DEVELOPMENTAL DYNAMICS 225:469–484 (2002)

Early Embryonic Expression of Ion Channels and Pumps in Chick and *Xenopus* Development

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ABSTRACT **An extensive body of literature implicates endogenous ion currents and standing voltage potential differences in the control of events during embryonic morphogenesis. Although the expression of ion channel and pump genes, which are responsible for ion flux, has been investigated in detail in nervous tissues, little data are available on the distribution and function of specific channels and pumps in early embryogenesis. To provide a necessary basis for the molecular understanding of the role of ion flux in development, we surveyed the expression of ion channel and pump mRNAs, as well as other genes that help to regulate membrane potential. Analysis in two species, chick and *Xenopus*, shows that several ion channel and pump mRNAs are present in specific and dynamic expression patterns in early embryos, well before the appearance of neurons. Examination of the distribution of maternal mRNAs reveals complex spatiotemporal subcellular localization patterns of transcripts in early blastomeres in *Xenopus*. Taken together, these data are consistent with an important role for ion flux in early embryonic morphogenesis; this survey characterizes candidate genes and provides information on likely embryonic contexts for their function, setting the stage for functional studies of the morphogenetic roles of ion transport.** © 2002 Wiley-Liss, Inc.

Key words: ion channels; ion pumps; chick; *Xenopus*; embryogenesis

INTRODUCTION

Electrical activity due to ion channel function has been extensively studied in the context of the nervous system. However, there exists a large but often little-recognized body of literature that supports a regulative role for endogenous ion flows and standing (DC) potential differences in many aspects of embryonic morphogenesis (Jaffe and Nuccitelli, 1977; Jaffe, 1981).

The idea that non-neuronal electrical activity is a controlling factor in biological growth and organization is an old one (Lund, 1947). The presence of a 24-hr chick embryo is detectable noninvasively by means of changes in conductivity and dielectric constant of the much larger egg (Romanoff, 1941). The discovery of strong endogenous DC electric fields within living systems have been augmented by functional experiments, suggesting that these fields have a causal role in physiology and development (Jaffe, 1981). Models of regulation of embryonic morphogenesis by ion flux are based on three main classes of observations: (1) most organisms, tissues, and cells undertake significant energy expense (in ATP used to power ion pumps) to produce complex standing electric fields and, thus, to induce ion currents through extracellular spaces; these currents are found in spatiotemporal patterns consistent with specific roles in development (e.g., Jaffe and Nuccitelli, 1977; Nuccitelli, 1986). (2) Interruption of the pattern of these fields and currents (by means of pharmacologic agents, simple electrical shunting/short-circuiting, or active reversal-of-field polarity) has very specific effects on biological processes (e.g., see Borgens and Shi, 1995). Finally, (3) cells, tissues, and organs exhibit specific physiological responses when exposed to exogenously applied electric fields (e.g., see McCaig and Zhao, 1997); these effects often occur only within sharp field parameter windows (Hotary and Robinson, 1992, 1994).

Excellent overviews summarize fields found in animal tissues, and in embryonic development in particular (Nuccitelli, 1988; Borgens et al., 1989; Robinson and Messerli, 1996). During gastrulation and neurulation, three-dimensional gradients of voltage provide coordinates for embryonic morphogenesis (Hotary and Robinson, 1992, 1994; Shi and Borgens, 1995). These gradients are likely to function by providing a coarse guide for galvanotactic cell migration such as that occurring in neural crest migration or the inflow of cells into an initiating limb bud (Borgens, 1983; Metcalf et al., 1994; Shi and Borgens, 1994). Ion flux is also likely to have

Grant sponsor: American Cancer Society; Grant number: RSG-02-046-01; Grant sponsor: The Medical Foundation; Grant number: New Investigator Award 2001; Grant sponsor: American Heart Association; Grant number: Beginning Grant in Aid 0160263T; Grant sponsor: Harcourt General Charitable Foundation.

