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Intercellular Communication and Tissue Growth: VIII. A Genetic Analysis of Junctional Communication and Cancerous Growth

R. Azarnia and W.R. Loewenstein

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

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Summary. Normal, proliferating cells are interconnected at their junctions by membrane channels through which molecules can pass from cell to cell (Loewenstein, W.R. 1966. *Ann. N.Y. Acad. Sci.* **137**:708). A channel-competent, normally growing cell (human fibroblast) was hybridized with a channel-incompetent cancer cell (mouse L-1d cell), and the segregant hybrid clones were analyzed in a genetic approach to the question of whether the junctional membrane channels are instrumental in transmission of growth-controlling molecular signals. The channel competence of the human parent was characterized by the ability to transfer small inorganic ions (electrical coupling) and fluorescein, and the growth patterns of this cell, by growth *in vitro* to low saturation densities and nontumorigenicity in immunosuppressed hosts. The mouse parent cell had the opposite characteristics. The early hybrid generations (which still had a large part of each parent chromosome complement) were of two classes: one class resembled the human parent cell in channel competence, *in vitro* growth pattern, and low tumorigenicity within 26 days; the other class presented an intermediate expression of channel competence characterized by transfer of small inorganic ions but not of fluorescein. As the hybrid generations lost human chromosomes, there was segregation of several biochemical and morphological traits, but no segregation of channel competence and normal growth traits. Among the segregants were 22 clones which had reverted to the channel-incompetent trait of the mouse parent. In every case, reversion to the channel defect went hand in hand with reversion to the growth defect, just as, in the early-generation hybrids, correction of the channel defect went hand in hand with correction of the growth defect. Thus, the human genetic factor that corrects the channel defect of the mouse parent cell seems closely linked, if not identical, with that correcting the growth defect. This genetic correlation encourages us in the belief that the channel defect may be an etiological factor in this particular cancer form.

The cells of normal, organized tissues capable of proliferation make permeable junctions (Loewenstein, 1966; Furshpan & Potter, 1968). The cell membranes at these junctions contain channels through which molecules up to 1200–1900 dalton can flow from one cell interior to another (Simpson, Rose & Loewenstein, 1977). The present paper is part of a series exploring the question of whether this intercellular flow of mole-

cules includes growth-controlling ones. The series is guided by the idea that if the junctional channels are indeed pathways for growth-controlling molecules, heritable alterations in channel permeability may give rise to uncontrolled growth (Loewenstein, 1966; 1968*b*). We have screened a variety of cell types for channel competence and have found that the channel-defective ones were in fact also cancerous. In one type of cancer cell the channel formation was abnormally slow and the channel permeability for a 300-dalton molecule abnormally low (Azarnia & Loewenstein, 1976), and three types seemed altogether incompetent to make the channels (Borek, Higashino & Loewenstein, 1969; Azarnia & Loewenstein, 1971; Azarnia, Michalke & Loewenstein, 1972; Azarnia, Larsen & Loewenstein, 1974).

Here we use one of the channel-incompetent cell types, a malignant derivative of a mouse L cell, to find out whether the incompetence of junctional communication correlates genetically with the incompetence of growth control. The general approach is to improve growth control by hybridizing this cell with a normal cell and to see whether the improvement is associated with improvement of junctional communication. The approach was prompted by the findings of Weiss, Todaro and Green (1968) and of Harris and Klein and colleagues (*cf.* Harris, 1971; Klein, Bregula, Wiener & Harris, 1971) that, in hybrids between several types of cancer cells and normal cells, the growth character of the normal parent cell is dominant. We chose a normally growing, junction-competent human cell, a skin fibroblast (Lesch-Nyhan), as the partner for hybridization. This hybrid system offers several experimental advantages: the two parent cells are genetically marked by enzyme defects permitting easy hybrid selection, the mouse and human karyotypes are readily distinguishable, and the hybrids lose chromosomes at a rate appropriate for segregant analysis. The system has the disadvantage that, being heterologous, one needs to use immuno-suppressed hosts to test its growth *in vivo*. The experiments consist of fusing the mouse and human parent cells *in vitro*, and of analyzing the properties of growth and of junctional communication of the resulting hybrid cells, namely the dependence of their growth on cell density in culture (density dependence), their ability to form tumors in animals (tumorigenicity), and to transfer small ions (electrical coupling) and fluorescein through junctional membrane channels. We show by segregant analysis that the foregoing properties of cellular growth control and junctional communication behave like genetically linked characters.

The present hybrid system offered also the opportunity for inquiring

into the question of the dominance of the genes determining the junctional membrane channels. We find segregants with intermediate expression of communication competence, suggesting that the dominance is incomplete. Some preliminary aspects of this analysis have already appeared (Azarnia, Larsen & Loewenstein, 1974).

Materials and Methods

Parent Cells

A nonmalignant strain of *human* skin fibroblasts and a malignant subline of *mouse* L cells, Cl-1D, were used as partners for hybridization. The human cell originated from a patient with Lesch-Nyhan syndrome; it is deficient in inosine pyrophosphorylase (Fugimoto & Seegmüller, 1970). The mouse cell is deficient in thymidine kinase (Dubbs & Kit, 1964).

Hybridization and Hybrid Selection

Hybrid cells were obtained from spontaneous fusion of the parent cells or from fusions promoted by inactivated Sendai virus (*cf.* Harris, 1971).

For hybrid selection, the cultures (except for the clones of series 10; *see* below) were kept in HAT medium (Littlefield, 1964). This medium contains hypoxanthine, thymidine and the antimetabolite aminopterin which blocks purine and pyrimidine synthesis. The two parent cells cannot grow in this medium, because the deficiency in inosine pyrophosphorylase renders the human cell incapable of utilizing the hypoxanthine of the medium for nucleic acid synthesis, and the deficiency in thymidine kinase renders the mouse parent cell incapable of utilizing the thymidine. The hybrid cells, in which the two enzymes are expressed, bypass the aminopterin block and grow. The hybrid cells were cloned, and all tests of junctional communication, growth *in vitro*, and tumorigenicity were done on such clones. The hybrid character of each clone was established by karyotype.

Some of the human parent cells survived in HAT medium when there were many hybrid cells presented capable of junctional communication. These parent cells presumably obtain the missing nucleotide derivative through "metabolic cooperation" with the hybrid cells (Subak-Sharpe, Bürk & Pitts, 1966; 1968; Cox, Krauss, Balis & Dancis, 1970). Since such metabolic cooperation appears to require junctional communcation (Gilula, Reeves & Steinbach, 1972; Azarnia, Michalke & Loewenstein, 1972) we passaged the cultures repeatedly (trypsin-EDTA) in HAT medium to break the junctional contacts. This eventually eliminated the human parent cell survivors. This problem did not arise with the mouse parent cells which are incapable of junctional communication; there were no survivors of these cells in HAT medium.

In one series of experiments (clone series 10) the hybrid cultures were kept in Eagle-Dulbecco medium after initial hybrid selection in HAT medium. This avoided selection against segregants deficient in thymidine kinase or inosine pyrophosphorylase. Thymidine kinase-deficient segregant clones (hence bromo deoxyuridine resistant) were obtained in medium containing bromo deoxyuridine.

