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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

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Summary. To study changes of junctional membrane permeability associated with transformation, the junctions and the nonjunctional membranes of quail embryo-, chick embryo- and mouse-3T3 cell cultures, infected with temperature-sensitive mutant Rous sarcoma virus, were probed with fluorescent-labelled glutamate. Junctional permeability fell in the transformed state. In the quail cells, the fall was detectable within 25 min of shifting the temperature down to the level (permissive) at which tyrosine-phosphorylation by the viral *src* gene product is expressed. This reduction of junctional permeability is one of the earliest manifestations of viral transformation. Normal permeability was restored within 30 min of raising the temperature to the nonpermissive level, a reversibility that could be displayed several times during the span of a cell generation. The reversal seems to reflect a reopening of cell-to-cell channels rather than a synthesis of new ones; it is not blocked by protein-synthesis inhibition. Treatments with cyclic AMP and phosphodiesterase inhibitor or with forskolin, which stimulate serine and threonine phosphorylation—the type of phosphorylation on which normal junctional permeability depends (Wiener & Loewenstein, 1983, *Nature* 305:433)—did not abolish, in general, the junctional effect of the virus; *src* tyrosine-phosphorylation apparently overrides the junctional upregulation mediated by cyclic AMP.

*Non*junctional membrane permeability was not sensibly affected by the virus. It was affected, however, by temperature: lowering the temperature from the nonpermissive to the permissive level caused the nonjunctional permeability to fall, and *vice versa*. This change was unrelated to transformation. Its secondary effect on junctional transfer is in the opposite direction to that produced by the temperature-activated viral transformation.

Key Words cell junction · cell-to-cell communication · cell-to-cell channel · gap junction · Rous sarcoma virus · transformation · cancer · growth control · tyrosine phosphorylation · *src* gene · protein kinase · $pp60^{src}$

Introduction

This series of papers is prompted by the hypothesis that the cell-to-cell channels, the elements connecting the interiors of normal cells, are conduits for growth controlling signals (Loewenstein, 1966; 1968*a*). It is part of a search for cell types that are deficient in this form of cellular communication. So far, the search has centered on radical defects of communication—complete blockage of the channels or complete lack of them—defects of a sort that can be readily detected by electrical measurements. The quest was rewarded by the finding of a number of such communication-lacking cell types, and the hypothesis drew strength from the fact that these cells invariably exhibited uncontrolled cancerous growth (Loewenstein, 1979).

That the growth of such fully uncommunicative cells be uncontrolled, is precisely a prediction of the hypothesis and its chief heuristic stimulus. However, *a priori* it is not necessary that, to decontrol growth, the communication defect be so drastic. Deficiencies affecting the conduction of only a range of the channel-permeant molecular species, say, the larger ones, could be sufficient, if the growth-controlling molecules (signals) fell in that range (Loewenstein, 1968*b*). One would then foresee two possible classes of growth-decontrolling defects of communication: one where the individual cell-to-cell channels are too small to transmit the signals, and another where the number of channels is too small to transmit signals at a sufficient rate.

The detection of such subtler deficiencies (but not their distinction) has come within our reach over the past years through the development of a spectrum of channel-permeant fluorescent probes (Simpson, Rose & Loewenstein, 1977; Schwartzmann, Wiegandt, Rose, Zimmerman, Ben-Haim & Loewenstein, 1981). Moreover, sensitive and efficient methods are now on hand for determining, in cultured cell populations, changes in the probes' junctional cell-to-cell transfer and *non*junctional membrane permeance (Flagg-Newton, Dahl & Loewenstein, 1981; Radu, Dahl & Loewenstein, 1982). In order to show an alteration of junctional

permeability, one needs information about nonjunctional permeance in addition to information about junctional transfer, because both, junctional and nonjunctional permeances, are primary determinants of junctional transfer.

We use such methods here to examine the junctional permeability of transformed cells that are electrically coupled. As permeability probes, we choose a series of polyaminoacids: mono-, di- and triglutamic acid. This series, graded in molecular size and negative charge, allows one to set up threshold conditions for junctional transfer and, thus, affords a sensitive means for detecting deviations in junctional permeability. The probes are covalently coupled to lissamine rhodamine B, a label whose fluorescence is insensitive to changes of pH, such as might occur intracellularly during the manipulations of the temperature of the cell cultures.

In the present set of papers we report the results obtained with cells transformed by Rous sarcoma virus (RSV) and simian virus 40 (SV40). Our choice was guided by the thought that the gene products of these viruses might interfere with the cyclic AMP-dependent phosphorylation on which the normal junctional permeability depends (Wiener & Loewenstein, 1983). These viruses, certainly RSV, foster a very different kind of phosphorylation. RSV requisitions the transcription machinery of the host cell to produce a protein kinase—the transforming 60,000-mol wt protein, $pp60^{src}$, encoded by the viral *src* gene—that promotes the phosphorylation of tyrosine residues (Collett, Purchio & Erikson, 1980; Hunter & Sefton, 1980; Gilmer & Erikson, 1981; McGrath & Levinson, 1982; *cf.* Bishop, 1982), instead of serine and threonine residues, as in cyclic AMP-dependent phosphorylation (Krebs & Beavo, 1979). And in the case of SV40 there are reasons to suspect that one of its gene products might act by such a phosphorylating mechanism, too. We will show that both types of virus, indeed, cause alteration in junctional permeability.

We use temperature-sensitive mutants of the virus in the work of the first and third paper of this set. These mutants allow one to rapidly undo the transformed state or to restore it, by simply shifting the temperature of the cell cultures by a few degrees, between the nonpermissive and permissive levels for the virus. The temperature response of RSV-transformed cells is particularly fast. Tyrosine phosphorylation sets in within half an hour of shifting down to the permissive temperature (Radke & Martin, 1979). One can thus experimentally control the expression of the transformed state within the span of one cell generation. Such speed of control is invaluable when one deals with subtle changes of communication in cell populations. It enables one to test for reversibility of the observed changes in junctional permeability. In the work of the second paper, we gave this advantage up for one provided by cells that reverted in certain cytoskeletal properties, from RSV transformation. In that work we compare parallel cell cultures, transformed and untransformed, and rely entirely on statistical tests of the data—an approach requiring a larger number of measurements of junctional transfer.

The present paper gives an account of our results obtained with temperature-sensitive mutant RSV. We show that the junctional permeability is reduced in three vertebrate cell types by action of this virus. The reduction is an early manifestation of transformation, a manifestation reversed rapidly and without need for new protein synthesis when the viral *src* gene is turned off.

While our study was in progress, Atkinson, Menko, Johnson, Sheppard and Sheridan (1981) reported that, in RSV-transformed NRK cells, the junctional transfer rate of the 443-mol wt, pH-insensitive dye Lucifer Yellow was slowed. This finding was suggestive and encouraging. It lacked, however, information on nonjunctional membrane permeability, and so it was not telling whether the change of transfer represented an alteration of junctional or nonjunctional membrane. A reduction in junctional transfer rate by itself does not imply a change in junctional permeability; it may merely reflect a change in nonjunctional membrane permeability, the other major determinant of junctional transfer.