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Received 29 July 2002; Accepted 26 August 2002
DOI 10.1002/dvdy.10180

an important regulatory role in establishing developmental polarity. For example, in the chick, voltage potentials between the epiblast and hypoblast may determine the dorsoventral polarity of the gastrulating chick embryo (Stern, 1982). In the regenerating planarian, anteroposterior polarity may be dictated by the electric potential between the two ends of the organism (Marsh and Beams, 1957). Data along the lines of the three classes delineated above also implicate endogenous ionic currents and potential fields in the determination of pattern during regeneration (Kurtz and Schrank, 1955; Borgens, 1984) and the fine growth and pattern control that distinguishes neoplasm from normal tissue (Marino et al., 1994a,b).

The older literature is very suggestive of important roles for ion currents and potential voltage differences in directing aspects of embryonic morphogenesis (in addition to the well-recognized role of membrane voltage in fertilization). However, the electrophysiology data now need to be augmented by modern molecular biology approaches to begin to fully understand what proteins' activity underlies the endogenous ion flux and, thus, helps control development. Characterization of expression of specific channel and pump genes at the protein and mRNA level is necessary to enable spatially targeted functional over- and underexpression studies.

Important advances in this direction have been made in a couple of cases, such as the role of Ca^{2+} flux in amphibian neural induction (Moreau et al., 1994; Drean et al., 1995; Leclerc et al., 1997, 1999, 2000; Palma et al., 2001). Calcium transients are generated by L-type Ca^{2+} channels during blastula and gastrula stages, before the morphologic differentiation of the nervous system. These fluxes are downstream of the neural inducer *noggin*, and over- and underexpression analysis strongly suggests that the activity of the L-type channels specifies dorsoventral identity of embryonic mesoderm.

Because the Na^+/K^+-ATPase is instrumental in generating the voltage gradients used by neurons, it more than others has been studied during development of several organisms, including gastrulating sea urchins (Marsh et al., 2000) and pregastrulation mammalian embryos, where it is thought to be involved in transtrophectodermal fluid transport (Watson and Kidder, 1988; Watson et al., 1990; Jones et al., 1997; Betts et al., 1998;). Similarly, it is likely that the activity of the Na^+/K^+-ATPase is involved in gastrulation and neuronal differentiation in amphibians (Burgener-Kairuz et al., 1994; Uochi et al., 1997; Messenger and Warner, 2000).

In ascidians, analysis of developmental calcium currents (Simoncini et al., 1988) has led to the identification of a novel role for early expression of channel and pump mRNAs. The ascidian blastomeres contain a maternal transcript of a truncated voltage-dependent Ca^{++} channel, which is able to reduce the activity of the full-length form, suggesting that mRNA expression may be used by embryos as an endogenous dominant negative to regulate the function of gene products (Okagaki et al., 2001). Ca^{++} also appears to control morphogenesis in hydra (Zeretzke et al., 2002).

One of the earliest patterning roles for ion flux is in the elaboration of the left–right (LR) axis. As early as 1956, it was reported that a DC electric current imposed across the chick blastoderm was able to induce a high number of cardiac reversals (Sedar, 1956). By using genetic and pharmacologic techniques, it was recently shown that H^+ and K^+ ion flux is asymmetric at cleavage stages in *Xenopus* and in the early primitive streak in chick and in both species functions upstream of the asymmetric expression of the LR gene cascade (Levin and Mercola, 1998, 1999; Levin et al., 2002). Ca^{++} flux may also be involved in LR asymmetry in amphibians (Toyoizumi et al., 1997) and chick (Linask et al., 2001), and an asymmetry in the response of calcium channels to Ca^{++} depletion has been reported at the two-cell stage in ascidians (Albrieux and Villaz, 2000). In mammals, a dependence of consistent *situs* on ion flux is likewise suggested by the laterality phenotype observed after genetic deletion of PCKD in mice (Pennekamp et al., 2002).

Standing potential differences and ionic current flows are generated by active ion pumps and shaped and regulated by ion channels. There are currently very little data available on the spatiotemporal distribution of these molecules in early embryos, before the development of the nervous system. We have performed a survey of the expression of known ion channel and pump genes in chick and frog (*Xenopus*) embryos at early stages of development to begin to unravel the roles of endogenous ion potentials and flows in controlling aspects of development, identify molecular reagents that will help characterize genes upstream and downstream of embryonically relevant ion flux, and to provide candidate genes for in-depth functional analyses. We find that, at the mRNA level, several ion channel and pump genes, as well as some accessory genes, are expressed in spatiotemporally specific patterns, suggestive of roles in early development. By examining very early stages of *Xenopus* embryos, we observe complex localization patterns of maternal mRNAs in cleaving blastomeres. These data are consistent with the idea that the action of these proteins is crucial for several aspects of embryonic development. The identification of early (preneuronal) expression patterns by genes that modulate ion flux and cell membrane voltage sets the stage for focused functional investigation into the embryonic roles of these processes by means of the synthesis of electrophysiology with molecular biology.