Two methods were used for obtaining channel-incompetent segregant hybrids. In one method, we took advantage of the multilayered growth of the mouse parent cell. The early generations of hybrid cells, like the human parent cells, grew in single layers. Upon continued cultivation, cell populations appeared which tended to pile up. Some of the piled-up cells could be freed by gentle shaking of the culture dishes; these cells were

Table 1. Clone lineage

In vitro[a]	*In vivo*[c]
$1a \rightarrow 1a_3 \rightarrow 1a_3p \begin{matrix} \nearrow 1a_3ps \\ \searrow 1a_3pk \end{matrix}$	$1a \rightarrow 1a_{T1} \rightarrow 1a_{T2}$
$1b \rightarrow 1b_1 \rightarrow 1b_1p \rightarrow 1b_1pn$	$1b \rightarrow 1b_{T1} \rightarrow 1b_{T2}$
1c	
$2b \rightarrow 2pb \rightarrow 2pbs$	
$2d \rightarrow 2dp$	
$10a \rightarrow 10ap$	
10b	
$10c \rightarrow 10cp$	
3D[b]	
9A through *9N*[b]	

[a] Clones derived *in vitro*. The heterokaryons of series *1* were products of spontaneous fusion; those of series *2, 3, 9* and *10* of fusion induced by Sendai Virus. Clones lettered *p* were selected among hybrid cells that piled up in culture. All clones were selected in HAT medium, except for those of series *10* which were selected in Eagle-Dulbecco medium.
[b] Clones of series *3* and *9* were selected at random among hybrid cells in culture for several generations.
[c] Clones derived from tumors (*T*) produced by cells *1a* and *1b*. Subscripts *T1* and *T2* denote cells from first tumors and re-transplants, respectively.

cloned and yielded the segregants. They are designated by the letter *p* in the tables. (The cells growing in single layers, in direct contact with the dishes, stuck more firmly.)

In a second method, segregant cells were obtained by cloning at random among the hybrid populations. The clones so obtained are designated by capital letters. The segregants obtained with the two methods gave the same results.

Table 1 lists the various hybrid clones tested and gives their lineage. The clones of series *1* were obtained by spontaneous fusion; those of series *2, 3, 9,* and *10,* with the aid of Sendai virus. All hybrid clones and their segregants originated *in vitro*, except those with suffix *T*, which were derived from explants of the tumors produced by the hybrid cells *1a* and *1b*.

Karyotyping

The karyotypes of all clones tested were examined on Giemsa-stained metaphase spreads of the cells (100 cells for each clone) treated with colcemid (0.2 g/ml, 3–5 hr). The mouse parent cell has a convenient marker chromosome (D) with a double constriction which has no equivalent in the human cell (Fig. 1). Furthermore, the human chromosomes, except for 6 large metacentric chromosomes (Denver Group A) and 4 small acrocentric ones (Group G) are distinguished by their size and shape from the mouse chromosomes. The 29 biarmed human chromosomes (Groups B, C, E, F, and X) are most readily distinguished; their numbers are tabulated (Table 2).

Cell Culture

The cells were grown in 60-mm dishes (Falcon, plastic) at 37 °C in an atmosphere of 5% CO_2 and 95% air: the parent cells and the hybrids of clone series 10 in Eagle-Dulbecco

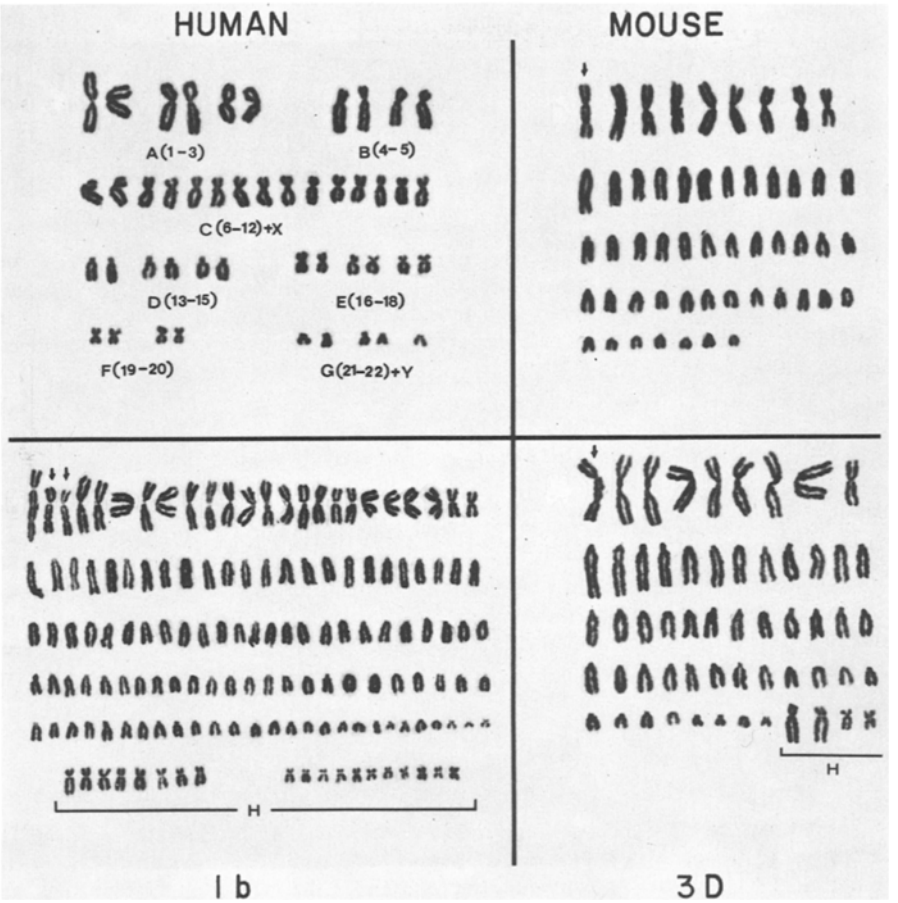


Fig. 1. Karyotype of human and mouse parent cells, and sample of early-generation hybrid cell (clone *1b*) and revertant hybrid cell (clone *3D*). Identifiable human biarmed chromosomes in the hybrid cells (Denver groups B, C, E, F, X) are bracketed (*H*). Arrows point to mouse marker chromosome D. Giemsa stain

medium (Vogt & Dulbecco, 1960) and all other hybrid cells in HAT medium. Both media were supplemented with 10% fetal calf serum. The cells were in their respective growth media during the tests of junctional communication and during the tests of *in vitro* growth.

Tests of Junctional Transmission

The general technique of the measurements of intercellular electrical coupling and of the tests of intercellular fluorescein transfer are described in the preceding paper of this series (Azarnia & Loewenstein, 1976). The cells into which the microelectrodes were inserted were contiguous or bridged by 1 or 2 cells in contact; in most cases the cells were contiguous. In the contiguous cases, we scored the number of cells transmitting or receiving current in each determination; in the noncontiguous cases, we scored only such cells in the shortest cell bridge between the electrodes. In both cases, we scored

all cells transmitting or receiving fluorescein. The values tabulated (Table 3) are the total scores of all determinations in each given clone.

The coupling ratios (V_{II}/V_I) in the coupling parent cells and hybrids ranged from 0.1–0.8; in most cases, about 0.4. The membrane potentials (at 0 current) of the various cell types ranged typically from 5–30 mV, inside negative.

Determination of Density Dependence of Cell Growth in Vitro

For determination of growth curves, the cells of the clone to be tested were seeded in 20–30 dishes of 33 mm diameter ($1–5 \times 10^4$ cells/dish) containing 2 ml of the respective growth medium. The dishes were incubated at 37 °C (5% CO_2 and 95% air), and the medium was changed each day. The cells were counted, 1 dish per day, in a hemacytometer (cell separation, by trypsine).

Tests of Cell Growth in Agar Methyl Cellulose

For tests of cell growth in semisolid medium, the cells were suspended in 4 ml of a mixture of growth medium (plus 10% fetal calf serum) and methyl cellulose (1.2% w/v) (Methocel, Dow Chemical Co.) (Friedmann & Shin, 1974; Rissner & Pollack, 1974). The cell quantities used for the various clones are given in *Results* (footnote 2). The suspensions were added to 60 mm dishes containing a thin layer of gelled agar (0.9%)-medium (and fetal calf serum). Fresh methyl cellulose-medium (4 ml) was added once a week. The cell colonies were observed in phase contrast and photographed twice a week. Observations were carried out for up to 3 weeks.

Tests of Tumorigenicity

Inbred male mice of the C_3H strain, 4–8 weeks old, were used as tumor hosts. The animals were injected with 0.2-ml doses of rabbit anti-lymphocytic serum (ALS, Microbiological Associates) 1 day before the inoculation of the cells to be tested; 1, 3, and 5 days after inoculation; and twice weekly thereafter. The animals were kept in a filtered-air environment; water, food and cages were autoclaved.

