Evidence for the Regulation of Left-Right Asymmetry in Ciona intestinalis by Ion Flux

Sebastian M. Shimeld and Michael Levin

Vertebrate embryos develop distinct left-right asymmetry under the control of a conserved pathway involving left-sided deployment of the nodal and Pitx2 genes. The mechanism that initiates asymmetric expression of these genes is less clear, with cilia, ion flux, and signalling molecules all implicated. Vertebrates share the chordate phylum with urochordates such as the sea squirt Ciona intestinalis. We have explored the role of ion flux in regulating left-right asymmetry in Ciona, using an assay in which perturbation of left-sided Ci-Pitx expression provides a read-out for the disruption of asymmetry. Our data show that omeprazole, which specifically inhibits H⁺K⁺ ATPase activity, disrupts asymmetry in Ciona. The vertebrate H⁺K⁺ ATPase is composed of two subunits, α and β. We identified one Ciona β ortholog and two Ciona α orthologs of the vertebrate H⁺K⁺ ATPase genes, and show that one of these is expressed in dorsal and ventral embryonic midline cells shortly before the activation of left-sided Ci-Pitx expression. Furthermore, we show that omeprazole exerts its effect on asymmetry at this point in development, and additionally implicate K⁺ channels in the regulation of asymmetry in Ciona. These experiments demonstrate a role for ion flux in the regulation of asymmetry in Ciona, and show a conserved, ancestral role for the H⁺K⁺ ATPase ion pump in this process. Developmental Dynamics 235:1543–1553, 2006. © 2006 Wiley-Liss, Inc.

Key words: asymmetry; Pitx; Ion pump; Ciona; protochordate; evolution

INTRODUCTION

Bilateral symmetry is a fundamental feature of many animal phyla, and is currently used to define a major taxonomic grouping, the Bilateria. Most members of the Bilateria, however, deviate from bilateral symmetry in a predictable manner. This is known as directional left-right (LR) asymmetry, and has been documented in arthropods, annelids, molluscs, vertebrates, and many other phyla (Palmer, 1996). The regulation of LR asymmetry has been studied in representatives of several phyla, and an intriguing finding that has emerged from this work is that, unlike the anteroposetrior and dorsoventral axes, there is currently little evidence for conservation of the genes or developmental processes regulating LR asymmetry in different phyla (for example see Palmer, 1996; Delattre and Felix, 2001; Hayashi and Murakami, 2001; Ligoxygakis et al., 2001). One interpretation of this lack of conservation is that directional asymmetry may have evolved repeatedly (Palmer, 1996, 2004).

Many studies have investigated the molecular control of LR asymmetry in vertebrates (reviewed by Levin, 2005). One key finding of these studies is that the homeobox transcription factor Pitx2 and the secreted signalling molecules nodal and lefty play a critical role in establishing differences between the left and right sides. Nodal gene expression is activated on the left side of the node, and appears to initiate a transcriptional cascade that results in the activation of nodal and Pitx2 gene expression to the left side of the embryonic

1Department of Zoology, University of Oxford, Oxford, United Kingdom
2Cytokine Biology Department, The Forsyth Institute & Developmental and Craniofacial Biology Department, The Harvard Medical School, Boston, Massachusetts

Grant sponsor: American Cancer Society; Grant number: Research Scholar Grant RSG-02-046-01; Grant sponsor: National Institute of Health; Grant number: 1-R01-GM-06227.