RESULTS

Expression of Ion Channels and Pumps in Early Embryos

Ion channel and pump subunits are transcribed in the gastrulating chick. By using in situ hybridization, we examined the expression patterns of several known genes encoding ion channels and pumps in

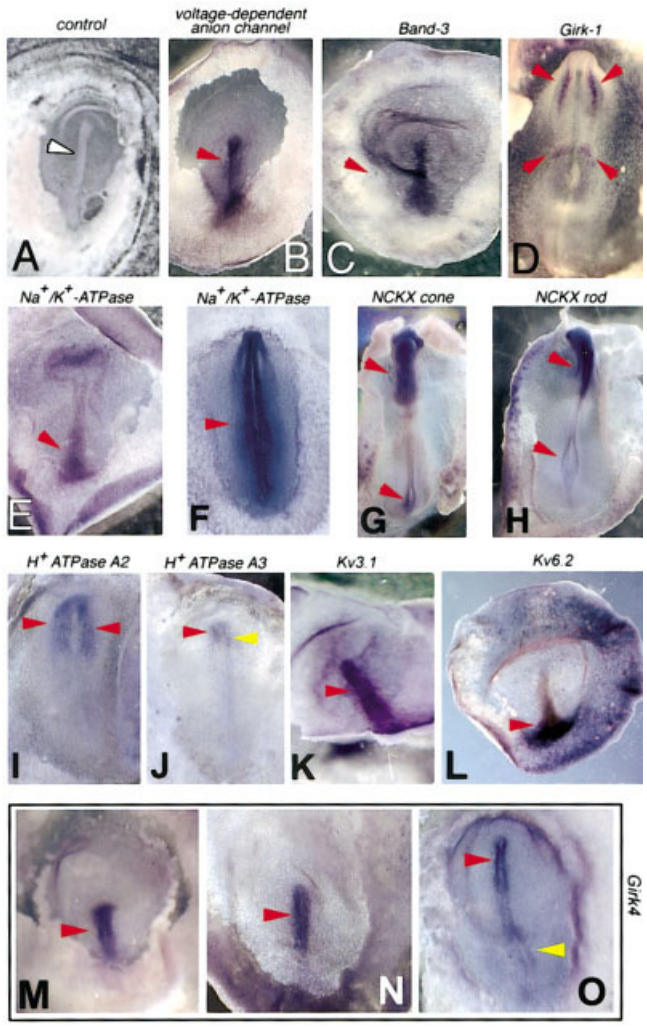

Fig. 1. Ion channel and pump genes expressed in chick. The spatiotemporal expression profile of several known chick ion channel and pump genes was investigated by in situ hybridization, and clones with specific expression are shown in this figure at select representative stages of embryogenesis. **A:** Embryos without specific expression show very low background even after a lengthy chromogenic reaction. **B:** A voltage-dependent anion channel is expressed in the streak at stage (st.) 3. **C:** Similar expression is observed for the chloride channel Band-3. **D:** Girk1 is expressed in the head folds of the neural tube and the developing somites of the st. 7–8 embryo. The β subunit of the Na^+/K^+ ATPase is expressed in the base (posterior third) of the primitive streak at st. 3^+ (**E**), but is strongly expressed in most embryonic cells at st. 8 (**F**). The NCKX cone (**G**) and rod (**H**) forms are expressed in the anterior neural tube and in the edges of the very posterior folds of the neural tube as it closes. **I:** The A2 isoform of the H^+ ATPase 116-kDa subunit is expressed outside of the notochord in the neural plate. **J:** The A3 isoform of the H^+ ATPase 116-kDa subunit is expressed anterior to Hensen's node (at the base of the notochord; yellow arrowhead indicates tip of node). **K:** The voltage-sensitive K^+ channel Kv3.1 is strongly expressed in the primitive streak at st. 2^+. **L:** In contrast, Kv6.2 is expressed in the base of the primitive streak only. The K^+ inward rectifier channel Girk4 is expressed in the whole primitive streak at st. 2^+ (**M**) and becomes restricted to the anterior half of the primitive streak by st. 4^- (**N**). At st. 4^+, it is expressed in the anterior third of the ridges in the primitive streak (**O**). Red arrows indicate regions of expression; yellow arrows indicate region of no detectable expression.