The cells to be tested were suspended in 0.2 ml growth medium and injected subcutaneously in the left hind leg. All cell types were tested with inocula of 10^6 cells; some cell types were tested, in addition, with other doses given in the *Results*. The animals were checked for tumor formation twice a week; the observations were carried out for up to 90 days.

In a small series of tests, athymic ("nude") mice obtained from Oxford Laboratory Animal Colonies, England, were used as hosts. The results are briefly described at the end of the *Results* section. The results tabulated and used in analysis are all from the tests with hosts treated with antilymphocytic serum.

Scoring of Tumor Incidence

Tumor incidence was scored at fixed times, namely at the 26th, 39th and 90th day after inoculation. Since some of the tumor-free animals died during the observation time, we expressed the tumorigenicity (tumor incidence) in terms of the cumulative tumor-bearing number relative to the sum of the tumor-bearing and surviving tumor-free animals. Thus,

$$\text{tumor incidence} = n_t/(n_t + n_f)$$

where n_t is the cumulative number of tumor-bearing animals at the various scoring times and n_f the number of surviving tumor-free animals at these same times. Here the individual that develops a noticeable tumor is counted whether it survives or not at the scoring time. Underlying is the assumption that the animals that died, without exhibiting tumors (and not scored), would have displayed, had they survived, the same tumor incidence as the scored population.

Results

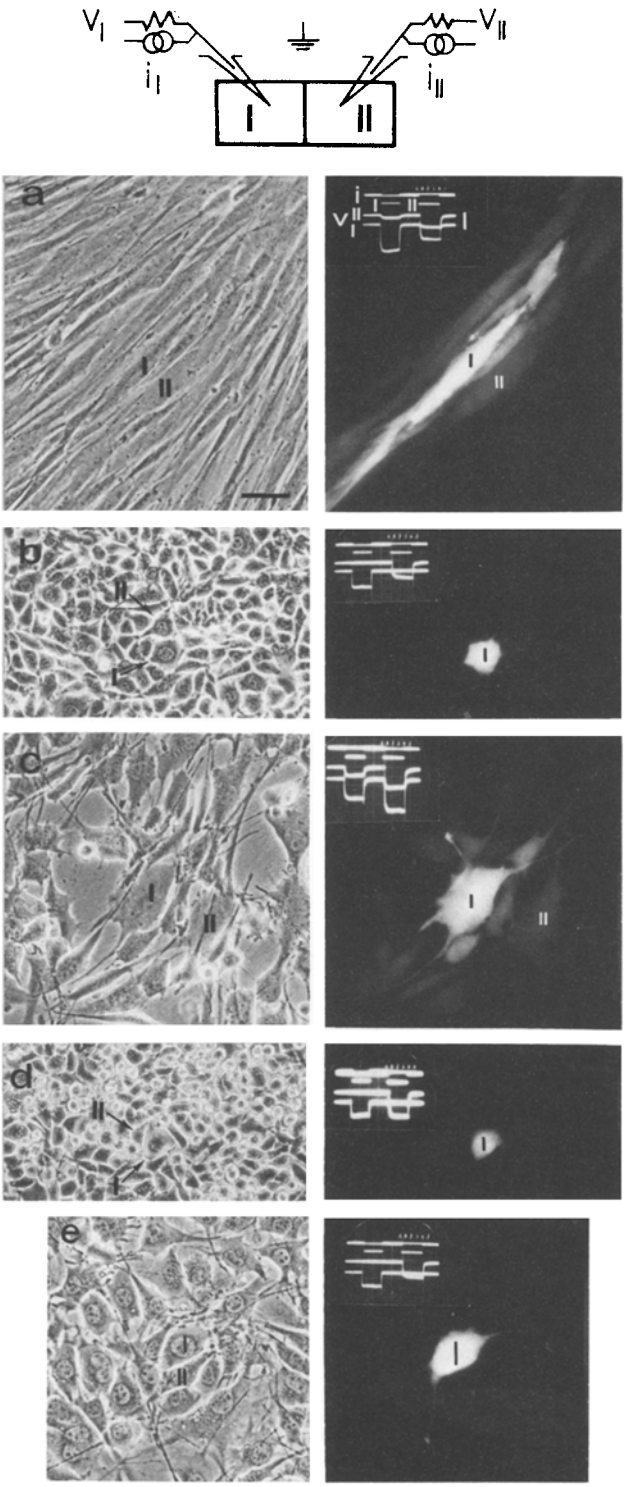
We examined the junctional communication, the growth properties and the chromosome constitution of the hybrids between the channel-competent and channel-incompetent parent cells. The corresponding tests were carried out on parallel clonal samples.

Hybrid Karyotype

The lineage of the various hybrid clones is given in Table 1 and their chromosome composition, determined at the time the junctional and *in vitro* growth properties were tested, is listed in Table 2. The clones of series *1* and *10* descended from the fusion products (heterokaryons) of 1 human cell and 2 mouse cells; they had two mouse D-marker chromosomes. All other hybrid clones descended from 1:1 mouse heterokaryons. The expected modal chromosome number for a hybrid cell containing complete parental chromosomes sets (Table 2) thus would be 152 and 99, respectively. All of the hybrid clones tended to lose preferentially the human chromosomes in the course of cultivation, as is typical of the human-mouse combination. The clones of series *1* and *10* had the most stable chromosome combinations; the early-generation hybrid clones of these series (*1a, 1b, 1c, 10a, 10b, 10c*) still contained a large part of the original parent cell complement (Fig. 1). The clones of series *2*, *3*, and *9* lost chromosomes more rapidly.

Junctional Communication

The results of the tests of junctional communication of the various types of cells are summarized in Table 3. All human parent cells examined were electrically coupled and transferred fluorescein (*channel-competent*) (e.g., Fig. 2*a*). The mouse parent cells, on the other hand, showed no detectable electrical coupling or fluorescein transfer (Fig. 2*b*).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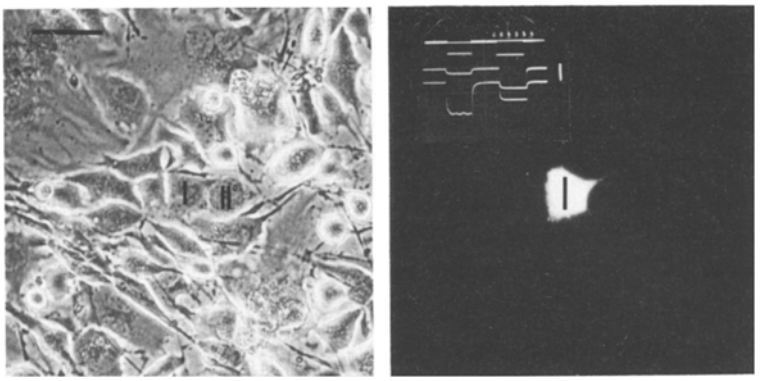

Fig. 3. Intermediate expression of junctional communication. A hybrid cell of clone *1b*$_1$*pn*, electrically coupled without transferring fluorescein. Calibrations 50 μm; 100 mV; current pulse duration 100 msec

The early generations of the hybrids between these cells (clones *1a, 1b, 1c, 10a, 10b, 10c*) took after the human parent in all these respects (Fig. 2*c*). The frequency of electrical coupling among these hybrid cells was as high as that of the human parent cell and the frequency of fluorescein transfer was nearly as high (Table 3).

