Negative Growth Control of HeLa Cells by Connexin Genes: Connexin Species Specificity[1]

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ABSTRACT

In order to examine whether different connexin gene species exert different degrees of tumor-suppressing activity, we characterized growth characteristics of a gap junction-deficient human cancer cell line, HeLa cells, before and after transfection with cDNA for three different connexins, connexin (cx) 26, cx 40, and cx 43. All transfected cell lines (3 clones transfected with the cx 26 gene, 2 clones with cx 40, and 1 with cx 43) showed establishment of gap junctional intercellular communication (GJIC). Two of the cx 26-transfected clones showed significantly slower growth compared with the parental HeLa cells. When transfectants were grown in soft agar, the three cx 26-transfected clones grew much less than the other transfectants and parent HeLa cells. When injected into nude mice, the two cx 26 clones which exhibited the highest amount of cx 26 transcript induced almost no tumors, whereas other transfectants, including the cx 26 clone which exhibited the lowest amount of cx 26 transcript, were tumorigenic. Among transfectants of various connexin genes, there was no good inverse correlation between their GJIC and tumorigenicity. GJIC levels were significantly higher in tumors induced in nude mice by clone cx 26 A and E transfectants. These results suggest that all of the connexin genes examined could induce recovery of GJIC of HeLa cells, but only the cx 26 gene exerts strong negative growth control on HeLa cells; thus, this connexin gene may have different functions from other connexin genes.

INTRODUCTION

Deregulation of cell-cell communication is often considered to disrupt homeostasis (1), which results in aberrant development (2) as well as malignant cell growth (3, 4). In particular, GJIC[3] has long been postulated to play an important role in maintenance of homeostasis because it is the only route for direct transfer of cytoplasmic compounds ($< M_r$ 1000) between cells (4–6). The gap junction is a channel composed of two connexons, one from each of two juxtaposed cells. Each connexon consists of six connexin protein molecules (7). Thus far, cDNAs from at least 12 different connexin genes have been cloned in rodents (8). While different combinations of connexins are expressed in different tissues, the biological significance of connexin diversity is not clearly understood (9).

In the past, two lines of evidence have been provided which support a role of disrupted GJIC in carcinogenesis: (*a*) various tumor-promoting agents have been shown to inhibit GJIC (10). This is consistent with the idea that blockage of GJIC can release "initiated" cells from growth control exerted by surrounding normal cells and thus allow their clonal expansion; and (*b*) aberrant GJIC has been shown to be a fairly common characteristic of transformed cells. For example, many cultured tumorigenic rodent (11, 12) and human (13) cells or cells transformed *in vitro* (14) show much lower GJIC ability than their

[3] The abbreviations used are: GJIC, gap junctional intercellular communication; cx, connexin.

normal counterparts. Using a newly developed simple method to determine GJIC in tissue slices, we have shown recently that reduced GJIC is associated with rat liver tumor progression *in vivo* (15). We have also found that human hepatocellular carcinomas have lower GJIC capacity than do their surrounding counterparts *in vivo* (16). The reduced level of GJIC was associated with lower levels of connexin mRNA or aberrant localization of connexin protein, suggesting that both transcriptional as well as post-translational regulation of connexins can be disturbed during carcinogenesis (17).

In addition to aberrant GJIC among tumor cells (homologous GJIC), a lack of GJIC between tumor and normal cells (heterologous GJIC) can be observed. For example, BALB/c 3T3 cells transformed by various agents, including chemical carcinogens and oncogenes, show similar or even higher levels of homologous GJIC but no GJIC with surrounding normal cells (18). Such a heterologous lack of GJIC was also observed in rat liver tumors (15) and human hepatocellular carcinomas *in vivo* (16); in the latter case, tumors were physically separated from surrounding cells due to encapsulation by connective tissue. Lack of heterologous GJIC is considered to help tumor cells maintain their malignant phenotypes by avoiding influence from surrounding normal cells.

More direct evidence for the role of GJIC in negative growth control or as a tumor suppressor element has come from experiments in which connexin genes were transfected into tumorigenic cells. Thus, cx 32 has been shown to retard *in vivo*, but not *in vitro*, growth of human hepatoma cells (19). When cx 43 cDNA was transfected, both *in vitro* and *in vivo* growth of rat glioma C6 (20, 21) and of chemically transformed mouse 10T1/2 cells (22, 23) were retarded. In order to further examine whether connexin genes exert negative growth control in human tumor cells and to determine whether different connexin genes have different abilities to control cell growth, we characterized HeLa cells transfected with cx 26, 40, and 43 cDNAs. HeLa cells do not exhibit extensive GJIC (24), but all transfectants showed recovery of GJIC. However, our results suggest that only the cx 26 gene exerts a strong negative growth control on HeLa cells *in vitro* and *in vivo*, when it is sufficiently transcribed.

MATERIALS AND METHODS

Cell Culture and Connexin cDNA Transfection. HeLa cells were cultivated in Dulbecco's MEM (GIBCO, Paisley, Scotland) complemented with L-glutamine, penicillin-streptomycin (GIBCO), and 10% FCS (Orgenics Ltd, Yavne, Israel). Cell cultures were maintained in a 37°C incubator, under a humidified 5% CO_2 atmosphere, and were routinely subcultured by trypsinization with a change of medium twice weekly. HeLa cells were transfected in the Bonn laboratory using DNA constructs containing the coding DNA of murine connexins 26, 40, and 43 under control of the SV40 early promoter in the pBEHpac18 vector (25). The resulting clones were characterized for expression of exogenous connexins at the mRNA as well as the protein level and used for analyses of homotypic *versus* heterotypic communication by dye transfer.[4] Aliquots of these clones were shipped after initial characterization to the laboratory in Lyon and grown up under conditions described in this paper for the experiments reported below. The selective pressure was maintained for all

[4] C. Elfgang *et al.*, manuscript in preparation.

transfected clones with puromycin (Sigma Chemical Co., St. Louis, MO) at concentrations of 0.5 (cx 26 clones and cx 40 clone A) or 1.0 (cx 40 clone B and cx 43 clone A) μg/ml.

***In Vitro* Dye Transfer Assay to Measure GJIC Capacity in Culture and Tumors.** To determine GJIC of cultured cells, a 5% (w/v) solution of Lucifer yellow CH (Sigma) in 0.33 M lithium chloride was transferred to a glass needle prepared from a capillary tube (A. M. Systems Inc., Everett, WA). Cells were impaled with needles and dye was injected continuously for 0.8 s under air pressure (200–400 hPa), using an Eppendorf microinjector Model 5242 (Hamburg, Germany); after 10 min the intercellular transfer of fluorescent Lucifer

Table 1 *GJIC of the connexin-transfected HeLa clones tested by the dye transfer assay*

	GJIC[a]	Dye transfer (%)[b]
HeLa	1.8 ± 0.3	0.0
Cx 26 A	9.6 ± 1.1	72.7
Cx 26 C	17.0 ± 1.6	80.0
Cx 26 E	6.1 ± 0.4	30.8
Cx 40 A	3.9 ± 0.6	17.4
Cx 40 B	7.0 ± 0.6	42.9
Cx 43 A	15.6 ± 2.3	75.0

[a] Cells (5 × 10⁵) were seeded in 60-mm Petri dishes, and the dye transfer assay was performed 4 days later when cell cultures reached confluence.
[b] Percentage of dye transfer passing beyond the first rank of cells surrounding the microinjected one.

yellow was estimated under an Olympus IMT-2 phase-contrast and fluorescence microscope as described previously (26). For each experimental point, at least 15 microinjections were performed.

The evaluation of GJIC in portions of tumor by microinjection of Lucifer yellow CH solution was performed with 0.5-mm-thick slices prepared from randomly chosen fresh tumor samples with a double-blade knife as described previously (15). Five min after injection, excess dye that did not enter the cells was removed by extensive washing in PBS. The injected slices were then embedded in 7% gelatin solution and frozen in liquid nitrogen. Later, they were cut into 5-μm semiserial sections on a 2800 Frigocut E Reichert cryostat. The sections were fixed by a brief microwave irradiation and examined in an Olympus Vannox T microscope with epifluorescent equipment. For estimation of GJIC permeability, an average of at least five injected spots per slice were selected and micrographed; the pictures were used for subsequent measurement of dye transfer surface area.