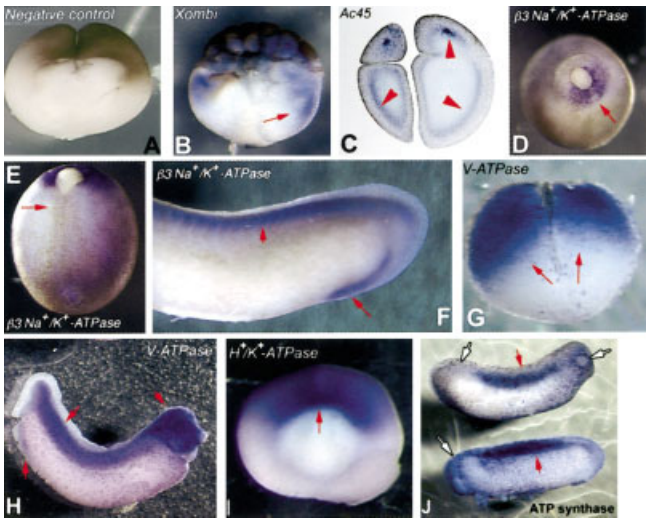

Fig. 2. Ion pump genes expressed in *Xenopus*. A variety of known ion channel and pump genes are also expressed during early *Xenopus* embryogenesis. Clones with specific expression patterns are shown here at representative stages. **A:** Sense probe controls show no signal. **B:** A probe for the maternal gene *Xombi* shows that whole-mount in situ hybridization can detect vegetal mRNA localization when it is present (arrow). **C:** Sectioning perpendicular to the animal–vegetal axis of a four-cell embryo stained in whole-mount in situ hybridization with a probe for the Ac45 V-ATPase subunit EST and embedded in JB4 shows (arrowheads) nuclear mRNA in the center of cells, as well as cytoplasmic mRNA. **D,E:** The neural β3 subunit of the Na^+/K^+ ATPase is detected at st. 11 in cells around the ventral margin of the blastopore (arrows). **F:** At st. 32, it is detected in the neural tube and in the posterior gut (arrows). **G:** Maternal mRNA encoding a subunit of the H^+ pump (V-ATPase) is present throughout the animal hemispheres of the four-cell embryo (arrows). **H:** It is later expressed throughout the neural tube and head of the tail bud stage embryo (arrows). **I:** mRNA for the H^+/K^+-ATPase (ion exchanger) is present in a more laterally restricted region of the two-cell embryo (arrow). **J:** mRNA for the 16-kDa proteolipid component of the H^+ synthase is expressed in bilateral stripes of deep tissue ventral to the neural tube, demonstrating that signal along the length of the neural tube and in the head is not obligatory or artifactual at tail bud stages. Red arrows indicate expression; white arrows indicate regions of no detectable expression. G is a photograph of the internal surface of two blastomeres of a four-cell embryo manually separated after in situ hybridization. I is a view of the internal surface of one blastomere of a two-cell embryo.

chick embryos. Control embryos hybridized to sense probes show extremely low background (Fig. 1A). The K^+ channels Kir6.1 and cSlo1, and the Ca^{++} channel Alpha1D were not detected in chick embryos between stages 0 and 11. The Ca^{++} pumps SERCA1 and SERCA2 and the α and β subunits of the Na^+/K^+ ATPase were weakly but ubiquitously expressed in embryos at these stages (data not shown; Table 1 summarizes the in situ findings because they are not discussed further in the text).

