

MUTATIONS OF THE *CONNEXIN43* GAP-JUNCTION GENE IN PATIENTS WITH HEART MALFORMATIONS AND DEFECTS OF LATERALITY

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Abstract *Background.* Gap junctions are thought to have a crucial role in the synchronized contraction of the heart and in embryonic development. Connexin43, the major protein of gap junctions in the heart, is targeted by several protein kinases that regulate myocardial cell–cell coupling. We hypothesized that mutations altering sites critical to this regulation would lead to functional or developmental abnormalities of the heart.

Methods. *Connexin43* DNA from 25 normal subjects and 30 children with a variety of congenital heart diseases was amplified by the polymerase chain reaction and sequenced. Mutant DNA was expressed in cell culture and examined for its effect on the regulation of cell–cell communication.

Results. The 25 normal subjects and 23 of the 30 children with heart disease had no amino acid substitutions

in *connexin43*. All six children with syndromes that included complex heart malformations had substitutions of one or more phosphorylatable serine or threonine residues. Four of these children had two independent mutations, suggesting an autosomal recessive disorder. Five of these children had substitutions of proline for serine at position 364. A seventh child, with a different heart condition, also had a point mutation in *connexin43*. Transfected cells expressing the Ser364Pro mutant *connexin43* sequence showed abnormalities in the regulation of cell–cell communication, as compared with cells expressing normal *connexin43*.

Conclusions. Mutations in the *connexin43* gap-junction gene, which lead to abnormally regulated cell–cell communication, are associated with viscerotaxial heterotaxia. (N Engl J Med 1995;332:1323-9.)

BIRTH defects are the leading cause of infant mortality in the United States,¹ and heart malformations are present in at least half of infants with such defects.² Reports of familial malformation syndromes, such as those involving an atrial septal defect,³ a hypoplastic left ventricle,⁴ and the tetralogy of Fallot,⁵ strongly suggest that genetic factors play a part in a number of heart defects. Recently, the DiGeorge syndrome (characterized by aortic-arch anomalies and hypocalcemia) has been mapped to a locus at 22q11,⁶ and the Holt–Oram syndrome (heart–hand syndrome) to 12q2.⁷ Mapping of this type led ultimately to the implication of the beta-myosin heavy-chain gene in hypertrophic cardiomyopathy⁸ and of the fibrillin gene in Marfan's syndrome.⁹

Among the genes likely to be involved in cardiac development, the gap-junction gene *connexin43* (α_1), which codes for a 43-kd gap-junction protein, is of particular interest. Like other members of the connexin gene family,¹⁰ *connexin43* forms membrane-spanning hexameric hemichannels (connexons) that, when in register in apposed cell membranes, couple to form a continuous hydrophilic channel through which ions, metabolites, and molecules involved in signal transduction can move from cell to cell.¹¹ An array of many such channels constitutes a gap junction. In the mammalian heart there are four known gap-junction proteins, of which *connexin43* is the predominant one,^{12,13} uniting ventricular cardiomyocytes in an electrical syncytium^{14,15} and participating in synchronizing the beat rhythm.¹⁶ As

the primary component of axial internal resistance in the myocardium, *connexin43* gap junctions are an important determinant of intraventricular conduction velocity.¹⁷

Although evidence for a role of *connexin43* and its homologues in morphogenesis is only beginning to accumulate,^{18,19} one proposed role is the formation of separate compartments for communication, in which cells that will share a distinct fate are coupled. In the eight-cell mouse blastula, for example, all cells are coupled by gap junctions²⁰; after implantation, however, the inner cell mass and the trophectoderm form distinct compartments,²¹ which fractionate progressively after gastrulation.^{22,23} The disruption of cell–cell communication by antibodies to gap-junction protein has been shown to result in developmental defects in mouse²⁰ and amphibian²⁴ embryos. More recently, a *connexin43* gene “knockout” in mice has been reported to result in fatal heart malformations involving pulmonic atresia and other conotruncal anomalies.²⁵

Several reports have shown that *connexin43* is phosphorylated on serine residues in vitro and in vivo.²⁶⁻²⁹ Furthermore, cell–cell communication in cultured primary cells expressing *connexin43* can be controlled rapidly and reversibly by the microinjection of active protein kinases or phosphatases that target serine or threonine residues.^{30,31} The cytoplasmic carboxyl terminal of *connexin43* contains several predicted consensus sites for phosphorylation by these enzymes.³⁰⁻³² Prominent among them are residues 362 to 376, which contain three tandem Arg-X-Ser-Ser sequences. In this study we demonstrate that mutations in this region that cause misregulation of cell–cell communication are associated with complex malformations of the heart and other organs.

