

# Adequate Connexin-mediated Coupling Is Required for Proper Insulin Production

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**Abstract.** To assess whether connexin (Cx) expression contributes to insulin secretion, we have investigated normal and tumoral insulin-producing cells for connexins, gap junctions, and coupling. We have found that the glucose-sensitive cells of pancreatic islets and of a rat insulinoma are functionally coupled by gap junctions made of Cx43. In contrast, cells of several lines secreting insulin abnormally do not express Cx43, gap junctions, and coupling. After correction of these de-

fects by stable transfection of Cx43 cDNA, cells expressing modest levels of Cx43 and coupling, as observed in native  $\beta$ -cells, showed an expression of the insulin gene and an insulin content that were markedly elevated, compared with those observed in both wild-type (uncoupled) cells and in transfected cells overexpressing Cx43. These findings indicate that adequate levels of Cx-mediated coupling are required for proper insulin production and storage.

**P**ROPER secretion of insulin from pancreatic islets is a multicellular event that depends on a communication network for coordination of the activity of individual insulin-producing  $\beta$ -cells. Several mechanisms that allow for cell-to-cell communication presumably participate in this coordination (LeRoith et al., 1988). Even though the nature of these mechanisms, and their relative contribution to the *in vivo* control of  $\beta$ -cells remain to be fully elucidated, the current view is that  $\beta$ -cell functioning is initiated by circulating nutrients and modulated by hormones and neurotransmitters (Samols and Stagner, 1991; Berggren et al., 1992). There is, however, evidence that other communication mechanisms are also operative in pancreatic islets. Hence, exclusive control by signals flowing through extracellular spaces cannot entirely account for several aspects of insulin secretion under both control and pathological conditions (Braaten et al., 1974; Patel et al., 1976; Raskin and Unger, 1978; Samols and Stagner, 1991). Also, insulin secretion remains normally regulated under *in vitro* conditions that perturb the native flow of these extracellular signals, provided  $\beta$ -cells are not physically separated from each other (Lernmark, 1974; Pipelers, 1984). When  $\beta$ -cells are separated, insulin biosynthesis and secretion are markedly reduced, particularly in response to glucose concentrations that physiologically stimulate pancreatic islets. These changes, and their rapid correction after cell reaggregation, suggest that a crucial regulatory mechanism of insulin secretion depends on

$\beta$ -cell contacts (Lernmark, 1974; Halban et al., 1982; Salomon and Meda, 1986; Bosco et al., 1989; Philippe et al., 1992).

The finding that pancreatic  $\beta$ -cells are connected by gap junctions (Orci et al., 1973) and are coupled, as shown by direct exchange of ions (Eddlestone et al., 1984) and gap junction-permeant molecules (Kohen et al., 1979; Meda et al., 1981; Michaels and Sheridan, 1981), raises the possibility that intercellular exchange of ions and molecules through gap junctions mediates such regulation. Previous experiments have shown that conditions stimulating insulin release increase  $\beta$ -cell gap junctions, the expression of their constitutive connexin protein (Cx43), and  $\beta$ -to- $\beta$ -cell coupling (Meda et al., 1979, 1983, 1991; Kohen et al., 1983; Eddlestone et al., 1984). In addition, conditions promoting gap junction formation improve insulin secretion and biosynthesis (Halban et al., 1982; Salomon and Meda, 1986; Schuit et al., 1988; Bosco et al., 1989, 1992; Philippe et al., 1992). Conversely, conditions disrupting  $\beta$ -cell gap junctions or blocking their channels have been shown to decrease insulin secretion (Lernmark, 1974; Halban et al., 1982; Salomon and Meda, 1986; Meda et al., 1990; Philippe et al., 1992). These findings suggest that connexin-mediated coupling is required for proper control of insulin secretion, presumably because it promotes the recruitment and synchronization of secreting  $\beta$ -cells which, in the absence of junctional communication, show marked differences in their ability to synthesize and release insulin (Salomon and Meda, 1986; Schuit et al., 1988; Bosco et al., 1989, 1992). Validation that connexin-mediated coupling participates to control insulin secretion awaits direct experimental testing, including the demonstration that insulin-producing cells that secrete abnormally have defects in

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connexin expression and junctional coupling, and that restoration of adequate junctional communication improves their secretory characteristics.

In this report, we first show that a variety of independent-insulin-producing cell lines (Gazdar et al., 1980; Santerre et al., 1981; Moore et al., 1983; Asfari et al., 1992; Hughes et al., 1992) that show multiple secretory defects, as judged by markedly lowered insulin contents and loss of responsiveness to the glucose concentrations that physiologically stimulate  $\beta$ -cells, have lost the ability to directly exchange cytoplasmic molecules by junctional coupling, due to lack of expression of Cx43. We also show that correction of these defects by stable transfection of the gene sequence coding for Cx43 (Beyer et al., 1987; Kumar and Gilula, 1992), modified the expression of the insulin gene, as well as the storage of insulin, in a way dependent on junctional coupling mediated by connexin expression. Cells exhibiting modest junctional coupling and Cx43 expression similar to those observed in native  $\beta$ -cells showed markedly improved secretory characteristics. These novel findings indicate that adequate expression of connexins and junctional coupling is required for proper functioning of insulin-producing cells.

## Materials and Methods

### Islet Cells

Islets of Langerhans were isolated from the pancreas of male Sprague-Dawley rats weighing 250–350 g, by collagenase digestion and purification on a Histopaque gradient (Giordano et al., 1993). The isolated islets were washed twice in PBS prepared without adding  $Mg^{2+}$  and  $Ca^{2+}$  and containing 0.2 mM EDTA, and then exposed for 6–7 min at 37°C to the same buffer supplemented with 0.16 mg/ml trypsin (1:250; GIBCO BRL, Gaithersburg, MD). This incubation was stopped by addition of 10 ml ice-cold sterile RPMI 1640 medium (GIBCO BRL), which was supplemented with 10% FCS, 110 U/ml penicillin, and 110  $\mu$ g/ml streptomycin, and centrifuged 10 min at 150 g. The rinsing in sterile medium and the centrifugation protocol were repeated three times before cell culture.

In experiments requiring purified  $\beta$ -cells, the trypsin incubation was stopped by addition of 10 ml ice-cold Krebs Ringer bicarbonate buffer (pH 7.4), supplemented with 0.5% BSA, 2.8 mM glucose, and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (control KRB). The resulting suspension, which comprised mostly single cells, was centrifuged 5 min at 130 g, and the pellet resuspended in control KRB at a final concentration of  $10^6$  cells/ml.  $\beta$ -cells were then sorted from the other islet cell types using a flow cytometer (Epics-V; Coulter Electronics, Hialeah, FL), equipped with an excitation argon laser (Innova 90; Coherent, Inc., Palo Alto, CA) tuned to 488 nm and operated at 500–600 mW output power (Giordano et al., 1993).

### Insulinoma Cells

Tumoral islet tissue was obtained from an insulinoma that was propagated by transplantation in inbred rats of the NEDH strain (Chick et al., 1977). For these experiments, the insulinoma was sampled 4–6 wk after subcutaneous implantation, at which time the tumor-bearing rats had plasma glucose levels below 50 mg/100 ml. The tumor was chopped into small fragments which were incubated 30 min at 37°C with 0.8 mg/ml Collagenase 1 (Serva, Heidelberg, Germany), under continuous shaking conditions. The resulting cell suspension was diluted in ice-cold Hanks' solution containing 0.3% BSA and passed through nylon filters to remove bundles of connective fibers. After repeated rinsing in sterile RPMI 1640 medium, cells were plated for culture as described above.

### Cell Lines

Aliquots of  $3 \times 10^5$  RIN (Gazdar et al., 1980), INS1 (Asfari et al., 1992), and HIT cells (Santerre et al., 1981) were plated in 8 ml RPMI 1640 me-

dium containing 10% FCS (heat inactivated for INS1 and HIT cells), 110 U/ml penicillin and 110  $\mu$ g/ml streptomycin, and supplemented with 10 mg/ml glutathione and 0.1 mM selenous acid (for HIT cells) or 50 mM 2 $\beta$ -mercaptoethanol and 1 mM sodium pyruvate (for INS1 cells). AtT20ins (Moore et al., 1983), CGT-5, and CGT-6 cells (Hughes et al., 1992) were similarly grown in DMEM H-21 medium also containing 10% FCS. All lines were kept at 37°C in a humidified incubator gassed with air and CO<sub>2</sub> to maintain medium pH at 7.4, fed at 3-d intervals, and passed by trypsinization once a week.

### Identification of Gap Junctions

For freeze-fracture EM, cells were grown on plastic tissue culture coverslips (Thermanox; Miles Inc., Kanakee, IL), fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.4), infiltrated in 30% phosphate-buffered glycerol, and frozen in Freon 22 that had been cooled with liquid nitrogen. Fracture and shadowing were carried out in a Balzers BAF301 apparatus (Balzers High Vacuum; Balzers, Liechtenstein). Replicas were washed in a sodium hypochlorite solution, rinsed in distilled water, mounted on Formvar- and carbon-coated grids, and examined in an electron microscope (EM301; Philips Eindhoven, The Netherlands).

