

Differential Regulation of Communication by Retinoic Acid in Homologous and Heterologous Junctions between Normal and Transformed Cells

Parmender P. Mehta and Werner R. Loewenstein

Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Florida 33101

Abstract. The permeability of junctions between cells of the same type (homologous junctions) is greatly increased by retinoic acid (10^{-9} – 10^{-8} M), a probable morphogen, and this responsiveness is shared by a variety of normal and transformed cell types (Mehta, P. P., J. S. Bertram, and W. R. Loewenstein. 1989. *J. Cell Biol.* 108:1053–1065). Here we report that the heterologous junctions between the normal and transformed cells respond in the opposite direction; their permeability is reduced by retinoic acid ($\geq 10^{-9}$ M) and its benzoic acid derivative tetrahydrotetramethylnaphthalenylpropenylbenzoic acid ($\geq 10^{-11}$ M). The opposite responses of the two classes of junction are shown to

be concurrent; in cocultures of normal 10T1/2 cells and their methylcholanthrene-transformed counterparts, the permeability of the heterologous junctions, which is lower than that of the homologous junctions to start with, falls (within 20 h of retinoid application), at the same time that the permeability of the homologous junctions rises in both cell types. Such a counter-regulation requires a minimum of three degrees of cellular differentiation. A model is proposed in which the differentiations reside in a trio of junctional channel protein. The principle of the model may have wide applications in the regulation of intercellular communication at tissue boundaries, including embryonic ones.

UNIQUE among the channels of cell membrane, the cell-cell channel of gap junctions is the product of two cells; each cell in the junction contributes one channel half (Loewenstein, 1987). Because of that, this channel can be regulated by either cell. Almost all we know about the mechanisms of its regulation comes from studies of junctions between cells of the same type (homologous junctions) where the channel presumably is made of symmetric halves. But the channels also form in junctions between cells of different type (heterologous junctions) (Michalke and Loewenstein, 1971; Epstein and Gilula, 1977). The mechanisms of regulation of such junctions remain largely ignored. Yet they are of great physiological and physio-pathological interest because junctional heterology is the prevalent condition at the boundaries between populations of different cells in organs and tissues. Here, we inquire into the regulation of heterologous junctions by retinoic acid, a naturally occurring retinoid, and show that they are regulated very differently from homologous junctions.

We used heterologous junctions of a special sort: junctions between normal and transformed cells. In a variety of such heterologous junctions, communication is low (Fentiman et al., 1976), and this property sets these junctions apart from the homologous junctions the corresponding normal cells make among themselves or the corresponding transformed cells make among themselves (Mehta et al., 1986; Yamasaki et al., 1987). For example, the heterologous communication between 10T1/2 cells and several of their transformed coun-

terparts is much lower than the respective homologous communications (Mehta et al., 1986). We have examined the communication response of such heterologous junctions to retinoic acid and its benzoic acid derivative tetrahydrotetramethylnaphthalenylpropenylbenzoic acid (TTNPB). Retinoic acid induces the transcription of various genes (Guiguere et al., 1987; Brand et al., 1988; Chioocca et al., 1988; Zelent et al., 1989; Vasios et al., 1989; Mangelsdorf et al., 1990) and increases the production of connexin43, a cell-cell channel protein, present in 10T1/2 cells (Rogers et al., 1990). At concentrations like those found in normal tissues, retinoic acid produces long-term enhancement of communication in homologous junctions of 10T1/2 cells in culture; the TTNPB derivative, which is not metabolized, is particularly powerful in this respect (Mehta et al., 1989). We analyzed the heterologous and the corresponding homologous communications side by side, in cell combinations in which the partners of the normal 10T1/2 cells were carcinogen, virus or oncogene transformed. We report that the responses of heterologous and homologous junctions can be diametrically opposite; heterologous communication can be reduced, even while the homologous communication of both cell partners is enhanced. This differential behavior points up a fundamental difference in the formation of the two kinds of junctions, which has profound biological implications.

1. *Abbreviations used in this paper:* MCA, methylcholanthrene; TTNPB, tetrahydrotetramethylnaphthalenylpropenylbenzoic acid.

Materials and Methods

Cell Culture

We used the following cell types: normally growing 10T1/2 cells (C3H 10T1/2 Clone 8); methylcholanthrene (MCA) transformed 10T1/2 cells, MCA10 and MCA4D (Reznikoff et al., 1973; Mehta et al., 1986); *v-mos* (Moloney sarcoma virus)-transformed 10T1/2 cells, EC8; and *v-mos*-transformed 3T3 cells, MA31 (Croy and Pardee, 1983). The EC8 cells are derived from C3H 10T1/2 clone 8 cells transfected with plasmid pM1 containing the complete coding region of *v-mos* cloned into plasmid pBR322 (Blair et al., 1980).

The cells were grown in basal minimal essential medium supplemented with 5% FBS (lot #1111581; Hyclone Laboratories, Logan, UT) and 25 µg/ml gentamicin at 37°C in an atmosphere of 5% CO₂, in 60-mm plastic dishes (Nunc, Roskilde, Denmark). The dish medium (5 ml), with or without retinoid, was changed every third day. (This protocol was adopted after a preliminary series of experiments showed that adding retinoid-containing medium every 2 or 4 h or every 3 d gave the same results.) For coculture, the normal cells were seeded at 5 × 10⁴ cells per dish and grown to confluence (7–10 d); the transformed cells were seeded, in fresh medium, on top of the confluent normal cell layer. The seeding density was 10,000 cells/dish, unless stated otherwise. At this density the dextran marker (see below) was not excessively diluted over the cell generations in the 2–3 d of the experiments. The cultures were protected from light <500 nm to prevent retinoid breakdown. Stock cultures were passaged once a week in 100-mm dishes, at 10⁵ cell/dish in 10 ml medium.

