The Actions of Retinoids on Cellular Growth Correlate with Their Actions on Gap Junctional Communication

Parmender P. Mehta, John S. Bertram, and Werner R. Loewenstein

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

Abstract. Retinoic acid (a possible morphogen), its biological precursor retinol, and certain synthetic derivatives of retinol profoundly change junctional intercellular communication and growth (saturation density) in 10T½ and 3T3 cells and in their transformed counterparts. The changes correlate: growth decreases as the steady-state junctional permeability rises, and growth increases as that permeability falls. Retinoic acid and retinol exert quite different steady-state actions on communication at noncytotoxic concentrations in the normal cells: retinoic acid inhibits communication at 10^{-10}–10^{-9} M and enhances at 10^{-9}–10^{-7} M, whereas retinol only enhances (10^{-8}–10^{-6} M). In v-*mos*-transformed cells the enhancement is altogether lacking. But regardless of the retinoid or cell type, all growth responses show essentially the same dependence on junctional permeability. This is the expected behavior if the cell-to-cell channels of gap junctions disseminate growth-regulating signals through cell populations.

THE retinoids, the natural or synthetic derivatives of vitamin A (retinol), comprise a family of compounds that exert a powerful action on cellular growth (Roberts and Sporn, 1984; Wolf, 1984). One of the first known actions of the vitamin itself was inhibition of growth (Wolbach and Howe, 1925). This growth effect has received the most attention because of its potential for cancer therapy. However, as a variety of retinoids became available, it transpired that they could both inhibit and stimulate growth (Lotan, 1980; Schroder and Black, 1980). The direction of the growth response seemed to depend, in some instances, on cell type; in others, on retinoid chemical structure (Lotan, 1980; Bertram, 1980); or in one cell type, solely on retinoid concentration (Haddox et al., 1979). No rationale has yet emerged for these disparate growth effects. We have searched here for one that is based on junctional intercellular communication.

This communication is altered by retinoids (Pitts et al., 1986), and several lines of evidence indicate that it may be instrumental in the control of growth (Loewenstein, 1987). The cell-to-cell membrane channels in the junctions transmit cytoplasmic molecules up to ~2 kD (Simpson et al., 1977), and it has been hypothesized that growth-controlling signals are among them (Loewenstein, 1966). The extent of the transmission in cell populations is determined by the permeability of the junctions. Thus, when the signals diffuse over some distance in the population to exert their effect, one would expect growth regulation to be governed by junctional permeability. A basic model predicts that an increase of junctional permeability would then lead to a decrease of growth, and vice versa (Loewenstein, 1979). We show here that this relationship, indeed, prevails when cells respond to retinoids.

Aside from growth, the retinoids profoundly influence cellular differentiation and development (Niazi and Saxena, 1978; Maden, 1982; Wolf, 1984; Sherman, 1986). Of particular interest are retinoic acid and retinol, which occur naturally in developing tissues. The receptors for retinoic acid belong to a family of receptors that selectively induce gene transcription, eliciting a cascade of events of differentiation (Giguere et al., 1987; Petkovich et al., 1987; Brand et al., 1988). Retinoic acid is a likely natural morphogen and retinol, its biosynthetic precursor, could be the morphogen-gradient source (Tickle et al., 1982; Thaller and Eichele, 1987). We report here that these two retinoids exert different effects on junctional communication.

Materials and Methods

Cell Culture

The following cell types were used: normally growing 10T1/2 cells (C3H 10T1/2 Clone 8; passage 7-20) (Reznikoff et al., 1973); 3T3, A31 (Aaronson and Todaro, 1968); methylcholanthrene-transformed 10T1/2 cells, MCA4D and MCA10 (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 cell types are derivatives of C3H 10T1/2 clone 8 cells transfected with plasmid pMI containing the complete coding region of the v-*mos* cloned into plasmid pBR322 (Blair et al., 1980).

The cells were grown in basal minimum essential medium supplemented with 5% fetal bovine serum and 25 μg/ml gentamicin at 37°C in an atmosphere of 5% CO_2. The same lot of serum was used throughout. The retinol content in the serum was determined by HPLC; the content was < 1 ng/ml, the resolution of the method. 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 action of retinol

John S. Bertram's present address is the Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI 96813.

on junctional transfer or growth in our most sensitive cell type (see Fig. 6). The cells were protected from light of < 500 nm to prevent retinoid breakdown. Stock cultures were passaged once a week in 100-mm plastic dishes, at 10^5 cells/dish in 10 ml of medium.

Growth and Communication Assays

Communication and growth were assayed in parallel cultures. The cells were seeded at 5×10^4 cells/dish in 60-mm plastic dishes, and the medium (5 ml), with or without retinoid, was changed every third day. This schedule of medium renewal was adopted after a series of preliminary experiments with 10T1/2, MCA4D, and MCA10 cells showed that the effects of tetrahydrotetramethylnaphtalenylpropenylbenzoic acid (TTNPB), retinol, or retinoic acid were the same when the retinoid-containing medium was renewed every 2 h, 4 h, or 3 d.

The saturation density, the number of cells at which the growth curves indicated no further increase in cell number, served as index of growth control. The cell number was determined by means of an electronic Coulter Electronics Inc. (Hialeah, FL) counter. For the countings, the cultures were trypsinized (0.25% trypsin, 1 mM EDTA); each count (each datum point in the figures) is based on six to nine culture dishes, three independent counts per dish. Growth curves were generated from daily counts and the saturation density was operationally defined when the counts no longer increased over three consecutive days. For correlating the various effects of retinoid treatment, the saturation densities were normalized with respect to the corresponding untreated controls. The absolute values ranged (10^4 cells/cm^2): 1.8–2.3 for 10T1/2; 5–6.7 for 3T3; 6.7–8.3 for MCA10; 4.8–5.8 for MCA4D; 5–6.7 for EC8; and 8.3–10 for MA31.

Junctional permeability was probed with Lucifer Yellow CH. The fluorescent tracer was injected into cells of similar size with the aid of a micropipette by pulses of pneumatic pressure controlled by a solenoid valve. The microinjection and the cell-to-cell transfer of the tracer were video recorded by means of a television system coupled to a fluorescence microscope and played back for analysis (Yada et al., 1985). The total number of fluorescent neighbors of the injected cell 1 min after the injection served as index of junctional transfer. The data plotted in the figures are mean values of 16–24 individual injection trials, unless stated otherwise. Where plotted as percentages, the data are normalized with respect to the index of the untreated controls (100%). The growth and communication assays were done in "blind" experiments.

Tests of Nonjunctional Permeability

Measurement of the rates of Lucifer fluorescence loss were taken on cells without neighbors in contact (sparse cultures). Fluorescence was excited with pulses of light of 10-s duration, one per min. This protocol minimized photodamage to the cells. Intracellular fluorescence was measured by video analysis (Yada et al., 1985).

Cytotoxicity Assay

Cytotoxicity was measured according to Puck et al. (1956). The cells were seeded at clonal densities in 60-mm dishes (in replicates), treated 24 h later with retinoids, incubated for 10–14 d (medium containing the retinoid was changed every 4–5 d), fixed with 3.7% buffered formaldehyde, and stained with 0.2% buffered Crystal Violet. The number of colonies with more than 50 cells (n) were scored, and the plating efficiency (PE) and survival rate (%) were determined:

$$PE = \frac{n}{n_t}\ ;\ \%\ \text{survival} = \frac{\text{PE of treated cells}}{\text{PE of untreated controls}} \times 100$$

(n_t = total number of cells seeded). The cytotoxicity curves for the various retinoids and cell types are displayed in Fig. 1.

Statistics

The statistical significance of the effects was calculated by standard t test, using Bonferroni's correction in the case of multiple comparisons with the same control value. For tests of the correlations between saturation density and junctional transfer (Fig. 11), we used Kendall's nonparametric τ method, corrected for ties (Kendall, 1962). This method is based on numerical ranks and does not require knowledge of the mathematical function relating the variables.

1. *Abbreviation used in this paper*: TTNPB, tetrahydrotetramethylnaphtalenylpropenylbenzoic acid.

