DEVELOPMENTAL BIOLOGY **204,** 224–234 (1998)

Heart Malformations in Transgenic Mice Exhibiting Dominant Negative Inhibition of Gap Junctional Communication in Neural Crest Cells

R. Sullivan,* G. Y. Huang,* R. A. Meyer,*,† A. Wessels,‡ K. K. Linask,§ and C. W. Lo*,[1]

**Department of Biology, Goddard Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania 19104; †Biology Department, Rutgers University, Camden, New Jersey 08102; ‡Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, South Carolina 29425; and §Department of Cell Biology, University of Medicine and Dentistry of New Jersey, Stratford, New Jersey 08084*

Transgenic mice were generated expressing an α1 connexin/β-galactosidase fusion protein previously shown to exert dominant negative effects on gap junctional communication. RNase protection analysis and assays for β-galactosidase enzymatic activity showed that the transgene RNA and protein are expressed in the embryo and adult tissues. *In situ* hybridization analysis revealed that in the embryo, expression was predominantly restricted to neural crest cells and their progenitors in the dorsal neural tube, regions where the endogenous *α1 connexin* gene is also expressed. Dye-coupling analysis indicated that gap junctional communication was inhibited in the cardiac neural crest cells. All of the transgenic lines were homozygote inviable, dying neonatally and exhibiting heart malformations involving the right ventricular outflow tract—the same region affected in the *α1 connexin* knockout mice. As in the knockout mice, the conotruncal heart malformations were accompanied by outflow tract obstruction. Histological analysis showed that this was associated with abnormalities in the differentiation of the conotruncal myocardium. These results suggest that the precise level of gap junctional communication in cardiac neural crest cells is of critical importance in right ventricular outflow tract morphogenesis. Consistent with this possibility is the fact that cardiac crest cells from the *α1 connexin* knockout mice also exhibited a greatly reduced level of gap junctional communication. These studies show the efficacy of a dominant negative approach for manipulating gap junctional communication in the mouse embryo and demonstrate that targeted expression of this fusion protein can be a powerful tool for examining the role of gap junctions in mammalian development.

Key Words: **gap junctions; heart defect; neural crest; outflow tract; conotruncus; α1 connexin; connexin 43; gap junctional communication; Doppler echocardiography.**

INTRODUCTION

Gap junctions are membrane channels that mediate the transfer of ions and small molecules between cells (Beyer *et al.*, 1990; Bennett *et al.*, 1991; Kumar and Gilula, 1992; Bruzzone *et al.*, 1996). They have been proposed to mediate cell–cell signaling involved in the regulation of growth and development (Loewenstein and Rose, 1992; Warner, 1992; Lo, 1996). Gap junctions are composed of polypeptides encoded by the connexin multigene family. Recent studies suggest that the *α1 connexin* gene (also referred to as Cx43) plays an important role in cardiac morphogenesis. One indication of this is the finding of right ventricular outflow tract (RVOFT) obstructions in the *α1 connexin* knockout mice (Reaume *et al.*, 1995). This cardiac phenotype yielded little insight into the role of gap junctions in cardiac morphogenesis, as there is little α1 connexin detected in the outflow tract (Van Kempen *et al.*, 1991; Gourdie *et al.*, 1992; Moorman and Lamers, 1995). However, as development of the outflow tract is known to be dependent on the activity of neural crest cells (Kirby, 1993; Waldo and Kirby, 1995), one possibility is that the RVOFT heart malformations may arise from the perturbation of crest cells.

Neural crest cells are observed to migrate not as individual cells, but rather in groups organized in streams or sheets (Bancroft and Bellairs, 1976; Davis and Trinkaus,

[1] To whom correspondence should be addressed.

0012-1606/98 $25.00

1981). Several studies have indicated an important role for cell–cell interactions in modulating crest activity in development (Rovasio *et al.*, 1983; Raible and Eisen, 1996). Of particular significance is the fact that migrating crest cells express α1 connexins (Ruangvoravat and Lo, 1992; Lo *et al.*, 1997). They have also been shown to be functionally coupled via gap junctions (Lo *et al.*, 1997). In a recent study, transgenic mice were generated expressing an *α1 connexin* transgene driven by the cytomegaloviral promoter. In such mice (CMV43), transgene expression was restricted to subpopulations of neural crest cells, including the cardiac crest cells. Given that neural crest cells express the endogenous *α1 connexin* gene, it is not surprising that this resulted in an increase in gap junctional communication in the cardiac neural crest cells. RVOFT heart defects were observed in these mice, even though the CMV43 transgene was not expressed in the myocardium (Ewart *et al.*, 1997). These observations suggest that the role of α1 connexins in cardiac morphogenesis likely involves the modulation of cardiac neural crest cells (Ewart *et al.*, 1997). However, one puzzling aspect of these findings is how both the gain and loss of α1 connexin function can result in conotruncal heart defects. This raises some doubt as to the role of crest cells in heart defects in the *α1 connexin* KO mice.

To further elucidate the role of α1 connexins in cardiac development, in this study we examined the developmental effects of downregulating gap junctional communication in crest cells using a dominant negative approach. For these studies, transgenic mice were generated expressing an α1 connexin/β-galactosidase fusion protein construct driven by the human *elongation factor-1α* promoter. Previous studies showed that this construct can provide high-level fusion protein expression and inhibit gap junctional communication in cells with an abundance of endogenous α1 connexins (Sullivan and Lo, 1995). Surprisingly, very few viable transgenic animals were obtained with this construct. Nevertheless, three fertile founder lines were isolated, and all three were found to be homozygous inviable. RNA and protein analysis of these three lines showed that the fusion protein was expressed, but at a low level. In the embryo, expression was predominantly localized to the dorsal neural tube and subpopulations of neural crest cells. Dye-coupling analysis showed that gap junctional communication in the cardiac crest cells was reduced. Significantly, these transgenic mice exhibited heart malformations involving the RVOFT. Together with previous studies (Reaume *et al.*, 1995; Ewart *et al.*, 1997), these results indicate a central role for α1 connexins in right ventricular outflow tract development. More importantly, they further indicate that the role of α1 connexins in cardiac morphogenesis involves the modulation of cardiac neural crest cells. These studies also show for the first time that a dominant negative approach can be used effectively to manipulate gap junctional communication in the developing mouse embryo.

MATERIALS AND METHODS

Production and Genotyping of Transgenic Mice

To generate transgenic mice, the *Sal*I/*Hin*dIII fragment of the pEFZ plasmid (Sullivan and Lo, 1995) was injected into eggs obtained from the breeding of B6/SJL F1 mice (NICHD Transgenic Mouse Development Facility, DNX Inc., Princeton, NJ). Southern blot analysis was used to analyze and confirm transgenic founder animals identified by slot blot and PCR analysis. PCR analysis is carried out using primers (5′-TACCAACAGCGGATGGTTCGG-3′; 5′-GTGGTGGTTTATGCCCGATCC-3′) within the lacZ portion of the fusion protein construct (according to Echelard *et al.*, 1994).

RNA Isolation and RNAase Protection Analysis

RNA was collected from adult tissues and whole embryos using RNAzol B (Biotecx Laboratories, Inc., Houston, TX) according to the manufacturer's protocol. RNAase protection analysis was performed with a RPA I kit from Ambion Inc. (Austin, TX), using a ^{32}P-radiolabeled riboprobe to a portion of the lacZ coding sequence (*Eco*RV/*Cla* fragment, nucleotides 619–1108 of bacterial β-galactosidase). This probe gives a 489-nt protected fragment. In addition, a ^{32}P radiolabeled actin or 18S ribosomal riboprobe was included as an internal control to normalize for RNA loading. Briefly, RNA samples were hybridized with riboprobes overnight at 43°C (1×10^5 cpm). RNAase digestion was performed using RNAaseA (0.45 u/ml) and RNAaseT1 (900 u/ml). Protected fragments were subsequently loaded onto 8 M urea/6% acrylamide sequencing gels with DNA sequencing ladders loaded in parallel for size determination.