### Identification of Connexins

For immunofluorescence labeling (Meda et al., 1991, 1993), cells were grown on glass coverslips, fixed 3 min in either -80°C acetone or 70% ethanol, rinsed in cold (4°C) PBS containing 0.1% BSA, and incubated 2 h at room temperature with one of the following antibodies: (a) affinity-purified rabbit polyclonal against liver Cx32, diluted 1:100; (b) affinity-purified rabbit polyclonal against liver Cx26, diluted 1:50; (c) polyclonal rabbit antiserum against residues 314–322 of heart Cx43, diluted 1:100; (d) rabbit serum against residues 313–330 of heart Cx40, diluted 1:200; (e) rabbit serum against residues 346–358 of heart Cx40, diluted 1:100. After repeated rinsing, all cells were incubated a second time for 1 h at room temperature using fluorescein-conjugated anti-rabbit antibodies, diluted 1:200. After rinsing, sections were covered with 0.02% paraphenylenediamine in PBS-glycerol (1:2, vol/vol) and photographed on a microscope (Axiophot; Carl Zeiss, Inc., Oberkochen, Germany) fitted with filters for fluorescein detection.

In these experiments, positive controls were provided by parallel incubations of liver, heart, pancreas, and NEDH insulinoma sections. Small fragments of these organs were dissected from anesthetized rats and rapidly frozen by immersion in 2-methylbutane cooled with liquid nitrogen. The fragments were then stored in liquid nitrogen until cryostat sectioning on a Cryocut 3000 (Leica Instruments, Nussloch, Germany). Frozen sections were collected on gelatin-coated slides and processed for immunofluorescence as outlined above. Other controls included exposure of sections during the first incubation to one of the following reagents: (a) purified rabbit IgG; (b) preimmune rabbit sera; (c) the fluorescein-conjugated antibodies that were normally used during the second incubation step; (d) anticonnexin sera that had been preabsorbed with an excess of the synthetic peptide against which they were generated. None of these control incubations resulted in a specific labeling of the cells (not shown).

For Western blotting, cell cultures were homogenized by sonication in 0.02 M Tris-HCl, pH 8.0, supplemented with 20 mM EDTA, 1  $\mu$ g/ml pepstatin A, 1  $\mu$ g/ml antipain, 1 mM benzamide, 200 KIU/ml aprotinin, 2 mM PMSF, and 1 mM DFP. After a 10-min centrifugation of the sonicate at 3,000 g and 4°C, the supernatant was collected and centrifuged 60 min at 100,000 g and 4°C. Pelleted material was resuspended in PBS and stored at -80°C. Protein content was measured by either the Bradford method or the DC protein assay kit (Bio-Rad Laboratories, Glattburg, Switzerland). Samples of crude membrane preparations (50  $\mu$ g protein/lane) were fractionated by electrophoresis in a 12.5% polyacrylamide gel and immunoblotted as previously described (Meda et al., 1991). To this end, electrophoresed samples were transferred onto 0.22  $\mu$ m nitrocellulose (Schleicher & Schuell, Feldbach, Switzerland) or Immobilon membranes (Millipore AG, Walkerswil, Switzerland) for 18 h, at a constant voltage of 25 V and in the presence of 0.02% SDS. After checking for efficient transfer by Ponceau S staining, the nitrocellulose membranes were saturated at room temperature in BLOTTO solution (40 mM Tris-HCl, 0.1% Tween 20, and 4% dry milk) and then incubated 60 min with one of the affinity-purified antisera listed above (diluted in BLOTTO 1:50 for Cx43, 1:500 for Cx32, and 1:250 for Cx26) or a mouse monoclonal antibody against heart Cx43 (Zymed Laboratories Inc., San Francisco, CA), diluted 1:500. After repeated rinsing in BLOTTO, the nitrocellulose im-

munoblots were incubated for 60 min at room temperature with a biotinylated serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) against either rabbit (diluted 1:1,000) or mouse Ig (diluted 1:500), where appropriate. In the former case, the filters were rinsed, incubated for 1 h at room temperature with peroxidase-labeled streptavidin (Jackson ImmunoResearch Laboratories, Inc.), diluted 1:2,500, and eventually processed for detection of peroxidase activity using 4-chloronaphthol. In the latter case, filters were incubated 45 min at room temperature with alkaline phosphatase-labeled streptavidin (Amersham International, Little Chalfont, UK) diluted 1:5,000, rinsed repeatedly in 40 mM Tris-HCl supplemented with 0.5 M NaCl and 0.1% Tween 20, and eventually processed for detection of alkaline phosphatase activity using bromochloroindolyl phosphate-nitro blue tetrazolium as substrate.

Heart, liver, pancreas, and NEDH insulinoma were processed in a similar way and used as controls.

### Identification of Connexin Transcripts

Cell cultures were homogenized in 2.5 ml 0.1 M Tris-HCl, pH 7.4, containing 2M  $\beta$ -mercaptoethanol and 4 M guanidium thiocyanate. After addition of solid CsCl (0.4 g/ml), the homogenate was layered on 2 ml 5.7 M CsCl-0.1 M EDTA (pH 7.4) cushion and centrifuged at 35,000 rpm and 20°C for 20 h. Pelleted RNA was resuspended in 300  $\mu$ l 10 mM Tris-HCl, pH 8.1, supplemented with 5 mM EDTA and 0.1% SDS, extracted twice with phenol-chloroform, precipitated in ethanol, and resuspended in water. Probes for Cx32, Cx26, and Cx43 were constructed and used as previously described (Meda et al., 1993). For Northern blots, total cellular RNA was denatured with glyoxal, electrophoresed in a 1% agarose gel (5–10  $\mu$ g total cellular RNA/lane) and transferred overnight onto nylon membranes (Hybond N; Amersham International). Filters were baked under vacuum at 80°C for 2 h, exposed 30 s to 302-nm light, stained with methylene blue, and prehybridized according to either one of the following protocols: (a) 4 h at 65°C in a 50% Pipes-buffered (pH 6.8) solution of formamide supplemented with 2 mM EDTA, 0.1% SDS, and 100 mg/ml salmon sperm DNA; (b) 30 min at 65°C in 5  $\times$  SSC containing 0.1% SDS, 5  $\times$  Denhardt's solution, and 250 mg/ml salmon sperm DNA. Filters were then hybridized 18 h at 65°C with  $10^5$  cpm/cm<sup>2</sup> <sup>32</sup>P-labeled probe, and washed in one of the following ways: (a) two washings at 65°C in 3  $\times$  SSC, and 2  $\times$  Denhardt's solution followed by three washings at 70°C in 0.2  $\times$  SSC, 0.1% SDS and 0.1% sodium pyrophosphate, (b) two washings at room temperature in 2  $\times$  SSC containing 0.1% SDS followed by two washings at 65°C in 0.5  $\times$  SSC and 0.1% SDS. Filters were then exposed to film (XAR-5; Eastman Kodak Co., Rochester, NY) between intensifying screens at –80°C for either 1 or 7 d.

Samples of total cellular RNA were similarly extracted from heart, liver, pancreas, and NEDH insulinoma, and used as internal controls in all blots.

### Reverse Transcriptase (RT)<sup>1</sup>-PCR Amplification

For evaluation of Cx43 mRNA levels, 1–2- $\mu$ g aliquots of total cellular RNA were transcribed using 20 U AMV RT (Boehringer Mannheim, Rotkreuz, Switzerland) in 10 mM Tris-HCl buffer (pH 8.3) supplemented with 50 mM KCl, 0.001% gelatin, 4 mM MgCl<sub>2</sub>, and 100 ng oligo dT (Boehringer Mannheim).

