DEVELOPMENTAL BIOLOGY **203,** 90–105 (1998)
ARTICLE NO. DB989024

Gap Junctions Are Involved in the Early Generation of Left–Right Asymmetry

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Invariant left–right asymmetry of the visceral organs is a fundamental feature of vertebrate embryogenesis. While a cascade of asymmetrically expressed genes has been described, the embryonic mechanism that orients the left–right axis relative to the dorsoventral and anteroposterior axes (a prerequisite for asymmetric gene expression) is unknown. We propose that this process involves dorsoventral differences in cell–cell communication through gap junctions composed of connexin proteins. Global modulation of gap junctional states in *Xenopus* embryos by pharmacological agents specifically induced heterotaxia involving mirror-image reversals of heart, gut, and gall bladder. Greatest sensitivity was observed between st. 5 and st. 12, well before the onset of organogenesis. Moreover, heterotaxia was also induced following microinjection of dominant negative and wild-type connexin mRNAs to modify the endogenous dorsoventral difference in junctional communication. Heterotaxia was induced by either blocking gap junction communication (GJC) dorsally or by introducing communication ventrally (but not the reverse). Both connexin misexpression and exposure to GJC-modifying drugs altered expression of the normally left-sided gene *XNR-1,* demonstrating that GJC functions upstream of *XNR-1* in the pathway that patterns left–right asymmetry. Finally, lineage analysis to follow the progeny of microinjected cells indicated that they generally do not contribute the asymmetric organs. Together with the early sensitivity window, this suggests that GJC functions as part of a fundamental, early aspect of left–right patterning. In addition, we show that a potential regulatory mutation in Connexin43 is sufficient to cause heterotaxia. Despite uncertainty about the prevalence of the serine^{364} to proline substitution reported in human patients with laterality defects, the mutant protein is both a mild hypomorph and a potent antimorph as determined by the effect of its expression on left–right patterning. Taken together, our data suggest that endogenous dorsoventral differences in GJC within the early embryo are needed to consistently orient left–right asymmetry.

Key Words: **left–right asymmetry; connexins; gap junctions; laterality, heterotaxia.**

INTRODUCTION

The generation of consistent left–right (LR) asymmetry in animal morphogenesis is a fascinating problem linking issues of molecular chirality with large-scale embryonic patterning (Brown and Wolpert, 1990; Levin, 1997). Congenital laterality defects in humans [often associated with twinning (Cuniff *et al.,* 1988; Levin *et al.,* 1996; Nascone and Mercola, 1997)], including situs inversus (complete mirror-image inversion of the whole body), heterotaxia (uncoordinated inversions of some sided organs), dextrocardia (malposition of the heart), and isomerism (loss of asymmetry), involve errors in patterning the embryonic LR axis (Burn, 1991; Winer-Muram, 1995). Within the past few years, a variety of signaling molecules have been implicated in a pathway that regulates the anatomical aspects of LR asymmetry by providing cues for the sided development of visceral organs (Fujinaga, 1996; Levin, 1997; Wood, 1997). However, correct asymmetric expression of these signaling molecules can be considered late events as they themselves must reflect an earlier LR asymmetry. Recent experiments suggest that the orientation of the LR axis depends on prior dorsoventral (DV) patterning (McCain and McClay, 1994; Danos and Yost, 1995; Levin *et al.,* 1997; Nascone and Mercola, 1997). Thus, an important question is how LR orientation is linked to DV patterning.

One mechanism whereby an initial asymmetry at the level of a single cell (Brown and Wolpert, 1990) may be imposed on multicellular fields has been proposed to involve intercellular communication via gap junction channels (Levin and Nascone, 1997). Gap junction channels between cells permit the passage of small molecules (gen-

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erally <1 kDa); each channel is formed by a hexamer of connexin proteins within a cell membrane associated with a hexamer in an apposing cell (Bruzzone *et al.*, 1996b; Goodenough *et al.*, 1996). A large family of connexins exists and many contain a large cytoplasmic region postulated to confer conductance regulation by intracellular pH, voltage, and phosphorylation (Bruzzone *et al.*, 1996a; White and Bruzzone, 1996). Specific expression patterns as well as functional studies contribute to the idea that gap junctional communication (GJC) is involved in diverse processes such as tumor progression and embryogenesis (deLaat *et al.*, 1980; Fraser *et al.*, 1987; Guthrie and Gilula, 1989; Hotz-Wagenblatt and Shalloway, 1993; Yamasaki *et al.*, 1995; Davies *et al.*, 1996; Lo, 1996; Krutovskikh and Yamasaki, 1997). Measurements in 16- to 64-cell *Xenopus* embryos revealed that dorsal blastomeres have greater GJC than ventral blastomeres and that subtle differences in GJC exist between blastomeres on the left and right sides (Guthrie, 1984; Guthrie *et al.*, 1988; Olson and Moon, 1992). This supports the idea that the channels may be involved in coordinating global DV and LR polarity in the early embryo, although no experimental evidence has yet been presented in favor of such a model. A report that several unrelated patients with visceroatrial heterotaxia contain potential regulatory mutations within Connexin43 [Cx43 (Britz-Cunningham *et al.*, 1995)] could constitute circumstantial evidence in support of this view. However, phenotypes observed in transgenic mice over- and underexpressing Cx43 have been interpreted to suggest that heart formation per se is perturbed due to a local alteration of organogenesis involving neural crest or mesodermal cells that contribute to the conotruncal region of the heart (Reaume *et al.*, 1995; Ewart *et al.*, 1997). Thus, we have directly tested the hypothesis that early patterns of GJC are involved in orienting the LR axis in *Xenopus*.

MATERIALS AND METHODS

Assessing GJC

GJC in control and experimental embryos was monitored as follows. One cell in 8- or 16-cell embryos was injected with a 1:1 mix of Lucifer yellow (LY, 0.522 kDa, Molecular Probes) and rhodamine-lysinated dextran (RLD, 10 kDa, Molecular Probes). Ten minutes later, the embryos were fixed in 3% formaldehyde in PBS and observed using epifluorescence optics (as in Guthrie *et al.*, 1988). Since LY passes through gap junctions, while RLD does not, an instance of open gap junctional communication is represented by a pair of neighboring cells, one of which is seen to contain RLD and LY while the other contains LY only. This effectively rules out a possible source of artifacts of previous studies, since injections of LY alone can result in false positives due to the presence of cytoplasmic bridges or incomplete cleavage. GJC is expressed as percentage of embryos showing selective transfer of LY to neighboring cells during the 10-min period prior to fixation.

Drug Exposure

Embryos were treated by addition of reagents to their culture medium (0.1× MMR) at the following doses: anandamide, 5 mg in 1 ml of 50% DMSO and then diluted 1:33 (final concentration 1.5×10^{-3}); glycyrrhetinic acid, 0.1 g/ml glycyrrhetinic acid in DMSO vortexed at a 1:1000 dilution into 0.1× MMR and then diluted further 1:8 (final concentration 1.2×10^{-5}); Heptanol, 10 μl vortexed extensively into 10 ml of 0.1× MMR and diluted 1:100 (final concentration 1×10^{-5}); oleic acid, 200 λ vortexed with 800 λ DMSO and then diluted 1:1000 (final concentration 2×10^{-4}); and melatonin, 0.2 g vortexed into 1 ml DMSO and diluted 1:1000 (final concentration 2×10^{-4}). The doses of the drugs used are within an order of magnitude, and often less, than doses used in cell culture experiments (Takens-Kwak *et al.*, 1992; Goldberg *et al.*, 1996).

Scoring Embryonic Situs

The laterality phenotype of embryos was determined by scoring the situs of the heart, stomach, and gall bladder under a dissecting microscope in embryos immobilized with tricaine at stage 45. Only embryos with normal dorsoanterior development (DAI = 5) and clear left-sided or right-sided organs were scored. A heterotaxic embryo was considered to be one in which any of those three organs was reversed in its position.

Misexpression of Connexins

Synthetic mRNA was transcribed by the Sp6 polymerase from linearized SP64T plasmids containing the individual cDNAs. mRNA was mixed with 50 ng of RLD and 320 pg of mRNA encoding β-galactosidase (as lineage labels) and injected into cells in four- or eight-cell-stage embryos. The mRNA for the various connexin constructs was carefully titered such that no defects other than heterotaxia resulted in approximately 90% of the injected embryos in a given batch. The doses (per cell) were Cx26, 420 pg; Cx43, 640 pg; and H7, 59 pg.

Analysis of Lineage Contribution

Injected embryos were fixed in 4% formaldehyde at room temperature for 1 h after scoring organ situs. They were then washed twice for 10 min in PBS containing 2 mM $MgCl_2$ and processed for β-galactosidase staining using X-gal as substrate.

