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Heart and neural tube defects in transgenic mice overexpressing the Cx43 gap junction gene

J. L. Ewart[1], M. F. Cohen[1], R. A. Meyer[1,2], G. Y. Huang[1], A. Wessels[3], R. G. Gourdie[3], A. J. Chin[4], S. M. J. Park[1], B. O. Lazatin[1], S. Villabon[1] and C. W. Lo[1,*]

[1]Department of Biology, Goddard Laboratory, University of Pennsylvania, Philadelphia, PA 19104, USA
[2]Biology Department, Rutgers University, Camden, NJ 08102, USA
[3]Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, SC 29425, USA
[4]Department of Pediatric Cardiology, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA

*Author for correspondence (e-mail: clo@mail.sas.upenn.edu)

SUMMARY

Transgenic mice were generated containing a cytomegaloviral promoter driven construct (CMV43) expressing the gap junction polylpeptide connexin 43. RNA and protein analysis confirmed that the transgene was being expressed. In situ hybridization analysis of embryo sections revealed that transgene expression was targeted to the dorsal neural tube and in subpopulations of neural crest cells. This expression pattern was identical to that seen in transgenic mice harboring other constructs driven by the cytomegaloviral promoter (Kothary, R., Barton, S. C., Franz, T., Norris, M. L., Hettle, S. and Surani, M. A. H. (1991) *Mech. Develop.* 35, 25-31; Koedood, M., Fitchel, A., Meier, P. and Mitchell, P. (1995) *J. Virol.* 69, 2194-2207), and corresponded to a subset of the endogenous Cx43 expression domains. Significantly, dye injection studies showed that transgene expression resulted in an increase in gap junctional communication. Though viable and fertile, these transgenic mice exhibited reduced postnatal viability. Examination of embryos at various stages of development revealed developmental perturbations consisting of cranial neural tube defects (NTD) and heart malformations. Interestingly, breeding of the CMV43 transgene into the Cx43 knockout mice extended postnatal viability of mice homozygote for the Cx43 knockout allele, indicating that the CMV43 trangsene may partially complement the Cx43 deletion. Both the Cx43 knockout and the CMV43 transgenic mice exhibit heart defects associated with malformations in the conotruncus, a region of the heart in which neural crest derivatives are known to have important roles during development. Together with our results indicating neural-crest-specific expression of the transgene in our CMV-based constructs, these observations strongly suggest a role for Cx43-mediated gap junctional communication in neural crest development. Furthermore, these observations indicate that the precise level of Cx43 function may be of critical importance in downstream events involving these migratory cell populations. As such, the CMV43 mouse may represent a powerful new model system for examining the role of extracardiac cell populations in cardiac morphogenesis and other developmental processes.

Key words: gap junction, connexin, Cx43, CMV, transgenic, neural tube defect, heart defect, outflow tract, neural crest, mouse embryo

INTRODUCTION

Gap junctions are specialized cell junctions that allow the diffusion of ions and low molecular weight metabolites between cells. They occur in most cell types and are encoded by a multi-gene family known as the connexins (Bennett et al., 1991). Each connexin gene is expressed with a unique distribution, and encodes a polypeptide that oligomerizes to form hydrophilic membrane channels with different conductance and gating properties. As gap junctions are expressed in abundance throughout embryogenesis, they may play a role in development, perhaps by mediating the transmission of cell signaling molecules involved in patterning and differentiation (Caveney, 1985; Guthrie and Gilula, 1989; Warner, 1992; Lo, 1996).

The connexin 43 gap junction gene is expressed in a developmentally regulated manner in many regions of the embryo (Ruangvoravat and Lo, 1992; Yancey et al., 1992). Connexin 43 (Cx43) gap junctions appear to play a vital role in heart development, as the targeted disruption of the *Gja1* locus encoding the Cx43 gap junction gene (the Cx43 knockout mouse) resulted in neonatal lethality due to conotruncal heart defects (Reaume et al., 1995). A role for Cx43 in heart development is also indicated by the finding of Cx43 mutations in patients exhibiting heart malformations resulting from visceroatrial heterotaxia (VAH), a disease characterized by laterality defects. Cx43 mutations in these patients do not affect gap junction channel formation, but rather regulation in the gating of the Cx43 gap junctional channels (Britz-Cunningham et al., 1995). This would suggest that the precise level of Cx43-mediated gap junctional communication (GJC) may be critically important to heart morphogenesis.

To further explore the role of gap junctions in mammalian development, we used a gain-of-function approach to examine the developmental impact of elevating GJC via the overexpression of Cx43. This entailed making transgenic mice containing CMV43, a Cx43 expression vector driven by the cytomegaloviral promoter (MacGregor and Caskey, 1989). Unlike the ubiquitous expression seen for CMVconstructs transfected into tissue culture cells (Foecking and Hofstetter, 1986; MacGregor and Caskey, 1989), in transgenic mice, the CMV promoter provides for expression predominantly in the dorsal neural tube and subpopulations of neural crest cells (Koedood et al., 1995; Kothary et al., 1991). This tissue-restricted pattern of expression is consistent with that of viral activity associated with congenital human CMV infections (Koedood et al., 1995). As the endogenous *Cx43* gene is also expressed in these same regions (Ruangvoravat and Lo, 1992), the CMV43 transgene should result in Cx43 overexpression. CMV43 transgenic mice were found to be viable but with reduced postnatal viability. Examination of embryos at various stages of development revealed cranial neural tube defects and also heart malformations involving the conotruncal region of the heart. When the CMV43 transgene was bred into the Cx43 knockout mouse, it was able to achieve rescue of the knockout lethality. This demonstrates that Cx43 expression mediated by the CMV43 construct can complement the Cx43 deletion. Given the neural-crest-restricted expression pattern of the transgene and its ability to rescue the knockout lethality, we hypothesize that perturbation of neural crest cells underlie the heart malformations in the Cx43 knockout and CMV43 transgenic mice. These results further suggest that the precise level of Cx43-mediated GJC may be critically important in heart morphogenesis.

MATERIALS AND METHODS

CMV43 expression vector

The CMV43 expression vector (Fig. 1) was generated by replacing the *lacZ* insert of the CMVβ plasmid (MacGregor and Caskey, 1989) with the mouse Cx43 coding sequence (Sullivan et al., 1993). To facilitate the analysis of transgene expression, a 650 bp fragment derived from the 3′ untranslated region of the dihydrofolate reductase gene (*dhfr*) from *Toxoplasma gondii* (kindly provided by D. Roos; Roos, 1993) was inserted as a sequence tag immediately downstream of the stop codon. For DNA injections, this plasmid was digested with *Pst*I to release the CMV promoter and the downstream Cx43/dhfr/SV40polyA DNA sequence. For RNAase protection analysis, probes were generated to the 3′ sequence tag, spanning the region indicated as an open box in Fig. 1.

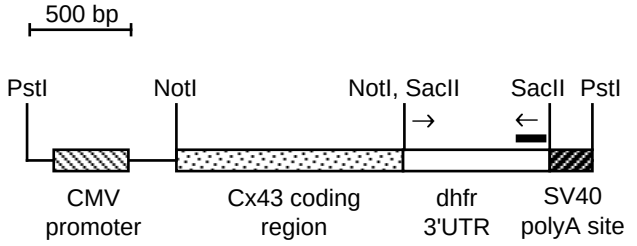

Fig. 1. CMV43 vector. The dhfr 3′UTR refers to a sequence tag derived from the 3′ untranslated region (UTR) of the dihydrofolate reductase gene (*dhfr*) from *Toxoplasma gondii* (Roos, 1993). Arrows indicate location of PCR primers used for genotyping. Black bar indicates protected fragment in RNAase protection analysis.

Production of transgenic mice

Transgenic mice were generated using eggs collected from superovulated SWR/J female mice mated with SJL/J males (Jackson Laboratory, Bar Harbor, ME). Some transgenic founders were generated by the NICHD Transgenic Mouse Development Facility (DNX, Inc., Princeton, NJ) using eggs obtained from the breeding of B6/SJL F_1 hybrid mice. To identify transgenic founder animals, blood samples were obtained from each mouse and screened by PCR amplification using primers spanning the 3′ sequence tag (Ts28: 5′-GTCAATCA-GAACGAAAGCGAG-3′; Ts47: 5′-TGTCACTGTAGCCTGCCA-GAACACTT-3′; denoted as arrows in Fig. 1). PCR amplifications were carried out in a Coy Tempcycler using 35 cycles of: 75 seconds at 94°C, 75 seconds at 62°C, and 75 seconds at 72°C. PCR-positive animals were further examined by Southern blotting analysis.