Amplification of the resulting cDNA was performed in 50  $\mu$ l 10 mM Tris-HCl buffer (pH 8.3), supplemented with 50 mM KCl, 0.001% gelatin, 0.6 mM MgCl<sub>2</sub>, 2.5 U Ampli Taq (Perkin-Elmer Corp., Norwalk, CT), 25 mM of each dNTP species (GeneAmp; Perkin-Elmer Cetus, Kusnacht, Switzerland), 2  $\mu$ Ci <sup>32</sup>P-dCTP, and 0.3 mM of both sense and antisense connexin-specific primers. For Cx26, we amplified a 247-bp fragment using as sense and antisense primers the oligonucleotide sequences 5'-GGG AAA ATC TGG CTC ACT GT-3' and 5'-TCT TTT CGT GTC TCC GGT AG-3', respectively. For Cx32 we amplified a 452-bp fragment using as sense and antisense primers the oligonucleotide sequences 5'-AGT GCC AGG GAG GTG TGA AT-3' and 5'-GGA ACA CCA CAC TGA TGA CA-3', respectively. For Cx43, we amplified a 334-bp fragment using as sense and antisense primers the oligonucleotide sequences 5'-CGG CGG CTT CAC TTT CAT TA-3' and 5'-AGA ACA CAT GGG CCA AGT AC-3', respectively. For Cx37, we amplified a 422-bp fragment using as sense and antisense primers the oligonucleotide sequences 5'-GGC

TGG ACC ATG GAG CCG GT-3' and 5'-TTC TGG CCA CCA TGG GGG GC-3', respectively. For Cx40, we amplified a 308-bp fragment using as sense and antisense primers the oligonucleotide sequences 5'-CTG GCC AAG TCA CGG CAG GG-3' and 5'-TTG TCA CTG TGG TAG CCC TGA-3', respectively. For Cx 46, we amplified a 332-bp fragment using as sense and antisense primers the oligonucleotide sequences 5'-GGA AAG GCC ACA GGG TTT CCT-3' and 5'-GGG TCC AGG AGG ACC AAC GG-3', respectively. For glucokinase, we amplified a 441-bp fragment using as sense and antisense primers the oligonucleotide sequences 5'-ATC GGA TCC TTT GCT GAG TCT GTC TAG AGG-3' and 5'-GCA GCT GGA ACT CTG CCA G-3', respectively. After a 3-min start at 95°C, amplification was carried out for 30 cycles, each comprising 30 s at 95°C, 30 s at 65°C, and 90 s at 72°C, in a PCR Thermal Cycler 9600 (Perkin-Elmer Cetus). From cycle 21 onward, tubes were removed at the end of each cycle, placed on ice, and the reaction mixture diluted with 95  $\mu$ l Tris-HCl (10 mM)-EDTA (1 mM). 1-ml aliquot from each tube was counted to determine total radioactivity. Another 90- $\mu$ l aliquot was incubated overnight at –20°C in the presence of glycogen and isopropanol. The precipitated DNA was size fractionated on a 5% polyacrylamide gel, using an 89-mM Tris-borate buffer (pH 8.0) supplemented with 2 mM EDTA. Amplified DNA fragments were identified after ethidium bromide staining using as markers jX174RF DNA/HaeIII fragments (Life Technologies AG, Basel, Switzerland). Radioactivity of cut gel bands was counted in a gel scintillation cocktail (Ready; Beckman Instruments, Palo Alto, CA). Accumulation of amplified products was calculated from these counts, as described in Wiesner et al. (1992).

For detection of Cx26, Cx32, and Cx43, 1  $\mu$ g cellular RNA was transcribed using 200 U superscript II Moloney murine leukemia virus (M-MLV) RT (GIBCO BRL) in 50 mM Tris-HCl buffer (pH 8.3) supplemented with 75 mM KCl, 0.1 mg/ml BSA, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 1 mM 4d (NTP), 15 U RNA sin (30,000 U/ml; Biofinex, Praroman, Switzerland), and 5  $\mu$ M pdN(6) polydeoxynucleotide (Boehringer Mannheim, Mannheim, Germany). Amplification of the resulting cDNA (5- $\mu$ l samples) was performed in 50 ml 10 mM Tris-HCl buffer (pH 8.8) supplemented with 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 0.2 mM 4dNTP (Finnzymes Inc., Epoo, Finland), 1 U Dynazyme (Finnzymes Inc.), and 0.25 mM of the sense and antisense connexin-specific primers listed above. After a 3-min start at 95°C, amplification was carried out for 30 cycles, each comprising 90 s at 95°C, 60 s at 65°C, and 75 s at 72°C in a Robocycler 40 (Stratagene, Zurich, Switzerland). Samples were then placed 5 min at 72°C. Amplified DNA fragments were separated in a 2% agarose gel and identified after ethidium bromide staining using as markers jX174RF DNA/HaeIII fragments (Promega, Wallisellen, Switzerland).

### Junctional Coupling

For microinjection, aliquots of 4.10<sup>5</sup> primary cells were suspended in RPMI 1640 medium and plated, as individual 40- $\mu$ l drops within 35-mm culture dishes. 1 d later, 2 ml of RPMI 1640 medium was added to each dish. Under these conditions, the cultures of primary islet cells to be injected comprised multiple subconfluent monolayer clusters, each comprising 5–30 cells. The other insulin-producing cell types were also grown at about the same density in RPMI 1640 medium, supplemented as detailed above.

Permeability of junctional channels was evaluated by impaling individual cells with a glass microelectrode (150–200 M $\Omega$ ) filled with Hepes-buffered (pH 7.2) 150 mM LiCl containing 4% Lucifer yellow CH (Sigma Chemical Co., St. Louis, MO). The microelectrode was connected to a pulse generator for passing current and recording membrane potential. After successful cell impalement, 0.1 nA negative square pulses of 900 ms duration and 0.5 Hz frequency were applied to the electrode for 3 min to ensure a reproducible injection of comparable amounts of the dye in each experiment. We have previously shown that this protocol allows for rapid detection of dye coupling across extensive cytoplasmic areas (Chanson et al., 1991), which largely exceeds those of the communication territories of islet cells (Meda et al., 1983, 1991). We have also reported that the incidence and extent of islet cell dye coupling are not affected by extending the duration of the Lucifer yellow injection, or that of the time interval between this injection and its photographic recording (Meda et al., 1983). In these experiments, each injected field was photographed under fluorescence illumination using filters for fluorescein detection (Meda et al., 1991).

Preliminary measurements of junctional conductance in some RIN and INS1 clones were performed by dual voltage clamp whole-cell recording, as previously described (Weingart, 1986).

1. Abbreviation used in this paper: RT, reverse transcriptase.

## Transfection of Cx43

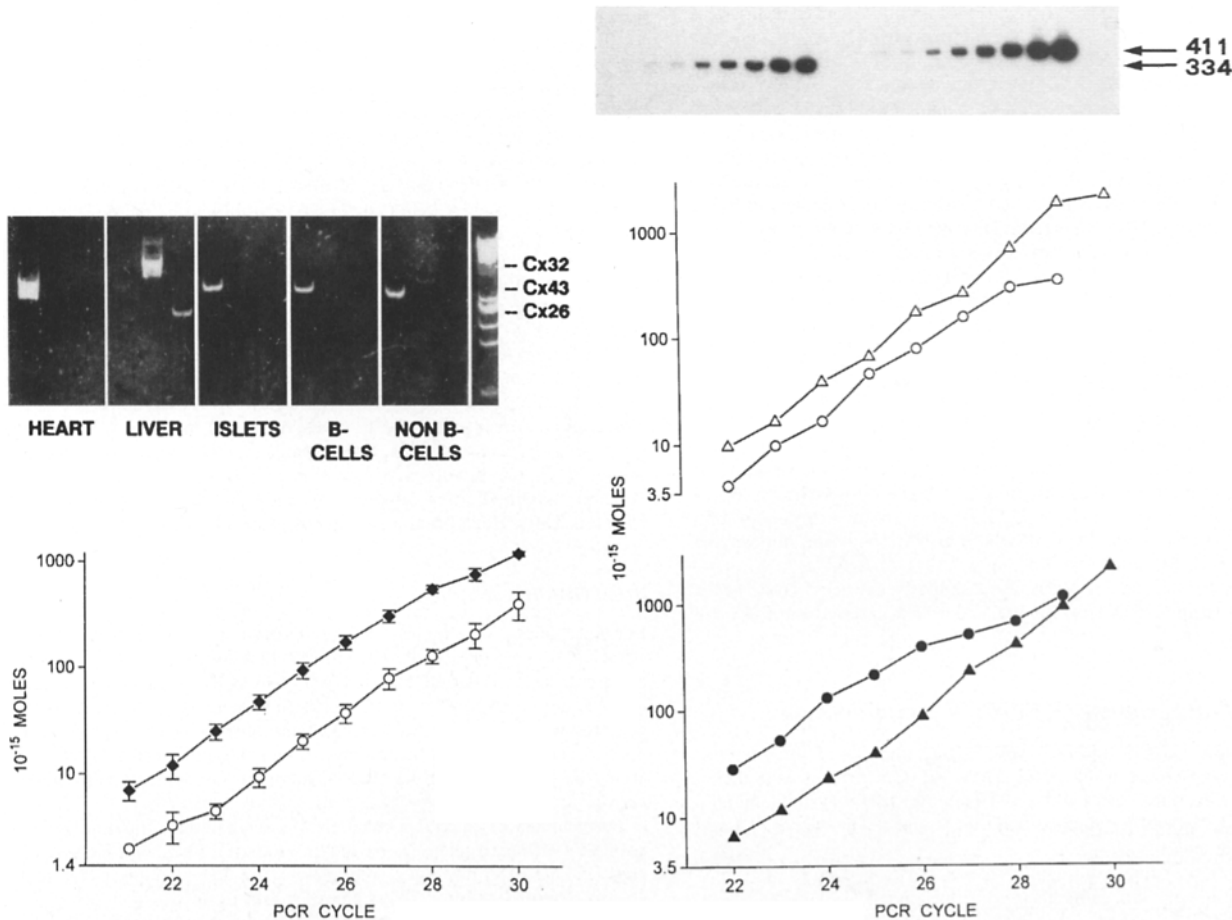
An expression plasmid was built by ligating in plasmid pSFFV, which contains the SV-40 early promoter region and the neomycin-resistance gene (Fuhlbrigge et al., 1988), the entire sequence coding for rat Cx43 (Beyer et al., 1987; Kumar and Gilula, 1992). A large portion of the 3' untranslated region of the endogenous Cx43 gene is missing in this construct, and is replaced by a shorter 3' untranslated sequence of SV-40, to allow for mRNA polyadenylation. As a result, the mRNA expressed after transfection is expected to be ~900 bp smaller than that expressed endogenously. Subconfluent cultures of INS1 cells were exposed for 12 h to 30  $\mu$ g Lipofectin<sup>®</sup> reagent (GIBCO BRL) mixed with 20  $\mu$ g plasmid pSFFV-Cx43. After the first passage, stable transfectants were selected in RPMI 1640 medium supplemented with 10% FCS, 110 U/ml penicillin, 110  $\mu$ g/ml streptomycin, and 200  $\mu$ g/ml Geneticin<sup>®</sup> (Life Technologies, Paisley, Scotland).

