J. Membrane Biol. 91, 53–64 (1986)

The Journal of
Membrane Biology

Modification of Gap Junctions in Cells Transformed by a Temperature-Sensitive Mutant of Rous Sarcoma Virus

Michael M. Atkinson, Susan K. Anderson*, and Judson D. Sheridan
Department of Anatomy, University of Minnesota, School of Medicine, Minneapolis, Minnesota 55455

Summary. Prompted by our observation that a reduction in junctional permeance is one of the earlier events in the process of neoplastic transformation of a cell line by Rous sarcoma virus, we analyzed the gap junctions from these cells to determine if the basis of the reduction is a loss of junctional channels. The cells (normal rat kidney, or NRK) are infected with a temperature-sensitive mutant of Rous sarcoma virus, allowing one easily to manipulate the cells into and out of the transformed state, and hence also to manipulate the junctional permeance. Using freeze-fracture electron microscopy, we found that the number and size of the junctions did not change in parallel with the permeance changes we had previously characterized. There is, however, a significant rearrangement of the junctional particles to a more random configuration when the cells are transformed and a reversal to the more ordered pattern when the cells are shifted back to the normal phenotype. These changes do parallel the changes in junctional permeance. We conclude that the permeance of existing junctional channels is modified and that the change in permeance may involve a change in the interaction of the junctional channels with each other and/or the surrounding lipid domain.

Key Words gap junctions · Rous sarcoma virus · cell transformation · temperature-sensitive viral mutant · gap junctional particle packing

Introduction

Most cells have the capacity to exchange low molecular weight substances in a direct and intimate manner via cell-to-cell channels (Kuffler & Potter, 1964; Loewenstein & Kanno, 1964; Kanno & Loewenstein, 1966; *see* Peracchia, 1980; Loewenstein, 1981, for recent general reviews; and various authors in the *Annual Review of Physiology*, 1985, for a number of specific topics on permeable junctions). This capacity for cells to be "coupled' suggests a form of intercellular communication distinct from those requiring an extracellular signal (e.g. Loewenstein, 1966; Potter, Furshpan & Lennox, 1966; Loewenstein, 1979; Sheridan & Atkinson, 1985).

It is now generally accepted that the gap junction is the structural counterpart of cell coupling (McNutt & Weinstein, 1973; Peracchia, 1980). Evidence for this identity is indirect, but in the aggregate quite compelling. For example, of the different membrane specializations present in various cell types, the gap junction most consistently with the presence of cell coupling (Revel, Yee & Hudspeth, 1971; Gilula, Reeves & Steinbach, 1972; Azarnia, Larsen & Loewenstein, 1974), and structural analyses of isolated gap junctions are at least suggestive of a pore in the center of a hexameric unit which spans the two plasma membranes and the intervening extracellular gap (Caspar, Goodenough, Makowski & Phillips, 1977; Makowski, Caspar, Phillips & Goodenough, 1977; Unwin & Zampighi, 1980; Zampighi, Corless & Robertson, 1980). Persuasive evidence of a different sort is the recent demonstration that antibodies to gap junction proteins apparently block cell coupling (Warner, Guthrie & Gilula, 1984; Hertzberg, Spray & Bennett, 1984).

One of the cellular functions hypothesized to involve or require gap junctions is control of cell proliferation (Loewenstein, 1968; Burton, 1971; Burton & Canham, 1973; Sheridan, 1976; Loewenstein, 1979). Since a fundamental property of tumor cells is unrestrained proliferation, the state of the junctions in various solid tumors and cultured tumor cells has been studied physiologically and morphologically (Sheridan & Johnson, 1975; Weinstein, Merk & Alroy, 1976; Loewenstein, 1979; Newbold, 1982). Early reports of a lack of cell coupling between certain tumor cells (e.g., Loewenstein & Kanno, 1967; Kanno & Matsui, 1968) were soon followed by reports indicating that cell coupling was

* *Present address:* Department of Biological Structure, University of Washington, Seattle, WA 98195.

present in other tumor cells (e.g. Furshpan & Potter, 1968; Sheridan, 1970). Hence it was evident early on that tumor cells frequently were uncoupled, but that the lack of coupling was by no means universal (e.g. Borek, Higashino & Loewenstein, 1969). Morphological studies of gap junctions similarly showed that gap junctions appear to be absent or reduced in number in some tumors (e.g. McNutt & Weinstein, 1969; McNutt, Hershberg & Weinstein, 1971) and tumor cell lines (e.g., Azarnia et al., 1974), but not in others (e.g. Johnson & Sheridan, 1971; Pinto da Silva & Gilula, 1972). These initial studies generally focused on answering the question of whether or not junctions exist in tumor cells. Thus, the more fundamental questions remained: are defective junctions a causative factor in tumorous growth, and might the junctional defect include partial reduction of junctional capacity as well as outright loss?

Some of the inherent ambiguities associated with these earlier approaches are avoided by using model systems in which neoplastic transformation can be induced *in vitro*. This allows one to monitor the status of cell coupling and of the gap junctions as the transformation process proceeds. For example, transformation of at least one cultured cell line by a tumor promoter has been shown to be accompanied by a reduction of gap junctions (Yancey et al., 1982) and of cell coupling (measured indirectly by passage of a lethal metabolite through the junctions to a susceptable cell—Yotti, Chang, & Trosko, 1979).

Neoplastic transformation of cells *in vitro* by retroviruses is another useful system in this regard. Two major advantages are that the transforming genes, and in many cases their gene products, have been identified (Bishop, 1983) and that virus mutants have been isolated in which the ability to transform cells is temperature-sensitive (e.g. Wyke, 1973). Thus, cells infected with these mutant viruses can be switched from the normal state to the transformed state by first growing the cells at a temperature which restricts expression of the transformed phenotype and then shifting the cells to a temperature which permits transformation.

Our approach has been to assess cell coupling in a cell line infected with a temperature-sensitive mutant of Rous sarcoma virus (RSV). We reported earlier (Atkinson et al., 1981; Atkinson & Sheridan, 1985) that the capacity for cell-to-cell transfer of a fluorescent molecule is significantly reduced when the cells are switched to temperatures permissive for transformation. The junctions respond very quickly to the temperature shift, and the temperature effect is reversed with a similar time course by switching the cells back to the original growth temperature. As we discussed in detail (Atkinson et al., 1981), our method of dye delivery ensured that changes in cell-to-cell transfer of dye were not explanable by changes in nonjunctional leakage (a possibility raised by Azarnia and Loewenstein, 1984), or in other nonjunctional parameters such as cell size, but were instead due to alterations in junctional permeance. In addition, our more recent studies (Atkinson & Sheridan, 1985), and the studies by Azarnia and Loewenstein (1984) as well as by Chang et al. (1985) provide additional support for our earlier conclusions.