Anchorage-independent Growth Assay. This test was performed in soft agar (Agar Noble; DIFCO Laboratories, Detroit, MI) by seeding 10⁴ cells from each line in 4 ml of 2× concentrated and complete Dulbecco's MEM containing 0.3% agar on a solidified (0.5% agar) basal layer (5 ml) in 60-mm dishes. Two weeks after seeding, the colonies containing at least 20 cells were counted in triplicate plates.

Northern Analysis. Total RNA of cell lines and tumors chosen for GJIC estimation was extracted by a single-step technique described as the RNA-zol-B method (Cinnas/Biotecx Laboratory International, Inc., Friendswood, TX). Twenty μg of total RNA from each sample were migrated by
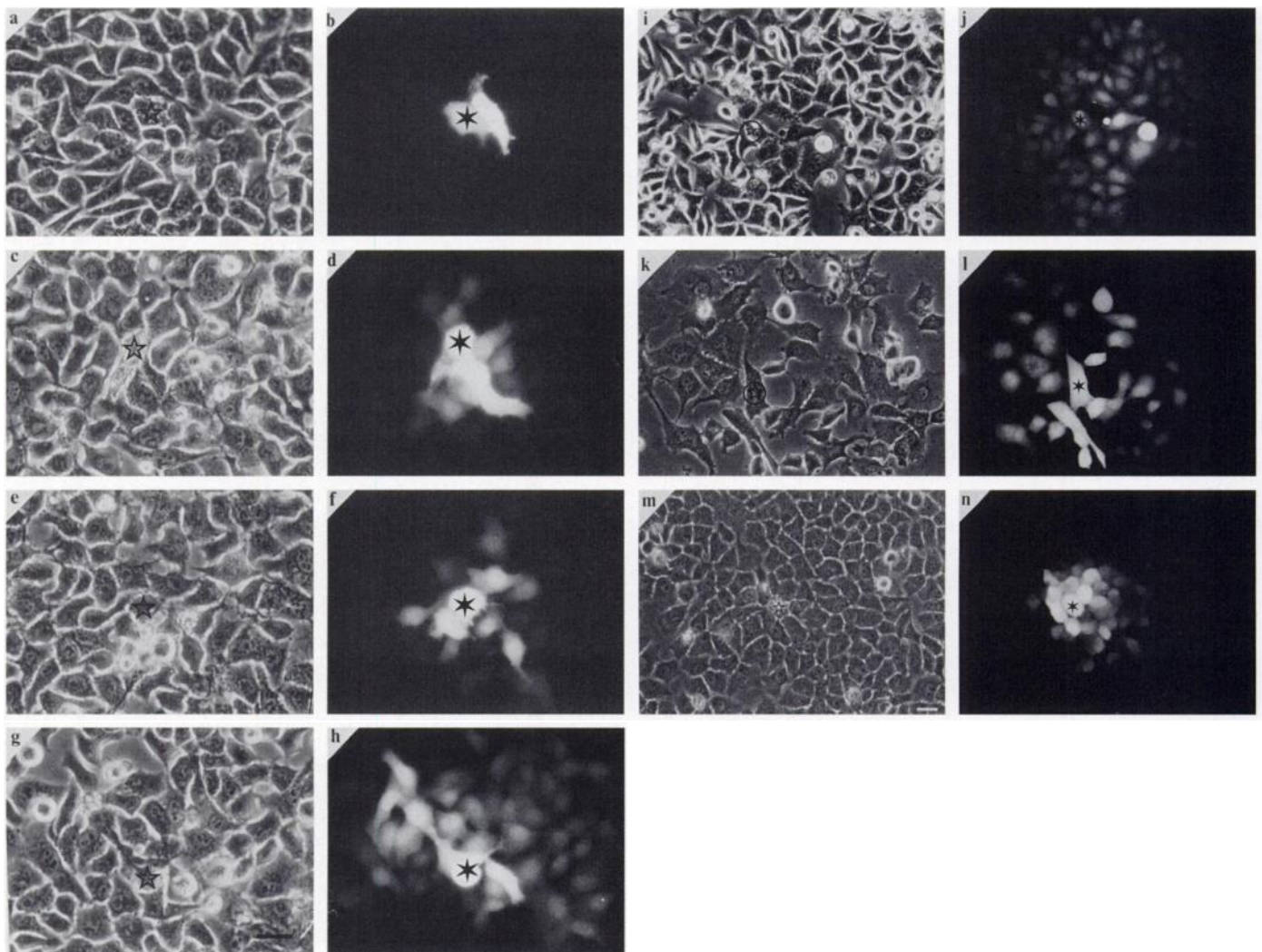

Fig. 1. Estimation of GJIC capacity by dye transfer assay of HeLa cells with transfected connexin genes or without. Lucifer yellow was microinjected into the *starred cells* in phase-contrasted cultures (*a, c, e, g, i, k,* and *m*) and the extent of dye transfer is shown in the corresponding fluorescent micrographs (*b, d, f, h, j, l,* and *n*). *a–b,* Parental HeLa cells; *c–d,* cx 40 A; *e–f,* cx 40 B; *g–h,* cx 43 A; *i–j,* cx 26 A; *k–l,* cx 26 C; *m–n,* cx 26 E clones. *Bar,* 20 μm.

electrophoresis (25 V, overnight) in denaturing formaldehyde agarose gels (1%). Gels were capillary blotted onto Hybond-N$^+$ nylon membranes (Amersham, Buckinghamshire, UK). Blots were pretreated with 50% formamide, 0.05 M Na$_2$HPO$_4$ (pH 7.4), 0.5 M NaCl, 1% SDS, and 10 μg/ml herring sperm DNA for at least 2 h at 42°C. Hybridizations were carried out for 24 h under high stringency conditions (formamide 50%, 42°C) with [γ-^{32}P]dCTP-radiolabeled cDNA probes for cx 26 (27), cx 40 (28), and cx 43 (29), which were prepared by using the rapid multiprime DNA-labeling system (Amersham). Blots were then washed twice in 2× SSC (1 × SSC is 8.8 g NaCl and 4.4 g sodium citrate per liter) and 1% SDS at 65°C for 30 min before exposure at −70°C to Hyperfilm-MP (Amersham) with an intensifying screen.

Western Analysis. The connexin-transfected clones were cultured in 35-mm dishes (Falcon) up to confluence. The confluent cultures were directly

Fig. 3. Western analysis of the connexin-transfected HeLa cells. The protein homogenates extracted from the HeLa clones were prepared as described in "Materials and Methods" and analyzed with the antibodies anti-cx 43 (A), anti-cx 40 (B), and anti-cx 26 (C). Lane 1, cx 43 clone A; Lanes 2 and 3, cx 40 clones A and B, respectively; Lanes 4 and 5, cx 26 clones E and A, respectively. Relative molecular weight (M_r) of proteins detected was determined by comparison with molecular weight standards. Mobility of molecular mass standards is indicated in kilodaltons on the left.

lysed in a sample buffer (62.5 mM Tris-HCl, pH 6.8-0.5% SDS) and the total protein concentration in homogenates was estimated with a protein assay kit (Bio-Rad, Richmond, CA). This assay is based on the principle that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G250 shifts from 465 to 595 nm when binding to protein occurs (30). Once the protein concentration was determined, the homogenate was completed with glycerol (10%, v/v), β-2 mercaptoethanol (1.5%, v/v), and bromophenol blue (2‰), which is used as a marker of electrophoretic progression. Ten μg of total protein extracts from each sample were then loaded on a 15% polyacrylamide gel. After electrophoresis at 100 V for 2 h, the proteins were blotted overnight at 30 V on a nitrocellulose membrane. The blots were incubated with rabbit polyclonal anti-cx 43 (1/2000), -cx 40 (1/500), or -cx 26 (1/500) antibodies for 1 h at room temperature on a shaker. Proteins detected by the antibodies were revealed by luminescence using the ECL system (Amersham). Light emission provoked by this assay was detected by a short exposure to blue-light sensitive autoradiography film (Hyperfilm ECL, Amersham). The antibodies directed against cx 26 and cx 43 were kind gifts from Dr. Y. Shibata (Kyushu University, Kyushu, Japan) and Dr. E. Rivedal (Institute for Cancer Research, Oslo, Norway), respectively. The characterization of cx 40 antibody has been described elsewhere.[5]

Indirect Immunofluorescence of Cultured Cells and Tumors. Cells were seeded on tissue culture chambers (Lab-Tek; Nunc Inc., Naperville, IL), washed with Ca^{2+}/Mg^{2+}-free PBS, and fixed in pure acetone for 5 min at −20°C. They were then washed and incubated in a blocking solution (3% BSA; Sigma) for 30–45 min. Primary rabbit antibodies against connexins were added in PBS to the cells and kept for 1 h at 37°C in a humidified chamber. Incubations with a secondary biotinylated antibody directed against rabbit immunoglobulin (Serva Feinbiochemica GmbH, Heidelberg, Germany) and then with a streptavidin-FITC conjugate (Serva) were both performed for 1 h at 37°C in a humidified chamber. After extensive washing in PBS, the preparations were kept in glycerol-PBS (90/10%) containing p-phenylenediamine (pH 8.0) (Sigma) and stored at −20°C before observation under a fluorescence/phase contrast Olympus Vannox T microscope. Micrographs were taken on Ilford HP5 (ASA 400) film. A similar procedure was used for tumor tissues.