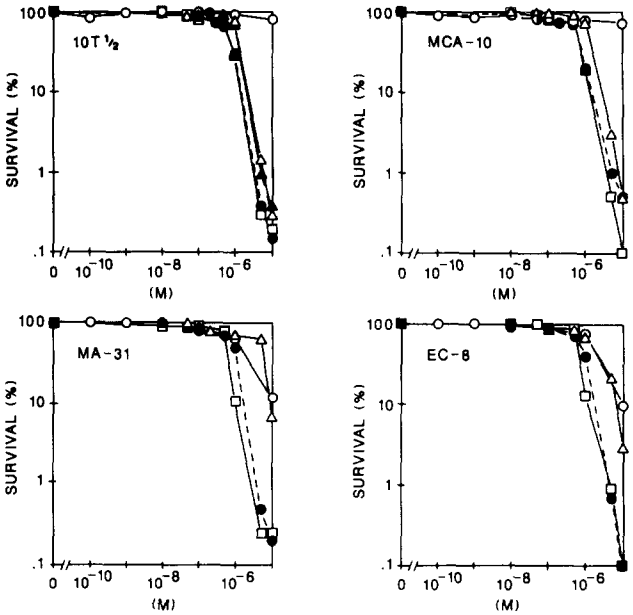

Figure 1. Cytotoxicity curves for the various retinoids in 10T1/2, MCA10, MA31, and EC8 cells. Abscissae, retinoid concentration; ordinates, percentage of surviving cell clones 10–14 d after retinoid treatment ([number of cell seeded/number of surviving clones] × 100). The curves for MCA4D (not shown) were similar to those for MCA10. (○) TTNPB; (▲) retinol; (△) retinyl acetate; (●) all-*trans* retinoic acid; (□) 13-*cis* retinoic acid.

Materials

BME (Gibco Laboratories, Grand Island, NY), fetal bovine serum (lot 1111581; HyClone Laboratories, Logan, UT), plastic dishes (Nunc, Roskilde, Denmark); all-*trans* retinoic acid (Sigma Chemical Co., St. Louis, MO); all-*trans* retinol, 13-cis retinoic acid, retinyl acetate, TTNPB (RO-13-7410/000) (Hoffman-La Roche Inc., Nutley, NJ); Lucifer Yellow (Molecular Probes Inc., Junction City, OR).

Stock solutions of the retinoids were in DMSO and aliquots were stored at −70°C in a nitrogen atmosphere, and used only once. Both test and control solutions contained the same concentrations 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 for the present work.

Results

We used normal mouse C3H 10T1/2 and 3T3 cells and their counterparts transformed by carcinogens or viral oncogenes, and treated them with retinoids, namely all-*trans* retinol, all-*trans* retinyl acetate, all-*trans* and 13-*cis* retinoic acid, or the benzoidal derivative TTNPB (Fig. 2). To correlate communication with growth, we examined the effects of the retinoids on junctional permeability in parallel with those on saturation density and restricted our observations to concentrations that did not significantly interfere with clonal growth and survival (see Materials and Methods; Fig. 1). Junctional permeability was probed with the 443-D fluorescent tracer Lucifer Yellow and indexed by the number of cells to which the tracer was transferred within 1 min of its microinjection into the test cell. As an index of growth control, we measured the saturation density, the cell density at which the cultures stopped growing at confluence. The results fell into two categories, as described below.

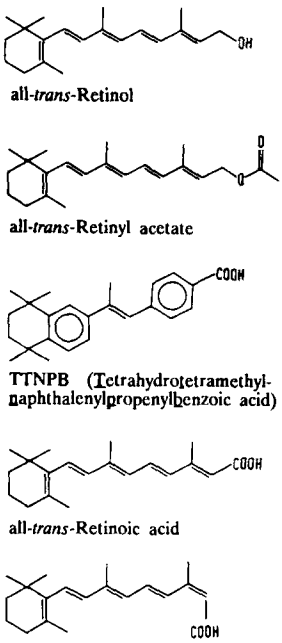

Figure 2. The retinoids used.

Normal 10T1/2, Normal 3T3, and Chemically Transformed 10T1/2 Cells

Communication. In normal 10T1/2 and 3T3 cells and in methylcholanthrene-transformed 10T1/2 cells, all five retinoids enhanced the junctional transfer over at least part of the concentration ranges tested. Fig. 3 gives an example for 10T1/2 cells treated with TTNPB for several days.

Increases of junctional transfer of this sort were detectable within 12–16 h of TTNPB application. They reached maxima within 3–5 d (Fig. 4, *A* and *B*) and were maintained for weeks when treatment was continuous (Fig. 4 *A'*). (The statistical significance levels, *P*, of the difference between test and control values were ≤ 0.03 at 12–16 h and <0.00001 at day 3 or later.) When there was a substantial base level, the transfer significantly declined during the first 1–4 h ($P \leq 0.0001$) before it rose (Fig. 4 *A'*). When the treatment was discontinued, the effect was slowly reversed (tested in 10T1/2): junctional transfer fell rapidly during the first 8 h ($P = 0.0007$ at 6 h), but then more slowly afterwards, with a residual enhancement lingering on for several days (Fig. 5 and Fig. 5, *inset*).

Dose–response curves were determined on day 4–5 of continuous retinoid treatment, when the cultures approached confluence. The enhancement of junctional transfer then had reached steady state. The curves were of two sorts: with enhancement of junctional transfer over the entire (noncytotoxic) working range of retinoid concentration (Figs. 6 *A* and 7 *A*); or biphasic, with inhibition of junctional transfer at low concentrations and enhancement at high concentrations (Figs. 6 *B* and 7 *B*). The curves for TTNPB, retinol, and retinyl acetate were of the former sort and those for all-*trans* and 13-*cis* retinoic acid, of the latter.

With TTNPB, the most powerful agent, the enhancement of junctional transfer was detectable at 10^{-12} M in 10T1/2 cells and at 10^{-9} M in MCA4D cells; the enhancement increased progressively with concentration, reaching a maximum at 10^{-8}–10^{-7} M, and then declined abruptly (10^{-7}–10^{-6} M) (Figs. 6, *A* and *a*, and 7 *A*). (The statistical significance level, *P*, of the difference between the test and control values was 0.02 at 10^{-12} M and 0.001 at 10^{-11} M in 10T1/2 [Fig. 6 *a*] and <0.00001 at 10^{-9} M in MCA4D [Fig. 7 *A*].) Retinol and its acetate had higher thresholds, and their maximal effects occurred at 10^{-6} M, their highest noncytotoxic dose (Figs. 6 *A* and 7 *A*).

The retinoic acids enhanced in the 10^{-9}–10^{-7} M range, like the other retinoids; but they inhibited in the 10^{-10}–10^{-9} M range and that inhibition was substantial: on the average, junctional transfer was reduced by 62% ($P < 0.001$) in 10T1/2 and by 22% ($P < 0.01$) in MCA4D (Figs. 6 *B* and 7 *B*).

Similar results were obtained with 3T3–A31 cells in experiments in which TTNPB, retinol, and retinyl acetate were tested over the 10^{-12}–10^{-5} M range, and the retinoic acids, over the 10^{-8}–10^{-6} M range (data not shown).

The aforegoing data all pertain to retinoid concentrations that were not cytotoxic (see Fig. 1). In the case of TTNPB, this range extended over six orders of magnitude in 10T1/2 cells, including the range between 10^{-8} and 10^{-6} M where the effect on junctional transfer declined. At cytotoxic concentrations ($>5 \times 10^{-5}$ M for TTNPB, retinol, and retinyl acetate, and $>10^{-7}$ M for the retinoic acids) junctional transfer of Lucifer Yellow was inhibited or abolished altogether (Fig. 6, *a* and *b*). (The action in the cytotoxic range was fast; for example, with 10^{-5} M TTNPB junctional transfer in 10T1/2 cells fell to ~60% within 20 min.)

Our choice of day 4–5 for testing junctional transfer for the various dose–response curves was dictated by the time the cells approached confluence. The study of the time course of the TTNPB response (10^{-7} M) in 10T1/2 cells had shown that the enhancement of communication then was close to steady state (Fig. 4). However, even at day 3 the dose–response curves were essentially like those at the later time (Fig. 6, compare *A* and *B* with *a* and *b*).

Nonjunctional Membrane Permeability. The permeability of nonjunctional cell membrane was not sensibly changed by the retinoids, as tested by measurements of the rates of loss of Lucifer Yellow fluorescence from the cells. This was so for TTNPB at various (noncytotoxic) concentrations effective on junctional transfer, as well as for all-*trans* retinoic acid at concentrations producing inhibition or enhancement of junctional transfer showing that, in either direction, the changes of junctional transfer reflect changes in junctional permeability. A sample of such a measurement is shown in Fig. 8, *inset*.

Growth. The parallel effects on growth are shown in Figs. 6, *A'* and *B'*, and 7, *A'* and *B'*. The corresponding curves of saturation density all exhibit an inverse relationship with steady-state junctional transfer. For example, in 10T1/2 cells, saturation density decreased as junctional transfer increased over the 10^{-10}–10^{-7} M concentration range of TTNPB, retinol, and retinyl acetate (Fig. 6 *A'*); moreover, in the case of TTNPB, saturation density increased as junctional transfer decreased over the 10^{-7}–10^{-6} M range, both attaining their respective control levels concomitantly (10^{-6} M) (Fig. 6 *A'*, open circles).