In contrast, other ion channels and pumps were specifically expressed in the chick embryo (see Table 2). A voltage-dependent anion channel is transcribed in the primitive streak of the gastrulating chick embryo (Fig. 1B). The Band-3 chloride channel, one of the most abundant proteins of the erythrocyte (Perlman et al.,

TABLE 1. Genes Not Specifically Expressed in the Chick Embryo[a]

Clone	Gene type	Reference	Expression
Kir6.1	ATP-sensitive K^+ channel	Lu and Halvorsen, 1997	Not detected
cSlo1	Ca^{++}-activated K^+ channel	Navaratnam et al., 1997	Not detected
Alpha1D	L-type voltage-activated Ca^{++} channel	Kollmar et al., 1997	Not detected
KCHIP4.2	Kv channel-interacting protein	Unpublished; accession no. AF508737	Not detected
SERCA2	Sarco/endoplasmic reticulum Ca^{++}-ATPase	Campbell et al., 1992	Nonspecific expression
SERCA1	Sarco/endoplasmic reticulum Ca^{++}-ATPase	Campbell et al., 1992	Nonspecific expression
Beta1	Na^+/K^+ ATPase β subunit	Takeyasu et al., 1993	Nonspecific expression
Alpha1	Na^+/K^+ ATPase α subunit	Yu et al., 1996	Nonspecific expression

[a]These transcripts were either not detected in embryos or were present at low levels in all tissues. As such, they give no clue to possible developmental roles.

TABLE 2. Genes Specifically Expressed in the Chick Embryo: Ion Channels[a]

Clone	Gene type	Reference
Kir3.4 (GIRK4)	→ G-protein–coupled inwardly rectifying K^+ channel	Thomas et al., 1997
Kir3.1 (GIRK1)	→ G-protein–coupled inwardly rectifying K^+ channel	Gadbut et al., 1996
cCNG	Cyclic nucleotide-gated cation channel	Timpe et al., 1999
pIRK522 (cIRK1)	Inward rectifier K^+ channel	Navaratnam et al., 1995
cCO6	Ca^{++}-activated K^+ channel	Oberst et al., 1997
pCCG6	$CNC\alpha_1$ (rod CNG channel)	Bonigk et al., 1993
pCCG8B	$CNC\alpha_2$ (cone CNG channel)	Bonigk et al., 1993
Kir6.1	ATP-sensitive potassium channel	Lu and Halvorsen, 1997
BIII1	→ Band3 anion exchanger	Kim et al., 1988
Kv6.2-114	→ Voltage-sensitive K^+ channel	Peale et al., 1998
Kv2.2	Voltage-sensitive K^+ channel	Unpublished (from Mark Bothwell's lab)
42A5	Voltage-dependent anion channel	Unpublished; accession no. AI981111
75G6	KvLQT1	Unpublished; accession no. AI982161

[a]The transcripts of these ion channel genes exhibited specific expression in embryos. Particularly interesting candidates are identified with an arrow; see figures and text for details of in situ analysis and significance.

1996), is similarly expressed (Fig. 1C). Girk1, a G-protein–coupled, inwardly rectifying K^+ channel (Isomoto et al., 1997), is present in the anterior folds of the neural tube, as well as in the first developing somite (Fig. 1D). The β subunit of the Na^+/K^+ ATPase, an ion pump that maintains voltage potential in most neuronal cells (Taniguchi et al., 2001), is expressed in the base (posterior third) of the primitive streak at stage (st.) 3^+ (Fig. 1E), and strongly in all embryonic tissues at st. 8 (Fig. 1F). The rod and cone forms of the potassium-dependent, sodium-calcium exchanger (Prinsen et al., 2000; NCKX) are expressed in the anterior neural tube and in the folds of the closing posterior edges of the neural plate (Fig. 1G,H).

The vacuolar ATPase (V-ATPase) is a proton pump consisting of approximately 12 protein subunits; it generates large electrochemical gradients at the expense of ATP when it is expressed in the plasma or vesicle membrane (Harvey, 1992; Harvey and Nelson, 1992). The A2 isoform of the 116-kDa subunit is expressed laterally to the notochord, in the neural plate at st. 6 (Fig. 1E). The A3 isoform of the H^+ ATPase 116-kDa subunit (Mattsson et al., 2000) is expressed anterior to Hensen's node (at the base of the notochord) at st. 4 (Fig. 1J).

