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Incorporation of the Gene for a Cell-Cell Channel Protein into Transformed Cells Leads to Normalization of Growth

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Summary. Incorporation of the gene for connexin43, a cell-cell channel protein of gap junction, into the genome of communication-deficient transformed mouse 10T½ cells restored junctional communication and inhibited growth. Growth was slowed, saturation density reduced and focus formation suppressed, and these effects were contingent on overexpression of the exogenous gene and the consequent enhancement of communication. In coculture with normal cells the growth of the connexin overexpressors was completely arrested, as these cells established strong communication with the normal ones. Thus, in culture by themselves or in coculture, the connexin overexpressor cells grew like normal cells. These results demonstrate that the cell-cell channel is instrumental in growth control; they are the expected behavior if the channel transmits cytoplasmic growth-regulatory signals.

Key Words intercellular communication · gap junction · connexin · growth control · cDNA · connexin43 · cell-cell channel · junctional communication · transformation · cancer etiology

Introduction

It is as yet unknown how cells in organs and tissues send signals to each other to coordinate their growth. The membrane channels of gap junctions are plausible conduits for signals that do not take the extracellular path (Loewenstein, 1981). These ubiquitous cell-to-cell channels directly link the interiors of adjacent cells and are wide enough (16–20 Å) to transmit cytoplasmic molecules of up to 1–2 kD (Flagg-Newton, Simpson & Loewenstein, 1979; Schwartzmann et al., 1981). Thus, it has been proposed that among the molecules transmitted are signals that regulate the growth of cell populations (Loewenstein, 1966; Nonner & Loewenstein, 1989). This hypothesis has drawn support from four kinds of evidence: (*i*) the intercellular communication these channels provide is reduced by the expression of a number of oncogenes (Atkinson et al., 1981; Azarnia & Loewenstein, 1984; Chang et al., 1985; Azarnia & Loewenstein, 1987; Azarnia et al., 1988; 1989; Rosen, Van Der Merwe & Davidson, 1988; Filson et al., 1990; Swenson et al., 1990) and by the activation of certain growth factor receptors (Maldonado, Rose & Loewenstein, 1988); (*ii*) the communication is weak or absent in a number of transformed cell types (Loewenstein, 1979; Musil et al., 1990*b*); (*iii*) the communication is abolished by tumor-promoting phorbol esters (Murray & Fitzgerald, 1979; Yotti, Chang & Trosko, 1979; Enomoto et al., 1981); (*iv*) and the communication correlates with growth control when it is experimentally varied by cyclic AMP, forskolin or retinoids (Mehta, Bertram & Loewenstein, 1986, 1989; Mehta & Loewenstein, 1991). Although that body of evidence is large, it is but circumstantial; communication is certainly not the only cellular function altered in transformation, nor are there reasons to believe that any of the experimental agents which were used to modify communication acted exclusively on that function. Missing was a demonstration specifically implicating communication in growth control. Here we have attempted to fill this gap by using a gene that encodes a cell-cell channel protein, to manipulate the communication.

Our approach was to incorporate the channel-protein cDNA into the genome of transformed communication-deficient cells and to ask whether the expression of this cDNA will normalize their growth. This strategy calls for transformed cells where the expression of channel protein is deficient but where the channel assembly process remains unimpaired. We chose MCA-10 cells, a line of methylcholanthrene-transformed mouse 10T½ cells (Mehta et al., 1986) in which, we will show, these conditions are fulfilled. Among the available channel protein cDNAs, we opted for that of connexin43 (Beyer, Paul & Goodenough, 1987), which is widely expressed in vertebrate cells (Beyer et al., 1989;

Gimlich, Kumar & Gilula, 1990), including 10T½ cells (Rogers et al., 1990).

Materials and Methods

Materials

All cell culture media were from GIBCO; calf and fetal bovine serum (Lot #2151948 and 1115933, respectively), from Hyclone Labs.; tissue culture plasticware from Nunc; Seakem GTG agarose, from FMC Bio Products; guanidine isothiocyanate, phenol (molecular biology grade), yeast tRNA, Poly (A), Poly (C) and salmon sperm DNA, from Boehringer Mannheim Biochemicals; ultrapure formamide, from Clontech Laboratories; Lucifer Yellow CH, from Molecular Probes; all other reagents (molecular biology grade or highest purity), from Sigma. Immobilon P membranes were from Millipore; Gene Screen Plus nylon membranes, [α-^{32}P]dCTP, from NEN; restriction enzymes were from Boehringer Mannheim and from New England Biolabs.

Cell Culture

The following cell lines were used: normal 10T½ cells, C3H10T½ clone 8; methylcholanthrene-transformed 10T½ cells, MCA-10 (Reznikoff et al., 1973; Mehta et al., 1986); and packaging cell line PA317 (Miller & Buttimore, 1986) for production of recombinant retrovirus (ATCC # CRL 9078). All cells except PA317 were grown in basal minimal essential medium supplemented with 5% serum—2.5% calf and 2.5% fetal bovine serum—and 25 μg/ml gentamicin. PA317 were grown in Dulbecco's Modified Eagle medium with 10% serum—5% calf and 5% fetal bovine—and 25 μg/ml gentamicin. All cells were grown at 37°C and 5% CO_2/95% air. For growth assays, cells were grown in 60 mm dishes with 6 ml medium. Stock cultures were passaged once/week at 10^5 cells/100 mm dish with 10 ml medium. For coculture assays 5×10^3 normal 10T½ cells were seeded and grown to confluence for 18–21 days before other cells were seeded on top of them. Medium was changed every 4th day.

Plasmids and Expression Vector Construction

Vectors pXT1 (Boulter & Wagner, 1987), PSG5 (Green, Issemann & Sheer, 1988), pMC1*neo* and pMC1*neo* poly A (Thomas & Capecchi, 1987) were purchased from Stratagene and vector pCMVβ (MacGregor & Caskey, 1989), from Clontech; vector DOL (Korman et al., 1987) was a gift from Dr. Eric Beyer (Beyer et al., 1987); a plasmid containing 1.5 kb rat liver connexin32 (*cx*32) cDNA, a gift from Dr. David Paul (Paul, 1986); plasmids containing 1.1 or 2.6 kb inserts of rat liver connexin26 (*cx*26) cDNA, a gift from Dr. Bruce Nicholson (Zhang & Nicholson, 1989); and plasmid pHCGAP3 (Tso et al., 1985) containing 0.9 kb human glyceraldehyde-3'-phospho-dehydrogenase, a gift of Dr. S. Påhlman.

The construction of plasmid DOL has been described (Korman et al., 1987). Briefly, DOL contains two Moloney murine leukemia virus long terminal repeats (LTRs) flanking a retroviral packaging sequence, a multiple-cloning-site polylinker, and the *neo* resistance gene driven by a Simian virus 40 promoter. DOL43 was constructed by ligating a BamHI-Sal I fragment containing the rat heart connexin43 cDNA described by Beyer et al. (1987) into the DOL polylinker.

Vector pXT1-43 was constructed by inserting the 1.4 kb *cx*43-containing fragment into the cut-and-filled BamHI site of vector pSG5. The resulting plasmid was cut with EcoRI and Bgl-II to yield an approximately 1.4 kb fragment containing *cx*43 cDNA, where *cx*43 cDNA is flanked on the 5' and 3' sides by EcoRI and Bgl-II sites, respectively. Plasmid pXT1 was cut with NsiI and EcoRI to obtain a 2.25 kb fragment containing 5'LTR, with viral packaging signal psi and viral splice donor and acceptor sites; pXT1 was cut again with NsiI and Bgl-II to obtain a 6 kb fragment containing the viral 3'LTR and complete pBR322 plasmid. These two fragments were then ligated with the 1.4 kb EcoRI-Bgl-II *cx*43 cDNA. The resulting plasmid containing *cx*43 cDNA between two LTRs was cut with XhoI and a 1.09 kb XhoI-SalI fragment with TK promoter and *neo* gene from plasmid pMC1NEO was inserted into XhoI site to give plasmid pXT43.

In all cases of vector construction, correct orientations were verified by polyacrylamide and agarose gel electrophoresis of several restriction enzyme digestions of the constructs. All plasmids were grown in *E. Coli* strain HB101 (Maniatis, Fritsch & Sambrook, 1989).

Retrovirus Production

The plasmids were transfected into the packaging cell line PA317 as described (Maniatis et al., 1989), and cells were selected for the *neo* resistance gene in medium containing 400 μg/ml active geneticin (G418). 10–20 G418 resistant clones were isolated, expanded and analyzed for correct retroviral structure by Southern blotting. Fresh PA317 cells were then infected with retrovirus of correct structure, produced by such selected G418 resistant clones. For this, the cells were exposed to medium conditioned by the transfected clones and containing the recombinant viruses; cells were then selected for G418 resistance, tested for correct virus structure and for virus titre. Virus titres were assayed on Morris hepatoma H-5123 cells (Borek, Higashino & Loewenstein, 1969) because of their fast growth, but similar titres were obtained with NIH 3T3 cells. Hepatoma cells were seeded at 3×10^5 cells/6 cm dish in 3 ml BME medium supplemented with 5% serum. After 24 hr the medium was replaced with 2 ml of serial 10-fold dilutions of medium conditioned for 24 hr by confluent cultures of G418-resistant (infected) PA317 packaging clones and containing 8 μg/ml polybrene (Aldrich). After 2–3 hr, this conditioned medium was replaced with 3 ml fresh medium, and 24 hr later with medium containing 400 μg/ml G418. After 7–10 days, cells were fixed, stained and colonies were counted. Virus titres were expressed as colony-forming units (cfu)/ml. The titres from the stablest and best producing PA317 clones ranged 10^3–5×10^5 cfu/ml and all of these clones had correctly arranged viral structure as judged by DNA as well as RNA blot analysis. (In contrast, only 10% of the transfected clones produced virus with correct structure.) The conditioned media from these clones, harboring the recombinant viruses, were stored frozen at −70°C.

Retrovirus Infection of Cells

Target cells were seeded in duplicate 60 mm dishes at 3×10^5 cells/dish. Medium was replaced after 24 hr with 2 ml of recombinant virus-containing medium and 8 μg/ml polybrene for 2–3 hr; cells were then washed twice with fresh medium and incubated in same for 2–3 hr. This protocol was repeated six times over a period of 36–40 hr. 24 hr after final infection and washing, cells

were grown in medium containing 400 μg active G418/ml for *neo* resistance selection. After selection and isolation, clones were maintained in medium containing 200 μg active G418/ml, a concentration at which cells not containing *neo* did not survive.

The studies of *cx*43 vector transcription and expression, including communication, were carried out on cells between the 4th and 10th passage after their cloning.

