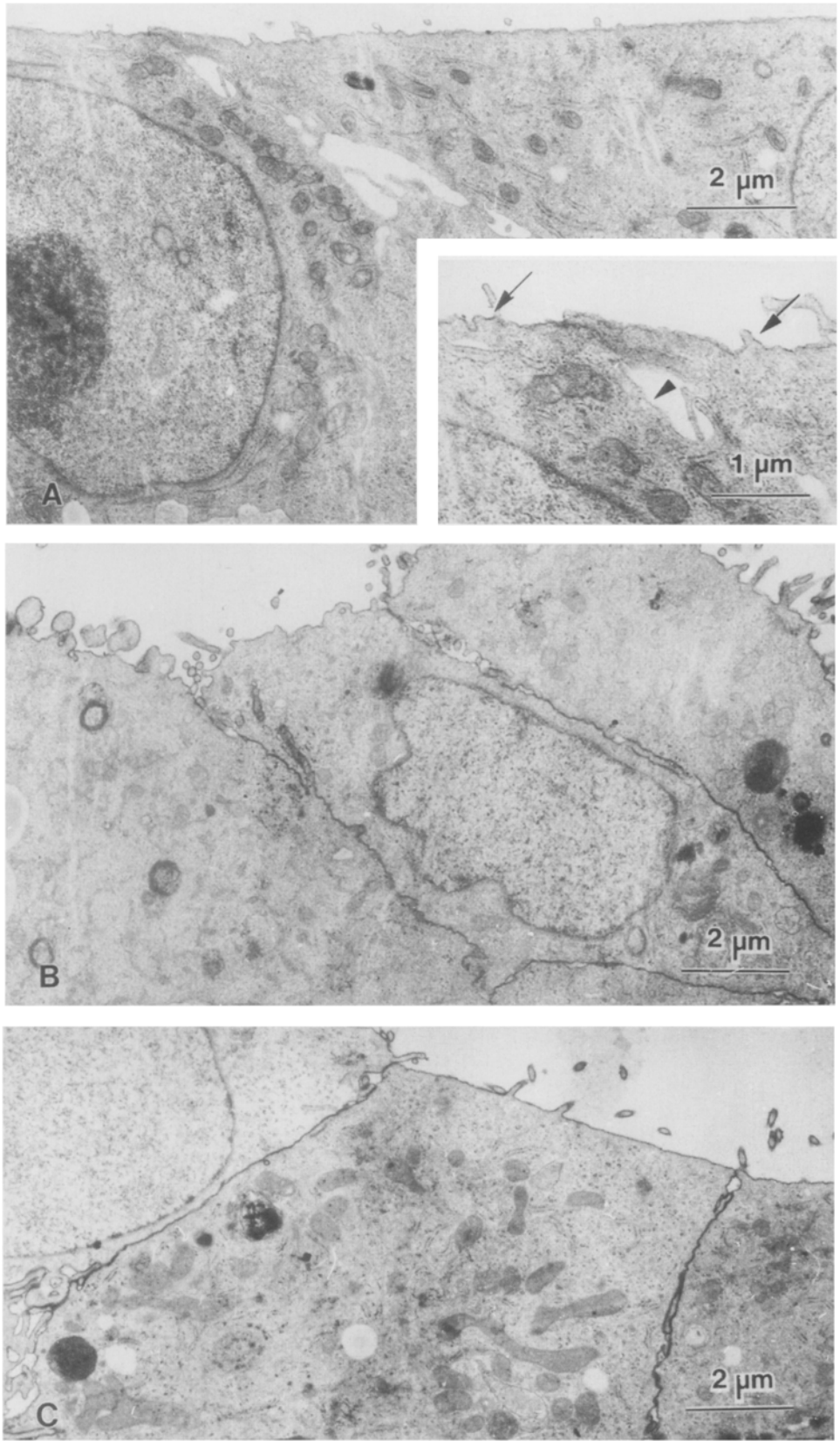

Fig. 5. Transmission electron micrographs of confluent monolayers of PTK_2 (*A*); MDBK (*B*); and MK_2 cells (*C*) treated with ruthenium red on their apical side, as in Fig. 4. The arrows in *A* show the apical surface stained with ruthenium red, and the arrowhead marks the intercellular space free of stain. Notice that the marker has free access to the intercellular region of the other two cell types