*Correspondence to: Sebastian M. Shimeld, Department of Zoology, University of Oxford, Tinbergen Building, South Parks Road, Oxford OX1 3PS, UK. E-mail: sebastian.shimeld@zoo.ox.ac.uk

DOI 10.1002/dvdy.20792
Published online 29 March 2006 in Wiley InterScience (www.interscience.wiley.com).
midline (Levin et al., 1995; Brennan et al., 2002; Saijoh et al., 2003). A central focus of recent studies has been the mechanisms that result in the initial asymmetric activation of *nodal*. In mouse embryos, node cells have an unusual sort of primary cilia. Unlike most primary cilia, which are immobile, these beat in a co-ordinated manner to generate a flow of liquid across the node, and experimental evidence suggests interfering or reversing this flow can predictably disrupt the development of asymmetry (Nonaka et al., 1998, 2002). Consistent with this are the results of several genetic studies, which show that mice mutant for intracellular components related to ciliary function have defects in LR asymmetric development (Supp et al., 1997; Marszalek et al., 1999; Okada et al., 1999; Takeda et al., 1999; Pennenkamp et al., 2002; Yoder et al., 2002). Cilia have been detected on the cells of the node equivalents of other vertebrates. However, alternative explanations for the role of nodal cilia in vertebrate asymmetry have been proposed (McGrath et al., 2003; Tabin and Vogan, 2003; Essner et al., 2005; Kawakami et al., 2005; Kramer-Zucker et al., 2005; Okada et al., 2005).

Additional evidence implicates the molecules that regulate ion passage across cell membranes in the regulation of asymmetry. Levin et al. (2002) identified an H⁺K⁺ATPase, the mRNA encoding which became asymmetrically localised during early cleavage stages in *Xenopus*. Pharmacological or genetic perturbation of the expression of the genes encoding this molecule in both *Xenopus* and chick disrupted LR asymmetry (Levin et al., 2002). Additional evidence suggests, at least in chick embryos, that asymmetric activity of the H⁺K⁺ATPase during gastrulation may result in the asymmetric localisation of extracellular Calcium ions, which in turn interact with the Notch pathway to stabilise asymmetric *nodal* expression (Raya et al., 2004).

Vertebrates share phylum Chordata with two other subphyla, the Cephalochordata (including amphioxus) and the Urochordata (ascidians and allies), known collectively as protochordates. The chordates form part of the deuterostomes, a taxon they share with the hemichordates and echinoderms. All members of the Urochordata and Cephalochordata have a characteristic chordate body plan at some point in their life cycle, including a dorsal, hollow neural tube and a notochord. Both also develop asymmetries that can be directly related to vertebrate asymmetries (Boorman and Shimeld, 2002a). Single *Pitx* and *nodal* genes have been characterised in amphioxus and ascidians, and both are expressed on the left side of the embryo at a developmental stage where the chordate body plan has been established (Yasu et al., 2000; Boorman and Shimeld, 2002b; Morokuma et al., 2002; Yu et al., 2002). Recently, omeprazole has been shown to disrupt LR asymmetry in sea urchins, suggesting ion flux may be conserved in all deuterostomes (Duboc et al., 2005).

In this report, we examine the role of ion flux in LR asymmetry of the ascidian *Ciona intestinalis* (hereafter referred to as *Ciona*). Our results demonstrate that pharmacological manipulation of ion flux can perturb LR asymmetric gene expression in *Ciona* embryos. Furthermore, they implicate the *Ciona* ortholog of the vertebrate H⁺K⁺ATPase in this process.