The underlying mechanism for these junctional responses presumably involves a change in the total cross-sectional area of open junctional channels. One possibility is that new channels are formed when the cells are shifted to the nonpermissive temperature, increasing the junctional capacity. Similarly, shifting the cells down to transformation-permissive temperatures could result in removal of junctional channels, leading to decreased junctional capacity. Another way the total cross-sectional area could be affected is by changes either in the bore size of the channels or in the proportion of open versus closed channels.

We analyzed the gap junctions from uninfected NRK cells and LA25-NRK cells using freeze-fracture techniques, and found that the number and size of junctions do not change in conjunction with the changes in junctional permeance, but that there is a marked and correlative change in the particle packing arrangements. A greater proportion of junctions displaying an "ordered" arrangement of particles is associated with the nontransformed state and greater junctional permeance. These changes in the arrangement of junctional particles, and the lack of a change in the number or size of gap junctions, strongly suggest that junctional permeance is being changed by direct modulation of existing junctional channels. Some of these results have been summarized elsewhere (Anderson, Atkinson, Sheridan & Johnson, 1981; Atkinson & Sheridan, 1985).

Materials and Methods

Cell Culture

Uninfected NRK cells and NRK cells containing the proviral form of a transformation-defective, temperature-sensitive mutant of Rous sarcoma virus (LA25) (Wyke, 1973) were grown in monolayer cultures using DMEM supplemented with 10% calf serum (heat-inactivated) and glutamine, and containing antibiotics. Cells to be processed for freeze-fracture were seeded into 60-mm plastic dishes and grown for two days at the appropriate temperature. At this time, cell monolayers were approximately

70 to 90% confluent. Stock cultures were maintained at 37°C and test cultures at 35 or 40.5°C in a humidified atmosphere of 5% CO_2/95% air.

Freeze Fracture

Cells to be processed for freeze fracture were rinsed with a phosphate buffered saline (PBS–in mM: NaCl, 136.9; KCl, 2.7; Ca_2Cl, 0.9; Mg_2Cl, 0.5; Na_2HPO_4, 6.5; KH_2PO_4, 1.5), fixed by exposing to 2.5% glutaraldehyde (in PBS) for 30 min, and glycerinated using 30% glycerol (in PBS) for 4 hr at 4°C. After additional rinses, the cells were scraped from the dish with a rubber policeman and fragments of the monolayer placed on gold double-replica specimen holders (Balzers). The holders were then plunged into semi-solid Freon 22 (DuPont) and, after approximately 10 sec, rapidly transferred to liquid nitrogen. The frozen samples were stored in liquid N_2 until fractured and replicated in a Balzers BA 360.

Electron Microscopy

Platinum-carbon replicas were viewed in a Phillips 201C electron microscope. For measurements of gap junctional areas, grid squares were systematically scanned for gap junctions, which we defined as aggregates of seven or more individual particles. Each junction was photographed, and the junctions occurring together on an interface were noted. ("Interface" here refers to a continuous membrane face—P and/or E—containing at least one gap junction.) No systematic search was performed for the gap junctions used in particle diameter measurements; instead, the entire replica was randomly sampled.

Magnifications were estimated from calibration grids. The photographs of the grids were processed in parallel with the photographs of the replicas.

Analysis

Areas were measured directly from photographs of junctions (80,000 magnification) using a Hewlett Packard digitizing board (HP 9874A). Particle diameters were measured directly from photographs of junctions (200,000 magnification) using a 7× magnified reticle (0.1 mm/division). Measurements were made perpendicular to the direction of shadowing. A maximum of 25 particles were measured per junction. For those junctions containing more than 25 particles, a grid was placed over the photograph and the size of the grid adjusted such that 25 points of intersection could just fit on the image of the junction. Normally, these points were then used as a reference, and the nearest particle was measured. Obviously deformed particles were excluded. The locations of the particles measured on each junction were recorded on transparent sheets.

Results

Due to the temperature sensitivity of the RSV mutant, LA25-NRK cells are transformed when grown at 33–35°C, but maintain a normal phenotype when grown at 39–40.5°C. As indicated in Fig. 1, the morphology of the transformed cells (Fig. 1*b*) is different from the nontransformed cells (Fig. 1*a*). However, the transition from one morphology to the other, induced by a temperature shift, is slow. Reciprocal temperature shift experiments show that the appearance of the cell is unchanged one hour after the growth temperature is decreased from 40.5 to 35°C (*compare* Fig. 1*a* with *c*), or increased from 35 to 40.5°C (*compare Fig.* 1*b* with *d*). Yet we have previously shown that the change in junctional dye transfer is maximally affected one hour after a temperature shift (Atkinson et al., 1981). Thus we effectively dissociated junctional changes from morphology changes by:

1) analyzing the gap junctions from LA25-NRK cells grown at 40.5°C (transformation-restrictive), when the cells readily transfer dye;

2) comparing them to the gap junctions from LA25-NRK cells subjected to a one-hour temperature downshift, when dye transfer is maximally reduced but morphology changes are not detectable; and

3) using the same approach on LA25-NRK cells maintained at 35°C (transformation-permissive) and shifted to 40.5°C for one hour.

Junctional Area

The mean junctional area of LA25-NRK cells grown at 40.5°C is about four times that of cells grown at 35°C (Table 1), which, as an isolated piece of data, could be an explanation for the differences in junctional dye transfer. However, the mean areas of gap junctions are virtually unchanged when the basis of comparison is cells grown at 40.5°C and cells that had been downshifted for one hour (Table 1), even though the latter's capacity to transfer dye is substantially reduced. Similarly, a comparison of the values from cells grown at 35°C and cells that have been shifted from 35 to 40.5°C indicates no significant change in the mean gap junctional areas, despite a large change in apparent junctional capacity for dye transfer. We also found that the mean gap junctional area of uninfected NRK cells grown at 35°C is significantly less than that of uninfected NRK cells grown at 40.5°C (Table 1), even though the junctional capacity to transfer dye is essentially unchanged (Atkinson et al., 1981; Atkinson & Sheriden, 1985). This provides an additional example of an apparent dissociation between mean junctional area and the functional state of the pre-fixed junctions and may also point up a growth temperature effect on junctional area. (Both LA25-NRK cells and NRK cells show a reduction of junctional area when maintained at 35°C.)