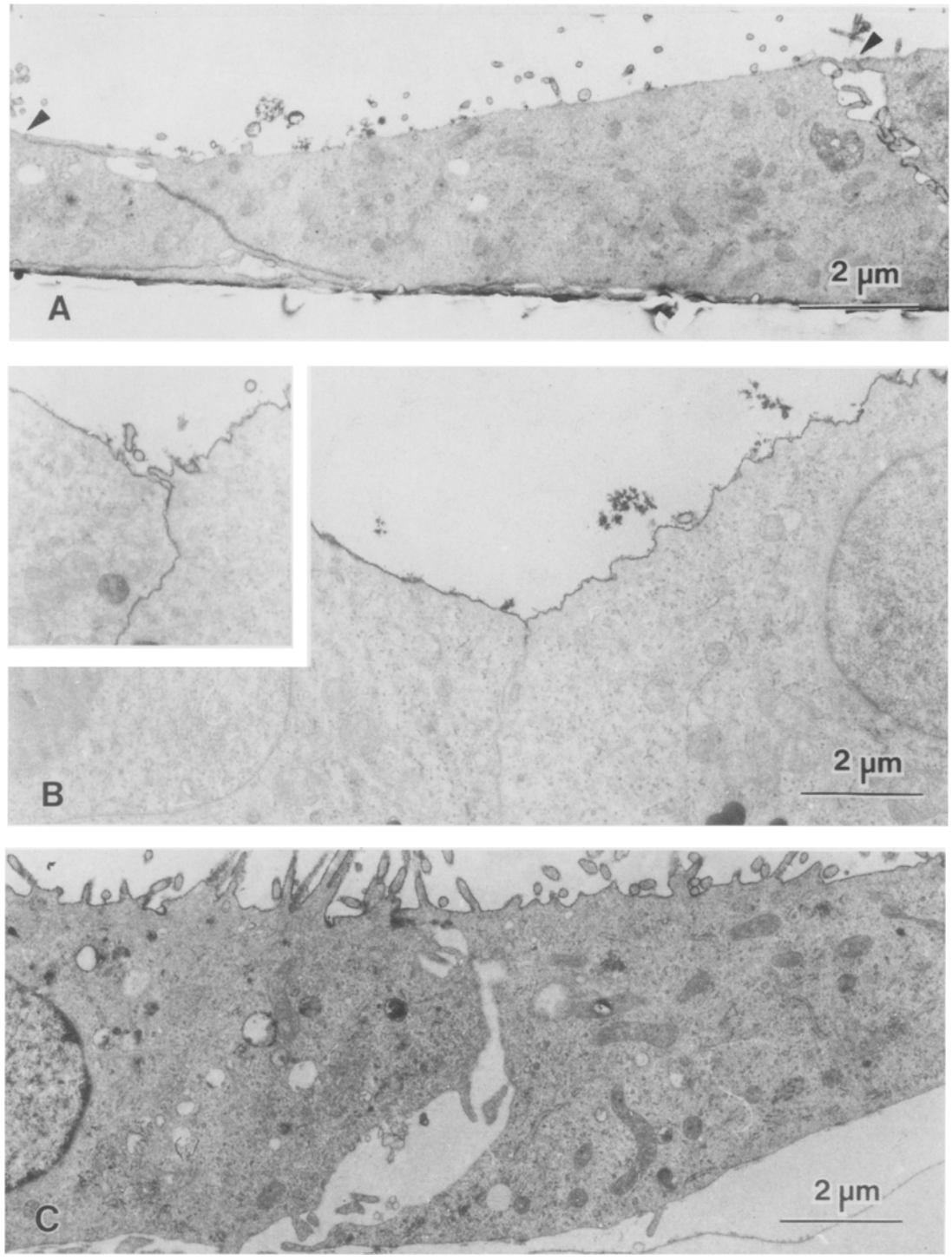

Fig. 6. Transmission electron micrographs of confluent monolayers of LLC-RK$_1$ (A); Ma-104 (*B*); and LLC-PK$_1$ cells (*C*) treated with ruthenium red on their apical side as in Fig. 4. In monolayers of the first type of cells, the marker readily penetrates the intercellular space, but in the case of LLC-PK$_1$ cells it does not. In the case of MA-104 cells though, some spaces are easily penetrated (*B*, *insert*), some others are not (*B*, *right*). Table 4 shows the percent of spaces in each type of monolayer that are permeable to ruthenium red

