15. M. E. Toaff, H. Scleyer, J. F. Strauss III, *Endocrinology* **111**, 1785 (1982).
16. P. Robel and E. E. Baulieu, *Trends Endocrinol. Metab.* **5**, 1 (1994); S. H. Mellon, *J. Clin. Endocrinol. Metab.* **78**, 1003 (1994).
17. J. A. Harikrishna, S. M. Black, G. D. Szklarz, W. L. Miller, *DNA Cell Biol.* **12**, 371 (1993).
18. We thank Y. He for technical assistance and M. Waterman for providing the vectors encoding bovine P450scc and adrenodoxin. D.L. was supported by Child Health Research Center grant HD 28825 and the Department of Pediatrics, University of California at San Francisco. This work was also supported by NIH grants HD 06274 (J.F.S. III), HD 07688 (B.J.C.), HD 17481 (D.M.S.), RR000847 (A.R.), and DK 37922 and DK 42154 (W.L.M.).

28 November 1994; accepted 6 February 1995

Cardiac Malformation in Neonatal Mice Lacking Connexin43

Andrew G. Reaume,* Paul A. de Sousa,† Sarang Kulkarni, B. Lowell Langille, Daguang Zhu,‡ Tyler C. Davies, Subhash C. Juneja, Gerald M. Kidder, Janet Rossant§

Gap junctions are made up of connexin proteins, which comprise a multigene family in mammals. Targeted mutagenesis of connexin43 (Cx43), one of the most prevalent connexin proteins, showed that its absence was compatible with survival of mouse embryos to term, even though mutant cell lines showed reduced dye coupling in vitro. However, mutant embryos died at birth, as a result of a failure in pulmonary gas exchange caused by a swelling and blockage of the right ventricular outflow tract from the heart. This finding suggests that Cx43 plays an essential role in heart development but that there is functional compensation among connexins in other parts of the developing fetus.

The connexins are a family of at least 12 proteins, each the product of a distinct gene, that make up the intercellular membrane channels of gap junctions (*1*). Gap junction channels composed of hexamers of connexins provide conduits for the direct passage of ions and other low molecular weight molecules, including second messengers, between cells (*2*), a property that has been postulated to be important for many physiological and developmental processes (*3*).

All of the connexins have four membrane-spanning domains, two extracellular loops, a cytoplasmic loop, and cytoplasmic NH_2- and COOH-termini. Sequence similarity is concentrated in the transmembrane domains and extracellular loops. In contrast, most of the sequence diversity and length variation among the connexins resides in the cytoplasmic loop and COOH-terminal tail, which are assumed to account for the distinct biophysical and regulatory properties that are exhibited by gap junction channels composed of different connexins (*1*). In addition, individual gap junctions may be composed of more than one type of connexin (*4*, *5*). Given the distinct developmental expression patterns of the different connexins, this could result in complex regulation of intercellular coupling. The biological significance of connexin diversity is, however, uncertain. Antibody and antisense studies in vitro show the general significance of gap junctional coupling (*6*), but mutational analysis is required to test the role of the different connexins in vivo. The first genetic evidence for a specific developmental role for a member of the connexin family was the demonstration that X-linked Charcot-Marie-Tooth disease, a neuropathological condition involving peripheral demyelination in humans, is associated with mutations in the gene encoding connexin32 (*7*).

We have created a null mutation in mice in the gene (*Gja1*) encoding another member of the connexin family, connexin43 (Cx43). The gene is expressed from the onset of zygotic transcription in early cleavage and supplies subunits for the first gap junctions to form in mammalian development (*8*–*10*). It is also widely expressed in postimplantation embryos and adult organs, often in conjunction with other connexins (*11*). A mutation was generated in the Cx43 gene by homologous recombination in 129 strain R1 (*12*) embryonic stem (ES) cells with a construct that replaced almost the entire coding sequence with the *neo*^{r} gene and that lacked a promoter (Fig. 1). This strategy was made feasible by the high level of Cx43 expression in ES cells (*13*). One in three *neo*^{r} clones was correctly targeted. Several ES cell lines that were homozygous for the Cx43 deletion were obtained by an increase in the G418 concen-

