Specificity of Gap Junction Communication Among Human Mammary Cells and Connexin Transfectants in Culture

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Abstract. In a previous paper (Lee et al., 1992), it was shown that normal human mammary epithelial cells (NMEC) express two connexin genes, Cx26 and Cx43, whereas neither gene is transcribed in a series of mammary tumor cell lines (TMEC). In this paper it is shown that normal human mammary fibroblasts (NMF) communicate and express Cx43 mRNA and protein. Transfection of either Cx26 or Cx43 genes into a tumor line, 21MT-2, induced the expression of the corresponding mRNAs and proteins as well as communication via gap junctions (GJs), although immunofluorescence demonstrated that the majority of Cx26 and Cx43 proteins present in transfected TMEC was largely cytoplasmic. Immunoblotting demonstrated that NMEC, NMF, and transfected TMEC each displayed a unique pattern of posttranslationally modified forms of Cx43 protein.

The role of different connexins in regulating gap junction intercellular communication (GJIC) was examined using a novel two-dye method to assess homologous and heterologous communication quantitatively. The recipient cell population was prestained with a permanent non-toxic lipophilic dye that binds to membranes irreversibly (PKH26, Zynaxis); and the donor population is treated with a GJ-permeable dye Calcein, a derivative of fluorescein diacetate (Molecular Probes). After mixing the two cell populations under conditions promoting GJ formation, cells were analyzed by flow cytometry to determine the percentage of cells containing both dyes. It is shown here that Cx26 and Cx43 transfectants display strong homologous communication, as do NMEC and NMF. Furthermore, NMEC mixed with NMF communicate efficiently, Cx26 transfectants communicate with NMEC but not with NMF, and Cx43 transfectants communicate with NMF. Communication between Cx26 TMEC transfectants and NMEC was asymetrical with preferential movement of calcein from TMEC to NMEC. Despite the presence of Cx43 as well as Cx26 encoded proteins in the GJs of NMEC, few Cx43 transfectants communicated with NMEC. No heterologous GJIC was observed between Cx26- and Cx43-transfected TMEC suggesting that heterotypic GJs do not form or that Cx26/Cx43 channels do not permit dye transfer.

GAP junctions (GJ)[1] are membrane structures that consist of clusters of channels connecting the cytoplasms of adjacent cells through which small molecules, less than about 1,000 D, can pass from cell to cell. Each channel consists of a ring of six monomeric connexin molecules joined end-to-end. Different connexins form GJs which display different unitary conductance values and channel gating properties. Each connexin exhibits a unique pattern of expression and multiple connexins can be expressed within a single cell (Loewenstein, 1981; Musil and Goodenough, 1990; Bennett et al., 1991).

Early studies of cell-cell communication were carried out either by measuring transmembrane electrical conductance or by detecting the transfer of [^{3}H]uridine between adjacent cells. Subsequently, dye transfer was detected by fluorescence microscopy, using injection of small fluorescent molecules such as Lucifer yellow into single cells of a monolayer, and observing its transfer to adjacent cells (Loewenstein, 1979, 1981). Subsequently, the use of membrane permeant dyes, such as carboxyfluorescein diacetate, were introduced to study gap junction intercellular communication (GJIC) (Goodall and Johnson, 1982). More recently, a simple method of scrape loading was developed, in which dye introduced into damaged cells by scraping or scratching a

1. *Abbreviations used in this paper*: Cx26, connexin 26; Cx43, connexin 43; GJ, gap junction; GJIC, gap junction intercellular communication; NMEC, normal mammary epithelial cells; NMF, normal mammary fibroblast; TMEC, tumor-derived mammary epithelial cells.

monolayer with a fine needle, is then transferred to neighboring cells via GJs (El Fouly et al., 1987). Despite the ease and qualitative results obtained using scrape loading, this assay is not quantitative (McKarns and Doolittle, 1992). Furthermore, scrape loading cannot be used for heterologous combinations.

Nonetheless, these methods have played a very important role in showing that GJs mediate cell–cell communication, and in correlating GJIC with growth control in certain model systems, principally rodent cell lines in culture (Mehta et al., 1986, 1992; Trosko et al., 1990; Yamasaki, 1990). Recent transfection studies of C6 glioma cells with Cx43 (Naus et al., 1992), human hepatoma SKHep1 cells with Cx32 (Eghbali et al., 1991), and transformed mouse fibroblasts with Cx43 (Mehta et al., 1991) have all shown that restoration of GJIC results in decreased growth rates in culture and in nude mice. These results support the hypothesis that GJIC can play a role in growth control, thus having tumor suppressor functions.

In previous studies we identified both Cx26 and Cx43 in human normal mammary epithelial cells (NMEC), and cloned and sequenced human Cx26 (Lee et al., 1992). Currently, with the use of low-stringency DNA hybridization and PCR technology, a number of new connexins have been identified (Bennett et al., 1991; Haefliger et al., 1992), but of those tested to date none are expressed in mammary epithelial cells (Lee et al., 1992). A series of patient-derived tumor mammary epithelial cell lines (TMEC) expressed neither Cx26 nor Cx43 at the mRNA level. Southern analysis showed that the Cx26 gene was still present in the tumor cells. The hypothesis that the connexin genes were present but down regulated in TMEC was confirmed by transiently inducing Cx26 mRNA in tumor cells by treatment with PMA (Lee et al., 1992).

In this paper, stable GJIC has been re-established in tumor cells by transfection with plasmids containing either rat Cx26 or rat Cx43 cDNAs driven by the cytomegalovirus promoter. It is shown here that stable transfectants expressed between 7- and 25-fold more protein than NMEC. For both connexins, the excess protein was largely cytoplasmic, making characteristic punctate membrane staining difficult to detect. The cells did communicate, but the extent of dye transfer was decreased compared to NMEC or normal mammary fibroblasts (NMF), as judged by scrape loading. These results indicated that improved methods of quantifying GJIC were highly desirable for further investigations.

This paper describes a quantitative method to measure homologous GJIC within a clone or cell line, as well as heterologous communication between different cell populations. In agreement with previous findings (Fentiman et al., 1976), we found excellent GJIC between NMEC and NMF. In addition, we observed communication between CX26 TMEC transfectants and NMEC, and between Cx43 TMEC transfectants and NMF. Transfer of dye between Cx26 TMEC transfectants and NMEC was asymmetrical with preferential movement from TMEC to NMEC. Only a few Cx43 TMEC transfectant cells were observed to communicate with NMEC, and no dye transfer was observed between Cx26- and Cx43-transfected TMEC. These results suggest that the particular connexin composition of a cell can influence heterologous communication.

Materials and Methods

Cell Culture and Cell Growth

NMEC strains 76N and 81N derived from reduction mammoplasty tissues (Band and Sager, 1989) were cultured in DFCI-1 (called D); TMEC line 21MT-2 derived from a metastatic breast tumor (Band et al., 1990) was cultured in D or in α-MEM plus 10% FCS supplemented with 1 mg/ml of insulin, 2.8 μM hydrocortisone, and 12.5 mg/ml EGF (called α+H+E); NMF strain 56NF (Anisowicz et al., 1991) was cultured in α+H+E. Transfectants were selected in α-MEM plus 5% FCS, but the TMEC used for dye transfer experiments were grown in D medium. NMF were grown in α+H+E and switched to D medium before measuring communication. To measure cell growth, cells pre-adapted for three passages in D or α+H+E, were seeded at 5×10^4 cell per 35-mm dish, harvested by trypsin every 1–2 d, and counted using a Coulter counter (Band et al., 1990).