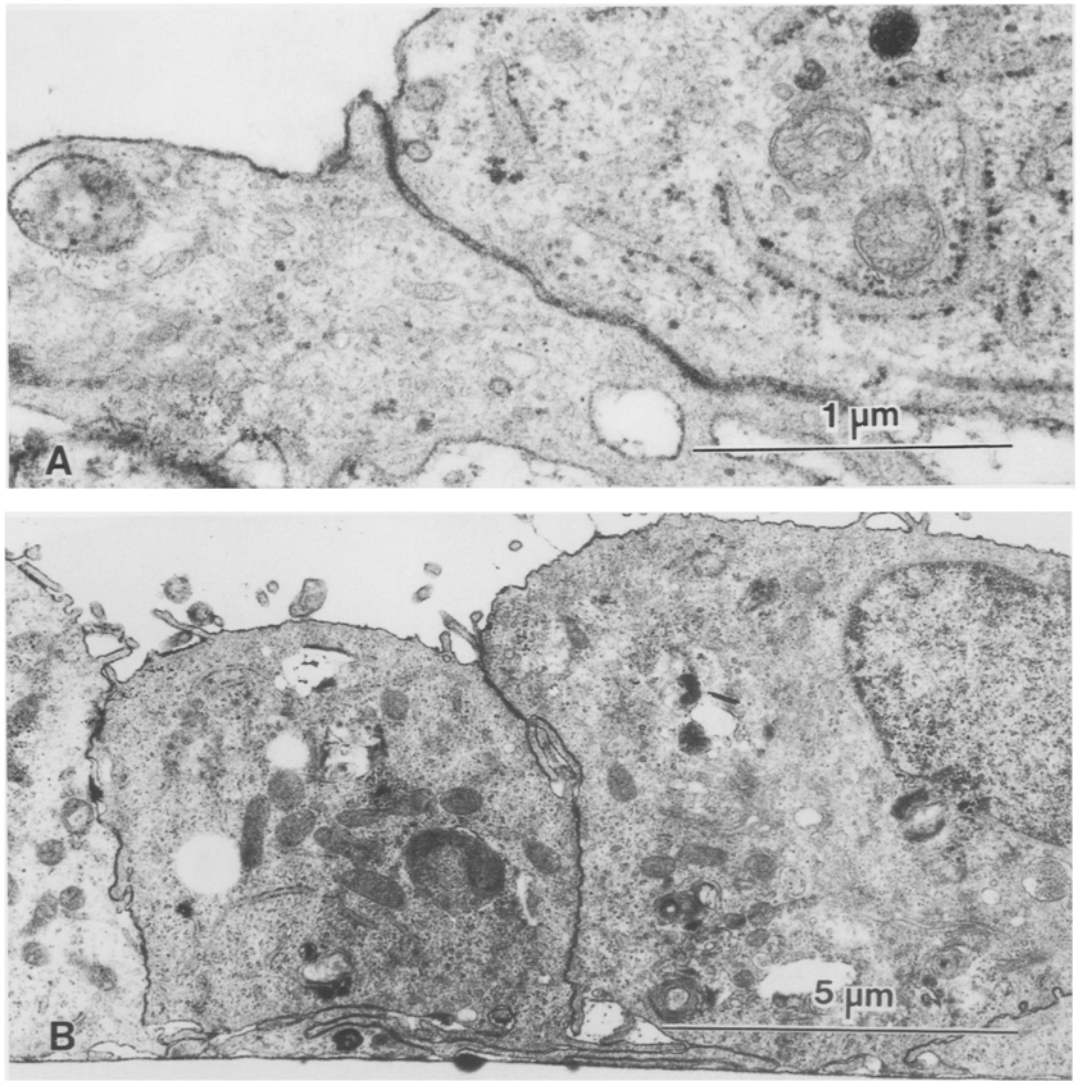

Fig. 7. Transmission electron micrographs of confluent monolayers of CPA_{52} (*A*) and VERO cells (*B*) treated with ruthenium red on their apical side as in Fig. 4. The first line is of endothelial origin and the second is of fibroblastic type. The intercellular space between them is freely penetrated by the marker

Discussion

Claude and Goodenough (1973) have found that the value of TER of different segments of the renal tubule keeps a close relationship with the number of strands in their TJs. Pursing that study, Claude (1978) compared the number of strands and values of TER of a variety of epithelia, and found that two epithelia with the same number of strands, but with a different number of cells per square centimeter, or with intercellular spaces with a different degree of interdigitation, would also have a marked difference in their values of TER. Accordingly, she introduced the linear amount of intercellular space as another important variable in the model. However, even when all these variables were taken into account, junctional resistance did not increase linearly with the number of strands, as it would be expected by the addition of resistors in series, but increased exponentially, as if it were traversed by channels that fluctuate between an *open* and a *closed* state. Only during the coincidence of open states in all the series of strands would a segment of TJ become conductive. In agreement with this possibility, in a previous work we found that when the temperature of incubation of monolayers of MDCK cells is lowered from 37 to 4°C there is no detectable change in the number and distribution of strands of the TJ, yet the value of TER increases reversibly by 305% (González-Mariscal, Chávez de Ramirez & Cereijido,

