

Expression of a Connexin 43/ β -Galactosidase Fusion Protein Inhibits Gap Junctional Communication in NIH3T3 Cells

Ruth Sullivan and Cecilia W. Lo

Biology Department, Goddard Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6017

Abstract. Gap junctions contain membrane channels that mediate the cell-to-cell movement of ions, metabolites and cell signaling molecules. As gap junctions are comprised of a hexameric array of connexin polypeptides, the expression of a mutant connexin polypeptide may exert a dominant negative effect on gap junctional communication. To examine this possibility, we constructed a connexin 43 (Cx43)/ β -galactosidase (β -gal) expression vector in which the bacterial β -gal protein is fused in frame to the carboxy terminus of Cx43. This vector was transfected into NIH3T3 cells, a cell line which is well coupled via gap junctions and expresses high levels of Cx43. Transfectant clones were shown to express the fusion protein by northern and western analysis. X-Gal staining further revealed that all of the fusion protein containing cells also expressed β -gal enzymatic activity. Double immunostaining with a β -gal and Cx43 antibody demonstrated that the fusion protein is immunolocalized to the perinuclear region of the cytoplasm and also as punctate spots at regions of cell-cell contact. This pattern is similar to that of Cx43 in the

parental 3T3 cells, except that in the fusion protein expressing cells, Cx43 expression was reduced at regions of cell-cell contact. Examination of gap junctional communication (GJC) with dye injection studies further showed that dye coupling was inhibited in the fusion protein expressing cells, with the largest reduction in coupling found in a clone exhibiting little Cx43 localization at regions of cell-cell contact. When the fusion protein expression vector was transfected into the communication poor C6 cell line, abundant fusion protein expression was observed, but unlike the transfected NIH3T3 cells, no fusion protein was detected at the cell surface. Nevertheless, dye coupling was inhibited in these C6 cells. Based on these observations, we propose that the fusion protein may inhibit GJC by sequestering the Cx43 protein intracellularly. Overall, these results demonstrate that the Cx43/ β -gal fusion protein can exert a dominant negative effect on GJC in two different cell types, and suggests that it may serve as a useful approach for probing the biological function of gap junctions.

GAP junctions (GJ)¹ are cell junctions containing membrane channels that allow the direct transfer of ions and small molecules (<1.0 kD) between cells. Gap junction channels are composed of hexameric arrays of proteins encoded by the connexin multigene family. All of the connexins share a common membrane topology, but differ in their unitary conductance and channel gating properties (for review see Bennett et al., 1991; Beyer et al., 1990; Dermietzel et al., 1990; Saez et al., 1993). It is suggested that gap junction mediated cell-cell communication may play important roles in cell-cell sig-

naling, including fostering "community effects" that could regulate and coordinate growth and development (Bennett et al., 1991; Guthrie and Gilula, 1989; Lo, 1989; Saez et al., 1993; Warner, 1992). Examination of these possibilities would be greatly facilitated if a dominant negative approach were available for inhibiting gap junctional communication.

Dominant negative strategies have been successfully used to characterize the function of both cytoplasmic and membrane proteins (Herskowitz, 1987; for example see Amaya et al., 1991; Govind et al., 1992; Hemmati-Brivanlou and Melton, 1992; Levine et al., 1994; Eyer and Peterson, 1994). Given the oligomeric nature of gap junctions, a mutant connexin polypeptide might exert dominant negative effects on GJC if it were to associate with wildtype gap junction proteins. To examine this possibility, our strategy entailed expressing a connexin 43 (Cx43)/ β -galactosidase (β -gal) fusion protein. The Cx43 protein was fused at its carboxy terminus with bacterial β -gal. This

Address correspondence to C. W. Lo, Biology Department, Goddard Laboratory, University of Pennsylvania, Philadelphia, PA 19104-6017. Tel.: (215) 898-8394. Fax: (215) 898-8780.

1. *Abbreviations used in this paper.* β -gal, β -galactosidase; cx43, connexin 43; dhfr, dihydrofolate reductase; GJ, gap junctions; GJC, GJ communication; TxR, Texas red.

should result in the positioning of the bulky β -gal polypeptide in the cytoplasmic compartment, and thereby allow the retention of β -gal enzymatic activity (Manoil, 1991; Silhavy and Beckwith, 1985). The possibility that such a Cx43 fusion protein will oligomerize with wildtype Cx43 is suggested by a recent study examining a gap junction fusion protein comprised of desmoglein fused to Cx32 (Trojanovsky et al., 1993). This fusion protein was found to be incorporated into gap junction plaques, thereby suggesting that at least some gap junction fusion proteins may retain the ability to undergo normal assembly and trafficking.

The rationale for selecting β -gal as the Cx43 fusion partner is based on the fact that others have shown that β -gal fusion proteins can function in a dominant negative fashion. β -Gal is a bacterial enzyme whose active form is a homotetrameric protein made up of 116-kD monomers (Langley and Zabin, 1976; Zabin, 1982). Others have shown that transgenic mice expressing a neurofilament/ β -gal fusion protein exhibited the dominant sequestration of neurofilament proteins in the perikarya, a result likely arising from high affinity interactions mediated by the β -gal moieties (Eyer and Peterson, 1994). Analysis in *Drosophila* transgenic embryos also showed that expression of β -gal fused with a DNA binding protein, *dorsal*, can lead to a dominant mutant phenotype, i.e., dorsalization of the embryo (Govind et al., 1992). In this instance, the dominant negative effect was attributed to steric hindrance (the β -gal moiety interfering with DNA binding) and/or sequestration of the wildtype *dorsal* protein as a result of tetramerization mediated by the β -gal moiety. In both examples, the use of β -gal as the fusion partner not only provided the means to exert a dominant effect on function, but also it was invaluable for tracking expression of the fusion protein, as the β -gal moiety retained enzymatic activity.

To examine whether a Cx43/ β -gal fusion protein can inhibit GJC in a dominant negative manner, we generated an expression vector encoding the Cx43/ β -gal fusion protein and transfected it into NIH3T3 or C6 cells. NIH3T3 cells express Cx43 in abundance and exhibit a high level of GJC (see below). In contrast, C6 cells express Cx43 but exhibit only low levels of Cx43 at the cell surface and only low levels of GJC (Naus et al., 1993; Zhu et al., 1991). NIH3T3 and C6 transfectants were clonally isolated and analyzed by Southern, Northern, Western, and immunohistochemical analyses. In the fusion protein expressing NIH3T3 cells, we observed a reduction in Cx43 localization at regions of cell-cell contact, while in the fusion protein expressing C6 cells, little or no Cx43 protein was detected at regions of cell-cell contact. Dye injection studies revealed that both fusion protein expressing NIH3T3 and C6 cells were inhibited in GJC, with only low levels of dye coupling detected in 3T3 cells and virtually no dye coupling observed in the control C6 cells. In light of these and other observations, we hypothesize that the inhibition of coupling by the fusion protein may arise from the intracellular sequestration of wildtype Cx43 due to perturbations in Cx43 trafficking to the cell surface. Overall, these studies showed that the Cx43/ β -gal fusion protein can exert a dominant negative effect on GJC, and suggest that this fusion protein could be used to probe for the biological function of gap junctions.