DNA Probe Synthesis

For synthesis of DNA probes for Southern and Northern blot analysis, the relevant DNA fragments were excised from the aforedescribed plasmids and gel purified and electroeluted two times. 100 to 200 ng were then nicktranslated with [α-^{32}P]dCTP to a specific activity of 10^8–10^9 cpm/μg DNA. The ^{32}P-labeled probes were precipitated twice with 2.5 M NH_4 acetate and ethanol to eliminate free nucleotides and used at 5–10 ng/ml hybridization buffer.

RNA Analysis by Northern Blots

Total RNA was extracted from one 15 cm or two 10 cm dishes of freshly confluent cells by the acid-phenol guanidine isothiocyanate procedure (Chomezynski & Sacchi, 1987) or, from rat liver, by the guanidine isothiocyanate procedure (Chirgwin et al., 1979). Integrity of extracted RNA was checked on minigels. For Northern blot analysis, 20 μg RNA/lane was electrophoresed in 1% agarose/formaldehyde gels (Lehrach et al., 1977) in 50 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.0, at 2V/cm for 12–16 hr at room temperature. RNA was then capillary blotted onto Gene Screen Plus membranes in 12.5 × SSC (1 × SSC: 0.015 M NaCl, 0.015 M trisodium citrate, pH 7.4) for 24–48 hr. Blots were prehybridized 1 hr or longer in hybridization buffer (5 × SSC, 0.01 mM sodium phosphate, pH 6.8, 0.2% SDS, 1 × Denhardt's reagent, 50 μg/ml yeast tRNA, 2.5 μg/ml each of poly A and poly C, 100 μg/ml sonicated salmon sperm DNA and 5–10% polyethylene glycol (mol wt 8000), and hybridized with nick-translated, $^{32}P_i$–labeled DNA probes for 16–48 hr at 44°C, and washed with 0.1 × SSC for 1–2 hr or until background radioactivity was negligible. Blots were exposed to Kodak X-Omat AR or X-Omat RP film with intensifying screen (Cronex Lightening Plus, Dupont) for 2 hr–2 d. After probing for *neo*, *cx*43, *cx*32 or *cx*26, blots were reprobed by hybridization with ^{32}P-labeled probes for glyceraldehyde-3'-phosphate dehydrogenase (GAPDH), an indicator for RNA gel loading.

DNA Analysis by Southern Blots

High molecular weight genomic DNA was isolated from the cells according to Ausubel et al. (1987) except that after ammonium-acetate precipitation genomic DNA was spooled out and was not treated with RNase. About 20 μg of genomic DNA was digested with the restriction enzyme XbaI, electrophoresed in 0.8% agarose gel for 40 hr at 1 V/cm in Tris-borate-EDTA buffer (Maniatis et al., 1989) and transferred by capillary action to Gene Screen Plus membrane according to manufacturer's instructions. After transfer, endogenous and vector-specific DNA species were identified by hybridization with nick-translated ^{32}P-labeled probes and exposure to X-ray sensitive film in the presence of intensifying screen.

Immunoblots

Freshly confluent cells from the various clones were rinsed once with cold solution A (30 mM HEPES, 122 mM NaCl, 3 mM KCl, 1 mM Na_2HPO_4 and 4 mM glucose, pH 7.5) and once with cold TBS (10 mM Tris, 130 mM NaCl, pH 7.4). Cells were scraped with a rubber policeman in 2 ml scraping buffer (TBS containing 1 mM protease inhibitor PMSF, 10 mM NaF_2, 10 μM vanadate and 5 mM each EDTA and EGTA) collected into 15 ml centrifuge tubes and spun for 5 min at 500 × *g*. Cell pellets were resuspended in 1 ml scraping buffer, spun in an Eppendorf microfuge for 1 min at room temperature, and then lysed and vortexed in hot lysis buffer (66 mM Tris, 2% SDS, 10 mM EDTA, 5 mM EGTA, pH 6.8; 35 and 100 μl for 35 and 60 mm dishes, respectively.) Tubes were spun for 10 sec, boiled 10 min, vortexed, and centrifuged at 14,000 rpm in an Eppendorf microfuge for 20 min. The supernatant was collected and stored frozen. Protein concentration was determined with BCA reagent (Pierce, Rockford, IL), using BSA as standard. Samples were run in 12% SDS PAGE gels, transferred to Immobilon PVDF membrane, blocked for 2 hr at room temperature with 1% Carnation milk in TBS, and incubated with polyclonal antibody specific for connexin43 (gift of Eric Beyer and Joan Brugge) for 1 hr in TBS containing 1% Carnation milk and 2% polyethylene glycol (6000 mol wt). Membranes were washed 3–4 times for 10 min each with TBS containing 0.05% NP40, and incubated with ^{125}I protein A (NEN, Dupont) at about 5 × 10^6 dpm/ml for 1 hr. After several washes with TBS, membranes were exposed to Kodak X-Omat-AR film for 1–2 days at −70°C.

Immunostaining

Cells were seeded on coverslips; at a barely confluent state, they were fixed at −20°C for 20 min in 50 : 50 acetone : methanol containing 5% glacial acetic acid, washed in phosphate-buffered saline (pH 7.2; PBS), incubated in blocking solution (PBS containing 10% Carnation nonfat dry milk or 1% goat serum) at 4°C for 1 hr, incubated in PBS/1% goat serum containing polyclonal antibody specific for connexin43 (rabbit serum at 1 : 100 dilution; gift of Joan Brugge) either for 6–16 hr at 4°C or 1–2 hr at room temperature, washed 3 × with PBS, incubated with rhodamine-labeled goat anti-rabbit IgG (1 : 100 dilution) for 1 hr and washed 3 × with PBS. Immunostained cells were immersed in 50% glycerin/PBS and examined on a Nikon Diaphot-fluorescence microscope with a 100× oil immersion objective. Images were captured on an optical disk, recorded with an SIT66 (Dage MTI) videocamera, and reproduced on a videoprinter (Hitachi).

Communication Assay

The fluorescent dye Lucifer Yellow CH (lithium salt, Molecular Probes; 5% aqueous solution) was injected by air pressure into the cells with the aid of micropipettes. Injections were done on a Nikon diaphot microscope equipped with fluorescence optics and a Dage MTI SIT 66 video camera. As index of junctional communication, we scored the total number of cells to which Lucifer Yellow had spread 1 min (for homologous junctions) or 2 min (for heterologous junctions)—after injection of a cell. Injections and cell-to-cell diffusion of the dye were video monitored together with a continuous time display and taped for later analysis. (In Figs. 7 and 9, the photographs of cells, injected in rapid succession and fixed, were taken directly through the microscope, in order to display the results of several injections in one frame.)

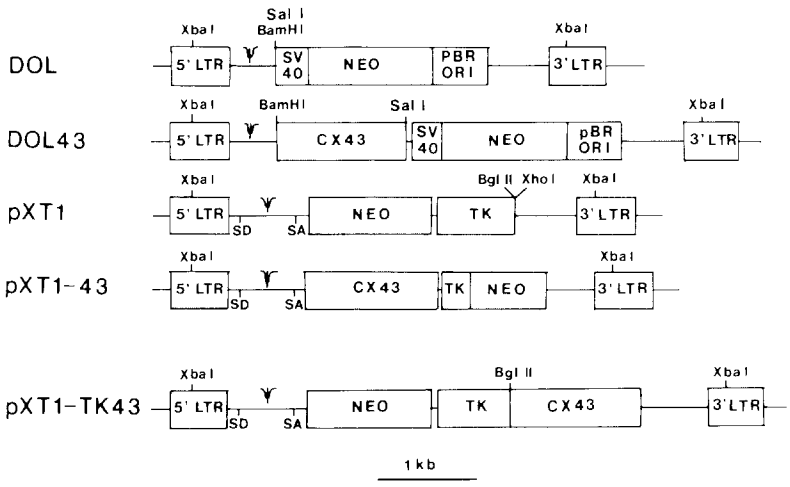

Fig. 1. Connexin43 expression- and control retroviral vectors. Only the relevant proviral regions are displayed. The plasmids contain the rat heart *cx*43 and/or the neomycin phosphotransferase (*neo*) genes inserted between transcriptional enhancers, promoters and polyadenylation signals provided by flanking Moloney murine leukemia virus long terminal repeats (5'LTR and 3'LTR). The locations of relevant restriction sites are shown. *SV40:* SV40 early promoter; *TK:* thymidine kinase promoter; *ψ:* retroviral packaging signal sequence; *SD/SA:* splice donor/acceptor.

Cell Labeling with di-I

The lipophilic tracer di-I (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes) was added at 3 μg/ml (sonicated in culture medium) for 30 min to confluent cultures of the cells to be labeled. Cultures were then washed several times with PBS, trypsinized, and 2×10^4 labeled cells were seeded onto confluent cultures of normal (unlabeled) $10T\frac{1}{2}$ cells. The tracer, which is retained in cells for many days (Honig & Hume, 1986), fluoresces red with green excitation light and thus is easily detectable and distinguished from yellow-green fluorescent (blue light excitation) Lucifer Yellow, the tracer we used in tests for junctional communication.

Cell Labeling by β-gal Expression

Normal $10T\frac{1}{2}$ cells were co-transfected with β-gal vector pCMVβ (MacGregor & Caskey, 1989) and the *neo* resistance vector pMC1neo poly A, and geneticin-resistant clones were tested for β-gal expression by incubation with 5-bromo-4-chloro-3-indoyl-β-D-galactoside, a substrate which is converted by β-galactosidase into a blue reaction product (MacGregor & Caskey, 1989). Cells expressing β-galactosidase thus can be identified and distinguished from other cells by the dark blue stain in their perinuclear regions. The β-gal expressing $10T\frac{1}{2}$ cells were indistinguishable from the parental $10T\frac{1}{2}$ cells in all other respects.

Results

Retroviral Expression Vectors

Replication-defective retroviral expression plasmids DOL43, pXT1-43 and pXT1-TK43 were constructed which used retroviral long terminal repeats (LTRs) and internal SV40 or thymidine kinase (TK) promoters for simultaneous expression of the connexin gene (rat-heart *cx*43) sequences and the selectable neomycin phosphotransferase gene (*neo*) (Fig. 1). Plasmids DOL (Korman et al., 1987) and pXT1 (Boulter & Wagner, 1987) which contained the *neo*, but not the *cx*43 gene, were used for controls. Replication-defective helper-free retroviruses were produced by transfecting these plasmids into an appropriate packaging cell line, as described in Materials and Methods. *Cx*43 and control retrovirus-containing supernatants were then used to infect MCA-10 cells for experimentation.

