Proc. Nat. Acad. Sci. USA
Vol. 71, No. 3, pp. 880–884, March 1974

The Membrane Junctions in Communicating and Noncommunicating Cells, Their Hybrids, and Segregants

(gap junction/coupling/genetic analysis/cell fusion/electron microscopy)

R. AZARNIA, W. J. LARSEN, AND W. R. LOEWENSTEIN

Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Florida 33152

Communicated by Stephen W. Kuffler, October 31, 1973

ABSTRACT Human Lesch-Nyhan cells, which are coupling and have gap junctions, were fused with mouse cl-1D cells, which are noncoupling and lack gap junctions. The resulting hybrid cells were coupling and had gap junctions while they contained the nearly complete complement of parent chromosomes. As the hybrid cells lost human chromosomes, clones appeared among the segregants, which had reverted to the noncoupling and junction-deficient trait of the mouse parent cell. The human cell appears to contribute a genetic factor to the hybrids that corrects the junctional deficiency of the mouse cell.

The cells of many tissues form coupled systems in which molecules of a certain size range can diffuse directly from one cell interior to another (1–5). This has been shown by electrical measurement and by studies with intracellular tracer molecules. The coupled cells behave as if connected by passageways that are sealed off from the exterior; the diffusion between cell interiors is rapid compared to outward diffusion (1, 6).

The search for the cell-membrane differentiation implied by this coupling has been the object of many ultrastructural studies. Among the junctional structures of cell membranes that have been distinguished in the electron microscope, the *gap junction* (7) appears a likely site for the coupling. This disk-shaped structure presents subunits that are often hexagonally arrayed (8–10); and, in freeze-fracture, subunits are seen as membrane particles closely packed and in register in the two membranes (11–15). The gap junction is likely to contain the coupling passageways, because (*i*) it has been seen in many kinds of coupling cells both in organized tissues and in culture (4, 5, 16, 17)—in two instances it is the only detectable differentiated junctional structure (18, 19)—and (*ii*) it has not been seen in the one noncoupling cell type hitherto scrutinized (19).

However, the argument implicating the gap junction in coupling, although attractive, is not entirely free of pitfalls: in many cases the gap junction coexists with other differentiated junctional structures. Even in the two known instances where the gap junction seems to be the only structure with a pattern, there are, besides, other junctional regions where the membranes are closely apposed. Although these regions appear unpatterned with the staining and fixation techniques used, there are no compelling reasons to exclude them as possible coupling sites. The fact that close membrane appositions occur in some noncoupling cell kinds is, of course, hardly conclusive evidence; there may be "unpatterned" close membrane appositions of different kinds that are at present not distinguishable from each other. The strongest argument for mediation of coupling by gap junction is the important finding by Gilula *et al.* (19) that a line of noncoupling cells in culture has no detectable gap junctions. Yet even here there is the remote possibility that the electron-microscopic result reflects differences in detectability of gap junction in different types of cells, rather than actual differences in junctional structure.

Thus, on the whole, the available evidence for the gap junction serving as a site of cell coupling is entirely circumstantial and, hence, entails some risks of being deceptive. We have, therefore, thought it useful to take a new approach, in which we attempt to establish a genetic correlation between junctional structure and coupling. We hybridize noncoupling with coupling cells, and examine the junctional structure and the coupling in the hybrids and their segregants. The approach is an outgrowth of the recent finding in our laboratory that the coupling defect in certain noncoupling cell strains behaves like a recessive character; the defect is corrected by hybridization with coupling cells (20).

For the present work we chose as partners for hybridization a coupling human cell in which the gap junction is the only discernible differentiated junctional structure, and a noncoupling mouse cell in which no differentiated junctional structure at all is detectable. The choice of these two cell types was guided, besides, by the following features: the cells are genetically marked by enzyme defects so that one can conveniently obtain pure populations of hybrid cells; the karyotypes of the cells are readily distinguishable; and the hybrid cells lose chromosomes at a rate appropriate for experimental analysis of segregants. For all these reasons, the two cell types are unusually suitable for the purposes of the present work.

MATERIALS AND METHODS

Parent Cells. We used *human* Lesch–Nyhan cells and *mouse* cl-1D cells. The human cells are a nonmalignant strain of skin fibroblasts that is deficient in inosine pyrophosphorylase (21). The cells grow to a single layer in their dishes. The mouse cells are a malignant subline of L cells that is deficient in thymidine kinase (22). These cells pile up in their dishes.