The later hybrid generations fell into two categories in terms of junctional communication. In one category, all cells failed to couple, by both electrical and fluorescein tests (clones *2bps, 3D, 1a*$_3$*ps, 2dp, 9A-N, 10ap, 10cp*) (Table 3). These clones seemed to have fully reverted to the channel-incompetent trait of the mouse parent (Fig. 2*d*). In another category, some cells in a given clone were electrically coupled, while others were not (clones *1a*$_3$*pk, 1b*$_1$*p, 1b*$_1$*pn, 2bp*). The electrically coupled cells here had the peculiarity that they did not detectably transfer fluorescein (Fig. 3); these segregants seemed to have an intermediate communication phenotype and will be referred to as *intermediate* hybrids. The electrically noncoupling cells present in these clones had fully reverted

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Fig. 2. Junctional communication. *Top:* Electrode arrangement. Current ($i=2.5\times10^{-9}$A, inward) is injected into Cell *I* and, with a 100 msec delay, into cell *II*; the resulting changes in membrane potential (*V*) are measured in the two cells. The microelectrodes are connected to balanced bridge circuits and serve for both current passing and potential recording. Fluorescein is iontophoresed into cell *I*. (*a*) human parent cell; (*b*) mouse parent cell; (*c*) early-generation hybrid cell (clone "*1b*"); (*d*) revertant hybrid cell (clone "*3D*"); (*e*) revertant hybrid cell (clone "*1a*$_3$*ps*"). *Left:* photomicrographs of the cells in phase contrast. *Right:* in darkfield, showing spread of fluorescein 5–10 min after injection. Calibration 50 μm. *Insets:* oscilloscope records of *i* and *V*. Calibration 100 mV; current pulse duration 100 msec

Table 2. Cell karyotype[a]

Cell type	Clone	No. of chromosomes		No. of marker (D) chromosomes	No. of biarmed chromosomes[b]
		Range	Mode		
Parental					
Human		37–46	46	0	29
Mouse		40–53	53	1	0
Hybrid					
Channel-competent	*1a*	51–133	104	2	10
	1b	40–151	124	2	14
	1c	43–141	106	2	14
	10a[c]	52–150	130	2	15
	10b	41–141	108	2	12
	10c[c]	46–148	128	2	14
Intermediate	$1a_3pk$	43–126	111	2	5
	$1b_1p$	78–132	117	2	8
	$1b_1pn$	45–112	53/86	2	3
	2bp	42–110	52/74	1	2
Channel-incompetent segregant	*2bps*	45–103	49/59	1	2
	3D	46–60	54	1	2
	$1a_3ps$	37–116	102	2	5
	2dp	38–103	55/76	1	3
	9K	51–64	58	1	3
	9A	52–68	56	1	3
	9B	52–62	52/58	1	3
	9C	51–64	58	1	3
	9D	51–62	58	1	3
	9E	52–67	58	1	3
	9F	50–63	58	1	3
	9G	48–63	58	1	3
	9H	49–64	58	1	3
	9I	49–64	58	1	3
	9J	52–64	58	1	3
	9L	48–66	58	1	3
	9M	48–66	58/60	1	3
	9N	49–68	58	1	3
	10ap	48–138	128	2	13
	10cp	37–131	120	2	13
	$1a_{T1}$	40–128	108	2	6
	$1b_{T1}$	42–126	100	2	6
	$1a_{T2}$	40–118	102	2	4
	$1b_{T2}$	39–112	100	2	4

[a] Data based on 100 karyotypes for each clone.

[b] Means; small biarmed human chromosomes, Denver Groups B, C, E, F, X.

[c] These clones had lost the human thymidine kinase marker. All other early hybrid, revertant and intermediate clones had the marker.

Table 3. Junctional properties of clones

Cell type	Clone	Electrical[a] coupling		Fluorescein[a] transfer		Gap junctions[b] per cell
		+	−	+	−	
Parental						
Human		12	0	137	0	10
Mouse		0	62	0	6	0
Hybrid						
Channel-competent	*1a*	6	0	75	5	[c]
	1b			88	6	5.4[c]
	1c			57	4	
	10a	38	0	96	4	
	10b	12	0	36	2	
	10c	30	0	104	6	
Intermediate	$1a_3pk$	50	18	0	31	0[c]
	$1b_1p$	6	16	0	3	0[c]
	$1b_1pn$	19	14	0	10	
	2bp	12	16	0	10	
Channel-incompetent segregant	*2bps*	0	38	0	10	0
	3D	0	16	0	40	0
	$1a_3ps$	0	42	0	4	0
	2dp	0	92	0	4	0
	9K	0	20	0	50	
	9A	0	32	0	48	
	9B	0	36	0	62	
	9C	0	32	0	56	
	9D	0	28	0	56	
	9E	0	44	0	68	
	9F	0	60	0	102	
	9G	0	45	0	48	
	9H	0	42	0	96	
	9I	0	62	0	48	
	9J	0	18	0	50	
	9L	0	31	0	18	
	9M	0	30	0	66	
	9N	0	31	0	10	
	10ap	0	68	0	88	
	10cp	0	59	0	76	
	$1a_{T1}$	6	14	0	50	
	$1b_{T1}$	4	16	0	50	
	$1a_{T2}$	0	20	0	24	
	$1b_{T2}$	0	16	0	38	

[a] Number of tested cells transmitting or receiving current or fluorescein; total scores for each clone (*see Materials and Methods*). The electrodes were in contiguous cells for determination of electrical coupling, except for a few hybrid cell cases where 1 or 2 contacting cells intervened between the two cells containing the electrodes.

[b] Number of gap junctions per equivalent cell. Data of freeze-fracture electron microscopy from the following paper of this series (Larsen, Azarnia & Loewenstein, 1977), except for clone *2dp* where data is from thin-section electron microscopy (Azarnia, Larsen & Loewenstein, 1974).

[c] Fibrillar junctional structures were present.

to the channel-incompetent mouse phenotype; like the electrically coupling-negative cells of all other clones, they were also fluorescein transfer-negative.

In general, the channel-incompetent segregant clones had lost more human chromosomes than the channel-competent intermediate hybrids. However, in two cases (clones *10ap, 10cp*), the channel-incompetent segregants had retained a large part of the original human chromosome complement (Table 2).

In Vitro Growth

The channel-competent human parent cell grew in single layers in the culture dishes, and its growth levelled off at densities of 7×10^4 cells/cm^2 (saturation density) (Fig. 4). The channel-incompetent mouse parent cell grew in multi-layers, reaching densities of $> 300 \times 10^4$ cells/cm^2. The growth of this cell did not properly level off even at these high cell densities (Fig. 4); the cell counts for constructing the growth curves were generally discontinued at these densities, because cell death or cell detachment became dominant.

The cells of the channel-competent, hybrid clones grew in the dishes much like the human parent cell: they formed single layers, and their growth plateaued at densities of $3–18 \times 10^4$ cells/cm^2.

The channel-incompetent, segregant clones took entirely after the mouse parent: they piled up on each other in the dishes and reached high densities ($> 30–140 \times 10^4$ cells/cm^2).[1]

The clones (*$1a_3pk$, $1b_1p$, $1b_1pn$, 2bp*) consisting of intermediate hybrid cells in mixture with channel-incompetent segregants behaved like the channel-incompetent segregants in their characteristics of piling up. Their densities ($> 20–35 \times 10^4$ cells/cm^2) were intermediate between the early hybrids and channel-incompetent segregants.

Fig. 4 gives the growth curves of the two parent cells, the early hybrid clones (*1a, 1b, 1c*), the intermediate clone *$1b_1pn$*, and three channel-incompetent segregant clones (*2bps, 2dp, 3D*), which are typical for all other clones in the respective categories; and Table 4 summarizes the growth traits of the various clones.[2]

1 The growth of the channel-incompetent segregant clone *2bps* was followed to day 24; that of all other segregant clones, to day 18. Table 4 lists the respective cell densities at these times; the number of the cells was still increasing.

2 Growth in agar methyl cellulose of the mouse parent cell and clones *1a* was tested with suspensions of 10^6, 10^5, 10^4 and 10^3 cells; that of clones *1b* and *1c* with suspensions of 10^6, 10^4 and 10^3 cells; and that of all other cell types, with suspensions of 10^6 cells. The results for a given cell type were the same at the various suspension levels.