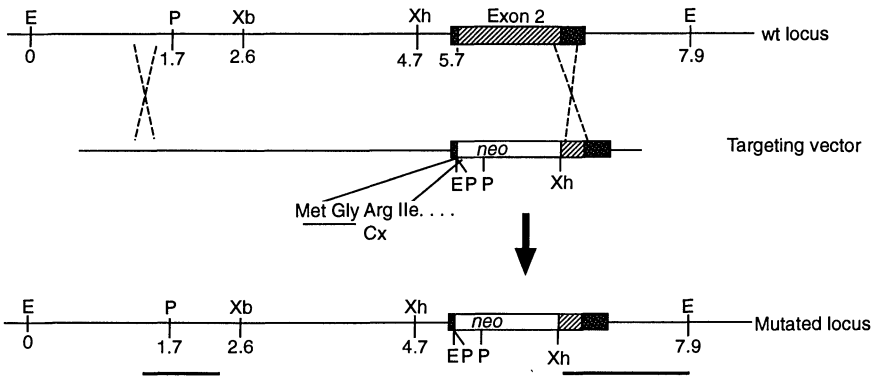

Fig. 1. Genomic structure of Cx43 and targeting strategy. A rat Cx43 complementary DNA probe (pG2A) was used to screen a 129Sv mouse genomic library. Six overlapping clones were isolated and the genomic structure determined. The coding sequence (striped box) is contained entirely within one exon, and there is an untranslated 5′ exon approximately 11 kilobases upstream (not shown). A targeting vector was designed to place the promoterless *neo* gene after the second amino acid of Cx43 and delete all the transmembrane regions of the Cx43 gene. This vector was introduced into R1 ES cells and *neo*^{r} clones isolated in the standard way (*39*) except that ES cells were grown in leukemia inhibitory factor (1000 U/ml) without feeders throughout. Homologously recombined clones were recognized by distinctive bands on Southern (DNA) blots after Eco RI digestion and probing with 5′ and 3′ probes, shown as bars under the genomic structure. E, Eco RI; P, Pst I; Xb, Xba I; and Xh, Xho I.

A. Reaume and S. Kulkarni, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada.
P. A. de Sousa, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto M5G 1X5, Canada, and Molecular Genetics Unit and Department of Zoology, University of Western Ontario, London, Ontario N6A 5B7, Canada.
B. L. Langille, Max Bell Research Centre, Toronto General Division, 200 Elizabeth Street, Toronto, Ontario M5G 2C4, Canada, and Department of Pathology, University of Toronto, Toronto, Ontario M5S 1A8, Canada.
D. Zhu, T. C. Davies, S. C. Juneja, G. M. Kidder, Molecular Genetics Unit and Department of Zoology, University of Western Ontario, London, Ontario, N6A 5B7, Canada.
J. Rossant, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada, and Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada.

*Present address: Cephalon, Inc., 145 Brandywine Parkway, West Chester, PA 19380, USA.
†Present address: University of Pennsylvania, Department of Biology, Leidy Laboratory of Biology, 38th and Hamilton Walk, Philadelphia, PA 19104, USA.
‡Present address: Immunex Corp, Seattle, WA 98101, USA.
§To whom correspondence should be addressed.

tration to 1 mg/ml, thus selecting for clones containing two mutated copies of the gene (*14*). Such cell lines were morphologically normal, proliferated well, and were capable of differentiating into embryoid bodies containing beating heart muscle and blood islands (*15*), although they showed complete absence of Cx43 by antibody staining (Fig. 2, A to C) (*16*). The dye-coupling ability of these cells was assessed by injection of carboxyfluorescein (*17*) and indicated a reduction, but not complete absence, of junctional communication (Table 1).

The ability of ES cells to proliferate and differentiate in the absence of Cx43 suggested that embryos lacking Cx43 might also be able to survive, despite high levels of expression in early embryogenesis. Heterozygous mutant ES cell lines were used to generate germline chimeras by injection into C57BL/6 blastocysts (*18*), and the phenotype of homozygous offspring from heterozygous crosses was analyzed. No viable homozygous offspring were produced from two separate cell lines, ES5 and ES24 (Table 2). Dead homozygous pups were occasionally observed in the cage shortly after birth. By delivery of pups close to term by cesarian section, the expected ratio of genotypes could be recovered. A slight deficiency of homozygotes was observed for the ES5 line but not for ES24, a difference that is still unexplained (Table 2). For the two mutant lines, the phenotype of homozygotes was identical. Although breathing was initiated and the lungs became expanded, the pups became cyanotic, suggesting a failure of pulmonary gas exchange. Most homozygotes died shortly after delivery, but occasionally pups were observed to survive up to 5 hours after birth. However, these pups remained cyanotic and exhibited labored breathing, as well as a swollen abdomen and stomach.