With the retinoic acids, the correlation was evident also for variations of junctional transfer below control level; the phase of inhibition of junctional transfer (10^{-10}–10^{-9} M retinoic

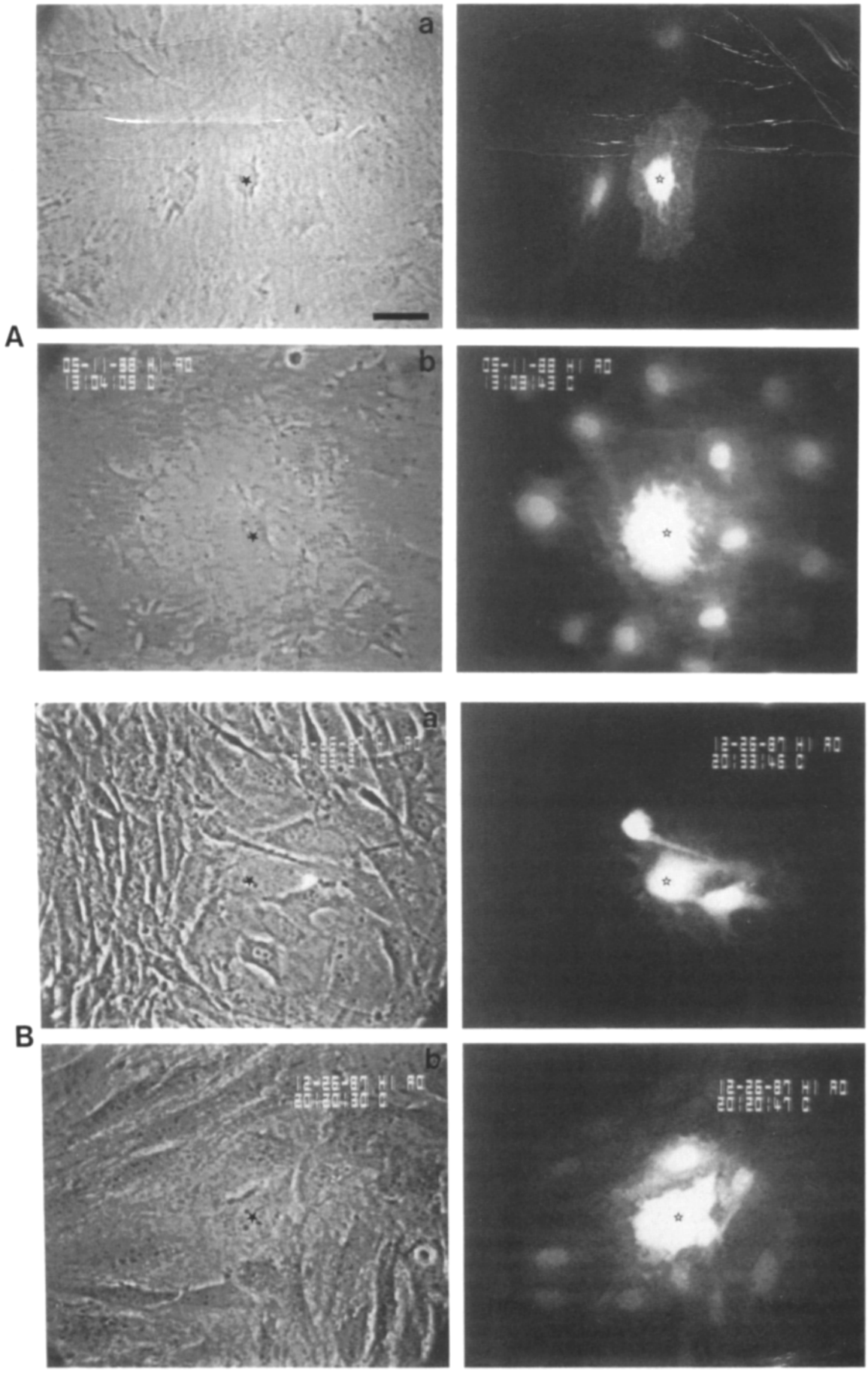
A
a
b
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Table I. The Saturation Densities of Normal and Chemically Transformed 10T1/2 Cells at Three Settings of Junctional Permeability

Junctional transfer	10T1/2	MCA4D	MCA10
Maximum	1.4–1.6	2.3–2.7	2.7–3.7
Control	1.8–2.3	4.8–5.8	6.7–8.3
Minimum	2.5–2.9	6.0–7.0	7.5–9.0

The ranges of absolute values of saturation density ($\times 10^4$ cells/cm^2) for the (untreated) control and the treated condition in which junctional transfer was at maximum (10^{-9} M TTNPB) and at minimum (10^{-9} M retinoic acid).

acid) was associated with an increase of saturation density above control level (an actual proliferative effect). The phase of enhancement of junctional transfer (10^{-9}–10^{-6} M retinoic acid) was associated with change of saturation density in the opposite direction (Figs. 6, *B* and *B'*, and 7, *B* and *B'*).

Aside from the correlation, it is of interest to compare the absolute values of saturation density, as they tell about the state of transformation of the cells. The saturation densities of the chemically transformed 10T1/2 cells (MCA4D and MCA10) were, on the average, about two to four times those of the normal 10T1/2 cells. By action of TTNPB (10^{-9} M), the saturation densities of the transformed cells were lowered to close to the control values of the normal cells as the junctional permeability of the transformed cells were raised maximally (Table I). However, conversely, the saturation density of the normal cells raised maximally by the retinoic acid (10^{-9} M) stayed well below the control values of the transformed cells. (The effects of retinoic acid and retinyl acetate on saturation density were comparable to those obtained by Mordan and Bertram, 1983).

V-mos-transformed 10T1/2 and 3T3 Cells

Communication. In 10T1/2 cells transformed by v-*mos* (EC8) and 3T3 cells transformed by the virus carrying v-*mos* (MA31), TTNPB and the retinoic acids inhibited junctional permeability (Fig. 8) (in the absence of change in nonjunctional membrane permeability; Fig. 8, *inset*). This effect was faster than the enhancement of junctional permeability in the preceding group of cells; it set in within 2–3 h of TTNPB treatment ($P = 0.03$), reaching a maximum within 3–4 h ($P < 0.005$) (Fig. 9). The inhibition was maintained for many days in experiments of continuous TTNPB treatment (12–18 d, data not shown).

Such inhibition of junctional permeability was the sole response exhibited by these cells over the whole retinoid concentration range tested (Fig. 9 *A*). This contrasts with the preceding group of cells where junctional permeability was enhanced by TTNPB at 10^{-12}–10^{-7} M and by retinoic acids at 10^{-9}–10^{-7} M. TTNPB and the retinoic acids inhibited in both EC8 and MA31 cells, but retinol and retinyl acetate inhibited significantly only in EC8 cells (Fig. 11 *E*), not in MA31 cells (Fig. 10 *A*).

Growth. The growth of these cells was stimulated when junctional transfer was inhibited (TTNPB and retinoic acid) and growth was unchanged when junctional transfer was unchanged (retinol and retinyl acetate in MA31 cells). The corresponding dose–response curves of saturation density mirrored those of junctional transfer: saturation density increased as junctional transfer decreased by action of TTNPB and the retinoic acids (Fig. 10 *B*). Despite the different responsiveness, the relationship between growth and communication in the EC8 and MA31 cells was essentially the same as in the preceding group of cells.

The Growth–Communication Correlation

Fig. 11 displays the correlation between saturation density and junctional transfer for the various normal and transformed cell types; each plot pools the data from all retinoid treatments for a given cell type. All plots show the same basic feature; an inverse relationship between saturation density and junctional transfer, regardless of the retinoid used and regardless of the direction of the retinoid action. The slopes of the relationship were steepest close to the control levels of junctional transfer, including the transfer below that level.

The relationships were subjected to nonparametric correlation analysis (see Materials and Methods). The five correlations were statistically highly significant ($P < 0.001$), including the correlation for EC8 cells where the data at low junctional transfer were disperse. The rank correlation coefficients (Kendall's τ) and the corresponding probability levels (P) are listed in Fig. 11 *F*.

Discussion

Junctional Communication and Growth

The most important result to emerge from this work is that the effect of retinoids on cellular growth control correlates with their action on junctional communication. With the five retinoids tested and the various cell types used, growth was inhibited when steady-state junctional permeability was increased, and growth was enhanced when that permeability was decreased. The direction and the intensity of the retinoid action on junctional permeability appears to determine the effect on growth. This dependence provides a rationale for the hitherto seemingly unrelated retinoid effects on growth.

The dependence is consistent with the hypothesis that the cell-to-cell channels transmit cytoplasmic growth-regulating signals (Growth control hypothesis; Loewenstein, 1966). It is the expected behavior if such signals must diffuse some distance through the interconnected cell community to exert their regulatory effect; as the junctional permeability deter-

Figure 3. Enhancement of junctional communication by retinoid treatment in 10T1/2 cells. (*A*) Normal 10T1/2 cells; (*B*) methylcholanthrene-transformed 10T1/2 cells, MCA10. Fluorescent Lucifer Yellow was injected into a cell (marked by star) in (*a*) the untreated control condition and (*b*) on day 3 of treatment with 1×10^{-8} M TTNPB. The video pictures show the test fields in phase contrast (left panels) and in fluorescence mode 1 min after the injection (right panels). Bar, 40 μm.