**RESULTS**

**Dechorionation Disrupts Asymmetric Gene Expression in *Ciona* Embryos**

In order to test the hypothesis that roles of ion flux in LR asymmetry extend to protochordates, we sought to establish an assay of asymmetry in embryos in which specific ion flows had been inhibited. *Ciona* eggs are enclosed by a chorion and, exterior to the chorion, by numerous small follicle cells (Fig. 1A). Removal of the chorion is a critical part of many established protocols for manipulating *Ciona* embryos, including blastomere ablation, transplantation, and electroporation. Test cells, which are maternally derived but differ from follicle cells and have a role in early tunic formation, come to lie between the chorion and the egg. Fertilised eggs develop within the chorion until the early larval phase, when hatching occurs. Fertilised eggs can be chemically dechorionated (Mita-Miyazawa et al., 1985), and develop normally up to the tailbud stage. Metamorphosis of dechorionated larvae is, however, typically abnormal, and this has been suggested to be caused by the absence of test cells, which are lost during dechorionation (Sato and Morisawa, 1999). We initially considered removing the chorion prior to experimentation, to improve access of pharmacological reagents to the embryo. However, since the removal of the chorion can disrupt asymmetry in some vertebrates (Fujinaga and Baden, 1991; Fischer et al., 2002), we first examined whether dechorionation disrupted asymmetry in *Ciona*. We dechorionated embryos, and allowed them to develop to the tailbud stage before fixation. Control embryos from the same batch were grown within their chorions, then dechorionated immediately prior to fixation. Both were examined for *Ci-Pitx* expression. Control embryos showed the previously reported pattern of *Ci-Pitx* expression, with transcripts localised to the buccal cavity and to left-sided epidermis (Fig. 1E) (Boorman and Shimeld, 2002b; Christiaen et al., 2002). Conversely, 84% of dechorionated embryos showed *Ci-Pitx* expression in both left and right epidermis (Fig. 1B–D) (n = 25). This result shows that dechorionation disrupts the normal asymmetric localisation of *Ci-Pitx*.

**Evidence for a Role for *Ciona* H⁺K⁺ATPase Orthologs in Left-Right Asymmetry**

Since dechorionation can disrupt asymmetry, we focused future experiments on *Ciona* embryos grown within their chorions. First, we exposed embryos to a number of compounds that affect different ATP-dependent ion pumps (Fig. 2A). In vertebrate embryos, it is important to consider the status of the midline in such experiments, as midline tissues act as barriers between left and right sides. Disruption of midline development leads to secondary disruption of LR asymmetry. It is also important to avoid the generalised teratogenic effects many compounds will induce if used at sufficiently high concentration. Consequently, all compounds
were screened in preliminary experiments to identify concentrations below which embryonic development appeared normal (with normal germ layer development and midline tissue morphogenesis) but above which non-specific developmental defects were observed; 4, the effect on development if the threshold concentration of the reagent were exceeded; 5, whether ectopic induction of Ci-Pitx expression was observed. Where ectopic expression was observed, the P value indicates the results of the chi-squared test to determine significance when compared to controls (not shown).

Fig. 2A: Compounds used in the initial screen and their activity. The columns from left to right are: 1, the name of the reagent tested; 2, the molecules predicted to be affected by the compound; 3, the concentration above which non-specific developmental defects were observed; 4, the effect on development if the threshold concentration of the reagent were exceeded; 5, whether ectopic induction of Ci-Pitx expression was observed. Where ectopic expression was observed, the P value indicates the results of the chi-squared test to determine significance when compared to controls (not shown). B,C: Examples of ectopic Ci-Pitx expression induced by exposure to omeprazole. Both embryos are at the tailbud stage and in dorsal view with anterior to the left. Embryos treated with ouabain or concanamycin A that developed ectopic Ci-Pitx expression were also similar to these examples.

Fig. 1. Dechorionation disrupts LR asymmetric expression of Ci-Pitx. A: Unfertilised Ciona egg showing the oocyte (o), chorion (c), and spikes of follicle cells (f). B,C: Embryos cultured following dechorionation shown in dorsal aspect with anterior to the left. Expression of Ci-Pitx is on both sides of the midline. D: Lateral view of embryo shown in B above, with anterior to the right, indicating normal AP and DV development, and activation of Ci-Pitx expression in the buccal cavity (bc), a normal site of expression (Boorman and Shimeld, 2002b; Christiaen et al., 2002). E: Dorsal view with anterior to the left of an embryo grown within its chorion. Note the left (L) sided restriction of Ci-Pitx. R, right.