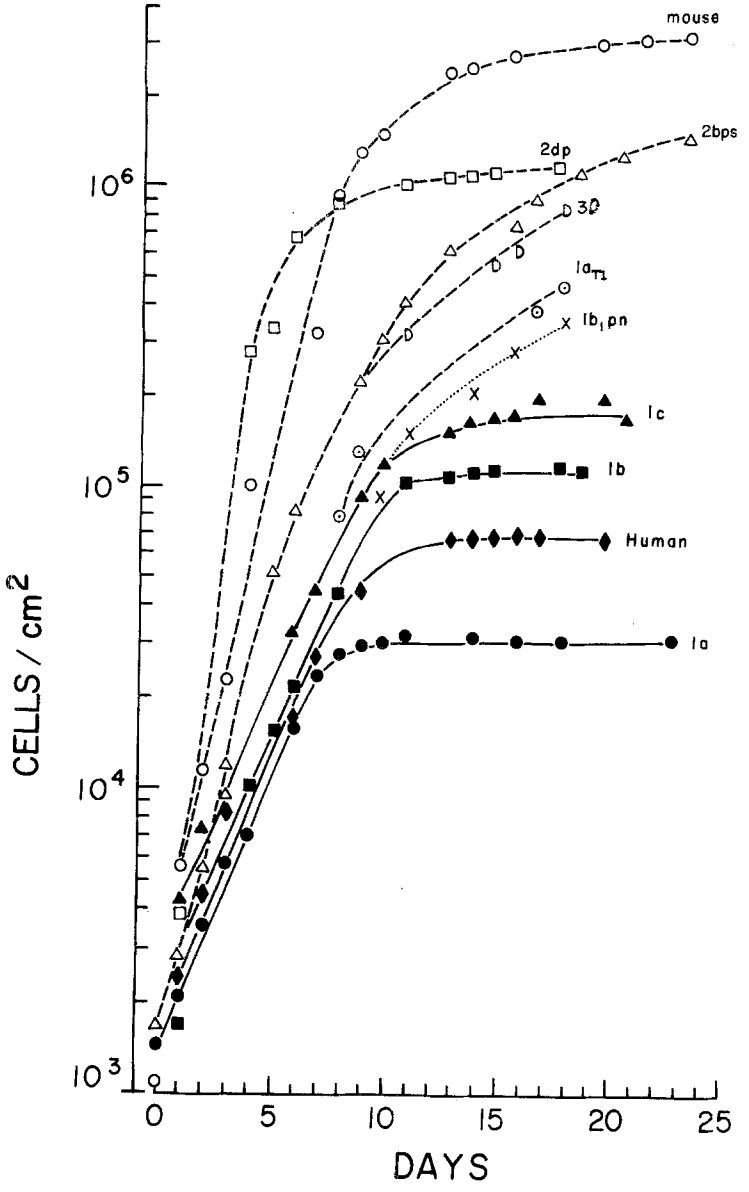

Fig. 4. Growth on solid surface. Growth curves of channel-competent human parent cell and hybrid clones (*1a, 1b, 1c*); channel incompetent, mouse parent cell and revertant hybrid clones (*2bps, 2dp, 3D,* $1a_{T1}$); and intermediate hybrid cell $2b_1pn$. *Ordinates:* cell density on the bottom of the culture dish. *Abscissae:* time after seeding. Cell density at time 0 was $1.5–4 \times 10^3$ cells/cm^2

Table 4. Junctional competence and cellular growth *in vitro* and *in vivo*

Cell type	Clone	Functional junction		Gap[c] junction	*In vitro* growth			Tumorigenicity[d] tumor incidence		
		Electrical coupling	Fluorescein transfer[a]		Maximum density 10^4 cells/cm^2	Multilayered	Agar M-C suspension[b]	% 26th day	% 39th day	% 90th day
Parent										
Human		+	+	+	7	−	−	0	0	0
Mouse		−	−	−	>300	+	+	100		
Hybrid										
Channel-competent	*1a*	+	+	+	3	−	(+)	0	25	100
	1b		+	+	11	−	(+)	18	50	100
	1c		+		18	−	(+)	20	90	100
	10a[j]	+	+		5	−		0	11	60
	10b	+	+		10	−				
	10c[j]	+	+		10	−		0	20	
Intermediate	$1a_3pk$	+[g]	−	−[h]	> 20	+		12	75	75
	$1b_1p$	+[g]	−	−[h]		+	+			
	$1b_1pn$	+[g]	−		> 35[i]	+	+	22	60	100
	2bp	+[g]	−		> 20	+	+	33	100	
Channel-incompetent segregant	*2bps*	−	−	−	>140[i]	+	+	100		
	3D	−	−	−	> 90[i]	+	+	100		
	$1a_3ps$	−	−	−	> 50[i]	+	+	100		
	2dp	−	−	−	>110[i]	+		66	100	
	9K	−	−	−	> 90[i]	+	+	100		
	9A	−	−		>100[i]	+	+	100		
	9B	−	−		> 95[i]	+	+			
	9C	−	−		> 50[i]	+	+			
	9D	−	−		> 50[i]	+	+			
	9E	−	−		> 90[i]	+	+	100		
	9F	−	−		> 30[i]	+	+			
	9G	−	−		> 70[i]	+	+			
	9H	−	−		> 60[i]	+	+			
	9I	−	−		> 30[i]	+	+			
	9J	−	−		> 70[i]	+	+	100		
	9L	−	−		> 35[i]	+	+			
	9M	−	−		> 70[i]	+	+			
	9N	−	−		> 50[i]	+	+			
	10ap	−	−		> 70[i]	+	+	100		
	10cp	−	−		> 70[i]	+	+	100		
	$1a_{T1}$[e]	[f]	−		> 50[i]	+	+	100		
	$1b_{T1}$[e]	[f]	−		> 45[i]	+	+	100		

[a] +, when ≧93% of cells tested transmitted or received fluorescein; detail in Table 3.
[b] −, cell types not growing in suspensions (agar methyl cellulose); +, growing in suspension; (+), growing in suspension but more sparsely and in dense clusters.
(*continued on opposite page*)

In Vivo Growth

The two parent cells also offered a simple contrast in terms of their ability to produce tumors in mice treated with anti-lymphocytic serum. The tumor incidence with inocula of 10^6 cells of the channel-competent human parent cell was 0% at all observation times ranging up to 90 days. The tumor incidence of the channel-incompetent mouse parent cell was already 100% by the 26th day; and even with inocula of only 10^4 cells, the incidence was 60% by the 26th day and 100% by the 39th day (Table 5).

The channel-competent early-generation hybrid clones were, in the long run, not entirely tumorigenicity-free, but they were initially clearly much less tumorigenic than the mouse parent. With inocula of 10^4 cells, the tumor incidence was 0% by the 26th and 39th day; and only inocula of 10^6 cells of clone *1a* and *1b* gave an 18–20% incidence by the 26th day.

The clones containing the intermediate cells (clones tested: *$1a_3pk$*, *$1b_1pn$*, and *2bp*) tended to have higher tumor incidence than the early generation hybrids.

The *in vitro* segregant channel-incompetent clones tested (*2bps, 3D, $1a_3bp$, 2dp, 9A, 9E, 9J, 10ap, 10cp*) showed as early and as high a tumorigenicity as the mouse parent cell. Thus, these clones had reverted to the mouse phenotype in respect to tumorigenicity, as they had in respect to junctional channel competence and *in vitro* growth.

In Vivo Segregation

Bregula, Klein and Harris (1971) have shown that hybrids between cancerous and normal cells with unstable chromosome constitution, can

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[c] +, gap junctions found in transmission electronmicroscopy and/or freeze-fracture. Data of this column from Azarnia *et al.*, 1974, and Larsen *et al.*, 1977. For details see Table 2 of subsequent paper of this series (Larsen *et al.*, 1977).

[d] Tested with inocula of 10^6 cells; detail in Table 5.

[e] Segregant clone obtained from a tumor of cells from clones *1a* or *1b*. All other segregants were obtained *in vitro*.

[f] In clone *$1a_{T1}$* and *$1b_{T1}$*, respectively, 30 and 20% of the test cells were electrically coupled; detail in Table 3.

