Specific Permeability and Selective Formation of Gap Junction Channels in Connexin-transfected HeLa Cells

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Abstract. DNAs coding for seven murine connexins (Cx) (Cx26, Cx31, Cx32, Cx37, Cx40, Cx43, and Cx45) are functionally expressed in human HeLa cells that were deficient in gap junctional communication. We compare the permeabilities of gap junctions comprised of different connexins to iontophoretically injected tracer molecules. Our results show that Lucifer yellow can pass through all connexin channels analyzed. On the other hand, propidium iodide and ethidium bromide penetrate very poorly or not at all through Cx31 and Cx32 channels, respectively, but pass through channels of other connexins. 4,6 Diamidino-2-phenylindole (DAPI) dihydrochloride shows less transfer among Cx31 or Cx43 transfectants. Neurobiotin is weakly transferred among Cx31 transfectants. Total junctional conductance in Cx31 or Cx45 transfected cells is only about half as high as in other connexin transfectants analyzed and does not correlate exactly with any of the tracer permeabilities. Permeability through different connexin channels appears to be dependent on the molecular structure of each tracer, i.e. size, charge and possibly rigidity. This supports the hypothesis that different connexin channels show different permeabilities to second messenger molecules as well as metabolites and may fulfill in this way their specific role in growth control and differentiation of cell types.

In addition, we have investigated the function of heterotypic gap junctions after co-cultivation of two different connexin transfectants, one of which had been prelabeled with fluorescent dextran beads. Analysis of Lucifer yellow transfer reveals that HeLa cells expressing Cx31 (β-type connexin) do not communicate with any other connexin transfectant tested but only with themselves. Two other β-type connexin transfectants, HeLa-Cx26 and -Cx32, do not transmit Lucifer yellow to any of the α-type connexins analyzed. Among α-type connexins, Cx40 does not communicate with Cx43. Thus, connexins differ in their ability to form functional heterotypic gap junctions among mammalian cells.

CURRENTLY 12 members of the murine connexin gene family that code for protein subunits of gap junctional cell-to-cell channels have been described (Willecke et al., 1991; Haefliger et al., 1992; Hennemann et al., 1992; White et al., 1993). It has been shown that each connexin gene is expressed in one or more cell types and many if not all cell types express more than one connexin (Nicholson et al., 1987; Willecke et al., 1991; Bennett et al., 1991; Dermietzel and Spray, 1993). The latter result suggests that gap junction channels formed by different connexins are functionally different or may be differently regulated. Furthermore, it makes it necessary to study homogeneous channels of defined connexins in order to understand their function in primary cells or tissues.

Two experimental systems have been exploited in several laboratories for functional characterization of isolated connexin genes. Initially, rat connexin cRNA was injected into Xenopus oocytes (Dahl et al., 1987). Functional expression of gap junction channels between oocytes placed in close contact was electrophysiologically studied under homotypic conditions where both hemichannels are composed of only one type of connexin protein. Later, two different murine connexin cRNAs were injected into oocytes and the function of heterotypic channels was investigated under conditions where each of the two hemichannels consisted of different exogenous connexins (Cx)[1] (Werner et al., 1989; Swenson

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1. *Abbreviation used in this paper*: Cx, connexin.

et al., 1989). These authors concluded that rat Cx43 and Cx32 channels can couple with each other. Later, it was reported that rat Cx32 and Cx26 (Barrio et al., 1992), as well as murine Cx40 and Cx37, (Hennemann et al., 1992) can form functional heterotypic gap junction channels in the presence of antisense Cx38 RNA. More recently, it has been shown that rat Cx40 and Cx43 (Bruzzone et al., 1993) as well as Cx43 and Cx50 (White et al., 1994*a*) do not form functional gap junctions in Xenopus oocytes.

As an alternative to the oocyte expression system, connexin DNAs have been expressed after transfection in cultured mammalian cells, for example in SK Hepl human hepatoma cells (Eghbali et al., 1991), C6 rat glioma cells (Zhu et al., 1991), N2A murine neuroblastoma cells (Beyer et al., 1992; Veenstra et al., 1994*a*, *b*), or HeLa human cervix carcinoma cells (Hennemann et al., 1992; Traub et al., 1994). None of these recipient cells is totally deficient in gap junctional communication when tested for junctional conductance, although they show little transfer of microinjected Lucifer yellow. This could either be due to expression levels below detectability of this technique or could reflect differential permeabilities of connexin channels. In this regard, Tomasetto et al. (1991) have shown that human Cx26 and Cx43 after transfection into human mammary carcinoma cells do not form functional gap junctions when assayed by transfer of fluorescent dyes. Recently, Steinberg et al. (1994) have reported that chick Cx45 channels, expressed after transfection in rat osteosarcoma cells, show little or no permeability when probed with Lucifer yellow, in contrast to rat Cx43 channels which pass Lucifer yellow relatively well.

So far, the Xenopus oocyte expression system and the mammalian cell expression system yielded comparable results, although one could argue that the expression of transfected mammalian connexins in mammalian cells may be closer to the real physiological situation in contacting cells than the interaction of exogenous connexin channels on Xenopus oocytes stripped of the vitelline envelope. We have decided to study seven murine connexin DNAs expressed in human HeLa cells in order to check the hypothesis that different connexin proteins, in particular when expressed in the same cell type, may form gap junctional channels of different permeability. Thus, we used five different tracer molecules of different size, charge, and rigidity for permeability studies which we compared with measurements of junctional conductance. Furthermore, we analyzed which of the seven connexin transfectants was functionally compatible or incompatible with the other connexin transfectants.

Our results show that the extent of dye transfer is different between several of the connexin transfectants, although the junctional conductance is similar. This suggests that different connexin channels may pass cellular molecules (i.e., ions, metabolites, or second messengers) to a different extent. Furthermore, we show, by transfer of Lucifer yellow, that β-type connexins (cf. Risek et al., 1990) Cx32 and Cx26 can form functional channels with each other but not with other α-type connexins tested. Transfectants expressing Cx31, another β-type connexin, pass Lucifer yellow only to themselves but not to other connexin transfectants analyzed. Finally, the α-type connexins tested can form functional heterotypic channels in mammalian cells, with the exception of Cx40 and Cx43 which are incompatible.

Materials and Methods

Cells and Culture Conditions

Human cervix carcinoma HeLa cells (ATCC CCL1; American Type Culture Collection, Rockville, MD) (cf. Eckert et al., 1993), rat fibroblastoid F208 cells (Quade, 1979), or human keratinocyte HPK I cells (Dürst et al., 1987) were cultured in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (standard medium). Cultured mouse hepatocytes have been previously described (Stutenkemper et al., 1992). HeLa transfectants were maintained in standard medium containing geniticin (G418, 1 mg/ml) in case of HeLa-Cx43 transfectants or puromycin (0.5 μg/ml; Sigma, Deisenhofen, FRG) in case of all other HeLa connexin transfectants. Cell cultures were maintained in a 37°C incubator in a moist atmosphere of 10% CO_2.

Northern Blot Analysis

Total RNA from cultured cells was isolated using the guanidinium isothiocyanate/acid phenol extraction method of Chomczynski and Sacchi (1987). Equal amounts (20 μg) of total RNA from each sample were separated by electrophoresis in 1.2% agarose/2.2 M formaldehyde gels, stained with ethidium bromide and capillary blotted onto Hybond-N nylon membranes (Amersham International, Amersham, UK). Hybridizations were carried out under stringent conditions (55% formamide, 0.5% SDS, 5× SSC, 10× Denhardt's solution, 42°C) using as probe either a labeled rat liver Cx26 cDNA fragment (HincII/SmaI; Zhang and Nicholson, 1989), rat heart Cx43 cDNA fragment (HindIII/NheI; Beyer et al., 1987), or rat liver Cx32 cDNA fragment (EcoRI; Paul, 1986). Filters were washed twice in 1× SSC/0.1% SDS at 60°C, and once in 0.5× SSC/0.1% SDS at 60°C. They were exposed to Kodak XAR-5 ray film (Eastman Kodak Co., Rochester, NY) at −70°C using an intensifying screen.