By external morphology, Cx43 homozygotes were essentially indistinguishable from wild-type, except for some swelling in the neck region. Autopsy revealed no gross anatomical defects, except in the heart, where the conus region overlying the right ventricular outflow tract was grossly enlarged. The topological arrangement of all the major vessels of the heart was undisturbed. Cx43 is expressed in many other tissues throughout development (*11*), but abnormal morphogenesis was observed only in the heart. Histological examination of other tissue types that express high levels of Cx43, such as brain, gut, skin, lungs, kidney, and limbs, revealed no overt abnormalities, although subtle cell type–specific or physiological effects cannot be excluded.

The conus defect in the heart was a consistent feature that allowed identification of all homozygotes at term and was also apparent earlier in gestation at 16.5 days post coitum (*19*). Scanning electron microscopy of the dissected hearts (*20*) confirmed the enlarged conus (Fig. 3B). When the outer wall of the conus was dissected away, the enlarged region was seen to be filled with intraventricular septae instead of the unobstructed cavity seen in wild-type pups. Serial histological transverse sections of the mutant hearts showed that the trabeculae of the right ventricle were somewhat more extensive than in heterozygous or wild-type mice, but the major morphological abnormality in the heart was observed in the outflow tract, where the internal septae divided the tract into separate, interconnected or blind-ended chambers (Fig. 3, C and D). Transmission electron microscopy (TEM) identified the primary cell type in these septae as myocytes and also showed that the cardiac myotube structure was apparently normal in the mutant heart (*21*).

Corrosion casts of the cardiac circulation were prepared by infusion of methyl methacrylate resin (*22*). With infusion into the

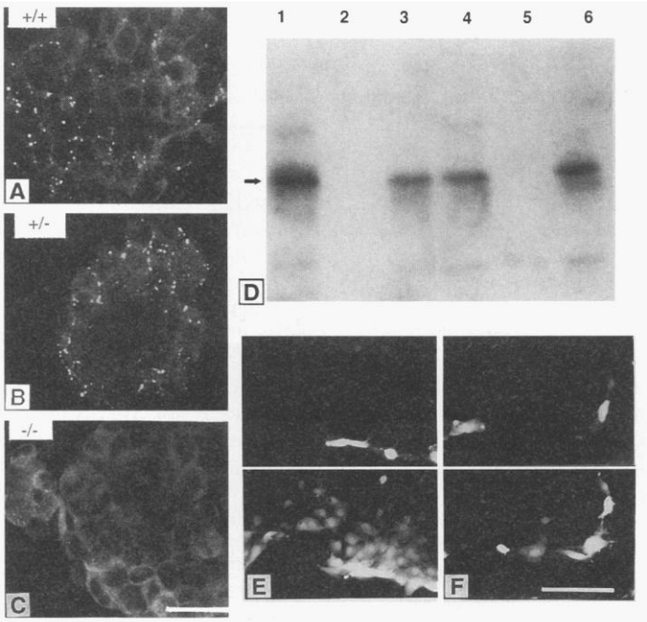

Fig. 2. (A to C) Confocal imaging of immunofluorescence localization of Cx43 in ES cells. (**A**) Wild-type cells; (**B**) $Cx43^{+/-}$ cells; (**C**) $Cx43^{-/-}$ cells. Punctate intercellular staining indicative of Cx43-containing gap junction plaques was detected only in stem cells with at least one normal Cx43 gene. The apparent heterogeneity of expression in $Cx43^{+/-}$ cells was not consistently observed. Confocal Z-series projections, 7 μm thick; scale bar, 25 μm. (**D**) Protein immunoblot analysis of Cx43 levels in primary embryo fibroblasts grown from 12.5-day-old fetuses. Arrow indicates the Cx43 band at ~46 kD. Lane 1, positive control (C43-transfected rat C6 glioma cells) (*38*); lane 2, negative control (untransfected C6 cells); lanes 3, 4, and 6, fibroblasts from wild-type fetuses; and lane 5, fibroblasts from $Cx43^{-/-}$ fetus. (E and F) Passage of Lucifer yellow through gap junctions in wild-type and homozygous mutant embryonic fibroblasts after scrape loading. (**E**) Rhodamine (top) and Lucifer yellow (bottom) images of wild-type cells show passage of Lucifer yellow from rhodamine-labeled cells at the scrape into cells located at a distance. (**F**) Rhodamine (top) and Lucifer yellow (bottom) images of $Cx43^{-/-}$ cells showing little spread of dye beyond the rhodamine-labeled cells.

