RESEARCH ARTICLE

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

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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.*

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

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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

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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; Pennekamp 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 (Yasui 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

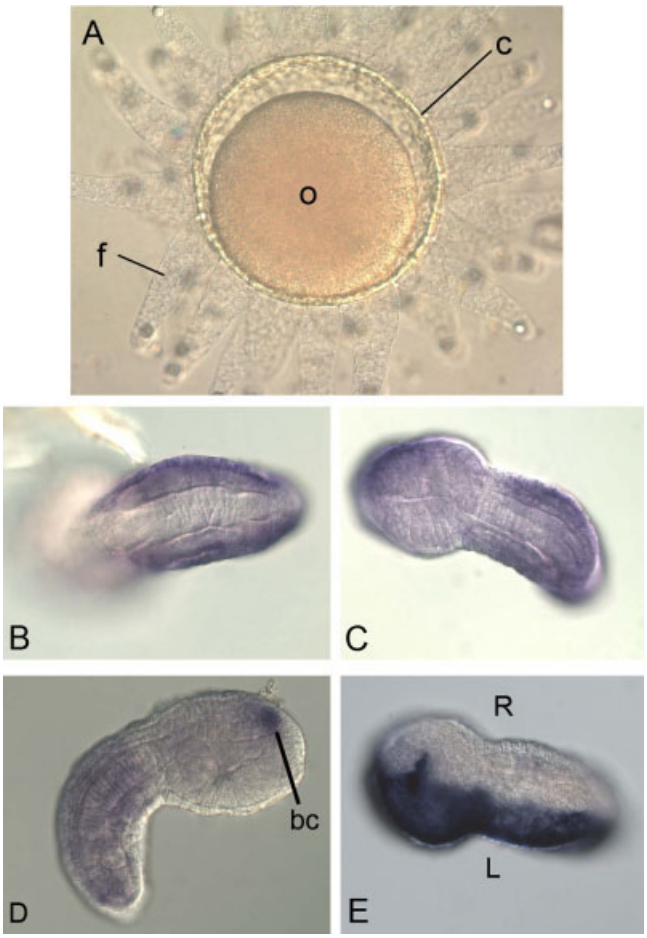

Fig. 1.

A

Compound and concentration	Activity or Target	Threshold concentration	Defect if threshold exceeded	Ectopic *Ci-Pitx* below threshold?
Oubain	Na^+K^+ ATPase inhibitor	12.5μgml^{-1}	Arrests in early cleavage	Yes (n=44, P>0.05)
Concanamycin A	vacuolar H^+ ATPase	0.5ngml^{-1}	Arrests in early cleavage	Yes (n=28, P>0.05)
Chromanol 293B	Iks Blocker	0.5μgml^{-1}	Deformed at gastrulation	No (n=36)
Omeprazole	H^+K^+ATPase inhibitor	40μgml^{-1}	Arrests at first cleavage	Yes (n=43, p<0.01)
Skelid	V-ATPase Inhibitor	25μgml^{-1}	Arrests at neurula stage	No (n=42)

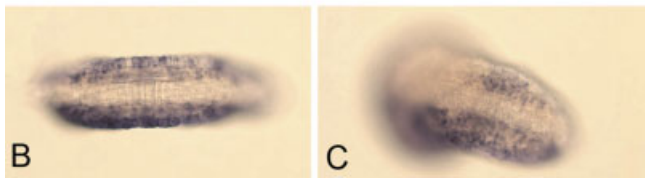

Fig. 2. **A:** Compounds used in the initial screen and their activity. The columns from left to right are: 1, the name of the reagent tested; 2, the molecules predicted to be affected by the compound; 3, the concentration above which non-specific developmental defects were observed; 4, the effect on 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 oubain 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.

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 development was compromised (Fig. 2A). Experiments were then conducted using reagent concentrations below this threshold.