Materials and Methods

Plasmid Vectors

The LacZ/SV40 polyA segments of the plasmid pGN (Le Mouellic et al., 1988; generously provided by Dr. Herve Le Mouellic, Institut Pasteur, Paris, France) were isolated and cloned into an expression vector containing the promoter and a portion of the 5' intron of the human elongation factor 1 α gene (pEF-BOS; generously provided by Dr. Shigekazu Nagata, Osaka Bioscience Institute, Osaka, Japan; Mizushima and Nagata, 1990), creating the plasmid pEZ (see Fig. 1 A). The Cx43 coding region was PCR amplified from the mouse Cx43 cDNA (plasmid p132; Sullivan et al., 1993) using the 5' primer CTAGCTCTAGAACTTCAG containing an XbaI site and 3' primer CAAGGATCCCCAATCTCC containing a BamHI site. The PCR product obtained contains 76 bases of DNA 5' of the Cx43 start codon and includes the Cx43 Kozak consensus sequence and the entire Cx43 coding region through the last amino acid, but excludes the stop codon. This PCR product was inserted upstream and in frame with the β -gal coding region of pEZ, creating pEFZ (Fig. 1 A). Insertion of the Cx43 coding sequence into pEZ eliminates the first AUG (Met) of the β -gal coding sequence, but otherwise retains the β -gal sequence in its entirety. This results in full-length β -gal coding sequence (minus the first Met) linked in frame at its NH₂ terminus with the COOH terminus of full-length Cx43 coding sequence. Note that fusions deleting up to the first 26 amino acids of β -gal have no impact on β -gal enzymatic activity (Silhavy and Beckwith, 1985). Expression of pEFZ results in a Cx43/ β -gal fusion protein in which the β -gal moiety is fused in frame with the last amino acid of the Cx43 protein. To ensure the fidelity of the Cx43 coding sequence, the Cx43 coding region was analyzed by double-stranded DNA sequencing.

The plasmid CMV43dhfr was generated by inserting the mouse Cx43 coding sequence in place of the β -gal sequence in the CMV β expression vector (Clontech, Inc., Palo Alto, CA; MacGregor and Caskey, 1989), and inserting a 600-bp fragment from the 3' untranslated sequence of the dihydrofolate reductase (*dhfr*) gene from *Toxoplasma gondii* (Roos, 1993) upstream of the SV-40 poly A insert. The *dhfr* sequences served as a tag that allowed the tracking of Cx43 transcript expression from this plasmid vector. The coding region of the Cx43 cDNA insert was checked by double stranded DNA sequencing.

Isolation of Transfectant Clones

NIH3T3 cells and C6 rat glioma cells were maintained in DME-HG with glutamine containing 10% fetal calf serum (Hyclone, Logan, UT) and 50 iu/ml penicillin, 50 μ g/ml streptomycin. The NIH3T3 cells were transfected with 20 μ g of either pEZ or pEFZ and 2 μ g of the selectable marker pREP4 (Fig. 1 B) (In Vitrogen, Inc., Del Mar, CA; see Groger et al., 1989; Hambor et al., 1988; Hauer et al., 1989) which carries the gene for hygromycin resistance. Transfection was performed using the calcium phosphate precipitation method followed by DMSO shock. Two days after transfection, cells were subjected to hygromycin selection (200 U hygromycin B/ml; Calbiochem-Behring Corp., La Jolla, CA). Resistant clones were selected and screened for fusion protein or β -gal expression by immunofluorescent staining using β -gal antibodies (Cappel Laboratories, West Chester, PA). Once these NIH3T3 clones were established, they were maintained by plating at 5×10^5 cells/10 cm dish and passaged every 3 d. Using the same procedure, except with a neomycin selection vector (pPGKneoBP4; generously provided by Dr. Alan Bradley, Baylor College of Medicine, Houston, Texas; Soriano et al., 1991), pEFZ expressing C6 transfectant clones were isolated and maintained for further analysis.

Southern Blot Analysis

Genomic DNA was isolated from tissue culture cells. Briefly, cells were lysed in lysis buffer (1% SDS, 0.1 M EDTA, 0.1 M Tris, pH 8.5) and incubated at 65°C for 30 min. Then potassium acetate was added to a final concentration of 0.3 M, and after 15 min on ice, samples were spun at 12,000 g for 30 min. Then the supernatant was recovered, and the DNA precipitated with ethanol. Genomic DNA was digested using BamHI and other restriction enzymes according to the manufacturer's specifications (New England Biolabs, Inc., Beverly, MA). DNA digests were separated electrophoretically on 0.8% agarose gels followed by Southern blotting. The blots were hybridized with nick translated ³²P-radiolabeled pEZ plasmid DNA. Following washing, blots were subjected to autoradiography on

Kodak XAR film at -70°C with a DuPont Lightning Plus intensifying screen.

Western Analysis

Protein was obtained from tissue culture cells by lysis in 1% SDS (200 μl 1% SDS per 10 cm plate). In some samples, 0.001 M PMSF was included in the SDS solution. The amount of protein was quantitated using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL) according to the method of Akins and Tuan (Akins and Tuan, 1992). Samples were subjected to SDS/PAGE followed by electroblotting onto nitrocellulose using a BRL Mini-V8.10 vertical gel electrophoresis apparatus. SDS/PAGE and blotting were performed according to the manufacturer's specifications with the exception that the electrophoresis buffer contained 50 mM Tris-HCl, 140 mM glycine, 1% SDS, pH 8.3, and the transfer buffer contained 50 mM Tris-HCl, 380 mM glycine, 0.1% SDS, and 4% methanol. Following blotting, nitrocellulose membranes were blocked overnight at 4°C in a PBS solution containing 5% nonfat dried milk, and 0.1% Tween 20. Western blotting was performed using either anti-Cx43 polyclonal antibody (generously provided by Elliot Hertzberg, Albert Einstein College of Medicine, Bronx, NY; directed to the carboxy terminus at amino acid residues 346-360; Nagy et al., 1992; Yamamoto, et al., 1990) or anti- β -gal polyclonal antibody (Cappel Laboratories, West Chester, PA). This was followed by incubation with goat anti-rabbit IgG peroxidase conjugated secondary antibody (Boehringer Mannheim, Indianapolis, IN). Immunoreactivity was detected using the ECL chemiluminescent detection kit according to the manufacturer's specifications (Amersham Corp., Arlington Heights, IL).

Northern Analysis

RNA was isolated from tissue culture cells using Tri-reagent LS (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. The RNA isolated was subjected to gel electrophoresis using 1.0% or 1.2% agarose gels made in 6% formaldehyde and $1\times$ E buffer (0.018 M Na_2HPO_4 , 0.002 M NaH_2PO_4). Following electrophoresis, gels were blotted overnight onto Duralose membranes (Stratagene Corp., La Jolla, CA), and then UV crosslinked with a Stratalinker (Stratagene Corp.), and baked 2 h at 80°C under vacuum. Hybridization was carried out using riboprobes generated with a transcription kit from Promega Corp. (Madison, WI). Membranes were hybridized with 5×10^5 cpm of riboprobe, then washed with several changes of $0.1\times$ SSC/0.1% SDS at 65°C , and exposed for 1-5 d at -70°C to preflashed Kodak XAR film with a Dupont intensifying screen. Quantitation of the Northern blots were carried out using a Molecular Dynamics phosphorimager (Sunnyvale, CA).

Immunofluorescence Analysis

Immunocytochemistry was performed using a Cx43 monoclonal antibody (Chemicon, Temecula, CA) and a β -gal polyclonal antibody (Cappel Laboratories, West Chester, PA). Detection was carried out using fluorescein (FITC)-conjugated goat anti-mouse IgG secondary antibody (GIBCO BRL, Gaithersburg, MD) and Texas red (TxR)-conjugated goat anti-rabbit IgG secondary antibody (Cappel Laboratories, West Chester, PA). Samples were analyzed using a BioRad confocal microscope.