Materials and Methods

Cells and Media

Quail embryo fibroblasts infected with temperature-sensitive RSV-NY68, Schmidt-Ruppin group A (QEF-*ts*NY68) or with wild-type RSV of the same Schmidt-Ruppin group (QEF-*wt*) (gifts of Drs. R. Farrell and V. Ingram), chick embryo fibroblasts and fibroblasts from the mouse 3T3 line infected with temperature-sensitive RSV-LA90, Schmidt-Ruppin group D, (CK-*ts*LA90, 3T3-*ts*LA90) or with the corresponding wild-type RSV (CK-*wt*, 3T3-*wt*) (gifts of Dr. R. Keane) were cultured in Dulbecco's MEM medium supplemented with 10% fetal calf serum (gentamycin 0.005%). The medium was renewed every 2–3 days, but not during the experiments nor during the 1-day period before the experiments, to avoid effects of serum on junctional permeability (Flagg-Newton & Loewenstein, 1981). The quail and chick embryo cells were primary cultures that became lines after their infection. The cells infected with temperature-sensitive virus were grown on the bottom of plastic dishes (Falcon, 35 mm) in an

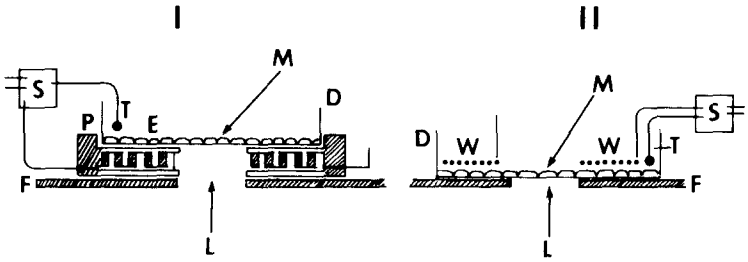

Fig. 1. Temperature-control apparatus. (*I*): *P*, modular thermoelectric device made of bismuth-thellurium junctions in series, embedded in ceramics; heat is transferred to a plastic culture dish (*D*) through a metal base. *T*, thermistor. *S*, servo-control. *M*, cells. *E*, medium. *F*, microscope stage. *L*, light path. (*II*): A variant in which an insulated wire coil (*W*) directly transfers the heat generated by an electrical current, to the medium of the dish

incubator, 6–8% CO_2 in air, at 33–35°C, the permissive temperature for the virus. (The cells infected with wild-type virus were grown at 37°C.)

Temperature Changes and Control

To shift their temperature to the nonpermissive level, the cultures were moved to an incubator at 41°C. Parallel subcultures were continued at the permissive temperature. During the junctional probings the dishes were on the microscope stage in room air. Their temperature was kept at the corresponding level by means of a thermoelectric device based on the Peltier effect, fitted to the microscope stage. A small thermistor, placed close to the cell layer in the dish, served to monitor the temperature of the culture and to supply the feed-back current for automatic control of the thermoelectric device, with an accuracy better than ±0.5°C in the steady state (Fig. 1,*I*).

Some of our experiments were done before we had adopted the thermoelectric system as a standard procedure. In these earlier experiments the temperature of the cultures was changed in the incubators, as described above, but junctional transfer was tested at 32°C room temperature. We included the data from those experiments in which the junctional test periods were limited to 15 min, in the tables of the Results. As our later experiments showed, junctional permeability is not much changed during the first 15 min of lowering the temperature from the nonpermissive to the permissive level. By including the earlier data, we only risked underestimating the difference between the junctional permeabilities in the transformed and untransformed states.

For studies of the time course of the junctional response, the temperature of the cultures was changed by means of the thermoelectric device, outside the incubator. (Dishes were of equal size containing 3 ml of medium.) The upshifts of temperature between the 34/35 and 41°C levels had time constants of about 50 sec, and the downshifts had time constants of about 40 sec. During heating cycles, the thermoelectric device underwent microscopic dimensional changes moving the cell layer out of focus. It was, therefore, necessary to re-focus before each test of junctional transfer, for which there was ample time.

However, in the case of the measurements of the rates of fluorescence loss, where a resolution of the order of 1 min was needed, we could not afford such adjustments. We heated the medium directly by flowing current through a coil of insulated wire submerged in the medium—a method suggested to us by Dr. John Barrett (Fig. 1,*II*). The thermistor feed-back system held the temperature at the desired levels ±0.5°C. Up- and downshifts of temperature had time constants of 15 and 48 sec, respectively (room temperature, 32°C).

Junctional Probing

Junctional permeability was probed with glutamic acid labelled with lissamine rhodamine B (LRB-Glu; 688 mol wt, 2 negative charges). The preparation and purification of this probe is described elsewhere (Simpson et al., 1977; Socolar & Loewenstein, 1979) (for molecular dimensions, *see* Schwarzmann et al., 1981). This red-fluorescent tracer was injected by means of micropipettes into the cells (*source cells*) by the pneumatic pressure of a syringe. The micropipettes were drawn from capillaries containing a microfilament (boro-silicate glass, A-M Systems, Inc.).

The general procedure was to individually inject, in rapid succession, 8–12 cells in different locations of each given dish. To minimize photo-bleaching of the label and harm to the cells due to light absorption, fluorescence excitation (560 nm) was intermittent (mercury lamp, HBO-100W); total period, <1 min. Within 5 to 10 min of their injection, we scanned the cells contiguous to the source cell (first-order neighbors) for fluorescence. Data from cells damaged by the microinjection, as indicated by the appearance of cytoplasmic granularity, opacity, or blebbing, were rejected. Such damage occurred rarely with the cell material used in the work of this paper and the last one of this series. (It posed a problem in the work of the second paper, necessitating a special probing method in that case.)

LRB-Glu (as well as the higher members of the glutamic-acid series labelled with LRB) is stable over the temperature range used. We checked this by means of paper electrophoresis at pH 8.4 (0.05 M NH_4HCO_3) (Simpson et al., 1977). The result was invariably a single spot corresponding to the mobility of LRB-Glu. (The unlinked LRB^- spots with different mobility.)

Scoring and Statistical Treatment of Junctional-Transfer Data

For *each individual* source cell, we scored the percentage of the fluorescent first-order neighbors—the *incidence of permeable interfaces*. Depending on cell density, the numbers of first-order neighbors per source cell ranged 5–10 in the quail cell cultures, 6–7 in the chick cells, and 4–10 in the 3T3 cell cultures.

Table 1. Incidence of LRB-Glu-permeable interfaces at various times after injection. Quail embryo cells (QEF-*ts*NY68), 41°C

Time[a] (min)	Permeable interfaces[b]	
	%	P[c]
2	35 ± 9	0.015
5	61 ± 5	0.47
10	62 ± 4	

[a] Time after injection.
[b] Mean incidence ± SE.
[c] Statistical confidence level of the difference between successive pairs of mean incidence values.

We chose to score the incidence of permeable interfaces within 5 to 10 min of the injection, not only because this period was experimentally convenient, but also because we had learned from a special run of experiments with quail embryo cells (QEF-*ts*NY68) at 41°C that there was no loss of information nor bias of the results during this span: once a first-order neighbor was fluorescent at 5 min, it was still so at 10 min, and there was no evidence for more fluorescent neighbors at 10 min than at 5 min (Table 1). Evidently, tracer dilution by cell-to-cell diffusion is not limiting the detectability over this span under the present conditions and, as will be shown (Results), the loss of intracellular LRB-Glu through nonjunctional cell membrane is small.

The mean values of incidence of permeable interfaces listed in the main tables of the Results are based on the examination of 10–48 individually injected source cells. (In the summary Tables 2*A* and 3*A*, the means are based on the incidence values of the individual source cells from the pooled experiments.) For the calculation of all statistics, including the significance of the differences between the means, *n* was the number of source cells. (We give the number or range of interfaces examined in the tables' footnotes for additional information, although the numbers don't enter into the statistics.) The statistical confidence levels (*P*) of the differences were calculated by standard *t* test.