Our choice of retroviral vectors was guided by the results of preliminary trials showing that the efficiency of gene transfer was much higher by this method than by transfection, and that rearrangement of the vector DNA in the cellular genome occurred rarely (whereas with transfection, the frequency of rearrangement could be as high as 90%). The efficiencies of gene transfer following retroviral infection, as given by the fraction of *neo*-resistant colonies, were about 75% for both the *cx*43 and the *neo* control vectors (Table 1). Thus, the effects of infections with the *cx*43 and control retroviruses can be directly compared.

Focus Formation is Curtailed upon Incorporation of the Connexin Gene into the Transformed Cells

MCA-10 cells, as is typical of transformed cells, grow on top of one another forming dense, visible foci (Mehta et al., 1986). Such focus formation was strikingly inhibited following infection with the *cx*43 expression vectors DOL43 and pXT1-43: at all seeding densities (10^3–10^5 cells/dish) the cultures exhibited few foci; in contrast, the controls infected with *neo* formed numerous foci (Fig. 2).

We quantified the inhibition for seedings of 1,000 and 5,000 cells/dish. The colonies were then sepa-

Table 1. Efficiency of gene transfer

Vector	Number of colonies/dish (Mean ± SE)		Gene transfer efficiency (%)
	Normal medium	Selection medium	
DOL	30 ± 2 (3)	21.8 ± 1.8 (4)	73
DOL43	28.7 ± 1 (3)	21.3 ± 1.3 (4)	74
pXT1	36 ± 2.6 (3)	27.3 ± 2.5 (4)	76
pXT1-43	37.3 ± 4 (3)	27.8 ± 4.1 (4)	75

10^3 cells infected with the *cx*43 (DOL43, pXT1-43) and *neo* control (DOL, pXT1) expression vectors were seeded on 6-cm dishes and grown in normal or selection (G418) medium. Efficiency of gene transfer = [number of colonies in selection medium/number colonies in normal medium] × 100. The number of dishes examined is given in parentheses.

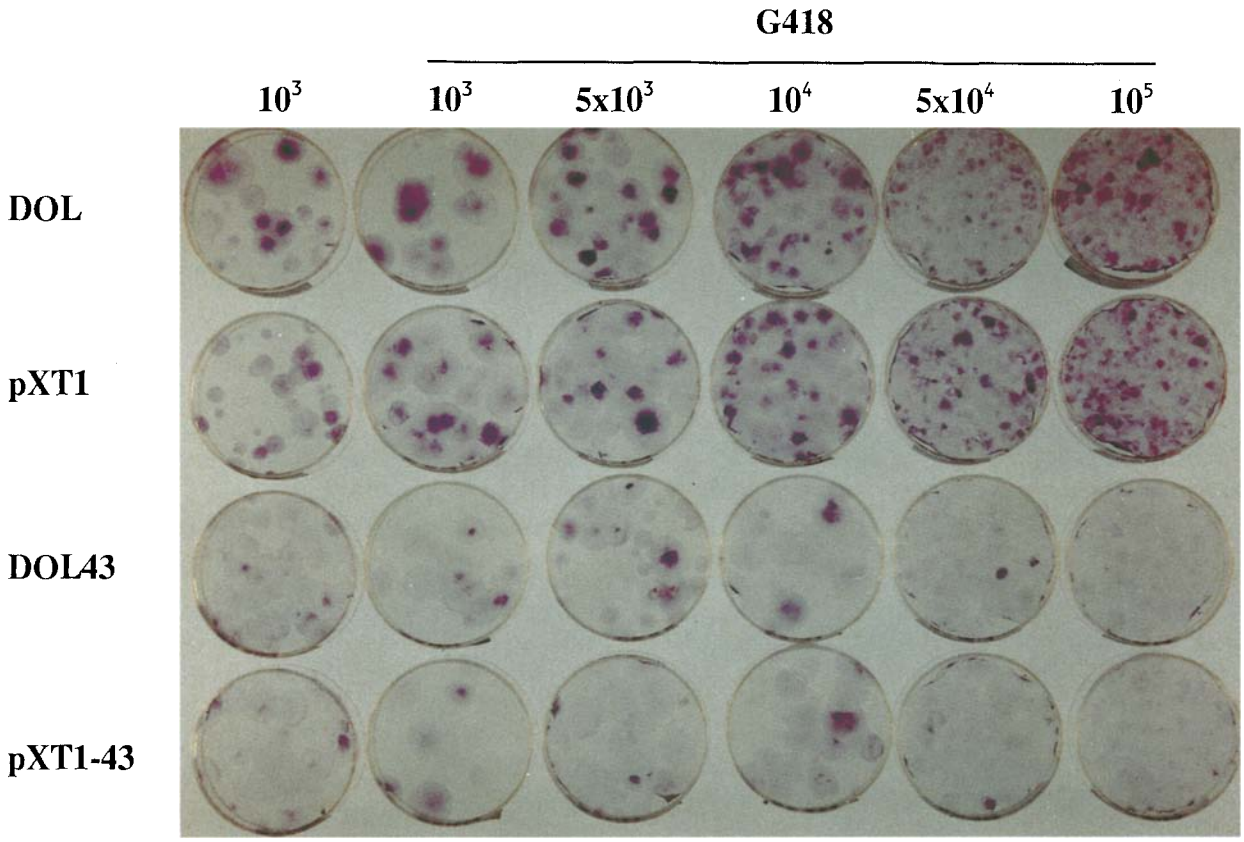

Fig. 2. Inhibition of focus formation by *cx*43 expression vectors. Dishes of MCA-10 mass cultures seeded at the specified densities and grown for 14 days after infection with the *cx*43 expression vectors DOL43 or pXT1-43 or after infection with the control vectors DOL or pXT1. At all seeding densities, the cultures infected with *cx*43 exhibit fewer foci (cell colonies with darkly stained centers) than the controls. All cultures were grown in medium containing 400 μg/ml G418, except those in the first column which were grown in normal medium, to index the efficiency of gene transfer (*see* Table 1; colony counts from the first two columns are included in that table.) Exponentially growing MCA-10 cells were infected (*see* Materials and Methods) with equal titres of recombinant virus. 24 hr after the last infection cycle, cells were trypsinized and seeded at the specified densities; 24 hr later G418 (400 μg/ml) was added to those cultures intended for *neo*-resistance selection. Medium was changed every 4 days, and cultures were fixed and stained with crystal violet after 14 days. Foci, which contain many cell layers, stain deeply blue, whereas monolayered colonies stain very weakly.

Table 2. Inhibition of focus formation and cell growth by *cx*43 expression vectors

Expt	Vector	Focus formation				Cell growth							
		10^3 cells seeded		5×10^3 cells seeded		10^3 cells seeded		2×10^4 cells seeded		5×10^4 cells seeded		10^5 cells seeded	
		Foci[a] (%)	Inhibition[b] (%)	Foci[a] (%)	Inhibition[b] (%)	Cells/dish ($\times 10^5$)	Inhibition[c] (%)	Cells/dish ($\times 10^5$)	Inhibition (%)	Cells/dish ($\times 10^5$)	Inhibition (%)	Cells/dish ($\times 10^5$)	Inhibition (%)
A	DOL	69 ± 5.8	0	72 ± 2.1	0	15.3 ± 0.1	0						
	DOL43	33 ± 9.4	52	24 ± 6.9	67	9 ± 0.2	41						
	pXT1	71 ± 5	0	61 ± 6.6	0	20.5 ± 0.5	0						
	pXT1-43	27 ± 6.4	62	24 ± 9	61	7.6 ± 0.7	63						
B	pXT1	78 ± 4.6	0	61 ± 2.9	0	8.8 ± 1.1	0	14.7 ± 0.3	0	22.3 ± 0.3	0	26.3 ± 1.2	0
	pXT1-43	25 ± 4.3	68	22 ± 2.2	64	3.3 ± 0.9	63	6.9 ± 0.7	53	10.5 ± 0.5	53	12.3 ± 0.3	53
	DOL43	31 ± 2.7	60	28 ± 2.4	67	3 ± 0.1	66	7.7 ± 0.3	48	12 ± 0.1	46	13.3 ± 0.9	49
	pXT1-TK43	69 ± 2.5	12	58 ± 6.3	5	7.9 ± 0.6	10	14.7 ± 1.3	0	23.7 ± 1.3	0	27 ± 0.6	0

[a] Percent of foci forming colonies; mean ± SE of 3 dishes with 10–20 and 20–50 colonies each at seeding density 10^3 and 5×10^3, respectively. (There were no significant differences between the means of the individual dishes.)

[b] Inhibition of focus formation is calculated as $[1 - (x/y)] \cdot 100\%$, where x is % foci from clones with *cx*43 vector and y is % foci from clones with respective control vector.

[c] Inhibition of growth is calculated as $[1 - (x/y)] \cdot 100\%$, where x is the number of cells with *cx*43 vector and y is the number of cells with control vector.

The data in *A* and *B* are from separate infection experiments, where cultures infected with the respective expression vectors were grown in parallel. Note that the inhibition of DOL43 and pXT1-43-infected cultures is the same in both experiments. Cells were counted in a parallel set of dishes at the time (14 and 12 days after seeding in *A* and *B*, respectively) other dishes were fixed and stained for determination of foci-forming colonies. The data in *A* are from the same experiment as in Table 1 and Fig. 2.

rate enough for counting, and the proportion of colonies that exhibited foci could be accurately determined. That proportion was reduced by 52–67% in the cells infected with the *cx*43-expression vectors, compared to the controls expressing *neo* alone (Table 2*A*); but the number of colonies themselves was not significantly reduced (Fig. 2 and Table 1). Individual cell counts by flow cytometry (Coulter counter) provided a further measure of the growth inhibition: the total number of cells in the dishes was reduced by 41-63% (Table 2*A*, right). The two *cx*43 expression vectors produced similar degrees of inhibition (Table 2*A*), a consistent finding also in other experiments. Thus, for further analyses we used only one of these vectors, DOL43.

Cultures infected with a third *cx*43 construct, pXT1-TK43, served as controls in addition to the cultures infected with *neo* alone. In this construct the positions of *cx*43 and *neo* were reversed, so that *cx*43 was driven by the TK promoter instead of the more powerful LTR promoter (Fig. 1). Thus, one would expect only low-level *cx*43 expression. As it turned out, there was no detectable transcription of exogenous *cx*43 at all (*data not shown*) and the growth of the infected cells was not significantly different from that of the parental MCA-10 cells (Table 2*B*).

Connexin43 is Overexpressed in Clones Infected with the Gene

From DOL43-infected mass cultures, like those above, 10 individual clones of G418-resistant cells were isolated and analyzed for *cx*43 DNA and RNA. Genomic DNA and total RNA were extracted from the cells and hybridized in Southern and Northern blots with radiolabeled *cx*43 or *neo*. All clones transcribed the *cx*43 vector DNA, eight at very high levels (Fig. 3*A*, *D-1* to *D-8*). The band corresponding to the predicted ~5.2 kb of the exogenous *cx*43 transcript was shown by all DOL43-infected clones; it was particularly strong in clones D-1 to D-8. *Cx*32 and *cx*26, two other vertebrate connexin genes (Kumar & Gilula, 1986; Paul, 1986; Zhang & Nicholson, 1989; Gimlich et al., 1990), were not detectably transcribed in these cells (Fig. 3*B*).