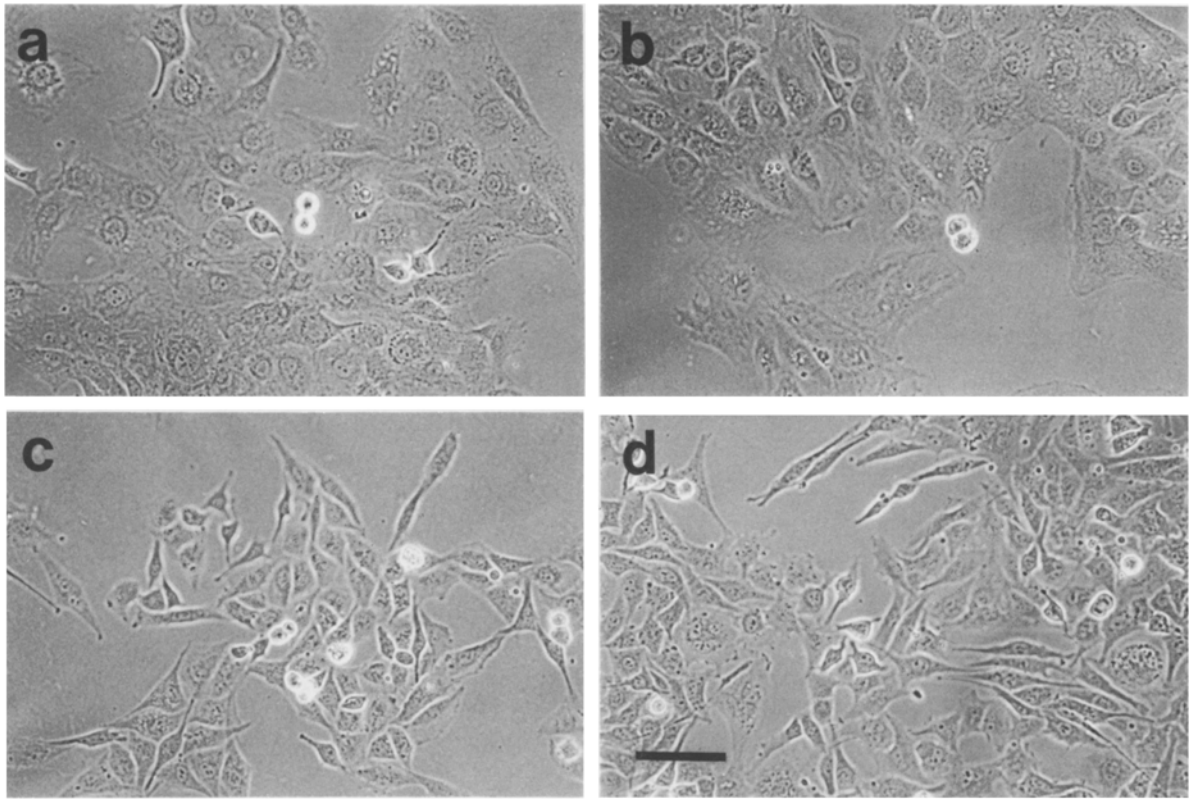

Fig. 1. Phase contrast microscopy of LA25-NRK cells showing lack of any obvious morphology changes one hour after shifting the cells to a new growth temperature. Four plates were seeded from the same stock culture and placed in a 40 or 33°C incubator: (*a*) cells grown at 40°C for >24 hr; (*b*) cells from a replicate plate which had been shifted to 33°C for 1 hr; (*c*) cells grown at 33°C for >24 hr; (*d*) cells from a replicate plate, which had been shifted to 40°C for 1 hr. Calibration bar: 100 μm

Table 1. Junctional Areas of Uninfected NRK cells and LA25-NRK cells

	Junction areas		Log junction areas		N^b
	Median ($\mu m^2 \times 10^2$)	Mean[a]	Median	Mean[a]	
LA25-NRK					
40.5°C	1.0	2.7 ± 0.5	−2.00	−2.07 ± 0.08	76
40.5°C → 35°C	0.8	2.8 ± 0.6	−2.11	−2.05 ± 0.07[c]	85
35°C	0.2	0.7 ± 0.2	−2.62	−2.58 ± 0.04	133
35°C → 40.5°C	0.2	1.0 ± 0.2	−2.72	−2.60 ± 0.03[c]	253
Uninfected NRK					
40.5°C	3.3	14.8 ± 3.2	−1.48	−1.47 ± 0.09	73
35°C	1.2	6.7 ± 1.8	−1.89	−1.78 ± 0.07[d]	87

[a] ±SE
[b] Number of junctions.
[c] Not significantly different from nonshifted counterpart.
[d] Significantly different ($P < 0.01$—Student's *t*-test) from uninfected cells maintained at 40.5°C.

The distributions of junctional areas are highly skewed, as indicated by the differences in the medians and means (Table 1). However, the distributions become more symmetrical and approximate normal distributions when the values are log transformed (Fig. 2; Table 1). As is suggested by the histogram in Fig. 2, no statistical differences are found between shifted cells and their nonshifted counterparts.

We next investigated the possibility that, al-

Table 2. Junctional area per interface of LA25-NRK cells before and after temperature shifts

Temperature (°C)	Junctional area per interface		Log junctional area per interface		N^b
	Median ($\mu m^2 \times 10^2$)	Mean[a]	Median	Mean[a]	
40.5	2.2	6.7 ± 1.9	−1.66	−1.68 ± 0.13	31
40.5 → 35	3.5	6.5 ± 1.5	−1.46	−1.47 ± 0.09[c]	36
35	1.0	3.3 ± 1.1	−2.00	−2.00 ± 0.13	29
35 → 40.5	0.8	4.4 ± 1.4	−2.18	−2.01 ± 0.10[c]	60

[a] ±SE

[b] Number of interfaces.

[c] Not significantly different from nonshifted counterpart.

though changes in junctional size are not evident, total junctional area per intercellular contact may be modified by additions or depletions of gap junctions. To test this, the number and area of junctions per interface were determined. As used here, "interface" is simply the experimental unit defined as continuous membrane face (P and/or E) that contains at least one junction. Since the relevant parameter is total junctional area between two cells, we must assume that the area of junctions per "interface" is a reliable sample of the total interfacial area. That is, that the probability of the fracture plane leaving and re-entering the membrane bilayers in a continuous area of the close contact between cells is either small or not different, at least for the experimental conditions used for comparison. In addition, it is assumed that the junctions identified and analyzed for any particular interface are either a random or constant sample of the total population existing between the two cells. Again, this should be true at least for the conditions used for comparison.

Although the distributions of junctional area per interface are skewed—*compare* means and medians, Table 2—the parameters of the distributions are again similar for cells grown at 40.5°C and cells that have been downshifted for one hour, and for cells grown at 35°C versus cells shifted up to 40.5°C for one hour. As for the distribution of junctional areas, the log transform of junctional areas per interface approximates a normal distribution, and statistical analyses indicate that the distributions as compared above are not different. The difference between nonshifted 40.5°C cells and nonshifted 35°C cells is statistically borderline at best, in contrast to the clear difference obtained when mean junctional areas are compared. This probably is a consequence of the apparent tendency for a greater number of junctions per interface in the 35°C cells (and the upshifted cells).