In the experiments dealing with the time course of the junctional response, each mean is based on the examination of 8–12 source cells, comprising a time span as denoted by the horizontal bars in the figures. The shortest span, i.e., the highest time resolution, was 10 min, as in Fig. 3.

Measurement of Fluorescence Loss

Intracellular fluorescence of the tracer was measured by means of a photodiode system onto which the image of the cells was projected through the microscope (Flagg-Newton et al., 1981). The measurements were taken on single cells, without neighbors in contact. Excitation parameters: 560-nm; pulses, 5 sec duration, 1/min. The cell image was larger than the area seen by the photodiode, and the microscope focal depth, smaller than the cell thickness. *Thus, cell dimensional changes, if they occurred at all, were of no consequence here.*

This choice of excitation parameters was based on a series of experiments designed to find conditions for minimal photobleaching. For these experiments we used capillaries as stand-ins for the cells. The capillaries were filled with LRB-Glu at various concentrations below the range of fluorescence quenching. The fluorescence was measured by means of the photodiode system. (The same light source was used.) With the parameters above, there was no fluorescence decay detectable over 10–20 min, periods comparable to those of our cellular measurements. Figure 5, *inset*, illustrates this for a run at 34°C.

Solutions of Dibutyryl cAMP, Caffeine and Cycloheximide

Stock solutions were in serum-free MEM medium, 1 mM for dibutyryl cAMP and 1 mM for caffeine, and 100 μg/ml for cycloheximide. The last solution was freshly prepared for each experiment; the first two were kept at 4°C for up to 1 week. They were added to the serum-containing medium of the dishes, to a final tenfold dilution.

Results

Choice of the Junctional Probe

In cultures with multiple cell contacts, the incidence of permeable cell interfaces is a convenient index of changes in junctional permeability, and a particularly sensitive one when the junctional probe is close to the detection threshold of cell-to-cell permeation (Radu et al., 1982). Thus, in runs preliminary to the experiments below, we determined first, for each cell type, which of the probes of the graded series, LRB-Glu, LRB-Glu-Glu, and LRB-Glu-Glu-Glu (Simpson et al., 1977), was closest to the threshold of cell-to-cell permeation at the permissive temperature for the transforming virus. This turned out to be LRB-Glu for all three types of cells, and so we adopted this probe for all the experiments.

Quail Embryo Cells

Junctional Transfer Undergoes a Decrease in the Transformed State

The cell cultures infected with temperature-sensitive virus (RSV-*ts*NY68) were habitually kept at 34 or 35°C, permissive temperatures for the virus mutants. When the temperature was raised to the nonpermissive range, 41°C, the incidence of permeable cell interfaces increased (Fig. 2 and Table 2).

We made the comparisons of incidence between parallel cultures, namely subcultures in identical medium and serum supplement, of similar densities and of the same history (several weeks) of

passaging and medium. Such constancy of experimental conditions is important because junctional permeability can vary otherwise (Azarnia, Dahl & Loewenstein, 1981; Flagg-Newton & Loewenstein, 1981; Flagg-Newton et al., 1981). For the strictest comparisons, we used only experiments which were run in parallel the same day and had the same 48-hr history of cell passaging and feeding before the measurements (in addition to the general culture parallelism above). The corresponding data are aligned horizontally in the main Tables 2–6. As it turned out, the junctional transfer in the RSV-infected cells showed little dependence on cell density, and the differences between the transformed and untransformed states were well apparent in comparisons attending only to the general parallelism. Such comparisons are made in Tables 2*A* and 3*A*.

Table 2 gives the results obtained with quail embryo cells. One member of a pair of parallel subcultures (or one set of members) was kept here at the permissive temperature and the other member (or set of members) was for 2–24 hr at the nonpermissive temperature. At the permissive temperature, the mean incidence of permeable interfaces was smaller by a factor of 1.2 to 7.1. The difference was in all cases statistically significant (the confidence levels, P, ranged $0.005 - <0.00009$).

To control for effects of temperature *per se*, we used quail embryo cells that were infected with wild-type virus from the same strain as the temperature-sensitive mutant (QEF-*wt*). There was no significant difference between the incidence of permeable interfaces at 35°C and 41°C (Table 2, *bottom*). The higher cell-to-cell transfer at the nonpermissive temperature in the cells transformed by the temperature-sensitive mutant is thus not attributable to the temperature change *per se*.

Table 2*A* summarizes these results. All categories of cell density and of nonpermissive-temperature duration are lumped here, as neither density nor that duration seemed to influence the junctional transfer over the experimental range.

In another kind of experiment, we followed the response to temperature in one and the same culture. Figure 3 is an example in which the temperature was shifted repeatedly between the permissive and nonpermissive levels. Successive up- and downshifts are spaced 30 and 60 min apart, intervals we can presume to be long enough for the cell-to-cell transfer to reach steady state (*see below*). The incidence of permeable interfaces then shifted between a high and a low, following the change in temperature. This kind of experiment showed the relation between junctional transfer and viral transformation, most directly.

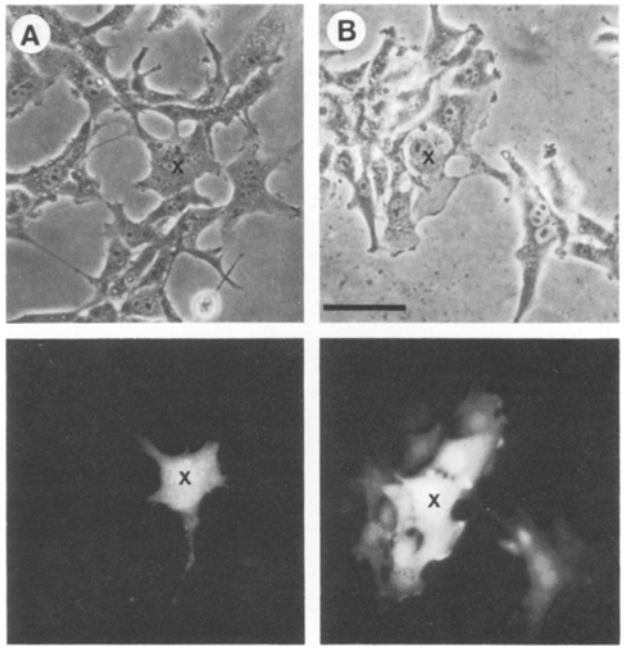

Fig. 2. Quail embryo cells infected with temperature-sensitive RSV (NY68) (*A*), at the permissive temperature (35°C) and (*B*), 30 min after raising the temperature to the nonpermissive level (41°C). *Top*, phase contrast photomicrograph. *Bottom*, same cells in darkfield, displaying LRB-Glu fluorescence. LRB-Glu was injected into the cell marked *x*. *A* and *B* are different cell groups. Calibration, 100 μm

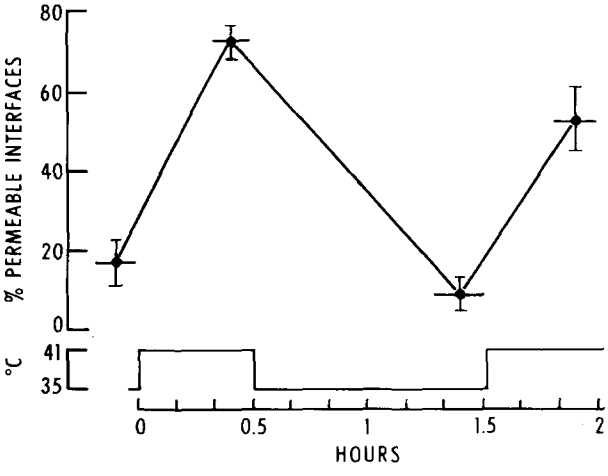

Fig. 3. Reversible changes in junctional transfer produced by temperature shifts between the permissive and nonpermissive levels of the virus, in quail embryo cells. The temperature of this culture (lower ordinates) was raised to the nonpermissive level (41°C), then lowered and raised again by the thermoelectric device (Fig. 1*I*). The upper ordinates give the mean incidences of LRB-Glu-permeable interfaces (± SE) determined before each temperature shift. The horizontal bars give the time span over which the incidences were determined

Table 2. Incidence of LRB-Glu-permeable interfaces at permissive and nonpermissive temperatures. Quail embryo cells.