Several other bands showed up in the Northern blots of the DOL43-infected clones, which were not present in the parental MCA-10 cells or the *neo* controls (Fig. 3*A*). These bands represent vector-derived transcripts; they were hybridized by both the *cx*43 and the *neo* probe (data for *neo* not shown). Possibly, these extra bands represent vector transcripts where the termination signal was overrun (*see*, e.g., Hantzopoulos et al., 1989). There also was an enhanced radioactivity visible at the position of the endogenous *cx*43 mRNA in the overexpressor clones in Fig. 3*A*. This was not seen in all blots and possibly stems from the superposition of radioactivity from partially degraded exogenous *cx*43 mRNA.

Southern blot analysis showed that the exogenous *cx*43 DNA had been incorporated in one piece into the genomic DNA; the genomes contained intact retroviral proviruses (Fig. 4).

Five of the overtranscribing clones (D-3 to D-7) were analyzed for translated product by probing

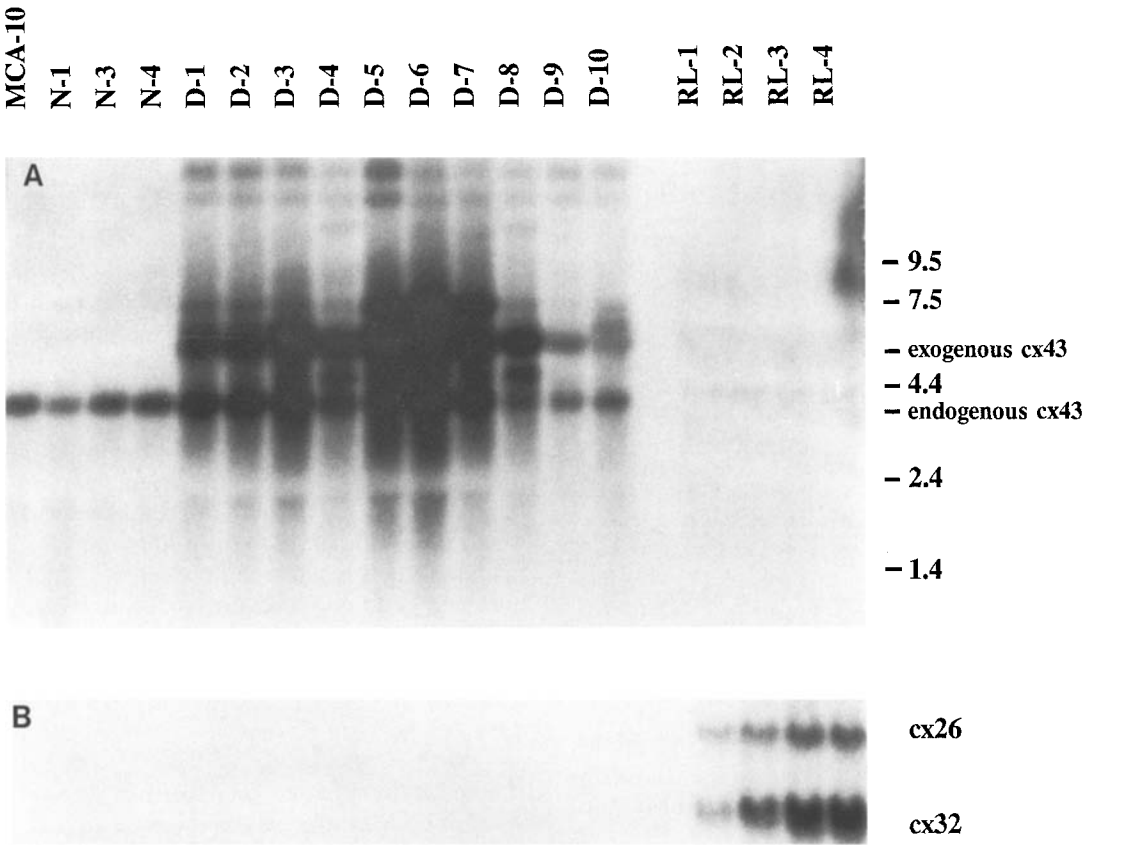

Fig. 3. Transcription of exogenous *cx*43 DNA. Northern blots of total RNA (20 μg) from clones infected with *cx*43 expression vectors (*D-1* to *D-10*) or *neo* control expression vectors (*N-1* to *N-4*), and from parental uninfected MCA-10 cells. (*A*) Probings for *cx*43 mRNA. All clones transcribe the endogenous *cx*43; the DOL43 clones, in addition, transcribe the larger exogenous *cx*43, eight of the clones (*D-1* to *D-8*) at a very high level. The cells on which the assays were performed were from about the 50th generation after *cx*43 infection. The additional bands hybridizing with the *cx*43 probe in lanes *D-1* to *D-10* are transcripts of the DOL43 vector, which presumably are improperly terminated (*see* text). (*B*) An identical blot to *A* but probed for *cx*32 and *cx*26 mRNA. No transcription of *cx*32 or *cx*26 is detectable except in the four lanes *RL-1* to *RL-4* on the right. These lanes contain 2.5, 5, 7.5 and 10 μg total RNA from rat liver, which is known to express *cx*32 and *cx*26, for a comparison.

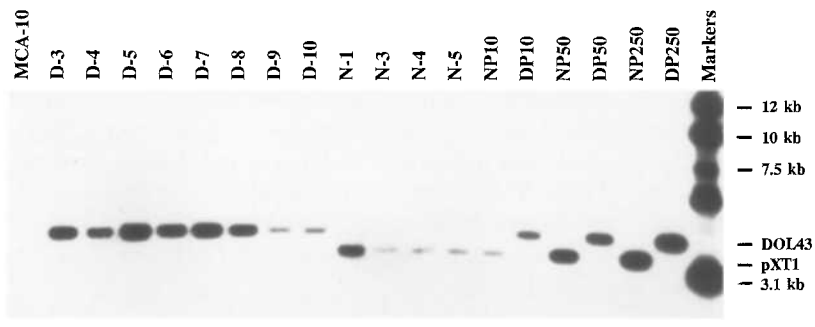

Fig. 4. Southern blots of genomic DNA (XbaI digests) from the DOL43-infected clones, *D-3* to *D-10*, and from *neo*-infected control clones, *N-1* to *N-5*. The blot was hybridized with ^{32}P-labeled, nick-translated *neo* probe from vector pMC1*neo*. Only a single band is seen in each clone; the band of the DOL43 clones comigrates with the DOL43 plasmid (*DP10* to *DP250*) and the band of the *neo* control clones comigrates with that of the *neo* plasmid (*NP10* to *NP250*), showing that in each clone the *cx*43 DNA had been incorporated intact. The plasmids were loaded at three concentrations: 10, 50 and 250 pg

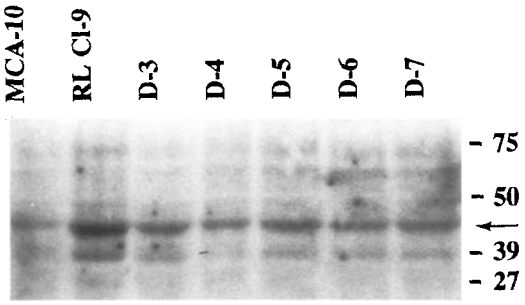

Fig. 5. Overexpression of *cx*43. Immunoblots of cell lysates from DOL43-infected clones *D-3* to *D-7* and of uninfected *MCA-10*, probed with a polyclonal antibody against *cx*43. For positive identification of *cx*43 protein (arrow), we included a lysate from a rat liver cell line (*RL CI-9*) that expresses *cx*43 to a high level, as seen by the strong band at 43–45 kD. In lanes *D-3* to *D-7* the corresponding band is stronger than in the *MCA-10* lane, showing that *cx*43 is overexpressed in all five clones compared to MCA-10. 75 μg total cell protein/lane.

Western blots with a polyclonal antibody raised against the amino acid region 252–271 of connexin43 (Beyer et al., 1989), the product of the very rat heart gene we had introduced into these cells. All clones expressed *cx*43 and, as expected from the transcription data, at a higher level than uninfected MCA-10 cells (Fig. 5).

Connexin43 is Overabundant in the Cell Junctions of Overexpressor Clones

Overexpression of the exogenous *cx*43 protein, as detected by Western blots of whole-cell lysates, is, by itself, no guarantee that the protein actually gets into the junctional membranes and forms functional channels there. For example, in BHK cells transfected with *cx*32, the overexpressed protein predominantly showed up out of place in nuclear and other intracellular membranes (N.M. Kumar and N.B. Gilula, *personal communication*). In the following experiments we show that the overexpressed *cx*43 protein is, in fact, localized at the cell junctions and that junctional permeability is increased.

*Cx*43 overexpressor clones and parental MCA-10 cells were immunolabeled with the aforementioned polyclonal antibody against connexin43. The junctions of cells with highly overtranscribed *cx*43, such as D-3 to D-7, exhibited abundant anti-connexin43 immunolabel. In D-5 cells, for example, the label virtually outlined the cell-cell boundaries (Fig. 6*a,b*). In weakly transcribing clones, such as D-9 and D-10, connexin43 occasionally was visible at cell junctions, too, but to a very much lower extent; the immunostain was then typically seen as scattered dots, occasionally as a dash, and the fluorescence was much fainter (Fig. 6*c,d*). The parental MCA-10 cells exhibited even less immunostain at cell junctions, if any at all (Fig. 6*e*).

There was strong immunostaining also in the interior of the overexpressor cells, staining which presumably represented connexin43 in transit to plasma membrane or abnormally localized connexin43. We rarely saw immunolabel in plasma membrane which was not in contact with another cell; in the few instances in which such nonjunctional-membrane labeling was found, it occurred only as isolated dots, never as a string of dots or a band.

But what really matters here is that the connexin43 found its way to the junctional membrane of the overexpressor cells and that it was much more abundant there than in the parental cells. This demonstrates—and more to the point than Western blots of whole cell lysates could ever do—that the connexin43 gene is overexpressed, and overexpressed where it counts.

Communication is Enhanced in the Overexpressor Clones

The next thing to see was whether this overexpression of connexin43 was functional, that is, whether the overabundance of that protein in the junctions would lead to an increase in junctional permeability. We probed the permeability of the cell junctions with the 443-D fluorescent dye Lucifer Yellow. The dye was microinjected into the cells, and the junctional transfer was indexed by the number of cells to which the fluorescent tracer spread within 1 min of its injection (Mehta et al., 1989).