RESULTS

Dorsal Cells Are Coupled, While Ventral Cells Are Isolated, in Early Embryos

To examine the spatial GJC properties of *Xenopus* embryos, as well as to ensure that the reagents used to modify GJC (see below) have the predicted effect *in vivo*, we developed a simple assay for GJC. One cell in each embryo was to be injected with a mixture of two fluorescent dyes: LY, which passes through gap junctions, and RLD, which does not (because of its large molecular weight). The embryos were then fixed and the cells examined under fluorescence. A cell showing both LY and RLD signal (the

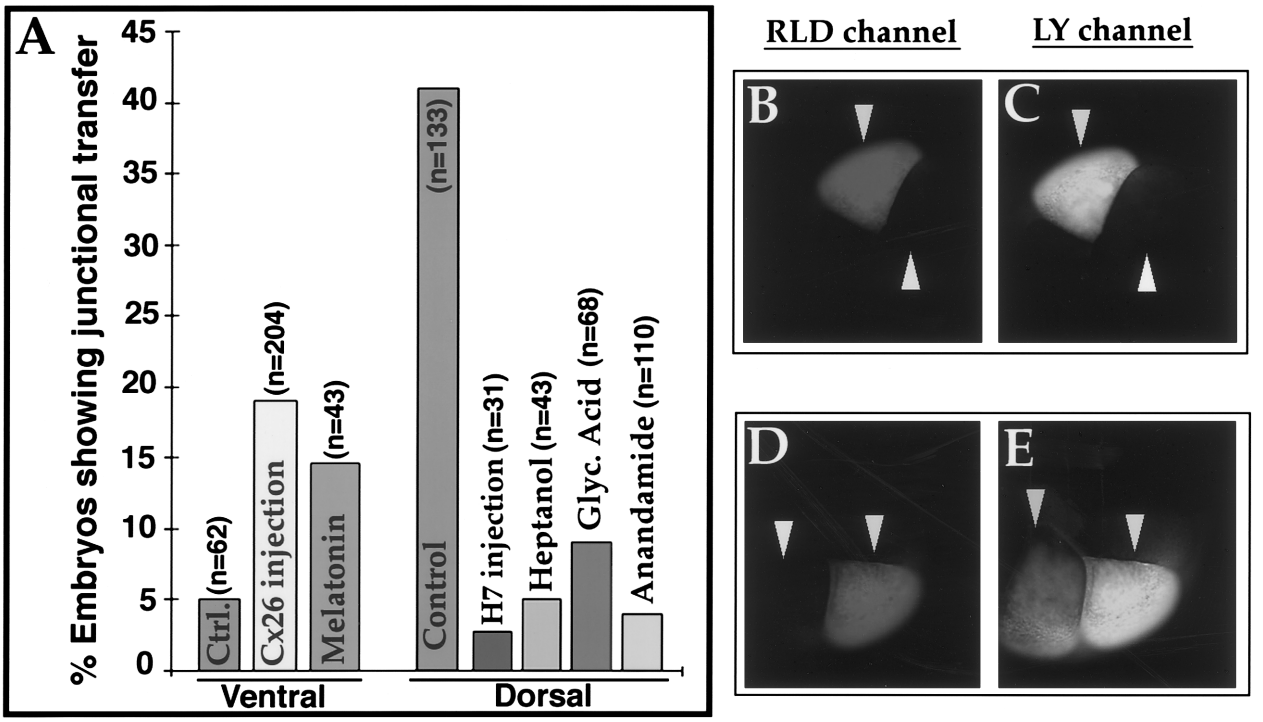


FIG. 1. Exposure to drugs and misexpression of connexin constructs alter DV differences in GJC in early *Xenopus* embryos. (A) When cells on the ventral side of embryos were tested for transfer of LY, only 5% of the embryos ($n = 62$) showed transfer of LY to neighboring cells. An example is shown in B and C. A cell was injected with RLD + LY (B, grey left arrowhead); the right neighboring cell shows no LY signal (C, white arrowhead). In contrast, expression of the Cx26 construct in ventral cells induced GJC communication in 19% ($n = 204$) of the embryos, as did exposure to melatonin (14%, $n = 43$). An example is shown in D and E. A cell was injected with RLD + LY (D, grey right arrowhead); there is LY signal in both the injected cell (E, grey right arrowhead) and a neighboring cell (E, grey left arrowhead). The fact that LY transferred, while there is no transfer of RLD to the left cell (D, white left arrowhead), shows that no cytoplasmic bridge connected the cells, thus demonstrating the presence of open gap junctions. The induction of GJC by Cx26 is significant (using the χ^2 test with Yates correction for increased stringency) to $\chi^2 = 6.26$, $P < 0.012$. Thus, as reported previously (Guthrie, 1984; Guthrie *et al.*, 1988; Olson and Moon, 1992), ventral cells are relatively isolated. Injection of Cx26 is sufficient to induce a significant degree of open GJC. In contrast to ventral cells, dorsal cells in control embryos showed a 41% ($n = 133$) incidence of open GJC. This difference is significant to $\chi^2 = 35.74$, $P < 2.3 \times 10^{-9}$. Two hours exposure to heptanol, glycyrrhetinic acid, and anandamide resulted in diminishing the level of GJC on the dorsal side to 5% ($n = 43$, $\chi^2 = 17.74$, $P < 2.5 \times 10^{-5}$), 9% ($n = 68$, $\chi^2 = 20.21$, $P < 6.9 \times 10^{-6}$), and 4% ($n = 110$, $\chi^2 = 43.26$, $P < 4.8 \times 10^{-11}$), respectively. Likewise, injection of the dominant negative construct resulted in a reduction of GJC on the ventral side to 3% ($n = 31$, $\chi^2 = 14.12$, $P < 1.7 \times 10^{-4}$).

injected cell) adjacent to a cell with LY signal only was counted as constituting gap junctional transfer (Figs. 1D and 1E, compare to Figs. 1B and 1C). Cases in which neighboring cells exhibited RLD signal in addition to LY were not counted. This is a more accurate assay than the traditional studies using LY alone (Guthrie, 1984; Guthrie *et al.*, 1988; Olson and Moon, 1992) because it avoids false positives due to cell division and cytoplasmic bridges which allow the passage of RLD.

Control (untreated) embryos were examined for GJC on the dorsal and ventral sides. We find that at the 8- and 16-cell stages, on the dorsal side, 41% of the embryos show GJC, while only 5% show GJC on the ventral side (Fig. 1A, see legend for statistical analysis). This pattern of relatively high dorsal coupling and ventral isolation confirms previous findings (Guthrie *et al.*, 1988; Guthrie and Gilula, 1989; Olson and Moon, 1992).

A number of reagents were used (see below) to rapidly modify GJC states (by protein level regulation) in living embryos. These include drugs such as anandamide, heptanol, and glycyrrhetinic acid (Davidson and Baumgarten, 1988; Chanson *et al.*, 1989; Lazrak *et al.*, 1994; Venance *et al.*, 1995) which decrease GJC and melatonin which increases GJC (Ubeda *et al.*, 1995). All of these substances have been shown to have the expected effects on GJC in cell culture. Also used in our studies were a number of wild-type and dominant negative connexin constructs (for mRNA injection).

To show that these reagents are effective in *Xenopus* embryos *in vivo*, embryos were exposed to working concen-