Characterization of the connexin 26 antibody used for the immunocytochemistry analysis has been described previously (31).

Tumorigenicity Assay in Nude Mice. Suspensions of 10^5 and 10^6 parental or transfected HeLa cells in PBS (200 μl) were injected s.c. into the back of
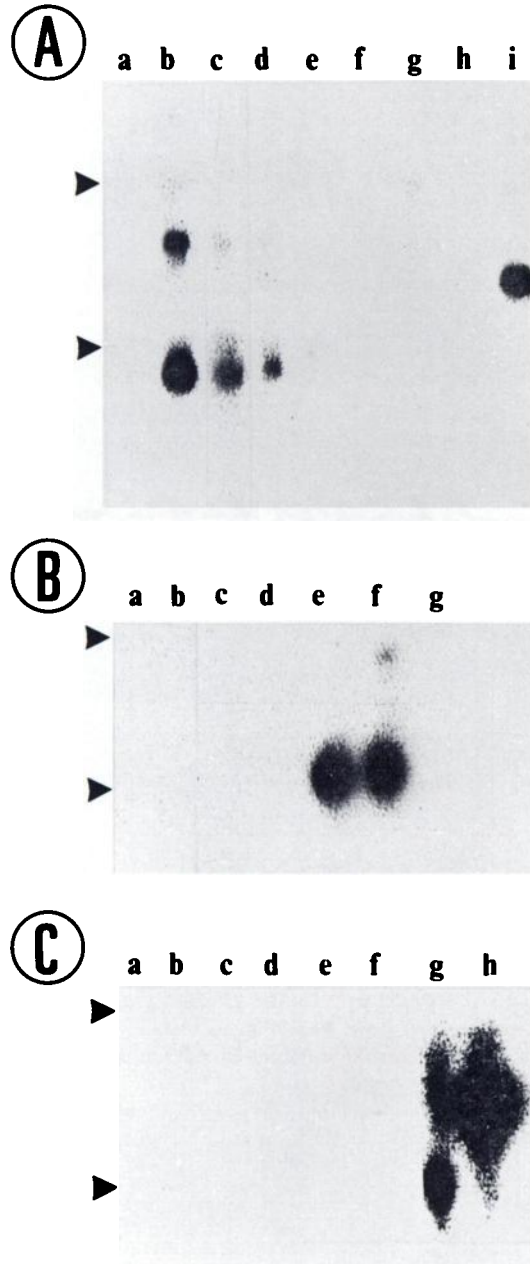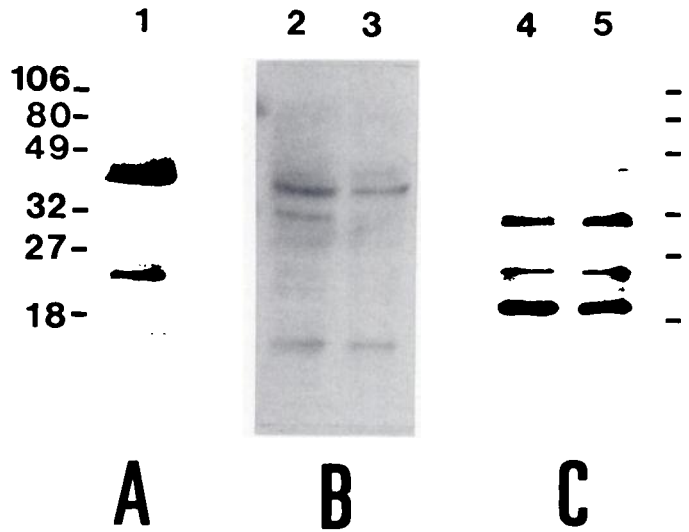
Fig. 2. Northern analysis of HeLa cells. Three blots performed under the same conditions (as described in "Materials and Methods") and containing the same samples were hybridized with radiolabeled probes for cx 26 (A), cx 40 (B), and cx 43 (C). Lane a, parental HeLa cells; Lane b, cx 26 C; Lane c, cx 26 A; Lane d, cx 26 E; Lane e, cx 40 A; Lane f, cx 40 B; Lane g, cx 43 A. Rat heart (Lane h) and rat liver (Lane i) were used as positive controls for cx 43 and cx 26 detection, respectively. No positive controls were used for the detection of cx 40. Arrows, rRNA bands (28 S and 18 S, respectively).

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[5] O. Traub, H. Lichtenberg-Frate, R. Eckert, B. Bastide, C. Elfgang, K. H. Scheidtmann, D. Hulser, and K. Willecke, submitted for publication.

six athymic nude mice (IFFA CREDO, L'Arbesle, France). Three groups of four mice used as negative controls were given, respectively: (*a*) no injection; (*b*) injection of 200 μl PBS; or (*c*) injection (10^6 cells) of the nontumorigenic human keratinocyte cell line (HaCaT) (32). After injection, each mouse was observed individually, and tumor growth was estimated by direct measurement (mm^3) every 2 days. Mice bearing tumors were killed and autopsied. Some tumors were analyzed for expression of connexin and GJIC capacity.

Estimation of GJIC between HeLa Transfectants and Primary Cultures of Skin Cells from Nude Mice. Keratinocytes were harvested from the dorsal epidermis of nude mice (6–9-week-old males) as described previously (11). Briefly, two sacrificed mice were soaked in 70% ethanol for 5 min. The dorsal skins were removed and placed in a beaker of cold PBS containing 2% penicillin-streptomycin. s.c. tissue was scraped off and the nearly translucent skins, cut into pieces, were incubated in a large 150-mm Petri dish (Nunc, Kamstrup, Denmark) containing 50 ml of sterile 0.25% trypsin. The skins were incubated hair-side-up in trypsin for 3 h at room temperature, then put into a Petri dish containing complete MEM-2 medium (GIBCO). The epidermis was carefully scraped off with a sterile scalpel and the thicker dermis was discarded and kept in trypsin under agitation for preparation of dermal fibroblasts. The epidermal material was transferred into a beaker containing the medium and left on a magnetic stirrer for 30 min at room temperature. Keratinocytes were resuspended in the medium by triturating the epidermis several times with a pipette.

Fig. 5. Indirect immunofluorescence staining showing the different locations of cx 26 in the cx 26-transfected clones A (*a*) and C (*b*). (*1*) No primary anti-cx 26 antibody; (*2*) with primary anti-cx 26 antibody; (*3*) the same at higher magnification.

Both keratinocyte and fibroblast preparations were diluted for counting viable cells by the trypan blue exclusion test. One million keratinocytes were seeded onto 60-mm dishes precoated with FAV [fibronectin, albumin, and vitrogen (Collagen Corp., Palo Alto, CA)]. After an attachment period of 24 h, the transfected HeLa clones (10^6 cells) were seeded. For coculture of fibroblasts and HeLa transfectants, fibroblasts (5×10^5 viable cells) were seeded onto 60-mm dishes precoated with FAV, and transfected clones (10^6 cells) were seeded 10 days later. All cultures were performed in complete MEM-2 medium at 37°C in a humidified incubator (5% CO_2). The dye transfer assay was performed in areas where skin cells and transfectants were easily recognized and in close contact.