The retinoid content in the serum was <1 ng/ml, the resolution of the method (HPLC). Thus, with 5% serum in the medium, the background concentration of retinol in all experiments was <1.5 × 10⁻¹⁰ M, three orders of magnitude below the threshold of effects of this retinoid on junctional communication in the four cell types (Mehta et al., 1989).

Communication Assay

Junctional permeability was probed with Lucifer yellow CH (Molecular Probes Inc., Junction City, OR). The fluorescent dye was microinjected into the cells and the cell-to-cell transfer of the fluorescence was video-recorded for analysis (DAGE MTI65 SIT camera). The junctional transfer was indexed by the total number of fluorescent neighbors of the injected cell (Mehta et al., 1989).

The transformed cells in the cocultures were marked with FITC-conjugated dextran (Sigma Chemical Co., St. Louis, MO), which the cells endocytosed. The dextran marker is too large (~40,000 D) to be admitted by the cell-cell channels (Schwartzmann et al., 1981), and the cells did not exchange the marker via the medium when care was taken to wash them free of nonendocytosed marker before coculture. This was ascertained in runs in which the transformed cell partner was labeled with the dextran (green fluorescent) and the normal partner with rhodamine-conjugated latex beads (red-fluorescent) (Polysciences, Inc., Harrington, PA). The cell marking did not affect the communication or growth rate of the cells.

The yellow fluorescence of the Lucifer dye and the green fluorescence of the dextran marker were set apart by interchangeable excitation and barrier filters during the measurements of junctional transfer.

Growth Assay

After the assays of communication, the cultures were fixed with 3.7% formalin in PBS, stained with 0.02% crystal violet, and the areas of the individual colonies were measured with the aid of a Ladd Graphical Digitizer coupled to a computer programmed to integrate areas from the digital coordinates. The colonies were projected with sixfold magnification for the measurements. The threshold of the system was set at 0.5 mm², corresponding to a colony size of ~50 transformed cells. The number of colonies above that threshold size was scored, and expressed as the percentage of the number of cells seeded (cloning efficiency).

The growth and communication assays were done "blindly"; the assays of growth and communication were carried out on coded dishes and video recordings, without knowledge of the treatment or cell type involved.

Retinoids

Stock solutions of the retinoids, namely All-trans-retinoic acid (Sigma Chemical Co.) and TTNPB (RO-13-7410/000; Hoffman-LaRoche Inc., Nutley, NJ) were in DMSO, and aliquots were stored at -70°C in a nitrogen

Table I. Response of Heterologous Junctions to Retinoic Acid: Heterologous Junctional Transfer*

Concentration	Junction		
	MCA10/10T1/2	EC8/10T1/2	MA31/10T1/2
<i>M</i>			
0	4 ± 0.4 (11)	5 ± 0.5 (11)	9 ± 1.0 (7)
10 ⁻⁹	2 ± 0.2 (17)	2 ± 0.2 (13)	5 ± 0.3 (18)
10 ⁻⁸	2 ± 0.2 (14)	0 ± 0 (11)	3 ± 0.4 (14)
10 ⁻⁷	0 ± 0 (9)	0 ± 0 (9)	0 ± 0 (9)
10 ⁻⁶	0 ± 0 (9)	0 ± 0 (8)	0 ± 0 (9)

* Mean values ± SE; in parentheses, the number of individual injection trials. Measurements were taken after 2 d in retinoic acid-containing medium or control medium. The individual values of junctional transfer were subjected to an analysis of variance. This showed that the trend of reduction in junctional transfer with rising retinoic acid concentration was significant at a level, $P < 0.0001$ in all three junctions. The difference between the mean values at 10⁻⁹ M concentration and 0 concentration was significant at $P < 0.02$ in all three cases.

atmosphere, and used only once. Both test and control solutions contained the same concentration of DMSO, in all cases <0.1%. We compared the effects of DMSO-free and DMSO-containing solutions; there were no differences detectable in any of the cell types used.

Statistics

Mean values were compared using one-way analysis of variance for computation of statistical significance (see Tables 1–4; Fig. 3). Bonferroni's correction was applied in the case of multiple comparisons with the same control value.

Results

Inhibition of Communication in Heterologous Junction

We used normal 10T1/2 cells in combination with MCA-transformed 10T1/2 cells (MCA10), *v-mos*-transformed 10T1/2 cells (EC-8), or *v-mos*-transformed 3T3 cells (MA31). The transformed cells, marked with fluorescent dextran (40,000 mol wt), were growing at low density on top of a confluent layer of the normal 10T1/2 cells. Junctional permeability was probed with the fluorescent dye Lucifer yellow (443 mol wt).

Table II. Response of Heterologous Junctions to TTNPB: Heterologous Junctional Transfer*

Concentration	Junction		
	MCA10/10T1/2	EC-8/10T1/2	MA31/10T1/2
<i>M</i>			
0	4 ± 0.5 (13)	7 ± 1.0 (14)	5 ± 0.5 (13)
10 ⁻¹¹	3 ± 0.2 (19)	4 ± 0.5 (13)	2 ± 0.2 (17)
10 ⁻¹⁰	0 ± 0 (7)	0 ± 0 (9)	0 ± 0 (11)
10 ⁻⁹	0 ± 0 (7)	0 ± 0 (8)	0 ± 0 (9)
10 ⁻⁸	0 ± 0 (7)	0 ± 0 (9)	0 ± 0 (7)
10 ⁻⁷	0 ± 0 (7)	0 ± 0 (7)	0 ± 0 (10)

* Mean values ± SE; in parentheses, the number of independent injection trials. Measurements taken after 2 d in TTNPB- or control medium. The trend of reduction in junctional transfer with rising TTNPB concentration is significant at $P < 0.0001$ in the three junctions (analysis of variance). The difference between the mean values of junctional transfer at 0 and 10⁻¹¹ M concentration is significant at $P < 0.02$ in EC-8/10T1/2 and MA31/10T1/2. In MCA10/10T1/2, the difference between the mean values at 0 and 10⁻¹⁰ M concentration is significant at $P < 0.0001$ (but not significant between 0 and 10⁻¹¹ M).