Transfection of the plasmid insert in the selected clones was verified by

Southern blotting. The induced transcript and the resulting protein were analyzed in Western and Northern blots, respectively (not shown).

## Insulin Secretion

RIN and INS1 cells were plated at the density of  $5 \times 10^5$  cells/35-mm dish (2 ml) and tested 3 d later. All dishes were preincubated 30 min at 37°C in a Krebs-Ringer bicarbonate buffer containing no glucose (control KRB), and then, incubated again 30 min at 37°C in 1 ml of the same medium supplemented now with either 2.8 mM, 11.2 mM, 16.7 mM, or 2.8 mM glucose plus 10 mM D-glyceraldehyde. At the end of this second incubation, the medium was collected, centrifuged 10 min at 150 g, and the supernatant rapidly frozen at -20°C for subsequent determination of insulin release. Cultures were thoroughly rinsed in control KRB, extracted for 24 h at 4°C in 1 ml acid ethanol and the extracts also frozen for subsequent determination of insulin content. Insulin was measured in media and extracts by a



**Figure 1.**  $\beta$ -cells express Cx43 and no other pancreatic connexin. (Top left) Cx32, Cx43, and Cx26 were identified by amplification of a product of 452, 334, and 248 bp, respectively. A product corresponding to Cx43, but not to either Cx26 or Cx32, was amplified in samples of intact pancreatic islets, purified  $\beta$ -cells, and non- $\beta$ -cells. The latter population represents a mixture of glucagon-, somatostatin-, pancreatic polypeptide-, and insulin-producing cells. Samples of heart, an organ which expresses Cx43 but no Cx26 or Cx32, and liver, which expresses Cx26 and Cx32 but no Cx43, served as controls. The starting material of these experiments was in all cases total RNA that had been extracted from cells lysed by sonication in a guanidium thiocyanate solution. (Bottom left) Evaluation of the amount of the 334-bp product after consecutive PCR cycles, starting from 500 ng islet cDNA and 5 ng heart cDNA, showed that the levels of Cx43 expressed by islet cells (open circles) were much smaller than those found in heart (solid diamonds). Values are mean  $\pm$  SEM of four (islets) to five (heart) experiments. (Top right) Glucokinase and Cx43 were identified in the same sample of purified  $\beta$ -cells, by amplification of a product of 411 and 334 bp, respectively. Adjacent bands show the accumulation of the two amplified products as a function of increasing PCR cycles. (Bottom right) Evaluation of these products in a typical experiment, showed a parallel amplification of the connexin (circles) and phosphorylating enzyme signals (triangles), in extracts of both isolated pancreatic islets (open symbols) and purified  $\beta$ -cells (solid symbols). These two preparations were extracted for analysis of Cx43 content immediately after purification, i.e., five (islets) to nine h ( $\beta$ -cells) after sampling the pancreas from the rats, and before any culture period.

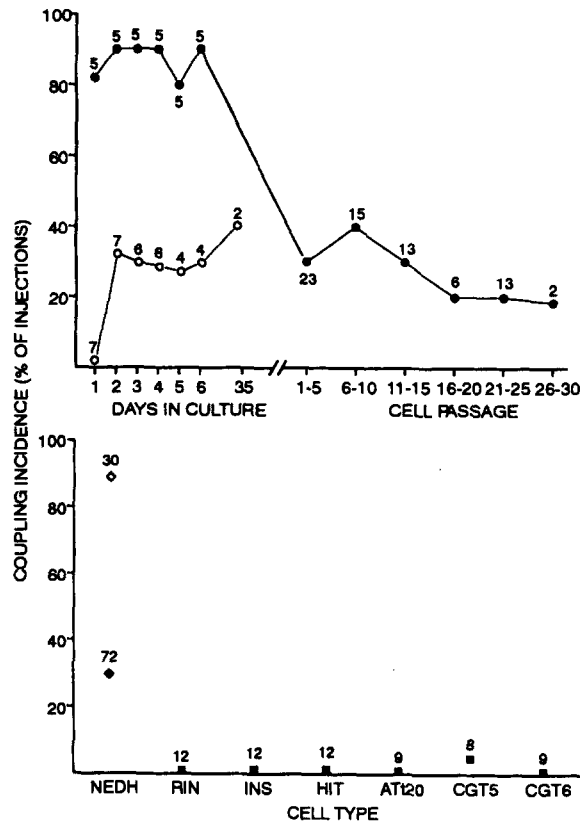
radioimmunoassay with a charcoal separation step using rat insulin as standard (Meda et al., 1990).

For analysis of insulin mRNA, total cellular RNA was extracted from INS1 cultures and processed for Northern blotting, as described above. Hybridizations were carried out using random-primed cDNA probes to hamster insulin and GAPDH, as previously reported (Philippe et al., 1992).

## Results

### Pancreatic $\beta$ -Cells Express Cx43 In Vivo and In Vitro

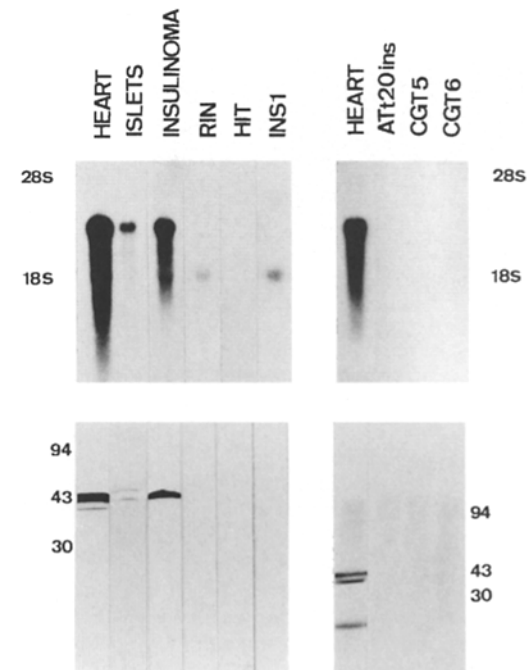
Connexins expressed by native insulin-producing  $\beta$ -cells were identified by examining freshly isolated pancreatic islets, as well as cells that were dispersed from these micro-