Hybrid Cells were derived from the fusion of the parent cells with the aid of inactivated Sendai virus (23) or from the spontaneous fusion of the parent cells (24). For selection of the hybrids, the cultures were kept in HAT medium (25) containing hypoxanthine, thymidine, and the antimetabolite aminopterin, which blocks purine and pyrimidine synthesis. The two enzyme-deficient parent cells, which cannot incorporate exogenous hypoxanthine or thymidine into nucleic acids, die in

TABLE 1. *Coupling and gap junctions*

Cell type	Clone*	Months after fusion	No. of chromosomes Range	No. of chromosomes Mode	No. of marker(D) chromosomes	No. of small biarmed chromosomes§	Coupling (no. of cells)¶ Electrical Coupling	Electrical Noncoupling	Fluorescein Coupling	Fluorescein Noncoupling	Gap junction transections frequency per 100 μm	Apposed membrane length scanned** μm
Parental												
Human			37–46	46	0	29	12	0	137	0	4.4	825
Mouse			40–53	53	1	0	0	62	0	6	0	5369
Hybrids	1a	1.5	51–133	104	2	10	6	0	75	5	0.37	1615
	1b	1.4	40–151	124	2	14			88	6	‖	
	1c	1.5	43–144	106†	2	14			57	4		
	$1a_3pk$	7.6	43–126	111	2	5	50	18	0	11		††
	$1b_1p$	7.2	78–132	117	2	8	6	16	0	3		††
	$1b_1pn$	7.8	45–112	53/86‡	2	3	19	14	0	10		††
	2bp	4.1	42–110	52/74‡	1	2	12	16	0	10		††
Revertants	$1a_3p$	5.6	37–116	102	2	5	0	42	0	4	0	3210
	2bps	7.1	45–103	49/54‡	1	2	0	38	0	10	0	3584
	2dp	6.4	38–103	55/76‡	1	3	0	92	0	4	0	6472

* Heterokaryons of series 1 were products of spontaneous fusion; those of series 2, of fusion induced by Sendai virus. Clones 1a, 1b, 1c, 2b, and 2d are derivatives of a separate fusion. The derivation sequences are: 1a → $1a_3$ → $1a_3p$ → $1a_3pk$; 1b → $1b_1$ → $1b_1p$ → $1b_1pn$; 2b → 2bp → 2bps; 2d → 2dp.

† This population still contained a small number of human parent cell survivors; see *Methods*.

‡ Two modes.

§ Means. Small biarmed human chromosomes, Denver groups: B,C,E,F,X.

¶ The electrodes were in contiguous cells for determination of electrical coupling, except for hybrid cells where the electrodes were in nonadjacent members of a series of three or four contiguous cells. For determination of fluorescein coupling, an injection into a single cell served generally to test coupling to and among several surrounding cells (Fig. 1). Tabulated as coupling are numbers of cells found to transmit or receive current or fluorescein, and as noncoupling, the numbers of cells found not to transmit current or fluorescein.

‖ Gap junctions were identified in this clone (see Fig. 2*c*); only a short stretch of membrane apposition was scanned.

** Total membrane length scanned where apposed membranes are ⩽ 800 Å apart.

†† See end of footnote to page 883.

this medium. The hybrid cells, which contain both inosine pyrophosphorylase and thymidine kinase, circumvent the aminopterin block by utilizing the thymidine and hypoxanthine from the medium and survive. The hybrid cells were then cloned, and all electric, fluorescent tracer, and electron-microscopic studies were done on such clones. The hybrid character of each clone was established by karyotype.

Some of the human parent cells survived in the HAT medium when there was a good number of coupling hybrid cells present in the culture. These parent cells presumably obtained the missing nucleotide derivative via coupling junctions with the hybrid cells (26, 27). Eventually, such cells were eliminated by repeated passaging in HAT medium. (The noncoupling mouse parent cells were never found to survive.)

The early generations of hybrid cells grew to single layers. Upon continued cultivation, cell populations appeared which, like the mouse parent cells, tended to pile up. Some of the piled-up cells could be freed by gentle shaking of the dishes; these cells were cloned and yielded the revertants. (The cells in direct contact with the dishes stuck more firmly.)