Transfection

The following DNA fragments containing connexin coding sequences were obtained from the clones described in the corresponding references and ligated into the expression vector pBEHpacl8 (Horst et al., 1991) that contains the SV-40 early promoter, a polyadenylatin signal and a gene conferring resistance to puromycin: 1,505-bp BamHI fragment of rat Cx26 cDNA (Zhang and Nicholson, 1989), 1,480-bp EcoRI fragment of rat Cx32 cDNA (Paul, 1986), 1,638-bp BamHI/XhoI fragment of mouse Cx31 cDNA (Hennemann et al., 1992*a*), 1,100-bp SacI/KpI fragment of mouse Cx37 DNA (Willecke et al., 1991), 1.2-kb KpI/BamHI fragment of mouse Cx40 DNA (Hennemann et al., 1992*b*), and the 1,678-bp SpeI/XhoI fragment of mouse Cx45 cDNA (Hennemann et al., 1992*a*). By choice of these connexin DNA fragments all ATG codons upstream of the corresponding start codons had been removed with the exception of EcoRI fragment of rat Cx32 cDNA. In this case an additional ATG codon, upstream of the start codon, was followed by a stop codon and was thus considered to be not relevant for expression.

HeLa cells were transfected with 20 μg DNA of the corresponding connexin plasmid in pBEHpacl8 using the calcium phosphate transfection protocol of Chen and Okayama (1987). Between 24 and 48 h after exposure to DNA, puromycin (1 μg/ml) was added to the medium. Clones were picked after 3 wk and grown under selective conditions.

The 1.43-kb XhoI/XbaI fragment of rat heart cDNA (Beyer et al., 1987) was cut from the pBluescript SK/Cx43 construct, containing a 1,393-bp Cx43 fragment, and ligated into the expression vector pcDNA I/Neo (Invitrogen, San Diego, CA) which contains the cytomegalovirus promoter, polyadenylation signal, and a gene conferring resistance to geniticin (G418). HeLa cells were transfected with 1 μg recombinant plasmid pcDNA I/Neo/Cx43 using the calcium phosphate transfection protocol of Wigler et al. (1979). 24 h after removal of the DNA precipitate, G418 (1 mg/ml) was added to the culture medium.

Immunochemical Analysis

The protein content of cells lysed in Laemmli sample buffer (Laemmli, 1970), was determined according to Lowry et al. (1951). Protein extracts of every sample were electrophoretically separated on SDS-polyacrylamide gel according to Laemmli (1979). Proteins were electrically transferred onto nitrocellulose membranes (0.45 μm; Schleicher and Schüll, Dassel, FRG) and incubated with affinity-purified antibodies to Cx26, Cx32 (Traub

et al., 1987), Cx40 and Cx43 (Traub et al., 1994), Cx31 (Butterweck et al., 1994*a*), Cx45 (Butterweck et al., 1994*b*), and Cx37 (Traub et al., 1995). Specific binding was detected after incubation with ^{125}I-labeled protein A (Traub et al., 1987) and autoradiography with Kodak XAR film at −70°C, using an intensifying screen.

HeLa connexin transfectants and HeLa parental cells were investigated at subconfluency for specific immunofluorescence using the same method as described previously (Dermietzel et al., 1984).

Modeling of Tracer Molecules

Space filling models of tracer molecules were generated using QUANTA CHARm (Molecular Simulations, Cambridge, UK) run on a Silicon Graphics 4D25TG workstation. Molecules were constructed using the ChemNote option from within QUANTA CHARm and then energy minimized with the CHARm force field (Adopted Basis Newton Raphson [ABNR] procedure, up to 500 cycles). All bond lengths, bond angles, and force constants are the default values in the CHARm parameter set.

Microinjection of Tracers

Glass micropipettes were pulled from capillary glass (Hilgenberg Glas, Malsfeld, FRG) with a horizontal pipette puller (PD-5; Narishige, Tokyo, Japan) and backfilled with tracer solution. Tracers were injected iontophoretically (Iontophoresis Programmer model 160; World Precision Instruments Inc., New Haven, CT). Dye-coupling was examined using an inverse microscope (IM35; Zeiss, Oberkochen, FRG) with fluorescence equipment (HBO 100; Zeiss). During injection, the cell culture dishes were kept on a heated block at 37°C and were gassed with a stream of CO_2 in order to maintain the pH near neutrality.

Lucifer yellow CH (Li^+salt) (Sigma Chem. Co., St. Louis, MO) as 4% (wt/vol solution) in 1 M LiCl was injected for 2–3 s using negative current of 20 nA. 5 min after Lucifer yellow injection, cell-to-cell-coupling was quantified by counting the number of direct neighboring cells that were fluorescent among the total number of first-order neighboring cells adjacent to the injected cell. 100% coupling means that all direct neighbors contained Lucifer yellow or one of the other fluorescent dyes used. Ethidium bromide (Merck, Darmstadt, FRG), propidium iodide (Sigma, Deisenhofen, FRG), and DAPI dihydrochloride were injected for 5 s as 5 mg/ml in 1 M LiCl with positive current of 20 nA. Transfer to first order neighbor cells was recorded 5 min after injection. Fluorescence analysis of Lucifer yellow or DAPI transfer was carried out using filter set 09 (Zeiss) in the microscope, whereas filter set 15 was used for analysis of ethidium bromide or propidium iodide.

Neurobiotin (*N*-2 [aminoethyl]-biotinamide hydrochloride; Vector Laboratories, Burlingame, CA) was iontophoretically injected for 2–4 s in 0.1 M Tris buffer, pH 7.6, using positive current of 20 nA. 5 min after injection, cells were washed twice with PBS, fixed for 10 min in 4% paraformaldehyde/0.2% picric acid in sodium phosphate buffer (0.15 mM, pH 7.4), washed twice with PBS, incubated in 0.4% Triton X-100, washed with PBS, incubated with horseradish peroxidase–avidin D (Vector Laboratories) for 2.5 h, washed with PBS, and incubated in 0.05% diaminobenzidine 0.003% hydrogen peroxide solution for another 15–20 min.

No difference in morphology was noticed during one series of microinjections (up to about one hour) in HeLa transfectants microinjected with any of the five tracer compounds mentioned before. For assay of heterotypic coupling, one cell type was stained with FITC dextran (0.0025 mg/ml; Sigma, Deisenhofen, FRG) in standard medium for about 18 h. After removal of the FITC-dextran containing medium, the cells were washed three times with standard medium, twice with PBS, trypsinized, and cocultivated with a 20-fold excess of unstained cells expressing a different connexin gene. Lucifer yellow injection was performed one day later into prelabeled cells. Under epifluorescence illumination, prelabeled cells could be distinguished from unstained cells. 10 min after Lucifer yellow injection, heterotypic dye transfer between the prelabeled microinjected cell and previously unstained first-order neighbors was recorded.

Statistics

All data are represented as mean ± SD. For the comparison of homotypic dye coupling samples of 19 measurements were drawn at random from the total measurements. Each of the samples was tested for normality using the Gauss grid method. The data were subjected to one factorial ANOVA using either the connexin type or the permeating dye as the independent factor. The samples within one factor group were subsequently compared using Tukey's protected *t*-test. Samples were considered to be significantly different if $P < 0.05$. For neurobiotin coupling and total junctional conductance, significant deviations from normality were observed. Therefore, in these cases Friedman's ANOVA was used instead.

Electrophysiology

For electrophysiological experiments, HeLa cells were grown in plastic Petri dishes on cover slips. Immediately before performing the measurements, the cover slips were removed from the dish, rinsed with PBS, and transferred to an experimental chamber containing PBS as bath medium.