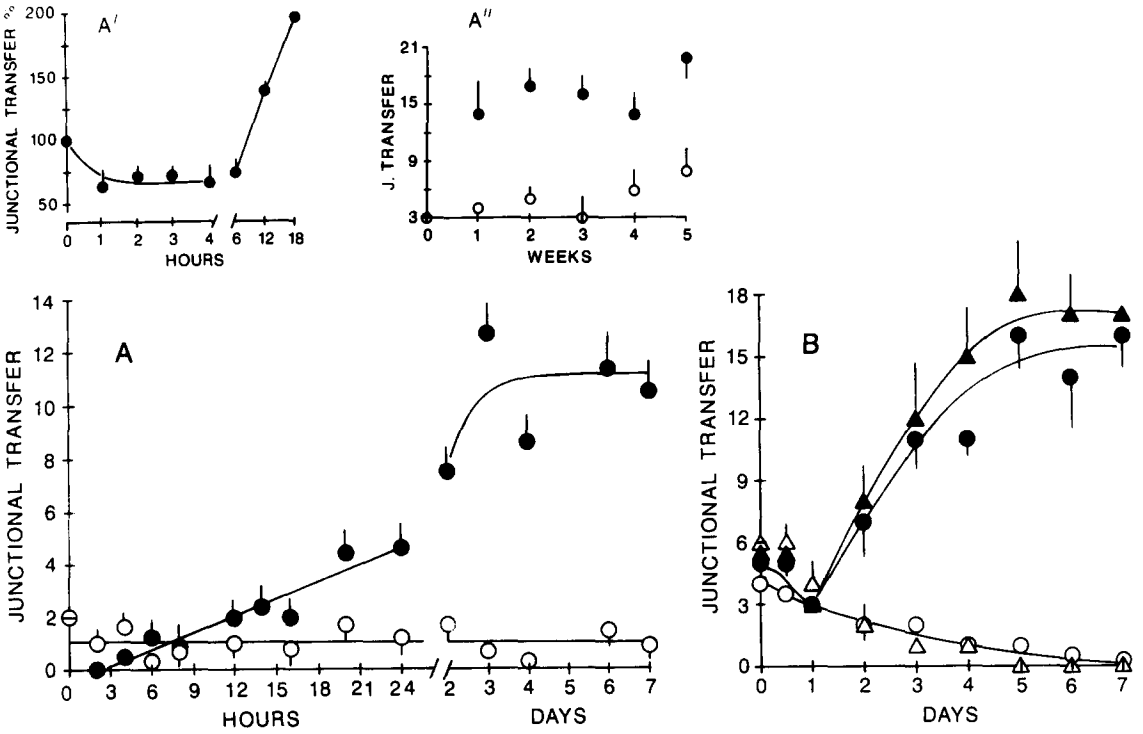


Figure 4. Time course of the TTNPB action on junctional communication. (*A*) 10T1/2 cells treated with 1×10^{-7} M TTNPB. Treatment started at time zero. Junctional transfer was indexed by the number of cells to which Lucifer Yellow was transferred 1 min after its injection into the test cell. The data plotted are mean values (±SEM) of 16–24 individual injection trials in this and all subsequent figures; SEM bars are omitted where less than the size of the symbols. Solid symbols are data from treated cultures; open symbols, from untreated sister cultures (controls). Note change of time scale. The basal junctional transfer was low in this culture. (*A'*) Data from a culture with a higher basal level of junctional transfer, displaying the transient inhibition preceding the enhancement of junctional transfer. Treatment with 1×10^{-6} M TTNPB. (*A''*) An experiment as in *A*, in which junctional transfer was followed for 5 wk. Fresh TTNPB was applied every 4 d. (*B*) Methylcholanthrene-transformed 10T1/2 cells treated with 1×10^{-8} M TTNPB: MCA4D cells (circles); MCA10 cells (triangles).

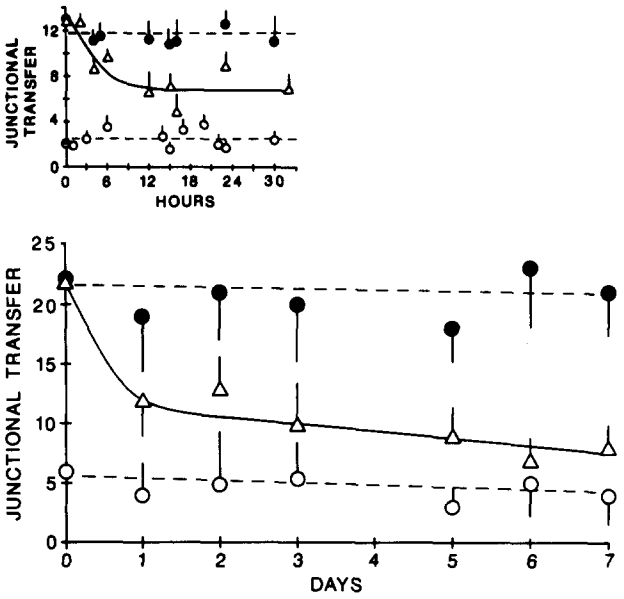


Figure 5. Reversal of the TTNPB effect. The 10T1/2 culture had been treated with 1×10^{-7} M TTNPB for 10 d. The retinoid was then removed (time zero), and measurements of junctional transfer were begun (△). (●) Data from a control sister culture in which the treatment was continued; (○) data from a second control sister culture untreated. *Inset*, data from another experiment, with higher time resolution, showing the initial reversal phase.

mines the extent of the diffusion, the growth regulation would be ruled by that permeability in an interplay with signal degradation and leakage (Loewenstein, 1979).

The present results indicate that the rule applies to both normal and transformed cells. This broadens our perspective and extends the range of applicability of models that predict an inverse relationship between growth and junctional permeability (Loewenstein, 1969, 1979; Burton, 1971). That range also encompasses the special, but biologically interesting, case of interactions between normal and transformed cells, where the growth of transformed cell clones depends on the permeability of the heterologous junctions between the normal and transformed cells (Mehta et al., 1986). As it is much smaller than the permeabilities of the homologous junctions, the heterologous junctional permeability there limits the intercellular diffusion and governs the relationship with growth.

To illustrate these notions and to show that communication-dependent growth control could, in principle, come about with either inhibitory or stimulatory signals, a model with discrete signal sources is presented in the Appendix. The model incorporates a rather unique topological property of junctional communication systems whereby the cell density in a population can be sensed and the population becomes amenable to autonomous growth control.

The correlation of saturation density with junctional communication stretched over a threefold range in the trans-