RESULTS

Gap Junctional Intercellular Communication and Connexin Expression in HeLa Cells Transfected with Connexin cDNAs. Three cx 26-transfected clones (cx 26 A, C, and E), two cx 40-transfected clones (cx 40 A and B), and one cx 43 clone (cx 43 A) of HeLa cells were characterized. All transfected clones acquired extensive dye-coupling capacity, in contrast to the parental cell line. The extent of GJIC varied among transfectants but was not correlated with
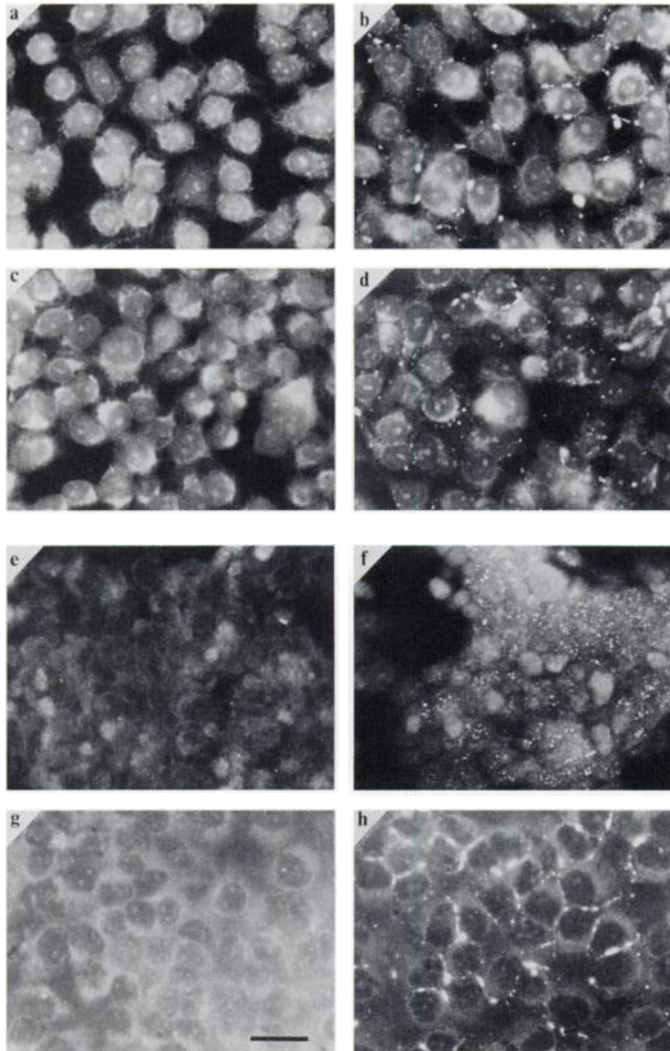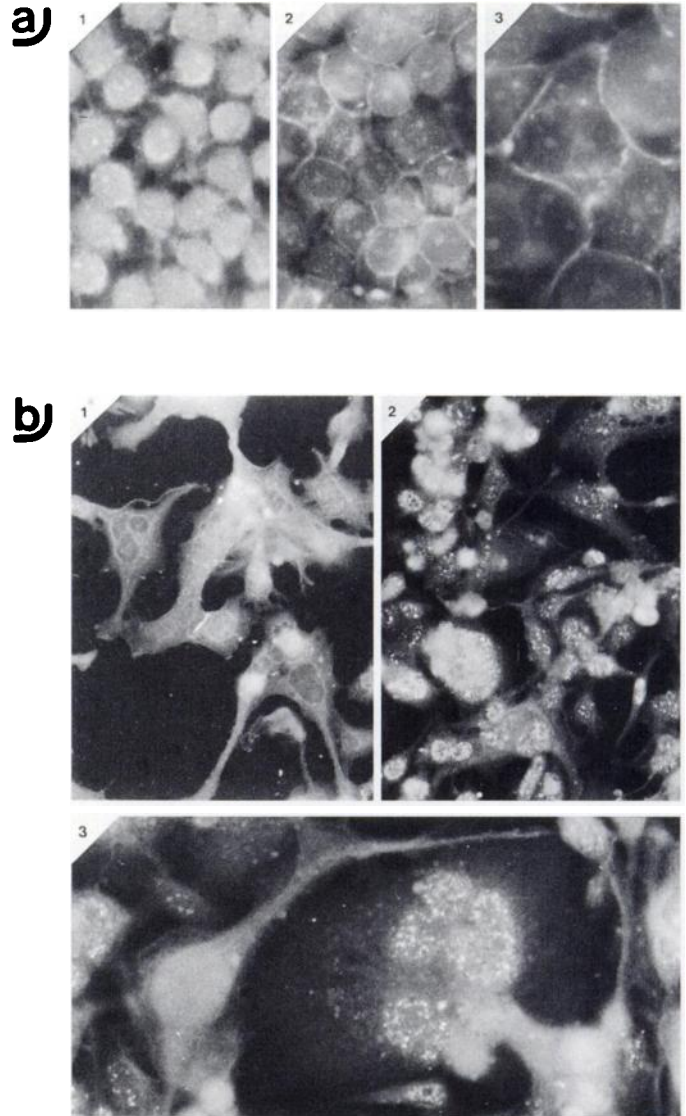

Fig. 4. Indirect immunofluorescence staining showing the expression of the transfected connexins in the HeLa clones. Confluent cultures of HeLa transfectants were incubated (*right*) or not incubated (*left*) with the corresponding anti-cx antibodies. *a–b*, cx 40 B; *c–d*, cx 43 A; *e–f*, cx 26 E; *g–h*, cx 26 A. *Bar*, 20 μm.

the type of connexin expressed (Table 1; Fig. 1); GJIC was highly dependent on the cell growth status (see below). As shown in Fig. 1, the induction of GJIC by connexin gene transfection did not induce morphological change, except that cx 26 C cells were somewhat large, flat, and polynucleated (Fig. 1, k and l).

Northern analysis showed that cx 40 A and B clones expressed similar amounts of a 2–2.2-kilobase transcript as a major cx 40 mRNA, instead of the 3.4-kilobase transcript which has been found to be expressed in normal rat tissue such as lung (28) (Fig. 2B). A lower level of 3.4-kilobase transcripts was found in the cx 40B clone. Two transcripts (3.0 and 1.8 kilobases) were found in the cx 43 transfectants. Similarly, in cx 26 transfectants, two transcripts, one major signal (1.5 kilobases) and another minor (3.0 kilobases), were found in all three clones. The amount of cx 26 transcript increased between clones in the order E < A < C (Fig. 2A).

The small size of the transcripts which were detected by Northern analysis in all transfectants corresponds to the coding sequence of the respective connexin cDNAs plus the SV40 slice and polyadenylation regions (25). Because of their appearent molecular weight, it does not seem that the larger sized transcripts that were also detected in most of the transfected clones correspond to the expression of endogenous connexins. Aberrant sizes of transcripts were frequently reported in connexin-transfected cells (21, 22).

In order to confirm that the connexin transcripts we detected were able to encode for normal sized connexins, we performed a Western analysis of the different clones we studied (Fig. 3). In the cx 43 transfectants, 2 major bands were detected by the anti-cx 43 antibody. The M_r 43,000–47,000 signal is, in fact, composed by 2 bands in which the M_r 47,000 band may be a phosphorylated form of the cx 43 (33, 34). The smaller and weaker M_r 25,000 signal is perhaps a degradation product of the cx 43. Since the major signal detected by the anti-cx 43 antibody corresponds to a normally phosphorylated cx 43, and since we did not detect any other major connexin such as cx
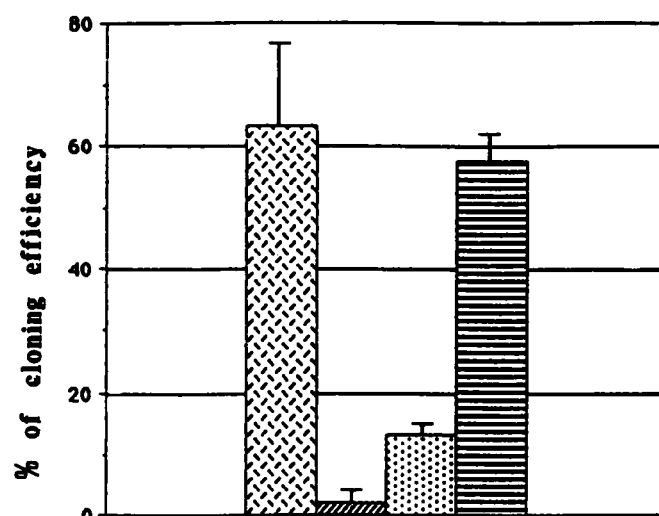

Fig. 7. Cloning efficiency of the cx 26 transfected clones A, ▨; C, ▤; and E, ▆ compared with the parental HeLa cells (criss-crossed column). Cells of each clone (200) were seeded in 60-mm dishes in triplicate, and the number of colonies was counted 10 days later.