Fig. 3. The effect on Ci-Pitx expression of different omeprazole concentrations. Embryos were incubated in their chorions from fertilisation, and scored by in situ hybridisation at the tailbud stage. The incidence of both ectopic right-sided expression and the absence of expression increase as omeprazole concentration increases. Numbers of embryos scored were as follows: Control, 90 embryos; 4 μg/ml, 81 embryos; 10 μg/ml, 87 embryos; 20 μg/ml, 111 embryos; 40 μg/ml, 69 embryos. The effective concentration is similar to that observed for Xenopus (Levin et al. 2002).
trol of excess stomach acid production (Vakil, 2003). We then conducted a more focused experiment, exposing embryos to different concentrations of omeprazole from fertilisation to the tailbud stage, at which point they were dechorionated, fixed, and assayed for Ci-Pitx expression. Embryos exposed to 4 µg/ml (n = 81) or 10 µg/ml (n = 87) omeprazole were not significantly different from controls (Fig. 3). Embryos exposed to 20 µg/ml (n = 111) or 40 µg/ml (n = 69) omeprazole showed a significantly increased disruption of asymmetric Ci-Pitx expression, such that at 40 µg/ml over 40% of embryos had ectopic right-sided epidermal Ci-Pitx expression (P < 0.01). Other aspects of embryonic development, including AP and DV morphology, midline tissue morphogenesis, and the expression of Ci-Pitx in the buccal cavity, appeared normal in these embryos, indicating the effect of the treatment was specific to LR development. This suggests that the Ciona equivalent of the vertebrate H+K+ATPase is involved in the development of LR asymmetry.

**Ciona Orthologs of the Vertebrate H+K+ATPase Genes**

In chick and Xenopus embryos, an H+K+ATPase has been implicated in the early development of asymmetry (Levin et al., 2002). The H+K+ATPase consists of two subunits, α and β. Vertebrate H+K+ATPase subunit genes share sequence identity with Na+K+-ATPases, which have been identified in several invertebrate species and are present in multiple copies in vertebrate genomes. We searched Ciona cDNA and genomic resources (Dehal et al., 2002; Satou et al., 2002) for genes with similar sequence to both subunits from both H+K+ATPase and the Na+K+ATPases. Our searches identified two α subunits and one β subunit (Fig. 4A,B). Similar searches of the genome of the congeneric urochordate Ciona savignyi (http://www.broad.mit.edu/annotation/ciona/background.html) identified the same gene complement, indicating we had identified the full set of subunit genes in Ciona (data not shown). To determine the evolutionary relationships of these vertebrate and invertebrate genes with the Ciona genes, we conducted molecular phylogenetic analyses. These showed that the Ciona α subunit genes were closely related to each other, and that both were basal to all the vertebrate H+K+ATPase and Na+K+ATPase α subunit genes (Fig. 5A). Similarly, the Ciona β subunit gene was basal to all the vertebrate H+K+ATPase and Na+K+ATPase β subunit genes (Fig. 5B). This demonstrates that the vertebrate H+K+ATPase and multiple Na+K+ATPase genes have evolved by gene duplications specific to the vertebrate lineage, after its separation from the lineage leading to Ciona. The C. intestinalis α and β subunit genes are, therefore, orthologous to all H+K+ATPase and Na+K+ATPase α and β subunit genes, respectively.

The expression of Ciona α and β Subunit mRNA and Protein During Embryogenesis

The Ciona EST database indicates that the Ci-αA and Ci-β subunit genes are expressed throughout embryogenesis, while the Ci-αB subunit gene was only detected in larvae and in adult tissues (Satou et al., 2002). We examined the distribution of Ciona α and β subunit protein using antibodies raised to vertebrate H+K+ATPase subunits (Matthews et al., 1995). In whole embryos, the distribution detected by both antibodies appeared enriched in the cortex or membrane on the outer surface of the embryo, consistent with a role as an ion pump (Fig. 6J,K; and data not shown). We also used in situ hybridisation to determine the pattern of expression of mRNA for all three subunit genes at key developmental stages (Fig. 6A-I). Preliminary experiments suggested the distribution of Ci-β and Ci-αA subunit mRNA was widespread, but especially prominent in the brain. Consequently, we then monitored the timing of staining to distinguish weak and strong expression (Fig. 6). Our results showed the Ci-β subunit gene and the Ci-αA subunit gene to be ubiquitously expressed from fertilisation, with expression particularly strong in the central nervous system of tailbud stage embryos (Fig. 6A–F). We did not, however, detect expression of the Ci-αB subunit gene until the tailbud stage, when transcripts were detected in ventral then dorsal midline epidermis (Fig. 6G–I).