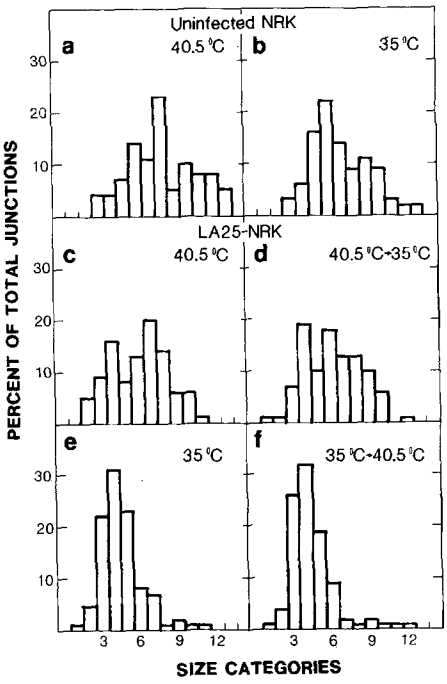

Fig. 2. Distribution of the log junctional areas for LA25-NRK cells and uninfected NRK. Junctions were grouped into 13 size categories according to the logarithm of their area. Each bin encompasses 0.2 log unit. The size categories break down as follows: 1. −3.74 to −3.46; 2. −3.45 to −3.15; 3. −3.14 to −2.86; 4. −2.85 to −2.55; 5. −2.54 to −2.26; 6. −2.25 to −1.95; 7. −1.94 to −1.66; 8. −1.65 to −1.35; 9. −1.34 to −1.06; 10. −1.05 to −0.75; 11. −0.74 to −0.46; 12. −0.45 to −0.15; 13. −0.14 to 0.14. Extremes for the areas measured are 1.34 μm^2 (log = 0.13), and 2.4×10^{-4} μm^2 (log = −3.62). (*a*) Uninfected NRK cells grown at 40.5°C, or (*b*) 35°C; (*c*) LA25-NRK cells grown at 40.5°C, or (*d*) 35°C; (*e*) LA25-NRK cells downshifted for 1 hr, or (*f*) upshifted for 1 hr

Distribution of Junctional Particles

To check the possibility that the particle packing arrangement might be correlated with the per-

Table 3. Packing arrangement of junctional particles

	Temperature (°C)	Ordered packing (%)	Nonordered packing	N^a
LA25-NRK				
	40.5	70	30	47
	40.5 → 35	30	70[b]	53
	35	18	82	34
	35 → 40.5	44	56[c]	48
Uninfected NRK				
	40.5	73	27	62
	35	79	21[d]	72

[a] Number of junctions scored for packing arrangement.
[b] Significantly different ($P < 0.001$—Chi-square) from cells maintained at 40.5°C.
[c] Significantly different ($P < 0.01$—Chi-square) from cells maintained at 35°C.
[d] Not significantly different from uninfected cells maintained at 40.5°C.

Table 4. Area of ordered junctions per positive interface and number of positive interfaces

Temperature (°C)	Mean area of ordered junctions/+interface[a] ($\mu m^2 \times 10^2 \pm$ SE)	Number of + interfaces[a]	N
40.5	5.7 ± 1.8	16	23
40.5 → 35	5.8 ± 2.6	7[c]	34
35	1.6 ± 0.6	4	16
35 → 40.5	9.4 ± 5.1[b]	14[d]	28

[a] Positive interfaces are those interfaces containing one or more junctions with ordered particle packing.
[b] Significantly different ($P < 0.05$—Mann-Whitney) from cells maintained at 35°C.
[c] Significantly different ($P < 0.001$—Chi-square) from cells maintained at 40.5°C.
[d] Not significantly different ($P > 0.25$—Chi-square) from cells maintained at 35°C.

meance of unfixed junctions, photomicrographs of junctions larger than 0.004 μm (approximately 30 particles) were scored blind for "ordered" or "nonordered" particle placement. Junctions were scored as ordered if the particles formed a regular pattern in their packing arrangement and if that arrangement predominated over the face of the junction. As shown in Table 3, a large proportion of the scored junctions from LA25-NRK cells grown at 40.5°C (Fig. 4*a*) and from uninfected NRK cells (Fig. 3) displayed an ordered particle arrangement. Conversely, only 18% of the scored junctions from LA25-NRK cells grown at 35°C were ordered (Table 3 and Fig. 4*c*). This apparent correspondence between ordered particle arrangements and the permeance of the junctions in their pre-fixed state is also found when the junctions from the temperature-shifted cells are scored (Table 3). That is, the proportion of ordered junctions decreases in downshifted cells and increases in upshifted cells (*see* Fig. 4).

If, as is suggested by the data, ordered junctional particle packing represents those junctions that were "open" in the pre-fixed state, then one would predict that the area of ordered junctions on an interface, and/or the number of interfaces with ordered junctions, would decrease when the cells are maintained under conditions of reduced junctional permeance. Moreover, the converse should also hold true for cells maintained under conditions of increased junctional permeance. The data presented in Table 4 suggest that this is indeed the case. The number of interfaces containing ordered junctions is decreased in downshifted cells (decreased permeance), although the mean area of ordered junctions per "positive" interface (i.e., interfaces containing at least one ordered junction) is unchanged. For cells shifted from 35 to 40.5°C (increased permeance), the mean area of ordered junctions per positive interface increases substantially, along with a smaller increase in the proportions of interfaces containing ordered junctions.

Junctional Particle Diameter

We next wanted to determine if the changes in particle packing were accompanied by changes in particle diameters. The mean particle diameters for all test conditions ranged from 7.6 to 8.3 nm, with junctions from uninfected NRK cells grown at 40.5°C, and LA25-NRK cells grown at 35°C possessing larger particles on average, relative to their counterparts (*not shown*). The reason why these two conditions should show an increase is not clear, but the differences do not bear a simple relationship to the different growth temperatures, nor to the permeance of the pre-fixed junctions, nor to the transformed state of the cells. The size of junctional particles appears to be independent of the packing arrangement (*not shown*).

Junctional Particle Density

We found that the density of particles is highly variable (*not shown*) and does not appear to be greater for junctions scored as ordered, apparently a consequence of the particle-free isles frequently associ-