Karyotyping. The karyotypes were examined on metaphase spreads of the clones treated with colcemid (0.2 μg/ml, 3–5 hr). The mouse parent cell has a convenient marker chromosome ("D") with a double constriction, which has no equivalent in the human parent cell. Moreover the human chromosomes, except for five pairs, are distinguishable by their size and shape from the mouse chromosomes (24). The 29 small biarmed human chromosomes are most readily distinguished; their numbers were tabulated (Table 1).

Culture Conditions. The cells were grown in 60-mm Falcon plastic dishes at 37°: the parent cells in Dulbecco's medium and the hybrids in HAT medium. The coupling measurements were taken in the dishes at 25–32°.

Coupling Measurements. Coupling was determined by electrical measurement and/or by fluorescent tracer diffusion. With the aid of two microelectrodes, current was pulsed between the interior of a cell (I) and the medium, and the resulting potential changes (V) were measured inside this cell and a contiguous one (II) (Fig. 1, top inset). The V_{II}/V_I ratio, resolvable down to 0.02 or 0.05, provided a convenient index of the electrical coupling (6). The microelectrodes were connected to balanced bridge circuits and served each for current passing and potential recording. This permitted us to measure input resistance in each cell tested to check membrane integrity, and, thus, to rule out membrane damage due to cell impalement as the cause of the coupling deficiency in the noncoupling cells. The V_{II}/V_I ratios in the coupling parental human or hybrid cells ranged from 0.1–0.8; in most cases they were about 0.4. The cell resting potentials of the various cell types ranged typically from 5 to 30 mV, inside negative.