Two members of the voltage-sensitive K^+ channel family (Pongs et al., 1999) were detected in different patterns at st. 2^+: Kv3.1 is strongly expressed in the primitive streak (Fig. 1K), and Kv6.2 is present in the base of the streak (Fig. 1L). The K^+ inward rectifier channel Girk4 is expressed in the whole primitive streak at st. 2^+ (Fig. 1M) and becomes restricted to the anterior half of the primitive streak by st. 4^- (Fig. 1N). At st. 4^+, it is expressed in the anterior third of the ridges in the primitive streak (Fig. 1O).

***Expression and subcellular localization of ion pump genes in* Xenopus.** In neural tissues, ion channels are known to be localized to different subcellular domains (Robertson, 1997; Bachmann, 1999; Caldwell, 2000). The localization of mRNAs in early cells of the frog embryo is known to be crucial in directing patterning mechanisms (Mowry and Cote, 1999; Kloc et al., 2000). Thus, we took advantage of the large early blastomere cells of *Xenopus* and examined the localization of ion channel and pump mRNAs in frog embryos, which have a radically different mode of gastrulation from chick and, thus, can be expected to provide added insight into possible embryonic function of electrogenic genes. *Xenopus* embryos feature two phases of gene expression during development: maternal mRNAs, and

those resulting from zygotic transcription after the midblastula transition (Yasuda and Schubiger, 1992). To detect maternal localization of ion channel mRNAs, we performed in situ hybridization of embryos at cleavage stages. Cleavage-stage embryos are not usually tested in expression profile studies; this results in a knowledge gap regarding the presence of transcripts of many important genes at cleavage stages, when fundamental patterning decisions are being made in the embryo. Cleavage stages are often absent from expression analyses for two reasons: difficulty in probe penetration due to the yolk, and the presence of the vitelline membrane, which can be difficult to remove at early stages. We addressed the second issue by fixing with a formaldehyde-based fixative before devitellinization, which renders the membrane brittle and easy to remove. To address the first issue, we carried out extensive controls to ensure that background signal was low and that penetration of probe could reveal signal anywhere within the embryo. Sense probes to several ion channel genes show no signal (Fig. 2A). A probe to the maternal mRNA *Xombi* (Lustig et al., 1996) demonstrates that even signal in the cells of the yolk-rich vegetal half of the embryo can be detected (Fig. 2B). Sectioning a four-cell embryo that was hybridized in whole-mount to a probe made with an expressed sequence tag (EST) of the Ac45 accessory subunit of the V-ATPase shows nuclear signal in the dorsal blastomeres but cytoplasmic signal in the ventral cells (Fig. 2C). This finding likewise demonstrates that mRNAs in the center of cells, as well as in the cytoplasm, can be detected by whole-mount in situ hybridization. We, thus, examined the expression of several ion pump mRNAs.

As in the chick, several were detected in specific patterns in the *Xenopus* embryo. The neural β3 subunit of the Na^+/K^+ ATPase is present during frog gastrulation at st. 11–12 in cells around the blastopore (Fig. 2D,E); of interest, it is present on the ventral surface, opposite to the site of the dorsal organizer. At tail bud stages, signal can be seen in the dorsal somites and posterior gut (Fig. 2F). Maternal mRNA for the V-ATPase proton pump is present throughout the animal half of the four-cell embryo (Fig. 2G); it is then is expressed strongly in neural tissues and particularly in the head at tail bud stages (Fig. 2H). Maternal mRNA for the alpha subunit of the H^+/K^+-ATPase (ion exchanger) is present in a subset of the animal pole of the two-cell embryo (Fig. 2I). Transcripts for the 16-kDa proteolipid subunit of the H^+ synthase are detected in stripes ventral to, and on both sides of, the neural tube at tail bud stages (Fig. 2J).

***Expression and subcellular localization of ion channel genes in* Xenopus.** Along with the expression of ion pumps in *Xenopus*, ion channel mRNAs were also detected during development. In contrast to the chick (Fig. 1M–O), the inward rectifier Girk4 (Kir3.4) K^+ channel (Wulfsen et al., 2000) is not present before neurulation in *Xenopus*, but is then expressed in neural tissue in hatched embryos and is specifically detected in the ear vesicle (Fig. 3A,B).