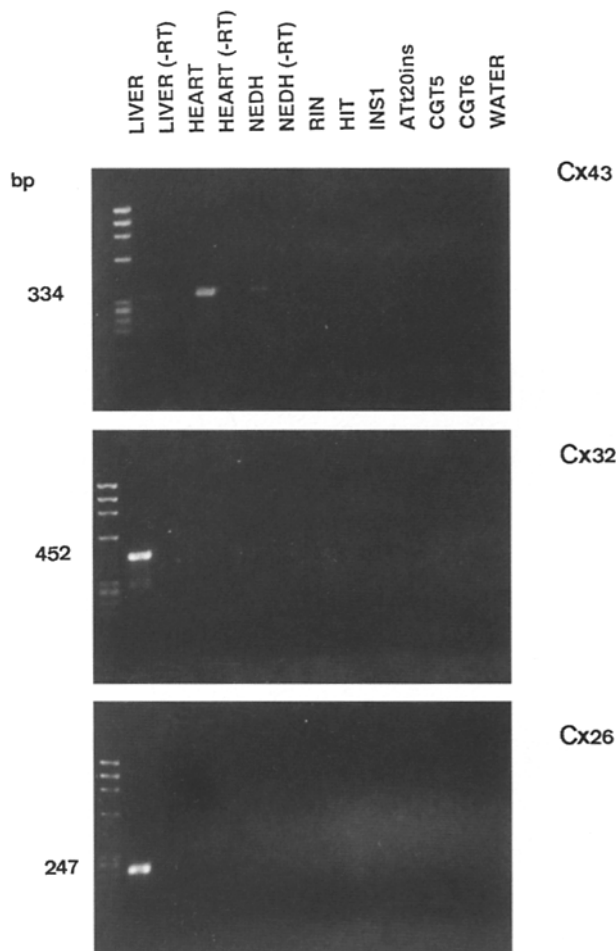
**Figure 2.** Incidence of dye coupling in insulin-producing cells. (Top) The incidence of coupling in primary  $\beta$ -cells (open circles) was minimal in 1-d-old cultures, but increased rapidly thereafter, being observed after 30–40% of the injections up to the 35th day of culture. 80–90% of the Lucifer yellow injections also revealed dye transfer between primary insulinoma cells (solid circles). Coupling incidence decreased with the first passage of these cells, and was thereafter observed in 20–30% of the cases up to the 27th in vitro passage. (Bottom) Transfer of Lucifer yellow was frequently observed between the glucose-sensitive cells of the NEDH insulinoma, whether these cells were tested immediately after dispersion of the tumor (open diamond) or after passage in culture (solid diamond). The figures plotted were calculated by pooling the data shown by the solid circles in the upper panel. In contrast, it was only exceptionally observed in the insulinoma-derived RIN and INS1 lines, as well as in four other cell lines which also featured glucose insensitivity. All data are median values for the numbers of experiments indicated, each experiment testing 8–20 cells.

organs and purified by flow cytometry. RT-PCR amplification of total RNA extracted from islets and purified  $\beta$ -cells, using primers for the three connexins found in the pancreas (Meda et al., 1993), revealed the expression of Cx43 (Fig. 1). In both islet and purified cell samples, the amplification of the Cx43 cDNA product increased in parallel with that of  $\beta$ -cell specific glucokinase, and was significantly less abundant than that observed in samples of heart (Fig. 1), the organ in which Cx43 was originally identified. In contrast, the RT-PCR procedure failed to detect Cx26 and Cx32 in all islet cell preparations (Fig. 1), even though these two connexins could be readily identified in samples of liver, which served as positive controls (Fig. 1).

After several days of culture, primary  $\beta$ -cells still expressed Cx43, as detected by RT-PCR, and remained dye coupled, as evidenced by cell-to-cell exchange of Lucifer yellow (Fig. 2). The incidence of this exchange was low in 1-d-old cultures of dispersed islet cells, but increased thereafter as these cells reassembled into clusters (Fig. 2).

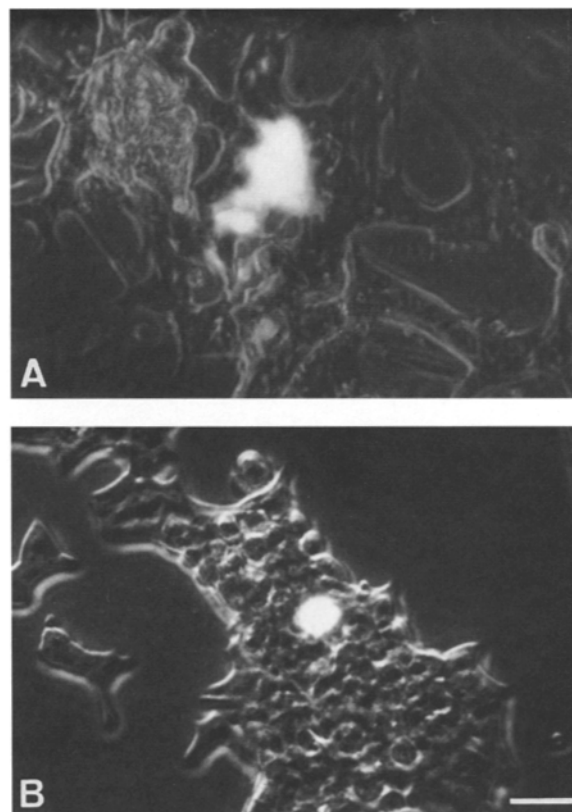


**Figure 3.** Cx43 expression in different types of insulin-producing cells. (Top) Northern blots of total RNA with either a cRNA (left) or a cDNA probe for Cx43 (right). Under both conditions, a single transcript was readily identified in heart, NEDH insulinoma, and islets, but not in the different insulin-producing cell lines tested. Lanes were loaded with either 5  $\mu$ g (HEART) or 10  $\mu$ g total cellular RNA (all other lanes). (Bottom) Immunoblots of membrane proteins separated by SDS-PAGE, and reacted with polyclonal (left) or monoclonal antibodies (right) recognizing different COOH terminus epitopes on the Cx43 molecule. This connexin was readily detected in heart and NEDH insulinoma, and at much lower levels, in freshly isolated pancreatic islets. In contrast, no Cx43 was detected in the RIN, HIT, INS1, AT120ins, CGT5, and CGT6 lines of insulin-producing cells. The band of apparent low mol wt found in heart samples reacted with mAbs was also observed when these antibodies were purposely omitted and hence, was considered to reflect some nonspecific binding. All lanes were loaded with 50  $\mu$ g protein.



**Figure 4.** Connexins are not detectable in lines of insulin-producing cells that show secretory defects. (*Top*) PCR amplification of a reverse transcribed RNA sample of heart, using primers specific for Cx43, resulted in the amplification of a single 334-bp product, seen here after ethidium bromide staining. A similar product was amplified, in lower amounts, from samples of NEDH insulinoma and liver RNA. In contrast, no product was detected in the six permanent lines of insulin-producing cells (*RIN*, *HIT*, *INS1*, *ATt20ins*, *CGT5*, *CGT6*) that were tested. (*Middle*) Running the RT-PCR procedure with primers specific for Cx32, resulted in the amplification of a 452-bp product in liver samples. In contrast, no product was detected in samples of heart, NEDH insulinoma, and in the six insulin-producing lines tested. (*Bottom*) Similar results were obtained with primers for Cx26, which resulted in the detection of a 247-bp product only in liver samples. In all panels, the left lane shows the size standards, and the right lane shows the water sample that was used as blank control. (-RT) indicates samples of heart, liver and insulinoma that were not reverse transcribed before PCR processing. As expected, no product was amplified in these samples.

Hence, from the second day of culture onward, transfer of Lucifer yellow between small groups of 2–4 cells was observed in ~30% of the injections, up to the end of the fifth week of culture (Fig. 2). The incidence and extent of dye coupling were similar in small and large islet cell clusters, and did not appear to be affected by the somewhat variable level of subconfluence of these clusters which was obtained in different culture dishes.



**Figure 5.** Secretory defective insulin-producing cell lines are essentially uncoupled. (*A*) Microinjection of Lucifer yellow within cultures of NEDH insulinoma cells revealed passage of the tracer from the injected cell into some of its neighbors. (*B*) In contrast, most injections performed in cultures of *INS1* cells failed to show such a cell-to-cell passage. In these cases, Lucifer yellow was retained within the single injected cell. Similar observations were made in cultures of *RIN2A*, *HIT*, *ATt20ins*, *CGT5*, and *CGT6* cells. Bar, 20  $\mu$ m.

#### ***Insulinoma Cells Express Cx43 and Coupling Similar to Native $\beta$ -cells***

Cells of the transplantable NEDH insulinoma, which exhibit several secretory features of native  $\beta$ -cells (Chick et al., 1977), also solely expressed Cx43 as judged at both transcript and protein level (Figs. 3 and 4). About 80% of the Lucifer yellow injections revealed that primary insulinoma cells remained coupled to three to four neighbors for several days in culture (Figs. 2 and 5). Coupling incidence decreased abruptly with the first passage of these cells, and was thereafter observed in 20–30% of the injections up to the 27th weekly passage (Fig. 2).

#### ***Secretory-Defective Insulin-producing Cell Lines Do Not Express Cx43 and Coupling***

Six independent lines of insulin-producing cells (*RIN2A*, *INS1*, *HIT*, *ATt20ins*, *CGT5*, *CGT6*), which differ markedly from  $\beta$ - and NEDH insulinoma cells with regard to both insulin content and glucose-induced release of the hormone (Gazdar et al., 1980; Santerre et al., 1981; Moore et al., 1983; Asfari et al., 1992; Hughes et al., 1992) failed to show detectable expression of Cx43, as judged at both

Table I. Gap-junctional Coupling and Secretory Characteristics of Insulin-producing Cells

Cell	Clone	Cx43 mRNA*	Coupling <sup>‡</sup>	Insulin-mRNA <sup>§</sup>	Insulin-content <sup>  </sup>	Glucose-induced insulin release <sup>¶</sup>	Group
PRIMARY $\beta$		+	++	+++	+++	+++	Limited coupling
NEDH Insulinoma		+	++	+++	+++	++	
INS-Cx43*	A	+	++	+++	+++	+	
	W	+	++	+++	+++	+++	
INS		-	-	+	++	+	No coupling
	G	-	-	ND	+	+	
	H	-	-	ND	+	-	
	J	-	-	ND	++	-	
	L	-	-	ND	++	-	
INS-Cx43**	B	+	-	+	+	-	
INS-neo**		-	-	ND	++	ND	
INS-Cx43**	I	+++	+++	+	++	-	Extensive coupling
	N	+++	+++	+	+	-	
	E'	+++	+++	ND	+	ND	
	H'	+++	+++	ND	+	ND	
	Q'	+++	+++	ND	+	ND	
	V'	+++	+++	ND	+	ND	
	K'	+++	+++	ND	+	ND	
	N'	+++	+++	ND	+	ND	

Cells were assigned to three different groups depending on the extent of their dye coupling (limited, nil, or extensive). Values of each parameter were ranked according to a nominal scale from -, undetectable to +, detectable, ++, intermediate and +++, large.