Gap junctional conductance was measured with the double whole-cell patch-clamp procedure (Neyton and Trautmann, 1985) using two List EPC-7 patch clamp amplifiers (List Electronics, Darmstadt, FRG). Patch-type pipettes were pulled from soft glass microcapillaries (CeeBee hematocrit; Chr. Bardram, Svendborg, Denmark) with a List L/M 3P-A vertical pipette puller in two steps (L/M 3P-A; List Electronics) to a final resistance of 1 MΩ when filled with intracellular solution. The pipettes were used without fire polishing and Sylgard coating. They were backfilled with intracellular saline containing 120 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA/KOH, 10 mM Hepes/KOH (pH 7.3) filtered through a 0.2 μm membrane filter to prevent pipette clogging. Total gap junctional conductance g_j was measured in two neighboring monolayer cells by keeping both cells at their resting potential and applying 10 mV voltage pulses to one of the cells. G_j was calculated from the current record of the neighboring cell without corrections for series resistance. The pipette series resistance in whole cell configuration was low, usually between 5–10 MΩ. However, due to the high G_j, the resulting error in the estimation of the real junctional conductance may be considerable (cf. Wilders and Jongsma, 1992). Thus, the numbers obtained as g_j underestimate the true conductance present in these cells. We assume that the current measured between two adjacent patch-clamped cells is flowing through direct gap junctions and gap junctions between first neighbor cells but is probably near or below resolution when it flows through other cells in the monolayer (cf. Gho, 1994).

Results

The Transfected HeLa Cells Express Murine Connexin Transcripts and Proteins

Human HeLa cells that show very low gap junctional conductance (Eckert et al., 1993) were tested by Northern blot analysis under reduced stringency of hybridization for expression of connexin transcripts using murine connexin DNA probes. No endogenous transcripts of connexins26, -30.3, -31, -31.1, -32, -37, -40, -43, and -45 were detected in total RNA of HeLa cells (cf. Fig. 1, for Cx26, Cx32, and Cx43; other data are not shown). HeLa cells exhibit stable membrane potentials and are well suitable for electrical measurements (Eckert et al., 1993). We decided to transfect into these cells DNAs coding for seven murine connexins to which we had antibodies available in our laboratory: Cx26, Cx32, Cx31, Cx37, Cx40, Cx43, and Cx45, in order to verify expression of the corresponding proteins in transfected cells. HeLa cells were transfected with connexin DNAs, inserted into appropriate vectors, and selected in the presence of antibiotics.

All HeLa transfectants together with HeLa wild type cells and control cells were characterized by Northern blot analysis, using total RNA and the corresponding connexin probe, under full stringency of hybridization. Fig. 1 (*A–C*) illustrates representative examples of Cx26, Cx32, and Cx43 transcripts. In HeLa-Cx26 (Fig. 1 *A*) and HeLa-Cx32 transfectants (Fig. 1 *B*) two different RNAs were detected, possibly due to different transcriptional termination sites of different integrated copies of exogenous, vector inserted DNA. RNA from mouse liver or appropriate cell lines served as

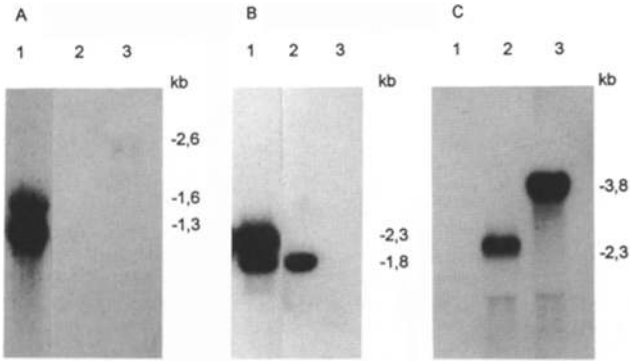

Figure 1. Northern blot hybridizations of RNA from selected HeLa-connexin transfectants. 20 μg each of total RNA were separated by electrophoresis, blotted onto nitrocellulose, hybridized specifically to the ^{32}P-labeled gene probes of murine Cx26- (*A*), Cx32- (*B*), and Cx43- (*C*) genes, and subjected to autoradiography. *A* (lane *1*) HeLa-Cx26; (lane *2*) HeLa, (lane *3*) HPK I (control); *B* (lane *1*) HeLa-Cx32; (lane *2*) mouse hepatocytes (control); (lane *3*) HeLa; *C* (lane *1*) HeLa; (lane *2*) HeLa-Cx43; (lane *3*) F208 (control).

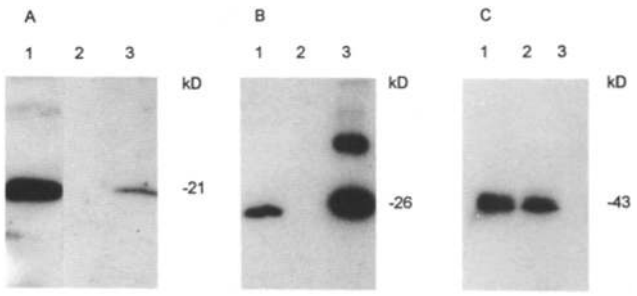

Figure 2. Immunoblot analyses of selected HeLa-connexin transfectants. 10 μl of protein extracts from confluent, cultured cells (~2.10^5) were electrophoresed, transferred to nitrocellulose, and incubated with specific antibodies to Cx26 (*A*), Cx32 (*B*), Cx43 (*C*) and ^{125}I-protein A. Autoradiographs are shown. *A* (lane *1*) mouse liver plaques (10 μg, control); (lane *2*) HeLa; (lane *3*) HeLa-26; *B* (lane *1*) HeLa-32; (lane *2*) HeLa; (lane *3*) mouse liver plaques (10 μg, control), *C* (lane *1*) F208 (control); (lane *2*) HeLa-Cx43; (lane *3*) HeLa.

positive controls. Expression of the corresponding connexin proteins in HeLa transfectants was analyzed by immunoblot. Fig. 2 (*A–C*) shows that the transfected cells specifically express Cx26, Cx32, and Cx43 protein. No connexin protein of the size recognized in transfected cells was detected in HeLa wild type cells (Fig. 2, *A–C*). Fig. 3 documents that HeLa-Cx26, -Cx32, and -Cx43 transfectants express punctate immunofluorescence with connexin specific antibodies on contacting membranes, the typical sites of gap junction plaques. In addition, cytoplasmic fluorescence is seen, probably due to overexpression of the transfected connexins in cytoplasmic vesicles. No punctate fluorescence on cytoplasmic or contacting membranes was detected in HeLa parental cells (not shown). Other HeLa connexin transfectants used in this study have been described together with characterization of specific antibodies in recent publications from our laboratory (Butterweck et al., 1994*a*,*b*), (Traub et al., 1994). In all these cases, the results of Northern blot analyses, immunoblots, and immunofluorescence support the notion that the corresponding murine connexin is specifically expressed in human HeLa cells.

Functional Reconstitution of Gap Junctional Communication in Connexin-transfected HeLa Cells

To check the hypothesis that different connexin channels may show different permeabilities we selected five low molecular weight tracer molecules of different charge and axial ratio. These properties are summarized in Table I. Space filling models of the tracers are depicted in Fig. 4. HeLa transfectants that express the corresponding mRNA and protein were characterized for gap junctional communication using the fluorescent tracer ions Lucifer yellow, propidium iodide, ethidium bromide, and DAPI (i.e., 4, 6-diamidino-2-phenylindole dihydrochloride). Dye transfer in HeLa wild type cells was always <6%. We do not know which endogenously expressed connexin causes the residual gap junctional communication in HeLa parental cells. The results are illustrated in Fig. 5. Homotypic transfer of fluorescent cationic dyes is illustrated in Fig. 6. Most transfectants analyzed transferred Lucifer yellow and DAPI dihydrochloride to about the same extent, except Cx43 and Cx45 channels. Cx45 transfectants show significantly more transfer of DAPI dihydrochloride than Lucifer yellow, whereas this situation is reversed in case of Cx43 transfectants.

In contrast, very little or no transfer of propidium iodide and ethidium bromide in HeLa-Cx31 or -32 transfectants is seen, similar to HeLa wild type cells. Furthermore, propidium iodide is weakly transferred in HeLa Cx43 transfectants relative to its more efficient transfer in Cx26-, Cx37-, or Cx40 transfectants. The data in Fig. 5 represent the levels of dye transfer to nearest neighbor cells 5 min after microinjection of the tracer. Since propidium iodide, ethidium bromide and DAPI dihydrochloride bind strongly to DNA they are quickly absorbed by the nuclei of the first order neighboring cells (cf. Fig. 6). Thus, we counted only first neighbor fluorescent cells, in order to compare the results with transfer of Lucifer yellow. The comparison of Fig. 5 with the data in Table I suggests that there is no general relation between passage of tracers through different connexin channels and size or charge of the molecules. The observed differences in dye transfer may reflect specific interaction of connexins with the penetrating tracer molecules.