Table 5. Theoretical *vs.* experimental TER in monolayers of mixed cell types

Cell line	% of cells in the mixture		TER	
	Plated	Experimental[a]	Theoretical[b]	Experimental[c]
			($\Omega \cdot cm^2$)	
PtK_2	50	47 ± 1 (6)	127 ± 8	119 ± 15 (7)
MDBK	75	72 ± 3 (4)	22 ± 1	35 ± 2 (8)
	50	54 ± 1 (8)	28 ± 2	31 ± 2 (19)
	25	34 ± 2 (4)	47 ± 2	82 ± 9 (7)
MK_2	50	45 ± 1 (8)	36 ± 5	31 ± 3 (8)
LLC-RK_1	50	55 ± 1 (8)	53 ± 8	84 ± 4 (8)
MA-104	75	72 ± 3 (4)	47 ± 5	35 ± 2 (8)
	50	54 ± 1 (8)	58 ± 5	31 ± 2 (19)
	25	34 ± 2 (4)	80 ± 6	82 ± 9 (7)
LLC-PK_1	75	77 ± 3 (4)	174 ± 10	184 ± 16 (21)
	50	51 ± 2 (4)	186 ± 9	185 ± 16 (22)
	25	23 ± 2 (4)	202 ± 8	239 ± 22 (15)
CPA_{52}	50	51 ± 2 (6)	15 ± 3	9 ± 3 (8)
VERO	75		7 ± 3	20 ± 6 (9)
	50	55 ± 3 (6)	10 ± 2	36 ± 5 (15)
	25		18 ± 1	55 ± 9 (10)

[a] Measured with ^{35}S-methionine at the moment of TER determination

[b] $\frac{1}{R_T} = \frac{f_1}{R_1} + \frac{f_2}{R_2}$

[c] Resistance was measured as in Table 1

1984). Furthermore, TJs in MDCK cells discriminate between cations and anions, and between different species of cations, and this ability does not develop after plating at confluence with the same time course as the development of TER, supporting the notion that sealing and ion-conducting elements are different entities, which are mounted at different times (Cereijido et al., 1978*b*). Therefore, the probability that TJ pores are in the open state should be of equal importance to strand number in determining TJ resistance.