Table 1. Dye coupling in homozygous mutant ES cell lines

Cell genotype*	*n*	Percent not coupled	Percent coupled	
			1° order†	2° order
+/+	8	25	25	50
+/−	34	53	15	32
−/−	28	82	11	7

*Two separate cell lines were analyzed for each genotype and the data pooled. †First-order coupling is indicated by transfer of dye to immediate neighbors of the injected cells; second-order coupling involves transfer beyond immediate neighbors.

Table 2. Observed genotype ratios from $Cx43^{+/-}$ heterozygous crosses. dpc; days post coitum

Target cell line	Background strain	Stage analyzed	No. analyzed	Genotype		
				+/+	+/−	−/−
ES5	129/C57BL	16 to 18 dpc	42	14	20	8
	129/C57BL	Term	104	39	59	6*
	129	16 to 18 dpc	33	8	19	6
	129	Term	121	39	75	7*
ES24	129/C57BL	16 to 18 dpc	101	26	48	27
	129/C57BL	Term	181	59	121	1*

*Dead pups only.

right ventricle, no passage of resin from the right ventricle to the pulmonary arteries was observed in mutant hearts (Fig. 3F). Conversely, back perfusion of the aorta resulted in minimal filling of the right ventricle, although such perfusion consistently crossed the ductus arteriosus and leaked back through the pulmonary valves to the right ventricle in wild-type pups (Fig. 3E). An obstruction of this sort is compatible with survival during fetal life, when central cardiovascular shunts allow blood to be rerouted via the foramen ovale and the ductus arteriosus (*23*). Postpartum closure of these shunts, combined with the need to deliver right ventricular outflow to the newly ventilated lungs, would lead to severe problems in blood gas transport in mutant neonates. Thus, we believe that the right ventricular defect caused the neonatal cyanosis and perinatal death.

Table 3. Dye coupling in embryonic fibroblasts.

Genotype	No. of cell lines analyzed	No. coupled	No. not coupled
+/+	6	6	0
+/−	13	11	2
−/−	3	0	3

In mammals, cardiac muscle is a major site of expression of Cx43, although other connexins, notably Cx40 and Cx45, are also expressed (*5*, *24*–*26*). The survival of Cx43 mutant mice to term indicates that functional cardiac conduction does not uniquely require Cx43 channels (*27*); either nonexpressed connexin genes are activated or coexpressed connexins can suffice or are upregulated in the absence of Cx43. We examined the latter possibility by measuring Cx40 and Cx45 mRNA levels in the mutant hearts, but could find no major changes (*28*). That Cx43 is not unique in its ability to mediate cardiac conduction is clear from its absence from the avian myocardium (*26*, *29*). Although primary conductance cannot have been severely impaired in the Cx43 mutants, cardiac function was ultimately affected. It is not clear why the defect should have been confined to the right ventricular outflow tract, especially because Cx43 is expressed only weakly in this region late in gestation (*25*). This area of the heart is subject to extensive remodeling during development (*30*), and failure of correct junctional communication could affect neural crest migration (*31*), proliferation of trabeculae, or the ordered apoptosis (*32*) that is essential for normal development. Alternatively, loss of Cx43 may alter conduction velocities (*25*) and local mechanical load on the myocardium, leading to abnormal morphogenesis (*33*).

We tested the ability of primary embryonic fibroblasts from mutant embryos to allow dye transfer by the scrape-loading technique (*34*) and could detect little or no effective dye coupling in cells from three different mutant embryos (Table 3 and Fig. 2, E and F). Protein immunoblot analysis of other mutant primary fibroblasts (*35*) revealed the complete absence of Cx43 protein (Fig. 2D). Combined with evidence of reduced coupling in ES cells (Table 1), this result suggests that the junctional permeability of cells was altered by loss of Cx43. However, loss of dye coupling does not necessarily imply complete loss of cell coupling, because ionic coupling may still occur utilizing different connexins with different conductance properties. The availability of normal tissue and cell lines lacking Cx43 should help address the physiologically relevant size range of molecules that pass between cells in different in vivo situations.