Junctional transfer was determined in six of the *cx*43 overtranscribing clones (D-3 to D-8). In all of these clones transfer was enhanced. Typically, the tracer spread to all neighbors of the injected cell and to cells far beyond, whereas in the controls—the uninfected clones or the clones infected with the *neo* vector alone—the transfer was limited to first-order neighbors and only to a fraction of them (Fig. 7). The increase in junctional transfer in the various overexpressor clones was large: 143 to 274% compared to the parental MCA-10 cells (Table 3).

The data tabulated all are from strictly parallel experiments where cells were seeded in identical conditions on the same day and junctions were probed in all clones on days 3 and 4 after seeding. These results were corroborated by probing individual clones not in parallel with one another. The results were in all cases essentially the same (*data not shown*).

We have shown before that the loss of the fluorescent tracer from the parental cells is negligible over the time of our communication measurements

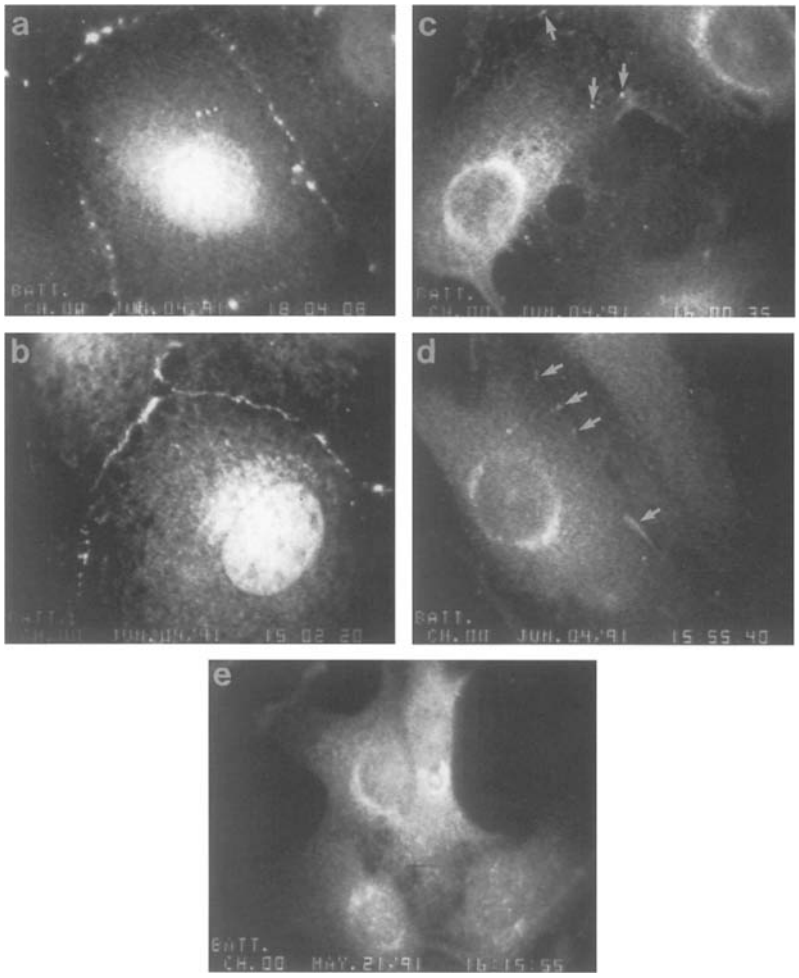

Fig. 6. Immunolocalization of connexin43 in cell junctions. The photographs show typical fluorescence images of cells immunolabeled with antibodies against connexin43, the product of the gene introduced into the cells. (*a,b*) The cell junctions in the highly overtranscribing clone D-5 are heavily decorated with the immunolabel. The immunostain outlines virtually the entire cell-cell boundaries. (*c,d*) The cell junctions of the weakly transcribing clone D-9 show fainter staining occurring in the form of dots (arrows) and a short bar (bottom arrow in *d*). (*e*) Parental MCA-10 cells.

(Mehta et al., 1986, 1989). Thus, significant contributions of changes of permeability in nonjunctional cell membrane are excluded; the enhanced junctional transfer in the overexpressor clones reflects an enhanced junctional permeability, that is, an increase in the number of open cell-cell channels.

The increase in junctional permeability also showed itself in a way not covered by our index of junctional transfer. Because the increase of permeability was so large, the underlying increase in the rate of junctional transfer was plainly visible in the fluorescence intensity of first-order neighbors of the injected cells. This fluorescence was always much more intense in the overexpressor clones than in the parental cells (*compare* Fig. 7*B*, *C*, *D* with *A*).

Growth is Inhibited in the Clones with Enhanced Communication

Next, we examined the growth of the clones. An equal number of cells from each clone was seeded

Table 3. Enhancement of communication in *cx*43 overexpressor clones

Clone	Junctional transfer[a]	Percent of control
MCA-10	6.5 ± 0.9 (41)	100
D-3	16.4 ± 0.8 (33)	252
D-4	13.3 ± 0.9 (36)	204
D-5	14.1 ± 0.9 (33)	217
D-6	17.8 ± 1 (37)	274
D-7	9.9 ± 0.8 (38)	152
D-8	9.3 ± 0.3 (34)	143
N-2	4.9 ± 0.5 (21)	75
N-3	5.2 ± 0.4 (42)	80
N-4	6.4 ± 0.6 (40)	98

[a] The number of fluorescent cell neighbors 1 min after injection of the Lucifer tracer into test cell; mean ± SE. The total number of injection trials from three dishes each are shown in parentheses. (There were no significant differences between the means of the individual dishes.) The MCA-10 cells and clones were grown in parallel and all were probed for communication on two consecutive days.

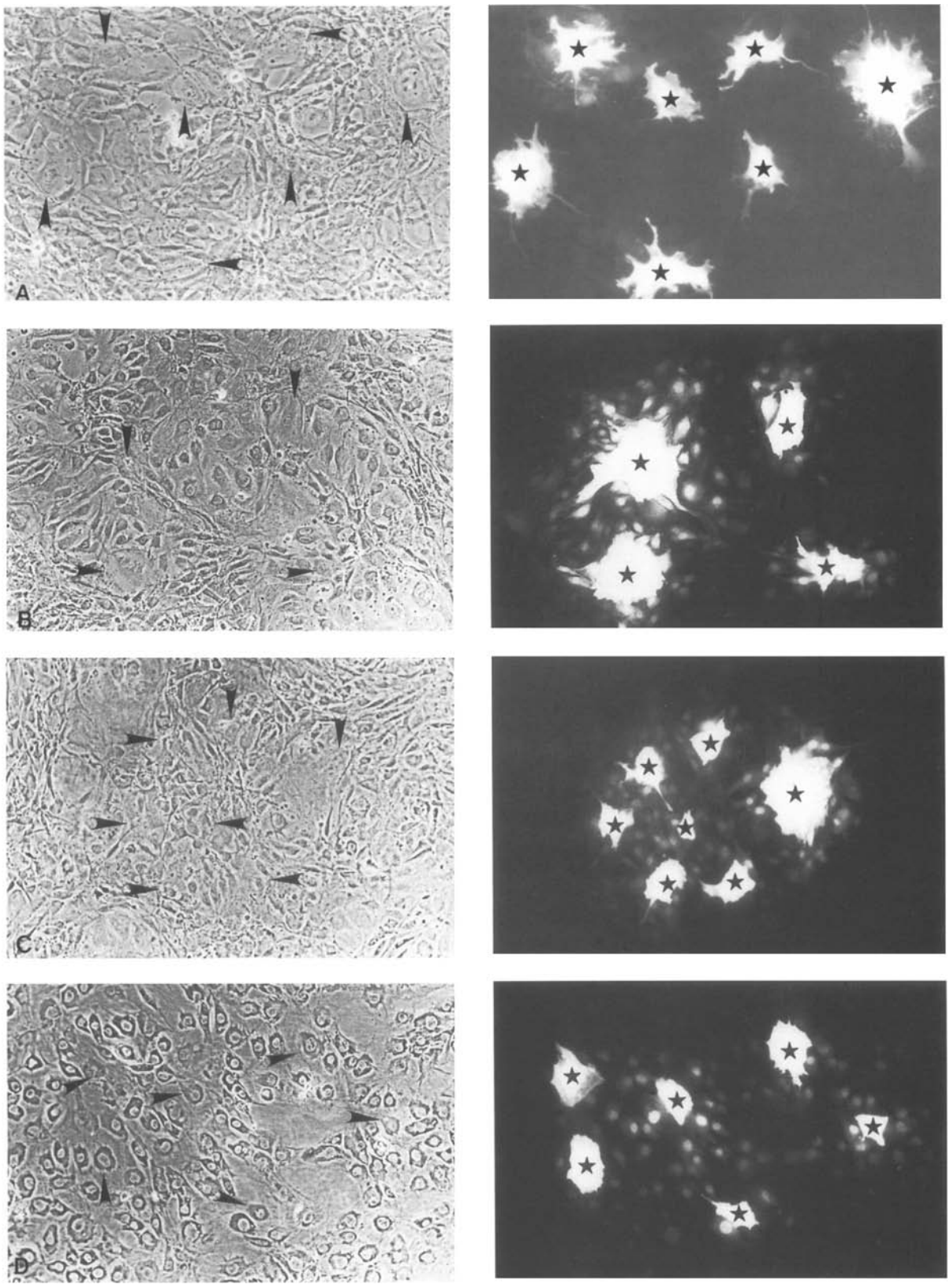

Fig. 7. Enhancement of communication in *cx*43-overexpressor clones. Samples from three overexpressor clones (D-3 to D-5). Cells were microinjected in rapid succession (6–7 cells/min) with the fluorescent tracer Lucifer Yellow, and the cell-to-cell spread of the tracer is shown in the fluorescence images (*right*) 1 min after the last injection. Injected cells are marked by arrows in the phase contrast

Table 4. Growth inhibition of *cx*43 overexpressor clones

Expt	Clone	2×10^4 Cells seeded		5×10^4 Cells seeded		10^5 Cells seeded	
		Cells/dish ($\times 10^5$)	Inhibition[a] (%)	Cells/dish ($\times 10^5$)	Inhibition[a] (%)	Cells/dish ($\times 10^5$)	Inhibition[a] (%)
A	MCA-10	19 ± 1.8 (5)	0	21.4 ± 0.8 (3)	0	17.6 ± 2.6 (3)	0
	D-1	13.2 ± 0.8 (5)	31	14.8 ± 0.3 (3)	32	12.1 ± 0.7 (3)	33
	D-2	12.7 ± 0.4 (5)	34	14 ± 0.6 (3)	35	12.2 ± 0.6 (3)	33
B	MCA-10			17.7 ± 0.4 (3)	0		
	D-3			7.1 ± 0.3 (3)	62		
	D-4			5.8 ± 0.7 (3)	69		
C	MCA-10	20.5 ± 0.3 (2)	0	20.9 ± 1.9 (2)	0	21.4 ± 1 (2)	0
	D-5	3.5 ± 0.3 (2)	84	2.3 ± 0.1 (2)	91	3.9 ± 0.3 (2)	86
	D-6	10.1 ± 0.6 (2)	51	8.8 ± 0.6 (2)	59	10.5 ± 1.1 (2)	53
	D-7	8.8 ± 3.5 (2)	58	5 ± 0.2 (2)	78	6.9 ± 0.9 (2)	71
	D-8			11.4 ± 0.1 (2)	47	9.3 ± 0.9 (2)	59

Cells were seeded on day 0 at the three densities indicated, and cell counts were taken on day 13 for seedings of 2×10^4; on days 8, 7 and 8 for seedings of 5×10^4 in *A*, *B* and *C* respectively; and on day 7 for seedings of 10^5 cells/dish. Medium (not containing G418) was changed on days 4 and 9. The data *A*, *B* and *C* are from three sets of experiments, each set with its own MCA-10 control. Data are means ± SE from the number of dishes listed in parentheses.