\*Screened by Northern blotting. The abundance of the Cx43 transcript was scored relative to the signal observed in samples of control rat heart (scored ++) that were processed in parallel.

<sup>‡</sup>Screened by microinjection of Lucifer yellow. The extent of coupling was scored relative to that observed in primary  $\beta$ -cells (scored ++) that exchanged the tracer with 3 to 4 companion cells in 60% of the injections.

<sup>§</sup>Screened by Northern blotting. The abundance of the insulin transcript was scored relative to the signal obtained in primary  $\beta$ -cells (scored +++).

<sup>||</sup>Screened by radioimmunoassay. The levels of stored insulin were scored relative to those of primary  $\beta$ -cells (scored +++).

<sup>¶</sup>Screened by radioimmunoassay after a 30-min increase in glucose concentration from 2.8 to 11.2 mM. The amounts of insulin released were scored relative to those of primary  $\beta$ -cells (scored +++).

\*\*Transfected cells.

transcript and protein levels (Figs. 3 and 4). These cells also did not express detectable levels of Cx26 and Cx32 as judged by both RT-PCR (Fig. 4) and Northern blotting (data not shown). Further RT-PCR screening also failed to detect expression of the genes coding for Cx37, Cx40, and Cx46 in both RIN and INS1 cells (not shown). All the lines tested were virtually always uncoupled, as assessed by injection of Lucifer yellow (Figs. 2 and 5). Dual voltage clamp whole-cell recordings also failed to detect junctional conductance in pairs of both RIN and INS1 cells (not shown).

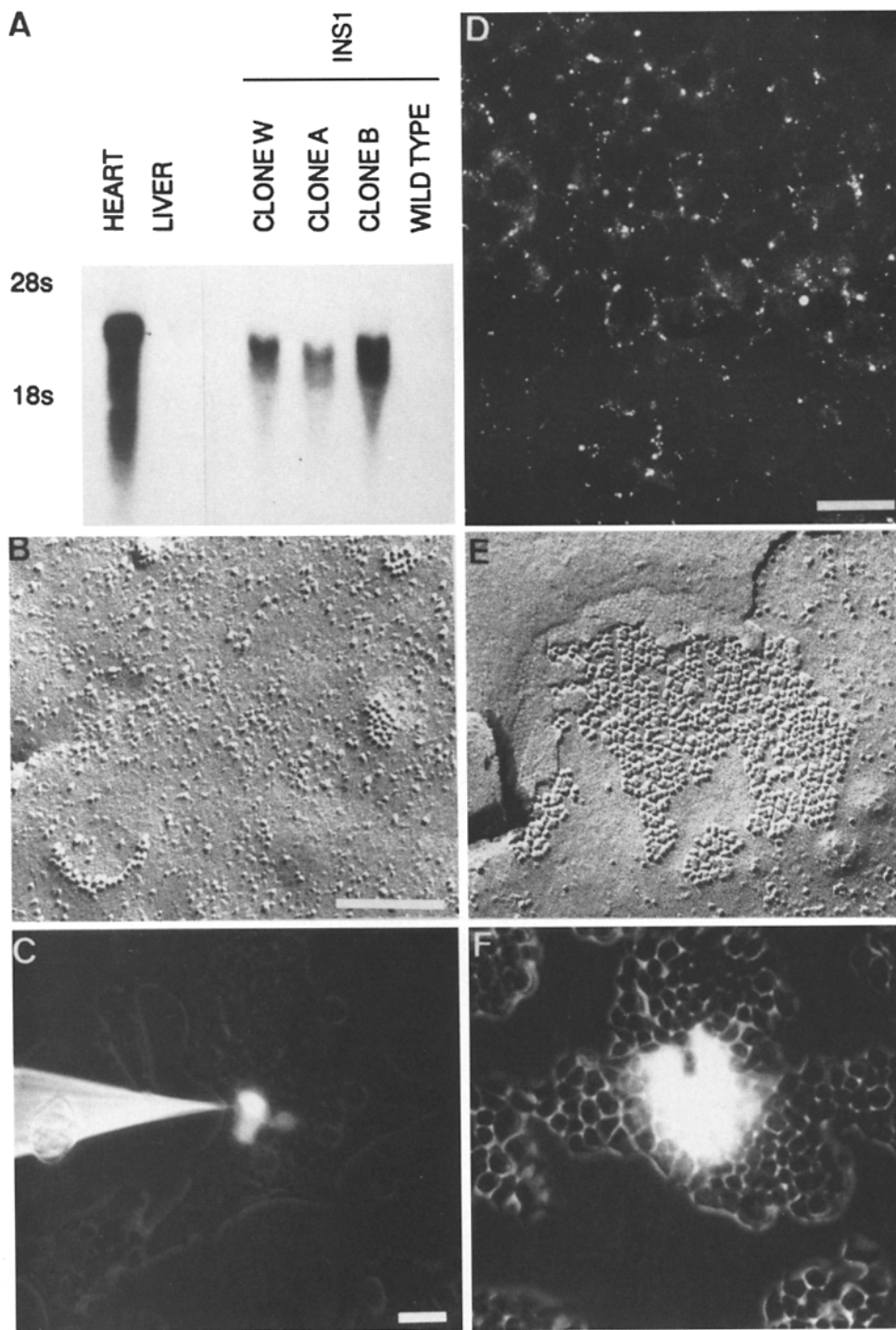
#### Clones of INS1 Cells Expressing Cx43 and Coupling Show Changes in Secretory Characteristics

The observation that the tumoral cells of primary NEDH insulinomas showed limited coupling and small, Cx43-made gap junctions, as do primary  $\beta$ -cells, whereas permanent cell lines do not, prompted us to assess the effects of a stable transfection of Cx43 on the secretory characteristics of a line of INS1 cells that was derived from the NEDH tumors.

As shown in Table I, several independent clones of INS1 cells were selected for acquired neomycin resistance after exposure to a plasmid which also contained the entire sequence coding for Cx43. As expected from the construction of this expression vector, all these clones expressed a Cx43 transcript that had a slightly higher mobility than that of the Cx43 mRNA observed in control heart samples

(Fig. 6 A). However, the levels of expression of this transcript varied from one clone to another, and, accordingly, these clones also differed in the number and size of gap junctions (Fig. 6, B and E), as well as in the degree of both dye (Fig. 6, C and F) and electrical coupling (Table I). None of the transfected clones expressing Cx43 showed detectable levels of Cx32 and Cx26 in Northern blots (not shown).

Two clones of INS1 cells expressed much less Cx43 mRNA than heart (Fig. 6 A), as it is also the case for native  $\beta$ -cells (Table I). These two clones formed small gap junctions, as detected by freeze-fracture electron microscopy (Fig. 6 B), and showed coupling between two and three adjacent cells after 50–60% of the Lucifer yellow microinjections ( $n = 30$ ; Fig. 6 C). When compared with the uncoupled cells of either the parental INS1 population, or to individual clones that had been derived from it, the frequently coupled cells of these two clones showed a markedly higher expression of insulin mRNA, an increased density of secretory granules (Fig. 7), and a larger content of stored insulin (Fig. 8). Hence, compared with the content of control INS1 cells ( $5.2 \pm 0.1 \mu\text{g insulin}/10^6$  cells,  $n = 75$ ), clones A and W showed an average insulin content of  $11.7 \pm 0.7$  ( $n = 75$ ) and  $13.7 \pm 0.4 \mu\text{g insulin}/10^6$  cells ( $n = 73$ ), respectively. These cells also retained the ability of parental INS1 cells to acutely increase insulin release during a 30-min rise of the glucose concentration from 2.8 to 11.2 mM. One clone (clone W) actually showed a sizable improvement of this function (Table I).