METHODS

Clinical Screening

We studied 30 patients with a mean age of 26 months (median, 7 months; range, before birth to 15 years) who had a variety of con-

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genital heart anomalies, including hypoplastic left and right heart syndromes, hypertrophic and dilated cardiomyopathies, asplenia and polysplenia syndromes, hypoplastic aortic arch, atrial and ventricular septal defects, trisomy 13, and the DiGeorge syndrome, for mutations in the carboxyl-terminal domain of *connexin43*. Most of the patients were children receiving heart transplants at Loma Linda University Medical Center. Twenty-five DNA sequences from normal subjects were also examined by random sampling of blood specimens from the clinical laboratory.

The cytoplasmic tail was targeted because this domain appears to contain most of the plausible sites for post-translational modification of connexin43, including all the consensus sequences of amino acids available for phosphorylation by the protein kinases that are known to regulate cell-cell communication.^{30,31} By sequencing the entire carboxyl-terminal domain directly, we avoided making a priori assumptions about which residues or mechanisms might be important within this region.

All samples containing apparent mutations after sequencing were cloned and sequenced again by allelic sequencing. Mutations observed after allelic sequencing were reconfirmed by allele-specific oligonucleotide hybridization of products of the polymerase chain reaction (PCR).

The study was approved by the institutional review board of Loma Linda University Medical Center. When applicable, informed written consent was obtained.

DNA Extraction

High-molecular-weight genomic DNA was isolated from heart tissue or buffy-coat preparations of whole blood, by established methods.^{33,34}

PCR Amplification

Each DNA sample was subjected to two rounds of nested PCR. The first round was designed to exclude any contribution from a processed pseudogene that has been identified in humans.³⁵ The entire coding region was amplified, plus a small portion of an intron in the 5' untranslated region of the gene (the coding region contains no introns). The second round was used to amplify a fragment of 400 base pairs (bp) that codes for most of the cytoplasmic-tail domain. In the first round of amplification (with primers AACAAACAAAA-CAAAACACTT and CACCCATCTACCCCATACACC), 1 μ g of the DNA sample was denatured at 94°C for 5 minutes and amplified for 20 to 25 cycles, each consisting of 45 seconds at 94°C, 45 seconds at 53°C, and 90 seconds at 72°C, followed by a 5-minute extension at 72°C. Then, 2.5 μ l of the first-round product was used in the second round of PCR (with primers GATGGTACCAGAGCGACCCCTTAC-CATGC and CCTGGATCCTGTGAGTACCACCTCCA). The product was denatured at 94°C for 5 minutes and amplified for 20 to 25 cycles, each consisting of 30 seconds at 94°C, 30 seconds at 53°C, and 30 seconds at 72°C, followed by a 5-minute extension at 72°C. The PCR reactions used 10 pmol of each primer in a 50- μ l reaction volume; the reaction also contained 1.5 mmol of magnesium chloride and 200 μ mol each of deoxyadenosine triphosphate (A), deoxycytidine triphosphate (C), deoxyguanosine triphosphate (G), and deoxythymidine triphosphate (T).

Sequencing

Sequencing was carried out with the Perkin-Elmer cycle-sequencing kit, according to the manufacturer's protocol. Two hundred femtomoles of purified second-round PCR product was used as template and was sequenced for 35 cycles of arithmetic (single-primer) amplification. Each cycle consisted of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. Both DNA strands were sequenced.

Allelic Cloning and Sequencing

The second-round PCR product was purified on a Magic PCR minicolumn (Promega), then ligated to PCR II vector (Invitrogen) and used to transform One-Shot bacterial cells, as described in the instructions to the TA cloning kit (Invitrogen). Multiple clones were isolated from each transformation and purified with Magic Miniprep columns (Promega), according to the manufacturer's instructions. Up to six insert-bearing clones were selected for sequencing, which