26, cx 32, and cx 40 in the cells (data not shown), we conclude that it is the protein encoded by the transfected cx 43 cDNA which is responsible for the dye transfer capacity of these cells. The anti-cx 40 antibody could detect a M_r 40,000 signal only in the two clones transfected with the cx 40 cDNAs (clones A and B) as reported previously (25, 35). A slightly larger amount of the cx 40 was detected in the clone A compared to the clone B (Fig. 3). Minor bands were also detected by the anti-cx 40 antibody as M_r 32,000 and 16,000 signals, which are probably degradation products of the cx 40. No band was detected when similar blots from cx 40 transfectants were exposed to antibodies directed against other major connexins such as cx 26, 32, and 43 (data not shown). The major bands we could detect in two of the cx 26 transfectant clones (clone C was not tested) exhibit a size of approximately M_r 21,000 as expected for the cx 26 (31). The larger sized bands that were detected in both cx 26 clones (M_r 25,000–26,000 and M_r 30,000) are probably dimers and tetramers of the cx 26 protein. Except cx 26, no major connexin (cx 32, 40, and 43) were detected in these two clones. None of the antibodies we used in that study recognized any signal in the nontransfected HeLa cells (data not shown).

In summary, all the clones we tested express a major protein which corresponds in size to the connexins expected to be expressed by the transfected vector. Because of the absence of such connexin in the nontransfected HeLa cells, the connexins we detected are probably responsible for the cell-cell communication capacity that we could only detect in the transfected clones.

Indirect immmunofluorescent staining of transfectants with the appropriate connexin antibodies confirmed the Northern and Western analyses showing that they expressed their respective transfected connexins (Figs. 4 and 5). At the cell-cell contact areas, large spots of connexins were found in the HeLa cells expressing cx 40 and 43, and some intracellular spots were also observed (Fig. 4, a–d). However, the three clones expressing cx 26 exhibited different patterns of cx 26 localization. Most of the cx 26 spots were intracytoplasmic in clone cx 26 E (Fig. 4, e and f), whereas they were localized in spots or thick lines between the cells of clone cx 26 A (Fig. 4, g and h) up to a continuous staining between cells in some areas (Fig. 5a). The cx 26 C cells, which expressed the highest level of cx 26 mRNA, exhibited most of the cx 26 spots in the nuclear region and presumably in the nuclear membrane
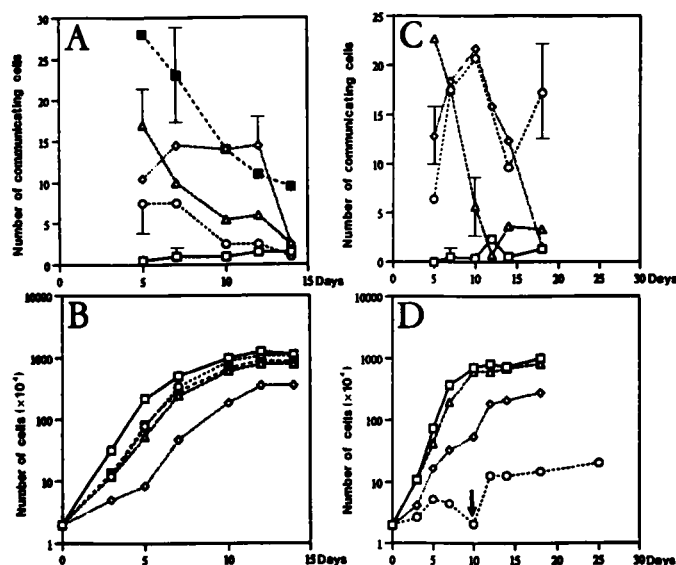

Fig. 6. Comparative study of GJIC (A and C) and cell growth (B and D) of HeLa cells transfected or not with cx cDNAs. Cells (2 × 10⁴) were seeded in 60-mm dishes in triplicate. From day 5, when intercellular contacts were sufficient, GJIC was estimated by the dye transfer technique in one of the three dishes just before trypsinization for counting the cells. (A) Number of communicating cells per microinjection of cx 26 A (◇), cx 40 A (○), cx 40 B (△), cx 43 A (⊠), and the parental HeLa (□) cells. Only the largest SE for each clone is shown, in order not to overload the graph. (B) Numbers of cells (× 10⁴) of the same cultures during 14 days. (C) GJIC and (D) cell growth of the three cx 26 clones (clone A, ◇; clone C, ○; clone E, △), compared as in A and B above with the parental HeLa cells (□). Only the largest SE for each clone is shown in C. The arrow in D shows the shift from 10% to 20% FCS for clone cx 26 C. SEs of the growth curves were not significantly different and are not shown.
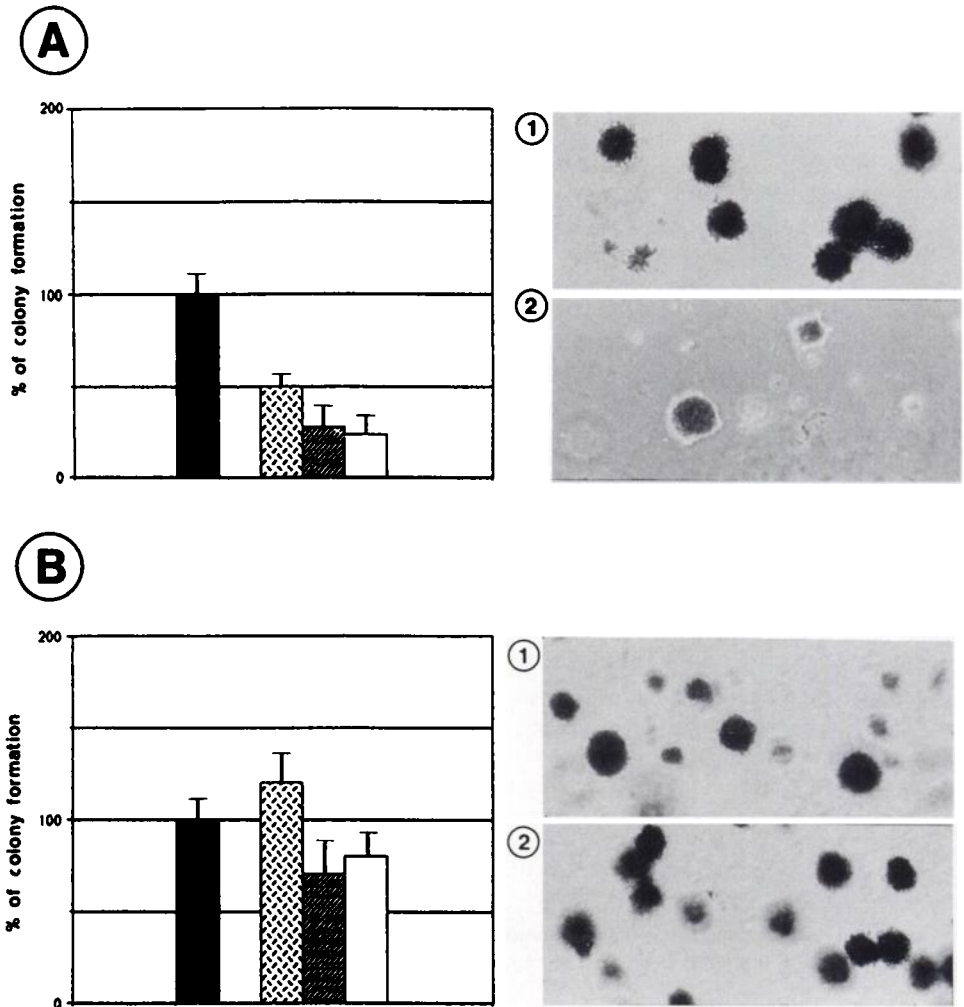
Fig. 8. Anchorage-independent growth capacity of the connexin transfectants. (*A*) Growth capacity in soft agar of the cx 26-transfected clones (*criss-crossed column*, cx 26 E; ▨, cx 26 A; ☐, cx 26 C) compared with the parental HeLa cells (■). The appearance of colonies of parental HeLa cells (*1*) and of cx 26 A transfectants (*2*) is shown. (*B*) Growth capacity in soft agar of the cx 40 (*criss-crossed column*, clone A; ▨, clone B) and cx 43 (☐)-transfected clones compared with the parental HeLa cells (■). The appearance of colonies of cx 43 A (*1*) and cx 40 A (*2*) transfectants is shown. The number of colonies of the transfectants is presented as the percentage of the number of colonies of parental HeLa cells.

(Fig. 5*b*). The reasons for different localizations of cx 26 in the clones A, C, and E are unclear. One possibility could be the selection of particular subpopulations due to the heterogeneity of the HeLa cells used for transfection experiments.