In Fig. 7 we have compared our results of total junctional conductance in monolayers with the transfer of Lucifer yellow and another small tracer molecule, neurobiotin (cf. the molecular dimensions in Table I and the space filling model in Fig. 4). Neurobiotin was microinjected into each connexin transfectant and allowed to spread for 5 min. After fixation of the cells, neurobiotin was complexed with avidin, coupled to horseradish peroxidase. The enzymatic reaction produced a reddish-brown precipitate in all cells that had received neurobiotin. Thus, this tracer is detected with higher sensitivity than the fluorescent dyes used. In most cases, 100% of the nearest neighbors had received transferred neurobiotin. Therefore, all labeled cells, not only the nearest neighbor cells, were counted. On the average, 6 HeLa parental cells were labeled with neurobiotin, whereas 24 cells (in HeLa Cx31 transfectants) and 50 to 135 cells were labeled in other

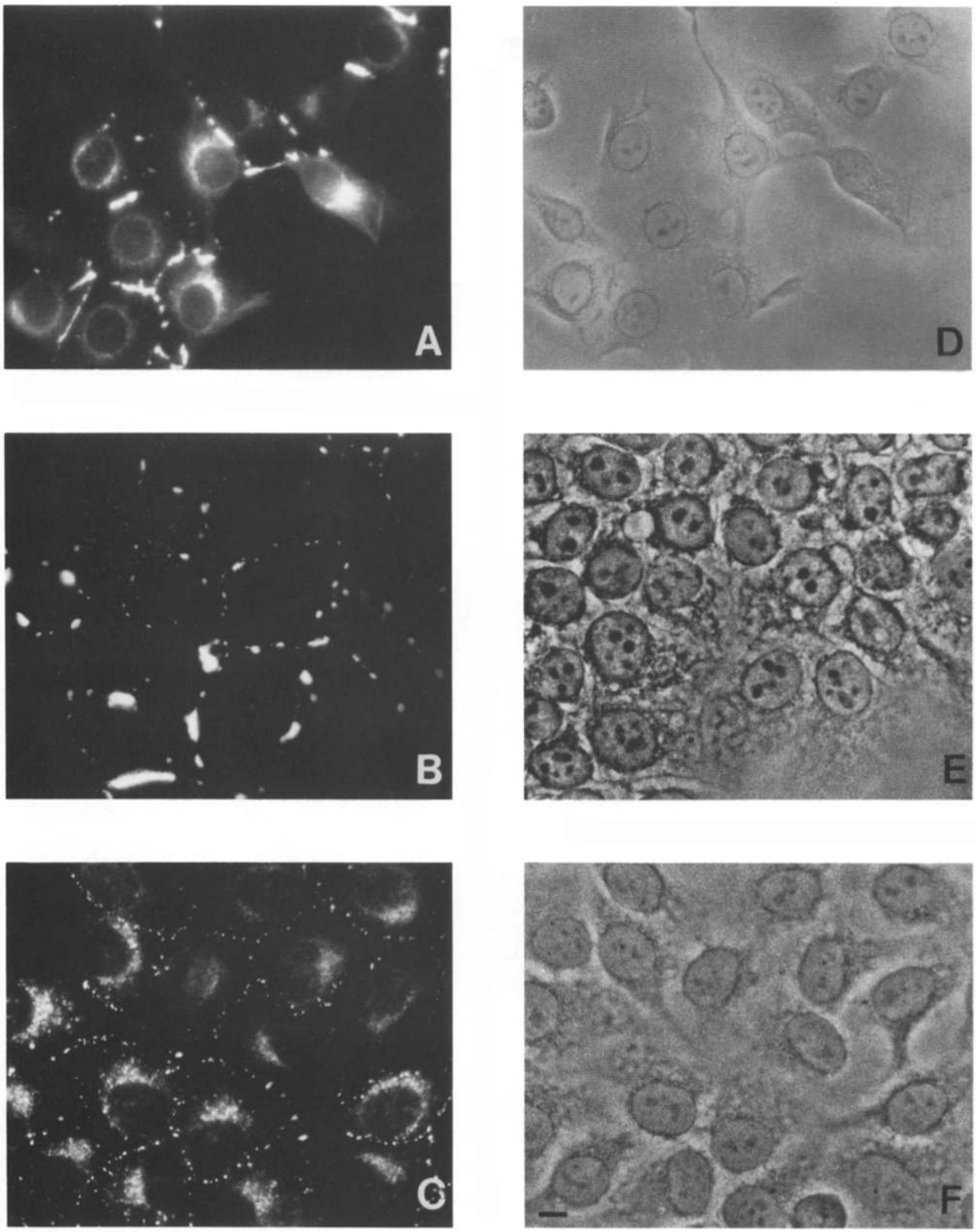

Figure 3. Immunofluorescence analyses of selected HeLa-connexin transfectants. Connexin transfected HeLa cells were fixed with ethanol, incubated with rabbit antibodies to Cx26 (*A*), Cx32 (*B*), or Cx43 (*C*) and FITC-labeled goat anti-rabbit secondary antibodies. (*A–C*) Immunofluorescence photographs. (*D–F*) corresponding phase contrast micrographs. Bar, 2 μm.