ATP-sensitive K^+ channels are found in the pancreatic β cells, cardiac myocytes, brain, and kidney (Ashcroft, 1988), where they couple cell metabolism with membrane electrical excitability. The K_{ATP} ion channel protein is an octamer, consisting of four subunits of the K^+ rectifier (KIR6.1 or KIR6.2) surrounded by four regulatory subunits. Metabolic changes in cells induce changes in the concentrations of ATP and MgADP, which inhibit and activate K_{ATP} channels, respectively; this has a profound effect on the functioning of many cell types. The inward rectifier Kir6.1 is detected as a maternal message in the animal half of vegetal cells during cleavage (Fig. 3C,D). It is expressed in the neural tissues at somite stages (Fig. 3E) but is also detected in the posterior gut. Maternal mRNA for the inward rectifier channel Kir3.1 is localized in the center of animal cells during cleavage (Fig. 3F,G) and around the blastopore lip during gastrulation (Fig. 3H).

Magainin is a *Xenopus* protein that has antibacterial properties, because it forms ion pores in cell membranes, causing a drain of the voltage potential and a K^+ efflux from bacterial cells (Matsuzaki, 1998). To ascertain whether this protein, which is normally expressed in adult frog skin, might have a role during development, we examined its expression pattern in embryos. We detect the presence of magainin mRNA in the cells of the animal cap during blastula stage (Fig. 3I).

Previous reports have indicated that the shaker-like (delayed rectifier) K^+ channel Kv1.1 is not expressed in early *Xenopus* embryos (Gurantz et al., 1996), but we detected its expression by using the more sensitive radioactive in situ hybridization in animal cells at st. 5 (Fig. 3J) and in cells undergoing ingression at the blastopore at st. 10 (Fig. 3K).

Comparison of Ion Flux Gene Expression in Two Species

Kv2.2 voltage-gated channel. Several channels have been cloned in both chick and *Xenopus*, which enables comparison of their expression in the two spe-

Fig. 3. Ion channel genes expressed in *Xenopus*. The inward rectifier Girk4 (kir3.4) K^+ channel is expressed in neural tissue in hatched *Xenopus* embryos (red arrowhead) and is specifically detected in a spot (green arrowheads) on the side of the posterior head (**A**, close-up in **B**). **C,D:** The inward rectifier Kir6.1 is detected as a maternal message in the animal half of vegetal cells during cleavage (arrowheads). **E:** It is expressed in the neural tissues at somite stages but is also detected in the posterior gut (arrowheads). Maternal mRNA for the inward rectifier channel Girk1 (Kir3.1) is localized in the middle of animal cells during cleavage (arrowheads, **F,G**), and around the blastopore lip during gastrulation (arrowhead, **H**). **I:** Magainin mRNA is detected in the cells of the animal cap of the blastula-stage embryo (arrowheads). The K^+ channel Kv1.1 can be detected by radioactive in situ hybridization in animal cells at st. 5 (arrowhead, **J**) and in cells undergoing ingression at the blastopore at st. 10 (**K**). A–I are chromogenic in situ hybridization, whereas J and K are radioactive sections (signal is lighter-colored, background is darker).

cies that have very different modes of gastrulation. We found that the mRNA for the voltage-regulated potassium channel xKv2.2 was detected in the nucleus in cleaving embryos (data not shown), and then expressed very strongly in the organizer during *Xenopus* gastru-