No such signals were detected in the nontransfected HeLa cells. If it is known that no major cx transcript, such as cx 32, 26, or 43, is detected in HeLa cells, even by reverse transcriptase-PCR (36), we cannot exclude the synthesis of other connexin type(s). However, their functional level would be limited compared to the transfected ones, which permit a drastic increase of GJIC in the transfectants.

In Vitro **Anchorage-dependent Growth Capacity of the Connexin Transfectants and Growth-dependent Changes in GJIC.** The effects on cell growth of the GJIC induced by connexin gene transfection was examined. Only cx 26 A and C clones showed much slower growth than did the other transfectants (Fig. 6, *B* and *D*).

When GJIC was measured during different growth phases of transfectants, we found that all clones except cx 26 A and C clones rapidly and permanently lost their GJIC before they reached confluence (Fig. 6, *A* and *C*). The GJIC capacity of cx 26 A and C clones was maintained somewhat longer than the others. However, the GJIC of cx 26 C clone was difficult to estimate since it was measured in small separated colonies because of the slow growth rate of that clone. All these colonies are like different cell populations which may have different communication capacities, as shown by the large variation of GJIC in this clone (Fig. 6*C*).

The loss of GJIC capacity of transfectants at confluence was not associated with a decrease in the level of mRNA of the transfected connexins (data not shown). However, the immunostainable spots of cx 40 and 43 were mostly localized intracellularly in very confluent cultures, and almost no staining was found at the cell-cell contact areas. In the cx 26 A clone, which lost GJIC only at full growth confluence, cx 26 was still localized all around the cells with almost no intracellular spots, even in high density areas (Fig. 5*A*).

The cx 26 C cells, which express the highest amount of cx 26 mRNA, did not grow well when cells were seeded at 2×10^4. However, when the FCS concentration was increased from 10 to 20%, there was a sudden growth stimulation (Fig. 6*D*). These results suggest that the growth capacity of this cell line is modifiable by external factors.

Among the three cx 26 transfectants, the growth ability of the clones seems to be inversely correlated with the level of the cx 26 mRNA (Fig. 2) and protein (Fig. 3) amounts. However, there is no correlation between the growth behavior of the cx 26 transfectants and their ability to communicate (Fig. 6*C*). The cx 26 A clone was tested in two different sets of experiments, as seen in Fig. 6, showing that the characteristics of that clone were stable.

Since the above results indicated a possible role of the cx 26 gene in negative growth control of HeLa cells, we examined the cloning efficiency of clones cx 26 A, C, and E. After seeding 200 cells, we observed that cx 26 E showed a cloning efficiency (58%) similar to that of the parental HeLa cells (64%). The other clones, however,

showed very low cloning efficiency [2% for the cx 26 A and 13% for the cx 26 C cells (Fig. 7)]. These results suggest that the growth of cx 26 A and cx 26 C cells is negatively controlled at clonal as well as at higher cell densities. However, it is difficult to argue whether some differentiation process has been initiated in the slowly growing colonies of cx 26 A and C transfectants. At least no obvious morphological changes were induced.

Anchorage-independent Growth Capacity of the Connexin Transfectants. In order to examine whether connexin genes alter anchorage-independent growth capacity of HeLa cells, we compared their ability to grow in soft agar (Fig. 8). All the clones expressing cx 26 had much lower ability to grow in soft agar compared with the parental HeLa cell line (Fig. 8A). The degree of reduced growth ability in soft agar among the three cx 26-transfectant clones was related to their anchorage-dependent growth ability; *i.e.*, the growth rate decreased in the order cx 26 E > cx 26 A > cx 26 C.

HeLa cells transfected with the cx 40 or cx 43 genes grew in soft agar better than cx 26 transfectants (Fig. 8B).

***In Vivo* Growth (Tumorigenicity) of the Different Connexin Transfectants.** In order to determine whether the expression of connexins decreases their tumorigenicity, we injected 10^5 or 10^6 transfected cells s.c. into nude mice. With 10^6 cells, tumors were produced within 1 month from all clones, except those expressing high amounts of cx 26, *i.e.*, cx 26 A and C. However, the tumorigenicity of the cx 40 and 43 transfectants was considerably diminished (2/3) when 10^5 cells were injected. One of 12 mice given injections of 10^6 cells of clone cx 26 A developed a tumor in the second month. The other mice injected with 10^6 or 10^5 cells of cx 26 A or cx 26 C clones showed no tumor up to at least 12 months (Table 2).

After injection of 10^5 cells of transfected clones, all transfectants except the cx 26 E clone produced less tumors than did the parental HeLa cells (Table 2). There was no correlation between GJIC capacity tested *in vitro* and tumorigenicity among these cells.

Fig. 9 shows the *in vivo* growth rate of the various transfected cells and the parent HeLa cells. Most transfectants, except the cx 40 B and
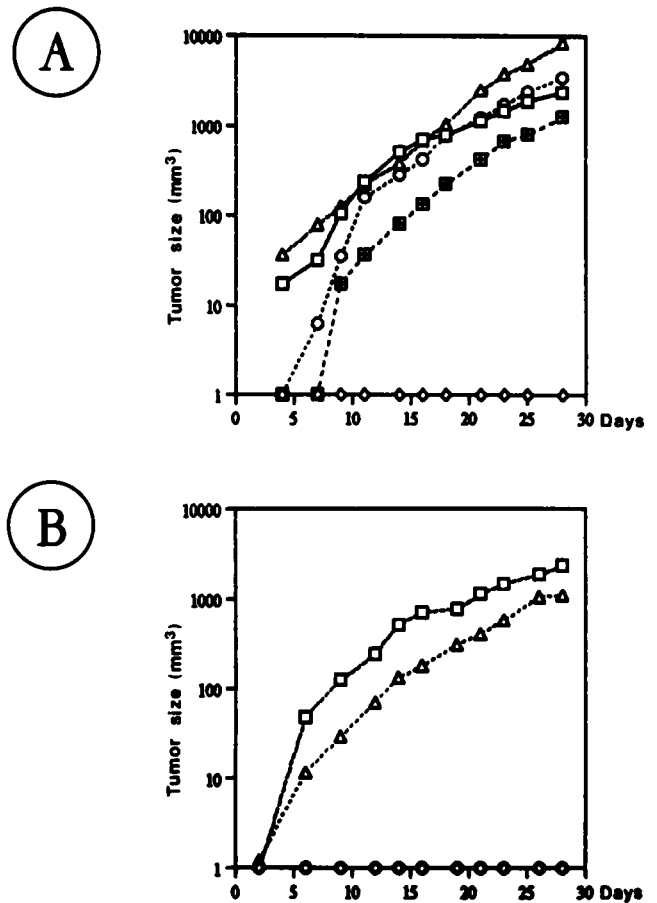

Fig. 9. *In vivo* growth of 10^6 HeLa cells transfected or not with cx cDNAs. The average of the tumor size (mm3) was estimated at the indicated days following the injection of 10^6 cells in the mice. (A) □, parental HeLa cells; ◇, cx 26 A; ○, cx 40 A; △, cx 40 B; ⊠, cx 43 A. (B) □, parental HeLa cells; ◇, cx 26 A; △, cx 26 E; ○, cx 26 C. For clarity, the SEs are not shown.

Table 2 *Tumorigenicity in nude mice of the HeLa clones expressing various types of connexins*

Experiments 1 and 2 were performed under the same conditions; however, the purpose of experiment 1 was to compare the tumorigenicity of transfectants expressing various types of connexin, while that of experiment 2 was just to compare the clones expressing Cx 26.

Clone	Tumorigenicity[a] (%)	Necrosis (%)
For 10^6 cells injected		
Experiment 1		
HeLa	6/6 (100.0)	33.3
Cx 26 A	1/6 (16.7)	0.0
Cx 40 A	6/6 (100.0)	16.7
Cx 40 B	6/6 (100.0)	16.7
Cx 43 A	5/6 (83.3)	60.0
Experiment 2		
Cx 26 A	0/6 (0.0)	
Cx 26 C	0/6 (0.0)	
Cx 26 E	6/6 (100.0)	0.0
For 10^5 cells injected		
Experiment 1		
HeLa	5/6 (83.3)	20.0
Cx 26 A	0/6 (0.0)	
Cx 40 A	2/6 (33.3)	0.0
Cx 40 B	2/6 (33.3)	0.0
Cx 43 A	2/6 (33.3)	0.0
Experiment 2		
HeLa	4/5 (80.0)	25.0
Cx 26 A	0/6 (0.0)	
Cx 26 C	0/6 (0.0)	
Cx 26 E	5/6 (83.3)	0.0

[a] Number of mice bearing a tumor/number of injected mice.

possibly cx 40 A clones, grew more slowly than the parent cells (Fig. 9A). Thus, similarly to the growth behavior *in vitro*, the *in vivo* growth of HeLa cells, expressing a sufficiently high amount of cx 26, was decreased drastically. The autopsy of the animals bearing a tumor did not reveal any metastasis.