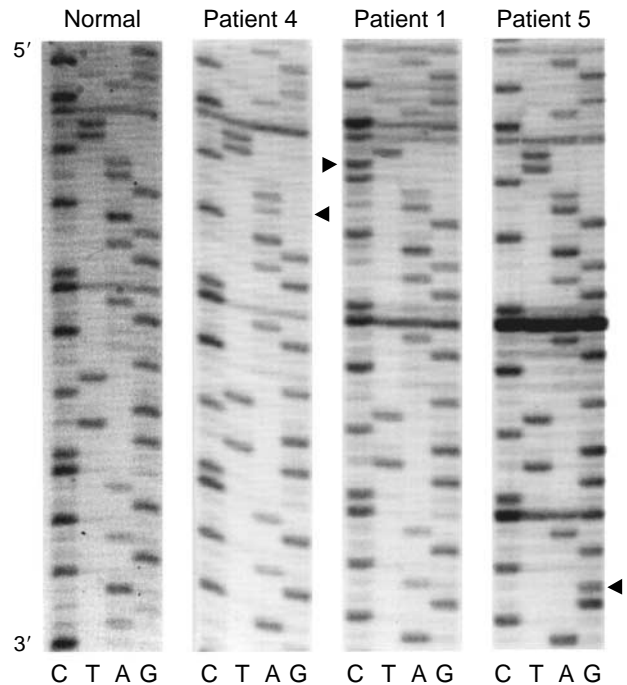


Figure 1. Comparison of Sequences in the Terminal Serine Box Region.

Deoxyinosine triphosphate was substituted for deoxyguanosine triphosphate in the sequencing reaction mixture to minimize compression artifacts; this resulted in occasional stops across all four lanes in places where the gel sequence should be read as deoxycytidine. The sites of mutations, indicated by arrowheads, are as follows: Patient 4, AGC (Ser365) to AAC (Asn); Patient 1, TCA (Ser364) to CCA (Pro); and Patient 5, AGC (Ser373) to GGC (Gly).

was carried out with a conventional, double-stranded dideoxy chain-termination method (U.S. Biochemical).

Hybridization with Allele-Specific Oligonucleotides

Two to six duplicate PCR reactions from each sample were blotted onto a Hybond-N membrane in a vacuum slot-blot apparatus (Vacu-systems). Separate membranes were prepared for each hybridization.

Oligonucleotide probes were end-labeled by 5'-phosphate exchange with [γ -³²P]ATP, catalyzed by T4 polynucleotide kinase, by standard methods.³⁶ All the oligonucleotides were 13 bases long and centered on the targeted mutation. Radiolabeled oligonucleotides were purified on NACS/Prepac (GIBCO BRL) columns, according to the manufacturer's instructions.

Membranes were prehybridized for 30 minutes at 42°C according to a standard protocol.³⁶ Hybridization was performed for two hours at the melting temperature of the probe, calculated from the formula

$$T_m = 4(G + C) + 2(A + T),$$

where T_m is the melting temperature in degrees Celsius and G, C, A, and T are the deoxynucleotides described above. The hybridization solution was 6 \times saline sodium citrate buffer (SSC) (1 \times SSC is 0.15 M sodium chloride and 0.015 M sodium citrate), 0.1 percent sodium dodecyl sulfate (SDS), and 0.8 pmol of labeled probe per milliliter of solution. After hybridization, the membranes were washed in two changes of 6 \times SSC and 0.1 percent SDS, at a temperature 4°C below the computed T_m of the probe. The washed membranes were blotted dry, covered with plastic wrap, and autoradiographed for one to four days.

Preparation of Expression Vectors

A full-length clone of the *connexin43* coding sequence was generated by two rounds of nested PCR and ligated to a constitutive eu-

karyotic expression vector, pCEP4 (Invitrogen), according to established methods.³⁶ After transformation and full-length sequencing, one clone, pM0.4, was selected for subsequent transfection and expression as the prototypic normal *connexin43* sequence. This clone was also used to prepare a chimeric mutant construct that carried the same substitution of proline for serine at position 364 that was identified in five of the six patients with heterotaxia. The mutant construct was prepared by excising and replacing a 235-bp restriction-digestion fragment at the extreme 3' end of the coding region with a fragment containing the Ser364Pro mutation. After transformation, several clones were sequenced in order to verify that only the targeted mutation had been introduced. One clone, pMC4, was selected for transfection. The resulting chimera consisted of a 1026-bp sequence encoding most of the structural domains of functionally normal *connexin43* and an engrafted 235-bp fragment containing the targeted substitution at Ser364.