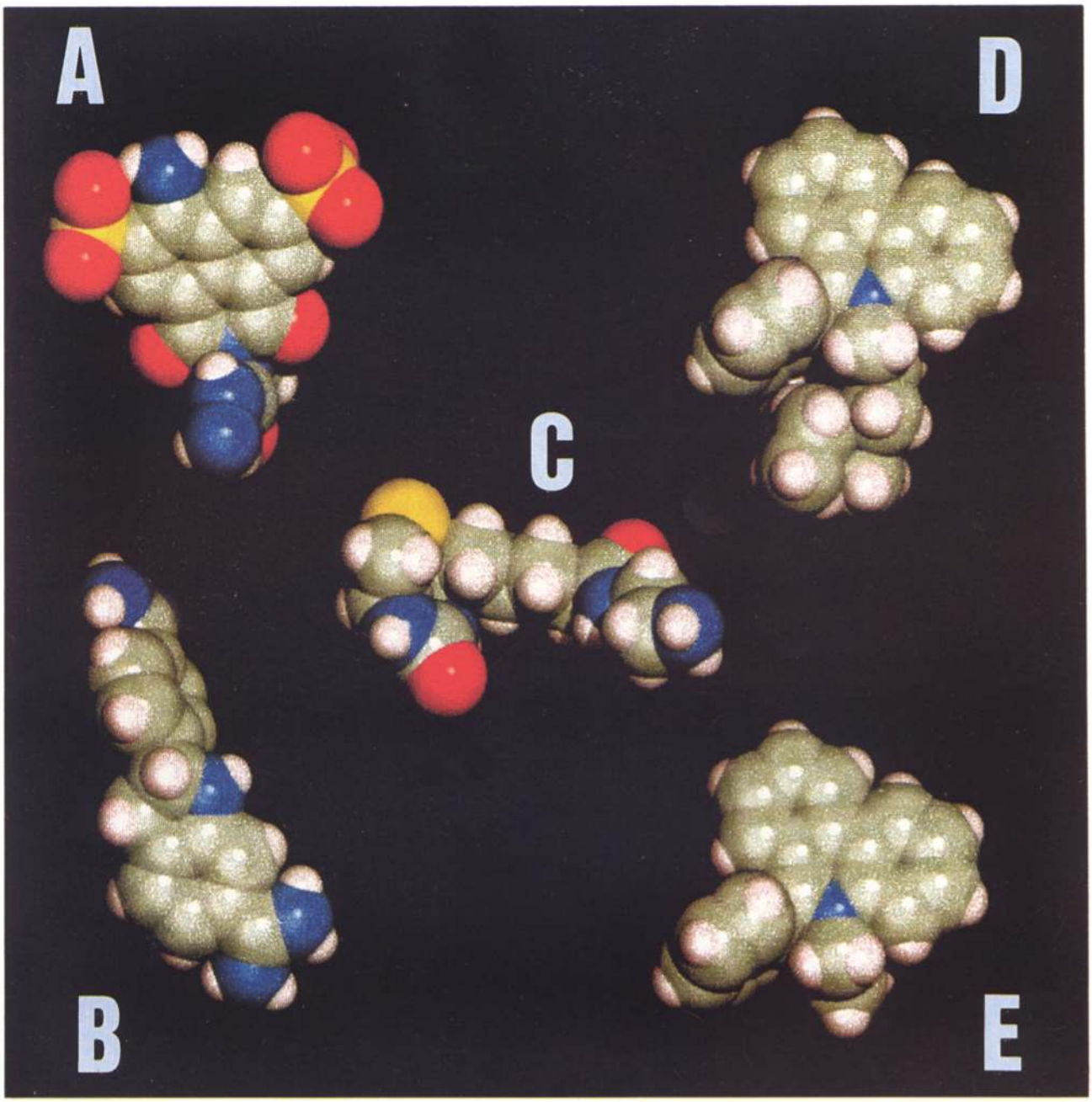

Figure 4. Space-filling models of five tracer molecules used in this study. The permeating ions analyzed in this study are shown and derived from the following chemical compounds: (*A*) Lucifer yellow CH; (*B*) DAPI; and (*C*) *N*-(2-aminoethyl)-biotinamide chloride (Neurobiotin); (*D*) propidium iodide; and (*E*) ethidium bromide. Space-filling models were drawn using the appropriate Van der Waal's radii. Atoms are color-coded: C, *grey*; H, *white*; O, *red*; N, *blue*; S, *yellow.*

connexin transfectants (cf. Fig. 5 and 7). All HeLa connexin transfectants used in this work show gap junctional conductances that are almost two orders of magnitude higher than the conductance measured in HeLa wild type cells (cf. Fig. 7). This result confirms the notion that functional reconstitution of gap junctions in transfected HeLa cells is due to expression of exogenous connexin genes.

Formation of Heterotypic Gap Junctions between HeLa Connexin Transfectants

We wished to analyze whether the different transfectants, each presumably expressing hemichannels consisting of only one type of connexin, can form functional, heterotypic gap junctions. One of the connexin transfectants was prelabeled

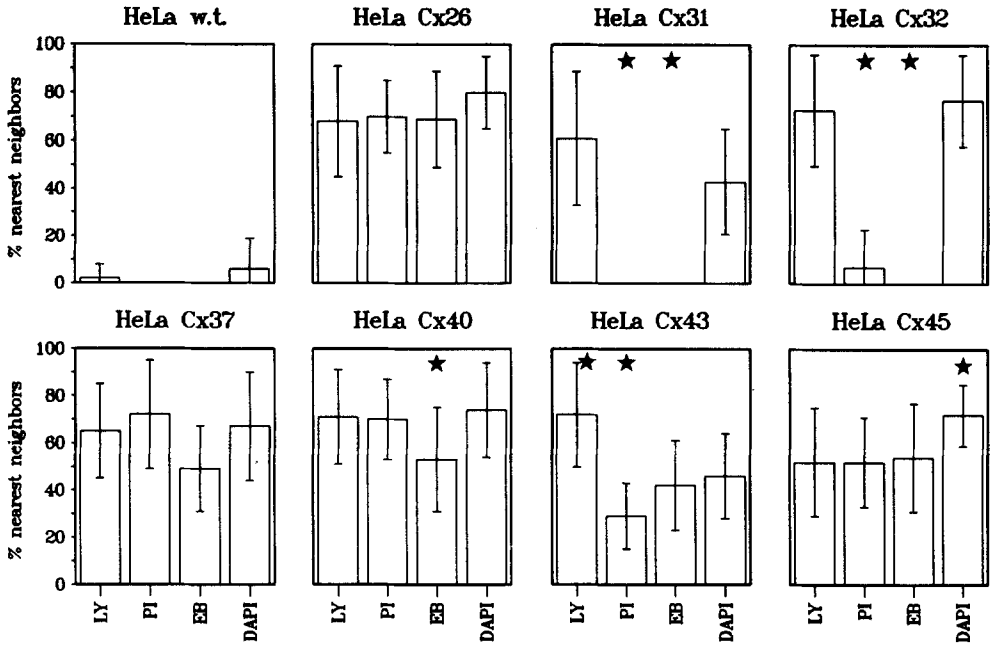

Figure 5. Homotypic coupling of first order neighbour cells after injection of several tracers. The columns represent the mean percentage of fluorescent nearest neighbor cells. The error bars are indicated as ±SD. At least 110 measurements were taken after Lucifer yellow injections. In all other cases, data of at least 20 microinjections were recorded. Fluorescence was monitored 5 min after microinjection of Lucifer yellow (*LY*), propidium iodide (*PI*), ethidium bromide (*EB*), or *DAPI*, HeLa wild type (*HeLa w.t.*). The data illustrated by star-labeled columns were significantly different ($P < 0.05$) from the other data in each group.

Table I. Physical Constants of Tracers Used in This Study

Name	Molecular mass of permeating ion analyzed (without counter ion)	Charge	Maximal minimal dimension (±0.2Å)		Axial ratio
	D				*max/min*
Lucifer yellow	443	2−	10.6	9.5	1.1
Propidium iodide	414	2+	12.3	9.3	1.3
Ethidium iodide	314	1+	11.6	9.3	1.3
DAPI	279	1+	15.4	6.0	2.6
Neurobiotin	287	1+	12.7	5.4	2.4

The maximal and minimal dimensions of the tracer molecules were calculated using the same software as for Fig. 4 (see Materials and Methods).

by incubation for 18 h with FITC-labeled dextran beads. Control experiments demonstrated that all cells showed uptake of the label. The labeled connexin transfectants were cocultured with a 20-fold excess of unlabeled transfectants for 24 h before microinjection with Lucifer yellow. An example of heterotypic dye transfer between different HeLa connexin transfectants is illustrated in Fig. 8. Table II shows that only certain combinations of connexin hemichannels allowed transfer of Lucifer yellow. Three β-type connexins (Cx26, Cx31, Cx32) and four α-type connexins (Cx37, Cx40, Cx43, and Cx45) are compared. Cx31 transfectants are unique, since they pass Lucifer yellow only homotypically and do not transfer it to any other connexin transfectant tested. Two other β-type connexins, Cx26 and Cx32, show heterotypic transfer of Lucifer yellow in both directions, i.e., from prelabeled to unlabeled cells and vice versa. However, they do not pass the dye to any of the α-type connexin transfectants assayed. On the other hand, the α-type connexin transfectants show heterotypic dye transfer among each other with the exception of Cx40 and Cx43 transfectants, which do not exchange Lucifer yellow. In addition, Table II shows that none of the connexin transfectants, when co-cultured with HeLa parental cells, transfers Lucifer yellow to these cells above background. Thus, the unidentified endogenous connexin channels in HeLa wild type cells apparently do not contribute to the results of our dye transfer experiments. Furthermore, dye transfer through plasma bridges, rather than gap junctions, is unlikely to obscure the results. Spreading of Lucifer yellow or any other fluorescent dye through plasma bridges between cells occurs so rapidly (within seconds) that it can be easily distinguished from the slower permeation through homotypic or heterotypic gap junctions. Rare cases where very rapid dye transfer was observed were not taken