A possible explanation for why highly communicating transfectants form tumors is that they may have lost their GJIC ability in nude mice. In order to test this hypothesis, we determined GJIC in tumors produced by transfectants. We found that most tumors showed measurable levels of GJIC. However, when we compared levels of GJIC among various transfectants, all tumors produced by cx 26 transfectants, including the unique tumor of cx 26 A, showed higher levels of GJIC than did other transfectants with cx 40 or cx 43 and parental cells. Results for the cx 26 A tumor in comparison to a HeLa tumor are shown in Fig. 10. As shown, the cx 26 transcript amount, as well as the immunostaining from the cx 26 A tumor, was weaker than in the cx 26 A cells. This tumor could have been raised from a subpopulation of the cx 26 A cells producing less cx 26 that could have facilitated its growth *in vivo*.

***In Vitro* Test of Selective Communication between Transfectants and Skin Cells from Nude Mice.** It has long been hypothesized that a lack of tumorigenicity could be due to extensive cell-cell communication between transformed cells and their normal counterparts. To attempt to explain the lack of tumorigenicity of clone cx 26
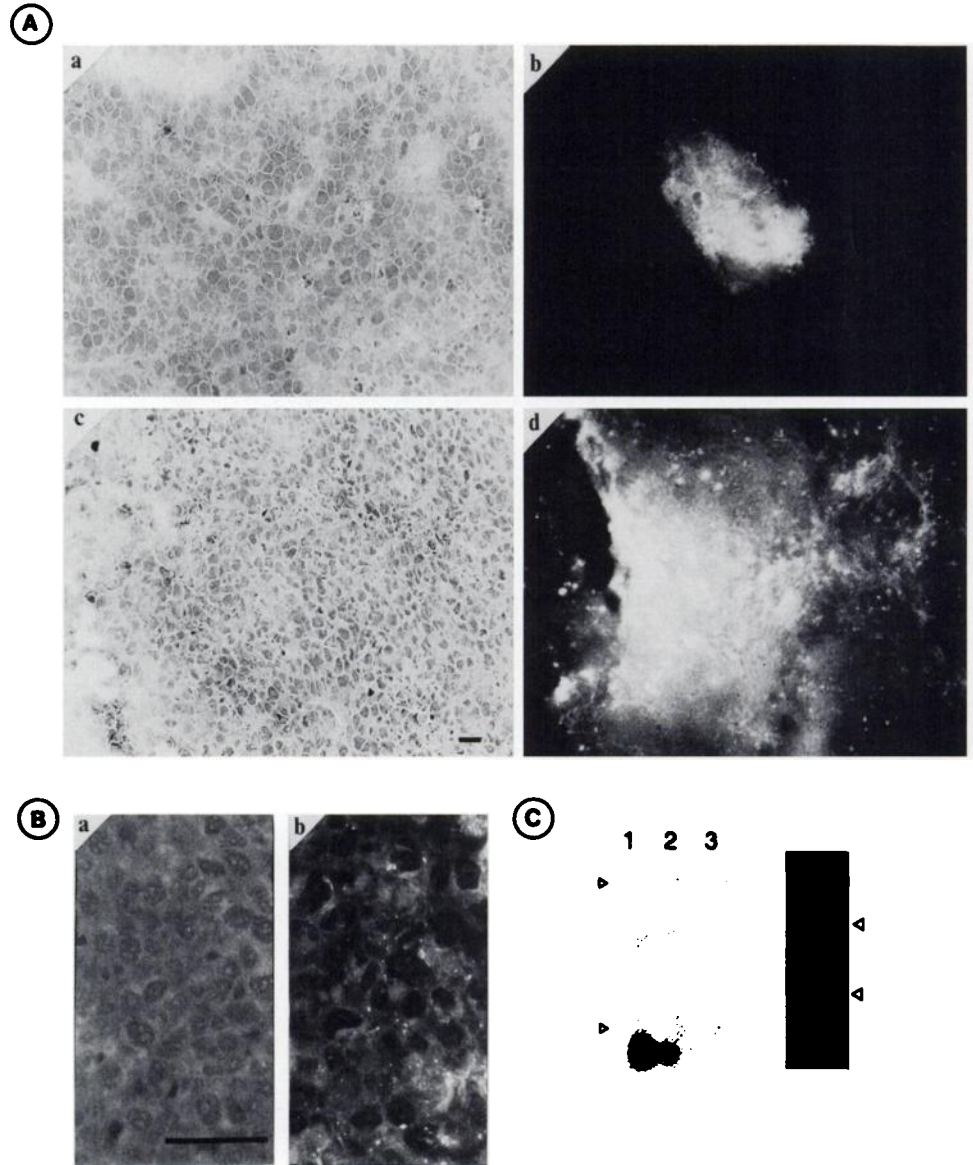
Fig. 10. Estimation of GJIC by the *in situ* dye transfer assay and of cx 26 expression in the cx 26 A tumor. (*A*) *In situ* GJIC of HeLa (*a* and *b*) and cx 26 A (*c* and *d*) tumors. *a* and *c*, micrographs of hematoxylin- and eosin-stained tumor sections. *b* and *d*, fluorescent micrographs showing the extent of dye transfer in parallel sections. (*B*) Indirect immunofluorescent staining of cx 26 in the cx 26 A tumor. *a*, without primary anti-cx 26 antibody; *b*, with primary anti-cx 26 antibody. (*C*) Northern analysis of the same tumors showing the presence of cx 26 mRNA in the cx 26 A tumor but not in the HeLa tumor. *Lane 1*, cx 26 A clone used as a positive control; *Lane 2*, cx 26 A tumor; *Lane 3*, HeLa tumor. *Right*, ethidium-bromide-stained gel shows that a similar amount of total RNA was loaded; *arrows*, rRNA bands; *bar*, 30 μm.

A, we cocultured this clone with primary cultures of keratinocytes or skin fibroblasts of nude mice. Cocultures were carried out in specific conditions (as mentioned in "Materials and Methods") to maintain keratinocytes for this short experiment (11). As shown in Fig. 11, no extensive dye transfer was observed from the cx 26 transfectants to skin fibroblasts or keratinocytes, or *vice versa*. We performed similar experiments with cx 40 and cx 43 transfectants and found that none of them were able to communicate with these cell types *in vitro*. Intriguingly, we did detect some transfer of Lucifer yellow from the skin fibroblasts to the cx 43 transfectants but not *vice versa*. Unidirectional dye transfer between other heterologous cell types has been reported recently (37). Thus, the lack of tumorigenicity of cx 26 A cells cannot be explained by extensive communication with the surrounding mouse skin cells, as tested by the dye transfer assay, but seems rather to be the consequence of a decreased growth ability of the cells due to high expression of cx 26.

DISCUSSION

We have found that expression of cx 26, but not of cx 40 and cx 43, exerts a potent negative growth control of HeLa cells *in vitro* as well

as *in vivo*. However, regardless of the type of transfected connexin (cx 26, 40, and 43), the extent of cell-cell communication was approximately the same in all HeLa transfectants. These results suggest that there may be qualitative differences in GJIC mediated by different connexin species. It has already been reported that different electrical coupling capacities exist between gap junctions mediated by different connexins (38). Different pore diameters in the connexons, different electrical charges of the connexin molecules, or post-translational modifications of the conformation of the connexins may also be responsible for subtle qualitative changes in cell-cell communication which cannot be visualized through a dye transfer assay. Such changes would be of fundamental importance when considered at both cellular and molecular levels.