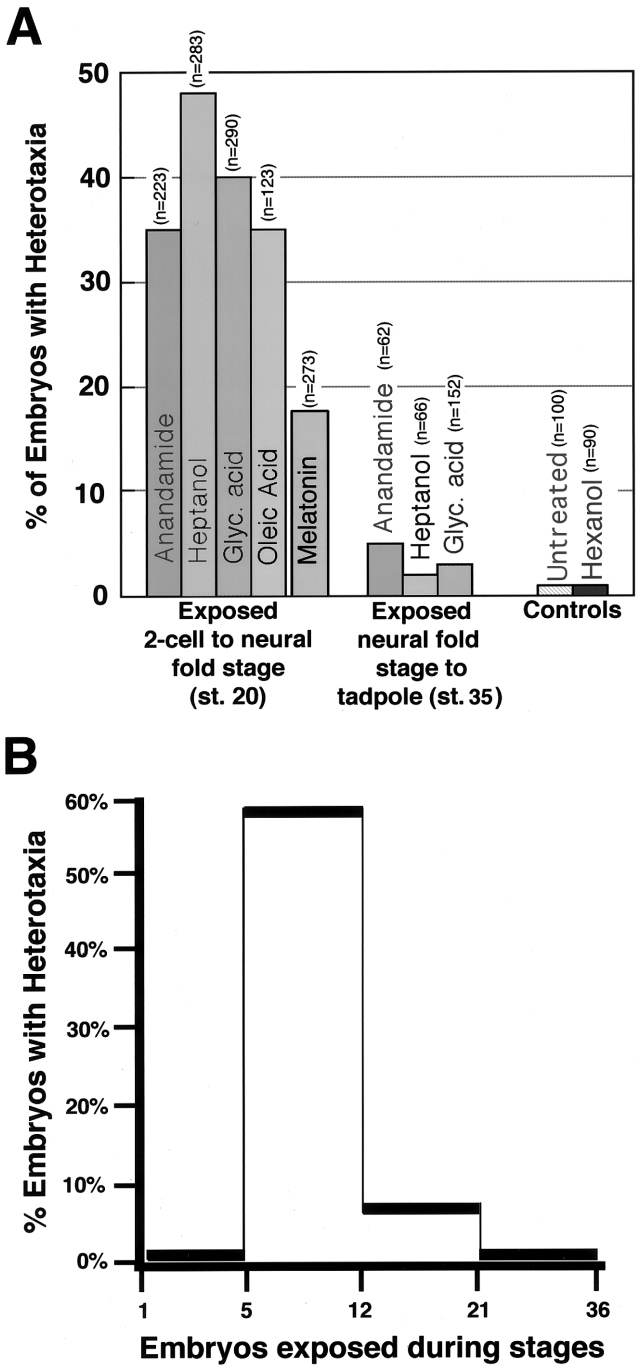


FIG. 2. Pharmacological modulation of GJC prior to organogenesis causes heterotaxia in *Xenopus* embryos. (A) After fertilization, embryos were cultured in 0.1× MMR containing the various drugs for the time period between the 2-cell stage to the neural fold stage (up tostage 22) or between stages 22 and 35 (organogenesis). They were then washed, transferred to fresh 0.1× MMR, and scored for the incidence of heterotaxia at st. 45 as in Fig. 1. Statistical significance of the results was judged by comparing the scores from the exposed batches to that of untreated controls using the χ^2 test with a Yates correction, as in Fig. 1. Exposure during the first 24 h to drugs that close gap junctions resulted in highly significant incidences of heterotaxia: anandamide, 35% ($\chi^2 = 43.5$, $P = 4.3 \times 10^{-11}$); heptanol, 48% ($\chi^2 = 73.3$, $P = 1.1 \times 10^{-17}$); glycyrrhetinic acid, 40% ($\chi^2 = 55.7$, $P = 8.3 \times 10^{-14}$); oleic acid, 35% ($\chi^2 = 41.1$, $P = 1.5 \times 10^{-10}$). Similarly, melatonin, a drug that opens gap junctions, caused a statistically significant 18% incidence of heterotaxia ($\chi^2 = 19.1$, $P = 1.2 \times 10^{-5}$). In contrast, early exposure to hexanol [which does not affect gap junctions (Chanson *et al.*, 1989)] does not cause heterotaxia (incidence of 1%, $\chi^2 = 0.003$, $P = 0.96$). Embryos exposed to the same concentrations of drugs between 48 and 72 h of development do not show statistically significant incidences of heterotaxia: glycyrrhetinic acid, 3% ($\chi^2 = 2.6$, $P = 0.1$); anandamide, 5% ($\chi^2 = 1.8$, $P = 0.1$), heptanol, 2% ($\chi^2 = 0.04$, $P = 0.8$). Untreated control embryos show <1% incidence of heterotaxia. (B) After fertilization, embryos were cultured in glycyrrhetinic acid during the stages indicated. They were then washed, transferred to 0.1× MMR, and allowed to develop until st. 43, at which point they were scored for *situs*. Embryos exposed to the drug prior to st. 5 exhibited 1% heterotaxia ($n = 33$) relative to controls. In contrast, embryos exposed between st. 5 and st. 12 exhibited 58% heterotaxia ($n = 14$). Embryos exposed between st. 12 and 21 exhibited 7% heterotaxia ($n = 23$). Embryos exposed between st. 21 and 36 exhibited 2% heterotaxia ($n = 46$).

trations (see below) of each drug or mRNA 30 min postfertilization, through the 16-cell stage. At that time, one cell in the dorsal or ventral side in each embryo was injected with a mixture of RLD and LY. Injection of the mRNA encoding the dominant negative construct H7 [a chimeric connexin shown in *Xenopus* cells to inhibit GJC (Paul *et al.*, 1995)] at the 1-cell stage or exposure to each of the drugs which decrease GJC leads to a 4- to 10-fold decrease of GJC on the dorsal side of the *Xenopus* embryo (Fig. 1A, see legend for statistical analysis). In contrast, injections of Cx26 mRNA or exposure to melatonin leads to a 3- to 4-fold increase of GJC on the ventral side. Thus, we conclude that our reagents have the expected effects on *Xenopus* embryos *in vivo*.

Modulation of GJC States Causes Heterotaxia

To test the hypothesis that GJC plays a role in LR patterning, we measured LR abnormalities in embryos treated with the drugs known to modulate junctional conductance [those examined in Fig. 1, as well as oleic acid which closes gap junctions (Lazrak *et al.*, 1994) and melatonin which facilitates gap junctional communication (Ubeda *et al.*, 1995)]; these drugs are believed to affect GJC through modification of connexon particle packing and docking within membranes (Burt, 1991; Goldberg *et al.*, 1996). Heart, gut, and gall bladder situs were examined in st. 45 *Xenopus* embryos exposed to drugs which diminish or increase GJC. Doses were titrated such that greater than 90% of the embryos in each batch exhibited a normal external appearance and dorsoanterior character (dorsoanterior index, DAI, equal to 5). While use of low doses

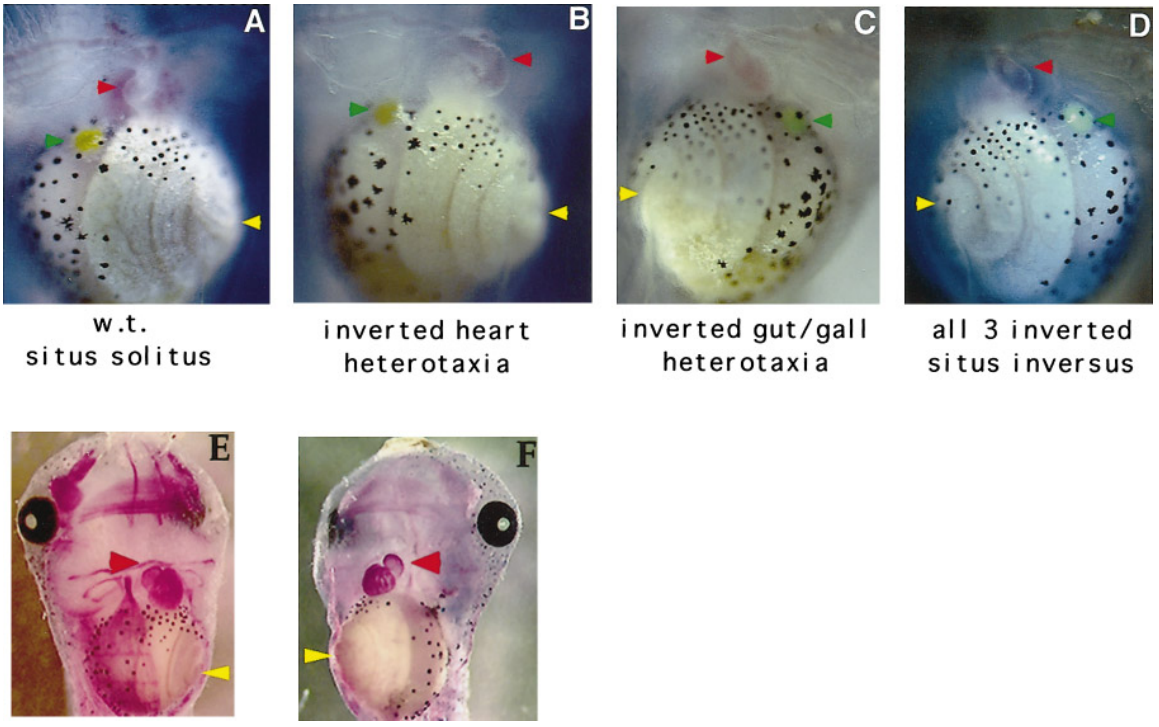


FIG. 3. Examples of laterality phenotypes induced by manipulation of GJC. All embryos are shown in ventral views (thus the embryo's right is the reader's left), with anterior toward the top. (A) A control stage 45 embryo. The heart loops toward the embryo's right, the axis of gut coiling points toward the left, and the gall bladder is on the right. (B–D) Embryos treated as in Figs. 2 and 4 that exhibited aberrant sidedness in any one of these three organs were scored as heterotaxic: inverted heart (B), inverted gut and gall bladder (C), and inverted heart, gut, and gall bladder (D). The clear hearts are difficult to photograph in living specimens (A–D); therefore, for clarity, staining with the MF20 antibody is shown in normal (E) and situs inversus totalis (F) embryos. Red arrowheads indicate heart loops; yellow arrowheads indicate gut loops; green arrowheads indicate gall bladders. Direction of the arrowhead indicates situs.

diminishes the efficacy of the treatment, it avoids effects on situs caused by reduction in DAI, as heart reversals can occur in embryos with a DAI of 3 or below (Danos and Yost, 1995). The doses found effective in embryos turned out to be somewhat lower, but within an order of magnitude of doses used in cell culture systems (Takens-Kwak *et al.*, 1992; Goldberg *et al.*, 1996).

Exposure between the 2-cell stage and st. 22 to anandamide, heptanol, oleic acid, or glycyrrhetinic acid each resulted in heterotaxia, with an incidence between 35 and 48% (Fig. 2).The difference between this incidence and the background of about 1% is highly statistically significant ($P = 10^{-17}$–$P = 10^{-10}$, χ^2 test with Yates correction). Treatment with melatonin during the same time period also resulted in a significant, albeit lower, incidence of heterotaxia (18%). The phenotypes resulting from these treatments are shown in Fig. 3. The high incidence of heterotaxia induced by each of these very different drugs in otherwise normal embryos, combined with the lack of effects (at up to 10-fold higher doses) of hexanol, a very similar reagent to heptanol except that it has been shown to be much less effective than heptanol in decreasing GJC (Chanson *et al.*, 1989), suggests that organ situs depends on patterns of endogenous gap junctional communication.

Embryos Are Sensitive to GJC Alterations Only Well before Organogenesis

To determine whether endogenous GJC is required for early events during LR patterning or whether it is involved specifically in organogenesis of the asymmetric organs, the effects of early and late drug treatments were compared. Exposure to five different GJC drugs prior to stage 22 (Nieuwkoop and Faber, 1967) was effective at causing heterotaxia, while exposure past stage 30 had no effect (Fig. 2A). For comparison, the heart, which is the first organ to develop LR asymmetry, forms a linear tube between stages 28 and 33 and loops between stages 33 and 36, whereas the gut begins to coil after stage 35. Thus, multiple organ sensitivity to these agents prior to, but not during, organogenesis indicates that gap junction permeability acts early in development to specify the orientation of the LR axis.

GJC Is Most Likely to Be Involved between Stages 5 and 12

To determine more precisely the developmental stage when GJC is involved in LR asymmetry, embryos were exposed at a number of shorter discrete time windows (Fig. 2B). To avoid inconsistency due to differential sensitivity to the drugs, the whole experiment (including a parallel control batch) was performed on a single fertilization, from which embryos were taken at various time points and exposed to glycyrrhetinic acid. The number of embryos at each data point is limited by the total number of eggs obtainable from a female at a single fertilization, as well as some embryonic mortality during development. We observed that the most sensitive period was between st. 5 and 12, where 58% of the embryos relative to controls exhibited heterotaxia. In contrast, embryos exposed prior to st. 5, or after st. 21, did not exhibit significant levels of heterotaxia, while exposure between st. 12 and 21 resulted in only a low incidence of heterotaxia (7%). Similar results were obtained using heptanol (data not shown). Treatment of embryos with glycyrrhetinic acid for shorter intervals between st. 5 and 12 yielded only a low incidence of heterotaxia (data not shown). The reason why heterotaxia is induced only by continued exposure between st. 5 and 12 is unclear but one explanation is that patterning dependent on GJC may occur through most or all of this window. The st. 5 to 12 interval precedes the developmental stages when left-sided expression of *XNR-1* (neural fold stage, after st. 15–16 but before st. 20; Lohr *et al.*, 1997; Levin and Mercola, 1998) and heart looping (neural tube to tailbud stage, st. 19–22; Danos and Yost, 1996) are likely to be specified and is well before either actually occurs (st. 23–24 and st. 33–36, respectively). Thus, we conclude that GJC is most likely to be involved in the early specification of LR pattern.