Embryo collection, histology and in situ hybridization analysis

To obtain embryos, plugged females were killed by cervical dislocation (plug date=E0.5) and the yolk sac was collected for PCR analysis (according to McMahon and Bradley, 1990). For in situ hybridization analysis, embryos were fixed in modified Carnoy's fixative and embedded in paraffin (MacDonald and Tuan, 1989). In situ hybridization was carried out as previously described (Ruangvoravat and Lo, 1992) using sense or antisense ^{35}S-radiolabeled riboprobes to the 3′ sequence tag. For standard histology and immunohistochemistry, fetal, neonatal, and adult hearts were fixed in Amsterdam's fixative (7:7:1:5 methanol:acetone:acetic acid:H_2O; Wessels et al., 1990).

RNA isolation and RNAase protection analysis

RNA was collected from whole embryos and from various adult tissues using RNAzol^{TM} B (Biotecx Laboratories, Inc., Houston, TX) according to the manufacturer's protocol. The RNA samples were analyzed for expression of the CMV43 transgene by RNAase protection analysis using a ^{32}P-radiolabeled riboprobe to the 3′ sequence tag (Fig. 1). This probe gave a protected doublet at 121-122 bp. In addition, in each reaction, a ^{32}P-radiolabeled 18S rRNA riboprobe (generated from plasmid pT7, Ambion Inc., Austin, TX; Hills and Dixon, 1991) was included as an internal control to normalize for RNA loading. The latter probe gave a predominant protected band at 82 bp. The 18S rRNA is expressed at the same levels in a variety of adult tissues and in the embryo, and thus is particularly appropriate for these quantitative analyses (Smith, 1995). To characterize the relative abundance of the CMV43 transgene transcript in hemizygote versus homozygote adult tissues, each RNA sample was subject to RNAase protection using three concentrations of rRNA probes (1×10^4, 5×10^4 and 1×10^5 cts/minute). The relative abundance of the protected product was subsequently quantitated using a Bioquant PhosphorImager.

Western blot analysis

To examine Cx43 protein expression, E11.5 and E12.5 embryos were harvested and homogenized in PBS containing 1 μg/ml leupeptin, 1 μg/ml pepstatin and 0.001 M PMSF. The embryo extracts were run on a 12.5% SDS/PAGE followed by electroblotting onto nitrocellulose. Then the nitrocellulose membranes were blocked at room temperature in PBS containing 5% nonfat dried milk, and 0.1% Tween 20 (PBST), then incubated with a Cx43 polyclonal antibody (18A-11; kindly provided by Dr Elliot Hertzberg). After extensive washing and further blocking in PBST, incubation was carried out with a goat anti-rabbit IgG peroxidase-conjugated antibody (Boehringer Mannheim, Indianapolis, IN). Immunoreactivity was detected using the Amersham ECL chemiluminescent detection kit according to the manufacturer's specification (Amersham Corp., Arlington Heights, IL).

Whole-mount immunostaining

To visualize developing neurons, whole-mount immunostaining was carried out as described (Hogan et al., 1994), using the anti-neurofilament antibody 2H3 (Dodd et al., 1988). Briefly, embryos were stained overnight with 2H3 monoclonal antibody (1:100), then washed in PBSMT and incubated overnight with peroxidase-conjugated goat anti-mouse IgG (1:200; Sigma, Inc., St. Louis, MO). Antibody binding was detected by further incubation of embryos in 0.6 mg/ml diaminobenzidine/0.6 mg/ml $NiCl_2$ for 30 minutes followed with 0.03% H_2O_2 for 3-5 minutes. Stained embryos were dehydrated, cleared in 1:2 benzyl alcohol:benzyl benzoate, and observed and photographed under a stereomicroscope.

Dye coupling studies

To examine gap junctional communication, embryos were harvested at E8.5, removed from their extraembryonic membranes and cultured in Dulbecco's modified Eagle's medium with 10% calf serum for up to 4 hours at 37˚C in a 5% CO_2 incubator. For each embryo, immediately before performing dye injections, the embryo was transected to remove the head, which was then further bisected longitudinally. Each half was immobilized in a drop of 0.8% low melting point agarose, with the neuroepithelium facing upwards. These head fragments were then maintained in L-15 medium with 10% serum on a heated stage and immediately used for dye injection studies. Impalements and injection of carboxyfluorescein was carried out using 2 nA hyperpolarizing current pulses of 0.5 seconds duration (once/second) held for 2 minutes.

RESULTS

A number of transgenic mouse lines were generated containing the CMV43 construct (Fig. 1). Analysis by Southern blotting showed that these mice carried varying numbers of copies of the CMV43 insert (data not shown). Six transgenic founder males were bred and each was found to provide for germ-line transmission. Extensive breeding of one transgenic line (line B) revealed the viability of both hemizygote and homozygote offspring (see below).

Transgene expression in these CMV43 transgenic lines was examined by RNAase protection analysis using the 3′ sequence tag in the CMV43 construct (see black box in Fig. 1). This analysis showed transgene expression in embryos and a variety of adult tissues (Fig. 2A). In one transgenic line (6926), expression was detected in the embryo but not in adult tissues (Fig. 2A). Analysis of transgenic line B showed an increase in transgene expression in homozygote versus hemizygote animals (Fig. 2B). To quantitate expression levels in hemizygote versus homozygote B animals, the hybridization intensity in the transgene-derived band was quantitated using a PhosphorImager, then normalized to that of the 18S rRNA band. With titration over a tenfold concentration range (10^4 cts/minute, 5×10^4 cts/minute, 10^5 cts/minute in lanes 1, 2, 3, respectively, in Fig. 2), transgene transcripts were found to be elevated threefold to ninefold higher in the tissues of homozygote animals (quantitation of lanes 1, 2 and 3 in Fig. 2B: testes - 3.4, 3.3 and 3.3; kidney - 5.8, 3.9 and 4.6; heart - 4.6, 9.3 and 8.1). However, the highest level of transgene expression was detected in line 6929, which also contained the highest copy number of the CMV43 transgene.

The developmental regulation of transgene expression was further examined by in situ hybridization. In E8.5 embryos, high levels of transgene transcript were detected in the dorsal neural tube and presumptive neural crest cells adjacent to the neural tube (Fig. 3A-D). At later stages, transgene expression was detected in tissues with neural crest contribution, including the outflow tract of the heart (Fig. 3E,F) and cells of the peripheral nervous system, including the cranial and spinal ganglia and nerves (Fig. 3G,H,M,N). The trigeminal ganglion and its associated nerves showed particularly abundant expression (Fig. 3M,N), with dense punctate signals distributed along the maxillary nerve (arrowheads in Fig. 3N). In both the cranial and spinal ganglia, the most intense labeling occurred in regions where nerves enter and leave the ganglia (arrows in Fig. 3G,H). We also observed CMV43 transgene expression in a number of other embryonic tissues, including the inner ear (Fig. 3K,L), the nasal epithelium (Fig. 3I,J) and the pituitary. This expression pattern is entirely consistent with those observed in transgenic mice containing a lacZ construct driven by the CMV promoter (Koedood et al., 1995; Kothary et al., 1991).

As the CMV43 transgene encoded a Cx43 protein identical to that of the wild-type protein, it was not possible to analyze for protein expression in the presence of the endogenous gene. Instead, the CMV43 transgene (from the B line) was bred into the Cx43 knockout mouse line, and the resulting compound heterozygous animals (heterozygous for the Cx43 knockout allele and the CMV43 transgene insert) were cross bred to obtain CMV43 transgenic embryos that did not contain the endogenous Cx43 gene. Western blot analysis of embryo extracts obtained from such matings indeed confirmed expression of the Cx43 protein in CMV43 transgenic embryos homozygous for the Cx43 knockout allele (Fig. 4).