Different connexin species or combinations of them are expressed in different tissues and/or cell types, suggesting that connexins have cell type-specific roles (39). This may partially explain why cx 26, but not cx 40 or cx 43, exerts a tumor-suppressing effect on HeLa cells. It is possible that the functions of the cx 40 or cx 43 proteins are not compatible with negative growth control of HeLa cells. Results from other laboratories appear to support this idea. For example, it has been
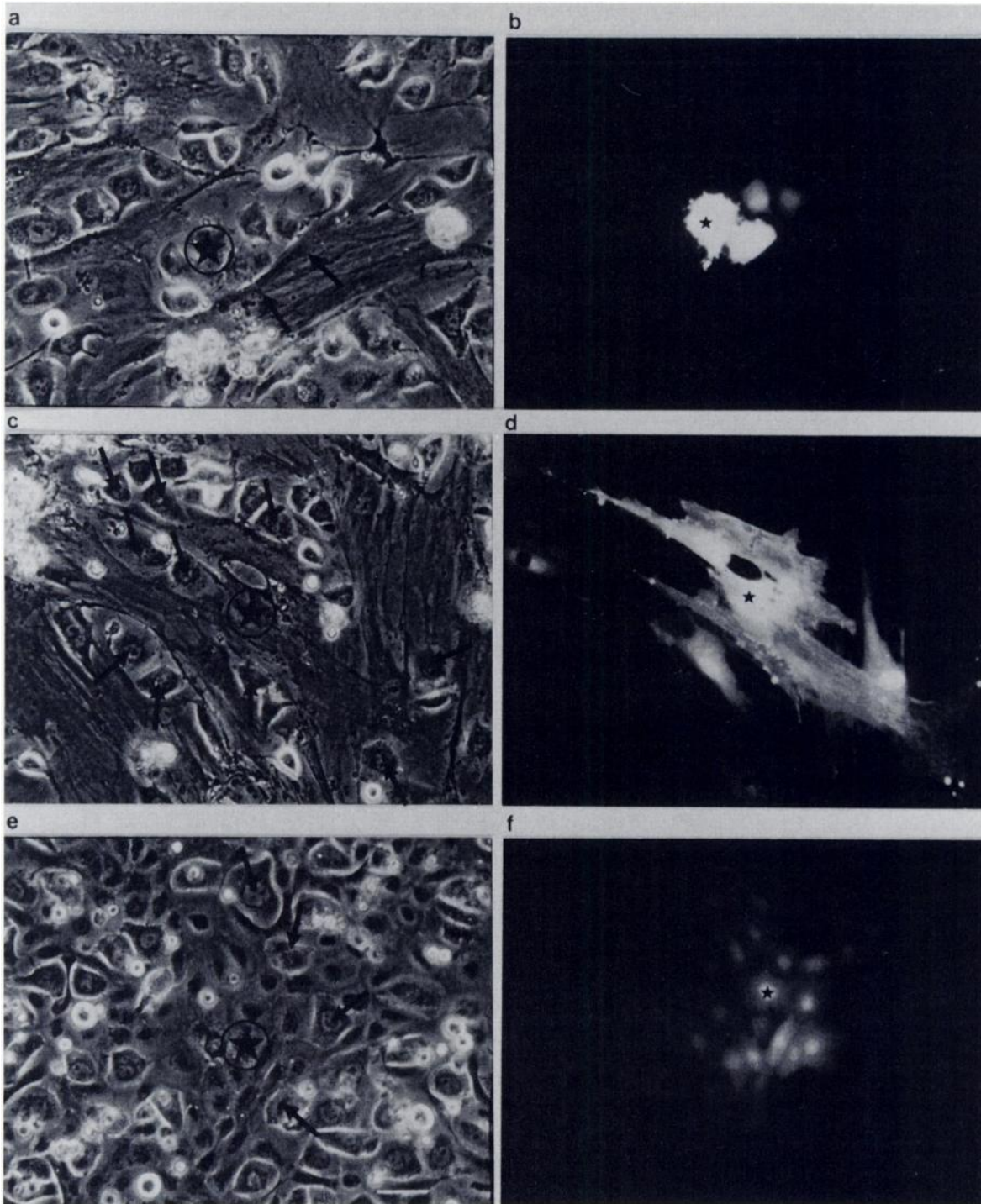
Fig. 11. Lack of dye transfer between cx 26-transfected HeLa cells (clone A) and skin cells from nude mice. Cocultures of cx 26 A cells with skin fibroblasts (*a–d*) or keratinocytes (*e–f*) were prepared as described in "Materials and Methods." Lucifer yellow was injected into the starred cells. Phase contrast micrographs (*a, c,* and *e*) show the distinct morphologies of the different cell types. The corresponding fluorescent micrographs (*b, d,* and *f*) show the extent of the dye transfer. *Arrows,* cells from a different origin of the microinjected cells. *a* and *b,* coculture of cx 26-transfected cells and skin fibroblasts (the microinjection was performed in the HeLa clone); *c* and *d,* same coculture with microinjection in the skin fibroblasts; *e* and *f,* coculture of cx 26-transfected cells and keratinocytes (microinjection in the HeLa clone). Note the lack of dye transfer between the cx 26-transfected HeLa cells and the others. *Bar,* 15 μm.

shown that the reinduced GJIC mediated by transfected cx 43 is associated with a loss of tumorigenicity of chemically transformed mouse fibroblasts (23) and decreased tumorigenicity of rat glioma cells (20); both normal mouse fibroblasts and rat glioma cells express cx 43. Moreover, decreased tumorigenicity of human hepatoma cells has been found following transfection of cx 32 cDNA, which is a

major connexin gene expressed in hepatocytes (19). Indeed, we found huge amounts of cx 26 transcript in rat cervix, the tissue from which HeLa cells originated (data not shown). These results are consistent with the idea that the connexin type expressed under normal physiological conditions plays an important role in the suppression of tumorigenicity. Thus, the lack of tumorigenicity of HeLa cells ex-

pressing a sufficiently high level of cx 26 may imply that cx 26 is expressed in the normal human cervical epithelium as it is in rat.

Among three independently cloned cx 26 transfectants, the tumorigenicity varied significantly. Two clones with higher amounts of cx 26 transcript (cx 26 A and C) were nontumorigenic, but the clone cx 26 E, with the lowest level of cx 26 transcript, was still tumorigenic. These three clones also showed different patterns of cx 26 protein localization inside the cells. The cx 26 E cells expressed cx 26 protein mostly in their cytoplasm. The cx 26 A clone, with an intermediate amount of cx 26 transcript, had most of the protein localized at the cell-cell contact areas. The cx 26 C cells expressed the highest amount of cx 26 transcript, but the protein was aberrantly localized in the nuclear region of the cells. Since these three cx 26 transfectants communicated to a similar extent, the difference in their tumorigenicity may be related at least in part to the pattern of localization of cx 26 proteins in the cells. In addition, in the case of cx 26 C clone, the cells may be overloaded with cx 26 proteins around the nuclear regions, which may have affected their growth properties.

We and others have long postulated that heterologous GJIC [between normal and (pre-)cancerous cells] plays an important role in negative growth control of (potentially) cancerous cells (14, 40). Therefore, we considered the possibility that the lack of tumorigenicity of cx 26 A clone was due to the establishment of GJIC between the transfectants injected into nude mice and the skin cells of the host. However, coculture studies failed to demonstrate dye transfer between transfectants and skin cells removed from nude mice. The lack of tumorigenicity of this clone cannot therefore be due to normalizing factors coming from the normal and surrounding skin cells through extensive GJIC, but we cannot exclude the possibility of some signaling ion transfer between the normal skin cells and the cx 26-transfected cells (clones A and C) since we did not estimate any electrical coupling. However, such a hypothesis is less probable than an intrinsic negative growth control acquired by the expression of cx 26 since the decreased growth ability is a general phenomenon observed in both *in vitro* and *in vivo* conditions. This intrinsic negative growth control may suggest that cx 26 has other roles than as a mediator of GJIC. This possibility is supported by the finding of Zhu *et al.* (41) that rat C6 glioma cells transfected with the cx 43 gene secrete a diffusible growth-inhibitory factor.

In conclusion, cx 26 expressed at a sufficiently high rate and correctly localized at the cell-cell areas (as the clone A) normalizes the behavior of HeLa cells by inhibiting their growth and their tumorigenicity in nude mice. These results are of particular interest since cx 26 has been postulated to be a tumor suppressor gene of human breast cancer (42). More recently, its expression was shown to be specifically decreased in malignant human bladder cancer cells (43). Taken together with other reports, it is becoming clear that connexin genes form a family of tumor suppressor genes. However, it is important to remember that all of the evidence has been derived from transfection experiments, which may select specific clones in which connexin genes were driven by an artificial constitutive gene promoter. Further studies are needed to clarify whether connexin genes exert a negative growth control effect under normal physiological conditions *in vivo*.

ACKNOWLEDGMENTS

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