[g] 27–73% of the tested cells were electrically coupled in these intermediate clones.

[h] Fibrillar junctional structures present; junctional structure of clones *$1b_1pn$* and *2bp* was not examined.

[i] Test discontinued while cells were still actively growing; the figure tabulated is the density at the 18th day, the last density tested.

[j] Thymidine-kinase negative.

Table 5. Tumorigenicity

Cell type	Clone	Inoculum (cells)	Cumulative tumor incidence[a]			Number of animals day 0	Incremental tumor incidence (Δn_t) and tumor-free incidence (n_f)					
			% 26th day	% 39th day	% 90th day		By 26th day		By 39th day		By 90th day	
							Δn_t	n_f	Δn_t	n_f	Δn_t	n_f
Parental												
Human		10^6	0	0	0	10	0	8	0	8	0	5
Mouse		10^6	100			10	10	0				
		10^5	100			10	10	0				
		10^4	60	100		10	6	4	4	0		
		10^3	0	0	67	10	0	8	0	8	4	2
Hybrid												
Channel-competent	*1a*	10^6	0	25	100	10	0	10	2	6	6	0
	1a	10^5	0	0	100	10	0	9	0	8	8	0
	1a	10^4	0	0	86	10	0	9	0	8	6	1
	1a	10^3	0	0	0	10	0	6	0	6	0	2
	1b	10^6	18	50	100	12	2	9	4	4	2	0
	1b	10^4	0	0	50	10	0	8	0	6	3	3
	1b	10^3	0	0	0	10	0	10	0	8	0	4
	1c	10^6	20	90	100	10	2	8	7	1	1	0
	1c	10^4	0	0	0	10	0	4	0	3	0	1
	1c	10^3	0	0	0	10	0	5	0	4	0	0
	10a *10b*	10^6	0	11	60	10	0	10	1	8	5	4
	10c	10^6	0	20		10	0	10	2	8		
Intermediate	$1a_3pk$	10^6	12	75	75	10	1	7	5	2	0	2
	$1b_1pn$	10^6	22	60	100	10	2	7	3	2	2	0
	2bp	10^6	33	100		10	3	6	6	0		
Channel-incompetent segregant	*2bps*	10^6	100			10	7	0				
	3D	10^6	100			10	7	0				
	$1a_3ps$	10^6	100			10	9	0				
	2dp	10^6	66	100		10	4	2	2	0		
	9K	10^6	100			10	10	0				
	9A	10^6	100			10	10	0				
	9E	10^6	100			10	10	0				
	9J	10^6	100			10	10	0				
	10ap	10^6	100			10	10	0				
	10cp	10^6	100			10	9	0				
	$1a_{T1}$	10^6	100			6	6	0				
	$1b_{T1}$	10^6	100			6	6	0				

[a] Cumulative tumor incidence $= n_t/n_t + n_f$; n_t = cumulative number of tumor bearing animals at scoring time; n_f = number of surviving tumor-free animals at scoring time.

undergo progressive chromosome loss inside animal hosts and that there is *in vivo* selection for the resulting cancerous segregants. The late tumor production by the present early-generation hybrid clones (*1a, 1b, 1c*), which by the 90th day after inoculation (at least at the higher inocula) approached that of the mouse parent, suggested just such an *in vitro* segregation. We tested this point by growing in culture the cells from the tumors produced by two of the early-generation hybrid clones. These cells ($1a_{T1}$, $1b_{T1}$), in fact, turned out to have fewer human chromosomes than the cells originally inoculated (Table 2). A small percentage of the cells was electrically coupled, but none transferred fluorescein (Table 3). The cells resembled the mouse parent cell, moreover, in their growth properties *in vitro*; and, when re-injected in turn into animals, the tumor incidence was high (Table 4). Finally, upon re-transplantation in the animals, the tumor cells ($1a_{T2}$, $1b_{T2}$; Tables 2 and 3) showed progressive reduction in human chromosomes, and their electrical coupling behavior (determined in cell culture) was entirely like that of the mouse parent cell or the channel-incompetent *in vitro* segregants. The small percentage of electrically coupled cells in the first transplant thus presumably reflected the presence of intermediate hybrids.

In conclusion then, the eventual tumor production by the early-generation hybrid cells seems to have been the result of *in vivo* segregation due to loss of human chromosomes.

It is instructive in this regard to compare these results with those obtained with hybrid cells of more stable chromosome constitution, a system of hybrids between a rat liver hepatoma cell and a rat normal liver cell (Azarnia & Loewenstein, 1973). These hybrid cells lost few or no chromosomes over several months of cultivation; and, in line with the foregoing considerations, the junctional communication was entirely normal at all times (like that of the normal parent cell), and the tumorigenicity was zero at all times after inoculation (*see also* Klein *et al.*, 1971).

The origin of the tumors produced by the clones containing the intermediate hybrid cells is more difficult to trace; whether selection for *in vivo* segregants had a primary role, is less clear. These clones also contained tumorigenic, *in vitro* segregants to start with. Thus, here *in vitro* as well as *in vivo* segregants may have contributed to the tumor production.

Enzyme Markers; Cell Shape

There was segregation among the hybrid clones in respect to biochemical phenotypic markers. In clone series *10*, which was raised in Eagle-

Dulbecco medium (all others, in HAT medium; *see Materials and Methods*) and hence was not selected for competence of thymidine kinase or inosine pyrophosphorylase, two clones (*10a* and *10b*) had lost the human thymidine kinase marker, as shown by their resistance to Bromodeoxyuridine.

Furthermore, there was segregation in respect to morphological traits. The channel competent, earliest-generation hybrid clones resembled in all-over shape the human parent cell. Later hybrid generations showed traits of both parents. For instance, the cells of the channel-competent clone *1b* were spindle-shaped like the human parent, but thicker, resembling the mouse parent in this regard (Fig. 2*c*). Finally, among the channel-incompetent segregant hybrid clones, some looked like the human parent or like clone *1b* (e.g., clone $1a_3p$; Fig. 2*e*), while others looked entirely like the mouse parent (e.g., clone *3D*; Fig. 2*d*).

Tumorigenicity Tests with Athymic Animal Hosts

A small series of tumorigenicity tests was carried out with athymic ("nude") mice as tumor hosts. These tests, run with inocula of 10^6 cells of the human and mouse parent and some of the channel-competent and channel-incompetent hybrid clones, gave results similar to the tests in which mice treated with antilymphocytic serum were the tumor hosts. Our principal study of tumorigenicity was done with the latter because we could not secure a supply of athymic mice sufficient for a full range of tests.

Tumorigenicity Controls with Other Human Cancer Cells

Since the present hybrid cell system is heterospecific, the question needed to be considered whether the antilymphocytic serum-treated mouse host was capable of bearing human tumors. We tested this point with three types of human cancer cell lines: HeLa, Intestine 407 (American Type Culture Collection CL6), and Minnesota E.E. (esophagus, CCL4). All three produced tumors; the tumorigenicities (inocula of 10^6 cells) were 100% by day 17, 22 and 61, respectively.

Since we did not know whether these cell lines had the full complement of human cell surface antigens, we also ran tumorigenicity tests with cells (epithelioid) from an explant of a human pancreatic carcinoma and from an explant of a human melanoma (kindly provided to us by Dr. A. Yunis),

in which the antigens were known to be expressed. The tumorigenicities of these cells (10^6) were 100% by days 62 and 39, respectively.[3]

Discussion

Communication Incompetence and Cancerous Growth

The aim of this work was to find out whether competence of intercellular communication by junctional membrane channels and growth regulation are genetically related. A straightforward way of demonstrating such a correlation would have been by means of one-step mutants. However, in practice, one-step channel-incompetent mutants are difficult to obtain; attempts so far have been unsuccessful (W. Michalke, *personal communication*; J. Pitts, *personal communication*). We have resorted to the use of segregants as provided by the spontaneous loss of chromosomes of the channel- and growth-competent parent cell of the present hybrid system.