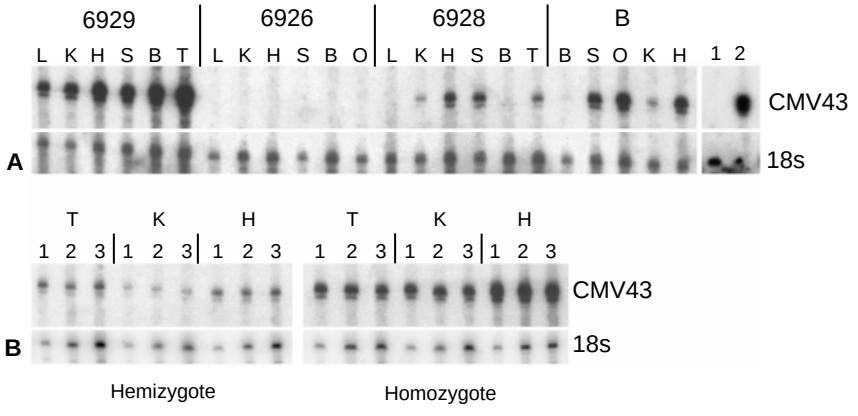

Fig. 2. RNAase protection analysis of transgene expression. (A) Analysis of adult tissues from four transgenic lines (6929, 6926, 6928 and B), and a transgenic (lane 2) and non-transgenic embryo (lane 1) from line 6926 (E11.5). Although line 6926 showed no transgene expression in adult tissues, expression was observed in embryos (lane 2). (B) Transgene expression levels increased in the tissues of hemizygote versus homozygote animals of transgenic line B. The 18S rRNA band (Smith, 1995), which provided an internal standard for normalizing RNA concentration, was varied over a tenfold range (lane 1, 10^4 cts/minute; lane 2, 5×10^4 cts/minute; lane 3, 10^5 cts/minute). L, lung; K, kidney; H, heart; S, stomach; B, brain; T, testes; O, ovary.

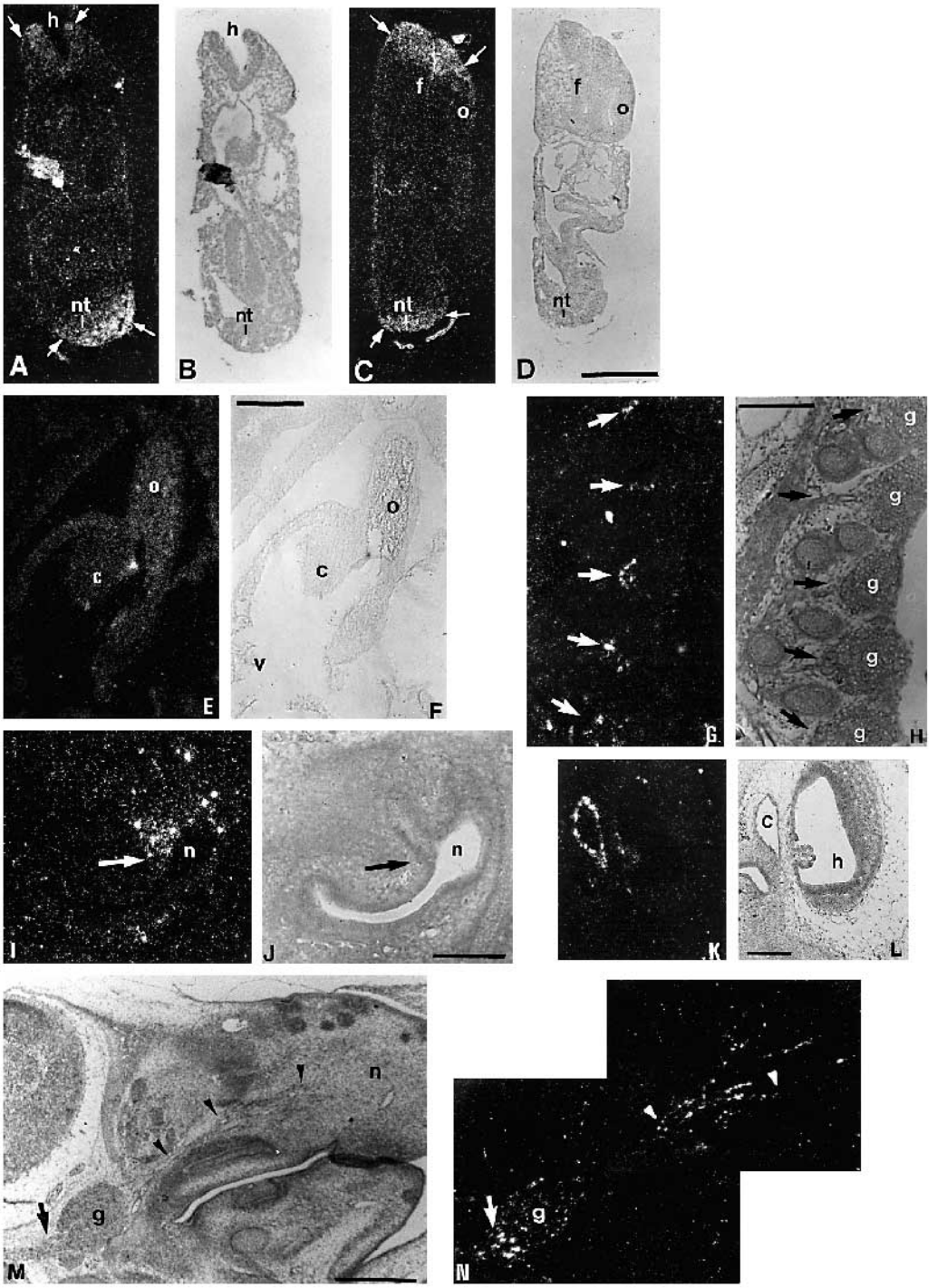

Fig. 3. In situ hybridization revealed transgene expression in the neural tube and neural crest cells. (A-D) Dark-field (A,C) and phase-contrast (B,D) images of two frontal sections of a normal E8.5 CMV43 transgenic embryo. Hybridization analysis revealed transgene expression in the neural tube (nt) and neural crest cells (arrows). Note the localization of hybridization signal dorsally over the hindbrain (h) and forebrain (f). o, optic vesicle. (E,F) Sagittal section of E11.5 embryo showing transgene expression in the outflow tract (o) of the heart. c, conus; v, ventricle. (G,H) Sagittal sections showing transgene expression at the sites where nerve tracts (arrows) leave the spinal ganglia (g) of a E14.5 embryo. (I-L) Sagittal section showing expression in the nasal epithelium (n) (see arrow in I and J) and cochlear duct (c) of a E14.5 embryo. h, hindbrain. (M,N) Sagittal section through the head of a E14.5 embryo showing transgene expression in the trigeminal ganglion (g), particularly where cranial nerve V enters from the hindbrain (arrow). Discrete spots of hybridization signal also can be seen along the maxillary nerve (arrowheads) entering into the nasal area (n). (A,C,E,G,I and K) Dark-field and (B,D,F,H,J and N) phase-contrast images. Bars: A-L, 200 μm; M,N, 500 μm.

Increased gap junctional communication in CMV43 embryos

To determine whether CMV43 transgene expression affected the level of GJC, iontophoretic dye injections were carried out in transgenic and nontransgenic embryos obtained from hemizygote CMV43 (line B) × CD1 matings. The genotypes of these embryos were determined after the completion of dye injections. For this study, microelectrode impalements were carried out into the dorsal hindbrain neural fold of E8.5 embryos, a region with high levels of transgene expression. This embryonic tissue also corresponds to the region from which 'cardiac' neural crest cells originate. The extent of dye spread was monitored by counting the number of dye-filled cells at the end of the injection period (2 minutes). This analysis revealed a significantly higher level of dye coupling in the CMV43 transgenic embryos than in their nontransgenic littermates (Fig. 5; Table 1). Statistical analysis showed that this difference was highly significant (Table 1). These findings demonstrate that the CMV43 transgene increased GJC in vivo.

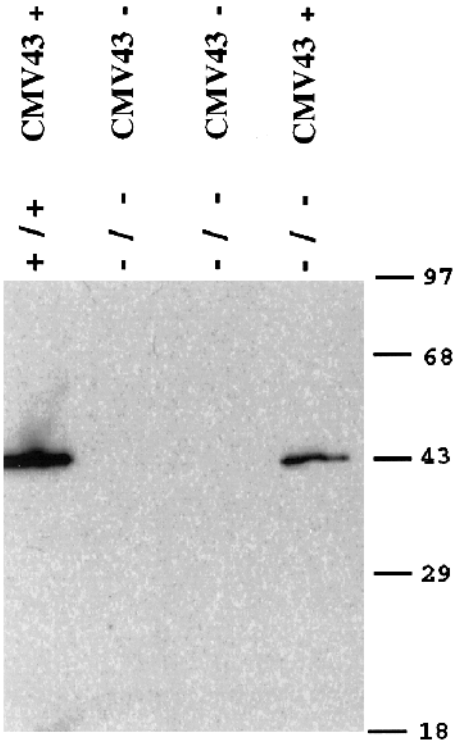

Fig. 4. Western blot analysis using a Cx43 antibody revealed Cx43 protein expression in a homozygous Cx43 knockout mouse embryo carrying the CMV43 transgene (–/–, CMV43 +). In contrast, no Cx43 protein was detected in homozygous knockout embryos that do not carry the CMV43 transgene (–/–, CMV43 –). Each lane contains 20 μg of extracts from a single E11.5 embryo (genotype indicated above each lane).