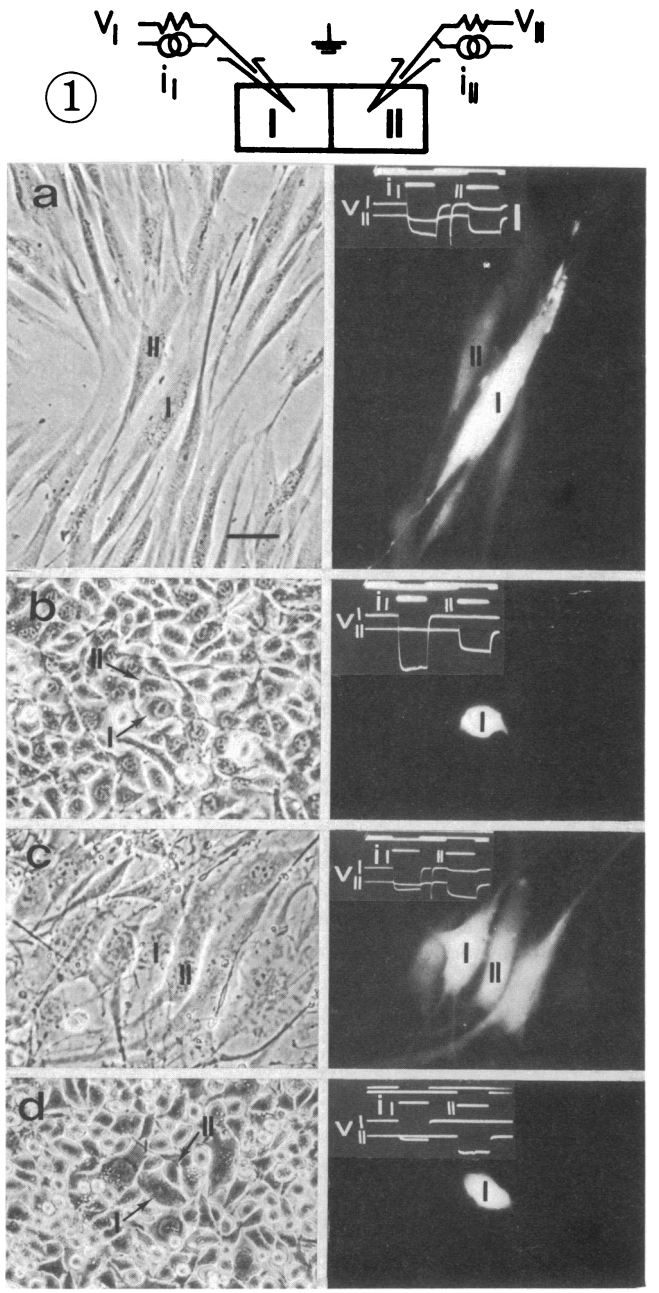

FIG. 1. Coupling. (*a*) human parent cell; (*b*) mouse parent cell; (*c*) early hybrids, (clone 1a); (*d*) revertants, (clone 2bps). Current (i = 2.5× 10^{-9} amp, inward) is injected into cell I and, with a 100-msec delay, into cell II, and the resulting voltage drops (V) are recorded in the two cells. Simultaneously, fluorescein is injected into cell I and the fluorescence is photographed 5 min thereafter in a darkfield. *Left column* shows micrographs of the cells in phase contrast; *right column*, in darkfield. Calibration, 50 μm. *Insets* show oscilloscope records of i and V. Calibration, 100 mV; current pulse duration, 100 msec.