β -Gal Histochemical Staining

For in situ histochemical detection of β -gal activity, plates of cells were fixed with 2% paraformaldehyde in PBS for 30 min at room temperature. After several washes in PBS, the cells were incubated in a solution containing 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl_2 , 0.02% NP-40, 0.01% SDS in PBS. After overnight incubation at 37°C , the plates were washed with PBS and the cells subsequently photographed using Ektar 100 or Agfa 200 color film.

Dye Coupling Studies

For the evaluation of gap junctional communication, the cells were plated at 200,000 cells/35 mm dish. Two days following plating, gap junctional communication was analyzed by dye injection. Microelectrode impalements were performed in L15 medium containing 10% serum on a heated stage. Single cells were impaled with glass microelectrodes containing 5%

6-carboxyfluorescein. Dye was iontophoretically ejected into the impaled cell using 2 nA hyperpolarizing current pulses of 0.5-s duration at a frequency of once per second. Impalements were held for either 1 or 3 min. Following the designated time, the microelectrode was removed and the preparation viewed under darkfield epifluorescent illumination. The number of dye filled cells (excluding the impaled cell) in the primary, secondary, and tertiary order of cell contacts were counted immediately after the impalement was terminated.

Results

An expression vector, pEFZ, was generated that encoded the full-length mouse Cx43 polypeptide fused at its COOH terminus to bacterial β -gal (Fig. 1 A). This plasmid is driven by the human elongation factor 1 α promoter, a promoter that is active at high levels in a wide range of tissue culture cells and provides ubiquitous expression in transgenic mice (Hanaoka et al., 1991; Kim et al., 1990). In parallel, we also generated a control vector, pEZ. This was made from the same parental plasmid containing the elongation factor promoter, but with only the β -gal coding sequences inserted. The efficacy of these constructs was tested by transient transfections into NIH3T3 cells. Examination of the transiently transfected cells by X-gal staining revealed strong blue staining, thus demonstrating that the β -gal or Cx43/ β -gal fusion proteins were expressed (data not shown).

To analyze the effects of fusion protein expression on GJC in NIH3T3 cells, we isolated stable transfectant clones so that homogeneous populations of cells could be examined. This was carried out by cotransfecting pEFZ with the positive selection vector, pREP4 (Groger et al., 1989; Hambor et al., 1988; Hauer et al., 1989), and selecting for transfectants in hygromycin containing medium.

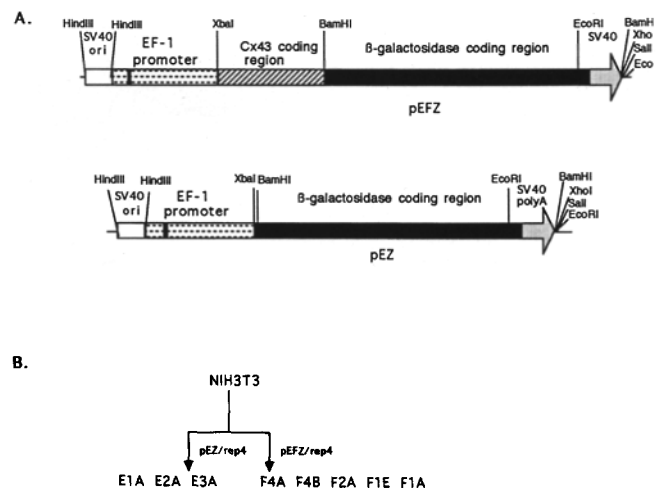


Figure 1. Cx43/LacZ Expression Vectors. (A) pEFZ and pEZ expression vectors. The pEFZ plasmid is driven by the human elongation factor 1 α promoter (Mizushima and Nagata, 1990) and contains the entire β -gal coding sequence fused in frame with the last amino acid of the Cx43 cDNA sequence. In the pEZ plasmid, only the β -gal sequences have been inserted downstream of the elongation 1 α promoter. (B) Lineage of NIH3T3 clones expressing various constructs. NIH3T3 cells were transfected with pEZ or pEFZ, and the hygromycin selection plasmid pREP4, after which stable clones were isolated.

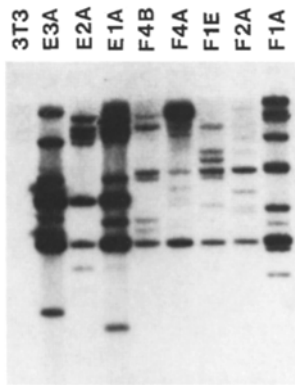


Figure 2. Southern blot analysis of pEFZ- and pEZ-transfected cells. Genomic DNA from the parental NIH3T3 cells, and pEFZ (F4B, F4A, F1E, F2A, F1A) or pEZ (E3A, E2A, E1A) transfected clones were digested with BamHI, and analyzed by agarose gel electrophoresis and Southern blotting. Hybridization was carried out using a radiolabeled pEZ probe. All clones exhibit the expected 3.6-kb BamHI fragment containing

the β -gal insert (arrow), and several other bands of varying sizes representing flanking vector/EF-1 α promoter sequences or inserts comprising of truncated copies of the pEFZ plasmid. Note no hybridization was observed in the parental NIH3T3 cells.

From this selection scheme, five pEFZ containing clones were isolated and further analyzed (F1A, F2A, F1E, F4A, F4B; see Fig. 1 B). In parallel, three control clones (E1A, E2A, E3A; see Fig. 1 B) containing the β -gal expression vector pEZ were also isolated. Genomic Southern blot analysis was carried out to examine the integration of the exogenous plasmid DNA. In the Southern blot of Fig. 2, genomic DNA from each of the clones was digested with BamHI, a restriction enzyme which should excise the 3.6-kb β -gal insert in pEFZ and pEZ. After hybridization with a probe comprising of radiolabeled pEZ plasmid DNA, the expected 3.6-kb fragment was indeed observed in all clones. In addition, a number of other hybridizing bands of varying sizes were detected. The latter likely correspond to flanking vector/EF-1 α promoter sequences or inserts comprising truncated copies of the pEFZ plasmid. The fact that each clone exhibits one or more unique hybridizing fragment(s) clearly demonstrates that each clone is independently derived.

Protein and RNA Expression in Transfectant Clones

Northern analysis showed that the parental NIH3T3 cells and all of the pEFZ and pEZ transfected clones expressed the native 3.2-kb Cx43 transcripts in similar abundance (Fig. 3 A, open arrow). In the pEFZ transfectant clones, there was also a larger transcript (Fig. 3, A and B, open arrowhead). This transcript was detected by both the β -gal and Cx43 hybridization probes, and thus must correspond to the fusion protein encoding transcript. Quantitation (with a phosphorimager) revealed that the level of fusion protein transcript expression is either comparable to or considerably less than that of endogenous Cx43 transcripts (compare upper/lower band intensities in Fig. 3 A). This is surprising given the strong promoter (derived from EF1- α ; Kim et al., 1990) used to drive expression of the fusion protein transcript. In comparison, expression of β -gal transcripts driven by the same promoter in control clones (pEZ transfected) was at least threefold higher than fusion protein transcript expression in pEFZ transfected cells (based on phosphorimager quantitation of upper bands vs the lower band in Fig. 3 B).