Disrupting Ventral Isolation of Cells Specifically Causes Heterotaxia

The observation that both a global reduction in GJC and a global upregulation of GJC cause heterotaxia suggests that the endogenous DV differences in GJC are important for correct LR patterning. To test this hypothesis, we made use of constructs which would allow spatially specific alterations in GJC profile in embryos. Previous studies (Guthrie, 1984; Guthrie *et al.*, 1988; Olson and Moon, 1992) and our analysis (Fig. 1A) have shown that cells on the ventral side of early *Xenopus* embryos were junctionally insulated from each other (schematized in Fig. 4A).

To examine the importance of this isolation for development of proper LR asymmetry, we misexpressed synthetic mRNA encoding wild-type connexins Cx26, Cx43, and Cx37 by bilateral injections into the two ventral-most animal-tier cells at the four- or eight-cell stage (Fig. 4B). Each mRNA was carefully titered beforehand to arrive at the highest dose which allows normal development (except for LR disturbances) in >90% of the embryos. Lower doses also resulted in LR phenotypes, but at a lower frequency. Cx26 was chosen because it lacks most of the intracellular regulatory region present in the larger connexins and thus is expected to form constitutively permeable junctions. Cx43 (which does contain this regulatory region) was used because mutant forms have been reported to occur in human patients with visceroatrial heterotaxia (Britz-Cunningham *et al.*, 1995). Cx37 was chosen because it is known to be unaffected (Paul *et al.*, 1995) by the dominant negative connexin H7 (see below).

Injected embryos were allowed to develop to st. 45 and scored for situs of heart, gut, and gall bladder (Fig. 4C). Ventral expression of each of the three connexins caused highly statistically significant incidences of heterotaxia (Fig. 4, see legend for statistical analysis): 33, 32, and 22% of the embryos misexpressing Cx37, Cx26, and Cx43, respectively, on their ventral sides exhibited heterotaxia, compared to 6% for β-galactosidase injections. The phenotypes produced were morphologically the same as shown in Fig. 3 (although there are differences in likelihood distributions discussed below). In contrast, dorsal injections of these same constructs did not produce significant levels of heterotaxia, suggesting that the effect is spatially specific. Thus, we conclude that ventral gap junctional isolation is needed for proper establishment of LR asymmetry.

Disrupting Dorsal GJC Specifically Causes Heterotaxia

Since isolation between ventral blastomeres appeared important for LR patterning, we next asked whether the relatively high dorsal gap junctional coupling (Fig. 4A) was likewise important. H7 is a chimeric connexin construct shown in *Xenopus* embryos to inhibit GJC by other connexins such as Cx32 and Cx43, but not Cx37 (Paul *et al.*, 1995). When this mRNA was injected into the two dorsal-most animal-tier cells at the four- or eight-cell stage (Fig. 4C), the resulting embryos exhibited a 29% incidence of heterotaxia (Fig. 4D, see legend for statistical analysis). In contrast, ventral expression of H7 had no effect on situs (3% incidence of heterotaxia). Control injections with equal amounts of β-galactosidase mRNA into dorsal blastomeres show a basal heterotaxia incidence of 2%. Importantly, dorsal coinjection of Cx37 mRNA with H7 lowered the incidence of heterotaxia to 9%, showing that a connexin which is not affected by this dominant negative (Paul *et al.*, 1995) is able to rescue the phenotype caused by H7. From the spatial specificity of the effects of H7 and the rescue, we conclude that dorsal gap junctional communication is also important for LR patterning.

All Types of Heterotaxia Are Represented Following GJC Modulation

To analyze in more detail the laterality phenotypes caused by alterations of GJC in embryos, we independently scored the occurrences of each possible combination of the sidedness of the heart, gut, and gall bladder. The results are

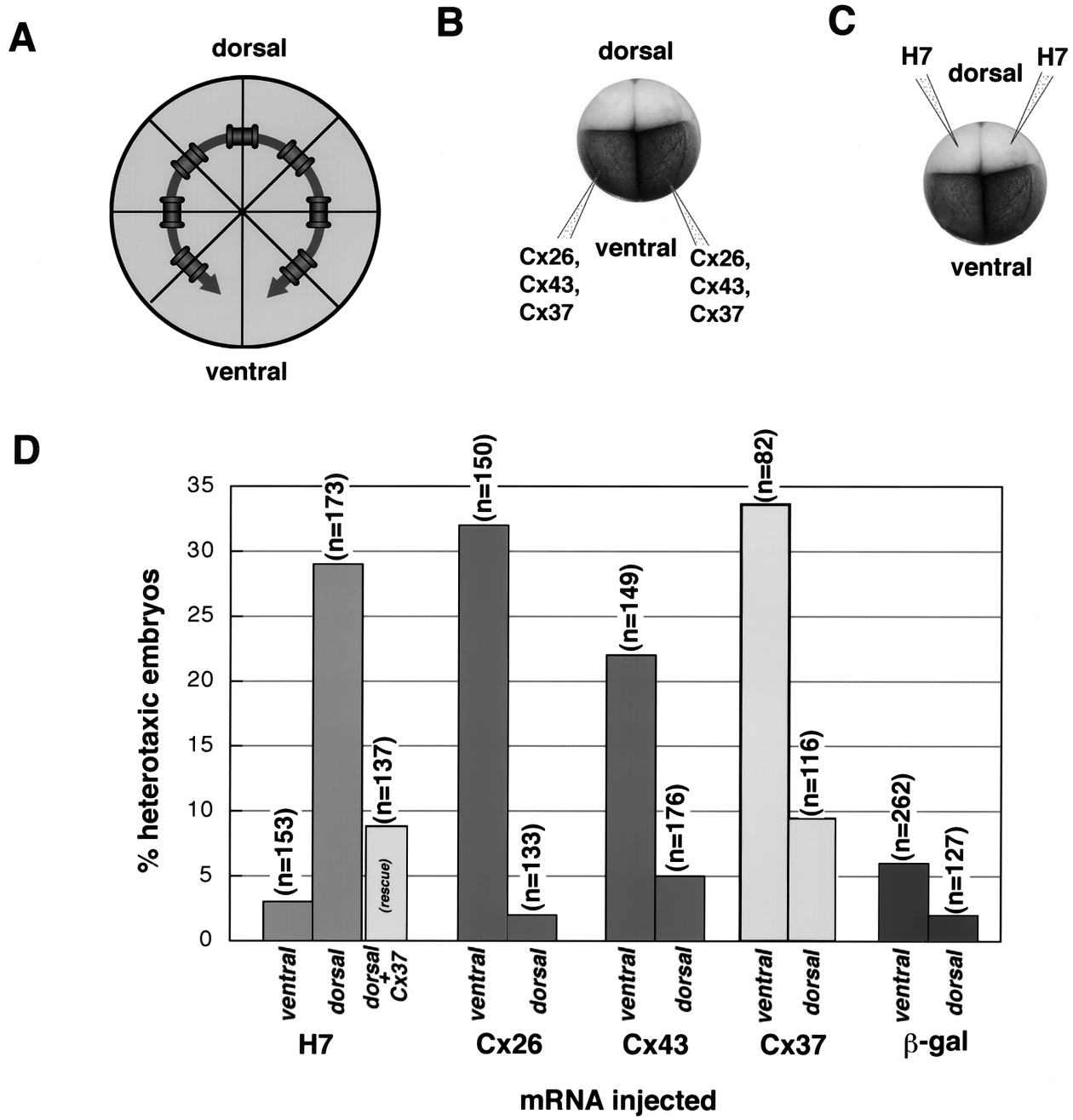


FIG. 4. Genetic modulation of the normal spatial pattern of GJC causes heterotaxia. To specifically disrupt the endogenous pattern of GJC (dorsal GJC coupling, ventral isolation, in animal tier of 16- to 32-cell embryos, shown schematically in A), 4- or 8-cell embryos were bilaterally injected with synthetic mRNA encoding one of the wild-type connexins in the ventral side (B) or with mRNA encoding the dominant negative connexin H7 into the dorsal side (C). All injections were done in the animal tier of cells. The results are shown in D. Note that the embryo diagrammed in A is highly schematic and that the results of Fig. 2 indicated that the critical developmental window extends between st. 5 to 12. Microinjection of Cx26 and Cx43 transcripts into ventral blastomeres (where there is low endogenous GJC) caused highly statistically significant incidences of heterotaxia (B): Cx43, 22% ($\chi^2 = 23.3$, $P = 1 \times 10^{-6}$); Cx26, 32% ($\chi^2 = 48.8$, $P = 3 \times 10^{-12}$); Cx37, 32% ($\chi^2 = 44.3$, $P = 2.8 \times 10^{-11}$). In contrast, dorsal (where there is already high endogenous GJC) injections of the same constructs or ventral injections of equal amounts of β-galactosidase mRNA did not cause heterotaxia: Cx43, 5% ($\chi^2 = 0.47$, $P = 0.48$); Cx26, 2% ($\chi^2 = 0.12$, $P = 0.72$); Cx37, 9% ($\chi^2 = 3.5$, $P = 0.06$). Microinjection of H7, a dominant negative connexin (Paul *et al.*, 1995), into both dorsal animal blastomeres (C) caused a 29% incidence of heterotaxia ($\chi^2 = 34.7$, $P = 4 \times 10^{-9}$). In contrast, ventral expression of H7 had no effect on situs (3% incidence of heterotaxia; $\chi^2 = 0.79$, $P = 0.37$). Coinjecting Cx37 dorsally with H7 rescued the effect, since only 9% of the embryos were heterotaxic, compared to 29% with H7 alone. This rescue effect is significant to the level of $\chi^2 = 17.4$, $P = 3 \times 10^{-5}$.