Table II. Percent Heterotypic Coupling in Connexin-transfected HeLa Cells

HeLa		Cx 26	Cx 31	Cx 32	Cx 37	Cx 40	Cx 43	Cx 45	HeLa w.t.
Cx26*	**M**	**68**	**2**	**67**	**1**	**0**	**0**	**0**	**0**
	SD	23	7	32	4	0	0	0	0
Cx31*	**M**	**3**	**61**	**3**	**0**	**2**	**3**	**0**	**0**
	SD	8	28	15	0	9	12	0	0
Cx32*	**M**	**58**	**0**	**73**	**0**	**2**	**0**	**1**	**1**
	SD	38	0	23	0	6	0	5	2
Cx37*	**M**	**2**	**5**	**1**	**65**	**29**	**51**	**30**	**0**
	SD	6	15	5	20	32	31	29	0
Cx40*	**M**	**2**	**0**	**1**	**38**	**71**	**2**	**24**	**2**
	SD	7	0	4	30	20	9	28	5
Cx43*	**M**	**2**	**0**	**0**	**65**	**0**	**72**	**32**	**2**
	SD	10	0	0	31	0	22	29	10
Cx45*	**M**	**4**	**2**	**0**	**40**	**36**	**39**	**52**	**1**
	SD	15	7	0	24	35	31	23	4
HeLa*	**M**	**0**	**0**	**2**	**0**	**2**	**1**	**1**	**1**
w.t.	SD	0	0	5	0	7	4	6	6

Cx-transfected HeLa cells or HeLa wild type cells (w.t.) were prelabeled (*) with FITC dextran and then cocultivated with a 20-fold excess of transfected HeLa cells expressing a different connexin gene. One day later, Lucifer yellow was microinjected into stained cells. Numbers in bold print represent the mean percentage of previously unstained, nearest neighbors to the microinjected cell that had received dye 10 min after microinjection. At least 20 microinjections were carried out for heterotypic combinations whereas more than 110 measurements each were averaged for measuring homotypic dye transfer.

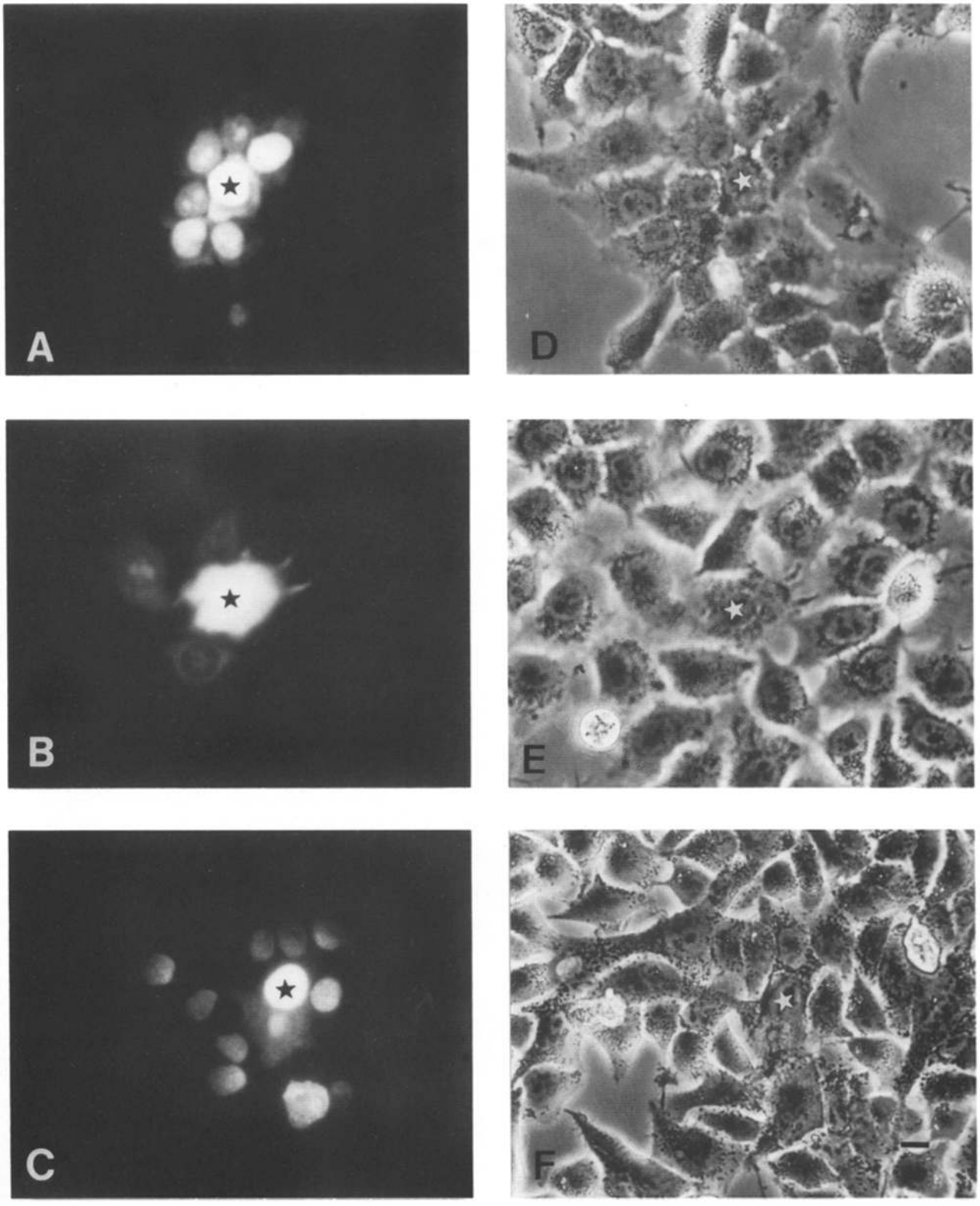

Figure 6. Intercellular, homotypic transfer of fluorescent cationic dyes microinjected into HeLa-connexin transfectants and photographed after 5 min. (*A*) Propidium iodide (HeLa-Cx26); (*B*) ethidium bromide (HeLa-Cx26); (*C*) DAPI dihydrochloride (HeLa-Cx43); (*D–F*) Corresponding phase contrast micrographs. Each microinjected cell is marked by stars. Note that these dyes bind to DNA and thus label nuclei preferentially. This is particularly obvious in case of DAPI which frequently labels only that side of neighboring nuclei which faces the microinjected cell. Bar, 4.3 μm.

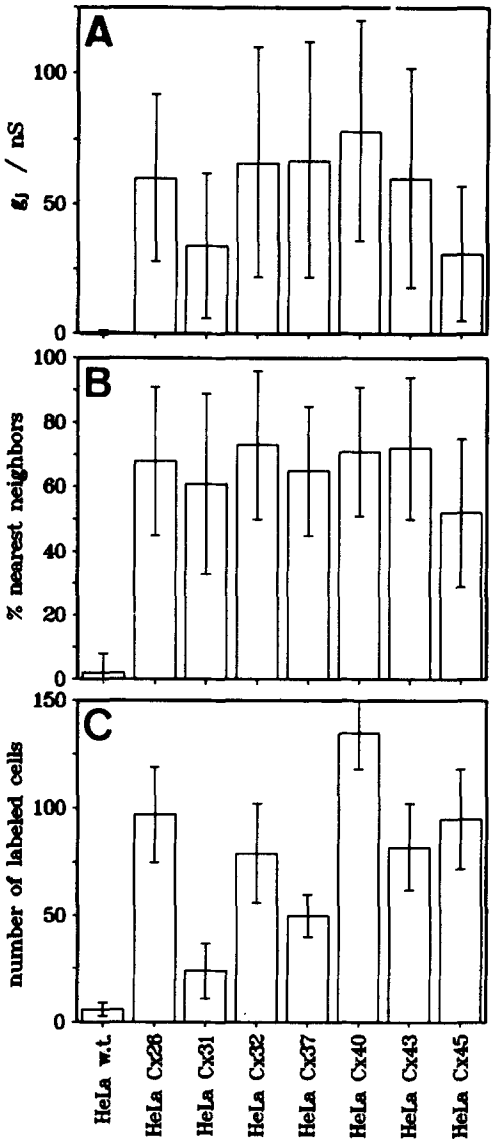

Figure 7. Comparison of homotypic gap junctional conductance with intercellular transfer of Lucifer yellow and neurobiotin. (*A*) total junctional conductance in monolayers; (*B*) spreading of Lucifer yellow to nearest neighbour cells; and (*C*) total number of cells to which neurobiotin has spread in monolayers.