There were no significant differences between the dishes.

[a] For determination of growth inhibition, the number of cells seeded is subtracted from the counts, thus inhibition = $\{1 - [(x - a)/(y - a)]\} \times 100\%$ where a is the number of cells seeded; x, the number of cells/dish of the overexpressor clone; and y, the number of cells/dish of the corresponding MCA-10 control. Sample cultures from this experiment for cell seedings of 5×10^4 cells are shown in Fig. 10*A* and *B*.

and allowed to grow for a specified period, at the end of which individual cell counts were taken. All *cx*43-overtranscribing clones exhibited significant growth inhibition; the number of cells was reduced by 31–91%, compared to the uninfected MCA-10 controls. The inhibition was evident at various seeding densities, and the hierarchy of the clones regarding inhibition was rather well maintained over the whole density range (10^4–10^5 cells/dish). Table 4 presents the data from eight *cx*43 vector-transcribing clones (D-1 to D-8) which include the six clones whose communication had been tested (the data are from four sets of parallel experiments, each set with its own uninfected MCA-10 control).

Representative culture dishes from these experiments are shown in Fig. 10*A*, 1st and 3rd columns, where the dishes of clones D-5 to D-8 show much fewer foci and less stain (i.e., fewer cells) than the MCA-10 dishes. In contrast, the growth of clones infected with *neo* control vector was not much different from the MCA-10 controls (Fig. 10*B*, left column).

The preceding data give measures of the growth inhibition for a single time point. In addition, we followed the whole time course of the growth of the two most growth-inhibited *cx*43 overexpressor clones, D-5 and D-7. Inhibition was pronounced by day 4, and the growth leveled off at significantly lower cell densities than with the MCA-10 (saturation densities) (Fig. 8*A*). The difference in saturation density was even more pronounced when the cells were grown in low (1%) serum medium. Clone D-5, for example, reached saturation density on day 4, whereas the MCA-10 controls were still growing by day 10 (Fig. 8*B*). On the other hand, the growth curves of the clones N-1 and N-2, infected with *neo* alone, were not significantly different from that of MCA-10 cells (Fig. 8*C*).

and by stars in the fluorescence photos. In the parental transformed MCA-10 cells (*A*) the tracer spread only to a few first-order neighbors. In the overexpressor clones D-3 (*B*), D-4 (*C*) and D-5 (*D*), the spread is extensive, encompassing all first-order neighbors and many higher-order ones. Note also the much higher fluorescence level in first-order neighbors of the overexpressor clones. That level is so high that the fluorescence image of first-order neighbors in some of the injections is as bright as that of the injected cell; the images are "fused" into one. In contrast, in parental MCA-10 cells the first-order neighbor fluorescence is barely visible (*A*). On the *left*, the cells are shown in phase contrast. Note the morphological normalization of the *cx*43-overexpressor cells, in particular of D-5 clone (*D*): cells are flatter, lack the multiple fine processes typical of the parental MCA-10 cells, and rarely grow on top of one another. (Cells here were fixed in 4% phosphate-buffered formaldehyde, 1 min after the last injection and photographed directly through the microscope to display the results of several injections in one frame.)

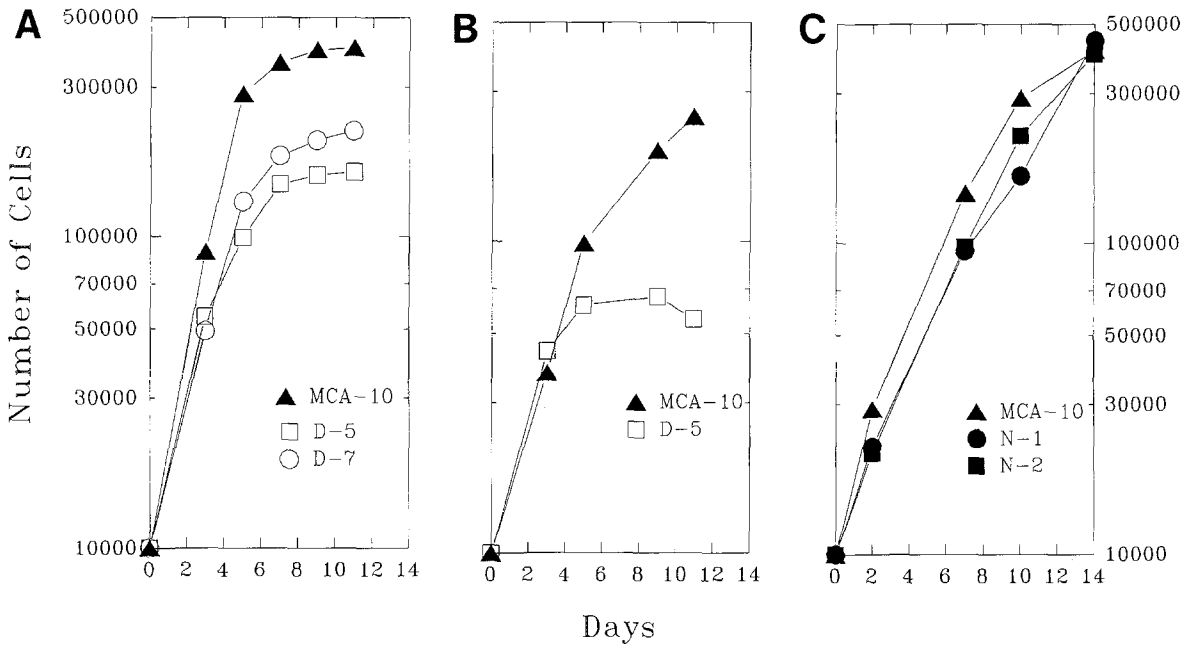

Fig. 8. Growth curves of *cx*43 overexpressor clones D-5 (squares) and D-7 (circles) and of the parental MCA-10 (filled triangles) in (*A*) 5% serum and (*B*) 1% serum. (*C*) Growth curves (5% serum) of two clones infected with *neo* vector alone: N-1 (filled circles), N-2 (filled squares); MCA-10 (filled triangles). In each case, 10^4 cells/dish were seeded on day 0; medium was changed every fourth day. Cell counts are averages of two dishes each. The cultures in each set of experiments (*A*, *B* and *C*) were grown in parallel, each set with its own MCA-10 control.

The growth inhibition was manifest mainly in the saturation density, but not in the rate of growth during the first three days when the cultures were still sparse and few cells were in contact. This is the expected behavior if junctional communication, which requires cell contact, is the controlling variable of growth here.

We note that for all tests of growth and communication the cells were grown in medium without geneticin to avoid possible side effects of this agent.

Apart from growth, the *cx*43 overexpressor clones also were morphologically different from the parental MCA-10 cells: the overexpressor cells tended to grow side by side, rather than on top of one another as the parental cells did. Moreover, they rarely displayed the fine processes typical of the parental cells (Fig. 7, fluorescent images).

Growth is Further Suppressed as the Communication of the Overexpressor Clones with Normal Cells in Coculture is Enhanced

We next examined the ability of the *cx*43 overexpressor clones to communicate with normal $10T\frac{1}{2}$ cells (heterologous communication). Here we used cocultures where the overexpressor cells were seeded on top of a confluent layer of normal $10T\frac{1}{2}$ cells. In this coculture condition the parental MCA-10 cells establish a low level of communication with the $10T\frac{1}{2}$ cells, and their growth, like that of a number of other transformed cells capable of heterologous communication, becomes inhibited to some extent (Mehta et al., 1986). So, the question was whether the heterologous communication would be enhanced by the *cx*43 overexpression and whether such enhancement of communication would lead to further growth inhibition.

Figure 9 gives samples of the probings of heterologous communication. 2×10^4 overexpressor cells, labeled with the lipid carbocyanine marker di-I (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; red fluorescent) for identification, had been seeded the day before over a layer of $10T\frac{1}{2}$ cells. To test communication, Lucifer Yellow (yellow-green fluorescent) was injected into overexpressor cells and its transfer to the normal cells was determined 2 min after injection. The heterologous junctional transfer was markedly enhanced by the *cx*43 overexpression; the Lucifer tracer was transferred to many normal cell neighbors, a pattern which stood in sharp contrast with the restricted transfer from the parental MCA-10 cells. Five overexpressor clones (D-3 to D-7) and four *neo* control clones (N-2 to N-5) were tested this way. In all cases