Quantitation of β-Galactosidase Enzyme Activity

β-Galactosidase activity was determined using the Galacto-Light chemiluminescent assay system of Tropix (Bedford, MA). For the analysis of enzyme activity in the embryo, individual E9.5 and E10.5 embryos (plug date = E0.5) were extracted in the lysis buffer provided by the manufacturer. For the analysis of adult testis or heart, or fetal heart samples, tissues were homogenized in the same lysis buffer using a Dounce homogenizer. For the quantitation of β-galactosidase activity, the reaction mixture was initially incubated for 60 min and then assayed for 10 s after injection of 200 μl Galacton-accelerator to the sample using the Monolight luminometer (Analytical Luminescence Laboratory). Each sample was assayed in triplicate and the β-galactosidase activity was normalized against the protein concentration.

Immunohistochemistry

For immunohistochemical analysis of transgene expression, adult animals were perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). Following perfusion, brain and testes or ovary were dissected and further fixed in 4% paraformaldehyde in PBS at 4°C overnight and then cryoprotected with 30% sucrose, frozen, and stored at −70°C until sectioning. Cryosections were mounted on chromalum–gelatin-coated slides. Immunostaining was performed using a β-galactosidase polyclonal antibody (Cappel Laboratories, West Chester, PA), with detection carried out with a fluorescein (FITC)-conjugated goat anti-rabbit IgG secondary antibody (Cappel Laboratories). Some tissue sections were also examined for β-galactosidase enzyme activity by histochemical

staining with X-gal. For standard histology and immunodetection of the marker α-smooth muscle actin, embryos were fixed in Amsterdam's fixative (Ewart *et al.*, 1997), and after paraffin embedding, and sectioning, the sections were examined using an indirect conjugated method which allowed detection of monoclonal antibody binding to mouse tissues (Huang *et al.*, 1998).

Section and Whole-Mount in Situ Hybridization Analysis

Embryos for *in situ* hybridization analysis were fixed in modified Carnoy's fixative and embedded in paraffin (Ruangvoravat and Lo, 1992). Ten-micrometer sections were cut and mounted on slides coated with 3-aminopropyltriethoxysilane. *In situ* hybridization was carried out as previously described (Ruangvoravat and Lo, 1992) using sense or antisense ^{35}S-radiolabeled riboprobes. After hybridization, slides were coated with Kodak NTB2 emulsion, exposed for 3 weeks, and developed. Slides were further stained with 0.1% toluidine blue and mounted with Permount. Sections were examined under phase-contrast and darkfield optics using a Leitz Ortholux upright microscope and photographed using Kodak T-Max 400 print film.

Whole-mount *in situ* hybridization of E9.5 and E10.5 embryos was carried out with digoxigenin–UTP-labeled lacZ riboprobes. For this analysis, embryos were fixed for 2 h in 4% paraformaldehyde at 4°C. Following fixation, embryos were washed in PBS containing 0.1% Tween 20 and stored in 100% methanol at −20°C. Embryos were bleached, dehydrated, treated with 20 μg/ml proteinase K for 5 min at room temperature, and postfixed. After further treatment with 0.1% sodium borohydride, prehybridization was carried out for 1 h followed by overnight hybridization. Embryos were then washed, treated with RNAase, and incubated with alkaline phosphatase-conjugated anti-digoxygenin antibody preabsorbed with embryo powder. After washes to remove unbound antibody, alkaline phosphatase activity was detected using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Quantitative Dye-Coupling Analysis

Dye coupling was monitored with microelectrode impalements into the dorsal hindbrain neuroepithelium of E8.5 embryos derived from FC or Cx43 knockout males mated to CD-1 females. This was carried out using a previously described method (Ewart *et al.*, 1997). Briefly, embryos were opened along the hindbrain neural fold and immobilized in a thin bed of 0.8% agarose. During microelectrode impalements, embryos were maintained on a heated microscope stage in phosphate-buffered L15 medium containing 10% fetal bovine serum. Carboxyfluorescein (2%) was iontophoretically ejected for a 2-min interval using 2-nA hyperpolarizing current pulses of 0.5 s duration at a frequency of one per second. The number of dye-filled cells was recorded immediately upon the termination of dye injection.

In Utero Doppler Echocardiography

Doppler analysis was carried out in pregnant females on E12.5 to E14.5 using previously described methods (Gui *et al.*, 1996). In brief, the pregnant female was sedated with Avertin (1.25 mg/5 g, i.p.). Echocardiography imaging and Doppler patterns were obtained using the Interspec XL (Conshohocken, PA) instrument by placing the ultrasonic transducer (7.5 MHz) over the abdomen and coupling the ultrasonic energy with an acoustic gel. Imaging at this frequency is adequate to locate the embryos and the beating heart and to orient the direction of the sagittal plane of the embryos. To obtain blood velocities, the sample volume (2–5 mm length) of the pulsed Doppler was placed over the entire heart. The high-pass filter was set at its lowest setting of 50 Hz. The same embryo was evaluated from several angles of insonation to determine the maximal blood velocities (Huhta *et al.*, 1990). The inflow and outflow waveforms were recorded on tapes using a Panasonic AG6300 videocasette recorder (Tokyo, Japan) and analyzed using an echocardiography analysis system (Digisonics, Inc., Houston, TX).

TABLE 1
Transgenic Animals

DNA construct	No. eggs transferred	No. live born pups	No. transgenic
pEFZ	363	26 (7.2%)	7[a] (1.9%)
pHXPL[b]	254	30 (11.8%)	13 (5.1%)
pHXPLDP[b]	171	29 (16.9%)	10 (5.8%)

[a] Two of these animals died, with one being hydrocephalic.
[b] pHXPL and pHXPLDP are lacZ reporter constructs.

RESULTS

Transgenic mice containing the α1 connexin/β-galactosidase fusion protein construct were obtained at a much lower frequency than expected (Table 1). Of 363 DNA-injected eggs transferred to foster mothers, only 7 transgenic founders were obtained (1.9%). This compares to a 5–6% transgenic efficiency achieved with parallel injections of two lacZ reporter constructs expressing wild-type β-galactosidase (Table 1). This result indicates that expression of the fusion protein is deleterious to the viability of the transgenic animals. Consistent with this possibility is the death of two transgenic founder animals prior to weaning, one of which was recovered after death and found to be hydrocephalic. Of the five viable founder animals, only three were fertile (lines FA, FC, and FE, collectively referred to as FZ). Mating of these three lines showed that they were homozygous inviable, with presumptive homozygous animals dying neonatally. It is also likely that some hemizygous transgenic animals are inviable, as 40% of the offspring from hemizygous X hemizygous crosses die before weaning.