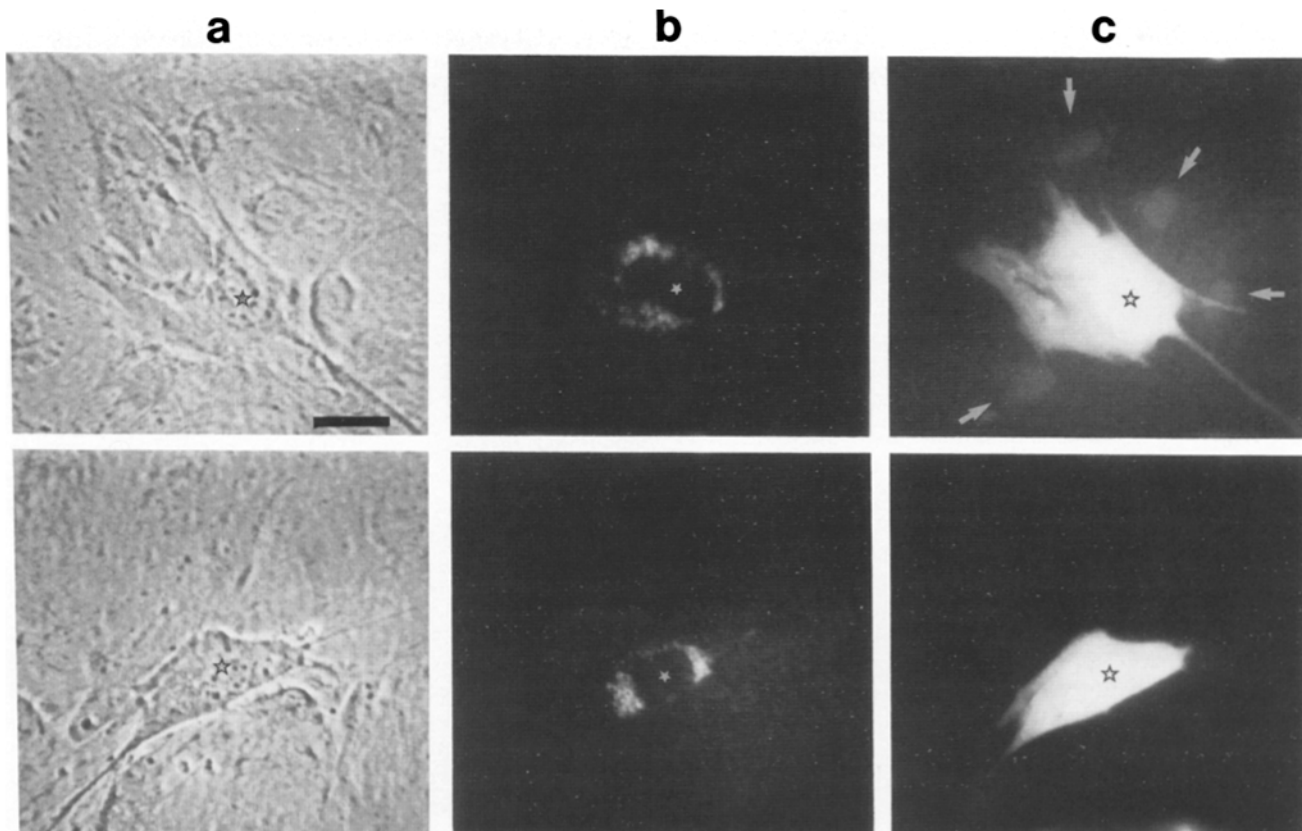


Figure 1. Inhibition of heterologous junctional communication by retinoic acid in the MCA10/10T1/2 system. Lucifer yellow, a 443-D fluorescent dye, was injected into a transformed cell (marked by *star*); the video pictures show the transfer of the dye to the normal cell neighbors in (*top row*) the basal (untreated) condition and (*bottom row*) after 48 h treatment with 1×10^{-8} M retinoic acid. The transformed cell is labeled with mol wt 40,000 FITC-dextran. (*from left to right*) Phase-contrast pictures showing the transformed cell on the confluent normal-cell layer; darkfield showing the green-fluorescent FITC-dextran label (concentrated around the nucleus) of the transformed cell; and darkfield showing the spread of the yellow-fluorescent Lucifer dye 5 min after the microinjection. In the basal condition the dye has spread to four normal cells (marked by *arrows*); in the treated condition it has spread to none. For samples of homologous junctional transfer in these cells, see Fig. 3 of Mehta et al., 1989. Bar, 40 μ m.

The dye was microinjected into transformed cells and its cell-to-cell transfer was video recorded for analysis. Cells that occurred singly were chosen for injection; each transformed test cell, thus, was completely ringed by normal

cells, offering a suitable geometry for assaying heterologous junctional communication by the number of normal cells to which the dye was transferred.