TABLE 1
Heterotaxia Phenotypes According to Treatment

Inverted	Ventral Cx43 (%)	Ventral Cx26 (%)	Dorsal H7 (%)	Anandamide (%)	Heptanol (%)	Glyc acid (%)
Heart	34	29	19	22	20	30
Gut coiling	13	16	6	7	6	3
Gall bladder	9	8	0	1	2	2
Heart and gut coiling	6	18	8	3	4	0
Heart and gall bladder	0	3	0	0	6	0
Gut coiling and gall bladder	9	13	29	17	17	31
Heart, gut coiling, and gall bladder	28	13	38	50	46	35

Note. St. 45 embryos were anesthetized with tricaine and scored for the situs of the heart, gall bladder, and direction of gut coiling. Incidence of the seven possible permutations of organ reversal is tabulated and expressed as a percentage of all embryos in that group with heterotaxia.

summarized in Table 1, shown as percentages of all of the embryos which exhibit heterotaxia in a given group. Drugs that inhibit GJC caused complete situs inversus most often (35–50%), but also frequently caused isolated heart reversals (20–30%) or isolated gut and gall bladder reversals (17–31%). The distribution was also similar for the embryos whose gap junctional communication was inhibited by the dominant negative construct H7.

In contrast, embryos whose ventral isolation was disrupted by injections of wild-type connexin constructs most commonly showed inversions of the heart (29–34%), although complete situs inversus was also well represented (13–28%). This analysis of the laterality of three morphological aspects of situs shows that the phenotype due to disruption of GJC is heterotaxia and not, for example, isomerisms or randomization of fully concordant situs. We also conclude that each organ can potentially make its own decision as to its laterality, since all possible combinations are represented in Table 1; however, the situs of the gut and gall bladder is fairly tightly linked.

GJC Isolation Must Extend across Midline to be Effective

Since dorsal communication and ventral isolation appear to be crucial for LR patterning, this suggests that a large-scale open communication path across the dorsal and lateral part of the embryo, terminating in the ventral cells (red arrow in Fig. 4A), is important in LR asymmetry. To test whether proper LR asymmetry is in fact due to the integrity of the full path (across the whole dorsal and lateral embryonic field), we compared bilateral injections (Fig. 4) to single-sided injections (Fig. 5). When Cx26 was injected on the right or left sides only of the ventral half of the four- or eight-cell embryo, no significant levels of heterotaxia were induced (3 and 5%, respectively, compared to 6% for control β-galactosidase injections). There was no significant difference between left or right injections (see legend to Fig. 5 for statistical analysis). In contrast, bilateral injections induced a 32% incidence of heterotaxia. Single-sided injections should not induce communication because functional gap junctions require connexins in the membranes of both cells. Therefore, since only bilateral, but not single-sided,

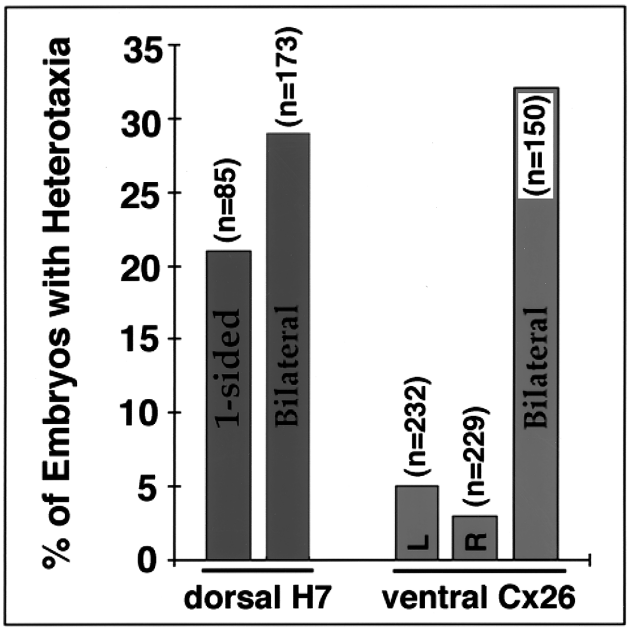


FIG. 5. Proper LR patterning depends on patterns of GJC across the embryonic midline. In contrast to the bilateral injections (Fig. 4), single-sided injections of Cx26 do not result in heterotaxia. When left- or right-side ventral animal blastomeres are injected with Cx26 constructs, the incidence of heterotaxia is insignificant compared to controls: 5% (χ^2 =0.08, P = 0.774) and 3% (χ^2 = 2.17, P = 0.14), respectively. In contrast, when 120 pg of H7 is injected into either left- or right-side dorsal cells, approximately 21% (χ^2 = 18.1, $P = 2 \times 10^{-5}$) of the embryos are heterotaxic.

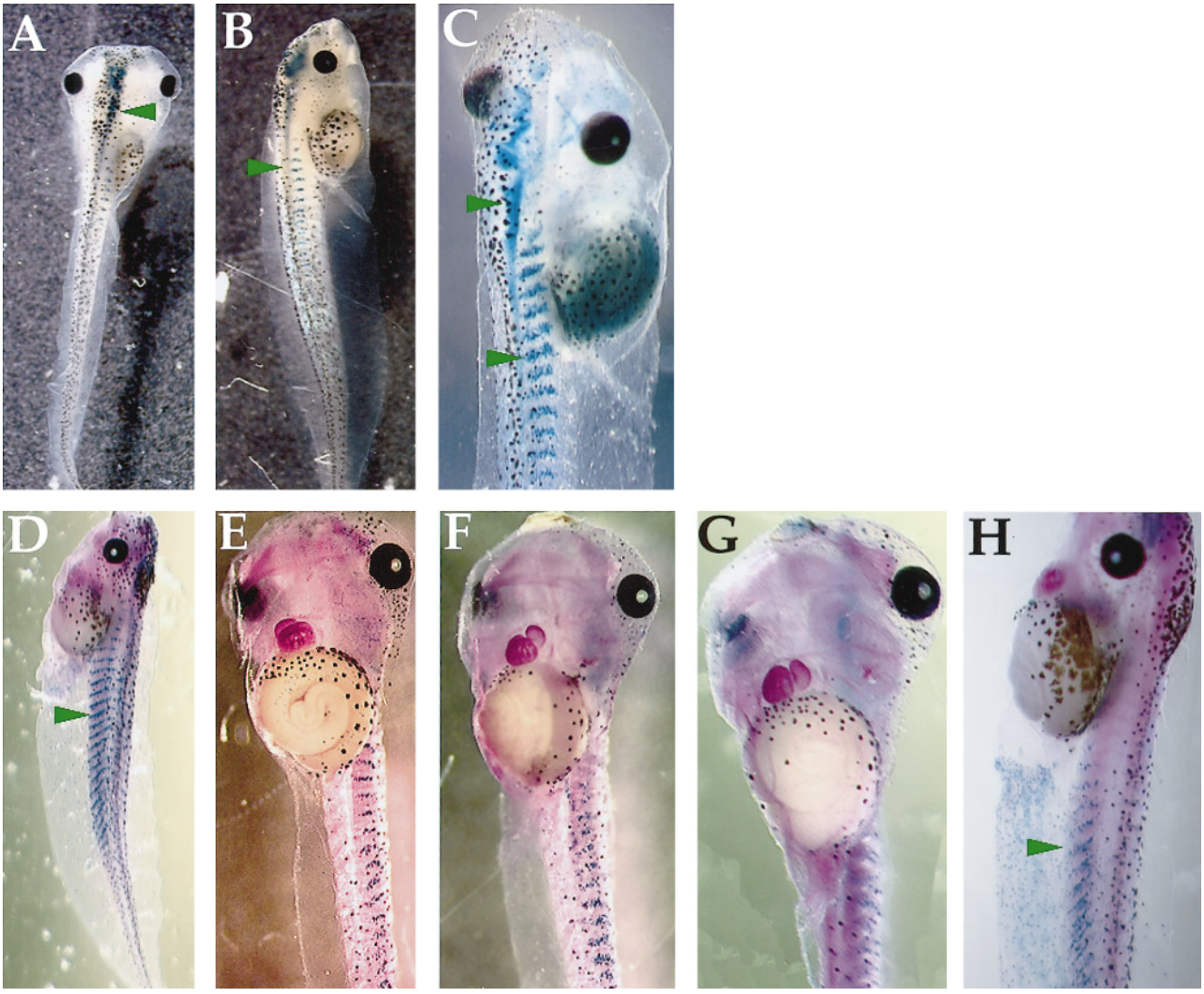
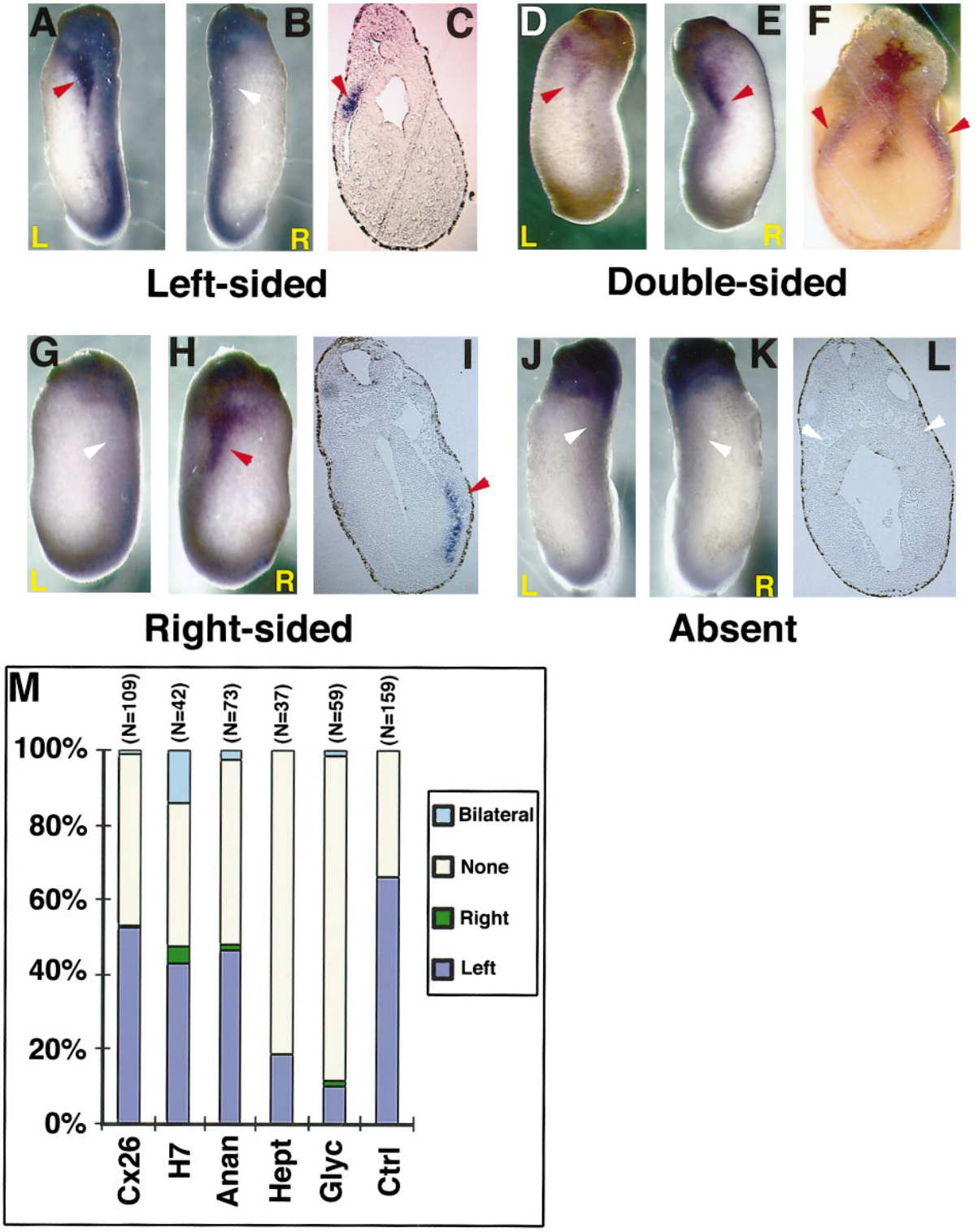