There is clearly considerable cross-talk among the members of the connexin gene family, because two mutations in individual, broadly expressed connexins, Cx32 in humans (*7*) and Cx43 in mice, have very tissue-restricted phenotypes. The cardiac defect in Cx43 mutant mice is reminiscent of some forms of pulmonary stenosis in humans. Mutations in the COOH-terminal portion of Cx43 have been reported in some cases of visceroatrial heterotaxia (*36*), and our results suggest that Cx43 defects could underline other congenital cardiac abnormalities as well.

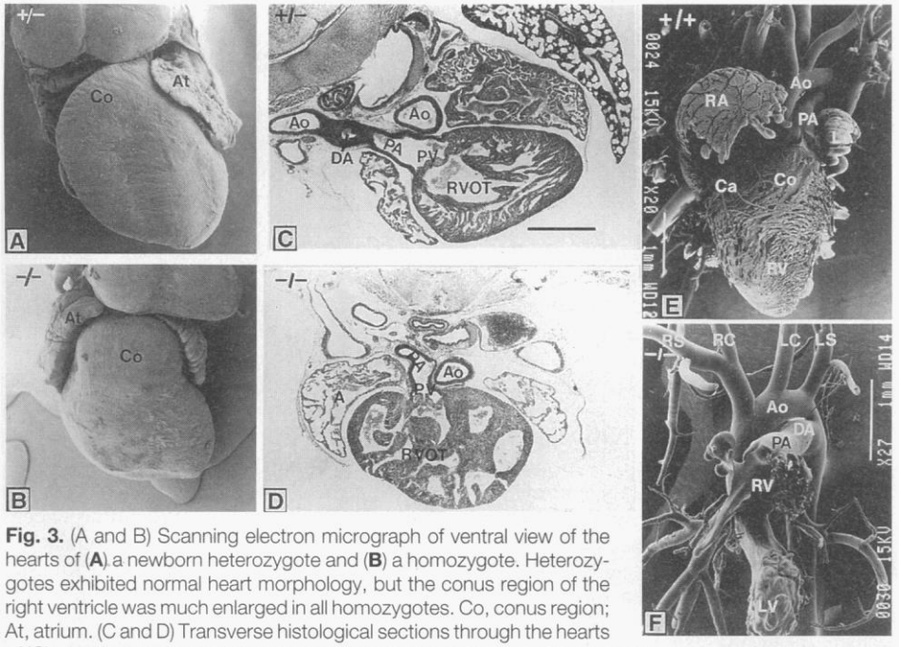

Fig. 3. (A and B) Scanning electron micrograph of ventral view of the hearts of (**A**) a newborn heterozygote and (**B**) a homozygote. Heterozygotes exhibited normal heart morphology, but the conus region of the right ventricle was much enlarged in all homozygotes. Co, conus region; At, atrium. (C and D) Transverse histological sections through the hearts of (**C**) a heterozygote and (**D**) a homozygote mouse at the level of the right ventricular outflow tract into the main pulmonary artery. Histology of heterozygotes was normal. In homozygotes, the right ventricular cavity was divided by septae into numerous interconnected subcavities that frequently were blind-ended. Ao, aorta; PA, pulmonary artery; DA, ductus arteriosus; PV, pulmonary valve; RVOT, right ventricular outflow tract; and A, atrium. Grid bar, 1 mm. (E and F) Corrosion casts prepared by retrograde perfusion of methyl methacrylate casting compound into the aorta of (**E**) normal and (**F**) homozygous mice. This compound consistently back-filled the right ventricular chamber (RV) of normal mice (E) despite the presence of pulmonary valves. However, only very small amounts of casting compound entered either the right ventricular cavity (RV) or left ventricular cavity (LV) of homozygotes (F). Lack of filling of the right ventricular cavity was consistent with other indications of blockage of the outflow tract. Filling of the left ventricle may have been prevented because emptying of blood from the ventricle via the atrioventricular valves was obstructed. RA, right atrium; Ao, aorta; PA, pulmonary artery; Ca, coronary artery; Co, conus; RV, right ventricle; RS, right subclavian artery; RC, right carotid artery; LC, left carotid artery, LS, left subclavian artery; LV, left ventricle; and DA, ductus arteriosus.