Origins of Probes

The probes specific for: (*a*) human Cx26, corresponds to a BamHI fragment created by PCR amplification from position −11 to +682 of the human Cx26 cDNA (Lee et al., 1992); (*b*) human Cx43 is an EcoRI fragment of partial cDNA clone (Tomasetto, C., and R. Sager, unpublished) corresponding to the position ~+400 to +1,200 of the human Cx43 cDNA (Fishman et al., 1990); (*c*) rat Cx26 corresponds to the BglII fragment released from SP64T-Cx26 (Zhang and Nicholson, 1989); (*d*) rat Cx43 corresponds to an EcoRI fragment released from clone G-2 (Beyer et al., 1987); (*e*) an RNA loading control suitable for human mammary cells is an internal (0.7 kb) PstI fragment of 36B4 (Masiakowski et al., 1982).

Plasmid Construction

pCMV-Neo was generated after removal (BamHI) of p53 sequences from pCMV-p53-Neo (Baker et al., 1990) and religation. pCMV-Cx26-Neo was obtained by excision (BglII) of rat Cx26 open reading frame from SP64T-Cx26 (Musil et al., 1990; Zhang and Nicholson, 1989) and introduction into the BamHI site of pCMV-Neo. Two steps of subcloning were used to obtain pCMV-Cx43-Neo. The first step was to subclone the rat Cx43 open reading frame from the clone G-2 (Beyer et al., 1987) by EcoRI digestion and cloning into the EcoRI site of pPolyIII-i (Lathe et al., 1987). In a second step, the excision of the Cx43 open reading frame from this construct using BamHI and BglII was ligated into the BamHI site of pCMV-Neo. The constructs are shown in Fig. 1.

Transfection

21MT-2 mammary tumor cells plated at 10^6 cells per 100-mm dish in α+H+E were transfected with 10 mg of linearized pCMV-Cx26-Neo or pCMV-Neo, by the calcium phosphate co-precipitation procedure (Zajchowski et al., 1990). Selection of G418-resistant clones was begun 48 h later with 400 mg/ml of G418. Transfection of Cx43 was performed by electroporation using 10^7 21MT-2 cells and 10 mg of pCMV-Cx43. Killing was estimated by trypan blue exclusion and cells were plated at 10^6 cells per 100-mm dish. Selection and cell cloning was performed as described for calcium phosphate transfection.

DNA and RNA Isolation and Analysis

Total genomic DNA was isolated from 21MT-2 and from stable transfectants (Lee et al., 1992). DNA samples (10 μg) were digested with EcoRI, fractionated by agarose gel electrophoresis, and transferred to nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, IL) (Southern, 1975). Total RNA from cells (80–90% confluent) was purified after guanidinium isothiocyanate lysis and centrifugation through CsCl (Chirgwin et al., 1989). RNAs were fractionated by electrophoresis on a 1% agarose, 2.2 M formaldehyde gel (Lehrach et al., 1977), transferred to nylon membranes (Hybond-N; Amersham Corp.), and immobilized by UV cross-linking.

Filters were prehybridized for 2 h at 37°C in 5× SSSC, 0.2% SDS, 0.1% BSA, 0.1% PVP, 0.1% Ficoll 400, 0.1% sodium pyrophosphate, 100 μg/ml of ssDNA. Hybridization was performed for 16 h in the same buffer in presence of ^{32}P-labeled probes (~$2–4 \times 10^6$ dpm/ml). Probes were labeled by random priming (~5×10^8 dpm/μg) (Feinberg and Vogelstein, 1987).

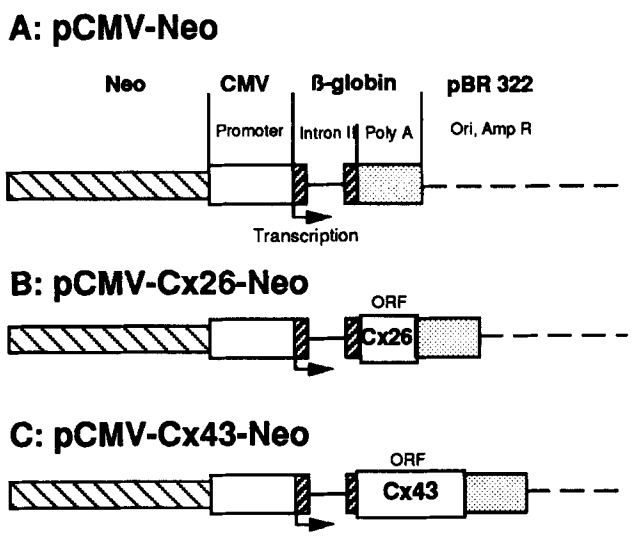

Figure 1. Schematic representation of the plasmids used. (*A*) The eucaryotic expression vector pCMV-Neo contains: the neomycin resistance gene (*Neo*), the cytomegalovirus promoter (*CMV promoter*), the rabbit intron II and polyadenylation sequences (*B globin, Intron II,* and *poly A*) and *Escherichia coli* origin of replication (*Ori*), and the ampicillin resistance gene (Amp^R). (*B*) The Cx26 expression vector pCMV-Cx26-Neo contains the complete open reading frame (*ORF*) of the rat Cx26-cDNA (*0.6 kb*) inserted into the unique BamHI site of pCMV-Neo. (*C*) The Cx43 expression vector pCMV-Cx43-Neo contains the complete open reading frame (*ORF*) of the rat Cx43 cDNA (1.4 kb) inserted into the unique BamHI site of pCMV-Neo.

Filters were washed 30 min in 2× SSC, 0.1% SDS at room temperature and 1 h in 0.1× SSC, 0.1% SDS at 60°C.

Western Blot Analysis

Confluent cell cultures (6 h post-plate in D media) were scraped into isolation buffer containing 4 mM $NaHCO_3$, 2 mM PMSF (Sigma Immunochemicals, St. Louis, MO), 2 mg/ml aprotinin (Sigma Immunochemicals), 2 mM EDTA, 5 mM diisopropylfluorophosphate (Sigma Immunochemicals), and the phosphatase inhibitors 100 mM sodium fluoride, 20 mM sodium phosphate, and 2 mM sodium orthovanadate (Sigma Immunochemicals) (Kadle et al., 1991; Musil et al., 1990). Samples were sonicated for 30 s at 50% power using a sonifier 250 (Branson Sonic Power Co., Danbury, CT) and protein concentrations were determined using DC protein assay (Bio-Rad, Richmond, CA). Proteins were solubilized in 2% SDS (Gallard-Schlesinger, Carle Place, NY) buffer containing 62.5 mM Tris-HCl, pH 6.8, 10% glycerol and 50 mM DTT (Sigma Immunochemicals) for 15 min at 70°C. Solubilized samples were separated by SDS-PAGE (Laemmli, 1970) using 4.5% stacking and 12.5% separating gels (10% for Cx43) cast in a minigel apparatus (Bio-Rad). Bio-Rad broad range SDS-PAGE standards were used for molecular weight determinations. After electrophoresis, the gels were rinsed with modified-Towbin transfer buffer containing 10% methanol (Kadle et al., 1991). Electroblotting of the protein to positively charged Immobilon-P membranes (Millipore Continental Water Systems, Bedford, MA) was performed at 300 mA for 90 min at 4°C. Residual gels were stained with Coomassie blue R-250 (Bio-Rad) to evaluate equal loading and transfer of proteins.

After electroblotting, non-specific protein binding of the membranes was blocked using filtered Blotto (5% Carnation non-fat dry milk powder in 40 mM Tris-HCl, pH 7.4, 0.1% Tween 20, 0.05% sodium azide) (Johnson et al., 1984). Antibodies against CX26 (rabbit polyclonal against amino acids 101–119 of rat Cx26 from David Paul, Harvard Medical School, Boston, MA) or Cx43 (against amino acids 252–270 of rat Cx43, Zymed Labs, San Francisco, CA) were diluted 1/1,000 in Blotto, and then incubated overnight at 4°C followed by washing in TBS (50 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.05% sodium azide). Antibody–connexin complexes were determined using the ECL detection system (Amesham Corp.). The relative amounts of the bands were evaluated using an LKB ultrascan XL enhanced laser densitometer (Biomed Instruments Inc., Fullerton, CA).