This is in agreement with EST data for this gene (Satou et al., 2002). Notably, the expression in dorsal and ventral midline epidermis is complementary to that described for Ci-Pitx, and initiates shortly before Ci-Pitx epidermal expression is first detected (Boorman and Shimeld, 2002b).

**The Timing of Omeprazole Disruption of Left-Right Asymmetry**

In vertebrates, Xenopus H+K+ATPase appears to affect asymmetry relatively early in development, at cleavage stages, while in chick embryos, H+K+ATPase activity appears to regulate asymmetry during the beginning of gastrulation, at the elongation of the primitive streak (Levin et al., 2002; Raya et al., 2003). Ciona embryos express the H+K+ATPase subunit from fertilisation through to the time when asymmetric gene expression is established, ruling out neither of these possibilities. Similarly, the H+K+ATPase α subunit is expressed from fertilisation. The H+K+ATPase αB subunit, however, is activated in midline cells shortly before activation of asymmetric gene expression, a time and location compatible with a late role in LR development, as seen in the chick.

![Fig. 4.](http://example.com/figure4.png)
LEFT-RIGHT ASYMMETRY IN CIONA

Fig. 4.
To investigate this further, we determined the window of embryonic development when omeprazole was able to disrupt asymmetry. Embryos were treated for specific time periods prior to the tailbud stage, then dechorionated, fixed, and assayed for Ci-Pitx expression (Fig. 7). Development to the tailbud stage takes 8 hr at 18°C. Embryos exposed only during the first 2 hr of development (from 1 cell to 16 cells) or the second 2 hours (16 cells to early gastrula) were not significantly different from controls (Fig. 7). Embryos exposed continuously for the first 4 hr of development were different from controls, with a small but significant increase in ectopic Ci-Pitx expression (0.05 < P < 0.01; Fig. 7). Embryos whose exposure included the 4- to 6-hr window but not the 6- to 8-hr window showed a significant increase in ectopic Ci-Pitx expression (P < 0.01). Finally, embryos whose exposure included the 6- to 8-hr window (from the early neurula onwards) showed a pronounced and significant increase in ectopic Ci-Pitx expression (P < 0.01). This demonstrates that embryos are most sensitive to omeprazole exposure relatively late in development.

**DISCUSSION**

Recent years have seen a rapid expansion in our knowledge of the mechanisms regulating LR asymmetry in model vertebrate species. Several important similarities between these species have been described, suggesting all vertebrates share some fundamental aspects of LR asymmetry, such as the role of nodal and Pitx in...
left-sided mesoderm. Ascidians, as a basal chordate group, offer an excellent system in which to examine proposed ancestral (i.e., primitive) mechanisms. As an outgroup to the vertebrates, they allow the deduction of the primitive vertebrate condition, thus potentially identifying mechanisms used by the ancestor from which all extant vertebrates have evolved. Here, we examine the effect of manipulating ion flux on the establishment of molecular LR asymmetry in Ciona.