Treated embryos were grown until the tailbud stage, then dechorionated, and subjected to in situ hybridisation for and scored for *Ci-Pitx* expression (Fig. 2B,C). The results show that only one tested compound, omeprazole, significantly disrupted asymmetric *Ci-Pitx* expression (Fig. 2A). Omeprazole blocks the H^+K^+ATPase, which has a role in regulating LR asymmetry in some vertebrates. Specifically, omeprazole forms a cationic sulfenamide in an acidic environment (such as that created internally by the H^+K^+ATPase) and binds covalently to the α subunit, blocking H^+K^+ATPase activity in organisms as diverse as vertebrates and protozoa (Mukherjee et al., 2001; Jiang et al., 2002; Levin et al., 2002). The specificity of action has made omeprazole a popular clinical treatment for targeting gastric H^+K^+ATPase activity in the con-

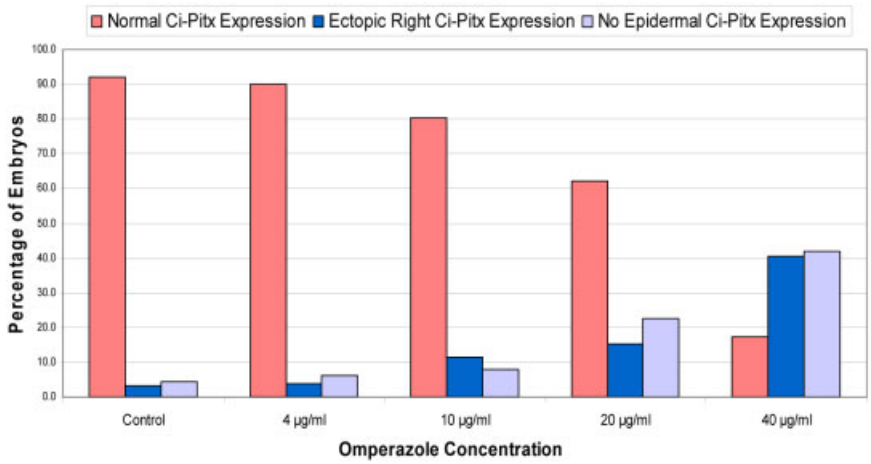

Fig. 3. The effect on *Ci-Pitx* expression of different omeprazole concentrations. Embryos were incubated in their chorions from fertilisation, and scored by in situ hybridisation at the tailbud stage. The incidence of both ectopic right-sided expression and the absence of expression increase as omeprazole 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 congenic 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. intestinails* 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*β subunit from fertilisation through to the time when asymmetric gene expression is established, ruling out neither of these possibilities. Similarly, the *H^+K^+ATPase αA* 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. A: Alignment of Ci-αA and Ci-αB with crustacean (*Artemia*) and flatworm (*Dugesia*) α subunits, zebrafish (Dr) Na^+K^+ATPase α1 subunit and *Xenopus* (Xl) H^+K^+ATPase α4 subunit. The 10 predicted transmembrane domains (M1–M10) are marked in bold, and the highly conserved phosphorylation site (marked by ATP) that is found in the extended cytoplasmic loop between M4 and M5 is highlighted. M5 and M6 form the core of the pocket through which transmembrane ion transfer occurs (Munson et al., 2000). **B:** Alignment of the Ci-β subunit with the mouse (Mm) H^+K^+ATPase (HK) β subunit, the mouse β1 subunit (an Na^+K^+ATPase), and their *Drosophila* (Dm) ortholog. **C:** Table indicating percentage amino acid identities between the sequences shown in A above. The first figure indicates overall similarity, the second figure similarity within the region from M1 to M10 inclusive. **D:** Table indicating percentage amino acid identities between the sequences shown in B above. The first figure indicates overall similarity, the second figure similarity within the more highly conserved region from position 25 to 141 inclusive. Species for A–D as follows: Ci (*Ciona intestinalis*), Dm (*Drosophila melanogaster*), Dj (*Dugesia japonicum*), Af (*Artemia franciscana*), Xl (*Xenopus laevis*), Mm (*Mus musculus*), Dr (*Danio rerio*).

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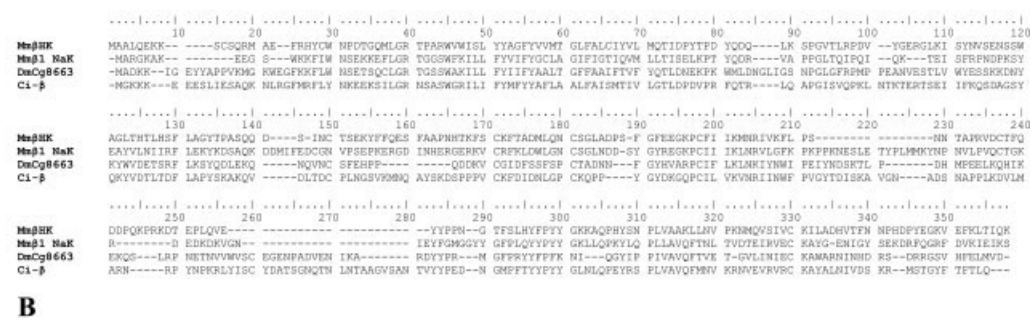