Cell system	Density (10^4 cells/cm^2)	Permeable interfaces %[a]				
		34°C	35°C	41°C	hr[b]	P[c]
QEF-*ts*NY68[d]	5.0 – 10.0		19 ± 4	67 ± 6	24	<0.00009
	9.6 – 12.0		20 ± 7	82 ± 6	2	<0.00009
	10.0 – 19.0		21 ± 5	59 ± 5	24	<0.00009
	11.0 – 18.0		18 ± 4	40 ± 5	5	0.0006
	15.0 – 22.0	10 ± 3		71 ± 2	5	<0.00009
QEF-*wt*[e]	1.7 – 2.4		55 ± 3	58 ± 4	24	0.236
	10.0 – 11.0		43 ± 7	53 ± 4	24	0.132

[a] Mean incidence of (first-order) permeable cell interfaces ± SE at the permissive—34, 35°C—and nonpermissive temperature—41°C. The total number of cell interfaces examined for each mean value ranged 67–254, and the number of culture dishes 2–4. (*See* Materials and Methods for the number of interfaces per source cell, the number of source cells (n), and statistical treatment of the data.)
[b] The cultures were habitually kept at the permissive temperature; this column gives the time they had been at the nonpermissive temperature, when the incidence of permeable interfaces was determined.
[c] Statistical Confidence level of the difference between the mean values at the permissive and nonpermissive temperatures (t test).
[d] Temperature-sensitive NY68 virus, Schmidt-Ruppin group A.
[e] Wild-type virus, Schmidt-Ruppin group A. Each horizontal line in this table and in Tables 3, 4, 5 and 6 gives the data from strictly parallel sets of experiments (*see* text).

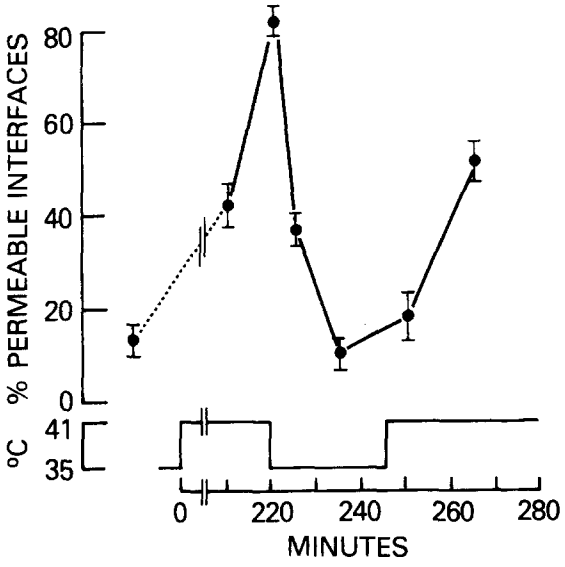

Fig. 4. A transient increase of junctional transfer produced by a lowering of temperature in quail embryo cells. The sequence of temperature changes is as in the experiment of Fig. 3, but the junctional transfer is tested here within 10 min of the temperature downshift (at time 220 min), resolving a rise in the incidence of permeable interfaces before the incidence falls to the level of the transformed state. The time span over which each mean is determined is 10 min

The responses to temperature were fast. Within the limits of our time resolution (10–15 min, *see* Materials and Methods), we found increases of transfer within 30 min of raising the temperature to the nonpermissive level (e.g., Fig. 3), and decreases within 25 min of lowering the temperature to the permissive level (e.g., Fig. 4). This fast reversibility of the response, which could readily be displayed several times within the span of one cell generation, provided the most straight-forward demonstration of the virus action on junctional transfer.

When we pressed the time resolution to the limit of our method (10 min), a further aspect became discernible in the temperature response in some cases: upon temperature downshift, the junctional transfer first rose before it fell to the steady-state level of the transformed state. In the example of Fig. 4, the sequence of temperature shifts was like that of Fig. 3, and the final levels of incidence of permeable interfaces at the permissive and nonpermissive temperatures were comparable. But, as we tested within 10 min of the temperature downshift, the transient rise in junctional transfer was resolved. This rise probably relates to a change in *nonjunctional* membrane, as will be analyzed below.

Junctional Permeability Undergoes a Decrease in the Transformed State

The rate of junctional transfer is directly related to junctional permeability and inversely to nonjunctional membrane permeability. Thus, our next prob-

Table 2A. Data summary. Quail embryo cells

Cell system	Permeable interfaces %			
	34°C	35°C	41°C	*P*
QEF-*ts*NY68		19 ± 2 (76)	62 ± 3 (77)	<0.00009
	10 ± 3 (21)		71 ± 2 (22)	<0.00009
QEF-*wt*		51 ± 3 (31)	57 ± 3 (30)	0.114

Mean value ± SE of the data of Table 2. In parentheses, the number of source cells injected, the *n* value on which the means and statistics are based.

lem was to resolve which of the two determined the observed changes of junctional transfer.

Nonjunctional Membrane Permeability and the Reduction of Junctional Transfer. We consider first the reduction in junctional transfer observed during the steady state of transformation. This reduction admitted two causal alternatives: a decrease of junctional permeability or an increase of nonjunctional membrane permeability. Changes of nonjunctional permeability can be determined in sparse cultures, in single cells not in contact with others. We measured, by microfluorimetry, the rate of loss of LRB-Glu fluorescence from such cells, at the steady permissive and nonpermissive temperature levels.

In principle, such loss of fluorescence can have two components here: (i) a loss due to photo-decomposition of the label (photo-bleaching) and (ii) a loss due to leakage of the molecules through the cell membrane—the component of interest here. The first component becomes negligible at low fluorescence excitation, and we chose excitation parameters such as to insure this condition. Thus, the fluorescence loss or the change of loss in the following experiments gives a measure of the LRB-Glu nonjunctional-membrane permeance. By LRB-Glu we mean the whole molecule; the LRB label is covalently linked to Glu and does not come off at these temperatures. The tests concerning the photobleaching and the stability of the LRB-Glu complex are described in Materials and Methods, and the absence of significant photo-bleaching is illustrated in Fig. 5, *inset*.

Representative outcomes of the measurements of fluorescence loss are shown in Fig. 5. The most common result was a virtual absence of fluorescence loss, over the 10–20 min period the measurements were taken, as exemplified by Fig. 5*A*. At stable temperature, the rates of loss were <0.2%/min at the permissive and nonpermissive levels. Such losses are negligible over the LRB-Glu junctional transit time (the longest transit times were about 85 sec) or even over the longest scoring times of permeable interfaces (10 min). A significant contribution by nonjunctional membrane to the observed changes in junctional transfer is thus excluded here.