FIG. 6. Lineage analysis of injected cells shows that they do not contribute to asymmetric organs. Embryos were injected with synthetic mRNAs (as in Fig. 4) and developed for β-galactosidase expression. Nuclear blue stain (indicated with green arrowheads) indicates cells which received injected mRNA. Dorsal injections (A–C) usually result in expression in the notochord and neural tube, and the dorsal halves of somites (green arrowheads), with an occasional contribution to the heart. Ventral injections (D–H) result in expression in the ventral half of somites (green arrowheads), with occasional stain in the gut. In more than 80% of the embryos with reversed organ situs, no β-galactosidase stain was observed in the heart, stomach, or gall bladder (e.g., F and G, compare to E). This determination was made in embryos whose hearts were unstained with MF20 antibody, to ensure that any β-galactosidase expression would be detected and not masked by red stain.

FIG. 7. GJC is upstream of *XNR-1* expression. Embryos were injected with synthetic mRNAs (as in Fig. 4) or exposed to drugs until st. 20 (as in Fig. 2), allowed to develop to st. 21–24, fixed, and processed for *in situ* hybridization with a probe for *XNR-1*. In 66% of wild-type embryos, stain is seen in the left flank (A), but not in the right (B). C shows the same in section. Double-sided (D–F) and right-sided (G–I) stain was not observed ($n = 159$). Stain could not be detected in 34% of the embryos (J–L). In contrast, embryos injected with Cx26 mRNA showed incidences of 52, 1, 46, and 1% of left-sided, right-sided, absent, and bilateral expression, respectively ($n = 109$). This is statistically different from the control embryos to $\chi^2 = 4.55$, $P = 0.03$. Embryos injected with Cx43 exhibited 68, 0, 27, and 5% of left-sided, right-sided, absent, and bilateral expression, respectively ($n = 78$). This effect was not statistically significant ($P = 0.8$). H7 injections resulted in 43, 5, 38, and 14% of left-sided, right-sided, absent, and bilateral expression, respectively ($n = 42$). This distribution of phenotypes is



statistically different from the control embryos to $\chi^2 = 6.57$, $P = 0.01$. Exposure to anandamide resulted in 47, 1, 49, and 3% of left-sided, right-sided, absent, and bilateral expression, respectively ($n = 37$). This is statistically different from the control embryos to $\chi^2 = 7.1$, $P = 0.008$. Exposure to heptanol resulted in 19, 0, 81, and 0% of left-sided, right-sided, absent, and bilateral expression, respectively ($n = 37$). This distribution is different from the control to $\chi^2 = 25.32$, $P = 4.85 \times 10^{-7}$. Glycyrrhetinic acid exposure resulted in 10, 2, 86, and 2% of left-sided, right-sided, absent, and bilateral expression, respectively ($n = 59$). This distribution is different from the control to $\chi^2 = 51.53$, $P = 7.05 \times 10^{-13}$. In A–L, red arrowheads indicate *XNR-1* expression domains, white arrowheads indicate lack of domain, and yellow letters "L" and "R" indicate left- and right-side views. M summarizes these numerical data.

injections caused heterotaxia, we conclude that junctional isolation across the midline is required for LR patterning. In contrast to wild-type connexins, the dominant negative H7 is active even when present on only one side of the membrane (Paul *et al.*, 1995). Consistent with this, single-sided injections of H7 on the dorsal side also produce significant levels of heterotaxia (21%, compared to 29% for bilateral injections and 2% for β-galactosidase injections).

Connexin mRNA Microinjection Causes Heterotaxia without Expression within Asymmetric Organs

To assess the contribution of cells which receive injected mRNA to the embryo, all mRNA injections included β-galactosidase mRNA. Analysis of β-galactosidase stain in st. 45 embryos with heterotaxia reveals that, consistent with established fate maps (Dale and Slack, 1987), dorsal injections labeled neural tube and notochord, as well as the dorsal halves of somites (Figs. 6A–6C). There was stain in the heart in approximately 15% of the cases. Ventral injections usually stained some ventral ectoderm and the ventral halves of somites (Figs. 6D and 6H), with an occasional (<20% of the cases) contribution to the gut. Many embryos resulting from dorsal injections were observed with complete situs inversus or heart reversal and absolutely no stain in the affected organs. Thus, we conclude that of embryos showing heterotaxia, the large majority has no contribution of cells expressing injected constructs within any of the asymmetric organs themselves.

GJC Functions Upstream of XNR-1 Expression in the LR Pathway

The only member of the asymmetric gene cascade in chicks which has also been described as asymmetrically expressed in *Xenopus* is *XNR-1,* the homolog of the left-sided TGF-β-related gene *nodal.* Aberrations in expression of this gene have been correlated with disturbances of *situs* in mice and frogs (Levin *et al.*, 1995; Collignon *et al.*, 1996; Lowe *et al.*, 1996). Moreover, misexpression of *nodal* on the right side of chicks (Levin *et al.*, 1997) and frogs (Sampath *et al.*, 1997) results in *in situs* defects including heart reversals. Thus, *XNR-1* is very likely to be a component of the LR signaling pathway upstream of morphological *situs.*

To begin to place the role of GJC within the events upstream of organ *situs,* we sought to determine whether GJC was upstream of *XNR-1* expression. Embryos were injected with Cx26 or H7 or exposed to the GJC-modifying drugs as above. Embryos were then allowed to develop to st. 21–24, fixed, and assayed for sidedness of *XNR-1* expression by *in situ* hybridization. The results are summarized in Fig. 7M.

Control embryos showed a 66% incidence of normal left-sided *XNR-1* expression (Figs. 7A–7C), with no stain (Figs. 7J–7L) detectable in 34% of the embryos ($n = 159$). No bilateral (Figs. 7D–7F) or right-sided (Figs. 7G–7I) *XNR-1* expression was observed. In contrast, embryos injected with Cx26 or H7 and embryos exposed to any of the GJC-modifying drugs exhibited absent, right-sided, and bilateral expression of *XNR-1* more frequently. These effects were statistically significant to P values between 0.05 and 7×10^{-13} and were strongest for glycyrrhetinic acid exposure (see Fig. 7M and legend for statistical data and analysis). The most frequent phenotype was absence of *XNR-1* expression (right isomerism). Since disruptions of GJC states caused alterations of the normal left-sided pattern of *XNR-1* expression, we conclude that GJC is upstream of that expression.

Connexin Mutation Found in Human Patients with Situs Anomalies Causes Heterotaxia in Xenopus Embryos

Having examined some aspects of the role of gap junctional communication in LR patterning, we then asked whether mutant forms of wild-type connexins had similar effects. One interesting case is a mutation of human Cx43. Mutation of serine^{364} to proline in a putative regulatory domain of Cx43 has been reported to occur in several unrelated human patients with heterotaxia (Britz-Cunningham *et al.*, 1995), although whether such a mutation causes laterality disturbances has never been tested.