To determine if the α1 connexin/β-galactosidase transgene was expressed in the three fertile FZ transgenic lines, RNAase protection analysis was carried out using a lacZ riboprobe. All three lines exhibited transgene expression in E10.5 and E11.5 embryos, albeit expression levels were very low (compared to tissue culture cell lines expressing the same construct; see Sullivan and Lo, 1995) (Fig. 1). Line FC and FE, but not line FA, also showed transgene expression in adult tissues (Fig. 1 and data not shown). Expression was detected only in the adult brain and gonads in line FC (see Fig. 1). In line FE, expression was most abundant in the

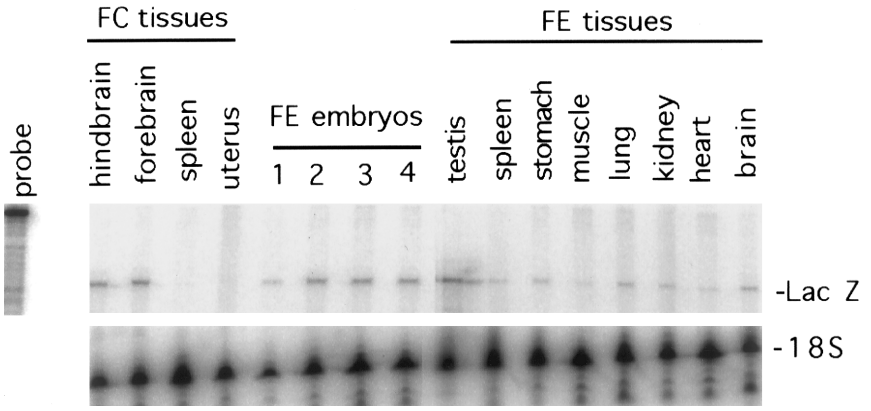

FIG. 1. RNAase protection analysis of transgene RNA expression. RNAase protection was performed using a ^{32}P-labeled lacZ riboprobe (indicated as LacZ) with RNA from various tissues obtained from adult animals of lines FC and FE and from FE embryos. An 18S ribosomal RNA probe was included to demonstrate RNA integrity (indicated as 18S). Note that a leak appeared in the well in which the FE testis sample was loaded, thereby resulting in faint bands appearing on either side of this lane.

brain and testis, although transgene expression was also observed in other adult tissues (Fig. 1).

To characterize the pattern of transgene expression during embryogenesis, whole-mount and section *in situ* hybridization analysis was carried out using E9.5 and E10.5 embryos (Fig. 2). These studies showed a surprisingly restricted pattern of transgene expression. Hybridization signal was predominantly detected in the dorsal neuroepithelium (see Figs. 2A and 2B, 2E and 2F, and 2G and 2H) and in presumptive neural crest cells. Neural crest expression is indicated by the presence of hybridization signal in cells situated dorsolateral to the neural tube (see arrowheads in Figs. 2E and 2G) and also in regions of the embryo known to be populated by neural crest cells—such as in the frontonasal process (Figs. 2C and 2D), the branchial arches (Figs. 2C, 2D, 2K, and 2L), and the dorsal root ganglia (Figs. 2I and 2J). In the heart, transgene expression was found in the outflow tract, a structure known to contain neural crest cells (Fig. 2L). In addition, hybridization signal was found in some regions of the pericardial sac (not shown) and at low levels in the trabeculated myocardium (Figs. 2M and 2N). It should be noted that much of the darkfield signal associated with the heart in Fig. 2M is not hybridization signal, but rather refraction of light due to red blood cells remaining associated with the heart chamber. Aside from expression in neural crest and crest-associated tissues, some transgene expression was also found in the limb bud mesenchyme (Figs. 2C and 2D) and the genital ridge (data not shown).

To examine for expression of the fusion protein, embryos and adult tissues were harvested and assayed for β-galactosidase enzymatic activity. Previous studies of tissue culture cells expressing this fusion protein construct had shown that the β-galactosidase moiety retained it enzymatic activity (Sullivan and Lo, 1995). Low levels of β-galactosidase activity were indeed detected in both the adult and embryo tissues, a result consistent with the low transcript levels seen by RNAase protection analysis (data not shown). This was further confirmed by histochemical staining with X-gal and immunohistochemical staining with a β-galactosidase antibody (Fig. 3). Such studies showed that in the adult testes, β-galactosidase protein and enzymatic activity were associated with the Leydig cells (Figs. 3A and 3C). In the brain, β-galactosidase protein can be observed in subpopulations of neuronal cells. This was seen by immunostaining (Fig. 3B), but not X-gal staining (data not shown), a discrepancy likely due to the lower fusion protein expression level associated with the brain compared to the testes. In comparison, the β-galactosidase antibody showed no specific immunostaining in brain sections from the nontransgenic littermates (Figs. 3D and 3E).

Quantitative Analysis of Gap Junctional Communication

To determine if expression of the fusion protein had any functional effects on gap junctional communication, dye-injection studies were carried out to quantitate the level of dye coupling in neural crest cells, a tissue in which fusion protein expression was detected. Microelectrode impalements were carried out into cells situated dorsolateral to the postotic hindbrain neural folds of E8.5 embryos obtained from nontransgenic females mated to hemizygous FC males. This postotic hindbrain neural fold is a region where both the transgene and the endogenous *α1 connexin* gene are expressed and also the region from which cardiac neural crest cells emerge (Kirby, 1993). The extent of dye spread was monitored by counting the number of dye-filled cells at the end of a 2-min impalement period. The genotype of the embryos was subsequently determined by PCR analysis of yolk sac DNA. These experiments revealed a reduction in dye coupling in the FC transgenic embryos compared to their nontransgenic littermates (Table 2; $P < 0.0001$). These findings demonstrate that expression of the fusion protein exerts a dominant negative effect on gap