Immunofluorescence Analysis

Cell cultures were grown on 8-well chamber slides (Nunc, Naperville, IL), rinsed twice with PBS, once with double-distilled H_2O, and then dried. Cells were fixed in 100% acetone for 10 min and then extracted with 0.2% Triton X-100 in PBS for 10 min at 20°C. After blocking with 1% BSA/5% normal goat serum (Zymed Labs) in PBS for 30 min, cells were incubated with 1/1,000 dilution of rabbit anti-Cx26 (against amino acids 101-119 of rat Cx26 from David Paul) or 1/50 dilution of mouse anti-Cx43 (against amino acids 252-270 of rat Cx43; Zymed Labs) overnight at 4°C. Antibody–antigen complexes were visualized using a 1/100 dilution of FITC-conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-mouse IgG (Zymed Labs) for 1 h at 20°C. The slides were washed two times with PBS before mounting with Fluoromount-G (Fisher Scientific Co. Allied Corp., Pittsburg, PA). Cells were examined and photographed using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Scrape Loading

Media was aspirated from confluent cell cultures grown in 35-mm dishes. Plates were washed twice with solution A (PBS 1× supplemented with 10 mM glucose and 30 mM Hepes) containing 0.1% BSA. A solution of 1% Lucifer yellow (Molecular Probes Inc., Eugene, OR) and 1% dextran-rhodamine (10,000 mol wt; Molecular Probes Inc.) in solution A was added to cells. The dye was introduced into the cells using a scalpel (El-Fouly et al., 1987). After 1 min the dye was removed followed by several washes with solution A. After 5 min the cells were examined using epifluorescence microscopy. Dye transfer was quantified by counting the number of Lucifer yellow–positive cells perpendicular to wounded cells (dextran rhodamine positive) (McKarns and Doolittle, 1992).

Permanent Labeling Using PKH26

Cell labeling with PKH26 was performed as recommended by the manufacturer (Zynaxis Cell Science Inc., Malvern, PA). Briefly cells were harvested in a single cell suspension by trypsinization, washed once with solution A, and transferred to a polypropylene tube. After centrifugation the cell pellets were gently resuspended in PKH26 diluent (buffer supplied by the manufacturer) at 2×10^6 cells per 100 μl and an equal volume of 2× PKH26 was added. Staining was carried out for 2 min in a 30 μM solution of PKH26 at room temperature with gentle agitation and terminated by addition of 1 vol of serum and 7 ml of 0.1% BSA. The cell suspensions were transferred into fresh tubes by layering on 3 ml of serum. Stained cells were recovered after centrifugation and three consecutive washes with 10 ml of a 10% serum-containing medium (α+H+E). Cells were replated at high density ($\sim 5 \times 10^4$ cells/cm^2) for 24–48 h during which time any unbound dye was bound or extruded.

Transferable Labeling Using Calcein

Staining was performed directly in cell culture dishes. After washing with solution A, a solution of 0.5 μM of Calcein AM (C-1430; Molecular Probes Inc.) freshly made up in solution A was added on the top of the cells for 30 min at room temperature. Unincorporated dye was eliminated by three consecutive washes with medium. Cells were then harvested by a brief trypsinization.

Double Dye Transfer Assay

PKH26- and calcein-stained cells were each harvested by trypsinization, counted, and resuspended in D media at 10^6 cells/ml (with the exception of the NMF at 5×10^5 cells/ml). Mixing was performed at an approximate 1:1 ratio and mixed cells were plated in a 35-mm dish or in a 24-well plate at $\sim 10^5$ cells/cm^2 for 4 to 5 h at 37°C. After 5 h ($t = 5$) co-cultured cells were harvested by trypsinization to obtain a single cell suspension in solution A. Aliquots of each stained population were mixed just before the flow cytometry to determine the pattern at time 0 ($t = 0$). In reporting cell mixture experiments, calcein-stained cells are listed first and PKH26-stained cells are listed second.

Flow Cytometry and Measurement of GJIC

Cell samples were measured on an Epics Elite™ (Coulter Electronics) flow cytometer. Calcein fluorescence was detected by using a 525-nm band pass filter and pKH26 was processed in a similar manner except a 610-nm-long pass interference filter was utilized. A 550- and 626-nm dichroic long pass filter combination was used to reflect calcein and PKH26 fluorescence, respectively. Fluorescent signals were processed over a four decade logarithmic range. Cell samples that were individually labeled were used to optimize signal detection and to adjust for fluorescence compensation (typically 10–15%). PKH26 and Calcein fluorescence were assayed on a gated forward vs. side scatter population. Two parameter dot plots were analyzed using a Quad-Stat™ statistic program.

Results

Selection and Properties of Transfectants

21MT-2 cells were transfected with pCMV-Cx-26-neo and with pCMV-neo controls by the calcium phosphate coprecipitation procedure. Four clones expressing Cx26 and two pCMV-neo control clones were selected for further study. Cx43 transfectants were produced by electroporation of 21MT-2 cells with pCMV-Cx43-neo and six clones were selected for further study. The yields of neo-resistant colonies per 10^6 cells were: neo-alone, 8; Cx26-neo, 7; Cx43-neo, 10, using linearized plasmids. Genomic DNA of each transfectant was digested with EcoRI excising Cx26 or Cx43 sequences which were identified by hybridization with the plasmid DNA containing rat Cx26 or Cx43. The same pattern was observed in DNA from control and transfectant TMEC (data not shown).

Two Cx26 transfectants (Cx26-D1 and Cx26-B6) and two Cx43 transfectants (Cx43-P1 and Cx43-4) were examined by Northern analysis, using total RNA from exponentially growing cells. As shown in Fig. 2, the two Cx26 transfectants expressed similar levels of exogenous (rat) Cx26 mRNA using the rat Cx26 probe, but human Cx26 transcripts were not detectable using the same probe. The two transcripts of human Cx26 were seen in NMEC (76N) using the human probe. Similarly the Cx43 transfectants expressed rat but not human Cx43 mRNA.

Western blotting demonstrated that the antibodies used in this study recognize both human (in NMEC/76N and NMF/56NF) and rat (transfectants) forms of Cx26 and Cx43 (Fig. 3). Comparison of connexin immunoreactivity in crude cell homogenates isolated 6 h postplating demonstrated that transfected TMEC overexpressed Cx43 (Fig. 3 *A*) and Cx26 (Fig. 3 *B*) compared with NMEC or NMF. Note that in Fig. 3 *A* (Cx43) transfectant lanes were loaded with only 1.5 μg protein, while the 76N lane received 35 μg and 56NF contained 40 μg. In Fig. 3 *B* (Cx26) the 76N and 56NF were loaded with 70 μg protein, while transfectant lanes received 20 μg protein. The high molecular weight bands seen in 56NF were also present in the preimmune control (data not shown). Densitometry showed that Cx43-P1 cells expressed a 25-fold increase in the level of Cx43 compared with 76N (100-fold more than 56NF), while Cx43-4 displayed 15 times more protein than 76N. In a similar manner, Cx26-D1 expressed 15 times and Cx26-B6 expressed seven times the level of Cx26 in 76N. No Cx43 or Cx26 immunoreactivity was observed in TMEC transfected without connexin insert (CMV-6).

In addition to quantitative differences in connexin expression, the abundance of various Cx43 immunoreactive bands was altered in transfected TMEC compared to 76N (42, 43, 45, and 46 kD) or 56NF (46 kD). Cx43-P1 expressed several strong immunoreactive bands at 43, 45, and 46 kD, while Cx43-4 exhibited a single 44-kD band. A low level of Cx43 was found at 46 kD in Cx43-P1 cells. Differences in migration were not due to non-specific protein–protein interactions since the pattern of immunoreactive bands was not modified when Cx43-P1 and 56NF crude homogenates were mixed prior to electrophoresis. In addition, no difference in Cx43 migration was observed if 56NF cells were plated in D or α+H+E (data not shown). Since the migration of Cx43 on SDS-PAGE depends upon the extent of posttranslational phosphorylation (Musil et al., 1990; Musil and Goodenough, 1991; Berthoud et al., 1992), our results suggest that each cell type modifies Cx43 in a different manner.