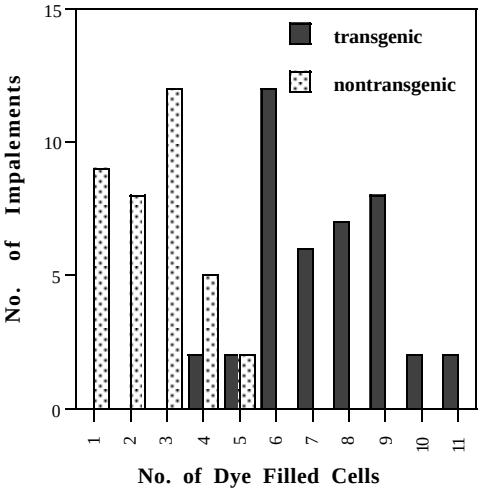

Fig. 5. Increases in gap junctional communication in the neural tube of CMV43 embryos. Impalements into the hindbrain neural fold of E8.5 transgenic embryos resulted in a greater number of dye-filled cells as compared to parallel injections of nontransgenic littermates (see Methods for dye injection technique). Statistical analysis using the Student's *t*-test show the difference is highly significant (see Table 1).

Decreased viability of CMV43 mice

CMV43 transgenic mice were generally hemizygote viable and, in the case of transgenic line B, they were also homozygote viable. However, the viability of homozygote B animals was greatly reduced. This was indicated by the finding that the proportion of transgenic animals arising from B/+ × B/+ crosses decreased to 54% at weaning, while over 80% were detected in utero (Table 2). This loss of transgenic offspring was due to neonatal or early postnatal lethality, as litter sizes do not decrease significantly from 1-2 days after birth until the time of weaning (data not shown). In contrast, in B/+ × CD-1 crosses, there was a more modest loss of transgenic offspring (Table 2). Similar analysis of CMV43 transgenic line 6929 revealed that they are homozygote inviable.

The long-term postnatal viability of the CMV43 trangsenic mice (hemizygote or homozygote) was also affected, as animals showing no visible signs of illness sometimes died suddenly. This premature lethality was particularly common among pregnant and nursing females and, in the case of transgenic line line B, was more frequently encountered in homozygote animals (40% dying by 6 months). Given that the loss of viability in these mice begins neonatally or in early postnatal life, we further examined these mice for evidence of developmental perturbations. For this study, embryos were harvested at various stages of development from transgenic line B and several other CMV43 transgenic lines. Such studies revealed that the CMV43 transgenic lines harbor two types of developmental anomalies, cranial neural tube defects (NTD) and heart malformations.

Table 1. Dye coupling in CMV43 embryos*

	Nontransgenic	Transgenic
No. of embryos	15	14
No. of cells impaled	36	41
Mean no. (±s.d.) dye filled cells†	2.53±1.2	7.37±1.7

*Impalements were into the hindbrain neural folds of E8.5 embryos from nontransgenic females mated with CMV43 hemizygote males. Genotyping carried out after dye injection.

†Student's *t*-test show $P<0.0001$.

Neural tube defects in CMV43 transgenic mice

A detailed examination of five CMV43 trangenic lines revealed that they all exhibited cranial neural tube defects (NTD) (Fig. 6). This was expressed with incomplete penetrance (9-30% incidence). NTD were observed primarily among embryos at E8.5-E14.5 and appeared to arise from a perturbation or delay in neural tube closure. Analysis of transgenic line B showed that the NTD phenotype is expressed in a transgene dosage-dependent manner. Thus, a 24% incidence of NTD was observed in offspring derived from homozygote B crosses (B/B

Table 2. Loss of transgenic offspring postnatally

	% Transgenic (n)	
Stage	B/+ × CD-1	B/+ × B/+
E8.5-E11.5	61% (143)	88% (33)
E12.5-E18.5	52% (432)	82% (50)
Weaning	47% (45)	54% (94)

n, total number of offspring examined.

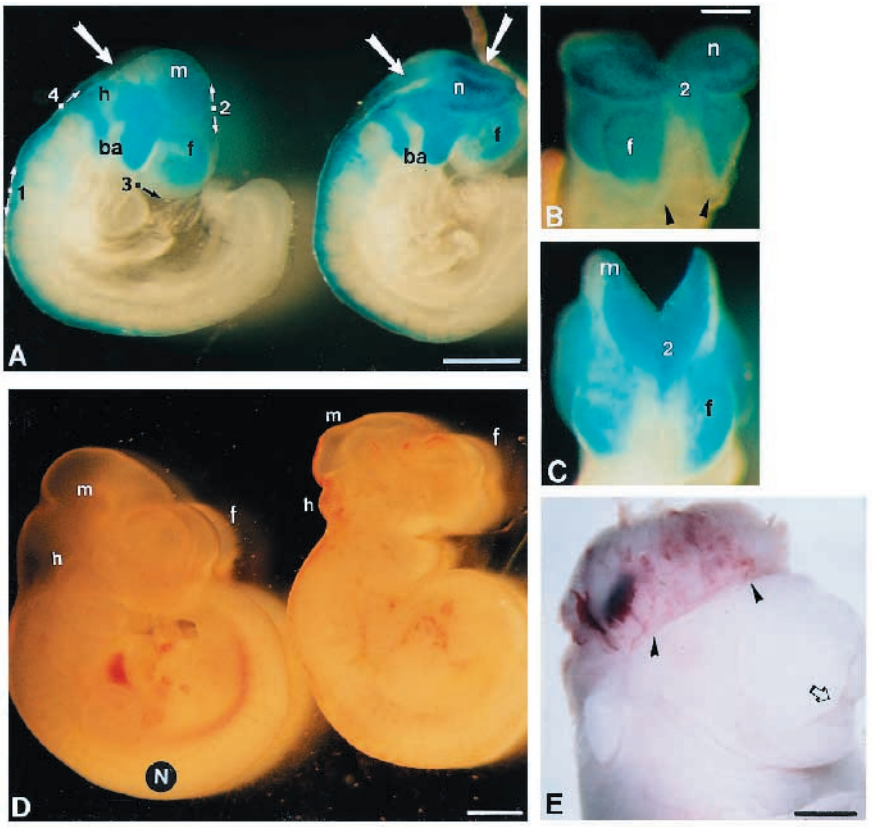

Fig. 6. CMV43 transgenic embryos exhibit neural tube defects. (A) A normal E8.5 embryo (left) and its littermate exhibiting NTD (right). The blue staining facilitates visualization of the neural folds and reflects X-gal detection of β-gal activity arising from a *lacZ* reporter gene driven by the *wnt-1* promoter/enhancer (Echelard et al., 1994). The small arrows and numbers indicated on the embryo to the left show the chronology and direction of neural tube closure. Neural tube closure in the normal embryo is complete except rostral of closure site 4 (white arrow). In contrast, the neural fold remains open along the midbrain and anterior hindbrain (two white arrows) in the embryo on the right. (B,C) Frontal views of the same two embryos shown in A. In the normal embryo (C), the neural folds over the midbrain are elevated and closely apposed. In the embryo with NTD (B), the neural fold remains wide open, exhibiting a flattened or open neural plate (n). Also note opening along the anterior neuropore spanning the region of the presumptive forebrain (arrowheads in B). (D) E10.5 normal (N) embryo and a littermate exhibiting NTD. Note the expanded brain ventricles in the normal embryo, while that of the abnormal littermate appears collapsed. (E) E17.5 embryo exhibiting severe exencephaly (arrowheads) and a cleft palate (open arrow). f, forebrain; m, midbrain; h, hindbrain. Bars: A, D, 500 μm; B, C, 200 μm; D, 2 mm.

× B/B), while hemizygote crosses (B/+ × B/+) gave a 14% incidence and B × CD1 matings, a 9% incidence.