Some cases, however, showed evidence of loss. The cells seemed leakier. The loss rate constants were then 1–3%/min, and a difference was discernible at the two temperature levels: at the lower level, the rates were smaller by about one third (Fig. 5*B*). This difference presumably reflects a direct ef-

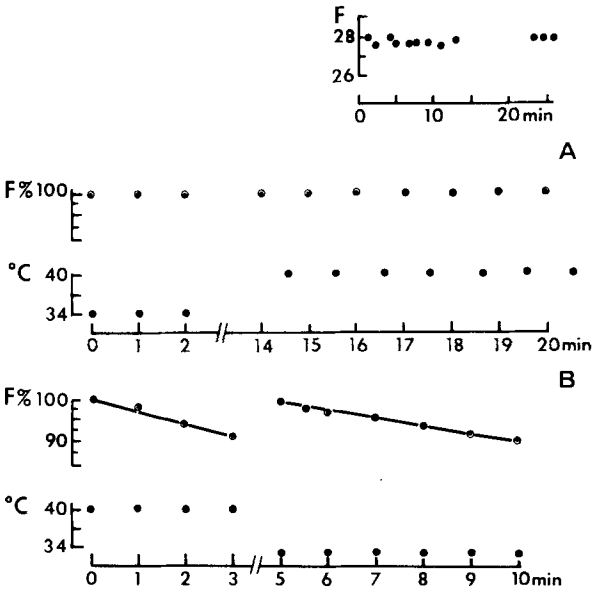

Fig. 5. Rates of fluorescence loss at the permissive and nonpermissive temperature. The fluorescence intensity (*F*), as a function of time. (*A*): A representative example of a quail embryo cell, exhibiting negligible losses at the two temperature levels. (*B*): An example of a leakier quail embryo cell. The rate constants of the curves are 2.7 and 1.8 min^{-1} at 40.5 and 34°C, respectively. *Inset*, absence of significant photo-bleaching, as determined *in vitro* (*see* Materials and Methods) with the excitation parameters used *in vivo*, over a 26-min observation period (34°C). *F* is the photodiode voltage (logarithmic scale) normalized in *A* and *B* in respect to the first value in each segment (100%). At time zero, *F* equals 12.8 and 16.0 units in *A* and *B*, respectively, on the unit scale of direct photovoltage reading in the *inset*

Table 3. Incidence of LRB-Glu-permeable interfaces at permissive and nonpermissive temperatures. 3T3 cells.

Cell system	Density (10^4 cells/cm^2)	Permeable interfaces %[a]				
		33°C	35°C	41°C	hr	*P*
3T3-*ts*LA90[b]	2.2 – 5.5		4 ± 1	27 ± 5	5	<0.00009
3T3-*ts*LA90	2.6 – 4.7		11 ± 5	74 ± 3	24	<0.00009
3T3-*ts*LA90	6.0 – 8.8	0.0 ± 0		61 ± 7	5	<0.00009
3T3-*ts*LA90	6.0 – 13.0		16 ± 6	69 ± 6	24	<0.00009
3T3-*ts*LA90	6.3 – 7.2		3 ± 1	37 ± 5	5	<0.00009
3T3-*ts*LA90	14.0 – 21.0		7 ± 3	20 ± 5	5	0.025
3T3-*ts*LA90 clone 1G9[c]	2.2 – 8.5		19 ± 5	79 ± 2	24	<0.00009
3T3-*ts*LA90 clone 1D7	2.4 – 9.0		17 ± 5	78 ± 3	24	<0.00009
3T3-*ts*LA90 clone 1C3	3.1 – 6.4		12 ± 7	48 ± 3	24	0.0002
3T3-*ts*LA90 clone 1C9	4.0 – 9.0		16 ± 8	55 ± 2	24	0.0005
3T3-*wt*[d]	3.5 – 4.0	80 ± 4		86 ± 4	5	0.16
3T3-*wt*	6.5 – 6.8		60 ± 5	70 ± 4	5	0.068
3T3-*wt*	11.0 – 17.0		59 ± 3	51 ± 4	24	0.042
3T3-*wt*	12.0 – 18.0		70 ± 5	67 ± 6	5	0.312
3T3-*wt*	22.0 – 24.0		21 ± 6	21 ± 6	24	0.495

[a] Means ± SE. Each mean value is based on the examination of 56-291 interfaces; 1–3 culture dishes.
[b] Temperature-sensitive virus, Schmidt-Ruppin group D.
[c] Clones derived from 3T3-*ts*LA90.
[d] Wild-type virus, Schmidt-Ruppin group D.

fect of temperature (not of transformation) on nonjunctional membrane, as discussed below. But the important point for our immediate question is that here, too, the nonjunctional membrane is excluded as a possible source for the changes in junctional transfer; the changes in nonjunctional-membrane permeability are in the wrong direction for that.

In conclusion, in terms of transformation: if the nonjunctional-membrane permeability to LRB-Glu changed at all, it decreased at the lower (permissive) temperature. A nonjunctional-membrane change is thus ruled out as a cause of the decrease of junctional transfer associated with transformation. *Clearly, it is the junctional membrane that is changed in the transformed state; its permeability is decreased. The actual decrease of junctional permeability may even exceed what one would estimate based on the junctional-transfer data alone; junctional transfer decreased despite the decrease seen in nonjunctional membrane permeability in the leakier cases.*

The following results will show that junctional permeability also is reduced in the other RSV-transformed cells examined in the present study, including mammalian cells. In this light, it will not seem far-fetched to suppose that the decrease of cell-to-cell transfer rate in RSV-transformed rat NRK cells found by Atkinson et al. (1981) reflects such a junctional change, too, specially in view of the fact that the transfer results, including reversibility and time of onset of the changes in transfer, are in good agreement.

Nonjunctional Membrane Permeability and the Transient Rise of Junctional Transfer. Cells behaving like the one in Fig. 5*B* constituted the minority in our samplings. Leakier, as they were compared to most, they still were relatively tight (and they survived and divided); their fluorescence loss rates were several orders of magnitude lower than those of cells with obvious injuries or cells deliberately punctured with a blunt micropipette (which did not survive). Nevertheless, the greater leakiness may possibly have been induced by the injection. We cannot distinguish between such an artifactual change and an intrinsic variability of membrane permeability. Be that as it may, the observation bears on the results of junctional transfer obtained with the same method.

In particular, it illuminates the phenomenon of an initial transient rise in junctional transfer, seen occasionally at higher time resolution, when the temperature was shifted down (Fig. 4). In the leakier cells, the fluorescence loss rate decreased within 0.5 min of the downshift (Fig. 5*B*). This fast tightening of nonjunctional membrane may explain why the junctional transfer first rose before it fell: the decrease in nonjunctional membrane permeability would determine an increase in the junctional transfer before the virus-induced decrease of junctional permeability sets in; the junctional transfer eventually would fall as the junctional permeability effect becomes dominant.

We have no reasons to believe that the effect on *non*junctional membrane permeability is related to

Table 3A. Data summary. 3T3 cells

Cell system	Permeable interfaces %			
	33°C	35°C	41°C	*P*
3T3-*ts*LA90		6 ± 1 (96)	37 ± 3 (99)	<0.00009
3T3-*ts*LA90	0.0 ± 0 (28)		61 ± 7 (27)	<0.00009
3T3-*ts*LA90 clone 1G9		19 ± 5 (19)	79 ± 2 (16)	<0.00009
3T3-*ts*LA90 clone 1D7		17 ± 5 (18)	78 ± 3 (16)	<0.00009
3T3-*ts*LA90 clone 1C3		12 ± 7 (9)	48 ± 3 (8)	0.0002
3T3-*ts*LA90 clone 1C9		16 ± 8 (9)	55 ± 2 (8)	0.0005
3T3-*wt*		57 ± 3 (46)	54 ± 3 (46)	0.282
3T3-*wt*	80 ± 4 (18)		86 ± 4 (19)	0.16

Mean value ± SE of the data of Table 3. In parentheses, the number of source cells injected (*n*).

viral transformation. The effect was present in untransformed cells, as well. Its direction and magnitude are what one might expect to find in a leaky cell membrane; it is probably a direct effect of temperature on membrane structure. But considered from the point of view of junctional communication, the effect provides an instructive instance where a change in junctional transfer may come about by a mere change in nonjunctional membrane and underlines the need for information about changes of nonjunctional membrane permeance in studies of junctional communication. Other instances of nonjunctional effects on junctional transfer are shown and analyzed in an elegant study of fluorescent-tracer transmission in *Chironomus* salivary gland by Zimmerman and Rose (1984).