B

	Dj	Xla Na/K	Dre H/K	Ci-αA	Ci-αB
Af	72.7/75.8	59.9/63.1	70.2/73.5	70.2/72.8	69.0/72.1
Dj	-	58.4/62.0	70.9/73.5	68.0/71.1	68.1/71.2
Xla1 Na/K		-	60.9/63.7	58.5/61.4	59.4/62.4
Dre H/K			-	70.1/72.7	69.8/72.6
Ci-αA				-	78.5/81.6

C

	Mmβ1 Na/K	DmCg8663	Ci-β
Mmβ H/K	26.7/30.8	20.2/29.1	23.1/32.1
Mmβ1 Na/K	-	24.0/34.5	25.7/34.0
DmCg8663		-	26.9/28.2

D

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.

Downstream of the H^+K^+ATPase: a K^+ Channel Blocker Disrupts Asymmetric Gene Expression in *Ciona*

In the contexts of LR asymmetry and mammalian gastric tissues, the Kir4.1 K^+ channel is a required partner for the physiological activity of the H^+K^+ATPase (Fujita et al., 2002; Levin et al., 2002; Chen and Levin, 2004). Correspondingly, we conducted a preliminary screen to identify potential types of channels involved in regulating LR asymmetry in *Ciona* (Table 1). Compounds affecting Ca^{2+} channels, Cl^- channels, and K^+ channels all produced a low incidence of ectopic right-sided *Ci-Pitx* expression. However, only the Barium chloride treatment was significantly different from controls, with approximately 25% ($n = 57$, $P < 0.05$, chi squared test) showing ectopic right-sided *Ci-Pitx* expression in tail epidermis (Table 1). Barium chloride blocks K^+ channels, implicating these in the regulation of asymmetry in *Ciona*.