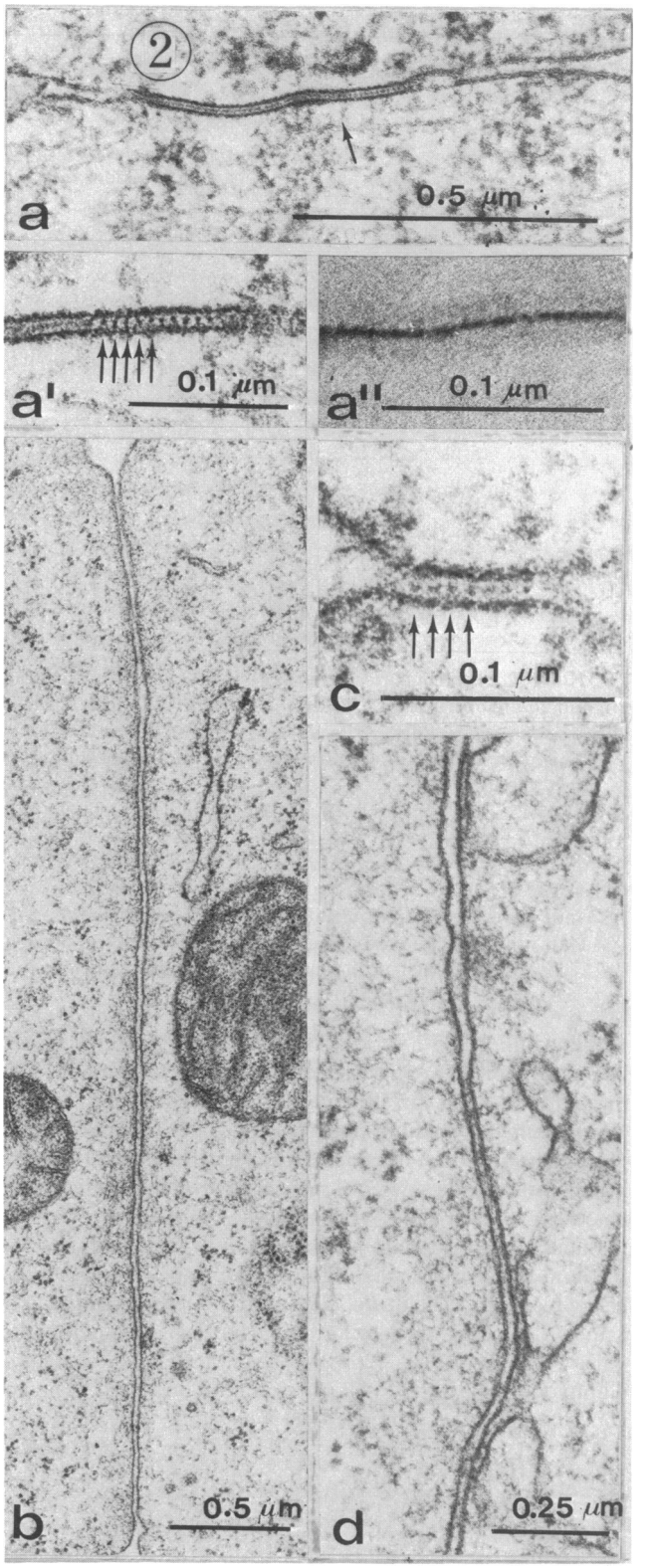

FIG. 2. (*a*) Electron micrographs of gap junction between two human parent cells. The membranes at the junction are closely apposed (*a*, 79,000×) and are further distinguished by a pattern of particles (*arrows*) with a periodicity of 80 Å (*a'*, 207,000×) and, after treatment with colloidal lanthanum, by a "gap" of 30–40 Å (*a''*, 295,000×). (*b*) Typical membrane apposition between two parental mouse cells. The membranes are separated by a rather uniform distance (200–400 Å) and show no differentiated junctional structures. 29,000×. (*c*) Gap junction between two early hybrid cells (clone 1b). Particles with the 80-Å periodicity are evident (*arrows*). 320,000×. (*d*) Typical undifferentiated membrane apposition between revertant cells (clone 2dp). Characteristically, the intercellular space is narrower than at the undifferentiated regions of membrane apposition of the two parental and early hybrid cells. 84,000×.

The microelectrodes were filled with KCl (3 M), or with fluorescein-Na(0.3 M) and KCl (0.1 M). For determination of coupling by fluorescent tracer, the fluorescein anion (330 molecular weight) was injected into the cell by electric current, and the spread of fluorescein was observed in a darkfield (6).

Electron Microscopy. The cell cultures were fixed in the dishes with a cacodylate buffered (0.05 M) solution of 2% glutaraldehyde and 2% sucrose, or in the same solution plus 1% lanthanum hydroxide (0.5–2 hr) (7) and then fixed in cacodylate buffered 1% osmium tetroxide. The cultures not treated with lanthanum were stained in block with uranyl acetate (5–15 hr). All cultures were embedded in araldite in the dishes, and thin sections (about 1000 Å), normal to the dishes, were stained in uranyl acetate and lead citrate. Several places in any given dish were sampled for sectioning; the places and sections studied were chosen at random. Regions of membrane apposition in noncoupling cells were scrutinized at 200,000× magnification (about 200 sections from each clone were used); 18,000× was sufficient for identifying gap junctions in the coupling cells.

RESULTS AND DISCUSSION

The two parent cells offer a simple contrast in their coupling. The human cells are electrically coupled, and fluorescein spreads from one cell to another (Fig. 1). Coupling between contiguous cells was found with a frequency of 100% (Table 1). In the mouse cells, the frequency was zero. No electrical coupling was detectable and fluorescein stayed within the confines of the injected cells. Table 1 summarizes the results.

The contrast in junctional structure between the parent cells is equally simple. The human cells have gap junctions, the mouse cells apparently do not. The gap junction transections of the human cells are roughly 4000 Å long, show particles with an 80-Å periodicity and, when infiltrated with lanthanum, a 30- to 40-Å "gap" Fig. 2). The gap junction was the only differentiated junctional structure discernible in these cells. We scanned systematically in the electron microscope the regions of cell-membrane apposition, that is, the regions where the cell surface membranes (unpatterned) were less than 800 A apart. We found 36 gap-junction transections in a length of 825 μm of membrane apposition in the human cells, or 4.4 junction transections/100 μm. In the mouse cells, on the other hand, we did not find a single gap junction in a length of 5370 μm of membrane apposition scanned.

The early generations of hybrid cells took after the human parent cell in both coupling and junctional structure. One series of clones (series 1) had initially nearly one complete set of chromosomes from the human parent cell and two sets from the mouse parent cell. The cells of the early derivatives of these clones (1a, 1b, and 1c in Table 1) were coupled. Electrical and fluorescein couplings were found nearly as frequently as in the human parent cells. These hybrid clones also had gap junctions. Clone 1a, for example, showed 6 gap-junction transections in 1615-μm length of membrane apposition.

Upon continued cultivation, the hybrid cells tended to lose chromosomes, preferentially the human ones. This is a general feature of these parent-cell combinations (24). We isolated several subclones in which many of the human chromosomes had been lost. These segregants fell in two classes in terms of coupling. In one class (clones $1a_3$pk, $1b_1$p, and $1b_1$pn), there were coupling cells and noncoupling cells. The coupling cells here had the peculiarity that while they were coupled electrically, they were not seen to transfer fluorescein. This peculiarity was shown most strikingly in experiments in which coupling was determined simultaneously by electrical measurement and fluorescein injection. Fluorescein did not spread detectably beyond the injected cell, whereas cell-to-cell spread of electrical current was detectable. The V_{II}/V_I ratios were usually lower than in the human parent cells or in the earlier hybrid generations, but the V_{II} values for any given current injected into cell I exceeded by at least three orders of magnitude the potentials recordable in extracellular locations.* (The noncoupling cells among these segregants were noncoupling in respect to both fluorescein spread and electrical current.)