Changes in Cell Shape

As other transformed cells, the quail embryo cells underwent changes in shape upon transformation. They became more refractile and rounder when the temperature was shifted to the permissive level. Such changes, however, are relatively slow; it took several hours for the first signs to become discernible. At 25–30 min, when reductions in junctional transfer were consistently present, there were no visible changes in morphology. This was so for temperature shifts in the opposite direction, too (Fig. 2).

Controls Against Transfer by Protoplasmic Bridges

LRB-Glu is not significantly taken up by the cells (Simpson et al., 1977; Flagg-Newton, Simpson & Loewenstein, 1979), and so we need not be concerned about its transfer from cell to cell via the extracellular medium. However, a possible contribution to the cell-to-cell transfer by (coarse) cytoplasmic bridges needed to be considered.

In some mammalian cell cultures such bridges (presumably the result of incomplete cell division or cell membrane fusion), indeed, play a role, as evidenced by the transfer of fluorescent-labelled fibrinopeptide or serum albumin (Flagg-Newton et al., 1981), molecules too large to go through the cell-to-cell channels (Flagg-Newton et al., 1979). In the normal cell types so far examined, the frequency of such bridging, when it occurred at all, was low, contributing a negligible fraction to the cell-to-cell transfer (Flagg-Newton et al., 1981; Radu et al., 1982). But we needed assurance in this respect also for transformed cell types, particularly the present one which divides rapidly (generation time, 29 hr).

Fibrinopeptide labelled with fluorescein isothiocyanate was thus injected into the cells, and its cell-cell transfer was tested (cell densities, 16×10^4 cells/cm^2). The frequencies of this transfer were 2.1% at 35°C and 1.0% at 41°C, negligible compared to the frequencies of the LRB-Glu junctional transfers. Clearly cytoplasmic bridging is not a factor in the temperature response of LRB-Glu-transfer.

Mouse 3T3 Cells

The mouse 3T3 cells, transformed by the temperature-sensitive RSV-L90 mutant, showed a marked reduction in junctional transfer in the transformed state. Table 3 gives the results of the comparisons between paired cultures at the permissive and nonpermissive temperatures—parallel subcultures or parallel subclones. The mean incidence of permeable interfaces was smaller by a factor of 3 to 12 at the permissive temperature. In the most dramatic case the incidence was zero at that temperature as against 61% at the nonpermissive temperature.

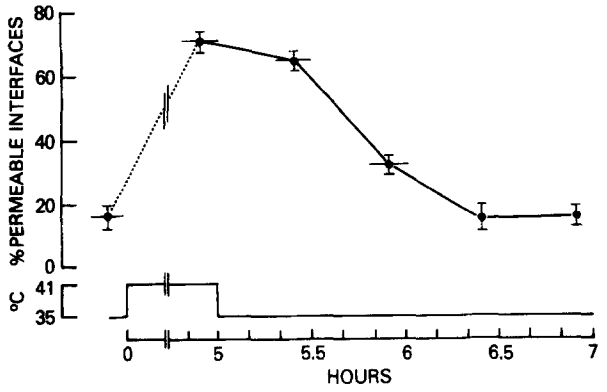

Fig. 6. Reversible changes in junctional transfer produced by temperature shifts between the permissive and nonpermissive levels, in the 3T3 cell system

Controls, carried out with 3T3 cells that had been transformed with wild-type RSV from the same Schmidt-Ruppin group D (3T3-*wt*), showed no significant difference at the two temperatures (Table 3, *bottom*).

The data are lumped in Table 3*A*, disregarding the durations at the nonpermissive temperature and the cell densities, which did not influence the results over the ranges examined.

The junctional response of the 3T3 cells transformed with temperature-sensitive virus was somewhat slower than in the quail cell system. By 30 min, when the quail cells exhibited a well-developed junctional response to temperature downshifts, the 3T3 cells still showed no significant change in junctional transfer. It took more than 1 hr for them to reach the transfer minimum (Fig. 6). (We have no information about the speed of the response in the other direction. In that direction our first tests were not made before 5 hr.)

The rates of fluorescence loss were determined as in the quail embryo cells. The rates were not significantly different at the two temperatures. Thus here, too, a reduction of junctional permeability is the primary event in the change of the cell-to-cell transfer at the permissive temperature.

Chick Embryo Cells

The chick embryo fibroblasts transformed by the temperature-sensitive RSV-LA90 strain showed the most striking change in junctional transfer of LRB-Glu, as here this probe was below the detection threshold of permeation at the permissive temperature. The mean incidence of permeable interface rose from zero at the permissive temperature, to 23% at the nonpermissive temperature. The corresponding controls with cells transformed by wild-type RSV (CK-*wt*) gave no significant difference in junctional transfer (Table 4).

Table 4. Incidence of LRB-Glu-permeable interfaces. Chick embryo cells

Cell system	Density (10^4 cells/cm^2)	Permeable interfaces %[a]			
		34°C	41°C	hr	*P*
CK-*ts*LA90	4.6 – 5.0	0.0 ± 0	23 ± 3	5	<0.00009
CK-*wt*	4.1 – 4.6	46 ± 3	50 ± 5	5	0.31

[a] Means ± SE. Each mean value is based on the examination of 47–77 interfaces; 1 culture dish.

The lag time of the junctional response was not determined for this cell system. The data in Table 4 are from cells that had been at the permissive temperature for days or weeks, or raised to the nonpermissive temperature for 5 hr.

Treatments with Dibutyryl Cyclic AMP/Caffeine and Forskolin

The junctional permeability of various normal cultured mammalian cell types increases when the intracellular concentration of cyclic AMP is elevated (Flagg-Newton & Loewenstein, 1981; Flagg-Newton et al., 1981). This upregulation of junctional permeability appears to be mediated by a protein kinase (Wiener & Loewenstein, 1983) which, presumably, like other cyclic AMP-dependent protein kinases (*cf.* Krebs & Beavo, 1979), phosphorylates serine and threonine residues on the target protein. Is such upregulation still operative in the RSV-transformed cells dominated by the *src*-gene protein kinase which phosphorylates tyrosine instead of serine? Or put differently, can the junctional *src*-gene effect be overridden by stimulation of the cyclic AMP-dependent protein kinase?

We explored this point by administering dibutyryl cyclic AMP (1 mM) plus the phosphodiesterase inhibitor caffeine (1 mM) to the RSV-transformed cells. This combination has proven effective in various normal cell types, including a highly junction-incompetent cancerous type, CL-1D, where increases in junctional permeability became detectable within 1–4 hr of the treatment and became maximal at about 3–24 hr in the various types (Flagg-Newton et al., 1981; Azarnia et al., 1981). We applied, for varying times, the two agents to quail embryo cells and 3T3 cells transformed by temperature-sensitive RSV virus at the permissive and the nonpermissive temperatures, and determined the incidence of cell interfaces permeable to LRB-Glu.