Cell Transfection

L929, a cell line with minimal cell-cell communication under standard conditions of culture,²⁹ was grown to 80 percent confluency, trypsinized, washed in Dulbecco's modified Eagle's medium (GIBCO), and resuspended in 2 ml of phosphate-buffered saline (pH 7.2). Then, 0.5 ml of each suspension was combined with 10 μ g of pM0.4, mutant DNA, or pCEP4 plasmid in a cuvette and electroporated in a gene pulser (BioRad) at 500 microF capacitance and 350 V. Afterward, the cells were transferred to flasks containing Dulbecco's modified Eagle's medium with 10 percent fetal-calf serum (Gemini Bioproducts). After two days' recovery, 200 μ g of hygromycin per milliliter of medium was added for the selection of transformants.

Immunocytochemical Analysis and Western Blotting

Immunocytochemical analysis was performed as described elsewhere,³⁷ with an antibody specific to residues 360 through 382 of *connexin43* that has been characterized²⁸ and provided by Drs. Dale Laird (McGill University, Montreal) and Jean-Paul Revel (California Institute of Technology, Pasadena).

Cell supernatants were prepared, and protein determinations performed, as described elsewhere.²⁸ Western blotting was done with described methods,^{27,28} except that the blocking reagents were those provided in the BioRad immunoblot kit.

To control for the specificity of antibody binding, we used a cognate blocking peptide that corresponded to residues 360 through 382 of *connexin43* that was synthesized by the Protein and Carbohydrate Structure Facility of the University of Michigan, Ann Arbor, and purified by high-performance liquid chromatography.

Microinjection Studies

After two weeks of culture in the presence of 200 μ g of hygromycin per milliliter of medium, transfected L929 cells were microinjected with 2 percent Lucifer yellow dye (Molecular Probes) or homogeneous purified protein kinases, prepared as described elsewhere.^{30,37} Chi-square analysis was used to

determine whether differences in the amount of dye transferred were statistically significant.

RESULTS

Genetic Screening

The 25 control sequences that represented a random sampling of blood specimens from the clinical laboratory had no amino acid substitutions in the region of interest when they were compared with GenBank sequence HSCGJP (human sequence cardiac gap-junc-