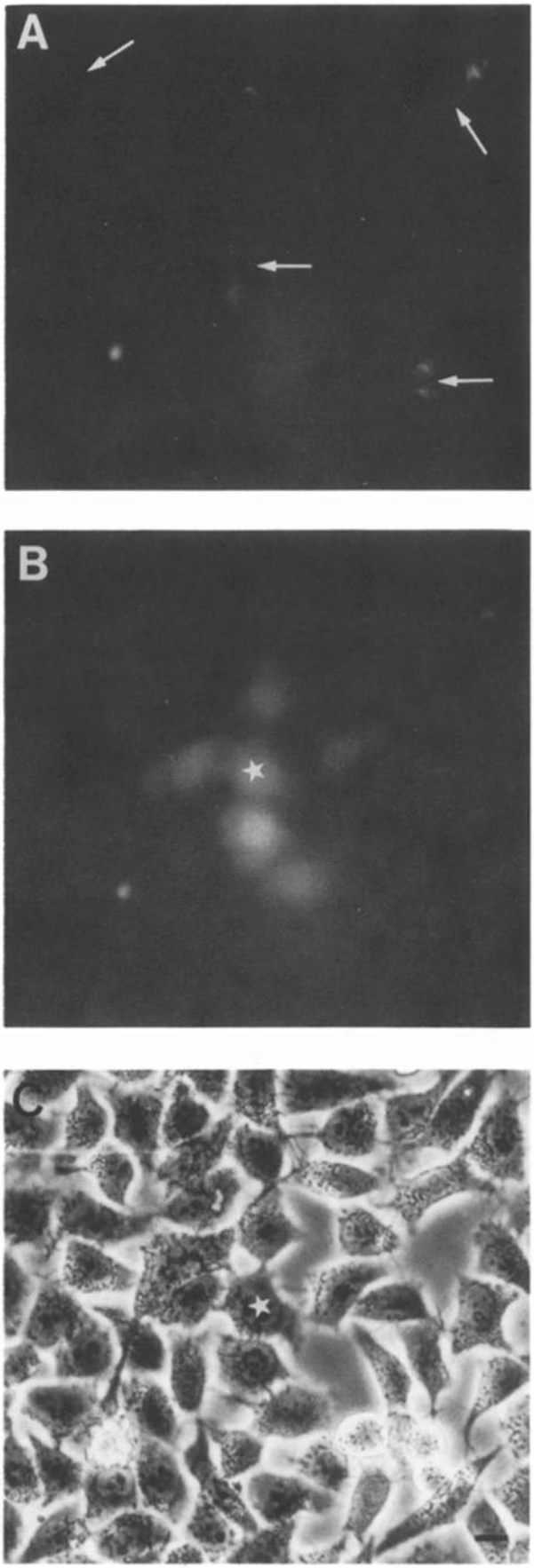

Figure 8. Heterotypic transfer of Lucifer yellow between different HeLa connexin transfectants. HeLa-Cx26 cells were prelabeled with FITC-dextran and then cocultivated with a 20-fold excess of unlabeled HeLa-Cx32 cells. One day later Lucifer yellow was microinjected into one of the prelabeled cells (*stars*). (*A*) Fluorescence photographs of co-cultured cells before microinjection. Note that after one day of coculturing most of the prelabeled cells had divided and had given rise to two neighboring prelabeled cells (*arrowheads*). Heterotypic dye transfer from the microinjected cell to unstained first order neighboring cells was recorded 10 min after microinjection. (*B*) Transfer of Lucifer yellow from a prelabeled microinjected cell (*star*) to neighboring cells. The dye had passed from the microinjected cell heterotypically into three previously unstained cells and homotypically into another prelabeled cell from where it diffused heterotypically into one previously unstained cell. (*C*) Corresponding phase contrast micrograph. The same view field is photographed in *A–C*. Note that the prelabeled cells in *B* show weaker fluorescence than in *A*, due to fading fluorescence during the second illumination when the *B* photograph was taken. Bar, 4.3 μm.

into account for calculation of coupling efficiencies in homotypic or heterotypic combination of cells. Finally, the data of Table II indicate that the transfer of Lucifer yellow, measured 24 h after plating the cells, is often less efficient in heterotypic versus homotypic combinations of compatible connexin transfectants. Probably, the formation of functional heterotypic gap junctions may take longer. The exchange of Lucifer yellow in compatible heterotypic combinations appears to be about the same in both directions, i.e., no directional dye transfer was detected in this system. Note that our recordings of Lucifer yellow transfer to first order neighbor cells, 10 min after microinjection, are likely to represent steady state conditions and do not allow to detect kinetic differences.

Discussion

Homotypic Connexin Channels

Our results show that cultured human HeLa cells can be induced to form functional gap junctions through expression on any of seven different murine connexin DNAs. Thus, the adhesion proteins on the surface of HeLa cells which were established from human cervix epithelium are sufficient to allow ectopic formation of functional gap junctions which are expressed in different tissues. This confirms the notion that the formation of gap junctions is not dependent on cell adhesion proteins that are specific for each type of connexin. Expression of certain cell adhesion proteins such as E-cadherin (Jongen et al., 1991), however, is likely to be a prerequisite for the stable formation of gap junctional channels. Since reconstitution of functional connexin channels in HeLa cells was accomplished to levels of about two orders of magnitude above that of endogenous gap junctional channels, one can be reasonably certain that the results are not influenced by expression of the unknown endogenous connexin channels. There is no indication that endogenous HeLa connexin channels contribute significantly to the properties of homotypic or heterotypic channel formation from transfected connexins—at least with regard to transfer of Lucifer yellow. We did not find any evidence for activation or recruitment of endogenous channels when HeLa connexin transfectants were co-cultured with HeLa wild type cells and analyzed for transfer of Lucifer yellow (cf. Table II).

Previously it has been concluded from cell-to-cell diffusion of ions and fluorescent dyes that the permeability through vertebrate gap junctions (of non-identified connexin type) in cultured cells is largely dependent on the size of the tracer molecule and shows little charge and ion selectivity (Flagg-Newton et al., 1979; Neyton and Trautmann, 1985; Imanaga et al., 1987). In this study we show that HeLa transfectants, expressing different, defined connexin channels pass the five different tracer molecules with different efficiencies. Gap junctions of Cx26, Cx37, Cx43, and Cx45 transfectants were permeable to ethidium bromide, whereas the ethidium-bromide permeability of Cx40 channels was reduced. Gap junctions of Cx26, Cx37, Cx40, Cx43, and Cx45 transfectants transferred propidium iodide, although the efficiency was lower in Cx43 transfectants. In contrast, Cx31 and Cx32 channels were not permeable to ethidium bromide and propidium iodide (see Fig. 5). The impermeability of Cx32 and Cx31 channels cannot be due to different total conductances, since comparable g_j values were measured in Cx26, Cx32, and Cx37 expressing cells (Fig. 7). Furthermore, our results show that the relative permeabilities of Lucifer yellow (Fig. 7 *B*) and neurobiotin (Fig. 7 *C*) through gap junctions of HeLa connexin transfectants are different and in most cases do not exactly correlate to the data of total electrical conductance (Fig. 7 *A*). This could indicate selective permeability even for molecules as small as neurobiotin. We conclude that permeability through gap junctions is a connexin-specific parameter, determined by the protein subunits constituting the channel. Since the tracers analyzed vary in other properties additional to charge and size, it is difficult to relate differences in dye transfer directly to charge and size of the tracer.