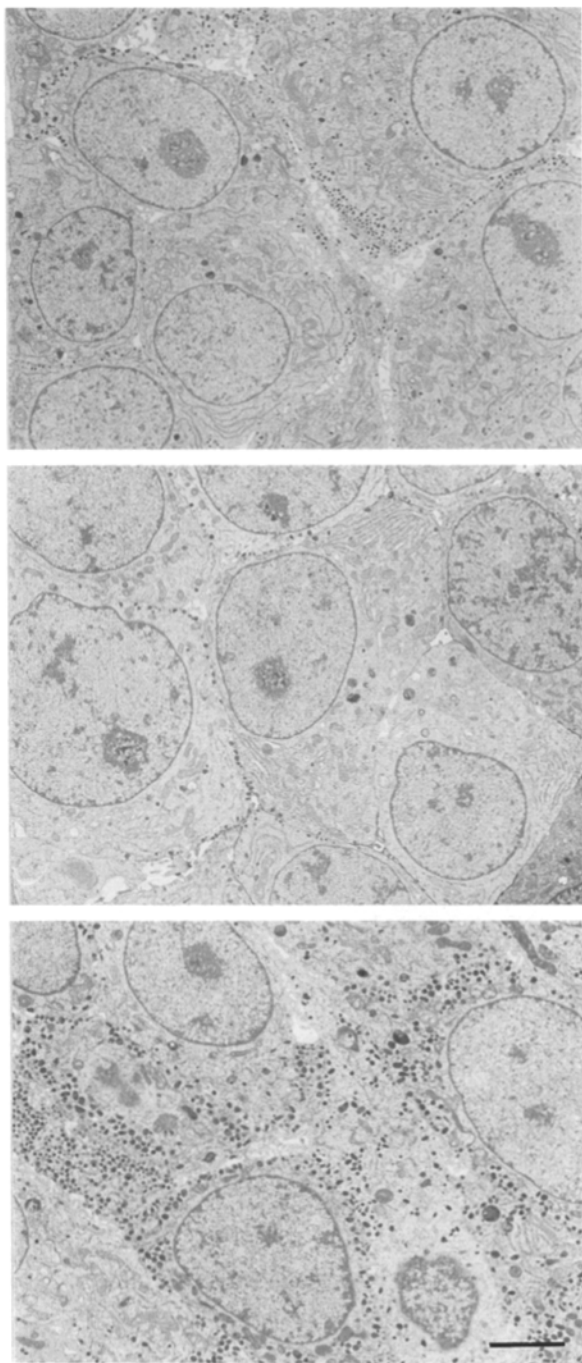
**Figure 6.** Transfected clones show variable expression of Cx43 and coupling. (A) The Northern blot hybridization of total RNA samples (2  $\mu$ g for heart and liver samples and 20  $\mu$ g for all INS1 samples) with a probe to Cx43, showed the transcript encoding this protein in heart and, in much limited amounts, in samples of INS1 clones W, A, and B. Note that, as expected from the constructed plasmid (see Materials and Methods), the Cx43 transcript observed after transfection had a slightly higher mobility than that found in native heart cells. In contrast, Cx43 mRNA was not detected in the parental, wild-type population of INS1 cells. (B) Cells of clone W were connected by minute gap-junctional plaques, like those observed between native  $\beta$ -cells. (C) These cells were also frequently coupled to small groups of two to four nearby cells. (D) Incubation of INS cells of clone I with a specific antibody against Cx43, resulted in abundant punctate immunostaining of many cell-to-cell interfaces. (E) Large gap-junctional plaques were detected by freeze-fracture EM at these interfaces. (F) At the end of a 3-min injection of Lucifer yellow within monolayers of the Cx43-transfected INS cells of clone I, diffusion of the gap junction tracer was seen to extend to >20 adjacent cells. Bar, 20  $\mu$ m in top and bottom panels, and 200 nM in the middle panels.

These changes were not seen in a third clone of INS1 cells (clone B), which was derived from the very same set of transfections, and also expressed limited amounts of Cx43 mRNA, still showed coupling after only 3% of the dye injections (Table I). As compared with the two clones that expressed limited coupling, this uncoupled clone showed a markedly lower expression of insulin mRNA, a much smaller insulin content, and a loss of glucose-induced insulin release (Table I). Lack of improved insulin synthesis and secretion was also observed in several clones of wild-type, nontransfected INS1 cells, as well as in an INS1 clone

that had been only transfected with the sequence coding for neomycin resistance (Table I).

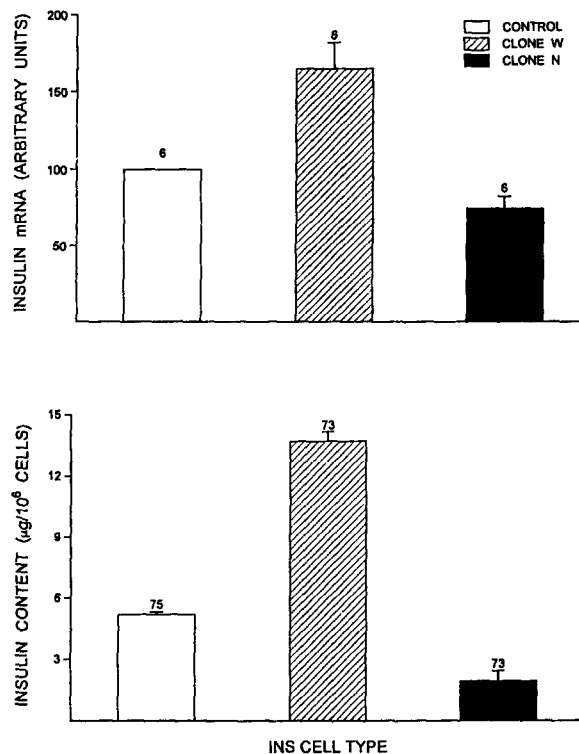
Comparable secretory characteristics were also found in eight stably transfected clones of INS1 cells that showed dye coupling between 20 and 30 cells after most Lucifer yellow injections (Fig. 6 F). Three of these clones, which were further tested by dual voltage clamp whole-cell recording, showed macroscopic junctional conductances in the 4–20 nS range, that were reversibly blocked by heptanol (not shown). All these clones expressed immunoreactive Cx43 at cell-to-cell interfaces (Fig. 6 D) and gap





**Figure 7.** Ultrastructural appearance of INS1 cells expressing variable levels of Cx43. (*Top*) The control INS1 cells used for the transfection experiments contained a modest amount of secretory granules. (*Middle*) A comparable low granule density was observed in the extensively coupled cells of clone N. (*Bottom*) In contrast, cells of clone W, which showed a spatially restricted coupling, contained significantly larger numbers of secretory granules. Bar, 3  $\mu\text{m}$ .

junctional plaques that were unusually large (Fig. 6 E), compared with those found between native  $\beta$ -cells. None of these clones showed the improved secretory characteristics that were observed in the clones exhibiting limited dye coupling (Table I). Rather, when compared with the



**Figure 8.** The secretory characteristics of INS1 cells vary with the levels of expression of Cx43 and coupling. (*Top*) Compared with the essentially uncoupled cells of the control INS1 population (*open column*), cells of clone W, which showed limited expression of Cx43 and coupling (*hatched column*) expressed higher levels of insulin mRNA. In contrast, this  $\beta$ -cell-specific transcript was reduced in cells of clone N, which overexpressed Cx43 and coupling (*solid column*). (*Bottom*) Analogous differences were observed in terms of the insulin content of the different INS1 clones. Data are expressed as mean  $\pm$  SEM values for the number of experiments indicated above each column. Data of the top panel are ratios of insulin to GAPDH mRNAs (the levels of the latter transcript were not modified in transfected clones), expressed relative to control values.

wild-type INS1 population, seven of the eight clones exhibiting extensive coupling, showed markedly reduced contents of secretory granules (Fig. 7) and insulin (Fig. 8 and Table I). Thus, insulin content averaged  $5.2 \pm 0.1 \mu\text{g}/10^6$  cells ( $n = 75$ ) in the nontransfected wild-type population of INS cells (Fig. 8), and was significantly ( $P < 0.001$ ) reduced in clones N ( $1.9 \pm 0.04$ ,  $n = 70$ ), H' ( $1.4 \pm 0.08$ ,  $n = 30$ ), Q' ( $0.9 \pm 0.05$ ,  $n = 53$ ), V' ( $0.8 \pm 0.04$ ,  $n = 53$ ), K' ( $1.2 \pm 0.08$ ,  $n = 69$ ), and N' ( $0.2 \pm 0.01$ ,  $n = 51$ ). The levels of insulin observed in these clones were lower than those observed in several clones of nontransfected cells that had been derived from the original INS1 population ( $2.1 \pm 0.05$ ,  $\mu\text{g}/10^6$  cells,  $n = 28$  for clone G;  $3.4 \pm 0.2$ ,  $n = 28$  for clone H;  $3.7 \pm 0.1$ ,  $n = 30$  for clone J;  $4.3 \pm 0.2$ ,  $n = 10$  for clone L) and in a clone of INS cells that had been transfected only for neomycin resistance ( $4.4 \pm 0.1 \mu\text{g}/10^6$  cells,  $n = 15$ ). The eight clones of extensively coupled cells (clone I) did not feature a lowered insulin content (Table I). However, when further tested for acute secretory responsiveness, it failed to increase significantly insulin release during a 30-min rise of glucose from 2.8 to 11.2 mM,

similar to a clone (clone N) that displayed drastically reduced insulin content (Table I).