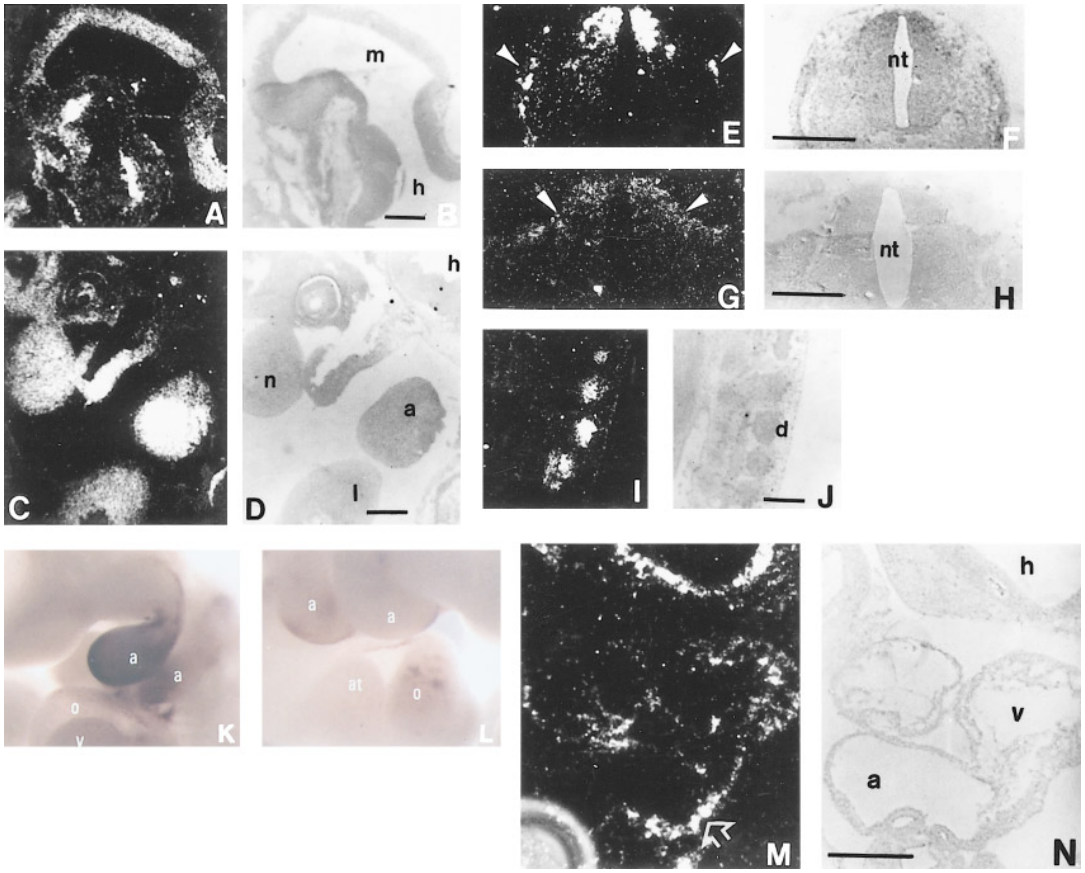

FIG. 2. *In situ* hybridization analysis of transgene expression in transgenic embryos. Transgenic embryos (E9.5 and E10.5) were examined by *in situ* hybridization analysis of histological sections using a ^{35}S-labeled lacZ riboprobe (A–J, M, N) or by whole-mount *in situ* hybridization using a digoxigenin-labeled lacZ riboprobe (K, L). (A–D) Sagittal sections of an E10.5 FA embryo demonstrate abundant transgene expression in the dorsal neuroepithelium of the midbrain (m) and hindbrain (h) as well as expression in the branchial arches (a), nasal placode (n), and limb (l). (E–H) Transverse sections through the spinal cord of an E9.5 FC embryo (E, F) and E10.5 FA embryo (G, H) showed transgene expression in the dorsal neural tube (nt) and in cells dorsolateral to the neural tube (arrowheads), likely presumptive neural crest cells. (I, J) Abundant transgene expression was also observed in the dorsal root ganglia (d). (K) An early E9.5 FC embryo exhibited abundant transgene expression in the branchial arches (a), while a lower level was seen in the outflow tract (o). (L) A late E9.5 FA embryo exhibited transgene expression in the branchial arches (a) and in the outflow tract (o). atria (at), ventricular (v), and outflow tract (o) regions of the developing heart. (M, N) Analysis of an E9.5 FC transgenic embryo showed abundant transgene expression in the hindbrain (h) neuroepithelium and in the atrial myocardium adjacent to the atrioventricular junction (open arrow). There also appears to be some expression in the trabeculated ventricular myocardium (v). Scale bar, 200 μm.

junctional communication in the presumptive cardiac crest cells. It is possible that this reduction in coupling is at least in part due to the inhibition of gap junctional communication mediated by α1 connexins, as this connexin isotype is expressed in neural crest cells (Lo *et al.*, 1997). To examine this question further, we examined dye coupling in cardiac crest cells in the *α1 connexin* knockout mouse. Such studies showed that in comparison to wild-type littermates, there was a marked reduction in dye coupling in the homozygous knockout embryos and an intermediate level of dye coupling in the heterozygous knockout embryos (Table 2). These results indicate a role for the endogenous *α1 connexin* gene in mediating gap junctional communication in cardiac crest cells. The fact that a low level of dye coupling persists in cardiac crest cells derived from the *α1 connexin* knockout mouse would suggest that other connexins are likely expressed in this crest cell population.

Heart Malformation in the FZ Transgenic Mice

Given the homozygote lethality of the FZ transgenic mice and the fact that heart defects are known to underlie the *α1 connexin* knockout lethality, the FZ transgenic mice were further examined for heart anomalies. These studies showed that all three FZ transgenic lines have right ventricular heart malformations (Figs. 4C and 4D). Typically the right ventricle was enlarged, resulting in a deepening of the interventricular cleft (see arrow in Figs. 4C and 4D).

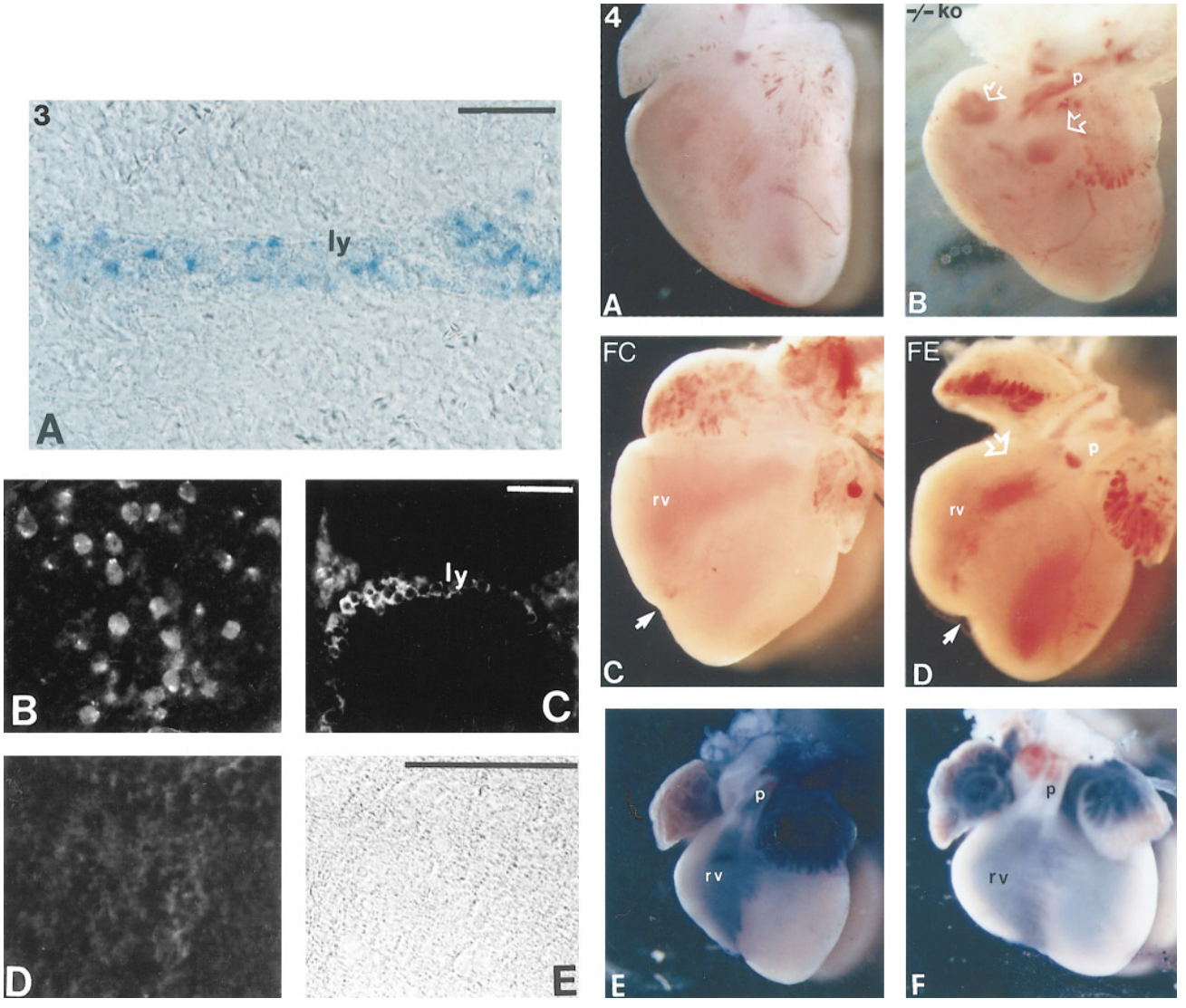

FIG. 3. Cx43/β-galactosidase fusion protein expression in FC adult brain and testis. (A) In the testis, Leydig cells (ly) exhibited X-gal staining. (B, C) In sections of the brain (B) and testis (C), immunostaining with an anti-β-galactosidase polyclonal antibody revealed fusion protein expression in both tissues. In the testis, expression is localized to Leydig cells (ly), the same as that seen by X-gal staining (see A). (D, E) Nontransgenic mouse brain immunostained in a parallel manner showed no staining with an anti-β-galactosidase antibody. Scale bars, 100 μm. The image in B is at the same magnification as the image in C.