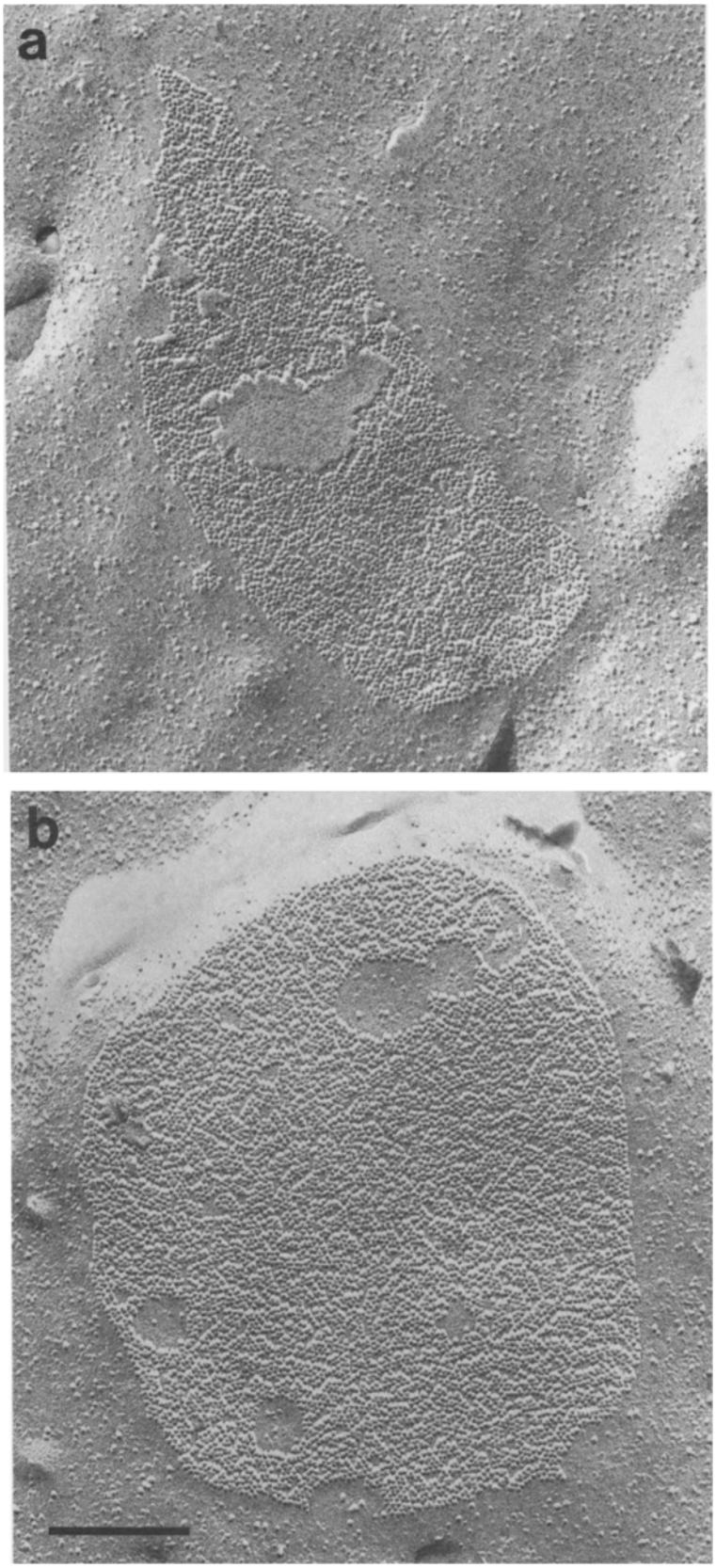

Fig. 3. Gap junctions, visualized by freeze-fracture electron microscopy, from uninfected NRK cells. Shown are the typical ordered arrangements of junctional particles, frequently sectioned into groups by particle-free isles. (*a*) Cells grown at 40.5°C; (*b*) cells grown at 35°C. Calibration bar = 0.25 μm

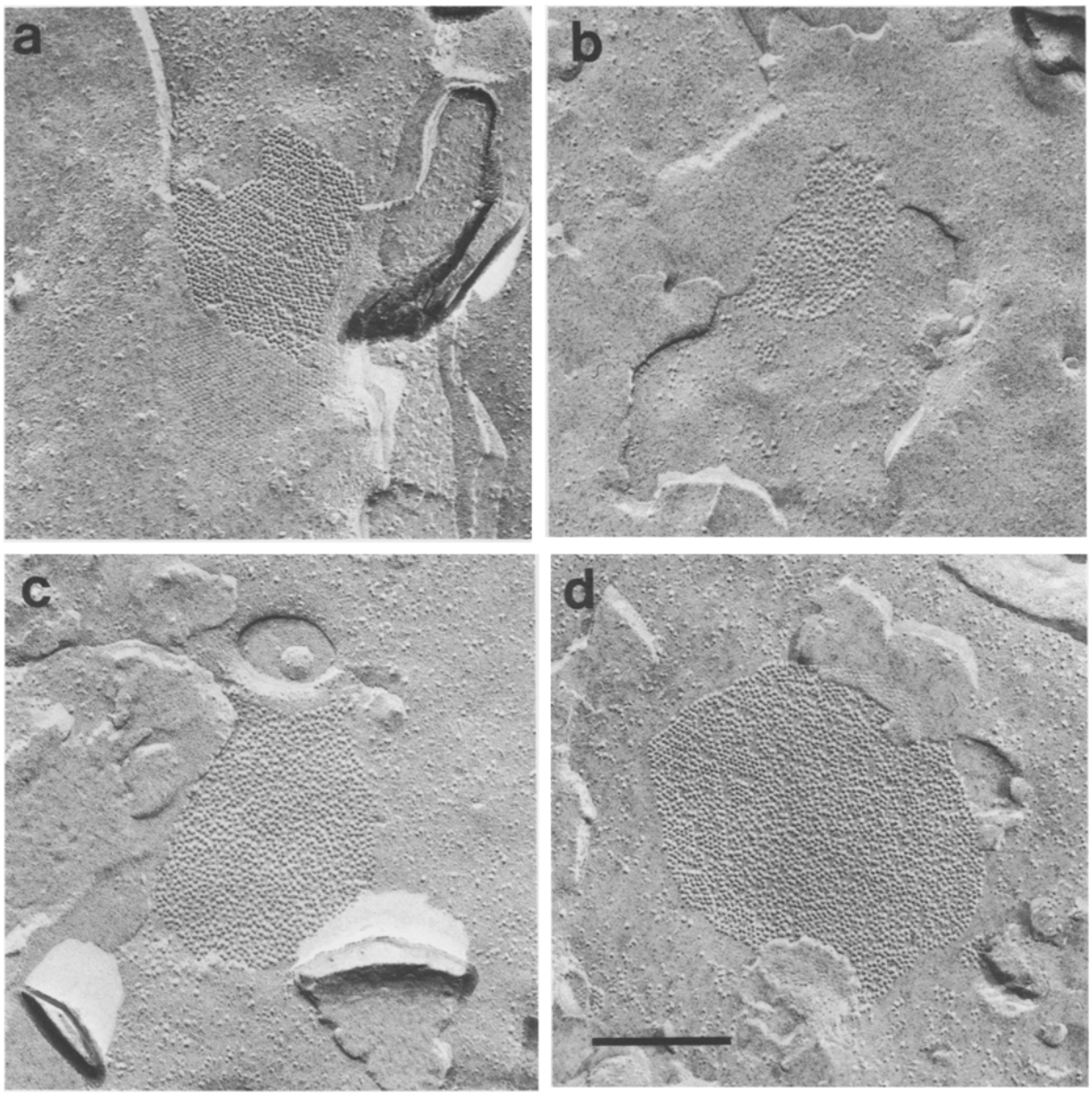

Fig. 4. Gap junctions, visualized by freeze-fracture electron microscopy, from LA25-NRK cells. Shown are examples of the predominant form of particle arrangements (*see* Table 3), except for *d*. (*a*) Cells grown at 40.5°C; (*b*) cells grown at 40.5°C and shifted to 35°C for 1 hr; (*c*) cells grown at 35°C; (*d*) cells grown at 35°C and shifted to 40.5°C for 1 hr. In *d*, note that the junction particles form both ordered and nonordered domains. Since the junction is predominantly in the nonordered configuration, the junction was scored as nonordered. This type of "mixed" junction was found in only a few cases. Calibration bar = 0.25 μm

ated with these junctions (*see* Fig. 3 for an example).