Consistent with the results of the Northern analysis,

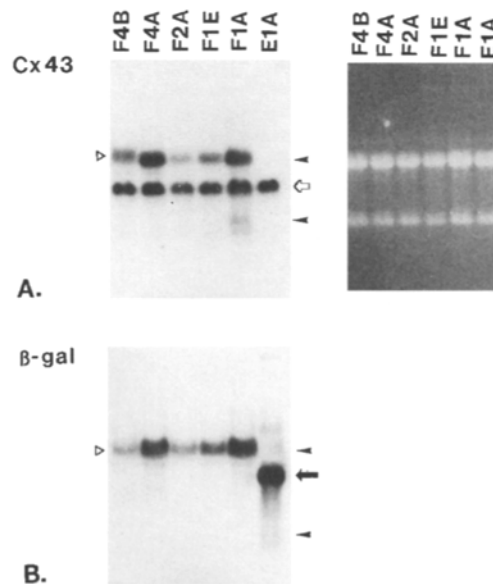


Figure 3. Northern blot analysis of Cx43 and β -gal transcript expression. (A and B). All five pEFZ transfected clones (F4B, F4A, F2A, F1E, F1A) and a control clone (E1A) transfected only with the β -gal expression vector, pEZ, were examined for the expression of Cx43 (A) and β -gal (B) transcripts. The endogenous Cx43 transcript (A, open arrow) and the authentic β -gal transcript (B, closed arrow) were detected in all clones. In addition, the expected β -gal and Cx43 hybridizing fusion protein transcript was detected in all of the pEFZ transfected clones (open arrowhead). Note that RNA loading is similar amongst all samples as indicated by ethidium bromide staining of the gel prior to blotting (upper right panel). The position of the large and small ribosomal RNAs are denoted by arrowheads.

Cx43 protein expression was detected in the parental NIH3T3 cells and all of the transfectant clones by Western immunoblotting analysis (Fig. 4). This is indicated by the detection of a strong band at 40–46 kD with a Cx43 antibody (Fig. 4, A and B, see open arrow). In the pEFZ transfected clones, two additional high molecular weight protein bands were detected (Fig. 4 A, arrowheads). These represent the Cx43/ β -gal fusion proteins, as they were recognized by both the Cx43 and β -gal antibodies (Fig. 4, A and B, left panels, arrowheads). There were large differences in the relative abundance of the fusion protein in the various clones, while any apparent difference in the abundance of the endogenous Cx43 protein were generally accounted for by differences in protein loading (see Coomassie-stained gel in right panel of Fig. 4 A; also data not shown). A comparison of the Western blot with the Northern analysis revealed good agreement between the level of Cx43 and Cx43/ β -gal transcript abundance vs Cx43 and fusion protein abundance (compare Figs. 3 A and 4 A). Note that although the endogenous Cx43 protein band is seen as a single broad band in Fig. 4 A, in shorter exposures of similar blots, the latter resolves as a doublet (Fig. 4 C). Analysis by alkaline phosphatase treatment revealed that the higher molecular weight Cx43 band corresponded to the phosphorylated forms of Cx43 (data not shown), a result consistent with the observations of others (Laird et al., 1991; Laird and Revel, 1990; Musil et al., 1990a,b; Musil and Goodenough, 1991; Puranam et al., 1993).

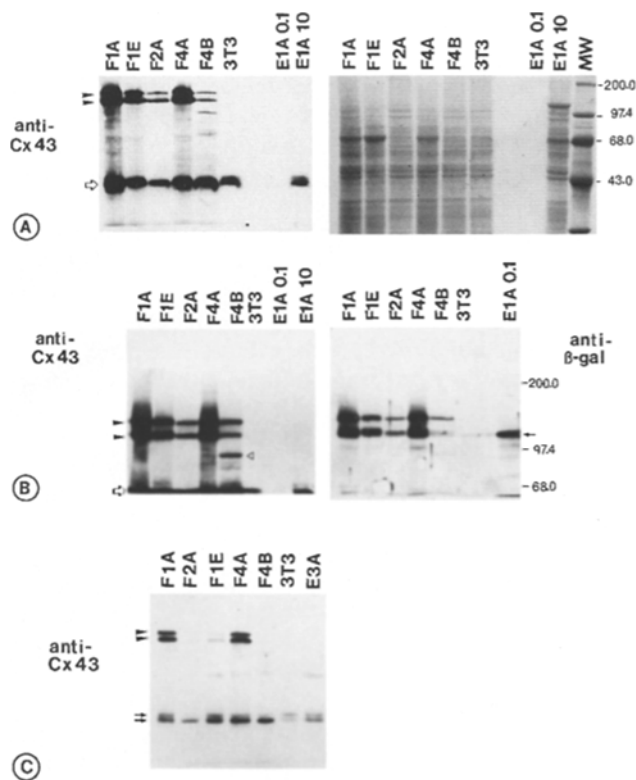


Figure 4. Western analysis of Cx43 and Cx43/ β -gal fusion protein expression. Aliquots of protein extracts (10 μ g, except 0.1 μ g in E1A) collected from the pEFZ and pEZ transfected clones, and the parental NIH3T3 cells were subjected to SDS/PAGE in 10% (A and C) or 6% (B) polyacrylamide gels. After transfer to nitrocellulose membranes, the blots were probed using either anti-Cx43 or anti- β -gal antibodies, with detection carried out by chemiluminescence. (A) Cx43 antibody detection revealed a strong band at 40–46 kD, corresponding to the endogenous Cx43 polypeptide (*open arrow*), and also two higher molecular weight bands at 120–160 kD (*arrowheads*). On the right is an identical gel stained with Coomassie blue to show protein loading. In the control E1A clone, only the endogenous Cx43 band is observed. MW = molecular weight markers. (B) Both the Cx43 and β -gal antibodies detected the same two high molecular weight bands in all of the pEFZ transfected clones as was seen in A (*arrowheads*). In the E1A control clone, anti- β -gal antibody detected only a single band (denoted by *arrow*) which corresponds to the authentic β -gal polypeptide, while the anti-Cx43 antibody detected only the lower molecular weight band corresponding to endogenous Cx43 (trapped at the gel front; denoted by *left open arrow*). In clone F4B, there is also another band of even lower molecular weight which likely represents truncated fusion protein (*open arrowhead*). (C) Short exposure of a western blot processed with a Cx43 antibody provided resolution of the endogenous Cx43 band as a doublet (see *arrows*). Note that in the fusion protein expressing clones such as F4A, there is a marked increase in the relative abundance of the lower Cx43 band (compare lower vs upper band denoted by *arrows*). Also observed are the two fusion protein bands which are denoted by the arrowheads. Although only some clones appear to express the fusion protein bands, in fact longer exposures of this blot show that all of the pEFZ transfected clones contain both fusion protein bands.

The finding of two fusion protein bands was unexpected, since the Northern analysis revealed only a single Cx43/ β -gal transcript. The larger of these two fusion protein bands is 160 kD, approximately the combined molecular weight

of the 43-kD Cx43 and 116-kD β -gal polypeptides. The lower molecular weight band is similar in size to that of authentic β -gal polypeptide (denoted by arrow in right panel of Fig. 4 B), but as it is also detected by the Cx43 antibody (Fig. 4, A and B), it likely is a truncated fusion protein product. It should be noted that these two bands are not likely to represent phosphorylated/nonphosphorylated forms of the fusion protein, since alkaline phosphatase treatment did not shift the mobility nor alter the relative abundance of these two fusion protein bands (data not shown). Moreover, random deletions or rearrangements of the inserted plasmid DNA is not likely to account for the lower molecular weight fusion protein band, as all of the pEFZ transfected clones contain both fusion protein bands. In clone F4B, an abundant lower molecular weight protein band was observed, probably the product of a truncated plasmid insert (*open arrowhead* in left panel of Fig. 4 B).