Before discussing the results of the segregant study, we should like to make clear the scope of the term "incompetence" (or "defect") as used in a genetic sense throughout the paper. We mean by it a cell-inherited alteration; that is, an alteration that (in the present case of cells originating from differentiated ones) may stem from a stable regulatory state as well as from a mutation in the DNA.

The results of the analysis of the channel-competent hybrids and channel-incompetent segregants show a close correlation between defective junctional communication and cancerous growth: correction of the junctional channel defect of the mouse parent cell went hand in hand with correction of the growth defect; and reversion to the junctional defect went hand in hand with reversion to the growth defect. The correlation held for two expressions of cancerous growth, lack of density-dependence *in vitro*, and tumorigenicity *in vivo*; and for two indicators of junctional communication incompetence, lack of electrical and fluorescein coupling (in the following paper, the correlation is also extended to junctional morphology; Larsen, Azarnia & Loewenstein, 1977).

The correlation between channel incompetence and cancerous growth

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3 In the case of the hybrid clone series 10, differences in antigenicity seemed unlikely to be a problem in the first place. There were no reasons to suppose that the tumorigenic, channel-incompetent segregant clones *10ap* and *10cp* had fewer human antigens than the nontumorigenic, channel-competent clones *10a* and *10c* which had nearly the same complement of human chromosomes (Table 2).

seems to be genetic and not simply a consequence of bulk loss of chromosomes: some of the channel-incompetent segregant hybrid clones (*10ap*; *10cp*) had retained, in fact, as large a number of the human chromosomes as the channel-competent hybrid clones (*1a, 1b, 1c*). Moreover, the hybrid cells had clearly segregated in several phenotypic respects: (a) Some hybrids of the class raised in Eagle-Dulbecco medium had segregated in respect to the human thymidine kinase marker; the channel- and growth-competent early-generation hybrid clones *10a* and *10c* had lost the thymidine kinase competence. (b) The hybrid cells with intermediate expression (*$1b_1p$, $1b_1pn$* and *2bp*) originated after loss of human chromosomes, not simply upon chromosome combination during hybridization. (c) Several early-generation hybrids (*1a, 1b, 1c, 10a*) resembled morphologically (spindle shape) the human parent (e.g., Fig. 2), although they had lost many biarmed chromosomes. Besides, among the channel- and growth-incompetent revertants, some looked like the mouse parent, whereas others, more like the human parent (Fig. 2). Yet, there was no instance of segregation of channel incompetence and growth incompetence, or of the respective opposite traits. We infer therefore that the human parent cell supplies a genetic determinant that corrects the junctional communication defect of the mouse parent cell, and this determinant is closely associated, if not identical, with that correcting the growth defect. The determinants may lie on the same human chromosome(s). However, the present analysis, based on segregants in which entire chromosomes are lost, of course, does not demonstrate identity of the genetic determinants of junctional communication and normal growth.

A correlation between defective junctional communication and cancerous growth has already been shown in a less complete way for another cell system, a system of hybrids between a rat liver tumor cell and a normal rat liver cell (Azarnia & Loewenstein, 1973). It was not feasible to carry out segregant analysis in this homologous system, but, because of its more stable karyotype, the system offered the advantage that the tumorigenicity of many generations of hybrids could be tested without the complication of segregation *in vitro* or in the animals. The result was then a simple all or none answer; the tumorigenicity, 100% in the cancerous parent cell, went to 0% in the hybrid cells at all observation times.

Thus, the result with the more stable hybrid system reinforces the argument that the growth and junctional defects may arise from the same chromosome deficiency, and encourages us in the belief that the junctional communication defect may be an etiological factor of the present cancer form. In fact, this is to our knowledge the first instance in which

a close and genetic correlation has been established between a possible causal cell-phenotype abnormality and malignant growth in any cancer form. However, we wish to emphasize, as we have done already on other occasions (Loewenstein, 1968*a*; 1972; 1974; 1975*c*) that we expect such an etiological role only in *some* cancer forms. Cancer is an umbrella term for a great many sorts of uncontrolled cellular growth. Controlled cellular growth, like any controlled biological system involves (i) control-signal generation, (ii) signal transmission, (iii) signal reception, (iv) signal comparison, and (v) effector processes (Loewenstein, 1968*a*; 1974). Hence, uncontrolled growth can, in principle, arise from a genetic defect at any of these four elements; or to state this differently, defects at each of these four levels may constitute independent cancer-etiological categories. We expect, therefore, to find junctional incompetence only among those categories in which uncontrolled growth stems from defects at level *ii*, but not in those categories in which the primary defect resides at any of the other four elements of control. In fact, some cancerous cell types have become known which present good junctional communication, at least in respect to junctional transfer of inorganic ions and fluorescein (Potter, Furshpan & Lennox, 1966; Furshpan & Potter, 1968; Loewenstein, 1968*b*; 1974; Borek, Higashino & Loewenstein, 1969; Sheridan, 1970; Johnson & Sheridan, 1971). This is, in the light of the aforegoing considerations, entirely consistent with the idea of a possible cancer-etiological role of defective junctional communication. It would be inconsistent only in the unlikely event that the many cancer forms were all due to one cause.

It would have been of great interest to establish the growth characteristics of the intermediate hybrid cells. However, this proved to be not possible because of the instability of the chromosome composition. By the time the cells had reached a sufficient density *in vitro* for determination of density dependence of growth, part of the intermediate hybrid cell generations had invariably progressed to segregation to the channel-incompetent trait. This was also generally the case by the time a sufficient number of cells were available for determination of tumorigenicity. Therefore, it was not feasible to test growth properties on uniform clones or subclones of intermediate hybrid cells, and we do not know whether the intermediate expression of junctional communication is associated with cancerous growth.[4] The limitation here lies in the methods of de-

4 *In vitro* segregation was not such a major difficulty in the case of the channel-competent hybrid cells. These were of earlier generations than the intermediate hybrid cells, and the populations were still uniform in respect to junctional communication in the tests *in vitro* and, at least at the outset, of the tests *in vivo*.

termination of the growth properties, not in those of junctional communication: the former require relatively large cell populations involving several cell generations; the latter, only a few cells.

A property of *in vitro* cancer cell growth that has come to the fore in recent years is the capacity of forming colonies in suspension in semisolid medium (methyl cellulose) (Macpherson & Montagnier, 1964; Stoker *et al.*, 1968). In contrast to normal cells, many cancer cell types are capable of growth in such suspension, and this "anchorage independence" thus has been a useful index of cancerous behavior (*cf.* Rissner & Pollack, 1974; Friedmann & Shin, 1974; but *see also* Stiles *et al.*, 1976). The cancerous mouse parent cell here shares this property. Thus, we examined some of the hybrid clones also in this regard, although there are no obvious reasons why this single-cell property should be related to junctional communication if channel incompetence is the primary membrane defect causing the aberrant growth. All the channel-incompetent segregant hybrid clones as well as the mouse parent cell turned out to grow in suspension, whereas the human parent cell did not grow (Table 4). The behavior of the early-generation hybrid clones (*1a, 1b, 1c*) was less clear. These cells grew, though more sparsely, forming dense colonies (Fig. 5). We were unable to test the capacity of junctional transmission and the tumorigenicity of these colonies. Hence, we cannot decide between two rather different possibilities, a cosegregation of anchorage independence with tumorigenicity and channel incompetence, or an early reversion with respect to anchorage independence alone.