Discussion

These studies were undertaken to determine if the changes in junctional permeance of LA25-NRK cells (Atkinson et al., 1981; Atkinson & Sheridan, 1985), elicited by raising or lowering the growth temperature, result from changes in the number of junctional channels, or from an alteration of the channels themselves. To distinguish between these possibilities, we analyzed the gap junctions of test and control cells, using freeze-fracture techniques. The rationale was that any change in channel numbers sufficient to account for the magnitude of change in junctional permeance would be detectable from measurements of the number and areas of gap junctions. Relating cell-to-cell channel number to

gap junctions in this way involves three assumptions:

1) The aggregates of large particles, which are seen in freeze-fracture replicas and which define the gap junction, are in fact manifestations of the channels mediating permeation of dye and other small molecules.

2) All such channels occur in gap junctions; or at a minimum, extra-junctional channels do not contribute significantly to the intercellular passage of small molecules.

3) The total population of gap junctions is represented by the sample used for the analysis.

Evidence for a correspondence between permeable junctions and gap junctions is quite ample and persuasive and has been recently reviewed (more recently by Peracchia (1980) and Loewenstein (1981)). In addition, the identity of the structure (gap junction) with function (permeation) is greatly strengthened by the recent data on junctional blockade by antibodies to gap junctional protein (Warner et al., 1984; Hertzberg et al., 1984). An alternative to, or complication of, the present dogma is the possibility that many of the cell-to-cell channels may exist outside the confines of a gap junction; and they may be formed within and removed from the interfacial membrane in a number sufficient to effect the change in permeance. Theoretically there are two possibilities: the channels could occur in some other junctional structure (e.g., tight junctions), or they could be dispersed throughout the interfacial membrane. Since the gap junction is the sole junctional specialization we find in NRK cells, only the latter possibility is pertinent to this discussion.

There are few studies that provide experimental support for the idea that a physiologically relevant number of cell-to-cell channels can exist in a diffuse, dispersed state (Williams & DeHaan, 1981; Sheridan & Larson, 1982). In one of these studies (Williams & DeHaan, 1981), spheroidal aggregates of dispersed heart cells, which were cultured for extended periods of time in the presence of a protein synthesis inhibitor, remained electrically coupled, even though gap junctions were no longer detectable in freeze-fracture replicas. Although other explanations are possible, the implication is that the channels of low resistance junctions either disperse or otherwise become too sparse to be recognized as gap junctions. However, it can also be inferred from the study that, even if gap junctions are indeed severely reduced, they nevertheless constitute the favored state under more physiological conditions and that dissolution of that state requires rather drastic measures. The question remains whether a significant number of extra-junctional channels might co-exist with gap junctions.

Perhaps the most critical assumption of any freeze-fracture study is that the sampling procedures provide essentially unbiased samples of the total junctional population. This is particularly important since there are no approaches other than freeze fracture that can be used to independently assess junctional characteristics of the type reported here and thus provide an indication of sample bias. Fortunately, the use of a temperature-sensitive system and the particular characteristics of the LA25-NRK cells provide the means to either circumvent or reduce the likelihood of potential artifacts. In particular, two major advantages of the system need emphasis:

1) The experiments involve two cell states which are quite different, i.e., transformed and nontransformed, with the attending differences in physiology and morphology. Therefore, the central question of this paper is approached from two directions: one in which the junctions of transformed cells become more permeant, and the other in which the junctions of normal cells become less permeant.

2) Comparisons of the gap junctions are restricted to conditions where junctional permeance has been significantly altered (Atkinson et al., 1981; Atkinson & Sheridan, 1985), but cell morphology changes have not taken place (*see* Wang & Goldberg, 1979; *see also* Fig. 1). Consequently, the potential for errors caused by an influence of cell morphology on any number of factors (e.g. fracture patterns) should be minimized.

Changes in channel number could occur in two different ways. One possibility is that individual channels are formed within or removed from established gap junctions. If this mechanism were the basis of the permeance changes in LA25-NRK cells, one would then expect a change in mean junctional area or in the distribution of junctional areas, provided the channel density remained relatively constant. Neither junctional area nor the distribution of junctional areas change significantly for LA25-NRK cells after a one-hour temperature shift. This is true for temperature shifts in either direction. Since the distribution of junctional particle density does not change, these results indicate that individual channels are not inserted or removed from gap junctions in numbers great enough to support the changes in junctional permeance.

The second possible mechanism involves formation of new junctions and removal of entire gap junctions from the interfacial membrane. In this case, the mean junctional area or distribution of areas may or may not be changed, but the number of junctions must be affected. Again, no changes were detected. Thus it appears that the large swings in

junctional permeance which occur within the one-hour time frame (Atkinson et al., 1981; Atkinson & Sheridan, 1985) cannot be a consequence of channel formation or removal. The alternative, of course, is that junctional channels are somehow modified in a way that affects junctional permeance.

Supportive evidence for a mechanism involving modification of junctional channels is provided by our observation that differences in the packing arrangements of junctional particles are correlated with the two states of junctional permeance. Except for one case, an ordered pattern of junctional particles predominated when the permeance of the pre-fixed junctions was high and an unordered arrangement correspond to low permeance in the pre-fixed junctions. Even for the one exception—LA25-NRK cells shifted from 35 to 40.5°C (*see* Table 3)—the number of ordered junctions did increase from 18 to 45%, but the unordered pattern still predominated. This could indicate that the relationship between junctional particle packing and junctional permeance is sufficiently indirect that changes in the packing arrangement can, in some instances, lag behind the changes in junctional permeance. However, our data suggest that the modest increase in the number of ordered junctions is accompanied by an approximately fourfold increase in the area of ordered junctions on an interface (*see* Table 4). Thus, the two responses together might provide a change of sufficient magnitude to support the permeance change.