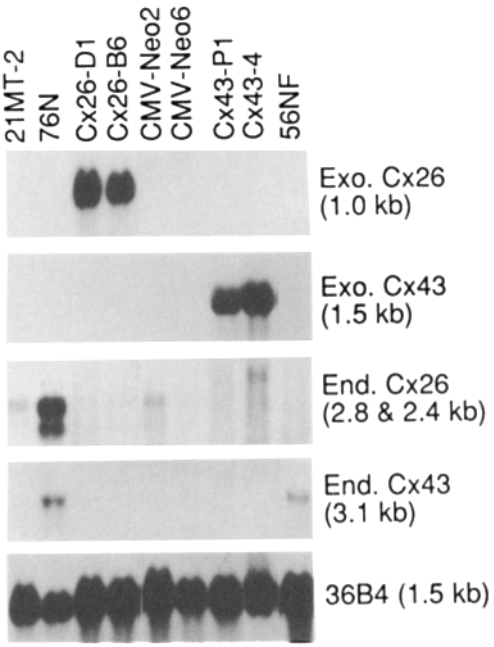

Figure 2. Northern analysis of connexin gene expression among mammary cells and transfectants. The exogenous connexin 26 (*Exo. Cx26*) and 43 (*Exo. Cx43*) transcripts are detected using the rat probes. Expression of the endogenous Cx26 (*End. Cx26*) and Cx43 (*End. Cx43*) mRNA are detected using human cDNA probes. 36B4 is used as an internal loading control. The sizes of the transcripts are indicated in parenthesis.

The cellular localization of Cx26 and Cx43 proteins was examined by indirect immunofluorescence using previously characterized antibodies (see Materials and Methods). Fig. 4, *A*, *C*, and *E* represent phase-contrast images of transfected cell cultures 6 h postplating (Fig. 4, *A* and *B*; Cx43-P1; *C–F*, Cx26-D1) incubated with either Cx26 antibody (Fig. 4 *B*), Cx43 antibody (Fig. 4 *D*), or pre-immune serum (Fig. 4 *F*). While occasional punctate fluorescence could be observed at cell–cell interfaces, intense granular immunoreactivity was observed in the cytoplasm, and of both Cx26- and Cx43-transfected TMEC.

Differences in the intracellular localization were apparent since the majority of Cx43 was perinuclear in location, while Cx26 was mostly restricted to the periphery of the cells. Similar staining patterns were observed in confluent monolayers of Cx43- and Cx26-transfected TMEC (data not

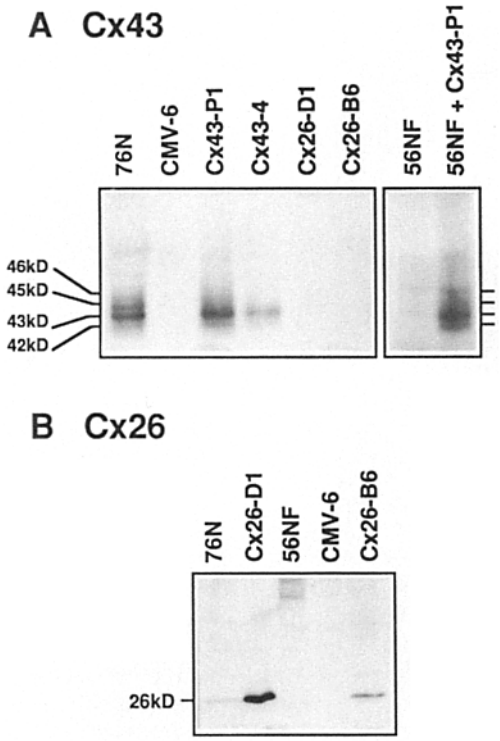

Figure 3. Western analysis of connexin protein expression in normal mammary cells and transfected TMEC. (*A*) Expression of Cx43 in total cell homogenates of 76N (*35μg*), 56 NF (40 μg), and transfected cell lines (*CMV-6, Cx43-P1, Cx43-4, Cx26-D1*, and *Cx26-B6*; 1.5 μg) using a mAb to amino acids 252-270 of rat Cx43 (Zymed Labs). Crude homogenates were isolated 6 h postplating and separated by SDS-PAGE (see Materials and Methods). The abundance of various Cx43 immunoreactive bands on SDS-PAGE was different in 76N, 56NF, and Cx43-transfected TMEC. The migration of various Cx43 immunoreactive bands was not changed after mixing 40 μg of 56NF and 1.5 μg of Cx43-P1 before electrophoresis. (*B*) Expression of Cx26 in total cell homogenates in 76N (70 μg), 56 NF (70 μg) and transfected cell lines (*Cx26-D1, CMV-6*, and *Cx26-B6*; 20 μg) using a polyclonal antibody to amino acids 101-119 of rat Cx26 from David Paul (Harvard Medical School).

shown). In contrast, the majority of Cx26 and Cx43 immunoreactivity in subconfluent NMEC is localized to cell–cell interfaces, with only limited cytoplasmic staining (Lee et al., 1992). Consistent with immunoblotting results (Fig. 3), weak punctate Cx43 staining was observed in NMF (data not shown). Incubation of Cx26-D1 cells with preimmune rabbit serum did not generate punctate fluorescence (Fig. 4 *F*). Immunoreactivity was not observed in 21MT-2 cells transfected with a vector without a connexin cDNA insert (data not shown), although a very low level of Cx26 in RNA was expressed in these cells.

Scrape Loading

Initially, transfectants were tested for GJIC by scrape loading (El-Fouly et al., 1987; McKarns and Doolittle, 1992). The extent of GJIC was clearly less in the transfectants than in NMEC or NMF (Table I). Scrape loading showed that homologous GJIC was restored by transfection in Cx26-D1, Cx26-B6, and Cx43-P1 cells. However, no dye transfer was observed in Cx43-4 cells. Heterologous communication between transfectants and normal cells could not be determined because the morphology of NMEC and TMEC is similar. The extent of homologous GJIC was later compared with that seen in the novel two-dye method which was developed to investigate this problem (Table I). We observed that both methods gave similar qualitative results, however, the two-dye method was quantitative.

Double Labeling and Transfer Assay

We have developed an assay to measure GJIC using two differentially stained cell populations, one as donor and the other as recipient. The donor cells are loaded with the dye calcein and the recipient cells are prelabeled with a permanent lipophilic dye, PKH26. Communication is measured as the percent of the recipient cell population that contains calcein. Control experiments showed that very little or no detectable calcein was released from cells within 5 h after the dye treatment.

Calcein was used as the transferable dye for several reasons. (*a*) Viable cells can be labeled to a high-fluorescence intensity which is suitable for epifluorescence microscopy and flow cytometry. (*b*) Calcein passes readily through gap junctions. (*c*) It is well retained inside the cells without passive diffusion for up to 6 h. (*d*) Calcein is non-cytotoxic at the concentrations used (Weston and Parish, 1990).

PKH26 was chosen as the permanent dye. (*a*) It labels viable cells to a high fluorescence. (*b*) It permanently binds to cytoplasmic membranes and does not pass through GJs or leak into the medium. (*c*) It is non-toxic and does not interfere with communication under the standard assay conditions used (Horan and Slezak, 1989). No change in growth rate of transfected cells was observed following PKH26 labeling (data not shown).

In a standard assay, two cell samples are labeled, one with calcein and the other with PKH26, equal numbers of cells from the two populations are mixed and then replated at high cell density to maximize cell–cell contacts. Mixed cells are co-cultured for 4 to 6 h. If GJIC occurs, calcein will be transferred from the donor cells to the PKH26-stained recipients, and the cells containing both dyes will be detected in the flow-cytometric analysis.