REFERENCES AND NOTES

1. E. C. Beyer, D. L. Paul, D. A. Goodenough, *J. Membr. Biol.* **116**, 187 (1990); J. C. Saez, V. M. Berthoud, A. P. Moreno, D. C. Spray, *Adv. Second Messenger Phosphoprotein Res.* **27**, 163 (1993).
2. J. C. Saez, J. A. Connor, D. C. Spray, M. V. L. Bennett, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2708 (1989); A. C. Charles *et al.*, *J. Cell Biol.* **118**, 195 (1992).
3. S. Caveney, *Annu. Rev. Physiol.* **47**, 319 (1985); C. R. Green, *Bioessays* **8**, 7 (1988); C. N. Dealy, E. C. Beyer, R. A. Kosher, *Dev. Dyn.* **199**, 156 (1994).
4. B. Nicholson *et al.*, *Nature* **329**, 732 (1987); K. I. Swenson, J. R. Jordan, E. C. Beyer, D. C. Paul, *Cell* **57**, 145 (1989); R. Werner, E. Levine, C. Rabadan-Diehl, G. Dahl, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5380 (1989).
5. H. L. Kanter, J. G. Laing, E. C. Beyer, K. G. Green, J. E. Saffitz, *Circ. Res.* **73**, 344 (1993).
6. S. lee, N. B. Gilula, A. E. Warner, *Cell* **51**, 851 (1987); A. Bevilacqua, R. Loch-Caruso, R. P. Erickson, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5444 (1989).