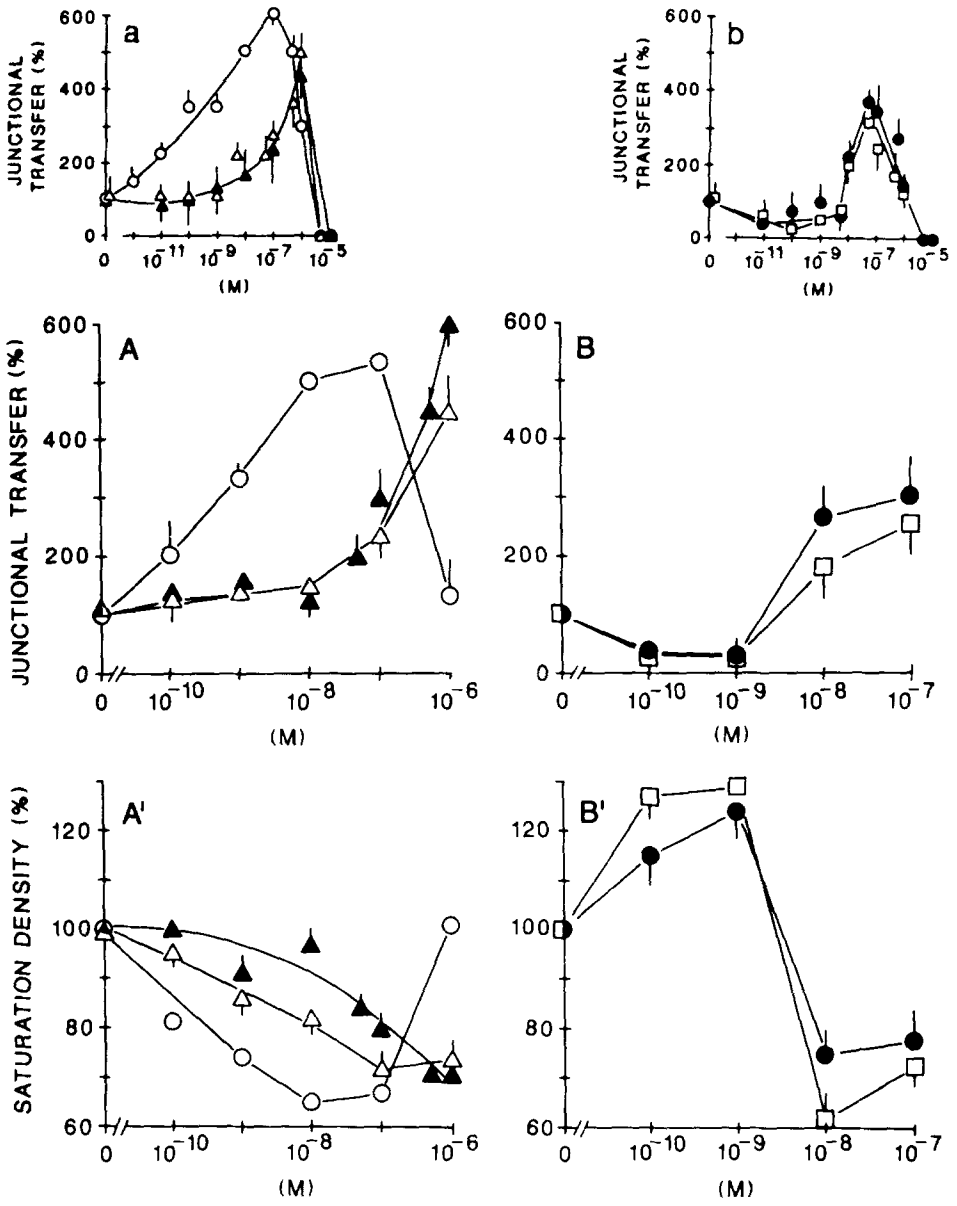


Figure 6. Correlated communication and growth responses in 10T1/2 cells. Dose-response curves for the various retinoids. (*A* and *B*) Steady-state junctional transfer. (*A'* and *B'*) The corresponding saturation densities. Both junctional transfer and saturation density are normalized with respect to the untreated controls (100%). The data are from parallel sister cultures for each retinoid. Junctional transfer was tested on day 4–5 of the retinoid treatment, as the cultures approached confluence. All data plotted pertain to the noncytotoxic concentration ranges (see Fig. 1). (*a* and *b*) The data here cover a wider concentration range, including the threshold concentrations for TTNPB and cytotoxic concentrations for the other retinoids. Junctional transfer here was determined on day 3 of treatment, before the cultures became confluent. (○) TTNPB; (▲) retinol; (△) retinyl acetate; (●) all-*trans* retinoic acid; (□) 13-*cis* retinoic acid.

formed 10T1/2 cells: from saturation densities of 2.3–7.0 (10^4 cells/cm^2) in MCA4D cells; and 2.7–9.0 (10^4 cells/cm^2) in MCA10 cells, at the respective minima and maxima of junctional permeability. At the maxima, the saturation densities were within the range of those of the normal 10T1/2 cells (even within the range of the values of untreated controls of the normal 10T1/2 cells, in the case of MCA4D) (Table I). Thus, in as much as saturation density characterizes normalcy and transformation in vitro, the MCA4D and MCA10 cells were normalized when their junctional permeabilities were maximally raised by TTNPB. In terms of the growth control hypothesis and the preceding premises concerning diffusion in cell communities, we would expect that such a rise of junctional permeability could produce the normalization, regardless of the means by which the rise of permeability is produced. In other words, junctional permeability would be the controlling variable and the retinoids would be the tools for changing its setting. We are further encouraged in this notion by the results of recent experiments in which a variation of the junctional-permeability setting was achieved in NIH 3T3 cells by a very different means, namely by point mutations and chimeric constructs of the cellular *src* gene which codes for a modulator of junctional communication. In that condition, too, the saturation density turned out to depend on junctional permeability and the inverse relationship was observable over an even wider range; the lowest saturation densities (those of cells with normal gene expression and normal growth phenotype) corresponded with high junctional permeability and the highest saturation densities (those of transformed cells with altered genes) corresponded with the lowest junctional permeability (Loewenstein and Azarnia, 1989).

The Dual Action of Retinoids on Communication

A further point to emerge is that retinoids can both inhibit

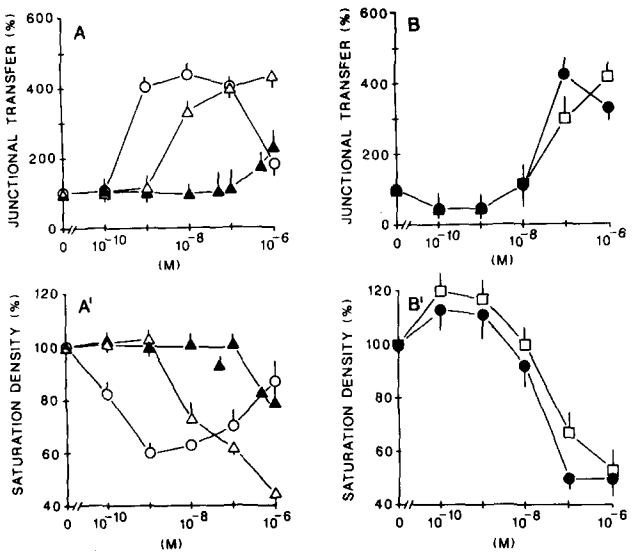


Figure 7. Communication and growth responses in methylcholanthrene-transformed 10T1/2 cells (MCA4D). Dose–response curves for the various retinoids. (*A* and *B*) Junctional transfer; (*A'* and *B'*) saturation densities. Junctional transfer and saturation density are normalized with respect to the untreated controls. Notation and general protocols as in Fig. 6. (○) TTNPB; (▲) retinol; (△) retinyl acetate; (●) all-*trans* retinoic acid; (□) 13-*cis* retinoic acid

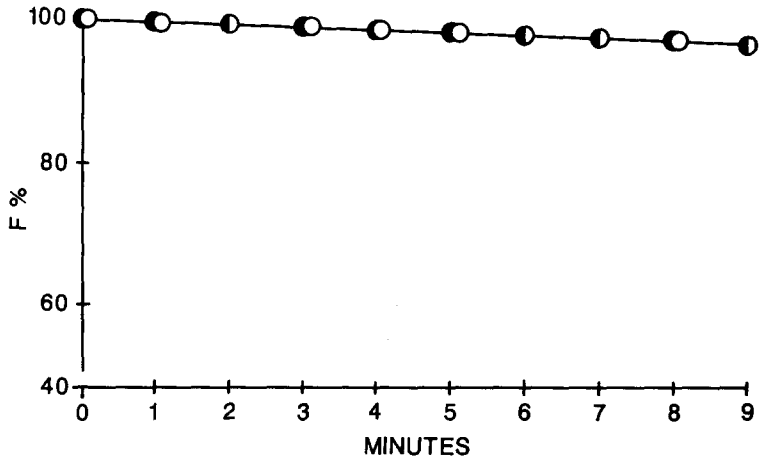
and enhance junctional communication, and that they can do so at low concentrations. TTNPB and retinoic acid were most potent; the threshold for enhancement of communication by TTNPB was of the order of 10^{-12} M and that for inhibition by retinoic acid was of the order of 10^{-10} M. In the v-*mos*-transformed cells the inhibition occurred singly, its full time course, a 2–4-h development, coming into view (Fig. 9). In all other cell types the two actions occurred together. The inhibition then preceded the enhancement which developed over several days (Fig. 4 *A'*). With retinoic acid, the inhibition and enhancement segregated at different concentrations in steady state (Figs. 6 *B* and 7 *B*), and the threshold for the inhibition was of the same order (10^{-10} M) as that in the v-*mos*-transformed case where inhibition occurred solely (Fig. 10).

All this suggests the presence of two distinct mechanisms: a fast mechanism (I) for the inhibition and a slow mechanism (E) for the enhancement of junctional permeability. Thus, at any given time, the junctional-permeability response to the retinoids would reflect E − I, the difference between the opposing mechanisms; and in the particular case of retinoic acid, the two phases of the response would reflect the different concentration dependencies of the two mechanisms, as diagrammed in Fig. 12. This way, the various retinoid effects can simply be accounted for by the preponderance of one mechanism or the other: in the actions of retinol, retinyl acetate, and TTNPB on normal and methylcholanthrene-transformed cells, the enhancement mechanism (E) would be preponderant, except for the initial 2 h; in the v-*mos*-transformed cells the inhibitory mechanism (I) would be preponderant at all times (E may be absent altogether); and in the retinoic acid response (where the threshold for inhibition of junctional permeability was evidently lower than that for enhancement), the predominance would shift with increasing concentration from I to E, crossing over as E = I.