The possibility of a direct relationship between junctional particle packing and junction permeance was first suggested by Peracchia (reviewed by Peracchia, 1980), although he proposed that the ordered packing arrangement corresponds to closed junctions, which is opposite to that suggested by our results. Others have reported that, if quick-freezing techniques are used on certain tissues, an ordered packing arrangement is obtained for junctions that had presumably been in the open state just prior to quick-freezing (Page, Karrison & Upshaw-Earley, 1983; Green & Severs, 1984). Junctions from cardiac papillary muscle, quick-frozen either *in situ* or immediately after the tissue is excised, show ordered particle packing (Green & Severs, 1984), suggesting that junctional particle arrays are ordered in the physiological state, for this tissue at least. It is also possible that the packing arrangement of junctional particles is related to the type of tissue, as well as to the methods of fixation (e.g., Raviola, Goodenough & Raviola, 1980). The question of how the technical issues of fixation might be relevant to our results needs to be pursued. It should be pointed out, however, that the rate of fixative penetration into LA25-NRK cells growing in a monolayer should be very rapid. Since the procedure of washing the monolayer before adding the fixative exactly matched the washing procedure used in the physiology experiments (i.e., before the cells were injected with dye), we know that the junctions were in their particular permeability state immediately prior to fixation. Also, since consistent differences in packing arrangements *were* observed, it is not likely that the fixation technique itself induces a particular arrangement of particles.

Junctional particle diameters have also been reported to change in conjunction with altered permeance (e.g. Peracchia, 1977; Dahl & Isenberg, 1980). However, we have been unable to detect any correspondence between particle diameter and junctional permeance in NRK cells. Because we were working close to the resolution of the replicas, small changes in particle diameter would have gone undetected.

LA25-NRK cells are transformed by pp60^{src}, the *src* gene product of RSV (Brugge & Erikson, 1977; Purchio, Erikson, Brugge & Erickson, 1978; Levinson et al., 1978). Several studies indicate that the transforming activity of pp60^{src} involves its protein kinase activity (e.g. Sefton, Hunter, Beemon & Eckhart, 1980; Rubsamen, Ziemiecki, Friis & Bauer, 1980). Temperature-sensitive mutants of RSV have been shown to be defective in this kinase activity at the nonpermissive temperature (e.g. Rubsamen et al., 1980; Sefton et al., 1980; Ziemiecki & Friis, 1980), and a similar defect appears to be present in the LA25 mutant (A. Goldberg, *personal communication*). For mutants of this type, the earliest appearance of an increase in activity occurs approximately 15 to 20 min after a temperature shift (A. Goldberg, *personal communication*), quite similar to the time a junctional response is first seen in LA25-NRK cells (Atkinson et al., 1981; Atkinson & Sheridan, 1985). These kinetics, and the relatively rapid change in junctional particle ordering, suggest that the junctional response may be closely coupled to the activity of pp60^{src}. The lack of a change in the number or size of gap junctions, and the correlation of junctional particle rearrangements with changes in junctional permeance, indicate that existing junctional channels are being modified in a way that affects their lateral association with membrane lipids and/or other channel proteins.

This work was supported by NIH grants CA 30129 and CA 16335.

References

Anderson, S.A., Atkinson, M.M., Sheridan, J.D., Johnson, R.G. 1981. Freeze-fracture studies of gap junctions in cells

infected with a temperature-sensitive mutant of avian sarcoma virus. *J. Cell. Biol.* **91**:121*a*

Atkinson, M.M., Menko, A.S., Johnson, R.G., Sheppard, J.R., Sheridan, J.D. 1981. Rapid and reversible reduction of junctional permeability in cells infected with a temperature-sensitive mutant of avian sarcoma virus. *J. Cell Biol.* **91**:573–578

Atkinson, M.M., Sheridan, J.D 1985. Reduced junctional permeability in cells transformed by different viral oncogenes. *In:* Gap Junctions. M.V.L. Bennett and D.C. Spray, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Azarnia, R., Larsen, W.J., Loewenstein, W.R. 1974. The membrane junctions in communicating and non-communicating cells, their hybrids and segregants. *Proc. Natl. Acad. Sci. USA* **71**:880–884

Azarnia, R., Loewenstein, W.R. 1984. Intercellular communication and the control of growth: X. Alteration of junctional permeability by the *src* gene. A study with temperature-sensitive mutant Rous sarcoma virus. *J. Membrane Biol.* **82**:191–205

Bishop, J.M. 1983. Cellular oncogenes and retroviruses. *Annu. Rev. Biochem.* **52**:301–354

Borek, C., Higashino, S., Loewenstein, W.R. 1969. Intercellular communication and tissue growth. IV. Conductance of membrane junctions of normal and cancerous cells in culture. *J. Membrane Biol.* **1**:274–293

Brugge, J.S., Erikson, R.L. 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. *Nature (London)* **269**:346–348

Burton, A.C. 1971. Cellular communication, contact inhibition, cell clocks, and cancer. *Perspect. Biol. Med.* **14**:301–318

Burton, A.C., Canham, P.B. 1973. The behavior of coupled biochemical oscillators as a model of contact inhibition of cellular division. *J. Theor. Biol.* **39**:555–580

Caspar, D.L.D., Goodenough, D.A., Makowski, L., Phillips, W.C. 1977. Gap junction structure. I. Correlated electron microscopy and X-ray diffraction. *J. Cell. Biol.* **74**:605–628

Chang, C., Trosko, J.E., Kung, H., Bombick, D., Matsumura, F. 1985. Potential role of the *src* gene product in inhibition of gap-junctional communication in NIH/3T3 cells. *Proc. Natl. Acad. Sci. USA* **82**:5360–5364

Dahl, G., Isenberg, G. 1980. Decoupling of heart muscle cells: Correlation with increased cytoplasmic calcium activity and with changes of nexus ultrastructure. *J. Membrane Biol.* **53**:63–75

Furshpan, E.J., Potter, D.D. 1968. Low resistance junctions between cells in embryos and tissue culture. *Curr. Top. Dev. Biol.* **3**:95–127

Gilula, N.B., Reeves, O.R., Steinbach, A. 1972. Metabolic coupling, ionic coupling, and cell contacts. *Nature (London)* **235**:262–265

Green, C.R., Severs, N.J. 1984. Gap junctions connexon configuration in rapidly frozen myocardium and isolated intercalated disks. *J. Cell Biol.* **99**:453–463

Hertzberg, E.L., Spray, D.C., Bennett, M.V.L. 1984. An antibody to gap junctions blocks gap junctional conductance. *J. Cell Biol.* **99**:343*a*

Johnson, R.G., Sheridan, J.D. 1971. Junctions between cancer cells in culture: Ultrastructure and permeability. *Science* **174**:717–719

Kanno, Y., Loewenstein, W.R. 1966. Cell-to-cell passage of large molecules. *Nature (London)* **212**:629–630

Kanno, Y., Matsui, H. 1968. Cellular uncoupling in cancerous stomach epithelium. *Nature (London)* **218**:775–776

Kuffler, S.W., Potter, D.D. 1964. Glia in the leech central nervous system: Physiological properties and neuron-glia relationships. *J. Neurophysiol.* **27**:290–320

Levinson, A.D., Opperman, H., Levintow, L., Varmus, H.E., Bishop, J.M. 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. *Cell* **15**:561–572

Loewenstein, W.R. 1966. Permeability of membrane junctions. *Ann. N.Y. Acad. Sci.* **137**:441–472