On the Genetics of Junctional Communication

The class of "intermediate" hybrids which are electrically coupled but do not detectably transfer fluorescein, is of great interest from both the physiological and genetic points of view. The intermediacy of the communication behavior admits two physiological interpretations at the level of resolution of the present methods: (*a*) The individual junctional channels made by these hybrids are normal, but their number, while providing a detectable degree of electrical coupling, is insufficient to give a detectable degree of fluorescein transfer. This is possible because, as Dr. S.J. Socolar analyzes in the *Appendix* for several cell topologies, the sensitivity of the electrical coupling measurements for discriminating differences in the number of junctional channels could well be rather low in many of the present instances. (*b*) The patency of the junctional channels is reduced in these hybrids; that is, the effective channel bore

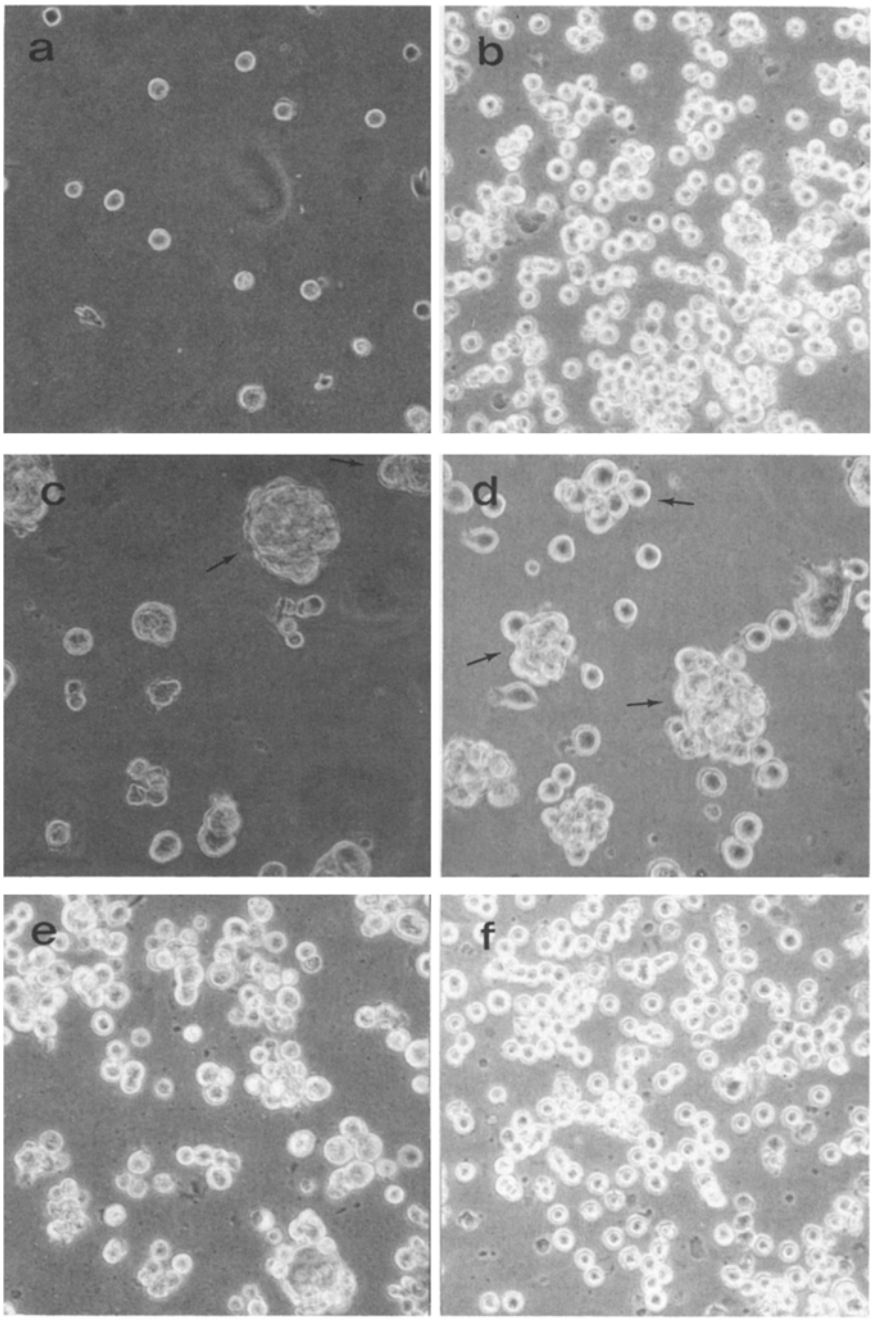

Fig. 5. Growth in suspensions in semi-solid medium (agar methyl cellulose). Phase contrast photomicrographs of the cell cultures 7 days after seeding 10^6 cells. The mouse parent cells (*b*) and the channel-incompetent segregant cells [clones 9K (*e*), and 3D (*f*)] proliferate; the human parent cells (*a*) do not, and only single cells are seen. Some cells derived from the early-generation hybrid cultures [*1b* (*c*) and *1c* (*d*)] proliferate too, forming clusters (arrows). The properties of junctional transmission and tumorigenicity of these clusters are unknown.

See Discussion

size is such as to permit the passage of small inorganic ions carrying the current, but not that of the 330-dalton fluorescein.

The occurrence of hybrids which take after the human parent and of intermediate ones which do so to a lesser extent implies segregation of the genes (or of gene copies) determining the junctional membrane channel; the dominance of the human character is not complete. In this light, we will now discuss the genetic origin of this expression of intermediacy in terms of the above modes *a* and *b*.

Intermediate expression in the number of channels (mode *a*) could be explained by assuming a mutation of the structural genes in the mouse parent cell coding for a protein necessary for the formation of the junctional channels; if the resulting defective protein enters into the formation process, a nonfunctional channel system would ensue. [The protein could be a building block of the channels themselves (*see* Goodenough, 1974; Duguid & Revel, 1975; Gilula & Epstein, 1976, for gap-junction protein components), or it could enter into other steps of channel assemblage.] Thus, in the hybrid cells, where the normal and the defective proteins would exist in mixture, functional channels would result when the channel system happened to be formed on the basis of the normal protein, but not when formed on the basis of defective protein or a mixture of defective and normal protein. The result would be a reduced expression of the phenotype of the communication-competent parent, that is, a reduced number of functional channels. So long as the total number of functioning channels made by the hybrids is relatively high, such a reduction may be difficult to determine with the present methods. With the present level of fluorescein injection and with the aforesaid sensitivity of coupling ratio to channel number (*Appendix*), the safety margin afforded for demonstrating coupling and fluorescein transfer is so large as to conceal variations by a factor of 2 or 3 in the number of functional channels in the parent and early hybrid cells. Thus, the hybrid cells (clones *1a, 1b, 1c, 10a, 10b, 10c*) may, in fact, have a reduced number of functioning channels. But a more drastic reduction in the number of channels should show up with the fluorescein method as diminished cell-to-cell transfer. This may be the case with the intermediate hybrid cells. The underlying shift to more defective channel protein in the intermediate hybrid cells could, for instance, result through the loss of one of the two human chromosomes carrying the gene(s) coding for the correct protein. (This would imply the existence of only one class of such intermediate segregants between the first generation hybrids and the fully junction-defective segregants.)

An alternative—still in terms of mode (*a*)—is that the mutation resides in a regulatory gene of the mouse parent cell, leading to a reduction or abolition of a protein involved in the formation of the junctional channel system. Viewed in this light, the expression of intermediacy of junctional behavior would indicate that the synthesis of the corresponding protein coded by the human parent is reduced by the regulatory defect. For instance: in the early hybrids, the corresponding human regulatory genes, in successful competition with the mouse genes, would give rise to abundant functioning channels. Intermediate hybrids would arise when the loss of one of the human regulatory genes caused a shift in dominance.

In the case of mode (*b*), it is immediately clear that two genes or two sets of genes would determine the permeability properties of the channel, viz., the structure of the channel. Here intermediacy in channel structure may be accounted for by a functional association between a defective channel protein and the correct one. In a simple model, the defect in one gene pair, that of the mouse parent, would lead, as in mode (*a*), to production of a channel protein with an altered structure, rendering it incapable of assembly of functioning channels by itself; but when associated with the correct channel protein coded by the human genes, it would become capable of such assembly, namely assembly of channels with intermediate permeability characteristics. Thus, the intermediate junctional behavior would result from loss of one gene of the human gene pair. This model acquires particular interest in view of the finding of an exclusive hybrid junctional structure, described in the companion paper (Larsen *et al.*, 1977).

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