In the second class of segregants (clones $1a_3$p, 2bps, and 2dp), neither electrical nor fluorescein coupling was detectable. These cells thus seemed to be segregants of the original hybrids which had reverted to the noncoupling trait. These cells had no discernible gap junctions.

Another series of hybrid cells (series 2) contained originally one set of chromosomes from each parent cell. These cells lost the human chromosomes too rapidly to be useful for coupling studies in early hybrid generations. By the time the first hybrid cells could be cloned and isolated, most of them had lost some 20 chromosomes. These cells, nevertheless, seemed to follow the coupling pattern of series 1: the clones with the highest number of human chromosomes (clone 2bp) still contained some electrically coupled cells, and those with fewest (clones 2bps and 2dp) were revertants, in that neither coupling nor gap junctions were found (Table 1). (The latter clones, as well as clone $1a_3$p, were, in addition, revertants in respect to to the trait of piling up.)

The lengths of membrane apposition scanned in the noncoupling parent and revertant cells were 130–260 times greater than the mean membrane length (about 25 μm) between gap junction transections in the coupling cells (Table 1). Hence, we conclude that if gap junctions do occur at all in the noncoupling cells, they are less abundant, per unit appositional area, by a factor of at least 130^2 to 260^2, or 17,000 to 70,000, than in the coupling cells.

Failure to find a structure in electron microscopy affords, of course, no certainty that the structure is absent. As pointed out above, this is precisely the uncertainty that afflicted even the best and most suggestive earlier evidence implicating the gap junction in coupling. However, this uncertainty is immensely smaller in the present study where the coupling correlates with gap junction and the noncoupling with its ab-

* The coupling behavior of this class of cells resembles that of certain early embryonic cells (28, 29). Possible explanations for the apparent lack of fluorescein coupling are: (*a*) the cell-to-cell passageways are too few for detection of fluorescein transfer in cell II; (*b*) the passageways are altered; flow of fluorescein, but not of the smaller inorganic ions that carry the cell-to-cell current, is blocked. The further possibility that fluorescein and inorganic ions take altogether different routes seems less likely, because electrical uncoupling is invariably paralleled by fluorescein uncoupling in several experimental situations of junctional blockage (1, 30, 31). We have not examined this class of cells in the electron microscope. We will attempt to get quantitative information on gap-junctional area by the use of freeze-fracture techniques.

sence, not only in the very different parent cells but also in the hybrids and, most importantly, in their segregants. In the segregants we chose for study, the phenotypic trait of non-coupling had separated from a number of other traits that originally occurred together in the mouse parent cell: For example, clone 1a and the revertant $1a_3p$, which have opposite properties of electrical coupling, both contain, for example, inosine pyrophosphorylase and thymidine kinase. Thus, in view of the fact that we had no difficulties in finding gap junctions in the 1a clone, it is unlikely that we should have missed these junctions in the revertant clone had such junctions been there.

The results show a genetic correlation between the occurrence of coupling and of gap junction: They reveal that the human cell contributes a factor to the hybrids that corrects the junctional deficiency of the mouse cell. This factor is probably linked to one or more human chromosomes, since loss of human chromosomes in the hybrids resulted in reversion to junctional deficiency.

The gap junction thus appears to contain the coupling passageways in the present cell systems. However, this should not be taken to mean that the gap junction is the universal or sole mediator of coupling. Here and in a number of other vertebrate cells, there are no other obvious structural candidates for mediation of coupling. But in invertebrate cells, gap junctions coexist with the highly differentiated septate junction which, like the gap junction, displays, in freeze-fracture, an organized particle array (32); and in, at least, three such cell types the coupling is particularly close (6, 33, 34). There are no *a priori* or experimental reasons at present for discarding the septate junction as another possible site of coupling.

Note Added in Proof. We are now also studying the cell junctions with freeze-fracture technique. Thus far we have examined three cell types, the parent human and mouse cells and the revertant clone 2bps. In all three, the results fully confirm our findings obtained with thin-section electron microscopy.

We thank Dr. Howard Green for providing us with the cl-1D cells and for valuable suggestions for cell selection. This work was supported by the U.S. Public Health Service research grant CA 14464 and National Science Foundation grant, GB 36763 X 1.

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