In summary, the two subsets of clones generated in the transfection experiments did not show similar changes in insulin synthesis, storage, and release. Two lines of evidence indicate that this variability does not reflect a random selection of intrinsically different clonal cells, independent of their connexin expression, but rather, is related to their different level of junctional communication. First, no major change in insulin expression, storage, and release was observed in several clonal populations derived from either nontransfected INS1 cells or from INS1 cells transfected only for neomycin resistance (Table I). Second, a comparable improvement in the secretory features characterizing  $\beta$ -cells was observed in both primary and Cx43-transfected tumoral cells that exhibited limited coupling. In contrast, a similar loss of these characteristics was seen in clones of INS1 cells exhibiting extensive coupling (Table I). The most striking and consistent difference between these two subsets of transfected clones concerned insulin content, which, as compared with the values observed in nontransfected INS1 cells, was significantly elevated in the two clones featuring modest coupling (clones A and W), but not in either the eight clones showing extensive coupling (clones I, N, E', H', Q', V', K', and N'), or the six other clones (clones G, H, J, L, B, and neo) of essentially uncoupled cells (Table I). Statistical analysis of this opposite distribution using the Fisher exact probability test, a nonparametric technique that allows for analysis of data from two independent samples of small size (Siegel, 1956), showed that clones expressing moderate coupling were significantly different from both the clones expressing extensive coupling ( $P < 0.001$ ) and the uncoupled clones ( $P < 0.03$ ), inasmuch as the improvement in the level of stored insulin (+++, scored in Table I) was concerned.

## Discussion

Connexins form a widely distributed family of high turnover, nonglycosylated proteins that form membrane channels for direct intercellular exchanges of cytoplasmic ions and molecules (Bennett et al., 1991; Kumar and Gilula, 1992). The physiological function of these exchanges in adult, differentiated tissues that are not specialized for contraction remains a matter of debate (Loewenstein, 1981; Bennett et al., 1991). Here we provide novel experimental evidence that connexin-mediated cell-to-cell communications contribute to proper control of insulin production.

We first report that, at variance with many other cells (Bennett et al., 1991; Kumar and Gilula, 1992; Meda et al., 1993), normal pancreatic  $\beta$ -cells express only one gap junction protein, referred to as Cx43. Since different connexins impart distinctive conductance and permeability characteristics to gap junction channels (Bennett et al., 1991; Brissette et al., 1994; Steinberg et al., 1994; Veenstra et al., 1994a,b), and ensure a stringent selectivity to their functional establishment (Bruzzone et al., 1993; White et al., 1994), it is conceivable that this exclusive expression is required to ensure proper  $\beta$ -cell coupling.  $\beta$ -cells are frequently but not obligatorily coupled to companion cells, and their junctional communication consistently is re-

stricted to small groups of adjacent cells (Kohen et al., 1979, 1983; Michaels and Sheridan, 1981; Meda et al., 1981, 1983, 1990; Eddlestone et al., 1984). We also show that pancreatic  $\beta$ -cells retain in culture the same pattern of connexin and junctional coupling they show in situ. In view of previous reports that rapid changes in the natural pattern of connexins parallel in vitro alterations of cell differentiation (Stutenkemper et al., 1992; Salomon et al., 1994), this stability suggests that appropriate expression of Cx43 is required for maintenance of  $\beta$ -cell-specific secretory characteristics.

Consistent with this hypothesis, we have observed that Cx43 is still expressed, in vivo and in vitro, by the cells of a transplantable rat insulinoma, which, in spite of a high growth rate, retain several of the major secretory landmarks of normal  $\beta$ -cells, including a high insulin content (Chick et al., 1977) and the ability to increase insulin release in the presence of physiologically relevant glucose concentrations (Masiello et al., 1982). In contrast, we have found that the cells of two insulinoma-derived lines (RIN, INS1) which show marked alterations of these functions (Gazdar et al., 1980; Asfari et al., 1992), do not express Cx43 and several other connexins, do not form gap junctions, and are essentially uncoupled. Comparable observations were made in four other cell lines (Santerre et al., 1981; Moore et al., 1983; Hughes et al., 1992), not related to the NEDH insulinoma, still also show markedly reduced insulin content and responsiveness to the glucose concentrations which stimulate normal  $\beta$ -cells. The cause of these secretory defects has been variably attributed to a multitude of causes, including inappropriate expression of  $\beta$ -cell-specific proteins that are thought to be essential for proper activation of the insulin secretory machinery by natural metabolizable nutrients (Newgard, 1992). So far, however, no protein has been shown to be similarly defective in all the lines that secrete insulin abnormally, providing no simple explanation for their essentially similar secretory defects. The present finding that Cx43 is expressed in native and tumoral insulin-producing cells that secrete normally, but is lost in several independent cell lines that share comparable secretory abnormalities, shows that absence of this junctional protein is at least a consistent landmark of secretory dysfunction. In view of previous observations that have implicated connexin-mediated junctional communications in the control of both insulin biosynthesis and release (Bosco et al., 1989; Meda et al., 1990; Bosco and Meda, 1992; Philippe et al., 1992), it is further possible that absence of Cx43 in proliferating lines be causally implicated in their loss of these  $\beta$ -cell-specific functions.

To test this implication, we have stably transfected the connexin- and coupling-defective INS1 line with the gene coding for Cx43. Expression of this sole protein in several independent clones resulted in the formation of typical gap junction plaques within the plasma membrane, and established coupling between the cells. Some of these clones showed modest Cx43 expression, small gap junctions, and a spatially restricted coupling, as observed in native pancreatic islets (Meda et al., 1979, 1983; Michaels and Sheridan, 1981), whereas others expressed much higher levels of Cx43 and formed much larger gap junctional plaques, providing for large junctional conductances and for dye

coupling between unusually large numbers of insulin-producing cells. When compared with the uncoupled cells of the parental, nontransfected population, the two sets of coupled clones featured a similar growth, but quite consistently showed marked changes in the expression of the gene coding for insulin and in the cellular stores of this hormone. These changes were not observed in multiple clones of either untransfected cells or cells transfected with other, connexin-unrelated sequences. However, these changes differed markedly depending on the level of connexin and coupling expression. Thus, clones expressing high levels of Cx43, gap junctions, and coupling, showed decreased expression of insulin mRNA, a drastically reduced insulin content, and a nil stimulation of insulin release in response to glucose concentrations (2.8–11.2 mM range) that physiologically stimulate normal  $\beta$ -cells. In contrast, clones selected for levels of connexin expression, gap junctions, and coupling similar to those observed in native islets, showed increased insulin mRNA levels, strikingly elevated amounts of stored hormone, and preserved glucose-induced insulin release. Statistics showed that at least the changes in insulin content were significantly different depending on the degree of coupling of different clones. The following informations may be derived from these data. First, stable transfection of Cx43 modifies the secretory characteristics of tumoral insulin-producing cells, independent of any effect on their growth *in vitro*. Second, this transfection alone is not sufficient to correct the multiple secretory defects of the lines we studied (Newgard, 1992), particularly with regard to insulin release in response to physiologically relevant glucose concentrations. This  $\beta$ -cell-specific characteristic is consistently altered in all the tumoral cell lines we investigated. Third, the striking changes in insulin gene transcription and storage observed in the transfected clones were dependent on the level of expression of Cx43 and coupling. The finding that cells coupled as seen in native pancreatic islets displayed improved secretory features, that were not observed in either uncoupled cells or cells showing a much more extensive junctional communication, indicates that adequate levels of Cx43 and coupling are required for proper insulin production. The reason for this requirement is unknown, and its elucidation awaits the identification of the signals exchanged through connexin-made channels, and able to influence the biosynthesis and release of insulin. Previous experiments have suggested that, when exchanged between limited cell numbers, such signals recruit  $\beta$ -cells into increased biosynthetic and secretory activity (Bosco and Meda, 1992; Bosco et al., 1989; Meda et al., 1990; Philippe et al., 1992). Conceivably, however, this positive effect may be lost whenever the cytoplasmic concentration of these signals is lowered excessively, as is expected after their diffusion into large numbers of coupled cells (Sheridan, 1973; Loewenstein, 1981).

We have demonstrated that loss of Cx43 expression and coupling are consistent landmarks of proliferating cell lines that secrete insulin abnormally. We have also provided evidence that correction of these defects promote  $\beta$ -cell-specific secretory characteristics, provided connexin-mediated coupling is restored to levels similar to those observed within native pancreatic islets. The availability of multiple transfected clones of insulin-producing

cells that stably express different levels of Cx43 should help identify the molecular mechanism underlying this effect.

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