Steinberg et al. (1994) have not found significant transfer of Lucifer yellow in rat osteosarcoma cells which express transfected chick Cx45. We conclude from our results that Lucifer yellow can pass through mouse Cx45 channels. The difference to the results of Steinberg et al. (1994) could either be due to the higher expression level of mouse Cx45 in transfected HeLa cells or to species specific differences. The HeLa-Cx45 cells show 10 fold higher junctional conductance (i.e., 31 nS) than the Cx45 transfected osteoblastic cells of Steinberg et al. (1994) which exhibit even less junctional conductance than the nontransfected cells. Our results of Lucifer yellow transfer through Cx45 and Cx31 channels correspond relatively well to electrical conductance (Fig. 7). However, the spreading of neurobiotin through Cx45 or Cx31 gap junctions is markedly different. Apparently, the positively charged neurobiotin passes Cx45 channels more readily than Cx31 channels. Furthermore, the cation DAPI penetrates through Cx45 gap junctions more readily than the Lucifer yellow anion. These results suggest that Cx45 channels in HeLa transfectants may be more permeable to cations than to anions. This conclusion is in accordance with results of Veenstra et al. (1994*b*) who showed that Cx45 channels in transfected mouse N2A cells are readily permeable to cations whereas permeability of anions is lower. Possibly, a negative charge in the pore of Cx45 channels could be responsible for the observed cation permeability (Veenstra et al., 1994*b*). Our data suggest that Cx43 channels may be partially selective for some of the tracers investigated. Possibly, the selective permeability of a given connexin channel could be altered by phosphorylation. Moreno et al. (1992) have reported that the unitary conductance of human Cx43 channels appears to depend on the phosphorylation state of this protein.

With regard to the specific permeability pattern of each connexin channel it is interesting to note that second messenger molecules appear to have cytosolic life times in the order of seconds (cf. Kasai and Petersen, 1994). For example, free or cytosolically buffered Ca^{2+}, a short range messenger, exhibits life times of less than a second, whereas inositol 1,4,5-triphosphate, cAMP, or cGMP are considered as long range messengers, since they show life times between 1 and 60 s, depending on the type and state of cells (Kasai and Petersen, 1994). Because of these short life times of second messengers and probably other metabolities as well, even relatively small differences in permeabilities of different connexin channels could be physiologically important.

The intracellular concentration of second messengers is dependent on the number of activated receptor molecules on

the plasma membrane or intracellular membranes. If connexin channels show specificity for transfer of certain second messengers they may serve to quickly equalize the concentration of second messengers within populations of the same cell type and may thus assure optimal coordinated response to external signals triggering or mediating cell differentiation or proliferation. One can assume that different cell types may require different concentrations of second messengers, ions, and metabolites for optimal function. Different connexin channels may have evolved in response to the need for optimal intercellular communication. These notions can now be tested by measuring the diffusion of second messengers and metabolites through different homotypic connexin channels in transfected mammalian cells.

Heterotypic Connexin Channels

In the second part of this paper we have investigated the potential of different connexin hemichannels to form functional heterotypic gap junctions (cf. Table II). Since the connexins are all expressed in the same parental HeLa cell type, any difference in heterotypic gap junctional communication should be due to the ability of different connexin hemichannels to dock to each other and to open up the pore between cells.

Interestingly, Cx31 transfected HeLa cells form homotypic gap junctions but no heterotypic ones with any of the other connexins tested. This means that Cx31 expressing primary cells (i.e., keratinocytes [Butterweck et al., 1994*a*] or trophectoderm cells (Dahl, E., E. Winterhager, B. Reuß, O. Traub, A. Butterweck, and K. Willecke, manuscript submitted for publication) form functional gap junction channels only with themselves. This could contribute to the formation and maintenance of communication compartments in epidermal differentiation (Pitts et al., 1988; Salomon et al., 1988) and in the 7.5 dpc mouse conceptus (Dahl, E., Winterhager, B. Reuß, O. Traub, A. Butterweck, and K. Willecke, manuscript submitted for publication), where the Cx31 expressing cells are in close contact with cells expressing other connexins, mainly Cx43. In this context it is interesting that the conserved cysteine residues are spaced in the presumptive second extracellular loop of rat and mouse Cx31 as CX_4CX_5C whereas the same motif on all other connexins is CX_5XC_5C (Hoh et al., 1991; Hennemann et al., 1992). It is possible that this difference in the second extracellular loop, which may be important for docking of gap junctional hemichannels, may contribute to the exclusivity of Cx31. Very recently, Brisette et al. (1994), have shown that the induction of Cx31 and Cx31.1 by addition of Ca^{2+} to cultured mouse keratinocytes is accompanied by decreased intercellular transfer of Lucifer yellow or neurobiotin. With the exception of Cx32, all other types of connexins studied in this paper are expressed in embryonic or adult murine epidermis in overlapping but distinct patterns (Risek et al., 1992; Butterweck et al., 1994*b*; Goliger and Paul, 1994). Homotypic and heterotypic interactions or incompatibilities of gap junctions may contribute to the control of proliferation and/or differentiation in skin and other tissues.

Our data of Table II further show that none of the β-type connexins (i.e., Cx26, Cx31, Cx32) shows transfer of Lucifer yellow to any of the α-type connexins tested. α-Connexins are distinguished from β-type connexins by insertion of about 18 amino acids into the putative cytoplasmic loop of the molecule (Risek et al., 1990; for comparative hydrophobicity diagrams see Willecke et al., 1991). We do not know whether these inserted amino acids are responsible for the difference in compatibility. Our observation is the first functional difference detected between several α- and β-type connexins in mammalian cells. There has been a preliminary report that the β-type connexins rat Cx32 or Cx26 can form conducting channels in Xenopus oocytes with rat Cx46, an α-type connexin (White et al., 1994*b*). The β-type connexins Cx32 and Cx26 formed functional heterotypic channels between HeLa transfectants that are permeable to Lucifer yellow. It is likely that heterotypic Cx32/Cx26 channels are also functioning in liver. All α-type connexins tested in this work (Cx37, Cx40, Cx43, Cx45) can form heterotypic gap junctions in the HeLa cell system, except Cx40 with Cx43. Previously, Bruzzone et al. (1993) have reported that rat Cx40 and Cx43 do not form heterotypic gap junctions in Xenopus oocytes but can both couple to Cx37.

Our results with connexin HeLa transfectants confirm the conclusions from connexins expressed in Xenopus oocytes. It should be mentioned, however, that Werner et al. (1989) and Swenson et al. (1989) had concluded, based on electrical measurements, that rat Cx43 and Cx32 can form functional heterotypic gap junctions in Xenopus oocytes. In the HeLa system, Cx32 does not form heterotypic gap junctions, permeable to Lucifer yellow, with Cx43. In this context it is noteworthy that all our data concerning compatible and incompatible connexins are currently based on transfer of microinjected Lucifer yellow. We cannot exclude that more sensitive measurements of electrical conductance between different connexin transfectants may reveal some heterotypic coupling between Cx43 and Cx32 hemichannels. Possibly, homotypic and heterotypic channels formed by the same connexins differ in their permeability. White et al. (1994*b*) have recently shown that the electrical properties of Cx46 hemichannels, expressed in Xenopus oocytes, were altered when this connexin was tested in heterotypic Cx26/Cx46 or Cx32/Cx46 channels. Moreover, heterotypic Cx32/Cx43 channels could be differently modified in Xenopus oocytes and in HeLa cells which may lead to different permeabilities. In co-cultures, none of the connexin transfectants transferred Lucifer yellow to HeLa wild type cells above background. Apparently, the expression of exogenous connexins did not increase expression of the endogenous HeLa connexin. Thus, the endogenous connexin channels of HeLa cells do not appear to contribute to the observed functional heterotypic channels.

Expression of incompatible connexins could lead to communication barriers between cells in close physical contact. For example, we (Bastide et al., 1993) and others (Peters et al., 1994; Gros et al., 1994) have shown that Cx40 is preferentially located in rat conductive myocardium where it is surrounded by working myocardium, mainly expressing Cx43. The incompatibility of Cx40 and Cx43 channels may lead to preferential formation of homotypic Cx40 gap junctions along the bundles of conducting tissue, a possibility which could well explain the electrical characteristics of this tissue. Bruzzone et al. (1993) have already pointed out that endothelial cells (expressing Cx40) and surrounding smooth muscle cells (expressing Cx43) in the vascular wall may not couple to each other because of the incompatibility of Cx40 and Cx43 channels.

In conclusion, the differences in permeability and compatibility between connexin channels could be important aspects towards understanding the structural and functional differences between members of this gene family in mammalian cells.

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