Flow Cytometric Analysis of GJIC

For the cytometric analysis of GJIC, mixed mammary epithelial cells are run at $t = 0$ and after co-culture at t = 5 h, as shown in Fig. 5 *A*. For each independent cell mixture, quadrant positions were defined using the cells mixed at time zero. The cursor is set with the mixture from $t = 0$ using the two parameter dot plot (log calcein vs. log PKH26) so that the calcein and the PKH26 stained cells are located in Q1 and Q4, respectively. The same cursor setting is then used to run the cell mixture from $t = 5$ h. Appearance of a third subpopulation above the PKH26 cursor (horizontal) in Q2 shows that these cells contain both calcein and PKH26 dyes. The number of communicating cells is measured by the distribution of fluorescence emission, that is represented by a two-parameter dot plot. The horizontal axis is the log emission of the fluorescent dye PKH26 and the vertical axis is the log emission of the fluorescent dye calcein. Two-dimensional analysis of log calcein vs. log PKH26 fluorescence

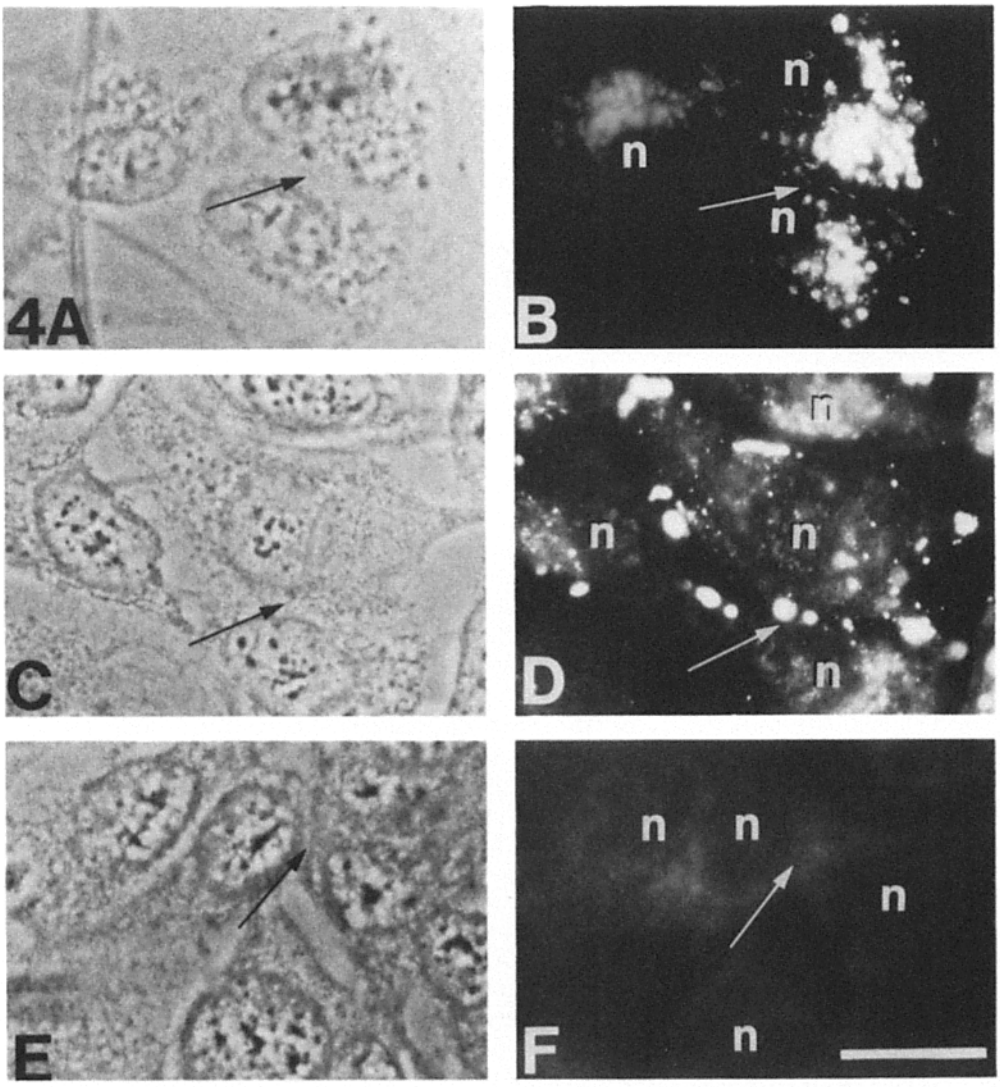

Figure 4. Localization of gap junction proteins in Cx26-D1 and Cx43-P1 transfected TMEC (21MT2) by indirect immunofluorescence. Cells 6 h postplating (D media) were fixed in acetone, permeabilized with 0.2% Triton X-100, and incubated with previously characterized antibodies to (*B*) Cx43 (monoclonal; amino acids 252-270 of rat Cx43), (*D*) Cx26 (polyclonal; amino acids 101-119 of rat Cx26), or (*F*) pre-immune rabbit serum. *A*, *C*, and *E* represent phase-contrast images. *n*, nuclei; *arrow*, areas of cell apposition. Bar, 10 μm.

profiles allows for the identification of three subpopulations in a given sample.

Thus, the fraction of cells in Q2 at $t = 5$ is a direct measure of dye transfer, which is calculated as:

$$\text{Percent of communication} = ([Q2/Q2 + Q4]_{t=5} \times 100).$$

Table I. Comparison of Scrape Loading and the Calcein/PKH26 2-Dye Methods to Quantitate GJIC

Cell type	Media	Scrape load	Two-dye method
NMEC	D	5 ± 1	87 ± 8%
56NF	α+H+E	6 ± 2	95 ± 3%
Cx43-P1	D	3 ± 2	64 ± 8%
Cx43-4	D	negative	ND
Cx26-D1	D	4 ± 1	51 ± 9%
Cx26-B6	D	3 ± 2	22 ± 4%
CMV-2	D	negative	4 ± 2%

Cell monolayers were immersed in a 1% solution of Lucifer Yellow and dextran rhodamine, wounded with a scalpel, and then monitored 5 minutes later for dye transfer (see Materials and Methods). Homologous communication was also measured using the two-dye method 5 hours after plating a 1:1 mixture of PKH26-labeled and calcein-loaded cells. ND, not determined. Values are presented as the mean (n > 3 experiments) ± standard error.

To show that this dye transfer assay is GJ dependent, we examined a pair of reciprocally labeled cell lines: communication-competent NMEC and communication-incompetent TMEC. We also examined NMEC alone as positive control. Fig. 5 *B* shows that when NMEC and TMEC are mixed no third subpopulation appears in Q2. However, when NMEC are mixed to examine homologous GJIC, a third subpopulation of double dye containing cells appears in Q2. Communication occurred in 85% of the NMEC in this particular experiment.