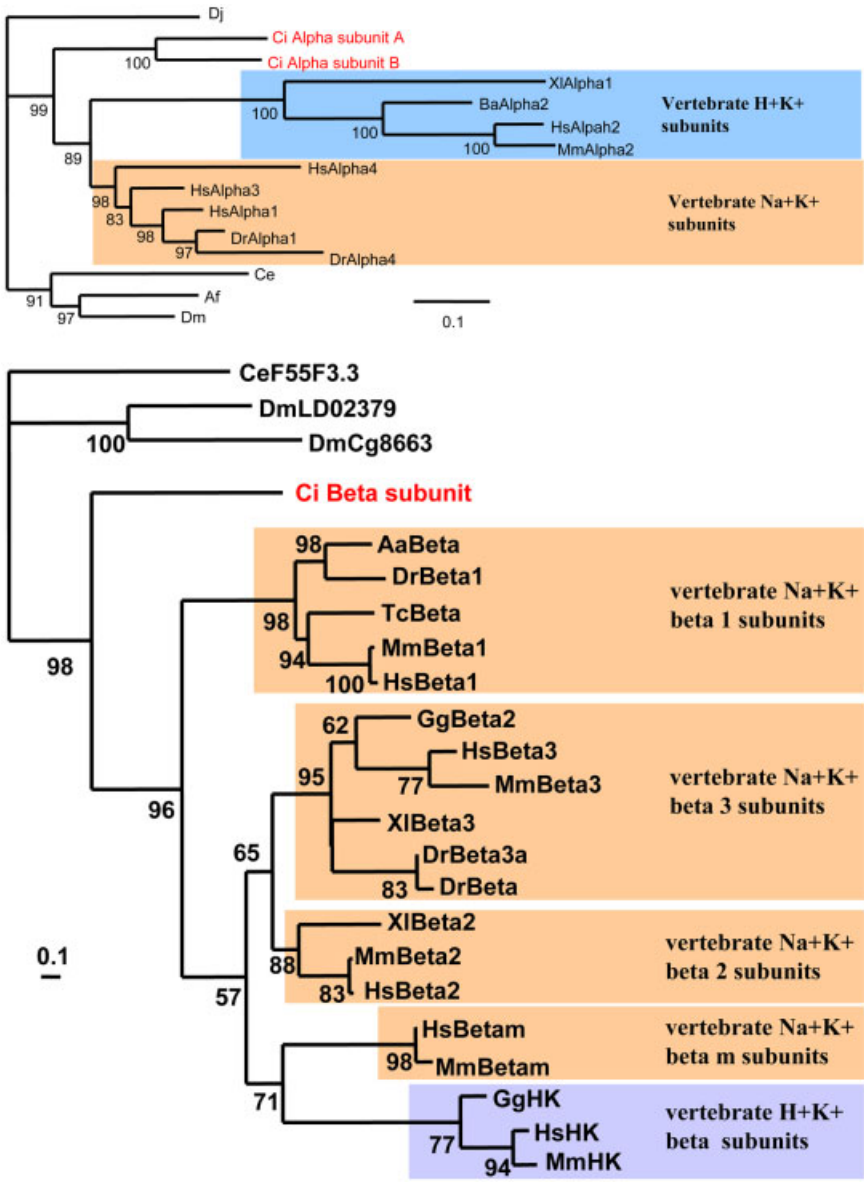

Fig. 5. **A:** Molecular phylogenetic analysis of the α subunits, based on the entire protein coding sequence. The tree is rooted with protostome sequences. The two *Ciona* sequences group robustly, indicating that they are closely related and likely evolved via a relatively recent duplication. The vertebrate H^+K^+ATPase sequences group robustly. Numbers next to nodes indicate percentage puzzling support values, and the scale indicates inferred substitutions per site. Species codes: Ci (*Ciona intestinalis*), Dm (*Drosophila melanogaster*), Ce (*Caenorhabditis elegans*), Dj (*Dugesia japonicum*, a flatworm), Af (*Artemia franciscana*, a crustacean), Xl (*Xenopus laevis*), Hs (*Homo sapiens*), Mm (*Mus musculus*), Dr (*Danio rerio*), Ba, (*Bufo marinus*, an amphibian). **B:** Molecular phylogenetic analysis of β subunits, rooted in protostome genes. The *Ciona* sequence falls basal to the vertebrate sequences. The vertebrate H^+K^+ATPase sequences group robustly. Numbers next to nodes indicate percentage puzzling support values, and the scale indicates inferred substitutions per site. Species codes: Ci (*Ciona intestinalis*), Xl (*Xenopus laevis*), Hs (*Homo sapiens*), Mm (*Mus musculus*), Dr (*Danio rerio*), Dm (*Drosophila melanogaster*), Ce (*Caenorhabditis elegans*), Aa (*Anguilla anguilla*, an eel), Tc (*Torpedo californica*, a ray), Gg (*Gallus gallus*). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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