Another important variable stems from the fact that natural epithelia usually have more than one cell type, and these cells establish a dissimilar number of strands. Therefore, in order to calculate the value of TER, Madara and co-workers added the resistances presented by each segment (segments with a given number of strands in freeze fracture replicas) as if they were resistors arranged in parallel (Marcial et al., 1984; Madara & Dharmsathaphorn, 1985). This was precisely the approach used in the present study. This analysis indicated that the difference between high and low resistance MDCK cells is not due to discrepancies in the number and arrangement of junctional strands, and that some other factor must be involved. According to Claude's view, this factor would be the ability of the different clones to express permeation channels across these strands. In other words, the variability in the values of TER exhibited by different clones may not reflect a difference in the sealing capacity of their strands, but in the number of permeating channels, or in the time these channels spend in the open state.

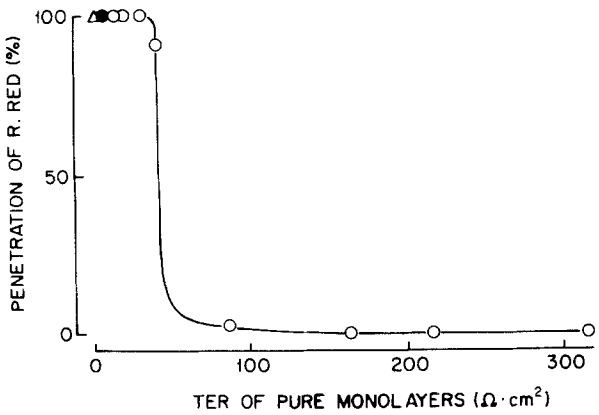

Fig. 8. Penetration of ruthenium red as a function of the electrical resistance across monolayers of a given cell type. Notice that not all experimental points represent epithelial lines, but the triangle and the filled circle correspond to VERO (fibroblasts) and CPA_{52} cells (endothelial). Monolayers with TER values below 40 $\Omega \cdot cm^2$ have all their space penetrated by the marker suggesting that their TER is not afforded by TJs, but reflects the restriction offered by the narrow and tortuous intercellular space

There are two major models of the molecular

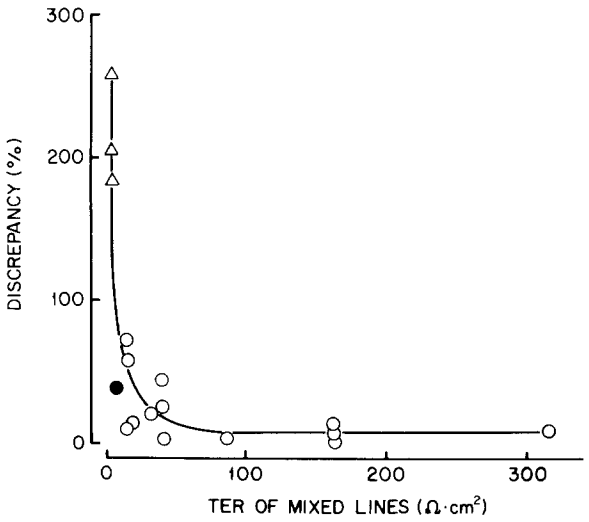

Fig. 9. Discrepancy between the value of TER predicted by Eq. (2) for mixed monolayers, and those measured experimentally, as a function of this experimental value. Discrepancy is calculated as:

$$\frac{TER_{Theoretical} - TER_{Experimental}}{TER_{Theoretical}} \times 100.$$

Mixed monolayers always contain cells of a given type plus wild MDCK ones. Triangles and the filled circle correspond to mixtures of MDCK cells with fibroblasts (VERO) and endothelial cells (CPA_{52}), respectively. The curve was drawn by eye. It is observed that when mixed monolayers are prepared with the two cell types that are able to make TJs in pure monolayers, they show a TER that has almost no discrepancy with that calculated with Eq. (2), indicating that TJs can be assembled and sealed by cells of different animal species. Discrepancy is high for mixtures with cell types that exhibit a small value of TER, and have intercellular spaces, which are permeable to runthenium red