Assays of GJIC in Various Mammary Cell Populations

Four cell populations exhibiting GJIC were examined in both homologous and heterologous combinations (Fig. 6). The cell types used were: (*a*) NMEC including two different isolates, 76N and 81N, which showed no differences in these studies; (*b*) NMF recovered from a reduction mammoplasty; (*c*) a clone of the TMEC line 21MT-2 transfected with the rat Cx26 cDNA (Cx26-D1); and (*d*) a 21MT-2 transfectant containing the rat Cx43 cDNA (Cx43-P1). Three selected pairs of cells which demonstrate heterologous GJIC are shown in Fig. 6. Donor cells treated with calcein are listed

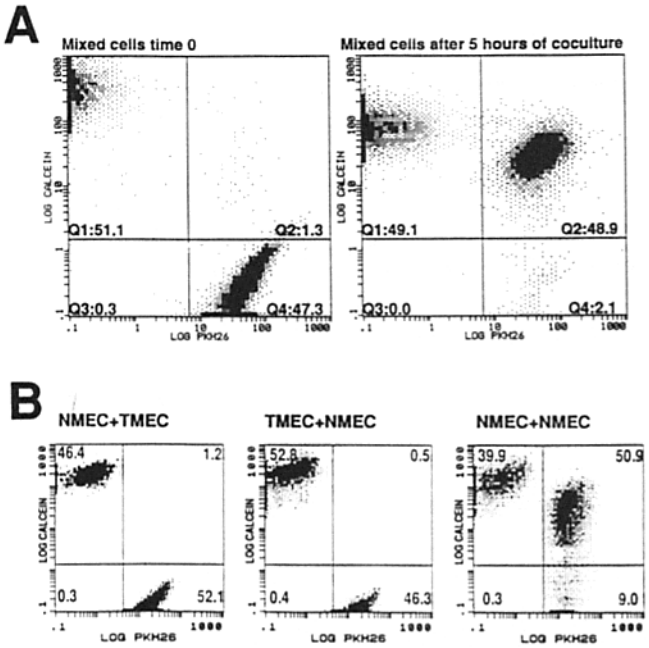

Figure 5. Double parameter dot plot analysis, log Calcein (Y axis) log PKH26 (*X axis*) of GJIC. (*A*) Setting the cursors. The cursors are set for the quantification using an aliquot of calcein-labeled cells and PKH26-labeled cells without co-culture (mixed cells at time 0). The same cursor setting is used for the quantification of the different cell subpopulations using a mixture of calcein and PKH26-labeled cells 5 h after co-culture. The percentage of cells present in each quadrant is indicated as Q values. (*B*) Analysis of GJIC in different cells mixtures after 5 h of co-culture. (*NMEC+TMEC*) Calcein-stained NMEC (communication competent) are mixed with TMEC (communication incompetent) PHK26 stained cells. (*TMEC+NMEC*) Reciprocal labeling experiment, TMEC are stained with calcein and NMEC are stained with PKH26. (*NMEC+NMEC*) NMEC are stained with calcein mixed with NMEC stained PKH26. The three combinations were each co-cultured in D media for 5 h, trypsinized and analyzed by flow cytometry. The percentage of cells in each quadrant is indicated.

first and the pre-stained PKH26 recipients are listed second. Each cell line was tested independently as donor and as recipient.

Fig. 6 *A* shows the result of mixing NMEC with NMF. High levels of heterologous GJIC were found for this mixed pair in reciprocal labeling experiments summarized in the histogram. This experiment shows that epithelial cells and fibroblasts are capable of efficient heterologous communication in both directions. Fig. 6 *B* shows the result of a GJIC experiment between NMF and the TMEC Cx43 transfectant (Cx43-P1). Both cell types express Ex43 and exhibit strong heterologous GJIC higher than homologous GJIC of the Cx43-P1 cells alone.

In Fig. 6 *C*, NMEC were mixed with TMEC Cx26 transfectants (Cx26-D1). In homologous GJIC, Cx26-D1 did not exhibit a high level of GJIC, only ~60% in this experiment, and 40, 46, and 55% in three other experiments listed in Table I. In heterologous GJIC, when Cx26-D1 was donor, and normal epithelial cells were recipients, the results were similar: 54% in the experiment shown, and 57 and 72% in two other experiments. However, in the reciprocal experiment, when the Cx26-D1 cells were recipients, the extent of communication was lower (25 and 33%). The coupled cells appear to be more efficient as donors of calcein than as recipients. The reasons why one direction may be preferential to the other in a reciprocal labeling experiment (compare in Fig. 6, *B-3* and *B-4*) are not clear. One factor may be differential retention of calcein by one of the two cell types. None of the other cell mixtures examined showed preferential transfer.

The results of two-dye quantitation experiments are summarized in Table II. Most combinations were repeated more than twice in different experiments, and the reproducibility was excellent. A clear distinction was seen between the high levels of homologous GJIC of the NMEC (containing Cx26 and Cx43) and NMF (containing only Cx43). Both Cx26- and Cx43-transfected TMEC exhibited 50–60% homologous GJIC. Most noteworthy is the high level of heterologous GJIC between NMEC and NMF, and between the NMF and Cx43 transfectants in which the GJIC was higher than that of the Cx43-P1 cells themselves. No GJIC was seen with the TMEC, which lack Cx26 and Cx43 mRNAs and proteins, or between Cx26 and Cx43 transfectants. Additional unlisted controls, such as between mixtures of Cx26-transfected TMEC with NMF, were all negative.

The NMEC showed weak GJIC with Cx43-P1 cells in both directions, a surprising result since both NMEC and the Cx43-P1 cells communicate strongly with NMF, using Cx43 channels (Fig. 3). These results suggest that there must be at least one other parameter involved in heterologous GJIC between NMEC and Cx43-P1. We designed an experiment: "Menage a trois," to examine this question. In this experiment we mixed three cell types, Cx43-P1, NMF, NMEC, and co-cultured them for 5 h in D medium. Initially, the Cx43-P1 cells were loaded with calcein, the NMF were unstained, and the NMEC were pre-stained with PKH26. As shown in Fig. 7 *A*, calcein is expected to move from the Cx43-P1 cells into the PKH26-labeled NMEC by traversing two kinds of junctions in the fibroblasts: junctions between NMF and NMEC, and junctions between NMF and Cx43-P1. The percentage of communication obtained in parallel mixed cells is summarized in Fig. 7 *B*. As controls we measured homologous communication in Cx43-P1 (Fig. 7 *B*, *Cx43-P1+Cx43-P1*) and NMEC (Fig. 7 *B*, *NMEC+NMEC*). The level of homologous communication of 53 and 90%, respectively, is comparable to the values found in other experiments (Table II). Measurement of heterologous communication between Cx43-P1 as dye donor and NMEC as a dye recipient was performed in the absence (Fig. 7 *B*, *Cx43-P1+NMEC*) and in the presence of fibroblasts (Fig. 7 *B*, *Cx43-P1+NMF+NMEC*). The level of heterologous GJIC of the NMEC between Cx43-P1 and NMEC was <15% (see Table I). However in the presence of NMF, heterologous communication was induced to 70%.

Discussion

In prior studies, we showed that NMEC express Cx26 and Cx43, and that each connexin is present in the GJs of these cells, as shown by immunostaining (Lee et al., 1992). In this paper, we have continued this investigation by examining the effects of transfecting each connexin gene into mammary tumor cells, which do not express either of these genes. We show that transfection of TMEC with either Cx26 or Cx43