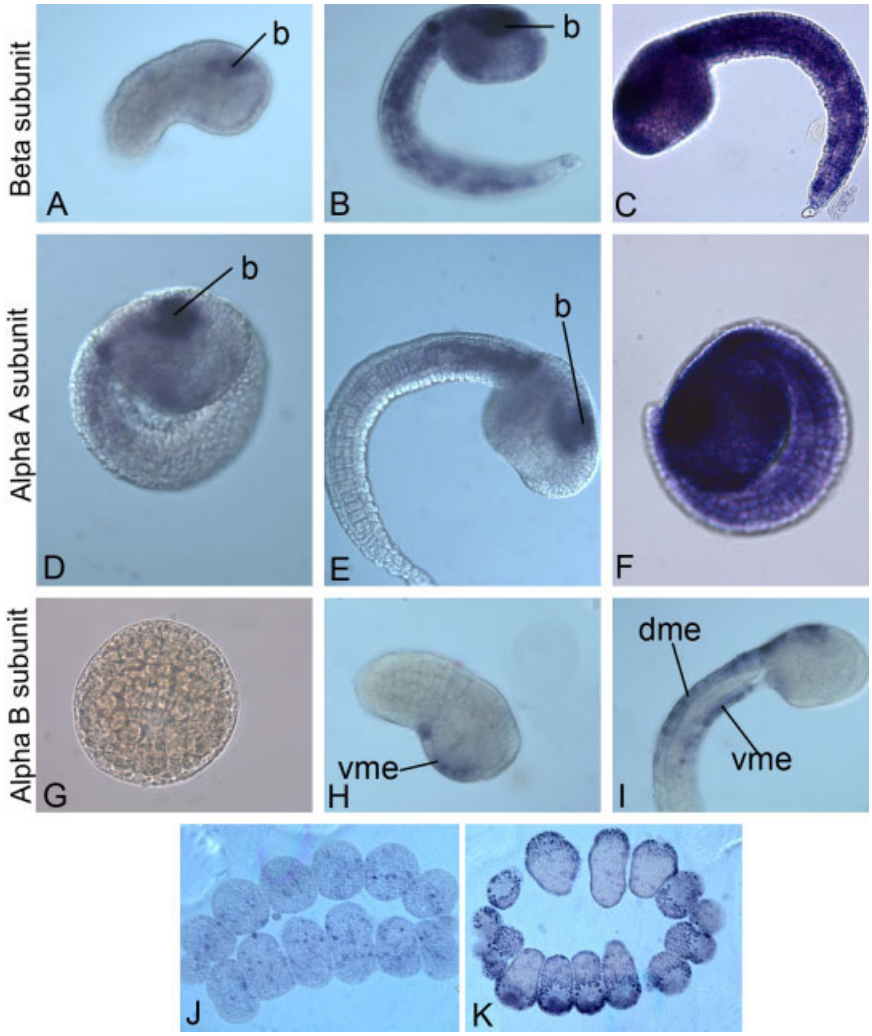

Fig. 6. Expression of α and β subunits in *Ciona* embryos. A, B, D, E, H, and I are oriented in right lateral view, with anterior to the right and dorsal to the top. C and F are oriented in left lateral view, with anterior to the left and dorsal to the top. G is orientated such that blastopore is uppermost. Additional examples of *Ci*-β and *Ci*-β*A* expression can be viewed at http://ghost.zool.kyoto-u.ac.jp/indexr1.html (Satou et al., 2002). **A–C:** *Ci*-β at early (A) and late (B) tailbud stages after 1.5 hr of staining time, and late tailbud stage (C) after 5 hr of staining time. Expression is especially pronounced in the brain (b), but revealed as ubiquitous after extended staining. **D–F:** *Ci*-α*A* expression at mid (D) and late tailbud (E) stages after 1.5 hr of staining, and at the late tailbud stage (F) after 5 hr of staining. Expression appears identical to the *Ci*-β subunit. **G–I:** *Ci*-α*B* expression at gastrulation (G), early (H), and late (I) tailbud stages. The embryo in G has been stained for 24 hr, and shows no evidence of expression. The embryos in H and I have been stained for 5 hr, and show expression is restricted to ventral (vme) and dorsal (dme) midline epidermis. **J, K:** Section through embryos stained with antibodies to vertebrate H^+K^+ATPase α and vertebrate H^+K^+ATPase β, respectively. J is a gastrula, with faint staining. K is a 64-cell embryo with staining focused to the cell membrane.

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*.

Manipulating Ion Flux Disrupts LR Asymmetry in *Ciona*

While asymmetry in ascidians does not become apparent morphologically until the larval stage (when it is visible in the organisation of cells of the anterior brain), molecularly it can be detected at the tailbud stage (Boorman and Shimeld, 2002b; Morokuma et al., 2002). Our initial experiments resulted in two pertinent findings. First, that manipulating some aspects of ion flux resulted in a significant disruption of asymmetric gene expression. Second, that dechorionation also affected LR patterning, as determined by *Ci-Pitx* expression. These results suggest that ion flux might play an in vivo role in the regulation of LR asymmetry in *Ciona*. The effect of dechorionation is, however, problematic, as it compromises many of the techniques, such as microinjection and electroporation, commonly used to manipulate *Ciona* embryos. The reasons why dechorionation affects asymmetry remain unknown; possible factors could be exposure of the dechorionated zygote to different mechanical forces, enzymatic activity or ionic environment, or the removal of the test cells that normally attached to the inside of the chorion. We note that, unlike the pharmacological manipulation discussed below, dechorionation resulted in most embryos expressing ectopic right-sided *Ci-Pitx*. Thus, dechorionation does not directly mimic the application of the H^+K^+ATPase blocker.