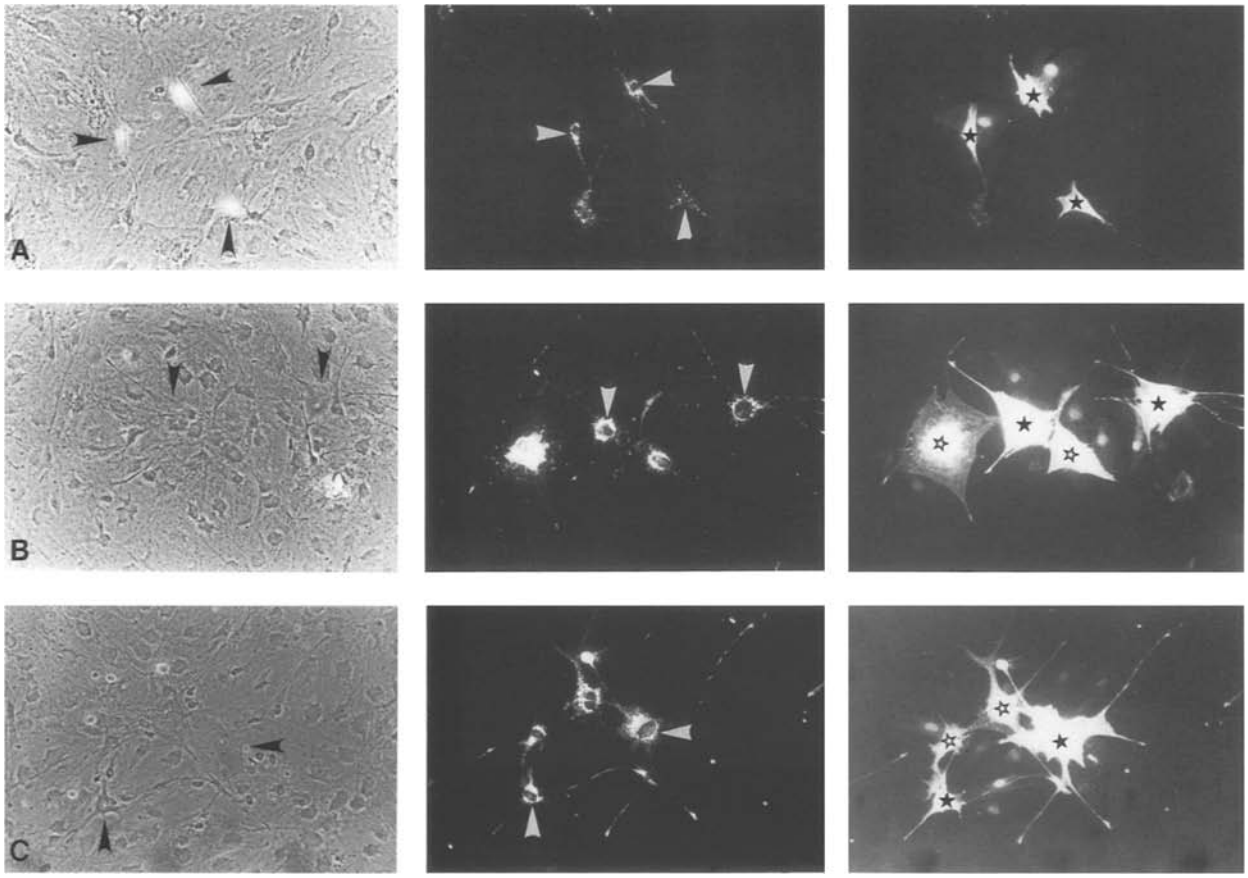

Fig. 9. Enhancement of communication between *cx*43 overexpressor cells and normal 10T½ cells (heterologous communication). 2×10^4 control MCA-10 cells (*A*) or cells from the overexpressor clones D-3 (*B*) and D-5 (*C*) labeled with the red-fluorescent lipid marker di-I were seeded on top of a confluent layer of normal 10T½ cells. On the following day, heterologous communication was tested by injecting Lucifer Yellow into the labeled cells. *Left:* phase contrast images of the cocultures. *Middle:* fluorescence images showing the di-I labeled cells. *Right:* fluorescence images showing spread of the green-fluorescent Lucifer tracer. The Lucifer tracer was injected in rapid succession into several cells in the microscope field. Two min after the last injection, the cells were fixed. The injected cells are marked by arrows in the left and middle panel, and by filled stars in the right panel. In the overexpressor clones Lucifer tracer has spread to many normal cell neighbors (as well as to homotypic overexpressor neighbors marked by open stars), whereas in the control MCA-10 cells, the tracer spread to only one or two normal cell neighbors. In *A* the blue excitation light was left on during photographic exposure to highlight the injected MCA-10 cells in the phase contrast image. Note that the morphology of the cells in coculture—the various overexpressor clones as well as MCA-10 cells—differs from that in solo culture (*compare* with Fig. 7).

the level of communication, as given by the number of normal cells to which the tracer was transferred, was much higher in the overexpressor clones than in the *neo* controls or MCA-10 cells (Table 5).

As in our probings of homologous communication, all data tabulated were from strictly parallel experiments. In addition, clones were probed individually not in parallel conditions. Invariably, heterologous communication in the overexpressor clones was greater than in the MCA-10 or *neo* controls (*data not shown*).

This enhancement of heterologous communication went hand in hand with a dramatic enhancement of heterologous growth inhibition. The inhibition was plainly visible in cocultures of a week or older. The MCA-10 or *neo* controls by this time had formed numerous and sizeable colonies on the 10T½ cell layer, whereas the overexpressor clones had formed few or none at all (Fig. 10*A* and *B* cocultures). The heterologous growth inhibition of the overexpressor cells was much more pronounced—in some cases so severe as to arrest growth completely (*see,* e.g., D-5, Fig. 10*A*)—than their self-inhibition in solo culture. A quantitative estimate of this heterologous inhibition was obtained by comparing, in parallel experiments, the number of the overexpressor cells in coculture with those in solo culture. The heterologous growth inhibition in the various clones by the normal 10T½ cells amounted to 64–100% (Table 6; and *see also* Table 6 legend for a definition of heterol-

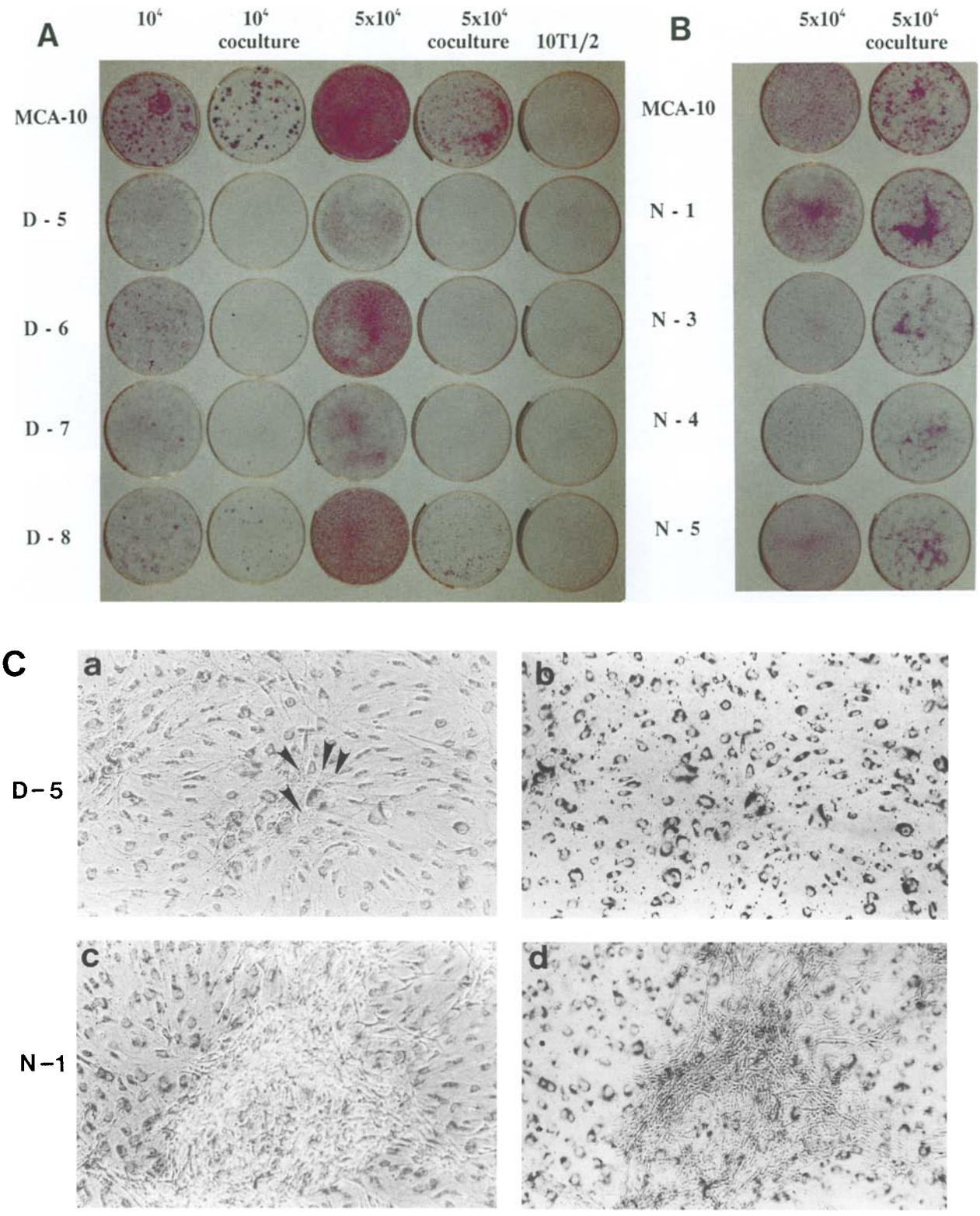

Fig. 10. Growth inhibition of *cx*43 overexpressor clones in coculture with normal 10T½ cells. (*A*) Culture dishes showing colonies of *cx*43 overexpressor clones *D-5* to *D-8* or of the parental *MCA-10* seeded on top of a confluent layer of normal 10T½ cells, and grown for 8 days (seedings of 5×10^4 cells) or 21 days (seedings of 10^4 cells). Shown also are the cultures of the corresponding cells grown in the absence of 10T½ cells (solo cultures; 1st and 3rd columns). In the overexpressor clones *D-5*, *D-6* and *D-7* there are virtually no colonies in the cocultures, and in *D-8* the number and size of colonies are greatly reduced compared to *MCA-10* or to the solo cultures. The 10T½ cell layer is not visible in these photographs; the last column (*10T½*) shows dishes with 10T½ cells alone. (*B*) Corresponding

Table 5. Enhancement of (heterologous) communication between *cx*43 overexpressor clones and normal 10T½ cells

Clone	Junctional transfer[a]	Percent of control
MCA-10	1.1 ± 0.1 (36)	100
D-3	4.4 ± 0.5 (31)	400
D-4	2.9 ± 0.3 (36)	264
D-5	5.5 ± 0.4 (40)	500
D-6	3.6 ± 0.4 (36)	327
D-7	3.8 ± 0.5 (28)	345
N-2	1.5 ± 0.1 (43)	125
N-3	1.8 ± 0.2 (45)	164
N-4	1.3 ± 0.1 (43)	118
N-5	1.5 ± 0.1 (42)	136

[a] Number of normal 10T½ cells to which Lucifer Yellow had spread 2 min after injection into an overexpressor cell or an MCA-10 cell; mean ± SE; in parentheses, the total number of injection trials from three dishes each. MCA-10 cells, *cx*43 overexpressor clones (D-3 to D-7) and neo control clones (N-2 to N-5) were prelabeled with red-fluorescent lipid marker di-I to distinguish them from the normal 10T½ cells (*see* Fig. 9). As in Table 3, all data are from cells grown in parallel and probed on two consecutive days.

ogous inhibition). And it was always greater than that of the MCA-10 or *neo* controls, as one might expect from the overexpressor clones' higher heterologous communication.