We tested the possible functional characteristics of such a connexin by injecting mRNA for the human Cx43^{Ser364Pro} generated by site-directed mutagenesis (Britz-Cunningham *et al.*, 1995) into *Xenopus* embryos at the four- or eight-cell stages (Fig. 8). When injected ventrally, where cells are in junctional isolation, Cx43^{Ser364Pro} led to a 16% incidence of heterotaxia. This is significant (see legend to Fig. 8 for statistical analysis), but somewhat less than caused by the wild-type Cx43 (22%). This suggests that Cx43^{Ser364Pro} is able to function as a connexin, albeit not as well as the wild-type form. Surprisingly, when misexpressed dorsally, where cells are already in good GJC, the mutant connexin also caused a significant incidence of heterotaxia (23%). In this it resembles the dominant negative, which caused a 29% incidence. Thus, we conclude that the Ser^{364}Pro mutant of human Cx43 mimics the dominant negative effect of H7 when misexpressed dorsally.

DISCUSSION

We examined the difference in gap junctional conductance between ventral and dorsal cells of the cleavage stage *Xenopus* embryo and found that ventral cells are relatively isolated, while dorsal cells were in good GJC with each other. The fact that not all of the embryos showed GJC on the dorsal side may be due to the fact that identification of the dorsal side by pigmentation is far from 100% accurate. We showed that drugs which modify gap junctional states in cell culture have the same effect when applied to the *Xenopus* embryo, as detected by increases or decreases of selective intercellular transfer of LY. The fact that melato-

nin exposure increases GJC on the ventral side suggests that the relative ventral isolation is not due to an absence of gap junctions ventrally but rather to gap junctions which are regulated to be closed. When applied prior to the neural fold stage, these drugs caused heterotaxia involving the heart, stomach, and gall bladder. The fact that heterotaxia was induced by a broad range of drugs which have in common only their effect on GJC and occurs in the absence of other defects or alterations of normal dorsoanterior development suggests that endogenous GJC is involved in LR patterning. Although highly statistically significant, the incidence of heterotaxia induced by these reagents was less than what would be expected if the situs of each of the three organs were completely and independently randomized (87.5%). This is most likely because we used doses of drugs and mRNA (see below) which were low enough to cause no developmental defects other than laterality phenotypes.

Interestingly, these drugs had no effect on situs when applied after the neural fold stage. The fact that the sensitive period is well before the generation of the organ primordia suggests that these reagents affect a basic and early LR system and not the morphogenesis of the asymmetric organs per se. Specifically, GJC-modifying drugs had their greatest effect between st. 5 and 12. In *Xenopus,* this time period includes cleavage stages as well as the midblastula transition and gastrulation. It also precedes specification of left-sided *XNR-1* expression (after st. 15–16 but before st. 20; Lohr *et al.,* 1996; Levin and Mercola, 1998) and specification of left–right orientation of the heart (st. 19–22; Danos and Yost, 1996). For comparison, actual left-sided *XNR-1* expression is first detected considerably later (st. 23–24) and heart looping occurs thereafter (st. 33–36). Importantly, exposure to the GJC-modifying drugs affected all organs examined, providing further evidence that GJC is required for early formation of overall LR pattern.

Targeted expression of wild-type and dominant negative connexins allowed us to address the importance of the specific dorsoventral difference in GJC found in embryos. Induction of GJC by wild-type connexins ventrally, where cells are normally isolated led to heterotaxia, while injection of wild-type connexins dorsally (where GJC is already present) had no effect. Conversely, inhibition of GJC by the H7 dominant negative in dorsal cells also led to heterotaxia, while injection of H7 ventrally had no effect. In both cases, heterotaxia was induced in the absence of other defects; in particular, dorsoanterior development [in which alterations have been shown to produce LR abnormalities in their own right (Danos and Yost, 1995)] is normal (DAI = 5). Heterotaxia caused by dorsal H7 expression was rescued by coinjection of a construct expressing a connexin that is not blocked by the dominant negative (Cx37, Fig. 4). These observations strongly suggest that the normal pattern of dorsal GJC and ventral junctional isolation are crucial for correct LR patterning.

Following the fate of injected blastomeres indicated that these cells generally did not contribute to the asymmetric organs. Combined with the sensitivity window identified by the drug study, this result suggests that heterotaxia was caused by a perturbance of overall LR pattern rather than by a local effect on an individual organ, consistent with the conclusion that GJC is needed to orient the LR axis prior to the onset of organogenesis.

The cell fate analysis also suggests that GJC is required only in certain tissues. Targeted injections combined with lineage analysis, as we have done, do not provide the spatial resolution necessary to map the crucial tissues in which specific GJC states are needed. This is because the injections need to be done at the four- to eight-cell stage to achieve uniform overexpression of either wild-type or H7 protein. Experiments using tissue-specific promoters in transgenic embryos will be invaluable in determining the precise spatial requirements for endogenous GJC.

We have also examined the expression pattern of *XNR-1,* normally present in the left lateral mesoderm (Lowe *et al.,* 1996), following drug treatment or connexin misexpression. Based on the timing window shown in our drug experiments, GJC would be expected to play a role upstream of *XNR-1* in LR patterning. Relative to controls, such embryos generally showed an increase in the incidence of loss of *XNR-1* expression (right isomerism with respect to *XNR-1*), as well as less frequently observed bilateral or right-sided *XNR-1* expression (Fig. 7). The relatively low frequency of bilateral and right-sided *XNR-1* expression is likely to reflect the fact that low doses of the drugs and mRNA were used to avoid the confounding influence of altered embryonic DAI. Thus, consistent with the timing and tissue lineage data, GJC appears to be upstream of asymmetric *XNR-1* expression. Interestingly, we observed that no stain may be detected in up to one-third of control embryos, siblings of which all (to 99%) develop correct LR symmetry. The reason for the imperfect concordance between correct *XNR-1* expression and morphological situs is unclear; however, it may be specific to the frog, as this phenomenon does not occur in chicks (unpublished data). One contributing factor is likely to be the imprecise temporal profile of *XNR-1* expression in frogs, where embryos at apparently equivalent morphological stages can show early or late phases of *XNR-1* expression. More specific placement of GJC in the LR gene cascade awaits characterization of other asymmetric genes in *Xenopus.*

In addition to the effects of wild-type and dominant negative connexins, we examined the functional activity of a mutation in Cx43. The $Ser^{364}Pro$ mutation, located in a putative control region of human Cx43, has been reported to occur in several unrelated human patients exhibiting visceroatrial heterotaxia (Britz-Cunningham *et al.,* 1995). However, other individuals with laterality defects apparently do not have this or other mutations in the *Cx43* gene (Casey and Ballabio, 1995; Gebbia *et al.,* 1996); this discrepancy may be due to the fact that aberrations in LR patterning can surely arise from mutations in many different genes. Thus, it is currently unclear whether $Cx43^{ser364pro}$ contributes to laterality defects in humans.

Nonetheless, Cx43 is likely to mediate numerous pro-

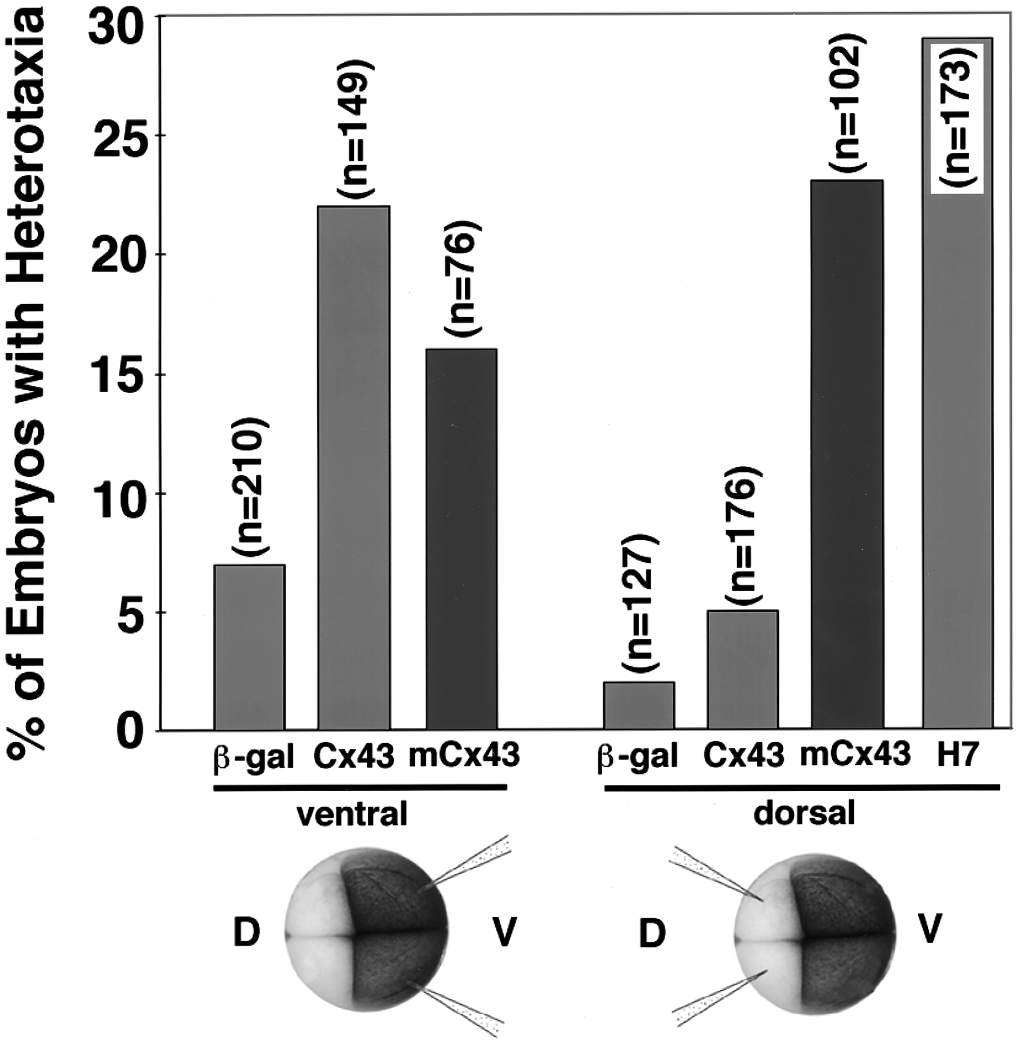


FIG. 8. A mutant form of Cx43, $Cx43^{ser364pro}$, induces heterotaxia. Embryos at the four- or eight-cell stages were injected with 430 pg of synthetic mRNA encoding human $Cx43^{ser364pro}$ and scored for organ situs at stage 45. Injections at the four- or eight-cell stage into relatively isolated ventral animal cells result in 16% incidence of heterotaxia ($\chi^2 = 4.57$, $P = 0.03$). Surprisingly, injections into dorsal animal cells (where there is already high endogenous GJC) also show a statistically significant incidence of heterotaxia (23%, $\chi^2 = 20.94$, $P = 4.7 \times 10^{-6}$). This suggests that the $Cx43^{ser364pro}$ mutation is an antimorphic as well as a hypomorphic protein.