FIG. 4. Heart malformations in transgenic mice. (A). Neonatal heart from a nontransgenic littermate. This heart and all others shown here are oriented with the right ventricle positioned to the left. (B) Neonatal heart from a homozygous *α1 connexin* knockout mouse. This heart shows double outpouchings (open arrows) flanking the subpulmonary space of the outflow tract (p). (C, D) Hearts from neonatal mice obtained from FC or FE heterozygous × heterozygous matings. Note dilation of the right ventricle (rv) and the resulting deepening of the interventricular cleft (solid white arrow). In the FE heart (D), it is possible to observe outpouching or bulging of the conotruncal region encompassing the subpulmonary space (see open arrow). P, pulmonary outflow tract. (E, F) Injection of trypan blue into the right ventricular (rv) chamber of the hearts from two littermates derived from a FE × FE cross. In the nontransgenic heart (E), the injected dye readily exited from the pulmonary outflow tract (p). However, in the transgenic heart (F), only a small amount exited from the pulmonary outflow tract (p), which is not visible in this photograph.

This was sometimes accompanied by visible distension of the infundibulum (see open white arrow in Fig. 4D). Such heart malformations were only found in offspring derived from hemizygous × hemizygous crosses and never in hemizygous × nontransgenic crosses, suggesting that the heart defects are associated with homozygous animals. This was confirmed in some cases by quantitative Southern blot analysis of the transgene inserts. It should be noted that the heart malformation is localized to the same region affected in the *α1 connexin* knockout mice, but the phenotype is

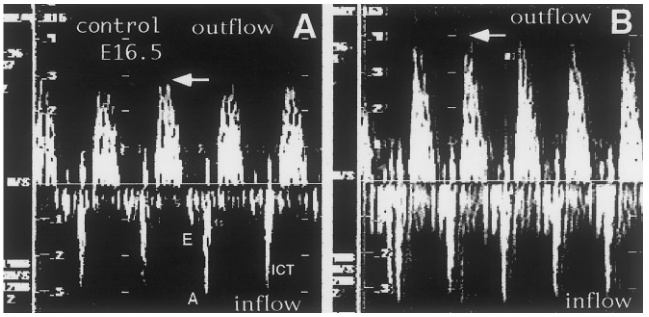

FIG. 5. Abnormal heart function detected by *in utero* Doppler echocardiography. (A) Cardiac blood flow seen by Doppler echocardiography in a normal E16.5 nontransgenic embryo. Peak systolic ejection velocity in this embryo is 0.28 m/s (see white arrow). A, A wave; E, E wave; inflow, inflow waveform; outflow, outflow wave form; ICT, isovolemic contraction time. (B) An E16.5 FE transgenic mouse embryo exhibited a peak systolic ejection velocity of 0.42 m/s (see white arrow), considerably higher than that in control nontransgenic embryos (see A).

distinct from that of the knockout mice. In the *α1 connexin* knockout animal, typically the right ventricular outflow tract defects are characterized by a double outpocketing at the base of the outflow tract (Fig. 4B), a phenotype never seen in the FZ transgenic mice.

Analysis of Cardiac Function

To determine if these heart malformations were accompanied by functional obstruction of the pulmonary outflow tract as had been observed in the *α1 connexin* knockout mice, we carried out trypan blue injection into the right ventricular chamber of E17.5 fetal hearts. In contrast to nontransgenic hearts, there is little dye movement out of the pulmonary outflow tract of the hearts of presumptive homozygous transgenic fetuses (Fig. 4F; compare to nontransgenic heart in Fig. 4E). Outflow obstruction was further indicated by *in utero* Doppler echocardiography of E17.5 fetuses derived from hemizygous × hemizygous matings. Such studies showed a significant increase in the peak systolic ejection velocity in 18% of the fetuses (Fig. 5; Table 3). A similar Doppler echo analysis of three litters derived from the mating of hemizygous FC males with nontransgenic females showed ejection velocity indistinguishable from that obtained with nontransgenic mice. The latter result suggests that cardiac dysfunction is exhibited only by the homozygous transgenic offspring. Given the expectation that 25% of the offspring would be homozygous, the finding of 18% with Doppler abnormalities would suggest that only three-quarters of the E16.5 homozygous offspring exhibit outflow tract obstruction. It should be noted that extensive breeding of these transgenic mice has consistently shown normal Mendelian segregation of the transgene.

TABLE 2
Dye Coupling in FC Transgenic and Cx43 Knockout Embryos*

Mouse strain	No. dye-filled cells
FC	
Hemizygous	3.25 ± 1.3 (65)
Nontransgenic	4.25 ± 1.08 (28)
	$P < 0.0001$*
α1 connexin knockout	
−/−	2.44 ± 0.73 (16)
−/+	3.63 ± 1.00 (48)
+/+	4.75 ± 0.94 (24)
	$P < 0.0001$**

*P value derived from Student's t test.
**P value based on ANOVA.

TABLE 3
Analysis of Cardiac Function by *in Utero* Doppler Echocardiography

Mouse strain	Fetal age	No. fetuses	IPSV[a]
FC	D12.5	4	1
	D16.5	9	2
FE	D14.5	6	1
	D15.5	10	1
	D16.5	5	1
Total		34	6

[a] Number of embryos showing increased peak systolic ejection velocity of 39.6 ± 3.6 m/s vs 22.9 ± 5.4 (P value < 0.0001).

Histological Analysis of the Heart Malformation

Histological analysis of FC and FE fetal hearts showed various abnormalities in the right ventricle and pulmonary outflow tract, but no abnormalities were detected in the left ventricle. Most surprising was the finding of cartilage nodules at the base of the semilunar valves in two E17.5 FC hearts (Figs. 6A and 6B). Although cartilage can be found in the hearts of older animals, the presence of cartilage is not a normal feature in fetal mouse hearts (S. Viragh, personal communication). Interestingly, smooth muscle actin expression in the myocardium was upregulated in the region surrounding the cartilage nodules (Fig. 6B and data not shown). Upregulation of smooth muscle actin was also observed in the myocardium surrounding the pouches in the outflow tract of *α1 connexin* knockout mouse hearts (not shown; Huang *et al.*, 1998). In line FE, hyperptrophy of the right ventricular and interventricular myocardium was occasionally observed (Figs. 6C–6E), and in some cases this resulted in a near complete occlusion of the right ventricular subpulmonary outflow tract (Fig. 6D). In some of the FC fetal hearts, the myocardium of the anterosuperior portion of the right ventricle appeared coarsely trabeculated (not shown). Abnormal myocardial "bulging" of the ven-