In normal embryos, neural tube closure begins at E8.0 with elevation and apposition of the neural folds. Fusion of the neural folds then proceeds from four sites, moving unidirectionally and bidirectionally in an ordered sequence (denoted in Fig. 6A), and is completed by E8.5 (Golden and Chernoff, 1983; Jacobson and Tam, 1982; Kaufman, 1979; MacDonald et al., 1989). In the CMV43 transgenic embryos, defects in neural tube closure were detected from E8.0 and are seen most frequently between closure sites 2 and 4. This region spanning the presumptive midbrain and anterior hindbrain, is the last portion of the neural tube to undergo closure. Embryos at E8.0 with a mild NTD phenotype usually exhibited small openings along the roof of the presumptive midbrain/hindbrain, reflecting a restricted defect in neural tube closure. In the more severely affected E8.0-E8.5 embryos, there was a complete failure in neural tube closure between closure sites 2 and 4 (Fig. 6A). In such

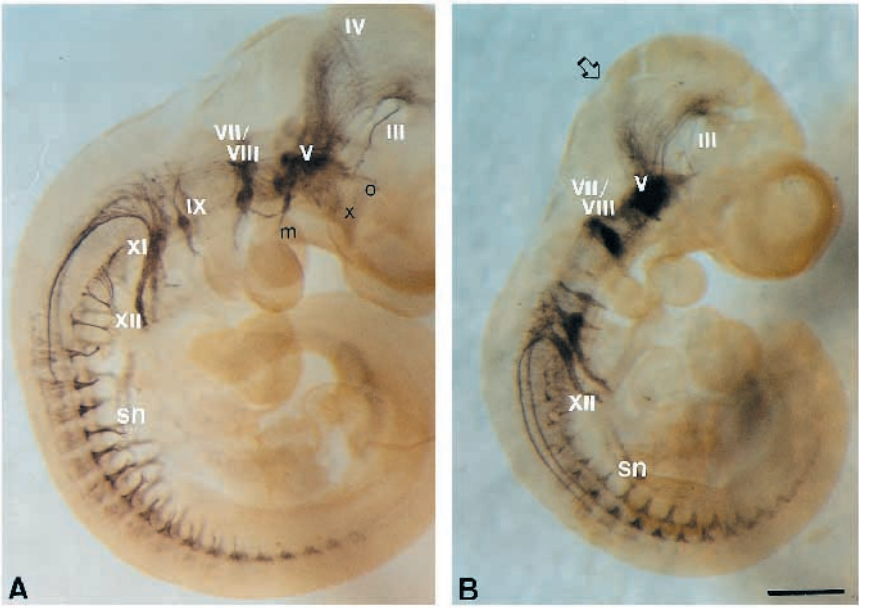

Fig. 7. Perturbations in the formation of cranial and spinal nerves in CMV43 embryos. Nontransgenic embryo (A) and its transgenic littermate with NTD (B) were immunostained with a neurofilament monoclonal antibody to visualize the cranial and spinal ganglia and nerves. In the embryo with NTD, the trochlear nerve (IV) is absent, and the oculomotor (III), trigeminal (V), facial/acoustico (VII/VIII) and hypoglossal (XII) nerves are reduced in size, as are the spinal nerves (sn). Note the absence of projections from the trigeminal ganglion which, in the normal embryo, gives rise to the ophthalmic (o), maxillary (x) and mandibular (m) nerves. In contrast, the glossopharyngeal (IX) and sympathetic (XI) nerves appear normal. Bar: 500 μm.

embryos, the neural plate encompassing the midbrain/anterior hindbrain failed to elevate and instead exhibited a flat configuration with a convex curvature and abnormal curling along the dorsolateral margin (see Fig. 6B and compare with normal littermate in Fig. 6C; blue staining reflects *lacZ* expression driven by the *wnt-1* promoter; Echelard et al., 1994). By E10.5-E11.5, the most prominent characteristic observed among embryos with NTD is the collapsed appearance of the brain ventricles (Fig. 6D; compare with normal littermate denoted as N). In some embryos, an opening persisted along the dorsal midline of the caudal midbrain/anterior hindbrain. By late gestation, nearly all fetuses appeared grossly normal, suggesting that neural tube closure eventually proceeded to completion in most embryos. However, partial or complete exencephaly was observed in a few embryos late in gestation, indicating that, in some instances, embryos may suffer from more severe closure defects (Fig. 6E).

Perturbations in the development of peripheral ganglia and nerves

In light of the cranial neural tube defects, we further examined transgenic embryos by whole-mount neurofilament antibody staining to determine if there were defects in the development of the nervous system. This analysis revealed perturbations in the formation of both cranial and spinal ganglia and nerves. Fig. 7 shows neurofilament staining of an E9.5 B transgenic embryo with NTD (indicated by collapsed brain ventricles) as compared to that of a nontransgenic littermate. The pattern of immunostaining revealed that, in the transgenic embryo, the oculomotor nerve (III) was reduced, while the trochlear nerve (IV) is absent. The trigeminal ganglion (V) is also reduced in size, with little or no evidence for the formation of the ophthalmic (o), maxillary (x) and mandibular (m) nerves. Nerve projections from the facial-acoustico (VII/VIII) ganglia are also reduced. The glossopharyngeal (IX) and accessory (XI)