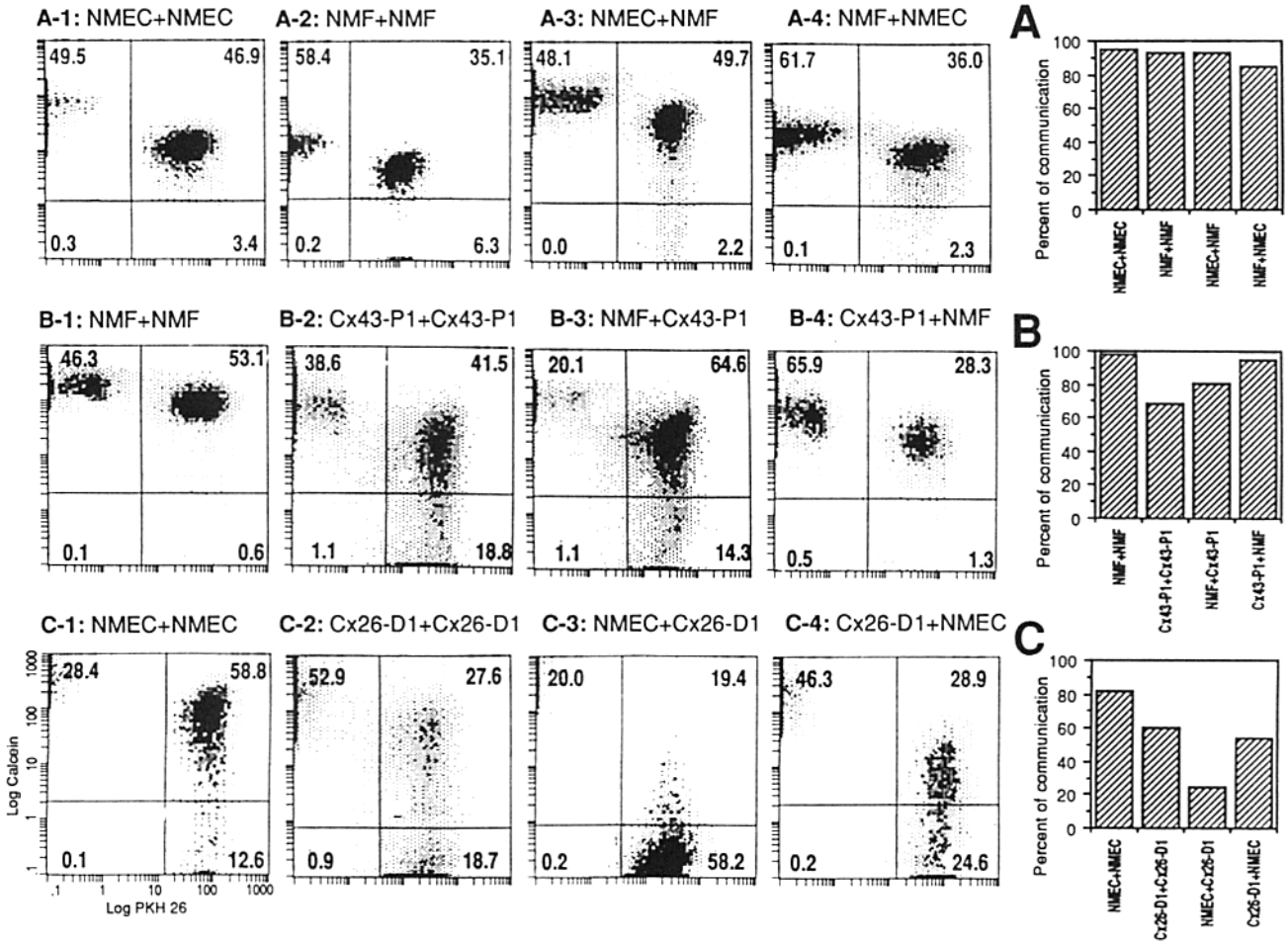

Figure 6. Double parameter dot plot analysis of homologous and heterologous communication in mixed cells after 5 h of co-culture and quantification. (*A*) Comparative study of homologous GJIC in NMEC (*A-1*) in NMF (*A-2*) and heterologous GJIC between NMEC and NMF by reciprocal labeling (*A-3* and *A-4*) within the same experiment. Histogram *A* summarizes the level of communication. (*B*) Comparative study of homologous GJIC in NMF (*B-1*) in Cx43-P1 (*B-2*) and heterologous GJIC between NMF and Cx43-P1 by reciprocal labeling (*B-3 and B-4*) within the same experiment. Histogram *B* summarizes the level of communication. (*C*) Comparative study of homologous GJIC in NMEC (*C-1*) in Cx26-D1 (*C-2*) and heterologous GJIC between NMEC and Cx26-D1 in reciprocal labeling (*C-3* and *C-4*) within the same experiment. Histogram *C* summarizes the level of communication. Calcein-stained cells are listed first and PKH26-stained cells are listed second. The same scale was used for the double parameter dot plots (see in *C-1*). Numbers in each quadrant are percentage of cells.

can restore mRNA (Fig. 2) and protein (Figs. 3 and 4) expression and most significantly, restore GJIC (Figs. 5 and 6). Western blot studies demonstrated that TMEC transfectants overexpress 7 to 25 times the level of the Cx26 and Cx43 compared to NMEC (Fig. 3). Immunofluorescence (Fig. 4) revealed that the majority of Cx43 and Cx26 protein in transfected TMEC is not present in GJs, but localized intracellularly. Similar results were obtained using several other preparations of Cx26 and Cx43 antibodies (data not shown).

Consistent with the observed intracellular accumulation of connexin protein in TMEC, scrape loading demonstrated that Cx43 and CX26 transfectants were less efficient in dye transfer than NMEC (Table I). Unexpectedly, we found that it was easier to re-establish GJIC in TMEC using constructs harboring Cx26 (5/5 communicated) rather than Cx43 (1/5 communicated) (Table I; R. Sager, unpublished observation). For example, Cx43-P1 which transfers Lucifer yellow and calcein (Table I) expresses posttranslationally modified forms of Cx43 (>43 kD) (Fig. 2 *A*), however, Cx43-4 failed to communicate and expressed only the 44-kD form of Cx43 (Fig. 2 *A*). The mechanisms responsible for altered posttranslational processing of Cx43 in TMEC is currently under investigation in our laboratory.

To facilitate studies of heterologous communication, we found it necessary to develop a quantitative assay using two dyes. Similar assays of double staining using fluorescent beads (Zidell and Loch-Caruso, 1990) or a different fluorescent dye (Mehta et al., 1992) to prestain the recipient cells have been reported, both using microscopic analysis. Our assay uses flow cytometry to determine the fraction of cells containing two dyes as a result of GJIC between pairs of cells each containing a different fluorochrome, one transferable and the other fixed as a marker of the recipient cell population. The advantages of this methodology include: (*a*) Heterologous transfer can be measured with morphologically similar populations of cells, since one permanent dye is used to distinguish them. This is particularly important with mammary epithelial cells because normal and primary tumor cells are difficult to distinguish by morphology when mixed in culture. (*b*) Small sub-populations with poor GJIC

Table II. Measurement of Homologous and Heterologous Cell–Cell Communication

Mixed cells stained with		Percent of communication (experiment number)						Average
Calcein (dye donor)	PKH26 (dye recipient)	1	2	3	4	5	6	±1
NMEC	NMEC	86	–	95	86	82	–	87
NMF	NMF	–	93	93	–	94	98	94
Cx26-D1	Cx26-D1	–	–	46	55	60	40	50
Cx43-P1	Cx43-P1	–	–	56	56	73	68	63
NMF	NMEC	–	–	85	–	76	–	80
NMEC	NMF	–	72	93	–	94	–	86
NMEC	Cx26-D1	33	–	–	9	25	–	22
Cx26-D1	NMEC	57	–	–	72	54	–	61
NMEC	Cx43-P1	–	–	–	18	15	–	16
Cx43-P1	NMEC	–	–	–	13	20	–	16
Cx43-P1	NMF	–	–	–	–	85	95	90
NMF	Cx43-P1	–	–	–	–	68	81	74
Cx26-D1	Cx43-P1	–	–	–	0.5	–	1	1
NMEC	TMEC	2	–	–	–	–	–	2
TMEC	NMEC	2	–	–	–	–	–	2

NMEC are normal mammary epithelial cells.
NMF are normal mammary fibroblasts.
TMEC are tumor mammary epithelial cells.
Cx26-D1 are TMEC transfected with a cDNA encoding the rat Cx26 protein.
Cx43-P1 are TMEC transfected with a cDNA encoding the rat Cx43 protein.
–, not tested.

can be detected because large cell populations are analyzed and the sensitivity of detection is high. (*c*) The relative frequencies of donor, potential recipient, and actual recipient populations can be determined simultaneously. It should be noted that this method does not measure the amount of dye transferred, but does quantitate the number of recipient cells. (*d*) Since the method measures one way transfer from donor to recipient, each cell line can be examined both as donor and as recipient in paired assays.