Tables I and II summarize the results of experiments in which the three cell combinations were treated for 2 d with various concentrations of retinoic acid or TTNPB. Both reti-

Table III. Response of Homologous Junctions to TTNPB in MCA10 and 10T1/2 Cells: Homologous Junctional Transfer*

Concentration	Homologous junctions	
	MCA10/MCA10	10T1/2/10T1/2
<i>M</i>		
0	3 \pm 0.2 (7)	3 \pm 0.5 (7)
10^{-11}	5 \pm 0.4 (7)	7 \pm 1.0 (7)
10^{-10}	11 \pm 0.9 (6)	12 \pm 1.2 (5)
10^{-9}	11 \pm 0.8 (7)	14 \pm 1.4 (5)
10^{-8}	13 \pm 1.7 (6)	18 \pm 1.5 (5)
10^{-7}	10 \pm 0.8 (9)	12 \pm 0.9 (7)

* Mean \pm SE; in parentheses, the number of independent injection trials. Measurements taken after 2 d in TTNPB- or control medium. Same batch of serum as in the experiments of Table II. The trends of increasing junctional transfer with rising TTNPB concentration in the two homologous junctions were significant at $P < 0.0001$ (analysis of variance), and the differences between the mean values of junctional transfer at 0 and 10^{-10} M concentration were significant at $P < 0.02$ in the two homologous junctions.

Table IV. Heterologous and Homologous Junctional Responses to TTNPB in MCA10/10T1/2 Coculture: Junctional Transfer*

Concentration	Heterologous junction	Homologous junctions	
	MCA10/10T1/2	MCA10/MCA10	10T1/2/10T1/2
<i>M</i>			
0	1.5 \pm 0.3 (11)	3.8 \pm 0.4 (8)	2.5 \pm 0.3 (6)
10^{-11}	0.5 \pm 0.7 (11)	3.8 \pm 0.4 (8)	6.1 \pm 0.6 (7)
10^{-10}	0.0 \pm 0 (7)	5.3 \pm 0.4 (8)	13.2 \pm 1.0 (5)
10^{-7}	0.0 \pm 0 (5)	6.4 \pm 0.8 (11)	18.0 \pm 2.0 (5)

* Mean \pm SE; in parentheses, the number of independent injection trials. Measurements taken after 2 d in TTNPB- or control medium. These experiments were performed in parallel with those in Table III. The trend of increasing homologous junctional transfer with increasing TTNPB concentration was significant at $P = 0.007$ in MCA10/MCA10 and at $P < 0.0001$ in 10T1/2/10T1/2 (analysis of variance).

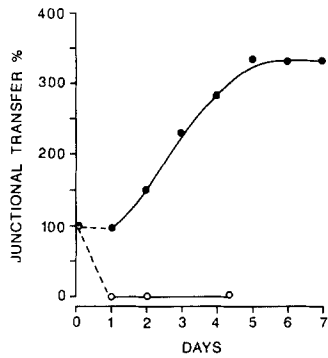


Figure 2. Time course of the changes of junctional communication in response to TTNPB in homologous and heterologous junctions in the MCA10/10T1/2 system. (●) Homologous junctional transfer between the MCA10 cells; (○) heterologous junctional transfer between MCA10 and 10T1/2 cells. The TTNPB treatment (1×10^{-8} M) began at time zero.

noids reduced heterologous communication. The reduction was detectable at 10^{-9} and 10^{-11} – 10^{-10} M concentrations of retinoic acid and TTNPB, respectively, and increased with rising concentration. At concentrations of $\geq 10^{-7}$ M of retinoic acid, or at $\geq 10^{-10}$ M of TTNPB, heterologous junctional transfer was absent altogether (Fig. 1). The trend of falling heterologous communication with rising retinoid concentration was statistically highly significant in all three cell combinations (Tables I and II, legends).

We have shown before that the permeability of the non-junctional cell membrane is not affected by the retinoids; the rate of dye loss from all four types of cells is negligible and not sensibly changed by retinoic acid or TTNPB at the concentrations used (Mehta et al., 1989). Thus, in either direction, the foregoing changes in junctional transfer reflect changes in junctional permeability.

Enhancement of Communication in Homologous Junction

The results obtained with EC-8/10T1/2 and MA31/10T1/2 combinations, by themselves, would have come as no surprise. Here, the homologous communication of the trans-

formed cell partners was known to be inhibited by retinoids (Mehta et al., 1989), and so the reduction of heterologous communication might have been explainable simply by an inhibition of the function of one channel half. The surprising result was that of the MCA10/10T1/2 combination, because in this combination the homologous communications of both cell partners were known to be enhanced by retinoids (Mehta et al., 1989).

But first of all, it was necessary to ascertain that the difference between heterologous and homologous junctional behavior was basic and not a fluke of serum composition, because serum factors profoundly affect junctional permeability (Flagg-Newton and Loewenstein, 1981; Maldonado et al., 1988). So, we tested the homologous and heterologous junctional responses to TTNPB in parallel experiments, using the same batch of serum throughout. The result was the same: the homologous junctional transfer in either cell partner was significantly increased by TTNPB (Table III).

Concurrent Counterregulation

One other possible source for the difference between the homologous and heterologous junctional behavior needed to be ruled out. In the preceding experiments, the homologous and heterologous junctions were tested separately, that is, in separate culture dishes. This way, junctional transfer could be assayed in optimal conditions, with each test cell completely surrounded by either homologous or heterologous cells. Although matching otherwise, the experimental conditions, thus, were of necessity unequal in one respect: in the homologous junctional testing, each cell type was in a culture dish by itself, whereas in the heterologous junctional testing the two types were cocultured. To rule out the possibility that this difference somehow had given rise to the differential behavior (for example, by a factor secreted into the medium), we tested the heterologous and homologous communications together. In these experiments (run in parallel with those in