cesses, in particular aspects of heart development, which is suggested by phenotypes of mutants lacking functional Cx43 (Reaume *et al.*, 1995) or overexpressing Cx43 under the control of a CMV promoter (Ewart *et al.*, 1997). Likewise, changes in gap junctional communication have been directly implicated in nongenotoxic tumor promotion (Hotz-Wagenblatt and Shalloway, 1993; Yamasaki *et al.*, 1995; Krutovskikh and Yamasaki, 1997). Thus, studies of protein level regulation of connexin function are important aside from left–right asymmetry.

The *Xenopus* system allowed us to examine one aspect of the structure–function relationship of *Cx43*. Ventral injection of $Cx43^{ser364pro}$ induced heterotaxia, suggesting that the mutant protein does produce functional junctions. That the resulting incidence is less than that caused by injections of wild-type Cx43 suggests that the junctions are not as effective as those made by wild-type Cx43 [as was also observed in cell culture (Britz-Cunningham *et al.*, 1995)]. Unexpectedly, heterotaxia is also caused by dorsal injection, where cells normally exhibit high GJC and where injection of wild-type connexins has no effect. Since dorsal expression of $Cx43^{ser364pro}$ had the same effect as H7, we suggest that the mutant protein interferes with endogenous conductance (albeit somewhat less so than H7). This implies that the $Ser^{364}Pro$ substitution is an antimorphic (dominant negative) as well as a hypomorphic mutation.

A dominant negative effect would predict that the $Cx43^{+/ser364pro}$ and $Cx43^{-/-}$ phenotypes would differ. Homozygous null mice [which exhibit obstruction of the cardiac outflow tract (Reaume *et al.*, 1995)] may survive fetal development because of maintenance of critical GJC by other connexins, either because of normal overlapping expression or by compensatory ectopic expression. In contrast, the $Cx43^{ser364pro}$ antimorphic activity might interfere not only with the function of wild-type Cx43 but also with other connexins in heterozygous $Cx43^{+/ser364pro}$ embryos. This could result in a broad range of phenotypes, including laterality defects. Such a dominant effect is also seen in a mutant Cx32 in Charcot-Marie-Tooth syndrome, and a similar mechanism has been proposed to account for it (Omori *et al.*, 1996).

Our functional experiments clearly showed that DV differences in GJC in early embryos are important in LR patterning. Moreover, to affect laterality, wild-type connexins must be expressed on both sides of the ventral midline, indicating that the generation of left–right pattern requires junctional isolation across the midline. In contrast, left or right misexpression of H7 caused heterotaxia. Based on these findings, we propose that an open junctional path across the entire dorsal side which terminates in ventral isolated cells is crucial for proper LR patterning.

This suggests a model by which large-scale (multicellular) asymmetry, a prerequisite to the asymmetric expression of genes such as *nodal*, can arise from subtle asymmetries at the level of a single cell and likewise, suggests a mechanism for orienting the LR axis with respect to the DV axis. Differences in GJC are set up after the initial patterning of the DV axis, as a likely consequence of signaling through the β-catenin–TCF/Lef pathway (Olson and Moon, 1992). Figure 9A shows a general model for how these differences can then be used to orient the LR axis. We postulate that asymmetric circumferential flow of small molecules (LR morphogens) leads to a segregation of determinants between left and right sides of the embryo (appearance of the blue to red gradient in Fig. 9A). This model at once combines aspects of linkage of DV to LR, as well as chirality and reflectional LR asymmetry.

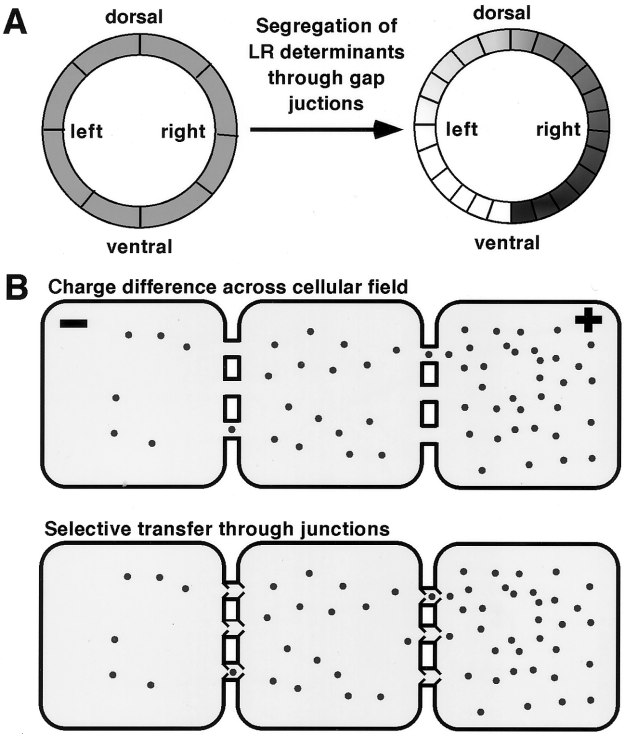


FIG. 9. Models for asymmetric transfer of LR morphogens through gap junction paths. Small determinants are envisioned to pass between cells such that they become selectively located to one side of the midline (segregation of black to the right side of the embryo, diagrammed in transverse section, in A). An important feature of the model is that communication must be restricted across the ventral midline. B shows two ways to achieve asymmetric transfer of small LR morphogens through gap junctions. A potential difference across a field of cells would result in a current flow through the conductive path. Thus, charged small molecules (black dots) would preferentially be electrophoresed in one direction. In contrast, a charge gradient is unnecessary if the gap junctions themselves are directional.

Asymmetrical morphogen flow (segregation of red determinants) can arise in several ways. We consider two possibilities here (Fig. 9B). The first is that a potential difference exists across a field of connected cells. Electrical potential differences have been detected between many kinds of embryonic cells, including *Xenopus* blastomeres (Palmer and Slack, 1970; Woodruff and Telfer, 1980; Marx, 1981; Hotary and Robinson, 1994; Metcalf and Borgens, 1994; Shi and Borgens, 1994, 1995). Given such a potential difference across the midline of an embryo, small charged molecules would be electrophoresed through the open GJC path and accumulate on one or the other side (depending on charge). A similar process is believed to occur in the lens of the eye, which maintains circumferential water and ionic currents by means of gap junctions (Mathias *et al.*, 1997).

An alternative model is more passive. Diffusion through gap junction channels (schematized by gates on each junction in Fig. 9B) could preferentially allow passage of small molecules in one direction. Such junctions have been found in other systems (Flagg-Newton and Loewenstein, 1980; Robinson *et al.*, 1993; Nedergaard, 1994; Zahs and Newman, 1997). In this scenario, diffusion-driven passage through asymmetric junctions oriented across the midline would serve to concentrate small molecules to the left or right side of the embryo. Of course many other models are possible. A common feature of these and other models is that DV differences in GJC restrict the flow of LR morphogens such that they partition across the midline. This asymmetry can then be transduced through induction and repression pathways to yield fields of asymmetric gene expression (Levin, 1997).

For either of these models, it is important to note that

while DV differences in GJC are necessary for the patterning of LR asymmetry, they are not, by themselves, sufficient to be the primary step of LR asymmetry. A prior asymmetry must exist to orient asymmetric junctions or to consistently assert potential differences between the left and right cells. Rather, GJC is likely to act downstream of such a mechanism and serve to impose cell-autonomous chiral information on cell fields.

The nature of the small molecule(s) serving as the LR morphogen is unknown; however, some common molecules believed to pass through gap junctions are Ca^{2+} (and other ions), cAMP, charged lipids, and oligonucleotides (Saez *et al.*, 1989; Giaume *et al.*, 1997). Precedents for morphogens which depend on GJC include the head-inducing factor in hydra (Fraser *et al.*, 1987) and the mesoderm-inducing factor in snails (deLaat *et al.*, 1980). Work is in progress to identify the factors which pass through gap junctions in patterning the LR axis and the tissues which they must traverse.

ACKNOWLEDGMENTS

We thank David Paul, Dan Goodenough, and Yosef Landesman for connexin constructs and many helpful discussions. We also thank S. H. Britz-Cunningham, M. M. Shah, and W. H. Fletcher for the $Cx43^{ser364pro}$ construct and members of the Mercola lab and Debbie Goff for their very helpful comments on the manuscript. This work was supported by the American Heart Association, NIH (HL59502), and the Council for Tobacco Research. The CT3 antibody developed by D. A. Fischman was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, University of Iowa (Iowa City, IA), under Contract N01-HD-7-3263 from the NICHD.

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Received for publication July 7, 1998
Accepted July 14, 1998