The slow development and slow reversal of the enhancement of junctional permeability suggest an action on biosynthesis of a junctional component. Further indicative here are electron-microscopic studies in tumor and skin tissues, showing that the gap-junction area increases after long term retinoic acid treatment (Prutkin, 1975; Elias and Friend, 1976; Elias et al., 1980). These studies provided no information on permeability, but in view of the present results it seems plausible that the enlarged gap-junction areas reflected increases in the number of (open) channels. Thus, we are led to believe that E promotes the synthesis of a critical element in the channel formation process. Retinoids can induce the expression of a number of proteins (Strickland and Mahdavi, 1978; Linnenbach et al., 1980; Fuchs and Green, 1981) and the recent discovery of DNA-binding domains in retinoic acid receptors, which are similar to the DNA-binding domains of steroid- and thryoid-hormone receptors (Petkovich et al., 1987; Giguere et al., 1987; Brand et al., 1988), point up a mechanism. Those receptors are known to induce transcription of specific sets of genes, producing a cascade of events leading to cellular differentiation (Yamamoto and Alberts, 1976). Thus, we suggest that enhanced expression of a protein involved in cell-to-cell channel formation is part of that cascade. Such a protein might be the channel polypeptide itself or a regulatory or accessory protein in the channel formation process. The apparent absence of E in v-*mos* transformation provides a lead which we are now pursuing. As to the inhibition of junctional permeability, this is fast enough to suppose that I operates on the channel closure mechanism.

From the physiological point of view, it is interesting to compare the actions of the two naturally occurring retinoids, retinoic acid and retinol. Two results stand out. First, the steady-state action of retinoic acid on normal cells was biphasic; retinoic acid inhibited communication in the 10^{-10}–10^{-9} M range and it enhanced it in the 10^{-9}–10^{-7} M range (Fig. 6 *B*). Second, the steady-state action of retinol was distinct from that of retinoic acid; retinol only enhanced (10^{-7}–10^{-6} M) (Fig. 6 *A*). These results may bear on embryonic development. Both retinoids are present in embryonic tissue at those concentrations (Thaller and Eichele, 1987) and junctional communication has been strongly implicated in development (Warner et al., 1984; Fraser et al., 1987). Retinoic acid is a likely morphogen; it forms a gradient of concentrations of the order of 10^{-8} M in the chick limb bud, which correlates with limb pattern formation (Thaller and Eichele, 1987) and it exerts potent morphogenetic effects when applied externally (Tickle et al., 1982). Retinol, its biosynthetic precursor and the likely source for the gradient, is present at $>10^{-7}$ M concentration (Thaller and Eichele, 1987). Two retinoic acid receptors of different affinities (Brand et al., 1988) and distinct cytoplasmic-binding proteins for retinoic acid and retinol (Chytil and Ong, 1984) have been identified.

Retinoic acid is metabolized faster than retinol by 10T1/2 cells (Rundhaug et al., 1987), as it is in a number of other cell types (Gubler and Sherman, 1985; Napoli, 1986). Thus, the question arose whether the differential actions of retinoic acid and retinol were due to differential depletion of these



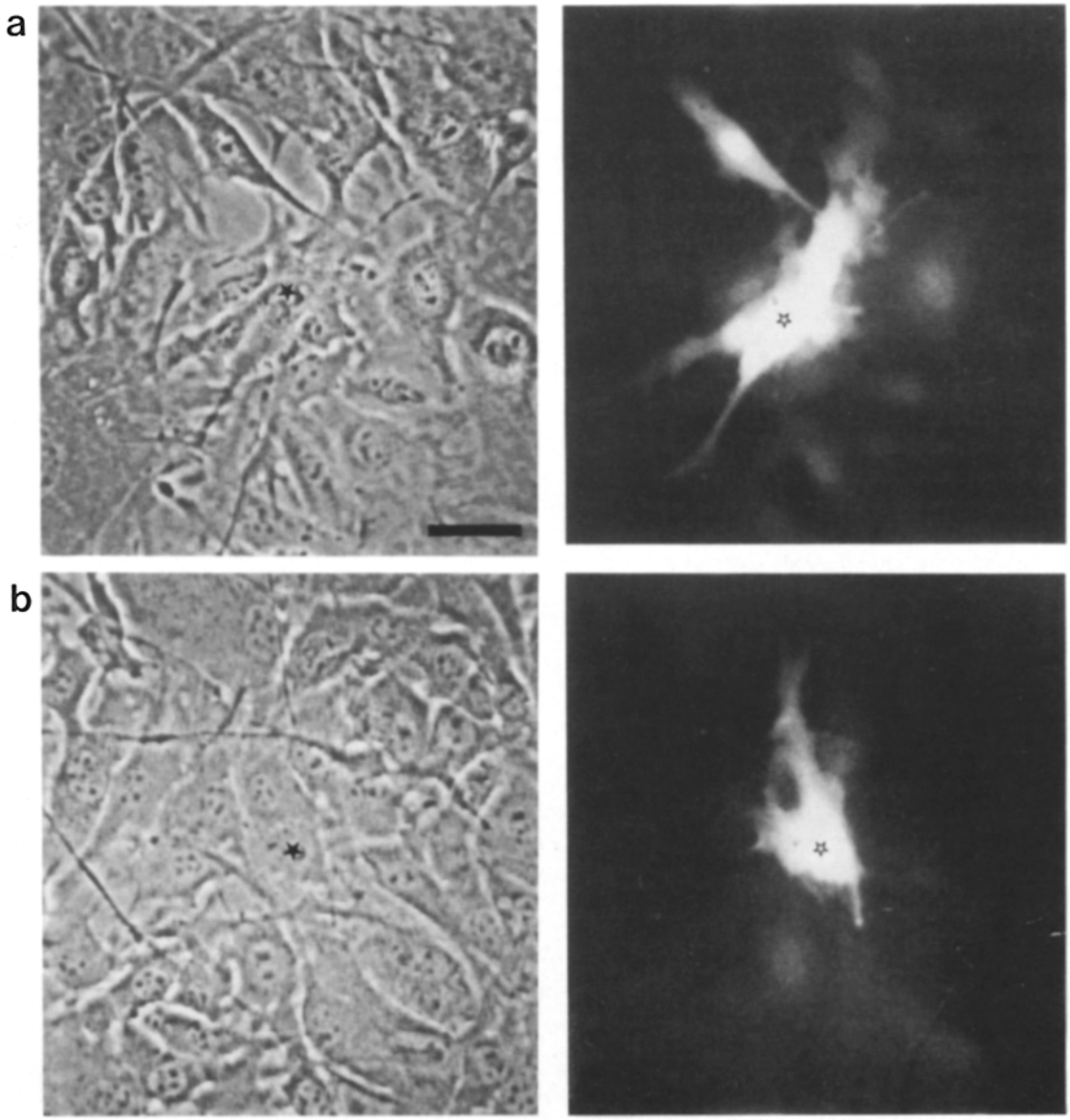


Figure 8. Inhibition of junctional communication by TTNPB in v-*mos*-transformed 3T3 cells, MA31. Video pictures of cell-to-cell transfer (*a*) untreated controls or (*b*) 8 h after application of 1×10^{-7} M TTNPB. The fluorescence picture shows the cell field 1 min after injection of Lucifer Yellow into the cell marked by the star. *Top panel*, rates of tracer loss. Photometric measurement of intracellular Lucifer fluorescence (*F*) from a MA31 cell, beginning at 8 h after retinoid application (within 5 s of the Lucifer microinjection). (●) Data for the treated cell (1×10^{-7} M TTNPB); (○) data for an untreated control cell. Bar, 40 μm.

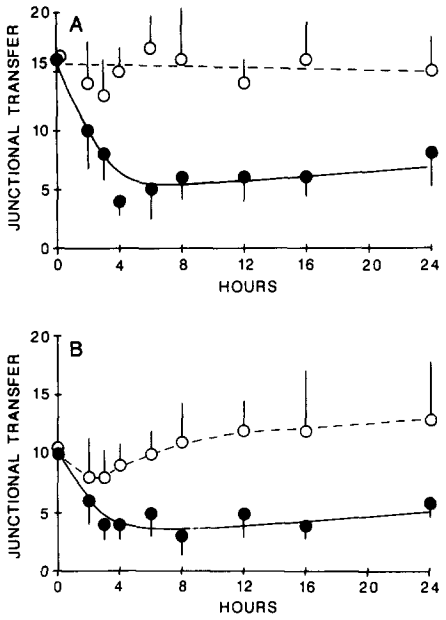


Figure 9. Time course of the inhibition of junctional communication by TTNPB. (*A*) EC8 cells; (*B*) MA31 cells. TTNPB treatment (1×10^{-8} M) started at time zero. Solid symbols, data from treated cultures; open symbols, from untreated sister cultures (controls).

retinoids in the medium. We performed experiments with 10T1/2, MCA4D, and MCA10 cells in which retinoic acid- or retinol-containing medium was added every 2 or 4 h, instead of every 3 d as in our usual protocol. For either retinoid, the dose–response curves (junctional transfer and growth) were not distinguishable from the corresponding curves obtained with the usual protocol. (These experiments were part of a preliminary series on the basis of which the protocol for our main experiments was adopted; see Materials and Methods.) There is practically no change in the concentration of retinoic acid or retinol in the 10T1/2 culture medium over 2–4 h (Rundhaug et al., 1987), and so depletion does not seem to be a factor in the differential actions of retinoic acid and retinol. Although a question of this sort does not arise regarding the enhancement of junctional permeability by retinol and the metabolically more stable synthetic retinoid TTNPB, we note that also in that case it did not matter whether retinoid-containing medium was renewed every 2–4 h or every 3 d.