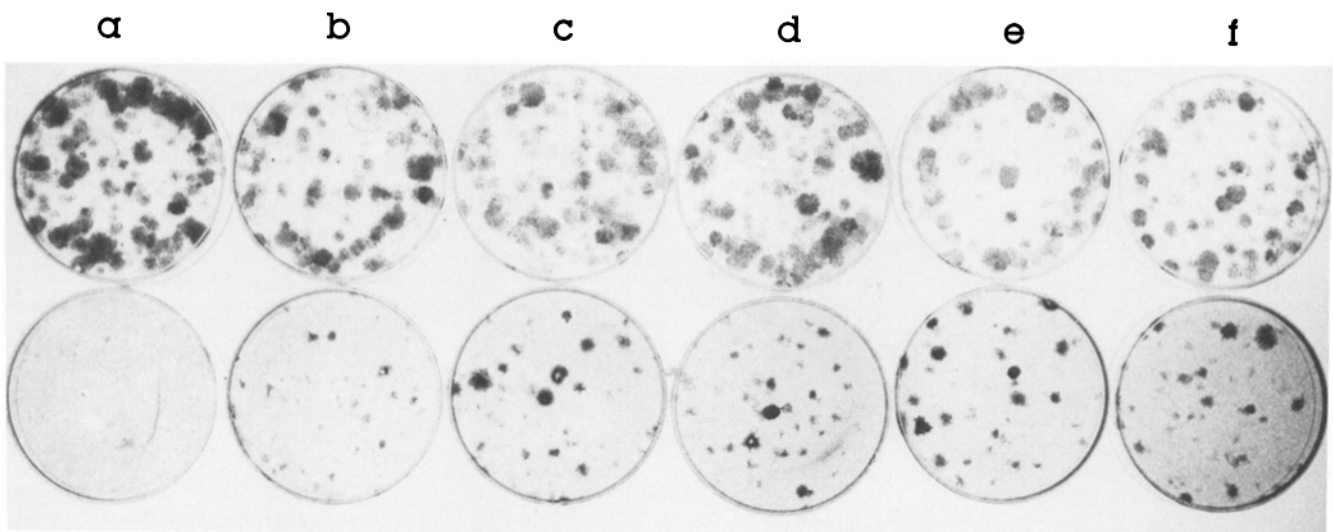


Figure 3. Transformed cell growth following inhibition of heterologous communication. Colonies of transformed MCA10 cells grown (top row) alone and (bottom row) on top of a layer of normal 10T1/2 cells. (a) In control medium; (b–f) treated with TTNPB (M): (b) 10^{-11} ; (c) 10^{-10} ; (d) 10^{-9} ; (e) 10^{-8} ; (f) 10^{-7} . The cultures were in the control- or TTNPB medium for 14 d, from the time of the transformed cells' seeding (100 cells/dish). The photographs show the culture dishes after staining of the transformed cell colonies with crystal violet; the single layer of 10T1/2 cells is not visible.

Table III), the transformed cells grew as colonies on top of the normal cell layer. The Lucifer dye was injected into transformed cells at the edge of the colonies (where the transformed cells were single layered), and the heterologous junctional transfer was determined by scoring the number of the normal cells to which the dye spread. Here, the transformed test cells were not completely ringed by normal cells, and so the number of heterologous fluorescent neighbors in the communication assay was smaller than in the preceding experiments. For tests of transfer between transformed cells, the dye was injected into transformed cells close to (but not at) the colony border, and for tests of transfer between the normal cells the injections were made into 10T1/2 cells in the confluent layer, some distance away from the transformed cell colonies.

The results are summarized in Table IV. They show the same basic feature as the preceding experiments: heterologous communication falls while homologous junctional communication rises. These opposite trends were statistically highly significant (Table IV, legend). Clearly, the heterologous and homologous junctions in the MCA10/10T1/2 cell combination respond in opposite directions, and this difference is not due to extrinsic factors in the medium nor to differences in experimental conditions. The two classes of junctions appear to be different in some fundamental way.

We corroborated these results for another methylcholanthrene-transformed 10T1/2 cell line, MCA4D. The basal level of MCA4D/10T1/2 heterologous communication was lower, but the inhibitory effect of retinoic acid on that communication (tested at 10^{-8} and 10^{-7} M) and the opposite effect on homologous communication (tested at 10^{-8} , 10^{-7} ,

and 10^{-6} M) were essentially like those in the MCA10/10T1/2 cell combination (data not shown).

Time Course of the Counterregulation

The retinoid action on the two classes of junctions had very different kinetics. Fig. 2 illustrates this for the MCA10/10T1/2 cell combination treated with 1×10^{-8} M TTNPB. Whereas the effect on heterologous junction was fully developed within 24 h, that on homologous junction took several days and reached a maximum only after 4–5 d.

Disinhibition of Growth

The inhibition of heterologous communication had a plainly visible sequel: transformed cell proliferation. The transformed cells began to grow when their communication with the normal cells was inhibited. Fig. 3 (*top row*) illustrates this for a set of experiments in which 100 MCA10 cells had been seeded on top of a confluent layer of normal 10T1/2 cells. The photographs show the cocultures after 14 d of treatment with various concentrations of TTNPB (*b–f*). In basal conditions, where the junctional heterologous junctions transfer was relatively high (see Table IV), there were no sizeable transformed cell colonies (*a*), but such colonies began to appear at a concentration of 10^{-11} M TTNPB, when heterologous junctional transfer was reduced (*b*).

MA31/10T1/2 and EC8/10T1/2 cocultures gave essentially the same growth response. Fig. 4 quantifies the response of a MA31/10T1/2 coculture (1,000 transformed cells had been seeded here over the 10T1/2 cell layer): the colonies increased in number (cloning efficiency) and size, as the heterologous junctional transfer fell from its basal value to below detectable level, by action of TTNPB. (0 on the bottom abscissa means transfer below the level of detection of our method, not complete disruption of transfer.)