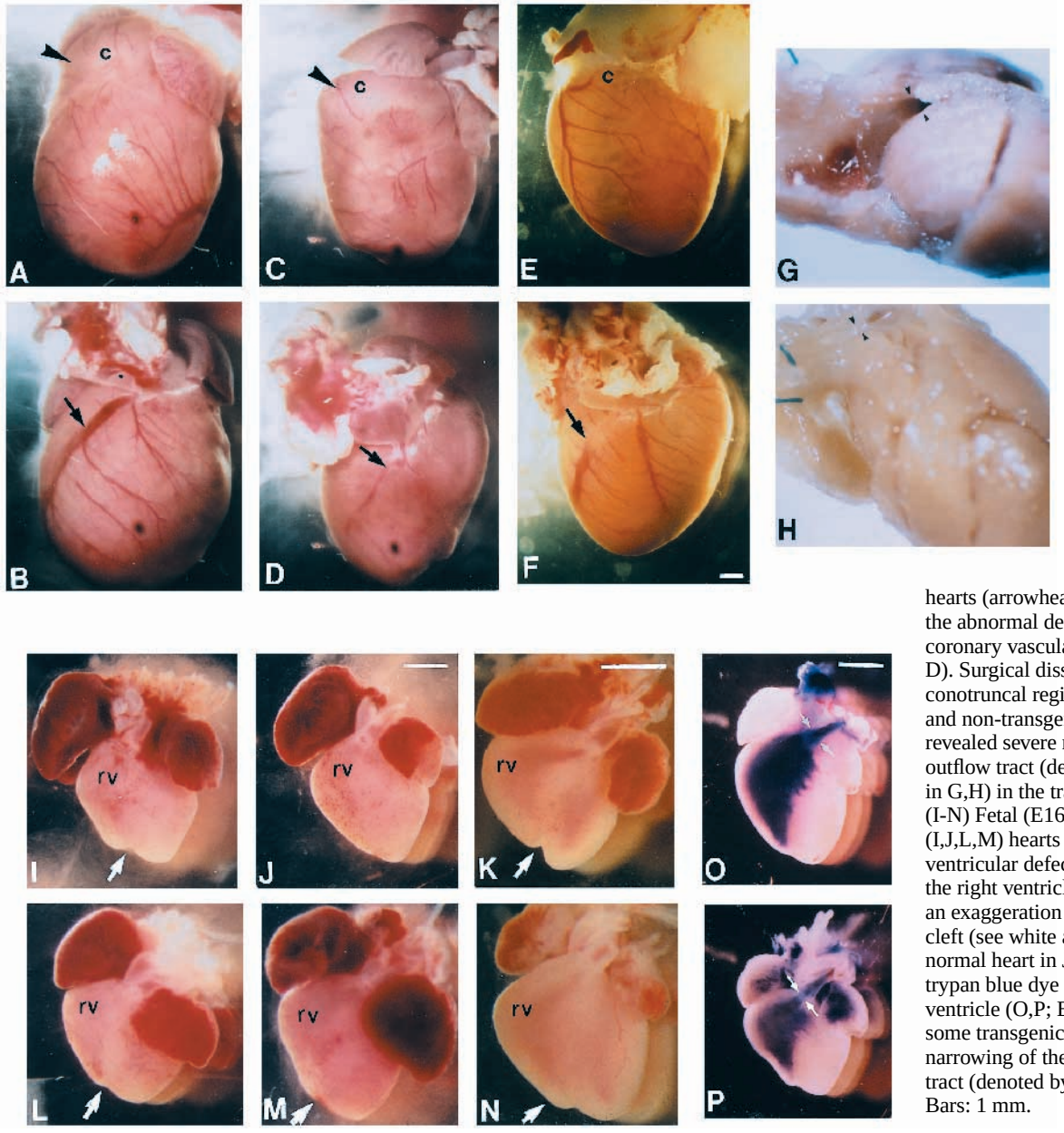

Fig. 8. Heart abnormalities in CMV43 mice. (A-H) Adult hearts from two CMV43 transgenic lines show similar right ventricular defects. Front (A,C,E) and back (B,D,F) views of adult hearts from transgenic line B (A,B), line 6929 (C,D) and age-matched control nontransgenic animal (E,F). A noticeable enlargement in the conotruncal region (c) is observed in the transgenic hearts (arrowhead in A and C). Note the abnormal deployment of surface coronary vasculature (arrows in B, D). Surgical dissection to expose the conotruncal region of a CMV43 (G) and non-transgenic (H) adult heart revealed severe narrowing of the outflow tract (denoted by arrowheads in G,H) in the transgenic heart. (I-N) Fetal (E16.5, K,N) and neonatal (I,J,L,M) hearts also exhibit right ventricular defects. Enlargement of the right ventricle is accompanied by an exaggeration of the interventricular cleft (see white arrow; compare to normal heart in J). (O,P) Injection of trypan blue dye into the right ventricle (O,P; E17.5) indicates that some transgenic hearts have a marked narrowing of the pulmonary outflow tract (denoted by arrows in P and O). Bars: 1 mm.

nerves appear normal, but the roots of the hypoglossal nerve (XII) and the spinal nerves (sn) appear to be poorly developed. Similar analysis of CMV43 transgenic embryos with no evidence of NTD also revealed similar ganglia deficiencies, suggesting that these ganglia deficiencies are not simply a byproduct of the neural tube closure defects. Also, note that the smaller size of the CMV43 embryo shown in Fig. 7B reflects a growth deficiency, not a developmental delay, as this embryo is at the same somite stage of development as the control litter mate in Fig. 7A. Such growth deficiencies are occasionally observed among the transgenic embryos and are not necessarily associated with NTD.

Heart abnormalities in CMV43 mice

As sudden death is often associated with cardiac pathology, we further examined two of the CMV43 transgenic mouse lines (B and 6929) to determine if the unexpected deaths of pregnant and nursing mothers may be due to congenital heart defects. In fact, both lines exhibited heart defects, which could be seen in fetal, neonatal and adult animals (Fig. 8).

The adult transgenic hearts showed abnormalities in the disposition of surface coronary vasculature (compare Fig. 8A-D with E,F). They also exhibited a marked dysmorphology associated with their overall shape (Fig. 8). However, most striking was the enlargement of the conotruncal region of the right ventricle (see Fig. 8A,C, and compare to control in Fig. 8E). This was characteristically seen as a hemicylindrical outpouching, which extended along the right atrioventricular groove and included the region just under the pulmonary arterial root. Surgical dissection of a homozygote B adult heart revealed a narrowing of the pulmonary outflow region (pulmonary stenosis) caused by apparent hypertrophy of the right ventricle (see Fig. 8G,H). Histological analysis further showed abnormalities associated with the apical portion of the heart, with some hearts exhibiting aneurysm-like pouchings of the ventricular lumen (Fig. 9H,I; see Discussion). As such ventricular aneurysms may arise as complications from acute myocardial infarctions (see Fig. 3.84 in Becker and Anderson, 1982), this finding together with the right ventricular and outflow tract abnormalities would suggest that heart defects may play a role in the observed adult mortality.

Examination of fetal and neonatal hearts also revealed abnormalities, some of which resembled those seen in the adult hearts. Thus in the fetal/neonatal hearts, defects were found primarily in the right ventricle. Characterisically the right ventricle exhibited varying degrees of enlargement, which resulted in an accentuation of the interventricular sulcus (as compared to non-transgenic heart in Fig. 8J). The severity of this phenotype appeared to vary with transgene dosage,

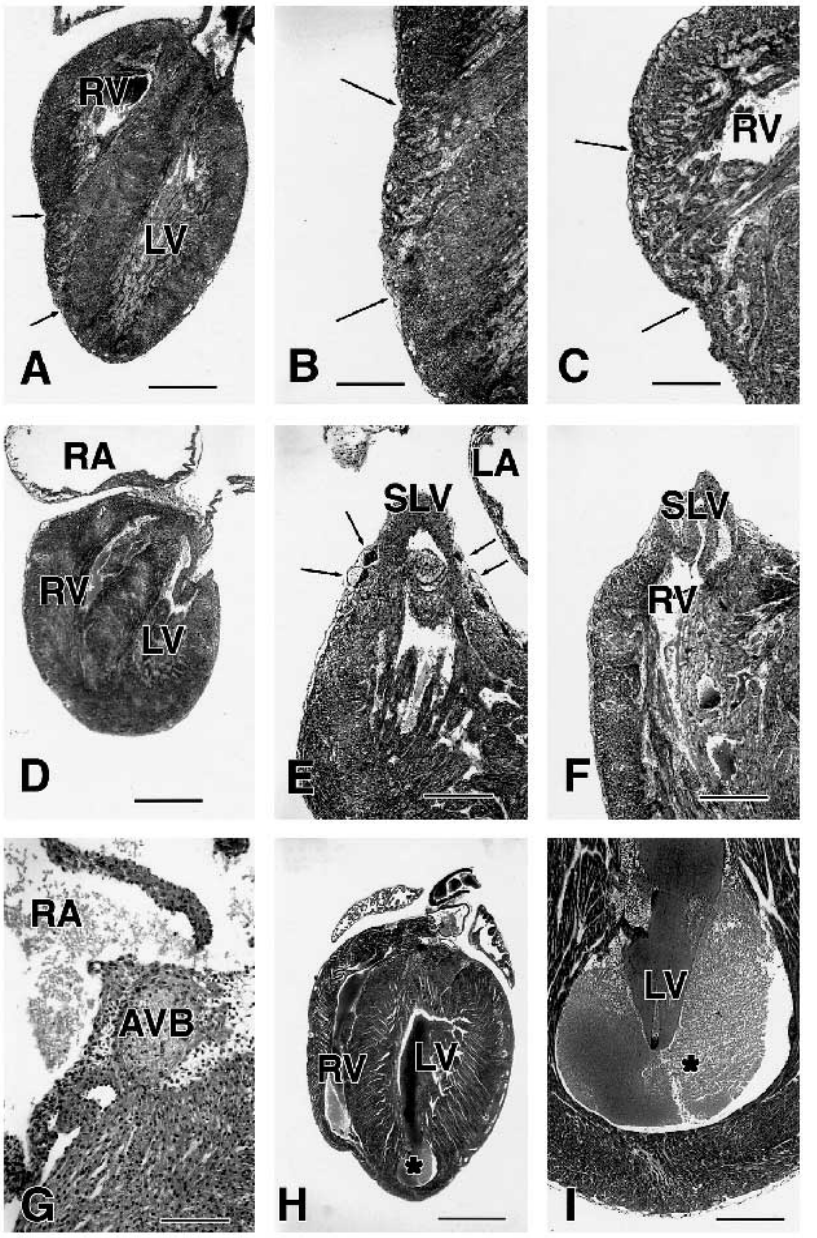

Fig. 9. Histological analysis shows various defects in the CMV43 transgenic hearts. (A-C). Neonatal transgenic hearts exhibit pronounced abnormalities in the myocardium of the right ventricle, which is restricted mostly to the apical portion of the heart. The region denoted by arrows in A is shown at higher magnification in B. A similar region of the right ventricle of another transgneic heart is shown in C. These sections show that, within the indicated area, the compact layer of the myocardium is virtually lacking and the trabeculae are not well organized. Scale bar in A, 500 μm, in B,C, 250 μm. (D). A severe hypertrophy of the right ventricle is observed in conjunction with some hypertrophy of the interventricular septum in this neonatal transgenic heart. Scale bar, 500 um. (E). Subepicardial coronary abnormalities are observed, as indicated by the dilation of the coronary vessels in this neonatal transgenic heart (see arrows). Scale bar, 250 μm. (F) Narrowing of the outflow tract can be seen in this neonatal transgenic heart section, likely due to the bulging of the ventricular septum into the lumen of the right ventricle. Scale bar, 250 μm. (G) This transgenic fetal heart (E17.5) show an unusually large and pronounced proximal portion of the atrioventricular conduction system. AVB, atrioventricular bundle. Scale bar, 125 μm. (H,I) This transgenic adult heart shows pouching of the apical left ventricular lumen (asterisk in H, enlarged in I). Scale bar in H, 2 mm; I, 400 μm. RV, right ventricle; LV, left ventricle; RA, right atrium; LA, left atrium.

as the most pronounced enlargement was found in embryos derived from B/+ × B/+ matings, while milder phenotypes were found in the offspring of B/+ × CD-1 matings. Injection of trypan blue into the right ventricles of the fetal or neonatal hearts showed evidence of subpulmonary stenosis (arrows in Fig. 8P, compare to normal heart in Fig. 8O). This phenotype resembled the right ventricular outflow obstruction seen in adult animals.