Effect of High Retinoid Concentrations

The retinoid concentrations in our experiments were not cytotoxic; i.e., they did not significantly affect clonal growth (Fig. 1). This was essential for our purpose of correlating junctional communication and growth. In earlier work dealing with retinoid actions on communication, the concentrations used were several orders of magnitude higher, and only inhibitory effects of communication were noted (Pitts et al., 1981, 1986; Wälder and Lützelschwab, 1984). Pitts and colleagues tested the effect of retinoic acid (10^{-4} M) at 2 min and at 3 h, and so the long term component of enhancement of communication was missed. The use of the high concentrations, however, does not lessen the importance of their discovery. Although cytotoxic, these concentrations seemed not toxic in a general sense over the short periods used; the inhibition of communication was reversible and nucleotide metabolism was reversible (Pitts et al., 1986). Thus, the usefulness of retinoids as agents for experimental modification of junctional communication may extend beyond their noncytotoxic concentration range.

Homologous Junctions vis à vis Heterologous Junctions

Our results revealed a surprising difference between junctions formed by cells of the same cell type (homologous junctions) and junctions formed by cells of different type (heterologous junctions). In heterologous junctions between normal and transformed cells, we found earlier that retinoids only inhibit communication (Mehta et al., 1986). Among the junctions showing that behavior were the 10T1/2/MCA10 and 10T1/2/MCA4D junctions: heterologous junctions formed by cell types whose respective homologous junctions gave just the opposite steady-state response. For example, in response to 10^{-7} M retinoic acid, the 10T1/2/MCA10 heterologous junctional communication is profoundly inhibited (Mehta et al., 1986), whereas the homologous junctional communication in either one of the cell partners is enhanced here. Clearly, this difference in responsiveness is not attributable to cell type nor to retinoid type (nor to retinoid concentration); it seems to reflect an intrinsic junctional property. Elsewhere we analyzed the heterologous junctional response in some

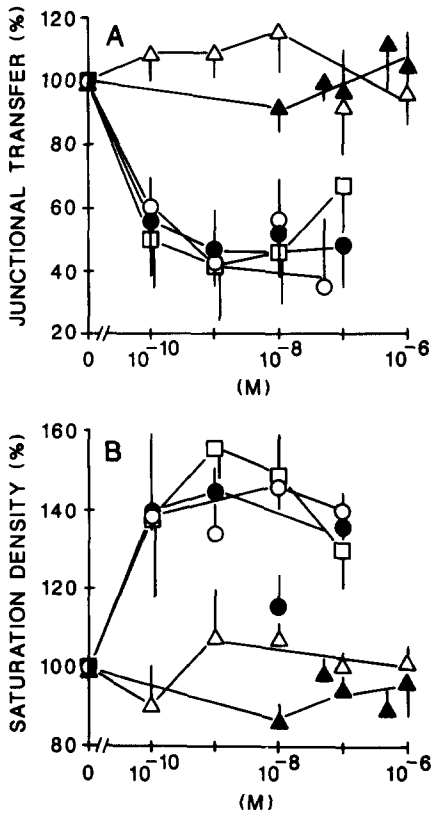


Figure 10. Dose–response curves of MA31 cells. (*A*) Junctional transfer and (*B*) growth at various retinoid concentrations. Growth and communication data for each compound (and cell type) are from parallel experiments on sister cultures. (○) TTNPB; (▲) retinol; (△) retinyl acetate; (●) all-*trans* retinoic acid; (□) 13-*cis* retinoic acid.

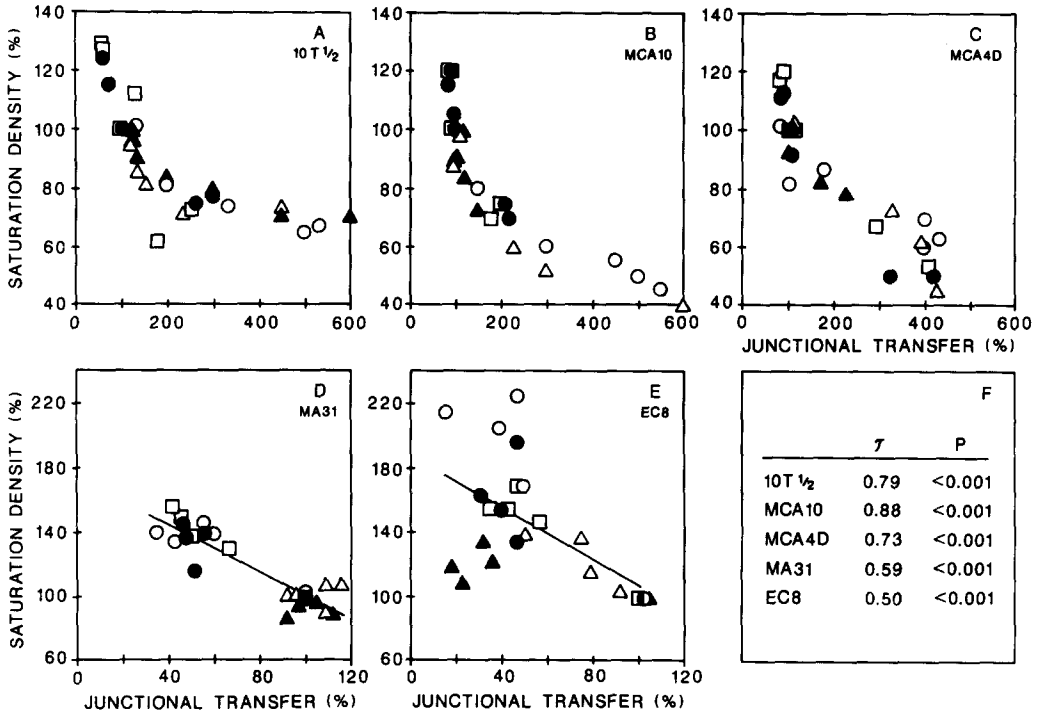


Figure 11. Saturation density vs. communication. Each plot pools the data of all experiments performed on a given cell type, that is of treatments with the various retinoids at the various concentrations. Plotted are the normalized mean saturation densities (ordinates) and the corresponding normalized mean number of communicating cells (abscissae). (*A*) 10T1/2; (*B*) MCA10; (*C*) MCA4D; (*D*) MA31; (*E*) EC8; (*F*) the correlation coefficients, Kendall's τ, and the corresponding statistical levels of significance, *P*, for each plot (see Materials and Methods). (○) TTNPB; (▲) retinol; (△) retinyl acetate; (●) all-*trans* retinoic acid; (□) 13-*cis* retinoic acid.

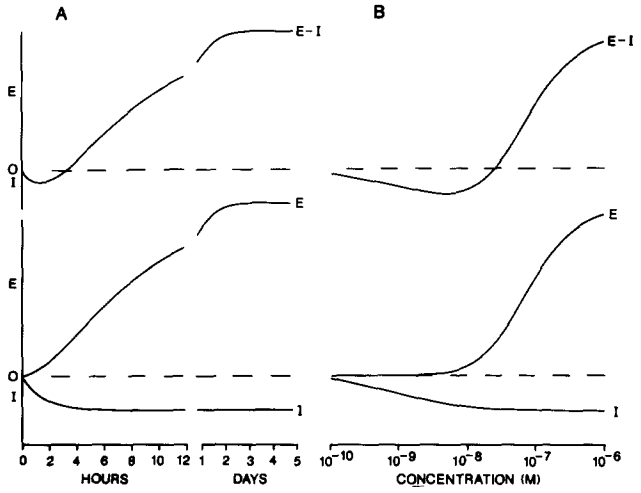


Figure 12. Diagrammatic representation of two opposing processes of different rates, E and I, in which the algebraic sum determines the retinoid response of junctional permeability. (*A*) The E−I curve (top), as generated by activation of both processes by a given retinoid (TTNPB, retinol, retinyl acetate), at constant concentration. The time courses of E and I are presented below. (*B*) The E−I curve, as generated by activation of E and I by retinoic acid at varying concentration (top). The individual dose-response curves of E and I are represented below. The diagrams refer to normal 10T1/2 and normal 3T3 cells and methylcholanthrene-transformed 10T1/2 cells.

detail, seeking a common ground with the present biphasic response in homologous junctions of normal cells and with the inhibitory response in homologous junctions of v-*mos* cells (Mehta, P., and W. R. Loewenstein, manuscript in preparation).