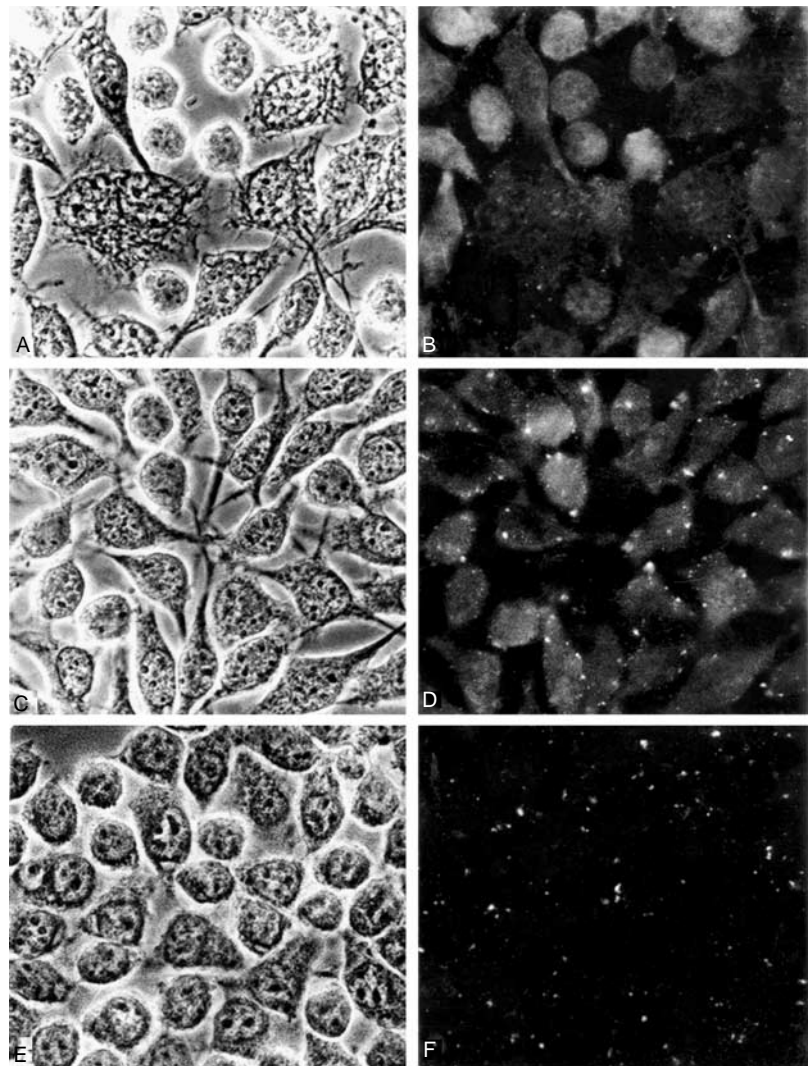


Figure 2. L929 Cells without Transfection and after Transfection with Normal and Mutant *Connexin43* ($\times 1400$).

A phase-contrast photomicrograph shows the nontransfected cells (Panel A). A fluorescence photomicrograph of the same cells (Panel B) demonstrates the binding of anti-*connexin43* primary antibody. Only occasional punctae are seen, and diffuse background staining is the predominant feature. A phase-contrast image shows cells transfected with wild-type *connexin43* (Panel C). A fluorescence photomicrograph of the same cells (Panel D), treated as in Panel B, shows abundant punctate staining, particularly around areas of cell-cell contact, which occurs frequently where cellular processes contact neighboring cells. A phase-contrast image of cells transfected with *connexin43* bearing a Ser364Pro mutation is shown in Panel E. A fluorescence photomicrograph of the same cells (Panel F) shows abundant punctate staining, particularly around areas of cell-cell contact.

tion protein). Similarly, of the 30 transplant recipients, 23 had no amino acid substitutions. One of the remaining seven patients, who had a familial atrial septal defect, had a Phe335Gln substitution that is under study (unpublished data). These results show that sequence polymorphisms in this region of the *connexin43* gene are rare, even in a group of patients with diverse racial and ethnic origins (black, Asian, white, and Hispanic).

Against this conservative background, however, a cluster of mutated sequences (Fig. 1) was isolated from six children of both sexes who had viscerotaxial heterotaxia syndromes in which complex cardiac malformations (including right or left atrial isomerism) were combined with abnormalities of visceral asymmetry. As a group, these 6 patients were distinct from the other 24, none of whom had viscerotaxial heterotaxia. Three patients (Patients 1, 2, and 3) had asplenia syndrome, two (Patients 4 and 5) had polysplenia syndrome, and one (Patient 6) resembled the other five with regard to atrial and bronchopulmonary isomerism and defects of laterality but had a single, small spleen. At the genetic level, the same mutation, Ser364Pro, was present in DNA from five of the six patients (Patients 1, 2, 3, 4, and 5) but in none of the 49 DNA samples sequenced from patients without heterotaxia. A second mutation, Glu352Gly, was present in two patients (Patients 1 and 6). Two independent mutations of *connexin43* were identified in four of the six patients: Patient 1, Ser364Pro and Glu352Gly; Patient 4, Ser364Pro and Ser365Asn;

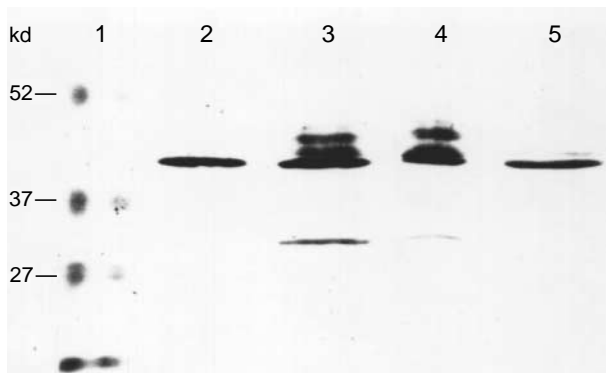


Figure 3. Western Blot of Connexin43 Immunoprecipitated from Supernatants of Parental and Transfected L929 Cells.

Lanes 2 through 5 were each loaded with 20 μ g of total protein. Lane 1 shows prestained low-molecular-weight standards (Bio-Rad). Lane 2 shows nontransfected cells with a prominent band at 42 to 43 kd. Lane 3 shows cells transfected with normal *connexin43*. The band at 42 to 43 kd is more prominent than that of the parental cells, with additional bands at 44 and 46 kd. Lane 4 shows cells transfected with *connexin43* bearing the Ser364Pro mutation. The immunoreactive bands are similar to those in lane 3 except that the 44-kd band is shifted slightly downward. Lane 5 shows cells transfected with the pCEP4 plasmid. There is a single prominent band at 42 to 43 kd, similar to that of nontransfected cells, and a very minor band at 44 kd. The minor band at 29 to 30 kd in lanes 3 and 4 has been attributed to a degradation product of *connexin43*.²⁸

Table 1. Transfer of Dye from Cell to Cell.