The results of the two-dye method show that the homologous GJIC of two TMEC transfectants, Cx26-D1 and Cx43-P1 is only about 2/3 as effective as that of NMEC. Furthermore, the heterologous GJIC results indicate that transfectants are poor partners with NMEC. As dye donor, Cx26-D1 is as effective with NMEC as recipient as with itself, but is a very poor recipient when NMEC are dye donor cells. Similar nonreciprocal transfer was observed between Cx26-B6 cells with two different NMEC populations (76N and 81N) (data not shown). Since the behavior is asymmetrical it suggests a structural effect of the heterotypic connexons. Differences in the amount of calcein loaded into the cell populations could not account for preferential transfer since equivalent amounts were loaded into both cell populations (Fig. 6 *C*). Since calcein has a relatively high molecular weight (623 D), the asymmetrical GJIC between NMEC and Cx26 TMEC transfectants may not be seen using smaller dyes or electric coupling. Previous studies show that asymmetrical GJIC in mammalian cell lines can be dependent on the molecular weight of the GJ permeable dye (Flagg-Newton and Loewenstein, 1980). Using *Xenopus* oocytes to express connexin cRNAs, heterotypic connexons can form GJs which are electrically coupled (Swenson et al., 1989; Werner et al., 1989; Barrio et al., 1991; Hennemann et al., 1992). However, the channel conductances of some heterotypic junctions exhibit asymmetry (Barrio et al., 1991; Hennemann et al., 1992).

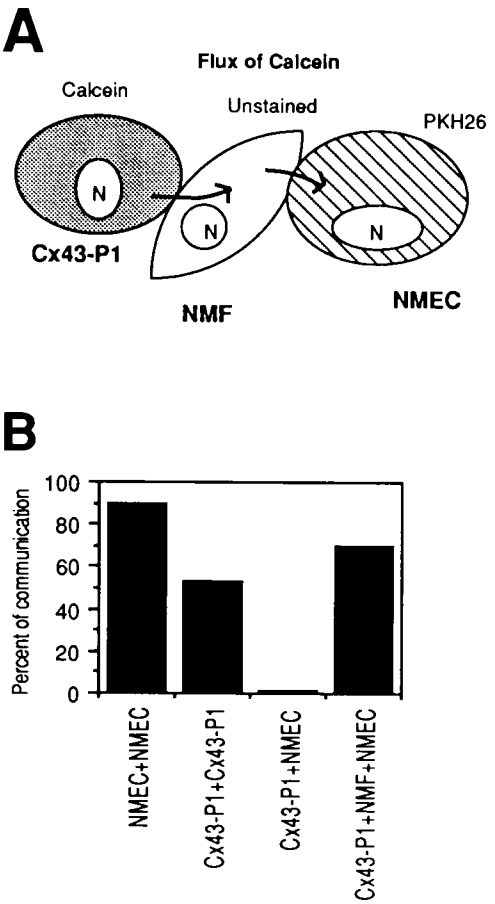

Figure 7. "Menage a trois." (*A*) Schematic representation of a mixture of three different cell types. At $t = 0$, Cx43-P1 cells stained with calcein are mixed with unstained NMF and with PKH26 labeled NMEC, in equal amounts. (*B*) Histogram representing the percentage of communication in different cell mixtures within the same experiment. After 5 h of co-culture the mixed cells are analyzed by flow cytometry, the percentage of communication is calculated as the percentage of PKH26 cells taking up calcein fluorescence.

The physiological role(s) of asymmetric GJIC may be in establishing communication domains within tissues. Throughout insect and mammalian development, compartments of cell communication are formed which allow electric coupling but are restrictive to the passage of Lucifer yellow (Warner et al., 1992). The boundaries are formed by a row of cells which exhibit GJs which are less permeable than the junctions between homologous cells (Blennerhassett and Caveney, 1984). We found that Cx26- and Cx43-transfected TMEC could transfer calcein homologously, but failed to transfer calcein in a heterologous manner. These results demonstrate that at least some communication compartments may be regulated by differential expression of connexin proteins.

Heterologous communication can also be modulated by differential expression of cell adhesion molecules. Transfection of S180 cells with either L-CAM or E-cadherin was sufficient to restore homologous GJIC, but no heterologous communication was observed (Matsuzaki et al., 1990). The calcium-dependent cell adhesion molecule E-cadherin has also been found to control GJIC in mouse epidermal cells

(Jongen et al., 1991). Both normal and tumor mammary epithelial cells used in this study express E-cadherin mRNA (R. Sager, unpublished observation).

An important finding is the high level of communication between mammary-derived epithelial cells and NMF which are normal fibroblasts of breast origin (Anisowicz et al., 1991). Both NMEC containing Cx26 and Cx43 as well as Cx43 TMEC-transfectants communicated with NMF. Weak heterologous communication was observed in both directions between Cx43-P1 transfectant and NMEC. This result is particularly surprising because of the high level of heterologous GJIC of the same Cx43-transfectant with NMF. These results suggest that there must be at least one other parameter involved in heterologous GJIC between NMEC and Cx43-P1. Heterologous GJs may form between NMEC and Cx43-P1 cells, but the intercellular channel may be closed due to altered conformation or posttranslational modification(s) of Cx43. It is noteworthy that Cx26 transfected TMEC exhibit heterologous GJIC with NMEC. Cx43 channels can be modulated by phosphorylation, but no such evidence exists for Cx26 (Bennett et al., 1991).

It is tempting to speculate that the different modified forms of Cx43 in NMEC, NMF and Cx43 transfected TMEC observed in Figure 3 may modulate heterologous communication. Our results are consistent with tissue specific differences in the migration of Cx43 on SDS PAGE (Kadle et al., 1991). Posttranslational modifications which alter the mobility of Cx43 have been extensively studied (Crow et al., 1990; Musil et al., 1990; Musil and Goodenough, 1991; Berthoud et al., 1992). The non-phosphorylated form of Cx43 was shown to accumulate in the cytoplasm of communication incompetent S180 cells. In contrast, transfection of the same cells with cDNA for L-CAM–restored GJIC and formation of the mature serine phosphorylated forms of Cx43 which localized to GJ plaques (Musil et al., 1990; Musil and Goodenough, 1991).

While the posttranslational modification(s) responsible for shifting the mobility of Cx43 in NMEC and NMF are not known, we observed that the presence of posttranslationally modified forms of Cx43 (Fig. 3) correlated with communication competence in Cx43-transfected TMEC. Cx43-P1 which expressed low levels of the higher molecular weight forms of Cx43 communicated via homologous and heterologous communication with NMF, however, Cx43-4 failed to transfer Lucifer yellow (Table I). Factors other than serine phosphorylation may also participate in the gating and turnover of Cx43 since the presence of the mature phosphorylated form of Cx43 does not always correlate with functional coupling (Berthoud et al., 1992). For example, the src oncogene can phosphorylate Cx43 on tyrosine residues resulting in closure of GJs (Crow et al., 1990; Filson et al., 1990).

Since Cx43-P1 TMECs did not communicate with NMEC, we designed an experiment to test whether NMF could mediate communication between two non-communicating cell types. We found that NMF dramatically facilitate heterologous GJIC between NMEC and Cx43-P1 cells (Fig. 7). These results are especially illuminating given previous observations that metastatic rodent mammary epithelial cells lose their ability to communicate with NMF (Nicholson et al., 1988; Hamada et al., 1988; Nicholson et al., 1990). Furthermore, highly metastatic rat mammary tumor cells lines were found to communicate with endothelial cells heterologously, while weakly metastatic clones lacked this property (El-Sabban and Pauli, 1991). We are currently investigating what factors present in NMF allow for heterologous communication between NMEC or Cx43-P1 cells.

Applications of the assay procedure described in this paper are being directed towards determining the effects of drugs and other treatments on the efficiency of GJIC to identify cellular factors that regulate GJIC especially in channels composed of Cx26 protein, in which no effect of posttranslational modification such as phosphorylation has been reported. The long-range goal of our studies is to determine what role GJIC plays in regulating or inhibiting growth of human mammary tumor cells in vivo.

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