It is interesting to note that in the pEZ-transfected clones, there was significantly more β -gal protein expression as compared to fusion protein expression in the pEFZ transfected clones, greater than would be predicted from the differences observed in the levels of RNA expression (see Fig. 3 and Fig. 4 B; of Fig. 4 B, right panel, note difference in protein loading 0.1 μ g protein in control E1A lane vs 10 μ g in all other lanes). In fact, this apparent discrepancy is consistent with the known perdurance (long half-life) of β -gal (Echelard et al., 1994) vs the short-half life of Cx43 (Bevilacqua et al., 1989; Laird et al., 1991; Musil et al., 1990a,b; Musil and Goodenough, 1991).

Fusion Protein Is Localized to Areas of Cell–Cell Contact

The pEFZ transfectant clones, and three pEZ control clones and parental NIH3T3 cells were analyzed by laser confocal microscopy after double immunostaining with a Cx43 monoclonal and β -gal polyclonal antibody. Thus Cx43 was detected as green via FITC-conjugated secondary antibodies, the β -gal as red via TRITC conjugated secondary antibodies, and the regions of overlap between the two are seen as yellow/orange. The β -gal polyclonal antibody showed no specific immunostaining in the parental NIH3T3 cells (only green fluorescence seen in 3T3 cells in Fig. 5). In contrast, the Cx43 antibody revealed a pattern of immunostaining in NIH3T3 cells which is typical for connexin polypeptides, i.e., punctate localization at regions of cell–cell contact (see green dots in 3T3 cells) and also perinuclear localization in the cytoplasm (see ring of green fluorescence around the nuclei of 3T3 cells). A comparison with the connexin immunocytochemical studies of others (Musil and Goodenough, 1991; Musil et al., 1990b; Hendrix et al., 1992; Musil and Goodenough, 1993; Puranam et al., 1993) suggests that the latter is likely to reflect connexin protein localization in the ER/Golgi compartment. Similar analysis of the control pEZ transfectant clones (E3A and E1A in Fig. 5; data not shown for E2A) also revealed punctate Cx43 membrane localization (green dots) as in the NIH3T3 cells. In addition, β -gal cytoplasmic staining was observed which was superimposed upon the perinuclear localization of Cx43 (see orange fluorescence in E3A and E1A). The latter is consistent with the widespread cytoplasmic expression of authentic β -gal protein.

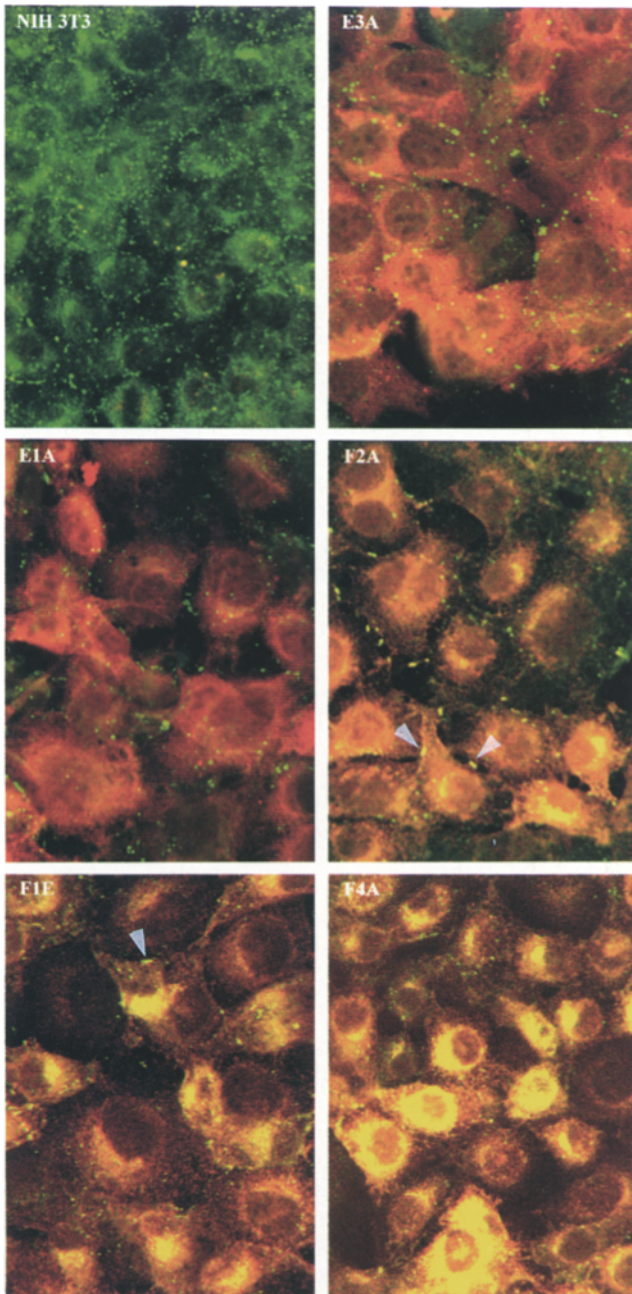


Figure 5. Characterization of fusion protein expression in transfected NIH3T3 cells by double immunofluorescence analysis. Confocal immunofluorescence microscopy was carried out to examine the expression of Cx43 and β -gal in the pEFZ and pEZ transfected NIH3T3 cells. This entailed using a mouse Cx43 monoclonal antibody in conjunction with a FITC conjugated anti-mouse secondary antibody, and a rabbit β -gal polyclonal antibody in conjunction with a Texas red-conjugated anti-rabbit secondary antibody. Thus green fluorescence denotes Cx43 localization, red fluorescence, β -gal, and yellow/orange, regions of Cx43/ β -gal colocalization. In the parental NIH3T3 cells and control pEZ transfected E1A and E3A cells, Cx43 is detected as punctate spots at regions of cell-cell contact (see punctate green fluorescence). Note that the yellow hue associated with some of the punctate spots in E3A is an artifact arising from the very strong underlying cytoplasmic β -gal immunostaining. In the Cx43/ β -gal fusion protein expressing cells, some Cx43 and β -gal were colocalized at regions of cell-cell contact (see punctate yellow spots denoted by white arrowheads). These cells also exhibited Cx43/ β -gal colocalization in the perinuclear region of the cytoplasm (see yellow ring of fluorescence around the nuclei). This is particularly abundant in clone F4A, a clone which showed little punctate cell surface localization of Cx43 or β -gal.

It should be noted that although some of the green punctate spots in the E3A cells exhibit a yellow hue, these same images when viewed with either the green or red channel alone showed only green and no red punctate spots. Thus, the yellow hue is simply an artifact arising from the strong red immunofluorescence resulting from the high level expression of β -gal in the E3A cells.

Parallel laser confocal analysis was carried out for the four fusion protein expressing clones F1A, F2A, F1E, and F4A (see Fig. 5; data not shown for clone F1A). β -Gal was observed together with Cx43 in a perinuclear compartment (denoted by a ring of yellow fluorescence around the nuclei). In addition, at some regions of cell-cell contact, Cx43 and β -gal were observed to be colocalized. This was indicated by the finding of punctate yellow spots (Fig. 5, white arrows), and also further confirmed by the fact that these same spots were observed when the images were viewed under either the separated red or green channels. Thus, at least some of the fusion proteins are likely transported to the cell surface and may be incorporated into gap junction plaques. However, it should be noted that in the fusion protein expressing cells, the punctate cell surface labeling typical of gap junctions was significantly reduced as compared to the parental or control cells (compare abundance of green/yellow punctate fluorescent spots in F1E, F2A, and F4A with green fluorescent spots in NIH3T3, E1A, and E3A cells). This was particularly prominent in clone F4A, which only infrequently exhibited punctate cell surface labeling, but exhibited abundant Cx43/ β -gal immunostaining in the cytoplasm (denoted by strong cytoplasmic yellow fluorescence; Fig. 5). These observations suggest that there is a reduction in gap junction plaques in fusion protein expressing cells such as clone F4A, and that this may be accompanied by an increased intracellular sequestration of the fusion protein.