Microscopic observations revealed that the *cx*43 overexpressor cells were present and attached on top of the 10T½ cell layer in short-term cocultures (Fig. 9), as well as in long-term coculture, including when the growth inhibition was 100% and there were no colonies visible at all (Fig. 10*C*,*a*,*b*).

Discussion

We show here that the incorporation of the cell-cell channel gene *cx*43 into the genome of a communication-deficient transformed cell type produces functional overexpression of the channel protein; the overexpressed channel protein gets into the junctional membranes and greatly increases permeability there. This enhancement of junctional permeability led to inhibition of cellular growth. The inhibition was manifest both in mass cultures of the cells and in clones. That the inhibition was demonstrable in mass culture is of great moment here. It showed that the inhibition was present in millions of *cx*43-infected cells which, apart from G418 resistance, had not been selected, but it was absent in mass cultures of *neo*-infected controls (Fig. 2). This ruled out the possibility that in our isolation of clones growth-inhibited revertants had been selected whose suppressed-growth trait was independent of communication—an ever present possibility in the case of clonal selection. A demonstration based on clones alone would have required statistical comparisons between large, if not unwieldy, numbers of *cx*43-expressing and *neo*-expressing control clones.

Analysis of the clones showed that the growth inhibition was contingent on the overexpression of *cx*43 and the consequent elevation of communication: the inhibition was present in all clones overexpressing the exogenous *cx*43, but not in the cells infected with the *cx*43 vector pXT1-TK43 that failed to express exogenous *cx*43, nor in cells expressing low levels of endogenous *cx*43, like the clones infected with the *neo* vector.

Thus, the results specifically implicate the cell-cell channel in growth control. They show that the normalization of growth regulation is *uniquely* tied to the communication function of the cell channels, a demonstration of specificity which was heretofore lacking. The channel is shown to play a role here in both homotypic and heterotypic cell interactions. Its sufficient functional expression in transformed MCA-10 cells was associated with growth inhibition when these cells were growing by themselves, and with additional inhibition when they were cocultured with 10T½ cells. The clones with the highest channel expression then behaved just like normal cells: their inhibition was so pronounced that their growth in solo culture leveled off at low cell densities and their growth on the 10T½ cell layer in coculture was completely arrested.

cultures of *neo* controls, 6 days after seeding. Neither solo cultures nor cocultures differ significantly in growth from the parental MCA-10 cultures. *A* and *B* belong to two separate sets of experiments. The cultures in each set were grown in parallel, with their own MCA-10 control. Tables 4 and 6 give quantitative data from the same sets of experiments. Cells were grown in medium without geneticin. (*C*) Higher magnification photos of 8-day-old cocultures of normal 10T½ cells with *cx*43 overexpressor clone D-5 (*a* and *b*) and *neo* control clone N-1 (*c* and *d*). Normal 10T½ cells from a *β-gal* expressing clone were grown to confluence before 2 × 10^4 D-5 or N-1 cells were seeded on top of them. Cultures were fixed 8 days later and reacted with 5-bromo-4-chloro-3-indoyl-β-D-galactoside, a substrate for β-galactosidase that is converted into a blue reaction product by cells expressing *β-gal*. All 10T½ cells, including those underneath N-1 or D-5 cells, show the blue reaction product (cells with darker perinuclear regions in the brightfield images *b* and *d*; phase contrast images of the same cell areas are shown in *a* and *c*). The 10T½ cells have therefore remained as a confluent layer of cells and not been displaced by the overlaying cells. Also evident is that the D-5 cells (some of which are identified in *a* by arrowheads) had, in fact, attached to the 10T½ cells but have grown little or not at all during the 8 days of coculture, whereas the N-1 control cells have multiplied rapidly and formed a focus.

Table 6. Heterologous growth inhibition of *cx*43 overexpressor clones in coculture with normal 10T½ cells

Expt	Cells	2×10^4 Cells seeded	5×10^4 Cells seeded	10^5 Cells seeded
		Inhibition	Inhibition	Inhibition
A	MCA-10	37 (3)	73 (3)	58 (3)
	D-1	73 (3)	73 (3)	64 (3)
	D-2	81 (3)	79 (3)	77 (3)
B	MCA-10		45 (3)	
	D-3		85 (3)	
	D-4		55 (3)	
C	MCA-10	45 (2)	84 (2)	60 (2)
	D-5	100 (2)	100 (2)	97 (2)
	D-6	99 (2)	100 (2)	66 (2)
	D-7	100 (2)	100 (2)	100 (2)
	D-8		98 (2)	72 (2)

Heterologous growth inhibition in coculture, using the corresponding solo culture, seeded and grown in parallel, as comparison standard. Inhibition = $\{1 - [(x - a - b)/(y - a)]\} \times 100\%$ where x is the total cell count in coculture; y, the total cell count (from Table 4) in corresponding solo cultures (x and y are means of the number of dishes indicated in parentheses; there was no significant difference from dish to dish); a, the number of cells seeded, and b, the number of cells in the confluent 10T½ cell layers, as determined by counts of three dishes of 10T½ solo cultures grown in parallel for each experiment *A–C*. The assumption that this (solo culture) 10T½ cell number applies also to the cocultures rests on the observation that 10T½ cells in coculture remained confluent underneath the overlaid cells (*see* Fig. 10*C* and Materials and Methods).

Such normalization of growth is the expected behavior if, as we hypothesize, the cell-cell channels transmit growth-regulating signals that originate within the connected cell population (Loewenstein, 1979). A recently proposed model shows that growth control of such a population can be achieved with minimal cellular differentiation, namely with growth regulatory signals produced at random cell sources scattered throughout the population; the distribution of signal in the population is ruled by the junctional permeability, and so growth is ruled by the number of channels (Nonner & Loewenstein, 1989). In this light one would expect normalization of growth by both homotypic and heterotypic cell interaction, if there are signal sources in both the transformed and normal cell populations; growth control would be restored when the channel deficiency is corrected in the homotypic or heterotypic junctions. Thus, in either cell interaction the extent of the control achieved, that is, the degree of growth inhibition, would increase with the number of (open) channels generated by the exogenous gene and, independent of that gene, with the number of cell sources. In heterologous interaction, the number of cell sources would come in as a factor. That number presumably is higher in normal cell populations than in the transformed ones here, and so the growth inhibition of the transformed cells in coculture with normal cells would be expected to go beyond that in solo culture. Indeed, this was the case in all cocultured overexpressor clones, and in those with the highest heterologous communication, such as D-5, D-6 and D-7, growth was completely arrested (Fig. 10*A* and Table 6).

That result was satisfying also in another way. Our demonstrations of growth inhibition depended on overexpression of the channel protein. This raised the question whether the overexpression by itself had influenced growth; the protein overexpression conceivably might have disturbed the cellular homeostatic balance enough to inhibit growth. Though remote, this possibility needed to be considered, because the overexpression of the channel protein was inherent in our experimental strategy. The results of the coculture experiments where growth of several *cx*43 overexpressor clones was completely arrested, ruled out this possibility: clearly the very same clones, though growth inhibited, were perfectly capable of growing in solo culture; and their extra inhibition culminating in complete growth arrest, correlated with the enhanced heterologous communication—exactly what is expected if heterologous communication is the controlling variable.

The demonstration of a causal involvement of the cell-cell channel in the modulation of the transformed phenotype raises the question of whether the present gene strategy may be useful for cancer

therapy. The evaluation of this prospect, like that of the prospects for other gene therapies, awaits the solution of the technical problem of targeting tumor cells with exogenous genes inside the organism. But it is good to bear in mind the *a priori* limitations of the present gene strategy. Obviously, the strategy is applicable only to the class of cancer cells in which communication is deficient. That class is large (Loewenstein, 1979), but the usefulness would per force be limited to a subclass: cell types in which the expression of the connexin gene, the shuttling of its product to the junction, the assemblage of channels, the open state of the channels, and the responsiveness to the putative signals are not impaired by the transformation. A broad screening of tumor cells eventually should give us an idea of the actual size of that subclass. Meanwhile, it may be useful to mention two cell types which seem to be excluded by evidence already on hand. One such type, a Morris hepatoma H-5123 cell line (Borek et al., 1969), can transcribe exogenous *cx*32 or *cx*43 at high levels, but fails to form open channels (P.P. Mehta, *unpublished results*). Another case, the SKHep1 line, can express exogenous *cx*32 and form open channels, but its growth (solo culture) is not corrected (Eghbali, Kessler & Spray, 1990). The failure of open channel formation in the first case may have been due to many reasons; for example, inappropriate connexin phosphorylation (Crow et al., 1990; Filson et al., 1990; Swenson et al., 1990) or deficient cell adhesion (Loewenstein, 1967; Musil et al., 1990*b*). And the failure of growth correction in the second case may have been due to defective signal production or defective signal responsiveness of the cells. Such negative results are not surprising in view of the aforementioned restrictions of the subclass and the pleïotropic nature of transformation. There also is a report of a positive result: rat C6 glioma cells were found to grow more slowly upon transfection with *cx*43 than the untransfected cells (Zhu et al., 1991). However, it is not clear whether this effect was due to expression of the exogenous connexin gene or to clonal selection of slowly growing revertants; the report was based on two clones expressing the exogenous gene and one that did not express it, and the possibility of revertant selection was not excluded.

Lee, Tomasetto and Sager (1991) have just shown by subtractive cDNA-mRNA hybridization that *cx*26 is expressed in normal human mammary epithelial cells, but not in the tumor counterparts. This finding raises the possibility that *cx*26 is a tumor suppressor gene and provides further support for our hypothesis. The subtractive hybridization procedure and the present gene strategy complement each other well; used together they should provide powerful tools for the analysis of communication-deficient cancer cell types.

Connexin genes are present throughout the phylogenetic tree, starting with some of the early multicellular organisms (Hoh, John & Revel, 1991), and so are cell-cell channels (Loewenstein, 1981). Thus, from early on in multicellular evolution, these channels may have been instrumental in the control of cellular growth by intracellular signals. That control seems to have developed in parallel with that by extracellular signals, the well-known "growth factors." The two modes of control have different regulatory functions, and we are just getting the first glimpses of interactions between them (Azarnia et al., 1988; Maldonado et al., 1988). It will be interesting to see how these interactions tie in with embryonic development and differentiation where—evidence is mounting (Guthrie & Gilula, 1989)—the channels play a role.

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