TREATMENT	TRANSFECTED WITH PLASMID ONLY*	TRANSFECTED WITH <i>CONNEXIN43</i>	
		NORMAL FORM	MUTANT FORM
		<i>no. of cells that transferred dye/no. injected with dye (%)</i>	
None	10/109 (9)	132/266 (50)	28/118 (24)
cAMP-dependent protein kinase	10/116 (9)	104/151 (69)	21/80 (26)
Protein kinase C	6/45 (13)	40/87 (46)	39/78 (50)

*The pCEP4 plasmid was used to transfect these cells.

Patient 5, Ser364Pro and Ser373Gly; and Patient 6, Glu352Gly and Thr326Ala.

Transfection and Dye Transfer

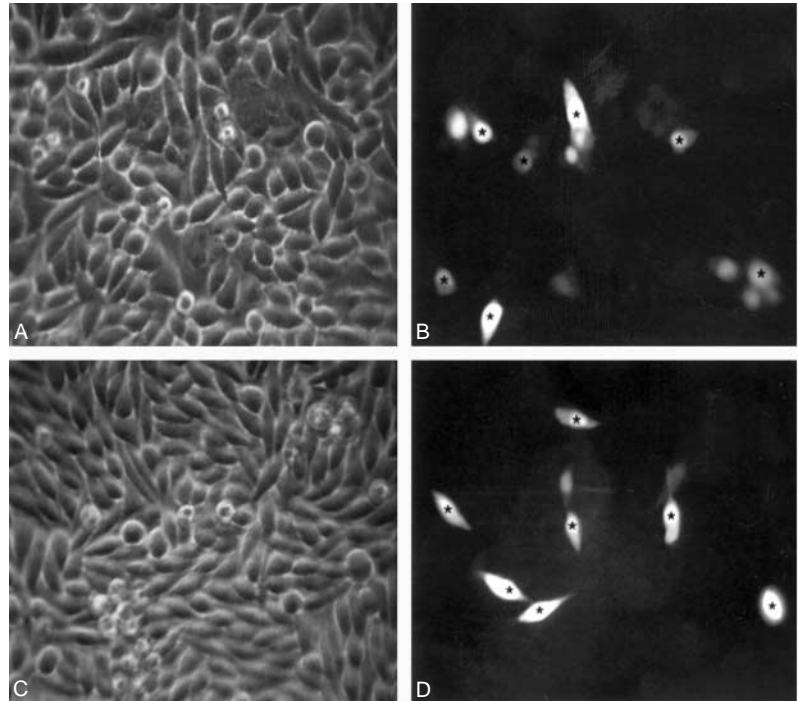
All the mutations involving serine residues altered potential consensus sites for phosphorylation by cyclic AMP (cAMP)-dependent protein kinase or protein kinase C, which have previously been shown to regulate *connexin43*-mediated cell-cell communication.^{30,31} To determine whether these mutations were functionally important, communication-deficient L929 cells were transfected with *connexin43* DNA containing the mutation that leads to the Ser364Pro substitution — the most common mutant sequence.

Although Northern blotting detected the *connexin43* transcript abundantly in transfected cell lines and in lesser amounts in parental L929 cells (data not shown), immunocytochemical analysis with an antibody highly specific for *connexin43*²⁸ revealed punctate staining typical of gap junctions in cells transfected with normal or mutant *connexin43* DNA, but not in nontransfected cells (Fig. 2). Western blotting with the same antibody showed that parental L929 cells and cells transfected with the pCEP4 plasmid both contained the nonphosphorylated parent species of *connexin43* and occasionally a barely detectable amount of a 44-kd form of the protein (Fig. 3). Cells expressing normal or mutant *connexin43* contained more of the parental form of this protein and a large amount of higher-molecular-weight species (44 and 46 kd) that probably correspond to the phosphorylated forms of *connexin43* described in several reports.²⁶⁻²⁹ Collectively, these results show that after transfection with normal or mutant *connexin43* DNA, L929 cells can produce *connexin43* and modify it after translation in sufficient quantity to form gap-junction-like membrane aggregates.