Retention of β -Gal Activity

Histochemical staining with X-gal was carried out to determine whether β -gal activity is retained by the fusion protein expressed in the pEFZ transfectant clones. In all cases, strong X-gal staining was found (for example see Fig. 6). These results indicate that the fusion protein (full length or truncated) has retained the ability to tetramerize through the β -gal moiety. It should be noted that all clones exhibited heterogeneity in the level of X-gal staining, including control clones transfected with the pEZ vector (or other β -gal expression vectors; data not shown) (Fig. 6). Such heterogeneity is routinely observed for β -gal expression in tissue culture cells, and is not a unique characteristic of cells expressing the fusion protein (MacGregor et al., 1987; also this study). Also note that the diffuseness of the X-gal staining pattern is at least in part due to the histochemical staining process, i.e., from diffusion of the

low spots denoted by white arrowheads). These cells also exhibited Cx43/ β -gal colocalization in the perinuclear region of the cytoplasm (see yellow ring of fluorescence around the nuclei). This is particularly abundant in clone F4A, a clone which showed little punctate cell surface localization of Cx43 or β -gal.

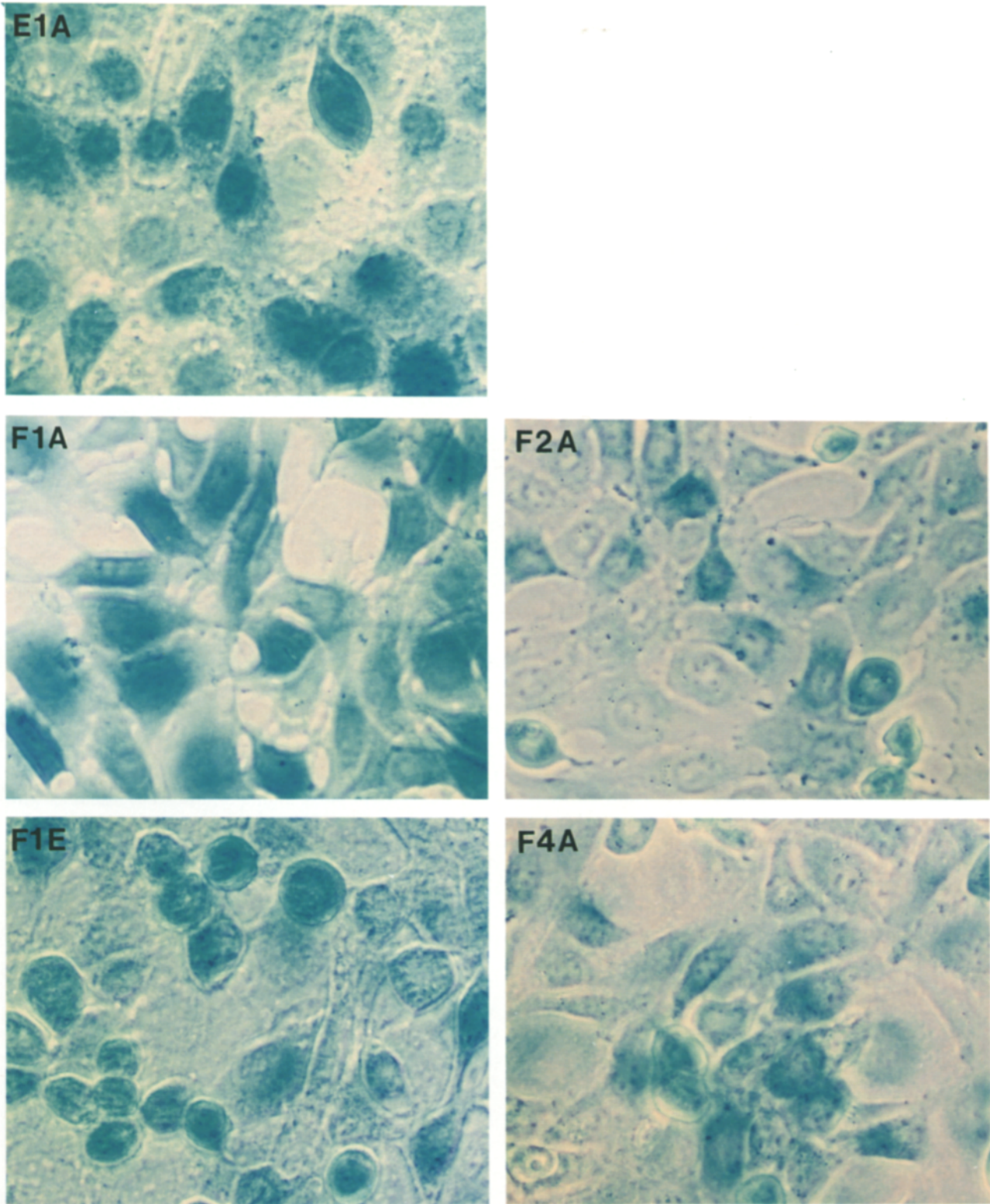


Figure 6. Expression of β -gal activity in pEFZ and pEZ expressing cells. All of the pEFZ or pEZ transfected cell lines express β -gal enzymatic activity. This is demonstrated by the abundant blue staining seen upon incubation of these cells with the β -gal substrate X-gal.

cleaved substrate prior to complexing with the chromogen nitroblue tetrazolium.

Gap Junctional Communication in Transfectant Clones

Gap junctional communication in the fusion protein ex-

pressing clones and each of the three control clones (E1A, E2A, E3A) was examined by monitoring dye coupling with microelectrode impalements. The low molecular weight fluorescent dye, 6-carboxyfluorescein, was iontophoretically injected and the extent of dye spread was

Table I. Dye Coupling Analysis of Fusion Protein Expressing Cell Lines

A. NIH3T3 transfectant clones examined with 3-min impalements:					
	Control	F1A	F2A	F1E	F4A
mean# dye-filled cells	10.8	10.9	11.4	7.7	4.2
SE	0.618	0.662	1.061	1.027	0.661
<i>n</i>	112	92	36	32	42
<i>P</i> values (Student's <i>t</i> -test)	–	0.876	0.639	0.016	0.000
B. NIH3T3 transfectant clones examined with 1-min impalements:					
	Control	F1A	F2A		
impalements with ≤ 1 dye-filled 2° cell	25 (52%)	23 (74%)	35 (78%)		
impalements with ≥ 2 dye-filled 2° cells	23 (48%)	8 (26%)	10 (22%)		
<i>n</i>	48	31	45		
χ^2	–	3.86	6.76		
<i>P</i> values	–	0.05	<0.01		
C. C6 transfectant clones examined with 1-min impalements:					
	Control	C6 F1C	C6 F2E		
impalements with ≤ 1 dye-filled 1° cell	30 (75%)	31 (100%)	23 (92%)		
impalements with ≥ 2 dye-filled 1° cells	10 (25%)	0 (0%)	2 (8%)		
<i>n</i>	40	31	25		
χ^2	–	9.02	2.95		
<i>P</i> values	–	0.0027	0.0857		