Pharmacological Inhibition of H⁺K⁺ATPase Disrupts Asymmetry

In both Xenopus and chick embryos, an H⁺K⁺ATPase has been shown to function in regulating LR asymmetry upstream of nodal (Levin et al., 2002; Raya et al., 2004). Since omeprazole inactivates H⁺K⁺ATPase activity in a wide range of organisms (Mukherjee et al., 2001), we assayed in detail the effect of this compound on the development of asymmetric Ci-Pitx expression in Ciona. Our results show a significant and concentration-dependent increase in ectopic right-sided Ci-Pitx expression in embryos treated with omeprazole. We also observed an increase in embryos showing no epidermal Ci-Pitx expression. However, we never observed embryos with reversed expression, that is with right-sided but no left-sided...
Ci-Pitx. The significance of this is discussed below.

Ciona Orthologs of Vertebrate H⁺K⁺ATPase Subunit Genes

The vertebrate H⁺K⁺ATPase is made from subunits deriving from two separate genes. The α subunit forms the core of the pump, and includes the binding sites for K⁺, H⁺, and ATP (Munson et al., 2000). The β subunit associates with the alpha subunit and is required for its function. Database searches identified two α and one β subunit genes in the Ciona genome. Molecular phylogenetic analysis confirmed the orthology of these genes to the vertebrate H⁺K⁺ATPase subunit genes, but also showed them to be equally related to the vertebrate Na⁺K⁺ATPase genes. While the different groups of vertebrate genes form clades with relatively strong support (Fig. 5), the relationship between these clades is less clear, marked by low support values. Thus, it is not possible to conclude on the basis of the phylogeny of the chordate genes whether H⁺K⁺ATPase activity or Na⁺K⁺ATPase activity is primitive. Notably, omeprazole has been reported as binding covalently to cysteine residues in the active region of the α subunit, which spans transmembrane regions M5 and M6 (Munson et al., 2000). Only one of the two predicted α subunit proteins in Ciona, CiαB, contains a cysteine in this region (Fig. 4A), suggesting this may be the target residue for omeprazole.

All three Ciona α and β subunit genes are expressed during embryogenesis. EST data (Satou et al., 2002) indicates two, the Ci-β subunit and the Ci-αA subunit, are expressed throughout development and our expression studies confirm this, showing ubiquitous low level expression and intense neural expression. The Ci-αB subunit is only expressed from the early tail bud stage onwards. This coincides with the activation of asymmetric gene expression in Ciona (Boorman and Shimeld, 2002b). Furthermore, expression is restricted to dorsal and ventral midline epidermis. Current data indicate that the epidermis is the first site of asymmetric gene expression.
expression in ascidians (Boorman and Shimeld, 2002b; Morokuma et al., 2002), and furthermore this pattern is complementary to that of Ci-Pitx. These data highlight regulation of Ci-αB activity as likely to be involved in establishing LR asymmetry in Ciona.

The Timing of H⁺K⁺ATPase Activity in Ciona

Studies in different vertebrates have resulted in conflicting data concerning the timing of H⁺K⁺ATPase activity in the regulation of asymmetry. In Xenopus, H⁺K⁺ ATPase activity becomes asymmetric very early in development, during cleavage stages (Levin et al., 2002). In chick embryos, H⁺K⁺ ATPase activity appears to regulate asymmetry relatively late in development, during gastrulation (Levin et al., 2002; Raya et al., 2004). To examine timing in Ciona, we conducted a time course of omeprazole treatment. The results showed that omeprazole affects LR asymmetry in Ciona relatively late in development. No effect was observed from treatment prior to gastrulation, with the strongest effect from treatment during the neurula and tailbud stages. Notably, this is the period when clear AP and DV axes become apparent in Ciona, including the formation of definitive midline structures such as the notochord. It also coincides with the activation of Ci-αB expression in the epidermis at the early tailbud stage, and precedes the activation of asymmetric Ci-Pitx. As such, it is more similar to the timing of H⁺K⁺ ATPase activity in the chick embryo than in the Xenopus embryo.