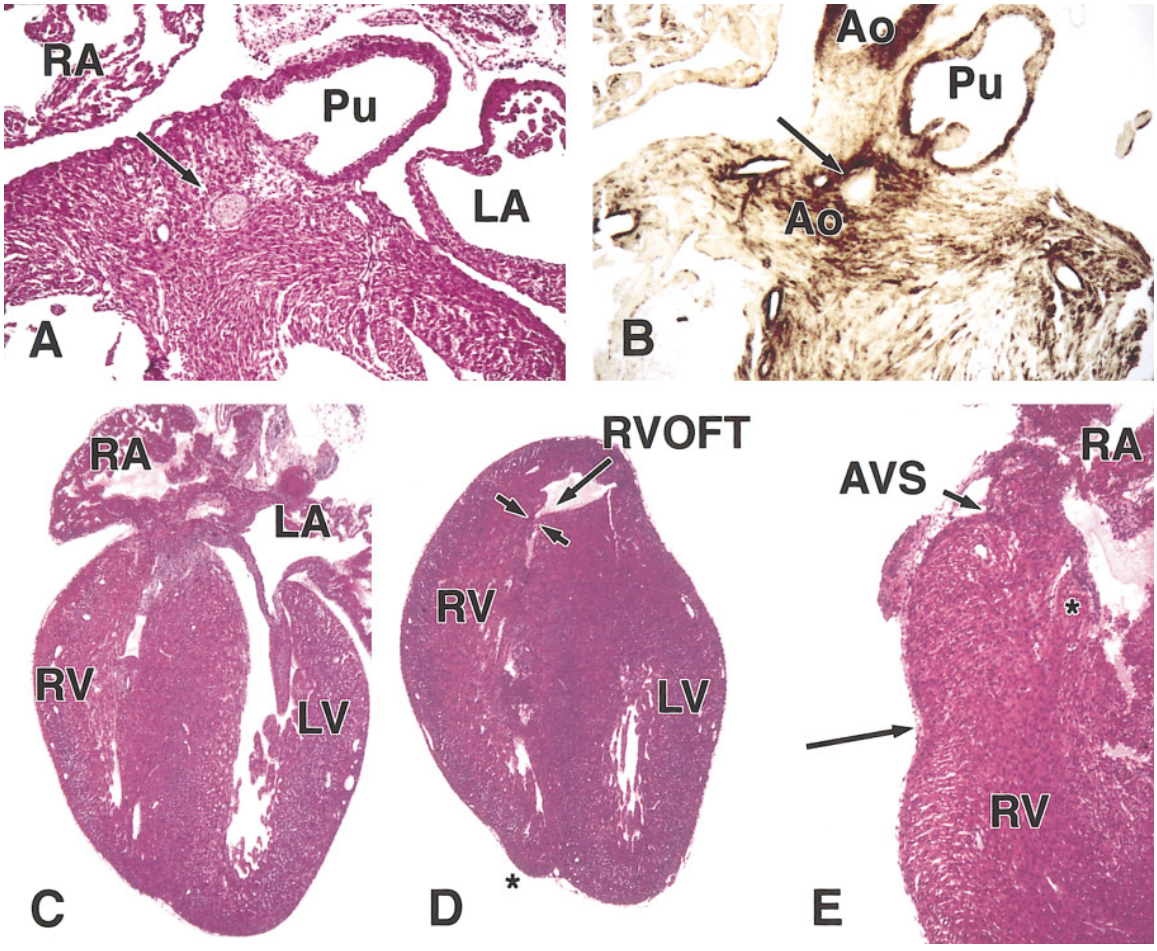

FIG. 6. Histological analysis of E17.5 FC and FE hearts. (A, B) Sections of the hearts of transgenic littermates from the FC line. The section shown in A is stained using hematoxylin–eosin, whereas the section shown in B was immunohistochemically labeled for smooth muscle actin. In both hearts, a cartilage nodule was observed at the base of the semilunar valves of the pulmonary trunk (arrows). Note the high level of smooth muscle actin expression in the surrounding myocardium in B. (C–E) Hematoxylin–eosin-stained sections of two FE hearts (D and E are sections from the same heart). The right ventricular myocardium was severely hypertrophied, resulting in the obstruction of the right ventricular inflow region (C and E) and the subpulmonary ventricular outflow tract (D). Some hearts also showed abnormal "bulging" of the ventricular myocardium (asterisk in D and area between arrows in E). The image in E also shows abnormal morphology in the atrioventricular region—note the abnormal tricuspid valve leaflet attachment (asterisk). It is important to note that the left ventricle is not affected in these hearts. Ao, aorta; AVS, atrioventricular sulcus; LA, left atrium; LV, left ventricle; Pu, pulmonary trunk; RA, right atrium; RV, right ventricle; RVOFT, right ventricular outflow tract.

tricular walls was also occasionally observed (Figs. 6D and 6E).

DISCUSSION

Transgenic mice were generated expressing an *α1 connexin*/β-galactosidase fusion protein under the regulation of the human *elongation factor-1α* promoter. Very few viable transgenic founders were obtained, suggesting that expression of this fusion protein is deleterious. This is not likely due to an intrinsic cell lethality, since tissue culture cells transfected with the same expression vector can exhibit high levels of fusion protein expression (Sullivan and Lo, 1995). In the viable transgenic lines, only low levels of transcript and fusion protein expression were observed, but nevertheless there was a significant reduction in gap junctional communication. These results indicate that this fusion protein can be highly effective in exerting dominant negative effects on gap junctional communication. The fact that coupling was not completely ablated by the fusion protein may indicate either an insufficient level of fusion protein expression or that the fusion protein is unable to interact with other connexin isotypes that are expressed.

Transgene expression was found to be restricted predomi-

nantly to the neural tube, subpopulations of neural crest cells, and various structures derived from neural crest cells. As this same embryonic expression pattern was detected in three independent transgenic lines, it likely represents expression mediated by the *elongation factor-1α* promoter construct. This result, however, differs markedly from the previous report of widespread lacZ reporter gene expression driven by the same promoter (Hanaoka *et al.*, 1991; Kim *et al.*, 1990). Given the potential for viability problems arising from expression of the fusion protein, a likely explanation is that the restricted expression pattern was selected based on its compatibility with postnatal survival and may reflect expression specified by a subset of regulatory element(s) in the promoter sequence.

All three transgenic lines expressing the fusion protein were found to be homozygote inviable, with presumptive homozygote animals dying at birth and exhibiting heart defects. Of particular significance is the fact that the heart malformation is localized to the RVOFT, the same region affected in the *α1 connexin* knockout mice. Also similar to the knockout mice is the finding of outflow tract obstruction in the FZ transgenic mice. In addition, we observed an abnormal persistence of smooth muscle α-actin expression in the conotruncal myocardium of the FC transgenic heart, an abnormality also seen in the conotruncal pouches of the *α1 connexin* knockout mouse heart (Huang *et al.*, 1998). The similarities in cardiac phenotype would suggest that the loss of function mediated by the fusion protein mimics the recessive loss of α1 connexin function in the knockout mice. However, we note that the heart phenotypes in these two mouse models, though similar, are not identical. This could be due to differences in the expression pattern of the fusion protein transgene compared to that of the endogenous *α1 connexin* gene. For example, *α1 connexins* are normally expressed in all neural crest cells (Lo *et al.*, 1997), but the transgene is only expressed in subpopulations of neural crest cells. Another possibility is that the difference stems from the fact that the fusion protein causes a reduction in the level of gap junctional communication in a qualitatively different manner from that of the null mutant mice.

Neural Crest Perturbation and Right Ventricular Heart Malformation

The pattern of transgene expression would suggest that crest perturbation is pivotal to the heart defects in the FZ transgenic mice. This possibility is supported by the fact that gap junctional communication in cardiac crest cells is significantly reduced in these transgenic mice. A role for crest perturbation has also been proposed for the heart defects in the *α1 connexin* knockout mice. This was based on the fact that restoration of α1 connexin function to cardiac crest cells via the CMV43 transgene can ameliorate the knockout heart phenotype (Ewart *et al.*, 1997). Together these observations suggest that a reduction of gap junctional communication in cardiac crest cells, whether from the dominant negative inhibition of gap junctional communication or the recessive loss of connexin function, is detrimental to RVOFT development.