Histological analysis of fetal/neonatal hearts revealed a variety of other defects, mainly located in the right ventricle. In the hearts shown in Fig. 9A-C, the apical myocardium of the right ventricle were highly abnormal, exhibiting a spongy appearance which was characterized by the near absence of the compact layer and disorganization of the trabeculae. In some hearts, there was also evidence of right ventricular hypertrophy and hypertrophy of the interventricular septum (Fig. 8D). This may at least in part account for the apparent narrowing of the outflow tract seen in the fetal heart shown in Fig. 9F. Abnormalities in the deployment of the subepicardial coronary vasculature were also frequently observed (Fig. 9E). Typically, this was associated with an unusual dilation of the vessels. In addition, some hearts exhibited an enlargement of the proximal portion of the atrioventricular conduction system, indicating aberrant development of the conduction system (Fig. 9G).

CMV43 transgene extends the viability of the Cx43 knockout mice

To explore the role of Cx43 in heart morphogenesis, we bred the CMV43 transgene into the Cx43 knockout mouse line. Cx43 knockout animals are normally cyanotic at birth due to right ventricular outflow obstructions and die within 15 minutes to a few hours of birth (Reaume et al., 1995). As the CMV43 expression pattern overlaps that of the endogenous Cx43 gene, transgene expression may partially complement the loss-of-function in the knockout mouse and, if this included Cx43 function required for heart morphogenesis, it may allow 'rescue' of the knockout lethality. To examine this possibility, the B transgenic mouse line was cross bred with the Cx43 knockout mouse line to obtain mice double heterozygous for the Cx43 null allele and the CMV43 transgene. Such mice were then mated to each other and four litters of offspring were obtained. Genotyping analysis showed that seven of these were homozygous for the Cx43 knockout allele and, of these, five also carried the CMV43 transgene. Both animals without the transgene died, one at birth and the other at 24 hours after birth. In contrast, all five of the Cx43 null mutant mice (homozygote for the knockout allele) with the CMV43 transgene remained alive for at least 36 hours after birth (the latest time when they were killed for analysis). At the time of killing, they were acyanotic, and appeared healthy, with milk in their stomachs and outwardly indistinguishable from their littermates. Nevertheless, an examination of the hearts of these 'rescued' animals revealed conotruncal enlargement similar to that described for the Cx43 knockout mice (Fig. 10) (Reaume et al., 1995), but this was accompanied by multiple bulges (see white arrows in Fig. 10A,B). Dye injection into the right ventricular chamber revealed that, unlike the Cx43 knockout homozygote, there was blood flow into the right ventricular outflow tract. However, this flow was associated with varying degrees of obstruction, with the most severe obstructions leading to backflow of the injected dye into the atrial chambers (Fig. 10B). Surprisingly, a network of fiber-like projections was detected over the surface of the hearts when harvested from animals 24-36 hours old (Fig. 10A,B). This was only found in the hearts of animals homozygous for Cx43 knockout allele and also carrying the CMV43 transgene, never in animals with only the Cx43 knockout allele or only the CMV43 transgene.

To determine the prospects for long-term viability of these animals, one litter was allowed to nurse without disturbance. None of these animals appeared cyanotic. As this litter of ten animals contained one pup which was particularly small, four of the normal-sized offspring were triaged (day 4) to help ensure survival of the runted individual. 11 days after birth, the remaining six offspring were photographed, tail clipped for genotyping and weighed. At this time, all appeared healthy, active and responsive. However, the next day, three of the offspring had died, two of which had already been partially cannibalized at the time of recovery. Genotyping carried out with DNA recovered from the carcasses showed that two of the

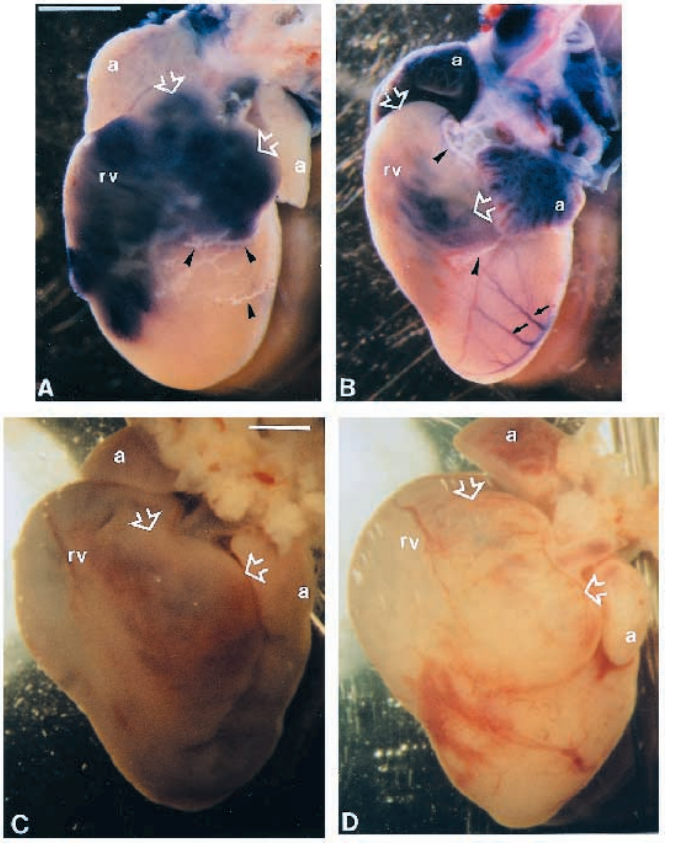

Fig. 10. Heart malformations in Cx43 knockout mice rescued by the CMV43 transgene. (A,B) Two hearts obtained 36 hours after birth was examined by dye injection into the right ventricle (rv). Both show bulges (open arrows) in the conotruncal region. The heart in B exhibits very little luminal space in the right ventricular chamber, as indicated by the small size and irregular outline delineated by the injected dye. This is likely accompanied by significant outflow obstruction, as the injected dye backflowed into the atria (a) and coronary vessels (small arrows). A network of white fibers (arrowheads) are typically seen along the surface of the heart, including in the region of the pulmonary outflow. (C,D) Two hearts obtained 11 days postnatally also exhibited significant bulging of the conotruncal region of the heart, suggesting significant outflow obstruction. Bar: 1 mm.

animals were Cx43 homozygote KO mice carrying the CMV43 transgene. The third animal, the runted individual, could not be completely genotyped (CMV43 transgene positive). However, examination of the hearts of these three animals showed that they all exhibited the same phenotype, with conotruncal enlargement similar to that found in the neonatal hearts (Fig. 10C,D). These results show that the CMV43 transgene can extend the life of the Cx43 knockout mice, but that complementation of the null mutation may be incomplete.

DISCUSSION

Transgenic mice were generated containing a mouse Cx43 expression construct driven by the CMV promoter. These mice exhibited neural tube closure defects, defects in the peripheral nervous system and heart malformations. These phenotypes were observed in several independently derived transgenic lines, including lines that were generated in different strain backgrounds (B line is SWR/SJL; all other lines B6SJL). For transgenic line B, breeding into a different inbred (C57BL6) or outbred background (CD1) provided the same phenotypes.