TTNPB had no growth-stimulatory action by itself. In the absence of normal cells, the growth of the transformed cells did not increase with retinoid treatment (Fig. 3, *top row* and Fig. 4). The stimulation of growth of the transformed cells following the disruption of their communication with the normal cells is the flipside of what we had shown before: inhibition of the growth following the induction of communication with normal cells (Mehta et al., 1986). Both effects are in keeping with the hypothesis that the cell-cell channels transmit cytoplasmic growth-controlling signals (Loewenstein, 1966) and are the expected behavior when heterologous junctional permeability limits that transmission (Nonner and Loewenstein, 1989). The present growth stimulation, thus, would represent a disinhibition, a release from the influence of the signals flowing between the normal and transformed cells.

Discussion

Differential Regulation of Communication

Retinoid-Induced Regulation. Our results disclose a basic difference between heterologous and homologous junctions of normal and transformed 10T1/2 cells: the two junctions are regulated in opposite directions by retinoic acid. This agent enhances the transcript level and the synthesis of connexin43, a cell-cell channel protein in 10T1/2 cells (Rogers et al.,

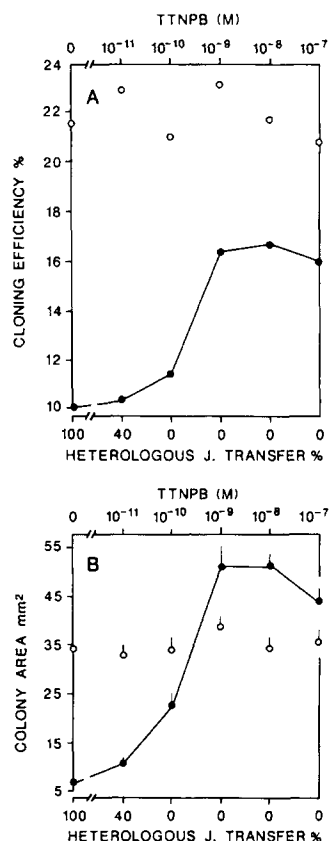


Figure 4. Size and number of transformed cell colonies. Data from the MA31/10T1/2 system. Ordinates: (A) mean number of transformed cell colonies, expressed as percentage of the number of transformed cells seeded (the cloning efficiency); (B) and mean colony area. Data (●) from MA31 cells growing in coculture with 10T1/2 cells and (○) from MA31 cells growing alone in the dishes. Each datum is the average of 69–101 individual colonies from three dishes; bars give standard errors. Abscissae: (*bottom*) heterologous junctional transfer normalized with respect to basal value (absolute value, 5); (*top*) TTNPB concentration. (Bottom abscissae apply only to ● data). The procedure and general conditions were like those in the experiments with MCA10 cells in Fig. 1, except that the MA31 cells were seeded 1,000 cells/dish.

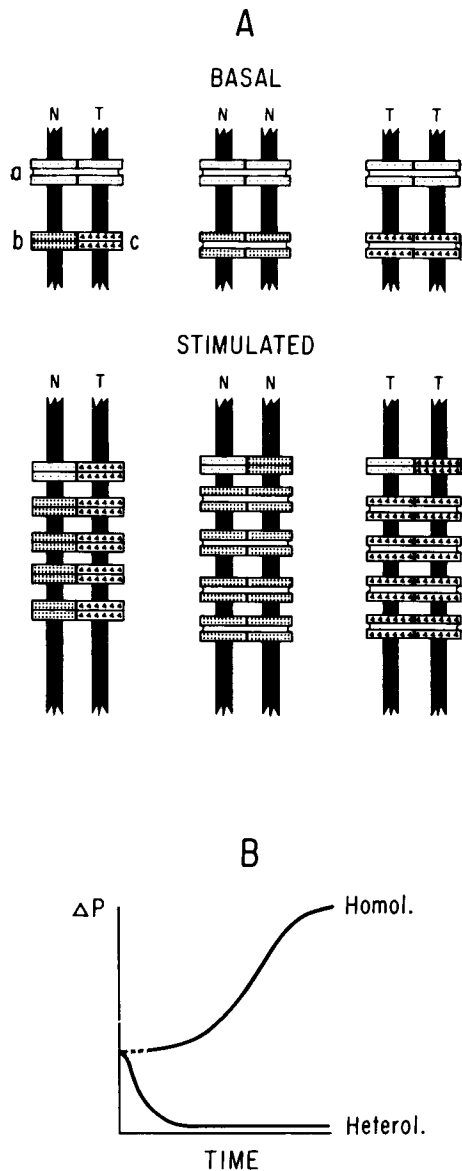


Figure 5. (A) Model for differential regulation of homologous and heterologous junctions. The normal (*N*) and transformed (*T*) cells make three hemichannel species, *a*, *b*, and *c*, which bind promiscuously but open up only in homophilic interaction. *b* and *c* productions are retinoid sensitive. The diagrams depict the formation of functional (*open*) and nonfunctional (*closed*) channels in the heterologous (*NT*) and homologous (*NN* and *TT*) junctions between these cells. The top diagram represents the basal situation: all three junctions have functional *a* channels; homologous junctions have functional *b* and *c* channels, in addition (providing these junctions with a higher degree of communication). The bottom diagram represents the situation after long-term retinoid stimulation: the proliferating *b*s and *c*s have bound the available *a*s in the three junctions by mass action; only the homologous junctions have functional channels, namely *b* or *c* channels (which are more abundant than in the basal condition). (B) The time course of the junctional-permeability responses (ΔP) to retinoic acid predicted from the model (time zero is the time of retinoid application). The permeability in heterologous junction declines as soon as the *b* and *c* population reaches the size of the *a* population. This can occur long before the *b* and *c* population density reaches saturation in homologous junction, and so the enhancement of homologous communication can lag behind the inhibition of heterologous communication.