It should be noted that RVOFT heart malformation arises not only from the loss of α1 connexin function, as it is also exhibited by the CMV43 transgenic mice in which coupling in crest cells is elevated due to α1 connexin overexpression. Thus, it is likely that critical to outflow tract morphogenesis is not merely whether α1 connexin function is present or absent, but rather the quantitative modulation of gap junctional communication in cardiac crest cells. Our recent finding that the heterozygous *α1 connexin* knockout mouse also exhibits RVOFT defect, but of reduced severity, is consistent with this notion (Huang *et al.*, 1998). As might be expected, dye-coupling analysis showed that gap junctional communication in cardiac crest cells of the heterozygous knockout mouse is intermediate to that of the wild-type and homozygous knockout mice.

α1 Connexin Function and Cell–Cell Signaling

It is interesting to consider what might be the basis for the quantitative requirement for *α1 connexin* function in cardiac crest cells in light of the permeability properties of gap junctions. We are currently investigating whether gap junctional communication may mediate cell signaling involved in the modulation of cardiac crest activity. With regard to this possibility, it will be interesting to examine the involvement of gap junctional communication in mediating cell signaling events linked to cytokines found along the cardiac crest migratory pathway (Lo and Wessels, 1998). Gap junctional communication may help to propagate extracellular signals received at the migration front to other cells in a migratory stream and in this manner facilitate the coordinate regulation of migration, proliferation, and/or differentiation of crest cells in response to environmental cues. The up- or downregulation of gap junctional communication may be expected to alter such cell–cell signaling and thus lead to the perturbation of crest activity. Our preliminary studies indicate that changes in the level of gap junctional communication can alter the migratory response of crest cells to cytokine stimulation (Huang and Lo, unpublished observations). Also of interest to note is our finding of abnormal smooth muscle α-actin expression in the conotruncal myocardium in the FC transgenic and *α1 connexin* knockout mice. This would suggest that gap junction perturbation in cardiac crest cells also may have effects on the differentiation of the myocardium. Whether this is mediated via a paracrine pathway or through coupling between crest cells and the myocardium remains to be determined.

Perturbation of Connexin Function and the Crest Ablation Heart Phenotype

Although our studies strongly suggest the involvement of crest perturbation in the RVOFT defects arising with the gain or loss of α1 connexin function, it is significant that the heart phenotypes exhibited by the FZ, CMV43, and

connexin knockout mice differ markedly from the classic cardiac crest ablation phenotype (Kirby *et al.*, 1983, 1985; Bockman *et al.*, 1987; Nishibatake *et al.*, 1987). With cardiac crest ablation, typically outflow septation anomalies are observed, but this is never seen with the loss or gain of α1 connexin function. This would suggest that α1 connexins are not involved in the maintenance of crest cell viability. Instead, it may be the migration, proliferation, and/or differentiation of crest cells that is being modulated by α1 connexin-mediated gap junctional communication. With regard to these possibilities, it is interesting to consider the finding of cartilage nodules in the heart of FC transgenic mice. It has been shown that cranial crest cells when transplanted to ectopic sites can give rise to cartilage (LeDourain and Teillet, 1974; Kirby, 1989). Thus, inhibition of coupling in neural crest cells by the fusion protein could have resulted in the inappropriate migration of noncardiac crest cells to the heart. Alternatively, it may be that the cartilage nodules are derived from cardiac crest cells that have differentiated inappropriately. Given these observations, it is interesting to consider whether clinically there may be heart anomalies involving crest perturbation that has not been recognized as such. One example might be heart anomalies described as pulmonary stenosis with intact ventricular septum (McQuinn, 1996).

Although our studies have focused on heart malformations associated with the perturbation of gap junctional communication in cardiac crest cells, it is likely that other crest lineages may be affected. Thus, we have observed cranial ganglia defects in the FZ transgenic and *α1 connexin* knockout mice, structures containing crest contributions that are also affected in the CMV43 transgenic mice (Ewart *et al.*, 1997; Lo and Sullivan, unpublished observations). Also possibly of relevance is our finding of cranial neural tube closure defects in both the FZ transgenic and *α1 connexin* knockout mice (Sullivan and Lo, unpublished observations), a phenotype shared by the CMV43 transgenic mice (Ewart *et al.*, 1997). Studies by others suggest that neural tube closure defects could arise in conjunction with or secondarily from defects in the deployment of neural crest cells (Morriss-Kay *et al.*, 1994; also see Moase and Trasler, 1989, 1990; Nieto *et al.*, 1994; Chen *et al.*, 1995).

Dominant Negative Approach for Modulating Connexin Function

Our studies show the efficacy of a dominant negative approach for manipulating gap junctional communication in transgenic mice. It should be noted that previously a hybrid mammalian connexin protein was observed to inhibit gap junctional communication in a dominant negative manner in *Xenopus* embryos, and its expression disrupted early events in *Xenopus* development (Paul *et al.*, 1995). Our studies represent the first example in which a dominant negative approach has been successfully used in mammalian embryos.

A dominant negative approach is a powerful tool for the analysis of gene function, as it provides the means for examining the effects of functional perturbation targeted to a restricted cell lineage or tissue. This may be particularly important for the analysis of genes that are widely expressed, such as the *α1 connexin* gene (Ruangvoravat and Lo, 1992). Thus, our study here provided evidence of neural crest perturbation as the basis for the heart malformations seen with the loss of α1 connexin function. A dominant negative approach also may be advantageous as the use of a suitable promoter to target expression may obviate the neonatal knockout lethality and allow α1 connexin function to be examined postnatally. It may also be possible to inhibit the function of multiple connexin isotypes simultaneously, thereby overcoming some of the functional redundancies inherent in a multigene family. However, whether the α1 connexin/lacZ fusion protein can interact with multiple connexin isotypes is not yet known, but heteromeric connexins are known to exist (Staufer, 1995).

In future studies, the role of gap junctions in mammalian development can be further explored by targeting expression of the α1 connexin/β-galactosidase fusion protein using different promoter constructs. By targeting tissues other than the cardiac neural crest, it may be possible to achieve long-term postnatal viability and thus allow the examination of α1 connexin function in other cells and tissues. The presence of the β-galactosidase reporter will allow the tracking of fusion protein expression, either on a cellular level by the histochemical localization of β-galactosidase activity or on a quantitative level by the biochemical determination of β-galactosidase enzymatic activity.

ACKNOWLEDGMENTS

We thank A. Phelps for excellent technical assistance and R. Gourdie and M. Kirby for helpful discussions and critical comments on the manuscript. This work was supported by NIH Grants HD36457 (C.W.L) and HL52813 (A.W.), NSF Grant IBN-06886 (C.W.L), and a grant from the American Heart Association, New Jersey Affiliate (K.K.L).

REFERENCES

Bancroft, M., and Bellairs, R. (1976). The neural crest cells of the trunk region of the chick embryo studied by SEM and TEM. *ZOON* **4,** 73–85.

Bennett, M. V. L., Barrio, L. C., *et al.* (1991). Gap junctions: New tools, new answers, new questions. *Neuron* **6,** 305–320.

Beyer, E. C., Paul, D. L., *et al.* (1990). Connexin family of gap junction proteins. *J. Membr. Biol.* **116,** 187–194.

Bockman, D. E., Redmon, M. R., Waldo, K., Davis, H., and Kirby, M. L. (1987). Effect of neural crest ablation on development of the heart and arch arteries in the chick. *Am. J. Anat.* **180,** 332–341.

Bruzzone, R., White, T. W., and Paul, D. L. (1996). Connections with connexins: The molecular basis of direct intercellular signaling. *Eur. J. Biochem.* **238,** 1–27.