It is significant that the developmental defects detected in the CMV43 transgenic mice corresponded to sites of transgene expression, ie. the neural tube and neural crest derivatives populating the peripheral nervous system and the heart. This pattern of transgene expression is identical to that observed in other transgenic lines containing CMV promoter driven constructs and correlates with known sites of congenital CMV infection in human fetuses (Kothary et al., 1991; Koedood et al., 1995). As these regions also are sites of endogenous Cx43 expression (Ruangvoravat and Lo, 1992), it is likely that overexpression of Cx43 is the underlying cause of these developmental anomalies. Consistent with this possibility, dye injection analysis revealed increased GJC in the dorsal neural epithelium, a region exhibiting both transgene and endogenous Cx43 gene expression. Furthermore, the incidence of NTD and the severity of heart defects were observed to increase with transgene dosage, further indicating that the developmental defects are elicited by Cx43 overexpression.

Cx43 and heart morphogenesis

The finding of heart defects in the CMV43 transgenic mice is significant, given that the deletion of Cx43 function in the Cx43 knockout mice also disrupts heart morphogenesis. In both the CMV43 and Cx43 knockout mice, heart defects are associated with the right ventricle and pulmonary outflow tract. Although the same regions of the heart are affected, it is important to note that the malformations are not identical. This is not unexpected, as in one there is loss of Cx43 function, and the other gain of Cx43 function. Moreover, developmental regulation of the CMV43 transgene is not likely to be the same as that of the endogenous gene. These results suggest that the precise regulation of GJC is critical to mammalian heart morphogenesis, in particular, as it relates to conotruncal development. This possibility is further indicated by the Cx43 mutations found in VAH patients (Britz-Cunningham et al., 1995). These mutations do not affect channel formation, but the regulation of the gap junctional channels. It is of importance to note that, in the study of Britz-Cunningham et al. (1995), the Cx43 mutations were associated with a distinct subclass of VAH patients, those with pulmonary stenosis. This may account for the differing results from two other studies in which the examination of VAH patients revealed no Cx43 mutations (Casey and Ballabio, 1995; Splitt et al., 1995). In the latter studies, the outflow tract phenotype of the VAH patients included in the analyses was not considered. Thus, in total, these observations strongly suggest that the proper regulation of GJC may be especially important in outflow tract morphogenesis.

It should be noted that, in the CMV43 transgenic mouse, transgene expression is restricted to just a subset of the regions where the endogenous gene is expressed, that is in the dorsal neural tube and in a subpopulation of neural crest derivatives, including those that migrate to the outflow tract of the heart. As cardiac neural crest cells are required for normal heart morphogenesis (for review see Kirby, 1993; Kirby and Waldo, 1990, 1995; Noden et al., 1995), including outflow tract septation (Kirby and Stewart, 1983; Kirby et al., 1985), we hypothesize that the heart defects in the CMV43 transgenic mice may arise from the perturbation of cardiac neural crest cells. However, neither the Cx43 knockout mice nor the CMV43 transgenic mice exhibit heart defects classically associated with cardiac neural crest ablations, such as aortic arch disruption, persistent truncus arteriosus, double outlet right ventricle and ventricular septal defects (Bockman et al., 1987; Kirby and Stewart, 1983; Nishibatake et al., 1987). This may indicate that the Cx43-related heart defects are not neural crest in origin. More probably, the differing phenotypes suggest that, in the CMV43 and Cx43 knockout mice, neural crest pertubations may arise not from ‘ablation’ of cardiac neural crest, but rather from more subtle changes in the migration, proliferation and/or differentiation of crest-derived cell populations. Distinguishing between these various possiblities will require direct examination of cardiac neural crest deployment in the CMV43 transgenic and Cx43 knockout mice.

It is significant that the finding of developmental defects in the coronary vasculature in the CMV43 transgenic mice is consistent with neural crest involvement, as deployment of the coronary vessels is dependent on neural-crest-derived parasympathetic innervation in the heart (Hood and Rosenquist, 1992; Waldo et al., 1994). Moreover, as the Purkinje conduction fibers codistribute with the forming coronary vasculature in avian embryos (Gourdie et al., 1995), the detection of conduction system abnormalities is not unexpected. The presence of cranial and spinal ganglia defects in the CMV43 transgenic mice also are of significance in this context, as these structures contain neural crest contributions. The CMV43 heart defects also included ventricular hypertrophy and other perturbations in the myocardium. Such pathologies may arise secondarily, as a result of the outflow tract obstruction, coronary vascular abnormalities or conduction disturbances. Alternatively, these other defects may reflect a dependence of cardiomycyte development on the differentiaton of normal patterns of innervation.

The finding that the viability of the Cx43 knockout mice is extended by the CMV43 transgene is particularly interesting in light of the above hypothesis. This result strongly suggests that heart defects in the Cx43 knockout mouse also may involve cardiac neural crest cells and that these crest cell perturbations are partially compensated by the CMV43 transgene via some as yet unknown mechanism. That is, we suggest that the lesion

in the Cx43 knockout mouse is not due to the perturbation of gap junctional communication between myocardial cells, but in neural crest cell populations involved in heart development. The fact that heart defects continue to be exhibited by the CMV43 transgenic/Cx43 knockout hybrid animals is perhaps not surprising, as the CMV43 transgene may not be expressed at sufficient levels for complete complementation of the Cx43 deletion, or its expression domain may not include all of the crest (and other) cell populations that require Cx43 function for normal heart morphogenesis. Overall, this study reinforces the notion that emerging extracardiac populations are central players in cardiac morphogenesis. The CMV43 mouse may serve as a powerful animal model system for understanding the interplay between the embryonic heart and migratory cells that participate in heart development.

Cx43 and neural tube defects

The CMV43 transgenic mice were also found to have cranial neural tube defects. These were expressed with partial penetrance. Interestingly, similar cranial neural tube defects were detected in the Cx43 knockout mouse line, and these were also expressed with partial penetrance (Sullivan and Lo, unpublished observations). Partial penetrance is commonly associated with NTD in both mice and humans (Campbell et al., 1986; Copp, 1994; Copp et al., 1990; Goulding and Paquette, 1994; Moase and Trasler, 1992), and may reflect the multifactorial nature of processes regulating neural tube closure (Campbell et al., 1986; Copp, 1994; Copp et al., 1990). As has been shown in other mouse mutants exhibiting NTD, we have also found that the NTD incidence in the CMV43 transgenic mice varied with strain background (Ewart and Lo, unpublished observations) (for review see Copp, 1994). These results suggests a role for Cx43 gap junctions in neural tube closure.

Another possibility is that neural tube closure defects in the CMV43 transgenic mice (and Cx43 knockout mice) arise secondarily from the disruption of neural crest cells. Several studies have indicated an association between the perturbation of neural crest delamination and neural tube closure defects, including studies of the *twist* knockout mouse (Chen and Behringer, 1995), the *Splotch* mutants (Franz and Kothary, 1993; Moase and Trasler, 1989, 1990) and chick embryos treated with *slug* antisense oligonucleotides (Nieto et al., 1994). The *Splotch* mutants are particularly relevant in this context, as they exhibit heart defects that are likely due to the perturbation of cardiac crest cells (Franz, 1989, 1993). In addition, they exhibit a large number of gap junctional vesicles in the developng neural tube, leading to the suggestion that the neural tube closure defects in these mutants may result from the perturbation of gap junctions (Wilson and Finta, 1979).

Gap junctions and neural crest cells

Although interactions involving direct cell-cell contact are not traditionally thought of as being important to migratory cell populations such as the neural crest, a number of observations, old and new, suggest otherwise. For example, neural crest cells have been described in numerous studies as migrating in streams, sheets or masses of cells (for example see Bancroft and Bellairs, 1976; Davis and Trinkaus, 1981; Tan and Morriss-Kay, 1985). Moreover, a time-lapse study of migrating neural crest cells showed that they pulsate synchronously, suggesting that they are functionally linked as a syncytium (Jaskoll et al., 1991). Further indications of the importance of direct cell-cell contact in neural crest migration are observations showing that the alteration of cell-cell adhesion can perturb cranial neural crest migration (Bronner-Fraser et al., 1992) and that high cell densities are needed for neural crest migration in vitro (Rovasio et al., 1983). It is also interesting to note that different members of the cadherin gene family are differentially expressed in subpopulations of neural crest cells (Kimura et al., 1995; Nakagawa and Takeichi, 1995). This further suggests a role for direct cell-cell interactions in the deployment of neural crest cells. In future studies, we will directly examine the migratory behavior of neural crest cells in the CMV43 transgenic and Cx43 knockout mice. Such studies are a necessary first step in elucidating the role of Cx43-containing gap junctions in neural crest development. Only then will it be possible to further consider what types of cell signaling processes may be mediated by gap junctional pathways.

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