7. J. Bergoffen *et al.*, *Science* **262**, 2039 (1993).
8. M. Nishi, N. M. Kumar, N. B. Gilula, *Dev. Biol.* **146**, 117 (1991).
9. G. Valdimarsson, P. A. De Sousa, E. C. Beyer, D. L. Paul, G. M. Kidder, *Mol. Reprod. Dev.* **30**, 18 (1991).
10. P. A. De Sousa, G. Valdimarsson, B. J. Nicholson, G. M. Kidder, *Development* **117**, 1355 (1993).
11. E. C. Beyer, D. L. Paul, D. A. Goodenough, *J. Cell Biol.* **105**, 2621 (1987); E. C. Beyer, J. Kistler, D. L. Paul, D. A. Goodenough, *ibid.* **108**, 595 (1989); P. Meda *et al.*, *Endocrinology* **133**, 2371 (1993); B. Risek and N. B. Gilula, *Development* **113**, 165 (1991); B. Risek, S. Guthrie, N. Kumar, N. B. Gilula, *J. Cell Biol.* **110**, 269 (1990); B. Risek, F. G. Klier, N. B. Gilula, *Development* **116**, 639 (1992); C. P. Ruangvoravat and C. W. Lo, *Dev. Dyn.* **194**, 261 (1992); G. Valdimarsson, P. A. De Sousa, G. M. Kidder, *Mol. Reprod. Dev.* **36**, 7 (1993).
12. A. Nagy, J. Rossant, R. Nagy, W. Abramow-Newerly, J. C. Roder, *Proc. Natl. Acad. Sci. U.S.A* **90**, 8424 (1993).
13. High levels of Cx43 mRNA were detected in RNA from R1 ES cells by Northern (RNA) blot hybridization (A. G. Reaume *et al.*, data not shown).
14. R. M. Mortensen, D. A. Conner, S. Chao, A. A. T. Geisterfer-Lowrance, J. G. Seidman, *Mol. Cell. Biol.* **12**, 2391 (1992).
15. A. G. Reaume *et al.*, data not shown. ES cells were cultured in suspension culture in medium lacking leukemia inhibitory factor for periods of 1 to 2 weeks.
16. ES cells grown on cover slips were processed for immunofluorescence as described (*9, 10*). The affinity-purified primary antibody was raised against a synthetic peptide corresponding to amino acids 360 to 382 of the COOH-terminal cytoplasmic domain (*37*) (provided by D. Laird, McGill University). The secondary antibody was fluorescein isothiocyanate–conjugated goat antibody to rabbit immunoglobulin G (IgG) (ICN Biomedicals). The cells were viewed with a Bio-Rad MRC 600 confocal laser scanning microscope.
17. J. R. McLachlan and G. M. Kidder, *Dev. Biol.* **117**, 146 (1986).
18. V. P. Papaioannou and R. Johnson, in *Gene Targeting: A Practical Approach*, A. L. Joyner, Ed. (Oxford Univ. Press, Oxford, 1993), pp. 107–130.
19. A. G. Reaume *et al.*, data not shown.
20. A midline thoracotomy was performed on killed newborn mice and the thoracic cavity was immersed in 3% paraformaldehyde in phosphate-buffered saline (PBS). After fixation the thorax samples were taken through an alcohol dehydration series and dried in a critical point dryer. The hearts were mounted on Cambridge SEM stubs and coated with gold-palladium in a sputter coater. Samples were viewed in a JEOL JSM-840 SEM.
21. Standard techniques were used to prepare transmission electron micrographs of the mutant hearts. Cardiac muscle cell ultrastructure was indistinguishable between mutant and wild-type hearts.
22. B. L. Langille and S. L. Adamson, *Circ. Res.* **48**, 481 (1981). Corrosion casts of the heart and central arteries were prepared with Batson's 317 methyl methacrylate casting compound (Polysciences, Warrington, PA). The descending thoracic aorta was catheterized retrogradely with PE10 polyethylene tubing that had been heat extruded to reduce its external diameter by 70%. The compound was infused immediately after mixing at a rate of 33 μl/min with a Harvard model pump.
23. W. J. Larsen, *Human Embryology* (Churchill Livingstone, New York, 1993).
24. B. Bastide *et al.*, *Circ. Res.* **73**, 1138 (1993); G. I. Fishman, E. L. Hertzberg, D. C. Spray, L. A. Leinwand, *ibid.* **68**, 782 (1991); R. G. Gourdie *et al.*, *J. Cell Sci.* **105**, 895 (1993); D. Gros *et al.*, *Circ. Res.* **74**, 839 (1994); H. L. Kanter, J. E. Saffitz, E. C. Beyer, *ibid.* **70**, 438 (1992); M. J. A. van Kempen, C. Fromaget, D. Gros, A. F. M. Moorman, W. H. Lamers, *ibid.* **68**, 1638 (1991).
25. R. G. Gourdie, C. R. Green, N. J. Severs, R. P. Thompson, *Anat. Embryol.* **185**, 363 (1992).
26. R. G. Gourdie, C. R. Green, N. J. Severs, R. H. Anderson, R. P. Thompson, *Circ. Res.* **72**, 278 (1993).
27. J. E. Saffitz, H. L. Kanter, K. G. Green, T. K. Tolley, E. C. Beyer, *ibid.* **74**, 1065 (1994); R. D. Veenstra, H.-Z. Wang, E. M. Westphale, E. C. Beyer, *ibid.* **71**, 1277 (1992).
28. Quantitative analysis by reverse transcription–polymerase chain reaction was performed on multiple samples of heart tissue isolated from mutant, heterozygous, and wild-type embryos recovered at 16.5 days of gestation.
29. R. Minkoff, V. R. Rundus, S. B. Parker, E. C. Beyer, E. L. Herzberg, *Circ. Res.* **73**, 71 (1993).
30. M. Vuillemin and T. Pexeider, *Am. J. Anat.* **184**, 114 (1989).
31. D.E. Bockman, M. E. Redmond, K. Waldo, H. Davis, M. L. Kirby, *ibid.* **180**, 332 (1987).
32. T. Pexeider, *Adv. Anat. Embryol. Cell Biol.* **51**, 6 (1975); J. M. Hurle and J. L. Ojeda, *J. Anat.* **129**, 427 (1979).
33. S. B. Bishop, P. G. Anderson, D. C. Tucker, *Circ. Res.* **66**, 84 (1990); E. B. Clark *et al.*, *Am. J. Physiol.* **257**, H55 (1989).
34. M. H. El-Fouly, J. E. Trosko, C.-C. Chang, *Exp. Cell Res.* **168**, 422 (1987). Primary embryo fibroblasts were grown to confluence in 35-mm tissue culture dishes, washed with PBS, and then scored with a sharp scalpel blade. The cultures were then incubated in a mixture of 0.2% Lucifer yellow and 0.05% rhodamine dextran (Molecular Probes) in PBS for 5 min in the dark. After rinsing thoroughly in PBS, culture medium was replaced and the cells were allowed to recover for an additional 5 min. Cells were rinsed again, mounted with PBS under glass cover slips, and examined under rhodamine and fluorescein excitation conditions in a Leitz photomicroscope.
35. Protein immunoblotting was performed as described (*38*). Blots were treated with an affinity-purified rabbit antibody directed against a synthetic peptide corresponding to amino acids 302 to 319 (*10*) (supplied by B. Nicholson, State University of New York, Buffalo). Bound rabbit antibodies were detected with ^{125}I-labeled goat antibody to rabbit IgG (ICN Biomedicals).
36. S. H. Britz-Cunningham, M. M. Shah, A.-M. Martinez, W. H. Fletcher, *Mol. Biol. Cell.* **4**, 329 (1993).
37. D. W. Laird and J.-P. Revel, *J. Cell Sci.* **97**, 109 (1990).
38. C. C. G. Naus, D. Zhu, S. D. L. Todd, G. M. Kidder, *Cell. Mol. Neurobiol.* **12**, 163 (1992).
39. W. Wurst and A. Joyner, in *Gene Targeting: A Practical Approach*, A. L. Joyner, Ed. (Oxford Univ. Press, Oxford, 1993), pp. 31–62.
40. We thank S. Lye and M. Bennett for useful discussions, K. Harpal for histological preparations, H. Christensen for TEM assistance, E. C. Beyer for pG2A probe, and B. J. Nicholson, D. L. Paul, and D. W. Laird for supplying complementary DNAs, antibodies, and the unpublished mouse Cx43 sequence. Supported by grants from the Medical Research Council (J.R. and B.L.L.) and the Natural Sciences and Engineering Research Council of Canada (NSERC) (G.M.K.). A.G.R. held a National Cancer Institute of Canada postdoctoral fellowship. P.deS. holds an NSERC postdoctoral fellowship. J.R. is a Terry Fox Cancer Research Scientist of the National Cancer Institute of Canada and an International Scholar of the Howard Hughes Medical Institute. The Cx43 mutant mice are available through the Induced Mutant Resource at the Jackson Laboratory, Bar Harbor, ME, USA.