monitored by counting the number of dye filled cells after 3 min of iontophoretic injection (Table I A). This analysis revealed abundant dye spread in the three control clones (E1A, E2A, E3A) (for example see E3A in Fig. 7). As the data obtained with these three clones were statistically indistinguishable, they were pooled (Table I A; also pooled in Table I B). In comparison, 3 min impalements of the fusion expressing clones revealed a lower level of dye coupling in clones F4A and F1E, with clone F4A showing the lowest level of coupling (Table I A). Although these 3-min impalements did not reveal the inhibition of dye coupling in clones F1A and F2A, dye coupling inhibition was in fact observed in both clones when dye injections were carried out with 1 min. impalements (Table I B). For these 1-min impalements, we monitored the number of dye filled cells that were one (first order), or two (second order) cell diameters away from the impaled cell. This analysis clearly showed a statistically significant decrease in the number of second order dye filled cells found in impalements into F1A and F2A cells as compared to the control clones (Table I B). Overall, these results demonstrate that all four fusion protein expressing clones (F1A, F2A, F1E, and F4A) are inhibited in dye coupling.

Expression of Cx43/ β -Gal Fusion Protein in C6 Cells

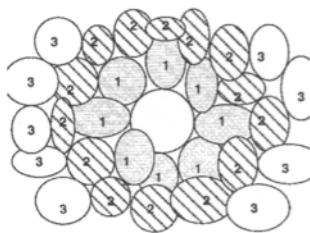
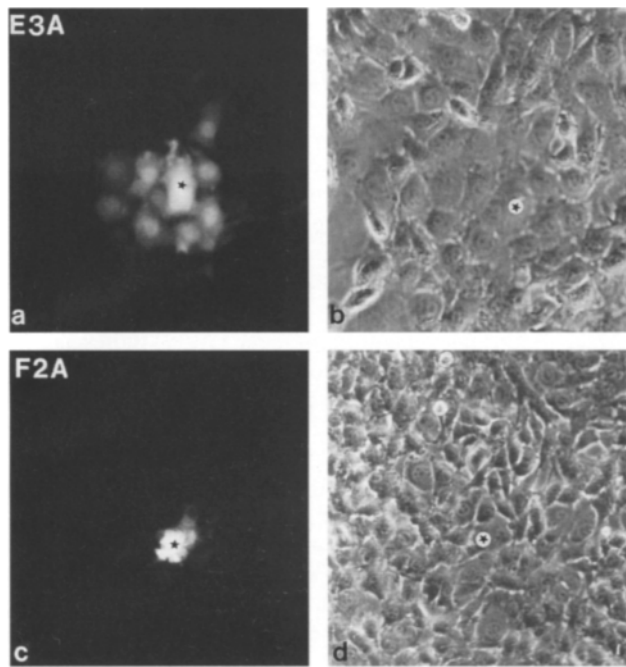
To examine if the fusion protein may inhibit gap junctional communication in cells other than NIH3T3, we transfected the pEFZ plasmid into the rat glioma cell line, C6. C6 cells are of particular interest as they express moderate levels of Cx43 transcript and protein, and yet exhibit only low levels of dye coupling and only occasional Cx43 immunostaining at the cell surface (for example, see Naus et al., 1993; Zhu et al., 1991). Nevertheless, these cells are able to generate functional gap junctions, as C6 cells transfected with a Cx43 expression vector exhibit greatly increased dye coupling in conjunction with increased expression of Cx43 at regions of cell–cell contact (Naus et al., 1993; Zhu et al., 1991).

To obtain stable clones for analysis, C6 cells were

cotransfected with the pEFZ plasmid and a neomycin selection vector, pPGKneo. After neo selection, a number of clones were isolated and analyzed, of which two (C6F1C, C6F2E) were found to contain the fusion protein expressing pEFZ plasmid. X-Gal staining showed that the fusion protein was indeed expressed in these two clones (data not shown). This was further confirmed by northern and western analysis (the latter showed the presence of both fusion protein bands as described above for NIH3T3 transfectants; data not shown).

To further examine whether the fusion protein was translocated to the cell surface, confocal microscopy was carried out after double immunofluorescence staining with antibodies to Cx43 and β -gal (Fig. 8; as in Fig. 5, green fluorescence corresponds to Cx43, red to β -gal, and yellow, Cx43/ β -gal colocalization). In the parental C6 cells, only Cx43 immunostaining was detected (green fluorescence in C6 cells of Fig. 8). This was seen most prominently in a perinuclear region in the cytoplasm, and also observed was a variable level of punctate labeling. In comparison, in the fusion protein expressing clones C6F1C and C6F2E, β -gal and Cx43 immunostaining were both detected. This was observed mostly as a diffuse pattern of immunostaining in the cytoplasm, with little punctate labeling detected (orange/yellow fluorescence in C6F1C and C6F2E cells of Fig. 8). This pattern of immunolocalization would suggest that little if any of the endogenous Cx43 or the fusion protein is translocated to the cell surface.

Fusion protein expressing and control C6 cells were further examined for dye coupling with 1-min impalements and scored for dye transfer to first and second order contact cells. This analysis revealed virtually no dye transfer to primary contact cells in the fusion protein expressing C6 clones (Table I C). This result contrasts with the finding of a consistent low level of dye coupling in the pPGKneoBP A expressing control C6 cells (transfected only with the selection vector; Table I C). These results show that the fusion protein also can inhibit GJC in fusion protein expressing C6 cells.



e

Figure 7. Dye coupling is inhibited in fusion protein expressing cells. Microelectrode impalements were used to iontophoretically inject carboxyfluorescein into individual cells to monitor the extent of dye coupling in the pEZ and pEFZ transfected clones. Parallel impalements and injections of dye into control clone E3A (*a* and *b*) vs the fusion protein expressing cell line F2A (*c* and *d*) revealed a notable decrease in the spread of dye in the fusion protein expressing cells. The impaled cell is denoted by an asterisk in the darkfield images (*a* and *b*) and in the corresponding phase contrast images (*c* and *d*). Following iontophoretic dye injection, the number of dye filled cells were counted, with the number of cells that are one cell (first order), two (second order), or three (third order) cell diameters away from the impaled cell recorded (labeled as 1, 2, and 3, respectively, in the schematic in *e*).

Discussion

Our studies showed that a Cx43/ β -gal fusion protein can inhibit GJC in a dominant manner when expressed in mouse NIH3T3 and rat C6 cells. As the fusion protein retains β -gal enzymatic activity, cells which express the construct are easily identified by X-gal staining. Confocal immunofluorescence analysis showed that in the transfected NIH3T3 cells, the fusion protein is localized both intracellularly and also as punctate spots at regions of cell-cell contact, a pattern similar to that of endogenous Cx43. However, the overall distribution of Cx43 at regions of cell-cell contact was reduced to varying extent (but not