Similarities and Differences Between Ciona and Vertebrate LR Patterning Mechanisms

Our data suggest a fundamental similarity in the mechanism used to initiate asymmetric gene expression in urochordates and vertebrates. It is possible this reflects convergent co-option of mechanisms in the two lineages, as suggested by Palmer (2004) for other aspects of the evolution of asymmetry. The most likely explanation for this similarity, however, is that it reflects conservation of a primitive mechanism that was present in the common ancestor of the chordates. The implication of this is that all living chordates have evolved from this starting point, suggesting it is likely to be present in vertebrate lineages that have yet to be fully studied at this level, such as elasmobranches and agnathans.

There are also differences between urochordates and vertebrates with respect to the development of LR asymmetry. First, our manipulation of ion flux in urochordates never resulted in full reversal of symmetry, whereas in some vertebrates reversal is observed. Omeprazole-treated embryos fell into three categories: embryos that appeared normal, embryos with ectopic right Ci-Pitx expression, and embryos with no Ci-Pitx expression. The latter could reflect embryos in which the left side had developed right-sided character. This range of phenotypes is, however, similar to that observed for Shh expression in chick embryos treated with omeprazole or with gap junction inhibitors (Levin and Mercola, 1999; Levin et al., 2002).

Second, in Ciona the mechanisms regulating asymmetry seem to operate in the epidermis, an ectodermal tissue, while in vertebrates the node and then mesoderm are the sites where asymmetry is regulated. The reason for these differences is unknown. However, many aspects of early Ciona development are very different from those of vertebrates. Cleavage is stereotypical, with a high reliance on cytoplasmic determinants and concomitant lineage-dependent mechanisms for determining major tissue types. Gastrulation occurs after only a few cell divisions, in embryos with a small number of large cells, and there is no direct equivalent of the node in Ciona. These differences probably reflect adaptive change in the urochordate lineage to produce an embryo capable of rapidly forming a motile larva, something relatively uncommon for organisms with planktonic eggs and presumably reflecting selection to avoid predation in the plankton. However, whether the differences in LR patterning mechanisms between Ciona and vertebrates reflect changes driven by similar selective pressures remains unknown, as without an outgroup it is impossible to determine whether vertebrates, urochordates, or either represent the ancestral state. Study of asymmetry mechanisms in other deuterostome phyla might help resolve this issue, and recent data from Duboc et al. (2005) suggest ion flux may indeed be primitive for deuterostomes. Additionally, it will be important to determine if other aspects of the molecular pathway controlling asymmetry in multiple vertebrate lineages, for example the use of Notch signalling, are also used by Ciona. Finally, our data implicate K⁺ channels in regulating LR asymmetry in Ciona. Future work will involve identifying the specific genes involved and investigating their relative importance in LR asymmetry.

EXPERIMENTAL PROCEDURES

Drugs and Embryo Treatments

Stock solutions of reagents were prepared as follows. Lanthanum Chloride 100 mM in deionised water; Oubain 62.5 mg/ml in 50% DMSO/50% deionised water; Barium Chloride 100 mg/ml in deionised water; Glipizide 250 mg/ml in DMSO; DMSO/50% deionised water; Omeprazole 20 mg/ml in DMSO. For pharmacological treatment of embryos, reagents were diluted to the desired concentration in 4 ml of filtered sea water in a 3-cm Petri dish. A further 1 ml of sea water containing 100–200 fertilised Ciona eggs was then added to the dish and quickly mixed. All embryos in a single experiment derived from the same batch of eggs. If the reagent needed to be removed before the tailbud stage, then the embryos
were washed 4 times with filtered sea water and returned to a clean 3-cm Petri dish. When embryos reached the tailbud stage, they were dechorion-ated as described (Mita-Miyazawa et al., 1985), and fixed in 4% MOPS-buff-ered paraformaldehyde at 4°C, before transfer to 70% ethanol for storage at −20°C. The Ci-Pitx probe and in situ hybridisation protocol were as previously described (Boorman and Shimeld, 2002b). Embryos were scored for Ci-Pitx expression under a dissecting microscope. Embryos showing disruption of normal AP or DV development, as judged by morphol-ology and buccal cavity and neural ex-pression of Ci-Pitx, were excluded from the counting. All embryos showing evidence of ectopic expression or no expression were further examined under higher magnification on a Zeiss Axioskop II. Statistical analyses were carried out using the embedded statistical functions of Excel.