To determine whether these gap junctions mediated cell-cell communication that could be regulated by phosphorylation, we used microinjections of the fluorescent dye Lucifer yellow to assess the kinetics and amplitude of communication, as in previous studies.³⁰ The results are shown in Table 1. Control cells transfected with the pCEP4 plasmid transferred dye to neighboring cells less than 10 percent of the time, and this finding was not substantially affected by the injection of cAMP-dependent protein kinase or protein kinase C. In contrast, cells expressing normal *connexin43*

Figure 4. Transfer of Dye by L929 Cells after Transfection with Normal and Mutant *Connexin43* ($\times 630$).

A phase-contrast photomicrograph (Panel A) shows cells transfected with normal *connexin43*. In the corresponding fluorescence photomicrograph (Panel B), the cells marked with asterisks were microinjected with 2 percent Lucifer yellow dye; four of seven cells transferred the dye to contacting cells (the faint mark in the lower center of the field is a leakage artifact). A phase-contrast photomicrograph (Panel C) shows cells transfected with *connexin43* bearing the Ser364Pro mutation. In the corresponding fluorescence photomicrograph (Panel D), two of seven marked cells transferred Lucifer yellow dye to contacting cells.



gap junctions transferred dye to neighboring cells much more often (Fig. 4A and 4B) than did control cells ($P < 0.001$), and this level of communication was further enhanced ($P < 0.001$) by the microinjection of active cAMP-dependent protein kinase (Fig. 5A), but not by protein kinase C (Fig. 5B). Cells transfected with *connexin43* bearing the mutation causing the Ser364Pro substitution also transferred dye to neighboring cells (Fig. 4C and 4D) much more frequently than did control cells ($P = 0.0033$), but significantly less ($P < 0.001$) than did cells transfected with normal *connexin43*. Dye transfer among cells transfected with mutant *connexin43* was unaffected by the injection of cAMP-dependent protein kinase (Fig. 5C) but was significantly increased by the injection of protein

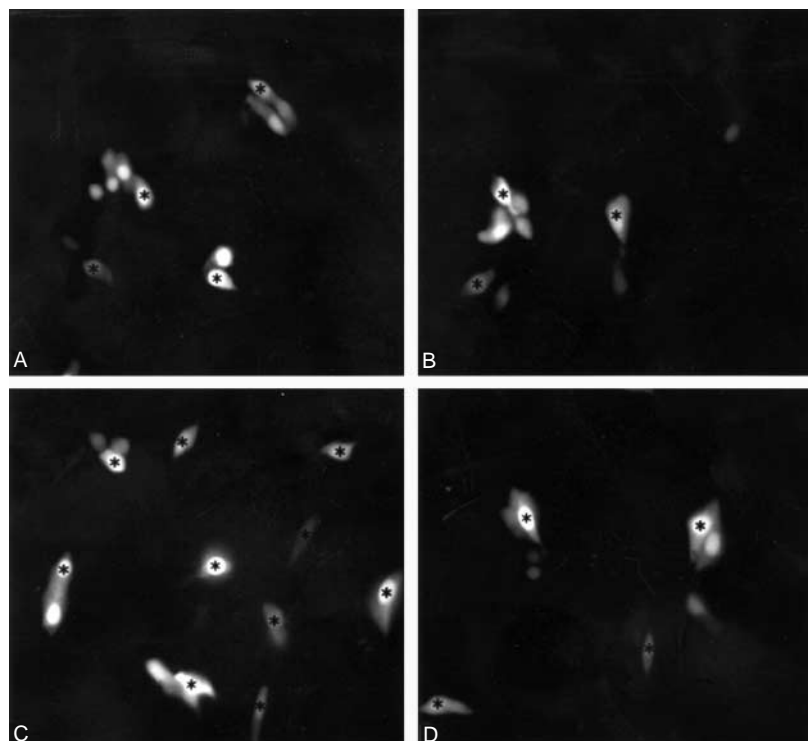
kinase C (Fig. 5D) ($P < 0.001$ for the comparison with untreated mutant cells).

DISCUSSION

Clinically, viscerotaxial heterotaxia represents a spectrum of conditions, some asymptomatic and some incompatible with life. The more severe asplenia and

Figure 5. Fluorescence Photomicrographs of L929 Cells Transfected with Normal *Connexin43* or with *Connexin43* Bearing the Ser364Pro Mutation ($\times 630$).