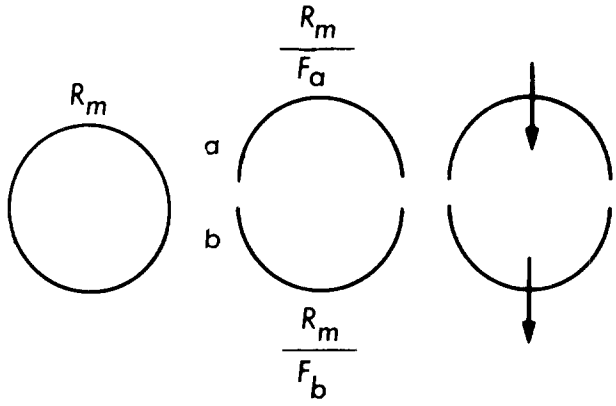

Fig. 10. Schematic representation of a hypothetical epithelial cell. R_m is the resistance of the whole membrane (*left*). To make a minimum estimate of the resistance across the transcellular route (*right*), it is assumed that R_m is equally distributed over the cellular membrane, so that fractions *a* and *b* have a resistance of R_m/F_a and R_m/F_b

structure of the TJ. The first one envisages the strands as formed primarily by a long chain of proteins (Staehelin, 1974; Bullivant, 1978; Hirokawa, 1982), and the second one considers them as long cylindrical lipidic micelles, with polar groups pointing toward the central axis, and with the hydrophobic chains fused to the lipidic matrix of the surface membranes of the two neighboring cells (Kachar & Reese, 1982; Pinto da Silva & Kachar, 1982; Meyer, 1983). The observation that TJs cannot be assembled if one of the partners does not form them in pure monolayers indicates that the two neighboring cells should participate in the establishment of a strand. It does not suffice that one cell would contact another and sink the hydrophobic chains of its lipid molecules in the lipidic matrix of the surface membrane of the neighbor. Therefore, the fact that fibroblasts fail to make TJs with epithelial cells might not be attributed to a mismatch of lipids in their membranes.

The observation that TJs may be established between cells of different animal species, provided they can make these junctions in monolayers of their individual type, indicates that the two cells should contribute a moiety, and that the molecules forming TJs should coincide. This situation is analogous to the one prevailing in the establishment of other types of cell-cell contacts. In fact, TJ formation may involve a similar underlying mechanism of recognition than the one necessary to make gap and adherents junctions. Thus, epithelial cells of different mammalian origin may establish communicating junctions between themselves (Epstein & Gilula, 1977), but not with fibroblast (Fentiman, Taylor-Papadimitriou & Stoker, 1976; Pitts & Burk, 1976). However, this does not seem to be a general condition of epithelial cells for lens (Fentiman et al., 1976) and *Xenopus* cells (Pitts, 1977) readily communicate with fibroblasts. Specificity for the establishment of gap junctions between epithelial and fibroblastic cells depends on the cell's origin. Thus, cells from an established cell line make gap junctions more selectively than cells from primary cultures (Hunter & Pitts, 1981). No significant amount of gap junctions seem to be established between heterologous insect cell lines and vertebrate ones (Epstein & Gilula, 1977). Communication may be also impaired when one of the partners is a malignant cell (Fentiman & Taylor-Papadimitriou, 1977). Alternatively, the making of a sealed TJ might not be reduced to the matching of complementary molecules in its strands, but may involve a long series of recognition and accommodation steps (Gumbiner, 1987). Thus, uvomorulin is a Ca^{2+}-dependent adhesion molecule