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

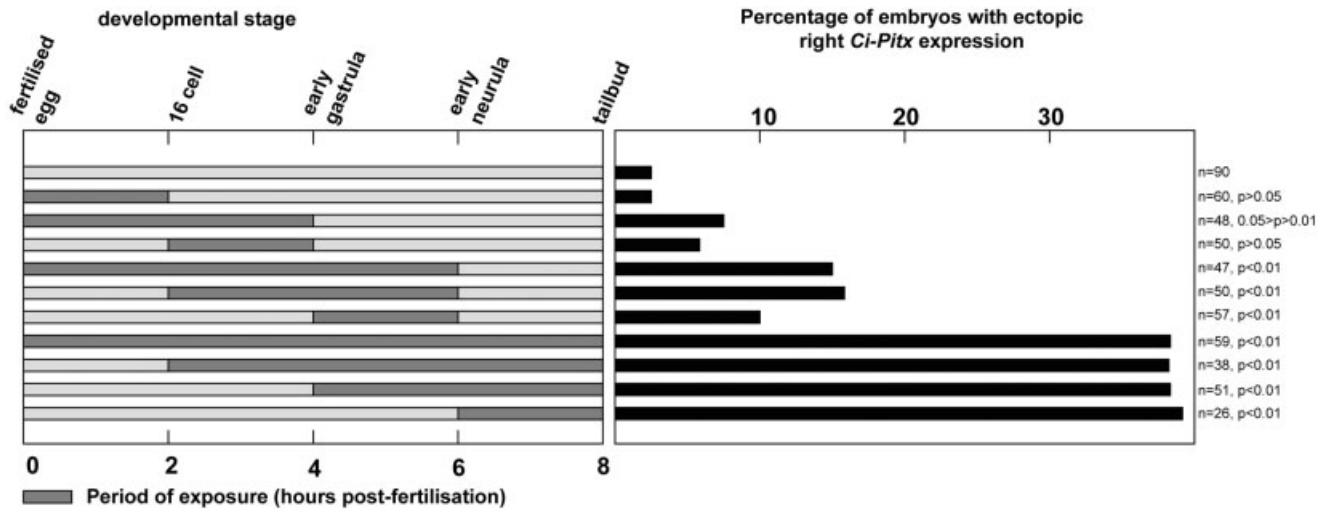

Fig. 7. Schematic representation of a time course experiment of developing embryos exposed to 40 μg/ml omeprazole for different periods of development. Dark shading on the left panel indicates period of exposure in 2-hr blocks, with the equivalent developmental stage shown above. The top bar represents untreated control embryos. Shown on the right are the percentage of embryos exhibiting ectopic right-sided *Ci-Pitx* expression. Values significantly different from controls are indicated, with *P* values and number of embryos scored shown.

TABLE 1. Compounds Used to Target Ion Channels, and Whether Their Application Induced Ectopic *Ci-Pitx* Expression

Compound	Activity or Target	Ectopic *Ci-Pitx?*
Lanthanum Chloride	Ca^{2+} Channel Blocker	Yes (n=54, p>0.05)
Barium Chloride	K^+ Channel Blocker	Yes* (n=53, P<0.01)
Glipizide	K^+ Channel Blocker (ATP sensitive)	No (n = 42)
9-anthrocene carboxylic acid	Cl^- Channels	Yes (n=49, P>0.05)
Bimakalim	K^+ Channel Opener	No (n=64)
Chromakalim	K^+ Channel Opener	No (n=56)
Pinacidil	K^+ Channel Opener	No (n=43)
Glibenclamide	K^+ Channel Blocker (ATP sensitive)	No (n=51
5-hydroxy decaonic acid	K^+ Channel Blocker (ATP sensitive)	No (n=72)

*Indicates significant difference from controls.

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*, Ci-αB, 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 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 common 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; Concanamycin A 0.125 μg/ml in DMSO; 9-anthrocene carboxylic acid 30 mg/ml in 70% DMSO/30% deionised water; Bimakalim 50 mg/ml in DMSO; Chromakalim 50 mg dissolved in 1 ml DMSO; Chromanol 293B 25 mg/ml in 75%DMSO/25% deionised water; Pinacidil 50 mg dissolved in 1 ml DMSO; Glibenclamide 1 g dissolved in 7 ml DMSO; 5-hydroxy decanoic acid 12.5 mg/ml in DMSO; Skelid 0.5 g dissolved in 4 ml 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 dechorionated as described (Mita-Miyazawa et al., 1985), and fixed in 4% MOPS-buffered 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 morphology and buccal cavity and neural expression 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 vertebrae *H^+K^+ATPase* and *Na^+K^+ATPase* α andβ gene sequences, and homologous sequences downloaded from these databases. Molecular phylogenetic analysis was performed using TREEPUZZLE, with 1 invariable and 8 gamma-distributed 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 R1CiGC30m03, 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 variety of controls in subsequent experiments. 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; Christiaen 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 described (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 $Na_3VO_4$5 mM EDTA; 10 mM Tris, pH. 7.6; 2 mM PMSF), 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. Immunohistochemistry was carried out as described (Levin, 2004). Briefly, embryos were fixed overnight in 4% paraformaldehyde 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 incubated overnight with primary antibody at a dilution of 1:500 at 4°C. After 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.

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