Table 5 gives the results. In general, the treat-

Table 5. Treatment with dibutyryl cyclic AMP-caffeine and with forskolin. Quail embryo cells and 3T3 cells

	Cell system	Cell density[a]	Temperature (°C)	Treatment duration (hr)	LRB-Glu-permeable interfaces %[b]		
					Untreated	Treated	*P*
d-cAMP-caffeine	QEF-*ts*NY68	19	35	5	13 ± 3	15 ± 3	0.251
	QEF-*ts*NY68	19	35	24	13 ± 3	7 ± 6	0.090
	QEF-*ts*NY68	12	35	24	12 ± 4	14 ± 4	0.313
	QEF-*ts*NY68	12	41	2	75 ± 3	76 ± 4	0.280
	QEF-*ts*NY68	19	41	5	73 ± 3	62 ± 2	0.05
	3T3-*ts*LA90	6.9	35	24	49 ± 5	76 ± 5	0.0003
	3T3-*ts*LA90	4	41	4	50 ± 3	51 ± 4	0.431
	3T3-*wt*	23	35	24	51 ± 3	47 ± 2	0.199
Forskolin	QEF-*ts*NY68	3.2	35	24	26 ± 2	25 ± 2	0.379
	QEF-*ts*NY68	3.6	41	24	46 ± 4	46 ± 2	0.231
	3T3-*ts*LA90	4.6	35	24	7 ± 4	9 ± 3	0.317
	3T3-*ts*LA90	7.1	41	24	40 ± 6	41 ± 5	0.481
	3T3-*wt*	15	35	24	66 ± 3	61 ± 3	0.225
	3T3-*wt*	19	41	24	61 ± 3	52 ± 3	0.047

1 mM dibutyryl cyclic AMP, plus 1 mM caffeine; 10 μM forskolin.
[a] (10^4 cells/cm^2).
[b] Means ± SE, each based on the examination of 63–81 interfaces.

ment had little effect at either temperature. There was no sign of increase in the junctional transfer in the quail embryo cells at either temperature, nor in the 3T3 cells at the nonpermissive temperature. Only one series of experiments gave a significant increase: a series with 3T3-*ts*LA90 at the permissive temperature. However, the corresponding series with 3T3-*wt* cells showed no increase of junctional transfer.

A further set of trials was performed with forskolin (10^{-5} M), an activator of adenyl cyclase, which powerfully increases junctional permeability in uninfected cells (P. Mehta and W.R. Loewenstein, *unpublished*). No change in junctional transfer was found (Table 5).

Treatment with Cycloheximide

The major protein of gap junction in mammalian cells—the protein probably embodying the cell-to-cell channel—turns over with a half life estimated at 5 hr (Fallon & Goodenough, 1981; Traub, Druge & Willecke, 1983) and is assembled to channels with times estimated at 3–4 hr (Revel, Yancey, Meyer & Nicholson, 1980; Dahl, Azarnia & Werner, 1981). Thus, the speed with which the junctional permeability changed when the temperature was shifted between the permissive and the nonpermissive levels (within 0.5 hr in the quail cell system) suggested a modification of the channels, rather than a synthesis of new ones. Indeed, this presumption was borne out by experiments in which the cells were treated with the protein-synthesis inhibitor, cycloheximide.

In these experiments the temperature of cycloheximide-treated cells was raised from the permissive to the nonpermissive level, and the junctional transfer was tested 0.5 to 5 hr after the upshifts. Parallel cultures untreated, kept at the permissive temperature, served as controls (column IV, Table 6). We used cycloheximide at 10 μg/ml, a concentration known to block protein synthesis by 95% in 1 hr in mammalian cells (e.g., Ennis & Lubin, 1964). The treatment with the metabolic inhibitor was begun at the permissive temperature (*pretreatment*) and continued at the nonpermissive temperature throughout the experiments, including the period of junctional testing. The treatment had to be long enough to insure effective inhibition of protein synthesis, but not too long to avoid actual diminution of cell-to-cell channels or general cell deterioration. The quail cells offered an advantageous system in this respect. Its response time to temperature was only 0.5 hr, and so the total time of cycloheximide treatment could be kept to a minimum, and a large part of the time could be invested into pretreatment. In two series of experiments (*1* and *2*, Table 6) the pretreatments were 1 and 2 hr long, respectively, and the whole treatments 1.5 and 2.5 hr, respectively—times over which no major reduction of junctional permeability, attributable to the treatment, was observed (*compare* column V with IV).

Table 6. Cycloheximide treatment. Quail embryo cells and 3T3 cells

Series #	Cell system	Density (10^4 cells/cm^2)	Treatment duration (hr)		LRB-Glu-permeable interfaces %[a]				
			I 35°C	II 41°C	III Before treatment 35°C	IV Untreated 41°C	V Treated 41°C	P[b] III *vs.* IV	P[b] IV *vs.* V
1	QEF-*ts*NY68	13	1	0.5	9 ± 4	59 ± 4	56 ± 5	<0.00009	0.30
2	QEF-*ts*NY68	13	2	0.5	9 ± 4	57 ± 5	60 ± 4	<0.00009	0.37
3	QEF-*ts*NY68	13	3	0.5	9 ± 4	54 ± 4	52 ± 3	<0.00009	0.34
4	QEF-*ts*NY68	19 – 21	1	1	13 ± 3	85 ± 2	79 ± 5	<0.00009	0.11
5	QEF-*ts*NY68	12	1	2	12 ± 4	75 ± 3	67 ± 3	<0.00009	0.03
6	QEF-*ts*NY68	19	1	5	13 ± 3	73 ± 3	70 ± 6	<0.00009	0.30
7	3T3-*ts*LA90	18	2	2		69 ± 4	44 ± 3	<0.00009	0.001
8	3T3-*ts*LA90	4	1	4		50 ± 3	43 ± 2	<0.00009	0.01

Schedule: (*i*) Exposure to cycloheximide begins (time zero) at 35°C and continues for varying times—the pretreatment periods (column *I*); (*ii*) temperature is shifted to 41°C and cycloheximide treatment is continued for various time periods (*II*); (*iii*) junctional transfer is tested in the treated cells (*V*) and in parallel untreated subcultures (*IV*). The junctional transfer also is tested on parallel subcultures just before time zero (*III*).

[a] Means ± SE, each based on the examination 58–74 interfaces.

[b] The statistical confidence levels of the differences between the means of columns III and IV and between those of columns IV and V.

In other series the pretreatment was extended to 3 hr or the total treatment duration varied over a range of 2–6 hr.

The results are summarized in Table 6. There was a clear increase in the mean incidence of LRB-Glu-permeable interfaces in the cycloheximide-treated cells when the temperature was raised to the nonpermissive level, in all cases ($P < 0.00009$). These increases were not significantly different from those of the untreated controls. (The data in columns III, IV and V of Table 6 are from parallel subcultures in each series of experiments.)

Experiments carried out with the 3T3 system gave similar results (Table 6). Here upon shifting the temperature to 41°C, the junctional transfer increased less in the cycloheximide-treated condition than in the untreated control. But even so the increase was significant, showing that also here protein synthesis is not required for the junctional response to temperature upshift.

Discussion

On the Mechanism of the Junctional Alteration

The present results show that Rous sarcoma viruses cause loss of junctional permeability in the host cells. In quail embryo cells, the loss sets in within 25 min of lowering the temperature to the level at which the virus *src* gene can express itself. This alteration is one of the first signs of the cells' transformation. It precedes by several hours other cellular alterations associated with transformation, such as changes in cell shape, cytoskeleton, fibronectin matrix, and glucose transport (Hynes & Wyker, 1975; Friis, 1978). The junctional change occurs about as early as the 36,000-mol wt phosphoprotein discovered by Radke and Martin (1979), one of the probable targets for tyrosine phosphorylation of the transforming pp60^{src} protein specified by the virus. This suggests that the junctional alteration is a primary event in the transformation process, and encourages us to believe that it might play a central role. We will return to this point in the last paper of this series, where we discuss the implications in cancer etiology (Azarnia & Loewenstein, 1984*b*).