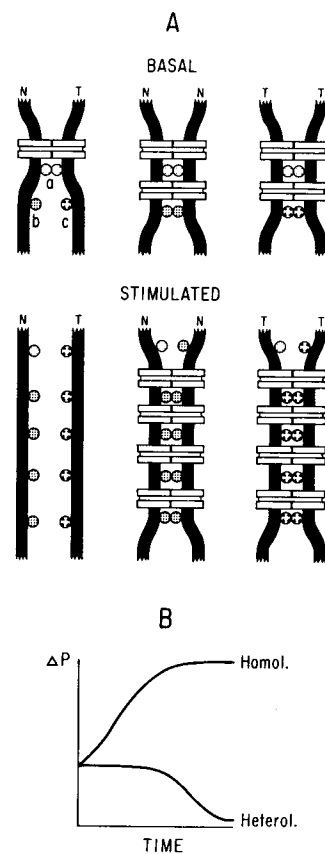


Figure 6. (A) Counterregulation through competition by specific cell adhesion molecules. *a*, *b*, and *c* are three species of homophilic adhesion molecules (*spheres*) whose interaction draws the cells together, facilitating channel formation. The productions of *b* and *c* are retinoid sensitive. The normal (*N*) and transformed (*T*) cells in this model form identical channels whose number depends on the interaction between matching pairs of adhesion molecules. (*top*) In the basal condition, all three junctions adhere by *a* interactions, and the heterologous junctions adhere by *b-b* and *c-c* interactions in addition; the number of channels formed is commensurate with the degree of adhesion. (*bottom*) Upon long-term retinoid stimulation, the *b* and *c* species overcrowd the three junctions, leading to reduced cell adhesion and reduced channel formation in the heterologous junction and to the opposite effects in the homologous junctions. (B) Time course of the

permeability responses (ΔP) to retinoic acid in this model. The permeability in heterologous junction starts to decline when the density of the *b* and *c* species approaches saturation; only then are the *a* species effectively crowded out. Because the *b* and *c* species then approach saturation also in the homologous junctions, the decline in permeability in heterologous junction lags behind the rise in homologous junction. This kinetic feature, which is shared by all models where determinants of permeability compete for junctional space, is incompatible with the results (see Fig. 2).

1990). The results show that the difference in responsiveness of the two junctions is not due to extrinsic factors in the medium nor to secreted factors, and that it is independent of the retinoid concentration. The difference seems to reflect an intrinsic junctional property, that is, a differentiation in the make-up of the junctions. The present data do not allow us to identify the differentiation in molecular terms, but they furnish enough constraints to infer the general conditions for the differentiation.

The combinations with methylcholanthrene-transformed cells, the MCA10/10T1/2 and MCA4D/10T1/2 systems, provide the keystone here. Their response pattern consisting of three classes of junctional response—basal, upregulated, and downregulated communication—imposes the severest constraints for modeling the regulatory interactions. The junctional responses are the product of the communication capacities of the cell pairs in a combination. Thus, the three classes of response demand a minimum of three communication capacities which amount to three degrees of differentiation. The distinct regulations of homologous and heterologous communication require two degrees of differentiation,

but it is the extraordinary combination of these opposite regulations with the presence of a basal heterologous communication, that demands at least one more.

These constraints, plus a kinetic one to be discussed further on, narrow the scope of possibilities sufficiently to construct a model in which the three degrees of differentiation are embodied by three species of cell-cell channel halves (Fig. 5 A): one species (*a*) is expressed by both cell partners and the other two species (*b* and *c*), whose production is more retinoid sensitive, are each expressed by only one partner. To the extent of our present knowledge, the formation of cell-cell channels may be envisioned as a two-stage process where the half channels first bind by their intercellular ends and then open up, but where the opening is not an obligatory sequel (Loewenstein, 1981). These features, which in sum represent a dissociation of channel function from half channel binding, are incorporated in our model. Thus, the model at once accounts for the basal homologous and heterologous communications and enhanced homologous communication, while the reduced heterologous communication comes out simply from mass action, if the binding between chimeric half channels has more heterophilic latitude than the formation of functional (*open*) channel has, that is, if different half channels can associate but may stay shut. This is illustrated schematically in Fig. 5 A. In the basal condition (*top*), communication is provided by *a-a* interactions in heterologous junction and by *a-a* plus *b-b* or *c-c* interactions in homologous junction. In the retinoid-stimulated condition (*bottom*), both types of junctions are swamped with *b* and *c* species, as the production of these species is enhanced; thus, the number of functional channels increases by *b-b* or *c-c* interactions in the homologous junctions, but decreases in the heterologous junction, as the *as* are prevented from forming functional channels (*a-a*) by their binding to the more abundant *b* and *c* species.

The premise of heterophilic binding in the model is guided by the knowledge that the coding sequences for the putative intercellular domain of a variety of channel proteins are highly conserved (Milks et al., 1988; Goodenough et al., 1988; Gimlich et al., 1990). The assumption of a more restrictive molecular interaction rule for the opening process is *ad hoc*. We wish to emphasize that this assumption implies merely less latitude, not general exclusion, of molecular heterology for the opening process; or, put in terms of the diagram, only the particular sorts of molecular heterology represented by the trio, but not all molecular heterologies, would preclude channel function. There is evidence that heteromolecular channels can form and be functional (Swenson et al., 1989; Werner et al., 1989), and the assumption does not conflict with that.