Identification and Analysis of *Ciona* H+K+ATPase Homologs

*Ciona* genomic and EST resources (Dehal et al., 2002; Satou et al., 2002) were searched with BLAST using vertebrate H+K+ATPase and Na+K+ATPase α and β gene sequences, and homologous se-quences downloaded from these data-bases. Molecular phylogenetic analysis was performed using TREFUZZLE, with 1 informative and 8 gamma-distrib-uted rates (Strimmer and von Haessler, 1996). Probes for the three H+K+ATPase subunit genes were obtained from the *Ciona intestinalis* gene collection release 1 (Satou et al., 2002). Clone numbers are as follows: β subunit R1CiGC30d02, αA subunit R1CiGC50m03, and αB subunit R1CiGC25L10. In situ hybridisation analysis of these genes was performed as described (Boorman and Shimeld, 2002b). Since preliminary experiments for two of the probes showed expression appeared ubiquitous, we included a va-riety of controls in subsequent exper-iments. These included sense controls (which did not show any staining when stained for the same length of time as used to reveal antisense probes) and Ci-Pitx, which revealed the previously-characterised pattern of transcription (Boorman and Shimeld, 2002b; Christi-aen et al., 2002), with no background, when stained for the same length of time.

Antibodies and Western Blots

Antibodies to α and β H+K+ATPase subunits have been previously de-scribed (Matthews et al., 1995). To test antibody specificity, we ran Western blots. Approximately 500 gastrula stage *Ciona* embryos (at which stage only one α subunit is expressed) were homogenised on ice in lysis buffer (1% Triton X100; 50 mM NaCl; 10 mM NaF; 1 mM Na2VO4; 5 mM EDTA; 10 mM Tris, pH 7.6; 2 mM PMSE), and stored at −80°C. Homogenates were then centrifuged at 13,000 rpm for 5 min at 4°C, and 20 μl of the supernatant used for Western blotting. Both antibodies detected single bands of sizes similar to that of the respective vertebrate subunits (data not shown), suggesting they recognise the correct endogenous protein. Immunohisto-chemistry was carried out as de-scribed (Levin, 2004). Briefly, embryos were fixed overnight in 4% parafor-maldehyde at 4°C. After washing three times in PBS/0.1% Triton X-100 (PBST), embryos were blocked with 20% goat serum +0.2% BSA, then in-cubated overnight with primary anti-body at a dilution of 1:500 at 4°C. Af-ter six washes with PBST, embryos were incubated overnight with the secondary antibody (an alkaline phosphatase conjugate). Six further PBST washes were carried out, followed by detection using BCIP and NBT.

ACKNOWLEDGMENTS

We thank N. Satoh and Y. Satou for the *Ciona* gene collection release 1 plates, Dayong Qiu for his assistance with the Western blots, and members of the Shimeld lab for discussion. We also thank two anonymous referees whose comments improved the manuscript. M.L. gratefully acknowledges support of the American Cancer Society (Research Scholar Grant RSG-02-046-01) and the National Institute of Health (1R01-GM-06227). Parts of this investiga-tion were conducted in a Forsyth Insti-tute facility renovated with support from Research Facilities Improvement Grant CO6RR11244 from the National Center for Research Resources, Na-tional Institutes of Health.

REFERENCES


Fujinaga M, Baden JM. 1991. Critical pe-riod of rat development when sidedness...
of asymmetric body structures is determined. Teratology 44:453–462.


Levin M, Mercola M. 1999. Gap junction-mediated transfer of left-right patterning signals in the early chick blastoderm is upstream of Shh asymmetry in the node. Development 126:4703–4714.