Loewenstein, W.R. 1968. Communication through cell junctions. Implications in growth control and differentiation. *Dev. Biol.* **190 (Suppl. 2)**:151–183

Loewenstein, W.R. 1979. Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta* **560**:1–65

Loewenstein, W.R. 1981. Junctional intercellular communication: The cell-to-cell membrane channel. *Physiol. Rev.* **61**:829–913

Loewenstein, W.R., Kanno, Y. 1964. Studies on an epithelial (gland) cell junction. I. Modification of surface membrane permeability. *J. Cell Biol.* **22**:565–586

Loewenstein, W.R., Kanno, Y. 1967. Intercellular communication and tissue growth. I. Cancerous growth. *J. Cell Biol.* **33**:225–234

Makowski, L., Caspar, D.L.D., Phillips, W.C., Goodenough, D.A. 1977. Gap junction structure. II. Analysis of the X-ray diffraction data. *J. Cell Biol.* **74**:629–645

McNutt, N.S., Hershberg, R.A., Weinstein, R.S. 1971. Further observations on the occurence of nexuses in benign and malignant human cervical epithelium. *J. Cell Biol.* **51**:805–825

McNutt, N.S., Weinstein, R.S. 1969. Carcinoma of the cervix: Deficiency of nexus intercellular junctions. *Science* **165**:597–599

McNutt, N.S., Weinstein, R.S. 1973. Membrane ultrastructure at mammalian intercellular junctions. *Prog. Biophys. Mol. Biol.* **26**:45–101

Newbold, R.F. 1982. Metabolic cooperation in tumor promotion and carcinogenesis. *In:* Functional Integration of Cells in Animal Tissues. pp. 301–317. Symposium 5 of the British Society for Cell Biology. J.D. Pitts and M.E. Finbow, editors. Cambridge University Press, Cambridge

Page, E., Karrison, T., Upshaw-Earley, J. 1983. Freeze-fractured cardiac gap junctions: Structural analysis by three methods. *Am. J. Physiol.* **244**:H525–H539

Peracchia, C. 1977. Gap junctions. Structural changes after uncoupling procedures. *J. Cell Biol.* **72**:628–641

Peracchia, C. 1980. Structural correlates of gap junction permeation. *Int. Rev. Cytol.* **66**:81–146

Pinto da Silva, P., Gilula, N.B. 1972. Gap junctions in normal and transformed fibroblasts in culture. *Exp. Cell Res.* **71**:393–401

Potter, D.D., Furshpan, E.J., Lennox, E.S. 1966. Connections between cells of the developing squid as revealed by electrophysiological methods. *Proc. Natl. Acad. Sci. USA* **55**:328–339

Purchio, A.F., Erikson, E., Brugge, J.S., Erikson, R.L. 1978. Identification of a polypeptide encoded by the avian sarcoma virus *src* gene. *Proc. Natl. Acad. Sci. USA* **75**:1567–1571

Ravioloa, E., Goodenough, D.A., Raviola, G. 1980. Structure of rapidly frozen junctions. *J. Cell Biol.* **87**:273–279

Revel, J.-P., Yee, A.G., Hudspeth, A.J. 1971. Gap junctions between electronically coupled cells. *Proc. Natl. Acad. Sci. USA* **68**:2924–2938

Rubsamen, H., Ziemiecki, A., Friis, R.R., Bauer, H. 1980. The

expression of pp60^{src} and its associated protein kinase activity in cells infected with different transformation-defective temperature-sensitive mutants of Rous sarcoma virus. *Virology* **102:**453–457

Sefton, B.M., Hunter, T., Beemon, T., Eckhart, W. 1980. Evidence that the phosphorylation of tryosine is essential for cellular transformation by Rous sarcoma virus. *Cell* **20:**807–816

Sheridan, J.D. 1970. Low-resistance junctions between cancer cells in various solid tumors. *J. Cell Biol.* **45:**91–99

Sheridan, J.D. 1976. Cell coupling and cell communication during embryogenesis. *In:* The Cell Surface in Animal Embryogenesis and Development. G. Poste and G.L. Nicolson, editors. pp. 409–447. Elsevier/North Holland, Amsterdam

Sheridan, J.D., Atkinson, M.M. 1985. Physiological roles of permeable junctions: Some possibilities. *Annu. Rev. Physiol.* **47:**337–353

Sheridan, J.D., Johnson, R.G. 1975. Cell junctions and neoplasia. *In:* Molecular Pathology. R.A. Good, S.B. Day, and J.J. Yunis, editors. pp. 354–378. Charles C. Thomas, Springfield, Ill.

Sheridan, J.D., Larson, D.M. 1982. Junctional communication in the peripheral vasculature. *In:* The Functional Integration of Cells in Animal Tissues. pp. 263–283. Symposium 5 of the British Society for Cell Biology. J.D. Pitts and M.E. Finbow, editors. Cambridge University Press, Cambridge

Unwin, P.N.T., Zampighi, G. 1980. Structure of the junction between communicating cells. *Nature (London)* **283:**545–549

Wang, E., Goldberg, A.A. 1979. Effects of the *src* gene product on microfilament and microtubule organization in avian and mammalian cells infected with the same temperature-sensitive mutant of Rous sarcoma virus. *Virology* **92:**201–210

Warner, A.E., Guthrie, S.C., Gilula, N.B. 1984. Antibodies to gap-junctional protein selectively disrupt junctional communication in the early embryo. *Nature (London)* **311:**127–131

Weinstein, R.A., Merk, F.B., Alroy, J. 1976. The structure and function of intercellular junctions in cancer. *Adv. Cancer Res.* **23:**23–79

Williams, E.H., DeHaan, R.L. 1981. Electrical coupling among heart cells in the absence of ultrastructurally defined gap junctions. *J. Membrane Biol.* **60:**237–248

Wyke, J.A. 1973. The selective isolation of temperature-sensitive mutants of Rous sarcoma virus. *Virology* **52:**587–590

Yancey, S.B., Edens, J.E., Trosko, J.E., Chang, C.C., Revel, J.P. 1982. Decreased incidence of gap junctions between Chinese Hamster V-79 cells upon exposure to the tumor promoter 12-*O*-tetra-decanoyl phorbol-13-acetate. *Exp. Cell Res.* **139:**329–340

Yotti, L.P., Chang, C.C., Trosko, J.E. 1979. Elimination of metabolic cooperation in Chinese hamster cells by a tumor promoter. *Science* **206:**1089–1091

Zampighi, G., Corless, J.M., Robertson, J.D. 1980. On gap junction structure. *J. Cell Biol.* **86:**190–198

Ziemiecki, A., Friis, R.R. 1980. Phosphorylation of pp60^{src} and the cyclohexamide insensitive activation of the pp60^{src}-associated kinase activity of transformation-defective, temperature-sensitive mutants of Rous sarcoma virus. *Virology* **106:**391–394

Received 30 July 1985; revised 13 January 1986