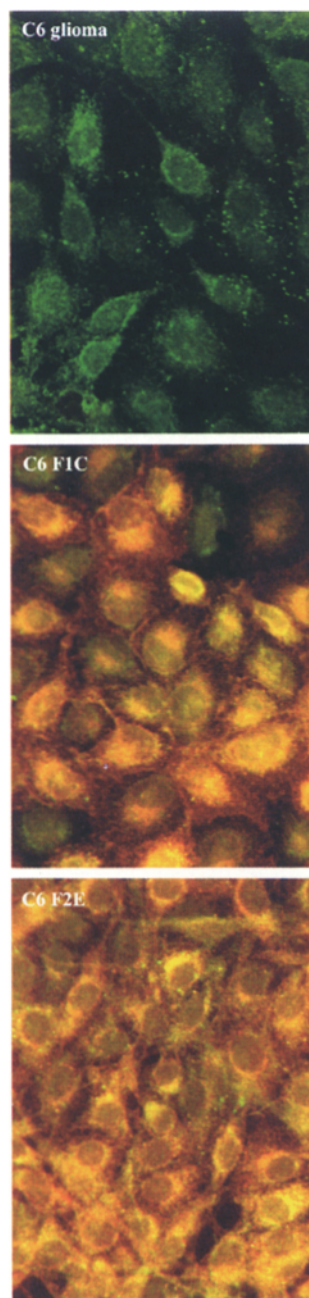


Figure 8. Localization of Cx43/ β -gal fusion protein in pEFZ transfected C6 cells by confocal immunofluorescence microscopy. Two fusion protein expressing C6 clones (C6 F1C, C6 F2E) and the parental C6 cells were analyzed by double immunofluorescence staining with Cx43 and β -gal antibodies using FITC (green fluorescence) and Texas red (red fluorescence) conjugated secondary antibodies, respectively. In the parental C6 cells, Cx43 was detected predominantly in the perinuclear region (ring of green fluorescence around the nuclei), and also as punctate localizations (see green spots). Note the absence of β -gal immunostaining in such cells as indicated by the absence of red fluorescence. In the fusion protein expressing C6F1C and C6F2E cells, both β -gal and Cx43 are colocalized in the cell cytoplasm (yellow/orange fluorescence), with the strongest labeling found in the perinuclear region. Note the absence or reduction in punctate immunostaining which was seen in the parental C6 cells.

abolished) in the fusion protein expressing 3T3 cells. Similar analysis of the C6 cells also revealed abundant fusion protein localization intracellularly. In contrast to the transfected 3T3 cells, there was little or no punctate Cx43 localization detected. In light of the latter observations, it is perhaps not surprising that there was more complete blockage of dye coupling in the fusion protein expressing C6 cells as compared to that of the 3T3 transfectant clones. These observations suggest the possibility that the fusion protein may inhibit GJC in NIH3T3 and C6 cells by sequestering the endogenous Cx43 protein intracellularly. Consistent with this possibility is the fact that in 3T3 clones highly inhibited in dye coupling, Cx43 localization at regions of cell-cell contact is markedly reduced. Also consistent with GJC inhibition arising from the sequestration of endogenous Cx43 protein is a comparison of the

properties of 3T3 transfectant clones F1A and F4A. Although both express similar levels of the fusion protein (and endogenous Cx43), clone F4A is more inhibited in dye coupling, and also exhibited a greater reduction in Cx43 immunolocalization at regions of cell-cell contact. The disparate properties of these two clones would indicate that the inhibition of coupling by the fusion protein may also entail interactions with proteins other than endogenous Cx43, perhaps proteins whose expression is differentially regulated in these two clones. As recent studies have shown that trafficking of the Cx43 polypeptide to the cell membrane entails oligomerization of the connexin polypeptides in the *trans*-Golgi compartment (Musil and Goodenough, 1993), perhaps the perturbation of connexin protein oligomerization by the fusion protein may play a role in the accumulation of Cx43 intracellularly.

Our analysis also showed a shift in the relative abundance of the phosphorylated vs. nonphosphorylated forms of Cx43 in the fusion protein expressing cells. Studies in various tissue culture cell lines have shown that phosphorylation of Cx43 to the P2 form is associated with connexin protein incorporation into gap junction plaques (Laird et al., 1991; Musil et al., 1990b; Musil and Goodenough, 1991; Puranam et al., 1993). Although our gel systems did not resolve the P2 vs. P1 forms of Cx43 (P2 vs P1; Musil et al., 1990b, 1991), the relative abundance of the nonphosphorylated form of Cx43 was clearly increased in the fusion protein expressing cells. Hence, in future studies, it may be of interest to further consider whether the fusion protein may exert a dominant negative effect on GJC by perturbing gap junction plaque assembly.

The possibility that perturbations in connexin protein oligomerization (or gap junction plaque assembly) may occur through interactions between the fusion protein and wildtype Cx43 is not unexpected, given the large size of the β -gal polypeptide and its ability to tetramerize. Thus, substantial steric effects are likely to arise from the β -gal moiety, with such steric effects possibly further exaggerated upon formation of the β -gal tetramers (which may occupy $175 \times 135 \times 90$ Å along a twofold axis; Jacobson et al., 1994).

That the inhibition of coupling is not likely due to competition for the intracellular machinery required for the synthesis/assembly of membrane/junction proteins is indicated by our finding that the fusion protein also blocks GJC in C6 cells. Thus, others have shown that when C6 cells are transfected with a Cx43 expression vector, abundant dye coupling is expressed in conjunction with extensive Cx43 immunolocalization at regions of cell-cell contact (Naus et al., 1993; Zhu et al., 1991). Of further note is the fact that most or perhaps all communication-deficient cell lines can be converted to communication competent cells when transfected with vectors expressing connexin genes (e.g., SKHep cells, BHK cells, and HeLa cells; Eckert et al., 1993; Kumar and Gilula, 1992; Fishman et al., 1991, 1990). This would also suggest that mammalian tissue culture cells have "excess capacity" with regard to the intracellular machinery needed for the synthesis of membrane (or gap junction) proteins. In addition, we note that in all of the fusion protein expressing clones, there was a close correlation between the abundance levels of the transcript vs protein for both the endogenous Cx43 and the exoge-

nously introduced fusion protein construct. This would further indicate that the inhibition in coupling is not likely the result of competition for the translational machinery in the cell.

We note that NIH3T3 cells expressing the fusion protein, although inhibited in GJC, nevertheless continue to exhibit a low level of dye coupling. Since such cells express abundant Cx43, perhaps there is insufficient fusion protein expression to completely "knockout" Cx43 function. Indeed, none of the fusion protein expressing NIH3T3 clones show complete loss of Cx43 immunostaining at the cell surface. Although such immunolocalization studies cannot distinguish between expression of the fusion vs. wildtype Cx43 protein, the results nevertheless are consistent with the possibility that some Cx43 containing gap junction plaques are present in all of the fusion protein expressing clones. The expression of other connexin isoforms could also account for the retention of dye coupling. In a preliminary screen, we have not detected the expression of at least two other connexin genes (Cx40, Cx26). Alternatively, coupling may persist if the fusion protein were able to form gap junction channels (albeit of a reduced permeability). This question is probably best answered in future experiments in which the coupling properties of the fusion protein are examined in the *Xenopus* oocyte assay system (Dahl, 1992). Overall, these results demonstrate that the Cx43/ β -gal fusion protein can exert a dominant negative effect in inhibiting cell-cell communication, and suggests that further studies are warranted to evaluate the mode of action of the fusion protein and its possible utility in probing the biological function of gap junctions. Further studies are also needed to characterize the truncated fusion protein coexpressed with the 160 kd full length fusion protein. This smaller protein contains epitopes to the COOH termini of both β -gal and Cx43. Our efforts in using various antibody reagents to map the portion of Cx43 present in this smaller protein product have been unsuccessful, possibly due to masking of epitopes (for example see De Sousa et al., 1993; Yeager and Gilula, 1992). Given the uncertainty as to the structure of this truncated fusion protein, it is difficult at present to assess its role in the inhibition of coupling.

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