Appendix

by W. F. Nonner and W. R. Loewenstein

A Model of Cellular Growth Control with Discrete Regulatory Centers

In our model growth-regulating signals diffuse through the cell population from discrete sources that are randomly distributed in space and time. The scheme operates with a minimal degree of cellular differentiation: all cells in the community can become signal sources and all cells can sense the signal (and always do) but, at any given time, only a few randomly scattered cells are active as signal sources and the ratio of signalling to sensing cells is constant within the same or successive generations. Such a cell population may be thought of as a composite of virtual clusters, each of which, on the average, includes one signalling cell as the center and $n - 1$ nonsignalling cells as the periphery. In the ensemble average, signals cross cluster boundaries equally fast in either direction (Fig. 13 *A*). The situation in the ensemble is then equivalent to that in a single isolated cluster with closed borders. Hence, such an isolated cluster is a representative statistical sample of the whole population.

The distribution of the regulatory signal within the cluster

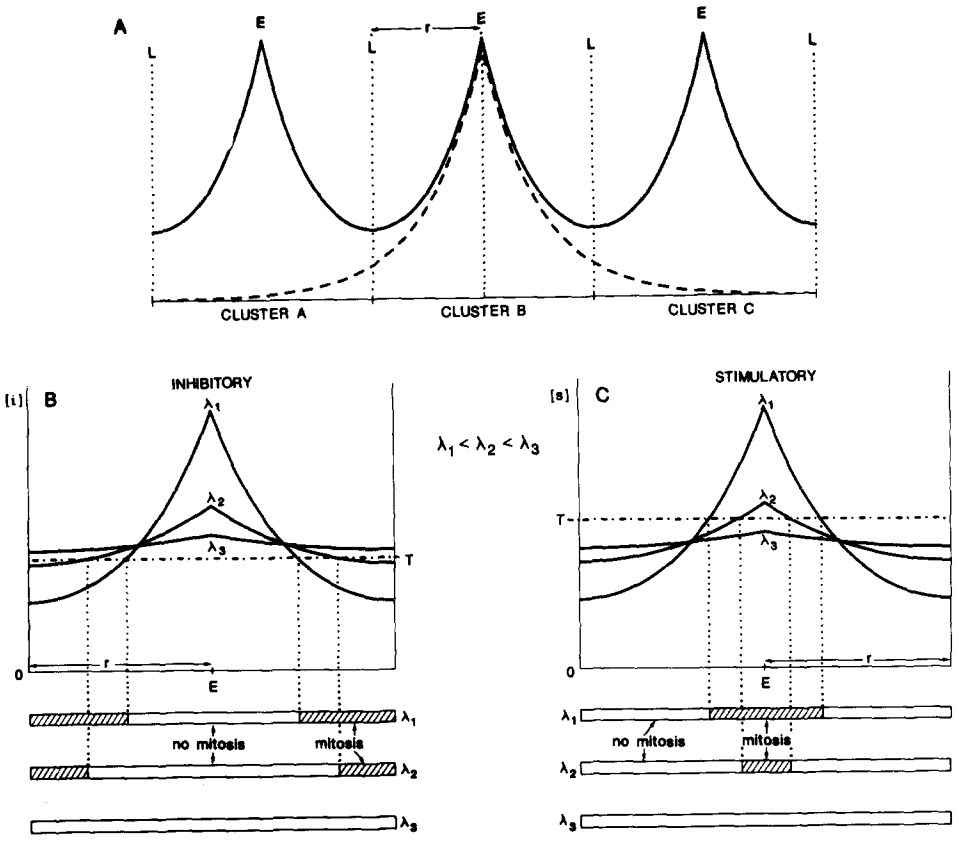


Figure 13. (*A*) Model with discrete regulatory centers. Cells acting as intermittent signal sources are randomly scattered throughout the cell population and all cells are signals sensors. Three sources (*E*) are represented in the diagram. With only one source emitting, the signal concentration will fall off with distance from the source, as indicated by the dashed line. There is some overlap of the fields of concurrently active neighboring sources, with the minima of the resulting concentration profiles (*solid lines*) defining borders (*L*) at which there is no net exchange of signals. These borders mark (virtually isolated) cell clusters of radius *r*. The average cluster then contains a central source cell and $n - 1$ nonsignalling cells. (*B* and *C*) Regulation in a cluster by *B*, an inhibitory signal (*i*); or (*C*) a stimulatory signal (*s*). The curves represent steady-state signal concentration profiles for three different settings of the characteristic length λ. The threshold levels (*T*) for inhibition and stimulation of mitosis conform to the restrictions described in the text. The bar diagrams below the curves indicate the extent of the mitotic (*hatched*) and nonmitotic (*open*) fields in the cluster. The concentration profiles are scaled to represent the conservation rule that the total amount of signal in a cluster is constant, due to an invariant balance between signal production and signal degradation/leakage.

is ruled by a charasteristic length, λ, relative to the cluster radius, *r*. Hence, λ determines the extent of the growth regulation. Fig. 13, *B* and *C*, illustrates that, with either stimulatory or inhibitory signals, an increase in λ can reduce the field of mitotic cells in the cluster and that (given a critical threshold, *T*) a sufficiently large λ will stop mitosis altogether. Since λ is a (increasing) function of junctional permeability, the field of mitotic cells and, hence, growth would be inversely related to that permeability over a range. It is important to note that this relationship holds regardless of the signal sign.

The operational prerequisites of the model can be specified for either signal sign: in the limit of homogeneous signal distribution (infinite λ), the concentrations of the inhibitory signals must be above the threshold for inhibition of mitosis (Fig. 13 *B*); whereas the concentrations of the stimulating signals must be below the threshold of stimulation (Fig. 13 *C*). With inhibitory signals the control situation, then, is simple; any increase of λ will reduce the field of mitotic cells. With excitatory signals, however, the effect of λ on the mitotic field may not be strictly monotonic; but the important point here is that from a minimal value onward an increasing λ will always reduce the mitotic field also, as exemplified in Fig. 13 *C*. So, a priori, the arrest of growth can be achieved with either inhibitory or excitatory signals. The present data set no constraints on signal sign.

It is now instructive to see to what endstage (e.g., saturation density) the cell population will develop when the total amount of signal in the cluster is fixed. This is the condition when the following parameters are constant: the ratio of signalling to sensing cells, the average signal production per source cell, and the degradation and leakage of signals. If we also fix the average junctional permeability per cell junction, there is then one degree of freedom by which λ could vary and govern mitosis: λ will increase as each cell in the growing population gains more partners for direct (first-order) junctional communication. Such an increase of first-order communicators, in general, would be expected as the cell density increases by mitosis in a population of cells in which long cell processes play no major role in the topography of cellular interconnections. As an increase in λ, in turn, will reduce growth (Fig. 13 *B*), a negative feedback loop is closed, and growth will be arrested at a critical cell density. Now, considering junctional permeability once again as the controlling variable, one would expect a higher junctional permeability to lead to growth arrest at a lower density. Thus, the saturation density would become inversely related to junctional permeability.

In summary, as the limiting factor in the conduction of signals in the communication network of the model, junctional permeability would rule cellular growth; and the limit to growth would be set by a negative feedback loop between cell density and the extent of conduction inherent in the topography (arborization) of the network.

This model is an extension of an earlier one (Loewenstein, 1979), but it is more general regarding signal sign and it identifies a topological factor as an important link in the feedback loop that controls growth. It accommodates the results

obtained with both normal and transformed cell populations reported here and, without further ado, it accommodates also the results obtained with mixtures of normal and transformed cells where the heterologous junctional permeability determines λ and rules the growth of transformed cell clones (Mehta et al., 1986). The closed loop inherent in the topology of a growing cell community is a unique feature of junctional-communication systems.

The analysis of the model was of necessity simplified, as there are only two observed quantities at hand. Thus, no detailed computations are justified at this stage. An essential aim was nevertheless achieved at the qualitative level: the identification of a plausible control loop of growth through junctional communication; by tracking the sign relationships of actions and reactions, we learn how a negative feedback loop for growth control can be formed through short-range cellular interaction, that the extent of first-order communication is pivotal in this control, and that the control can be exerted by inhibitory as well as excitatory signals. The statistical method of considering one cell cluster as pars pro toto justifies itself a posteriori, as all elements of the control loop are contained in any local group of cells and operate at short range. Stability of a cell population as a whole is a consequence of this local stability.

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