Chen, Z. F., and Behringer, R. R. (1995). *twist* is required in head mesenchyme for cranial neural tube morphogenesis. *Genes Dev.* **9,** 686–699.

Davis, E. M., and Trinkaus, J. P. (1981). Significance of cell-to-cell contacts for the directional movement of neural crest cells within a hydrated collagen lattice. *J. Embryol. Exp. Morphol.* **63,** 29–51.

Echelard, Y., Vassileva, G., *et al.* (1994). Cis-acting regulatory sequences governing *Wnt-1* expression in the developing mouse CNS. *Development* **120,** 2213–2224.

Ewart, J. L., Cohen, M. F., Meyer, R. A., Huang, G. Y., Wessels, A., Gourdie, R. G., Chin, A. J., Park, S. M. J., Lazatin, B. L., Villabon, S., and Lo, C. W. (1997). Heart and neural tube defects in transgenic mice overexpressing the Cx43 gap junction gene. *Development* **124,** 1281–1292.

Gourdie, R. G., Green, C. R., Severs, N. J., and Thompson, R. P. (1992). Immunolabelling patterns of gap junction connexins in the developing and mature rat heart. *Anat. Embryol.* **185,** 363–378.

Gui, Y. H., Linask, K. K., Khowsathit, P., and Huhta, J. C. (1996). Doppler echocardiography of normal and abnormal embryonic mouse heart. *Ped. Res.* **40,** 633–642.

Hanaoka, K., Hauasaka, M., Uetsuki, T., Fujisawa-Sehara, A., and Nabeshima, Y.-I. (1991). A stable cellular marker for the analysis of mouse chimeras: The bacterial chloramphenicol acetyltransferance gene driven by the human elongation factor 1 a promoter. *Differentiation* **48,** 183–189.

Huang, G. Y., Wessels, A., Smith, B. R., Linask, K. K., Ewart, J. L., and Lo, C. W. (1998). Alteration in connexin 43 gap junction gene dosage impairs conotruncal heart development. *Dev. Biol.* **198,** 32–44.

Huhta, J. C., Berges, A., Yoon, G. Y., Murdison, K. A., and Wood, D. C. (1990). Noninvasive ultrasonic assessment of chick embryo cardiac function. *Ann. N.Y. Acad. Sci.* **588,** 383–386.

Kim, D. W., Uetsuki, T., Kaziro, Y., Yamaguchi, N., and Sugano. S. (1990). Use of the human elongation factor 1α promoter as a versatile and efficient expression system. *Gene* **91,** 217–223.

Kirby, M. L. (1989). Plasticity and determination of mesencephalic and trunk neural crest transplanted into the region of the cardiac neural crest. *Dev. Biol.* **134,** 402–412.

Kirby, M. L. (1993). Cellular and molecular contributions of the cardiac neural crest to cardiovascular development. *Trends Cardiovasc. Med.* **3,** 18–23.

Kirby, M. L., Gale, T. F., and Stewart, D. E. (1983). Neural crest cells contribute to aorticopulmonary septation. *Science* **220,** 1059–1061.

Kirby, M. L., Turnage, K. L., and Hays, B. M. (1985). Characterization of conotruncal malformations following ablation of "cardiac" neural crest. *Anat. Rec.* **191,** 215–227.

Kirby, M. L., and Waldo, K. L. (1995). Neural crest and cardiovascular patterning. *Circ. Res.* **77,** 211–215.

Kumar, N. M., and Gilula, N. B. (1992). Molecular biology and genetics of gap junction channels. *Semin. Cell Biol.* **3,** 3–16.

LeDourain, N., and Teillet, M. A. (1974). Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neuroectodermal mesenchymal derivatives, using a biological cell marking technique. *Dev. Biol.* **41,** 162–184.

Lo, C. W. (1996). The role of gap junction membranes in development. *J. Bioenerg. Biomembr.* **28,** 379–385.

Lo, C. W., Cohen, M. F., Hunag, G. Y., Lazatin, B. O., Patel, N., Sullivan, R., Pauken, C., and Park, S. M. J. (1997). Cx43 gap junction gene expression and gap junctional communication in mouse neural crest cells. *Dev. Genet.* **20,** 119–132.

Lo, C. W., and Wessels, A. (1998). Cx43 gap junctions in cardiac development. *Trends Cardiovasc. Med.* **8,** 266–271.

Loewenstein, W. R., and Rose, B. (1992). The cell-to-cell channel in the control of growth. *Semin. Cell Biol.* **3,** 59–79.

McQuinn, T. (1996). Molecular biological approaches to genetic cardiac diseases. *Progr. Pediatr. Cardiol.* **6,** 1–18.

Moase, C. E., and Trasler, D. G. (1989). Spinal ganglia reduction in the splotch-delayed neural tube defect. *Teratology* **40,** 67–75.

Moase, C. E., and Trasler, D. G. (1990). Delayed neural crest cell emigration from *Sp* and Sp^d mouse neural tube explants. *Teratology* **36,** 335–343.

Moorman, A. F. M., and Lamers, W. H. (1995). Topography of cardiac gene expression in the embryo: From pattern to function. *In* "Developmental Mechanisms of Heart Disease," pp. 261–267. Futura, Armonk, NY.

Morriss-Kay, G., Wood, H., *et al.* (1994). Normal neurulation in mammals. *Ciba Found. Symp.* **181,** 51069.

Nieto, M. A., Sargent, M. G., Wilkinson, D. G., and Cooke, J. (1994). Control of cell behavior during vertebrate development by *Slug,* a zinc finger gene. *Science* **264,** 835–839.

Nishibatake, M., Kirby, M. L., and van Mierop, L. H. S. (1987). Pathogenesis of persistent truncus arteriosis and dextroposed aorta in the chick embryo after neural crest ablation. *Circulation* **75,** 255–264.

Paul, D. L., Yu, K., Bruzzone, R., Gimlich, R. L., and Goodneough, D. A. (1995). Expression of a dominant negative inhibitor of intercellular communication in the early *Xenpus* embryo causes delamination and extrusion of cells. *Development* **121,** 371–386.

Raible, D. W., and Eisen, J. S. (1996). Regulative interactions in zebrafish neural crest. *Development* **122,** 501–507.

Reaume, A. G., deSousa, P. A., *et al.* (1995). Cardiac malformation in neonatal mice lacking connexin 43. *Science* **267,** 1831–1834.

Rovasio, R. A., Delouvee, A., Yamada, K. M., Timpl, R., and Theiry, J. P. (1983). Neural crest cell migration: Requirements for exogenous fibronectin and high cell density. *J. Cell Biol.* **96,** 462–473.

Ruangvoravat, C. P., and Lo, C. W. (1992). Connexin 43 expression in the mouse embryo: Localization of transcripts within developmentally significant domains. *Dev. Dynam.* **194,** 261–281.

Staufer, K. (1995). The gap junction proteins β1-connexin (connexin-32) and β2-connexin (connexin-26) can form heteromeric hemichannels. *J. Biol. Chem.* **270,** 6768–6772.

Sullivan, R., and Lo, C. W. (1995). Expression of a Cx43/β-galactosidase fusion protein inhibits gap junctional communication in NIH3T3 cells. *J. Cell Biol.* **130,** 419–429.

van Kempen, M. J., Fromaget, C., Gros, D., Moorman, A. F., and Lamers, W. H. (1991). Spatial distribution of connexin 43, the major cardiac gap junction protein, in the developing and adult rat heart. *Circ. Res.* **68,** 1638–1651.

Warner, A. (1992). Gap junctions in development—A perspective. *Semin. Cell Biol.* **3,** 81–91.

Received for publication July 14, 1998
Revised September 10, 1998
Accepted September 10, 1998