Crucial for the choice of model was the time course of the events in the two kinds of junction (Fig. 2). These kinetics eliminated a whole slew of mechanisms where determinants of communication compete for a limited junctional space, and such determinants include, apart from the channels, specific cell adhesion molecules. A priori, that was a possibility to be reckoned with because cell-cell channel formation is facilitated by the adhesion provided by homophilic cell-surface glycoproteins and, in some instances, even seems to depend on it (Loewenstein, 1967; Keane et al., 1988; Mege et al., 1988; Matsuzaki et al., 1990; Musil et al., 1990). Thus, in junctions where the channel formation

is ruled by such an adhesion, a counterregulation of communication might be envisioned to arise from a competition between three different species of homophilic adhesion molecules for the junctional space, such as diagrammed in Fig. 6 A: the retinoid-insensitive species (*a*) would be crowded out from the available junctional space by the proliferating retinoid-sensitive species (*b*, *c*), and so the heterologous junction eventually would be cleared of interactive adhesion molecules and, hence, of channels, while the homologous junction would fill with both. However, the kinetics of such a counterregulation are incompatible with the results. The overcrowding by the *b* and *c* adhesive species in heterologous junction would be mirrored by an equivalent *b* and *c* crowding in the homologous junctions, and so the *b* and *c* density there would approach saturation by the time the *a* population in heterologous junction is effectively crowded out. Hence, the decline of heterologous communication could not begin before the enhancement of homologous communication is nearly saturated (Fig. 6 B)—a kinetic feature shared by the entire class of mechanisms where determinants of permeability compete for junctional space. In fact, the opposite was observed; the enhancement of homologous communication trailed the decline in heterologous communication by several days (Fig. 2). We further note that there were no changes in cell contact relation discernible in phase contrast. But the kinetic argument alone is sufficient to render the adhesion mechanism unlikely and is more compelling than a microscopic observation could ever be.

The observed kinetics, on the other hand, are precisely the ones predicted from our model (Fig. 5 B). Here, because the competitive interaction is in series with the channel function, an effective heteromolecular competition in heterologous junction starts as soon as the *b* and *c* population of half-channels builds up to the size of the *a* population, which may occur long before the *bs* and *cs* in homologous junction attain saturation. Such kinetics are precluded in an adhesion mechanism, because the competitive interaction is then, of necessity, in parallel with the channel function. An interaction in series with that function is something the bipartite cell-cell channel is eminently, if not uniquely, suited for. More than a clue, this kinetic constraint was nature's giveaway, one might say.

Basal Communication. Our model also accounts for another difference in the behavior of homologous and heterologous junctions: the disparity between their basal levels of communication. As in a number of other combinations between transformed and normal cell, the basal levels of MCA10/10T1/2 heterologous communication were lower than those of the corresponding homologous communications; the heterologous junctional transfer on the average amounted to 50–70% of either homologous junctional transfers. Even larger differences are found in other combinations where normal 10T1/2 cells are paired off with other MCA-transformed 10T1/2 lines. For instance, in MCA4D/10T1/2, MCA4B/10T1/2, and MCA/10T1/2 cocultures, the average heterologous junctional transfers only amount to 12, 8, and near 0% of the corresponding homologous ones (Mehta et al., 1986).

In the light of the model, these differences in basal communications would reflect the presence of a basal *b* and *c* type channel population. When that population approaches the size of the *a* channel population, the one common to both

cell types (as represented by the 1:1 ratio in Fig. 5 A, top), the chance for heteromolecular interaction (which does not result in open channels) becomes appreciable and then grows with the number of *bs* and *cs*. The magnitude of the difference between the basal heterologous and homologous communications, then, would reflect a relative *b* and *c* abundance.

Thus, in summary, the differential basal behaviors of homologous and heterologous junctions and the differential retinoid-induced behaviors of these junctions would be two of a kind; they would stem from half channel competitions that differ only by degree.

The Trio Principle. This analysis provides a new insight into the mechanisms of junctional communication. It intimates that communication may be inhibited by the interplay between different channel proteins, an interplay between three proteins giving rise to counter regulation in homologous and heterologous junction. Such a *ménage à trois* embodies a powerful principle of intercellular regulation at tissue boundaries, which may have wide biological applications. One possibility bears on embryonic development where evidence is mounting that junctional communication is involved (Guthrie and Gilula, 1989). In embryos, retinoic acid occurs at 10^{-8} M concentration (the range where communication was counterregulated in our experiments) and exerts a morphogenetic action (Tickle et al., 1982; Thaller and Eichele, 1987). Thus, the trio principle, we suggest, may be behind the formation of compartments. In insect embryos, junctional permeability is reduced at the compartment boundaries (Warner and Lawrence, 1982); the boundaries are formed by a row of differentiated cells (Lawrence, 1973), and the junctions these cells make with the compartment cells, heterologous junctions in the present terms, are less permeable than the junctions among the compartment cells themselves (Blennerhassett and Caveney, 1984).

The identification of the proposed channel differentiations awaits molecular analysis. Probing so far with the cDNAs for connexin 43 (Beyer et al., 1987), connexin 32 (Kumar and Gilula, 1986; Paul, 1986), connexin 26 (Zhang and Nicholson, 1989) have revealed only mRNA for connexin 43 in the MCA10/10T1/2 system (P. Mehta, unpublished work). But this surely does not exhaust the possibilities. The family of cell-cell channel proteins is probably large; three mRNA homologues have turned up for connexin 43 alone in broad genetic screening (Gimlich et al., 1990), and multiple connexin-gene expression is known to occur in one and the same cell (Nicholson et al., 1987; Gimlich et al., 1990). Besides, the differentiations need not be genetic for the entire trio. The model would work equally well if, for example, the differentiation of one of the trio members would stem from a posttranslational modification. Therefore, we have couched the model in the most general terms, and even so its heuristic value is high.

Apart from the inhibition of communication dealt with here, retinoic acid produces inhibition of another sort (Pitts et al., 1986). That inhibition sets in within 2 h with 10^{-8} M retinoic acid concentrations, and within a few minutes with $\geq 10^{-5}$ M concentrations (Mehta et al., 1989), whereas the present inhibition does not begin to develop before 20 h. So fast an effect is unlikely to be due to an enhancement of protein synthesis; it may reflect an action on the channel open state, and so is not comprehended by the model.

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