The reversal of the junctional deficiency, following the turning off of the *src* gene expression, is equally fast and proceeds even when protein synthesis is inhibited. No synthesis of channel protein seems therefore involved in the reversal. Thus, assuming that the permeability loss and reversal are symmetric processes, we are led to conclude that they are due to a modification of the open state of the cell-to-cell channel rather than due to degradation and synthesis of the channel protein. This does not necessarily exclude disassemblage and assemblage of channels from precursor elements (say, from channel protein subunits), but it makes it unlikely that degradation and new synthesis of such precursors are involved. A plausible mechanism would be a phosphorylation-dependent modification of a channel regulatory process. There is evidence that the normal junctional permeability is reg-

ulated by a cyclic AMP-dependent protein kinase (Wiener & Loewenstein, 1983), suggesting that a serine- or threonine-type phosphorylation is regulating here. Thus, it is attractive to think that the loss of permeability in the transformed state is due to a tyrosine phosphorylation of a regulatory protein, perhaps the same protein that is normally serine- or threonine-phosphorylated—the wrong kind of phosphorylation, so to speak—and that the reversal is due to de-phosphorylation. A mechanism of this sort also would account for the speed of the permeability loss and reversal. Tyrosine phosphorylation and de-phosphorylation are known to be fast. The 36,000-mol wt phosphoprotein, for example, appears within 20 min of shifting the RSV-transformed chick embryo cells from the nonpermissive to the permissive temperature, and is rapidly de-phosphorylated on shifting the temperature in the opposite direction (Radke & Martin, 1979; Radke, Gilmore & Martin, 1980). We have no clue as yet on the identity of the phosphorylated protein that is instrumental in the loss of junctional permeability. One possibility, of course, is that the channel protein itself is phosphorylated, and there is evidence that the 26,000-mol wt protein, the major protein of lens gap junction is phosphorylated by a cyclic AMP-dependent kinase (Johnson & Johnson, 1982; R. Johnson, *personal communication*). But the mechanism here need not be so simple. The key phosphorylation may well take place at a step of channel regulation removed from the channel itself.

We undertook the experiments with cyclic AMP hoping that they might throw light on this point. A positive result, namely a reversal by cyclic AMP treatment of the loss of permeability, indeed, would have spoken for a simple interaction between serine- (or threonine-) and tyrosine phosphorylation at the level of the same protein. However, a negative result, as we obtained on the whole, is not particularly revealing. It merely tells us that the channel deficiency is not easily overridden by stimulation of the cyclic AMP-dependent phosphorylation, and leads us to weigh the simple mechanism equally with the possibility of an action of the two types of phosphorylation on different target proteins. The simple mechanism is not ruled out.

The biochemical mechanism aside, the important fact remains that the action of *the virus* overrides the cyclic AMP stimulatory effect on junction, the prominent effect in normal cells (Loewenstein, 1984). Both, the treatment with the cyclic AMP-phosphodiesterase inhibitor combination and the treatment with forskolin, were, in general, ineffective in the infected cells (Table 5). A viral dominance of this sort also was noticeable regarding the junctional effect of cell density. In a broad variety of normal cell types, junctional permeability increases with the endogenous cyclic AMP level, when this is experimentally raised by lowering the cell density. There was no evidence for such density dependence in any of the cells infected with RSV (including wild-type), and this was so even at the nonpermissive temperature (Tables 2 and 3; *compare* with normal 3T3 cells, Fig. 5 of Flagg-Newton & Loewenstein, 1981). All these results are explainable as a dominance of *src* phosphorylation over cyclic AMP-dependent phosphorylation, if we may assume that the effect of this dominance lingers for a few hours after a temperature upshift.

Temperature-Sensitive vs. Wild Transformants

Although our experiments were not designed to compare temperature-sensitive with wild-type transformants and we made no special effort to create the parallel experimental conditions for such comparisons, one cannot escape being struck by the *consistent* differences in junctional transfer between the two. In all cases, the incidence of permeable interfaces of the wild-type cells was higher than that of the corresponding temperature-sensitive transformed cells at the permissive temperature (Tables 2–4). This may conceivably reflect a higher *src*-protein concentration or kinase activity in the temperature-sensitive transformants (the *src* gene integration patterns in the cellular genome, for instance, could be different).

We payed no regard to junctional differences between wild-type transformants and normal, untransformed cells. An approach comparing the two kinds of cells is not unfeasible but, to achieve the necessary experimental parallelism, would be much more difficult than our strategy using temperature as the (only) experimental variable.

Electrically Coupled RSV-Transformed Cells

By conventional electrical measurements, O'Lague and Dalen (1974) found that cells transformed by wild-type RSV (primary chick embryo fibroblasts) were well coupled and to a degree not very different from untransformed cells. This result was not surprising in terms of the hypothesis of growth control (Loewenstein, 1968*a*; 1979), nor do the present results disaccord with it. That the coupling coefficient (V_{II}/V_I) be high, is precisely what one would expect in cells transferring a molecule as large and negatively charged as LRB-Glu; and that it be not

very different in the two classes of cells, is what one would expect because it is high. The coupling coefficient is a direct but nonlinear function of junctional electric conductance or of ion permeability. For a wide range of cellular topologies, the nonlinearity is such as to render the coupling coefficient rather insensitive to changes in junctional permeability, except in the lower range of coupling coefficient (Socolar, 1977). Hence, subtle changes in junctional permeability are not expected to show up in measurements of electrical coupling (unless one boosts the resolution of the transfer voltage, V_{II}, far above that of the conventional method (Socolar & Loewenstein, 1979). Such limitations do not attach to the present measurements with fluorescent tracers. For these measurements—and precisely with the problem of nonlinearity in mind—we chose a junctional probe close to permeation threshold, insuring a sensitive setting.

A Nagging Question

In speculating about the processes that might link the *src* gene to the cell-to-cell channel, the thought comes up that a cytoskeletal protein serves such a coupling role. In particular, vinculin (a protein located at the sites which anchor microfilaments to the plasma membrane) presents itself as a natural candidate. Vinculin is tyrosine-phosphorylated by $pp60^{src}$ and may possibly be involved in the destabilization of the microfilament anchorage (Sefton, Hunter, Ball & Singer, 1981). It is true that the light-microscopically visible cytoskeletal changes—disorganization of microfilament bundles and rounding of cell shape—lag hours behind the junctional permeability change. However, this does not entirely dispel one's doubts as to whether an earlier *sub*microscopic change might be involved. One could conceive, for instance, a mechanism where a minute loss of microfilament anchorage impedes the critical membrane approximation between the cells that is necessary for cell-cell channel formation (Loewenstein, 1981)—a subtle mechanical cause for a subtle permeability effect. Such a mechanical coupling between the *src* gene product and the channel would make the junctional effect no less real, nor less important potentially in terms of the hypothesis of growth control. But it would put the mechanisms considered in the first section of this Discussion in a very different light and would turn the focus of $pp60^{src}$ action onto very different targets.

Because of this uncertainty, we waited with the publication of the present results (the basic ones were obtained in 1981) until the problem could be tackled in a cellular mutant that lacks those cytoskeletal disorganizations in transformation, at all times. The following paper describes the results obtained and brings the question to a satisfactory conclusion.

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