that has been localized to the adherents junction of the small intestinal epithelium by TEM immunocytochemistry (Boller, Vestweber & Kemler, 1985). Yet, when it is blocked in MDCK cells with antibodies during a resistance recovery assay developed by Martinez-Palomo et al. (1980), TJs do not reassemble (Gumbiner & Simons, 1986). In the case of a Ca^{2+} switch for *de novo* junctional assembly (González-Mariscal et al., 1985), TJ formation is inhibited until uvomorulin reappears at regions of cell-cell contact (Gumbiner, Stevenson & Grimaldi, 1988). In confluent established monolayers, anti-uvomorulin antibodies do not alter TER (Gumbiner et al. 1988). Thus, it seems that uvomorulin mediates an early adhesion event necessary for the establishment of TJs. It has been shown (Hirano et al., 1987) that uvomorulin concentrates at the boundary between different types of cells expressing an identical uvomorulin subclass, but no expression is detected between cells containing different uvomorulin subclasses. So, it appears that cell attachment mediated by Ca-dependent adhesion molecules may only be established by cells that express the same subclass of molecules. Thus, the lack of junction formation found in the present work between certain cells mixtures might be easily explained if the various cell lines used would express different cell-adhesion molecules.

In summary, the making of a TJ starts with recognition and accommodation of neighboring cells, and finishes with the insertion of sealing and ion-permeating elements contributed by the two partners. The universality of this process indicates that junctional components are well conserved, so that in a given epithelium TJs may be established by different cell types, and the whole epithelium may suffer transitions from one region of the organ to another, without dangerous gaps in the barrier between higher animals and the environment. Once established, TJs appear to behave as parallel resistances.

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Appendix I.
Differences in the values of TER is due to variations in the resistance of the TJ

Using the whole-cell clamp method, we have found that the electrical resistance of the cell membrane of a single MDCK cell (R_m) is around 2 GΩ (Bolivar & Cereijido, 1987). If we assume that this resistance is homogeneously distributed, a fraction (F_i) of cell membrane would have an electrical resistance of R_m/F_i (Fig. 10). Therefore, the electrical resistance of the transcellular route of a single cell will be given by the resistance of the apical fraction (F_a) plus the resistance of the basolateral (F_b) as follows:

$$\text{transcellular resistance of a single cell} = \frac{R_m}{F_a} + \frac{R_m}{F_b}. \tag{2}$$

The sum of the two fractions constitute the entire membrane:

$$F_a + F_b = 1. \tag{3}$$

So we can replace F_b in Eq. (2), and introduce the value of R_m found experimentally (2 GΩ):

$$\text{transcellular resistance of a single cell} = \frac{2\text{G}\Omega}{F_a - F_a^2}. \tag{4}$$

For example, if the apical fraction occupies 50% of the membrane, Eq. (4) will predict that the transcellular route across a single cell will have 8 GΩ. The 200,000 cells that occupy a square centimeter of monolayer would therefore offer a resistance ($R_{\text{transcellular}}$) of 40,000 Ω · cm². TER is given by:

$$\frac{1}{\text{TER}} = \frac{1}{R_{\text{Transcellular}}} + \frac{1}{R_{\text{Paracellular}}}. \tag{5}$$

Since TER is in the order of 600 Ω · cm² and $R_{\text{transcellular}}$ is in the order of 40,000 Ω · cm², Eq. (5) indicates that the paracellular route is some 100 times more conductive than the transcellular route. Notice that, if the electrical resistance of the cell membrane were not evenly distributed, and the fractions occupied by the apical and the basolateral sides were not identical (as we have assumed for this calculation), the resistance of the paracellular route would be still much lower than the transcellular one, so that the value of TER would always reflect mainly the resistance offered by the TJ.

Appendix II.
The electrical resistance offered by TJs composed by segments with a variable number of strands

Madara and coworkers have pointed out that the value of TER offered by these junctional belts can be calculated by adding in parallel the resistance of each segment (Marcial et al., 1984; Madara & Dharmshathaphorn, 1985). Therefore, the two variables involved are i) the length of each segment, which is proportional to the frequency (f_i) of finding segments with i strands in freeze fracture replicas of the TJ; and ii) the specific resistance (R_i) offered by segments with i strands, as follows:

$$\frac{1}{\text{TER}} = \sum_{i=1}^{i=n} \frac{f_i}{R_i}. \tag{6}$$