5 August 1994; accepted 11 January 1995

Kinectin, an Essential Anchor for Kinesin-Driven Vesicle Motility

Janardan Kumar, Hanry Yu, Michael P. Sheetz*

The membrane anchor for the molecular motor kinesin is a critical site involved in intracellular membrane trafficking. Monoclonal antibodies specific for the cytoplasmic surface of chick brain microsomes were used to define proteins involved in microtubule-dependent transport. One of four antibodies tested inhibited plus-end–directed vesicle motility by approximately 90 percent even as a monovalent Fab fragment and reduced kinesin binding to vesicles. This antibody bound to the cytoplasmic domain of kinectin, an integral membrane protein of the endoplasmic reticulum that binds to kinesin. Thus, kinectin acted as a membrane anchor protein for kinesin-driven vesicle motility.

Kinesin and cytoplasmic dynein are ubiquitous mechanochemical adenosine triphosphatases (ATPases) involved in powering diverse forms of intracellular organelle transport such as anterograde and retrograde movements of vesicles along microtubules and a variety of membrane-trafficking events (*1, 2*). Kinesin directs plus-end (anterograde) movement, whereas cytoplasmic dynein directs minus-end (retrograde) transport. Antibodies specific for kinesin heavy chain inhibit vesicle movements affecting axoplasmic transport or other cellular motile processes (*3*). These ATPases or motors interact with integral membrane proteins on vesicle surfaces (*4, 5*) that presumably contain the vesicle signals (*6*) regulating the directionality of vesicle movement on microtubules. Protein-dependent motor binding to motile vesicle populations has been quantitated (*5*), and a kinesin-binding, integral membrane protein has been isolated from motile microsomes (*7*). It is unclear to what extent motor binding is related to vesicle motility and which motor-binding membrane protein is directly involved in motility. Here we define the effects of specific monoclonal antibodies (mAbs) on the in vitro movement of membranous organelles along microtubules (*1, 4, 8, 9*) and the binding of kinesin to organelles, presumably through the tail portion of the kinesin heavy chain (*5*).

Monoclonal antibodies were raised against native epitopes on the cytoplasmic surface of carbonate-washed microsomal

Department of Cell Biology, P.O. Box 3709, Duke University Medical Center, Durham, NC 27710, USA.

*To whom correspondence should be addressed.