In Panel A, the cells marked with asterisks were microinjected with purified catalytic subunit of cAMP-dependent protein kinase, followed five minutes later by Lucifer yellow dye. All four cells transferred the dye to contacting cells. In Panel B, the cells marked with asterisks were microinjected with purified protein kinase C, followed five minutes later by Lucifer yellow dye. Three of four cells transferred the dye to contacting cells. In Panel C, the cells marked with asterisks were microinjected with purified catalytic subunit of cAMP-dependent protein kinase, followed five minutes later by Lucifer yellow dye. Three of 10 cells transferred the dye to contacting cells. In Panel D, the cells marked with asterisks were microinjected with purified protein kinase C, followed five minutes later by Lucifer yellow dye. Three of four cells transferred the dye to contacting cells.



polysplenia syndromes have an estimated incidence of 1 in 10,000 to 1 in 20,000 live births, or about 1 percent of all congenital heart defects. The occurrence is usually sporadic, but familial cases have been described,³⁸⁻⁴⁰ and most have been interpreted as demonstrating autosomal recessive transmission.³⁹ An X-linked form has recently been mapped to the region Xq24-q27.1,⁴¹ and other cases have been associated with chromosomal translocations (such as that between chromosomes 12 and 13) and deletions (involving chromosomes 10 and 13)⁴² and with monozygotic twinning.⁴³ A form of heterotaxia occurs in *iv/iv* mice and has been mapped to a region syntenic with human chromosome 14.⁴⁴ Taken together, this evidence strongly suggests that viscerotaxial heterotaxia is clinically and genetically heterogeneous and that normal laterality develops by a complex mechanism.

At present, most patients with heterotaxia are treated by palliative procedures rather than by orthotopic cardiac transplantation. Our group of transplant recipients was thus effectively preselected to represent the severe end of the heterotaxia spectrum. Despite the complexity of the malformations in these patients, they have an underlying anatomical similarity, as described above. The constancy of pulmonary atresia or stenosis is notable in view of the findings of a similar anomaly in mice with "knockout" of the *connexin43* gene. We suggest, therefore, that the *connexin43* mutations reported here define a distinctive subtype of viscerotaxial heterotaxia.

Five of the six patients with heterotaxia, who had no common kinship, share a Ser364Pro substitution, and in four patients a second *connexin43* mutation has been identified in the carboxyl-terminal region. Because the remaining two patients may well have mutations elsewhere in the *connexin43* gene (whose overall length is estimated at 15,000 bp),^{12,13,45} these data are consistent with recessive transmission of this syndrome, as is the report that a stillborn sibling of Patient 1 was afflicted with similar heart malformations.

Although the microinjection data show that the Ser364Pro mutation results in a strikingly altered response to regulation by two protein kinases, it is premature to propose a pathogenetic mechanism for heterotaxia, because little is known at present about how normal laterality develops. Nonetheless, it is worth pointing out that however the left and right sides may differ, asymmetry requires that there be a functional separation between them — that is, that the left and right sides constitute developmental compartments, as described earlier. In this context, it is relevant that in transgenic mouse embryos containing a *connexin43* promoter-driven:lacZ reporter gene, incipient *connexin43* expression (i.e., lacZ) shows an asymmetric left-right pattern in various tissues. Moreover, transgenic mice that overproduce *connexin43* have developmental perturbations, including defects in laterality such as embryonic turning and malrotation of the heart tube at the D-loop stage (Lo CW: personal communication). It appears, then, that gap junctions containing the

Ser364Pro mutant *connexin43* do not respond normally to regulatory phosphorylations. Such failure may perturb left-right boundary formation, allowing laterality to develop in a random manner that ultimately appears as viscerotaxial heterotaxia.

Since none of the mutations in the patients with heterotaxia affect putative channel-forming domains of the *connexin43* protein, we expect future work to show that the products of all these mutant genes are competent to form channels in appropriate circumstances. It is the regulation of channel function (i.e., cell-cell communication) that, according to our hypothesis, should be aberrant. Conceivably, then, the resulting disturbance of function may be more insidious than that occasioned by complete inactivation of the *connexin43* gene. We believe that the disturbances of internal asymmetry seen in our patients with heterotaxia offer an important clue to the timing and location of the regulatory events critical to the early stages of heart development in humans.

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