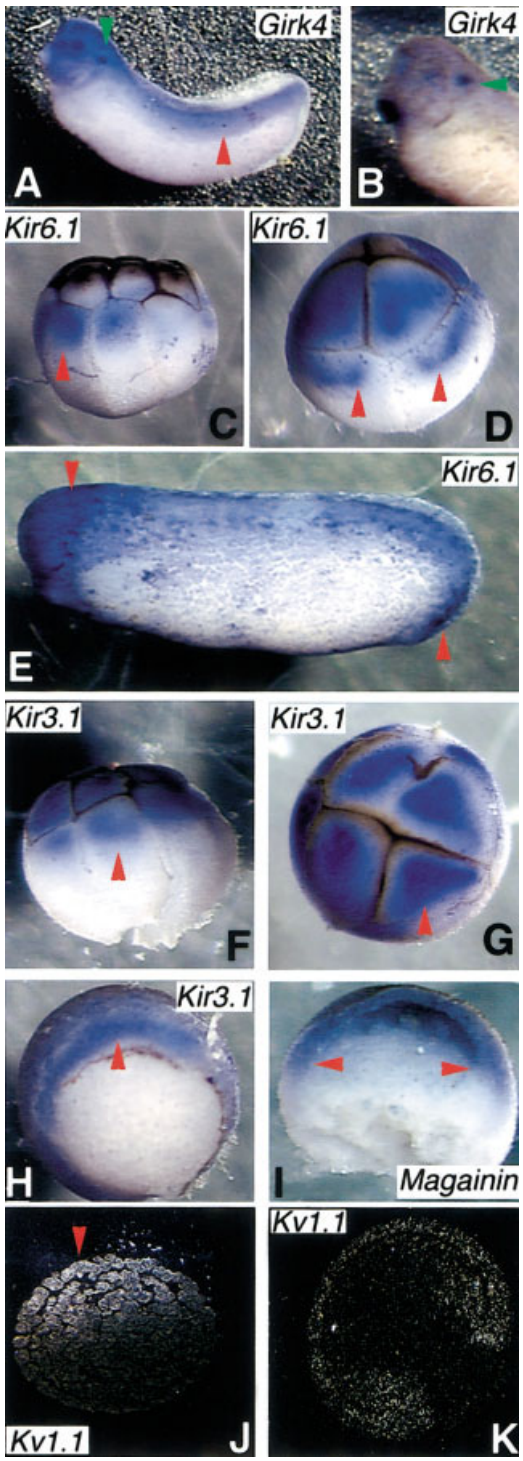

Figure 3.

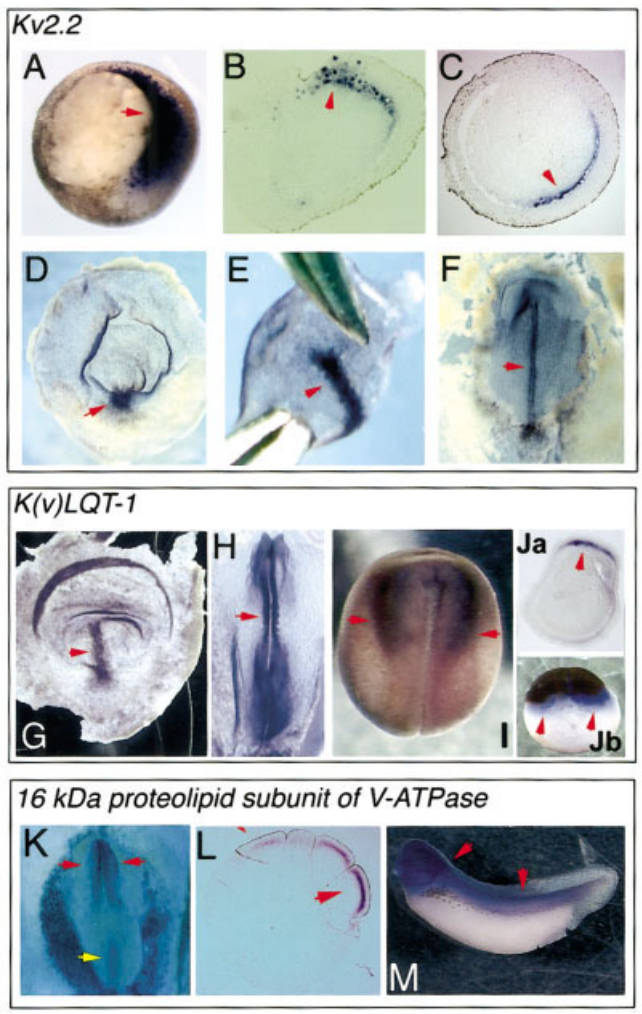

Fig. 4. Comparison of ion channel and pump gene expression in chick and *Xenopus*. We compared the expression of the same ion channel clones from chick and *Xenopus* by in situ hybridization. **A:** The K^+ channel xKv2.2 was expressed very strongly in the organizer during *Xenopus* gastrulation (arrow). **B,C:** Sectioning reveals staining deep in organizer cells (arrows). Similarly, in chick, cKv2.2 was expressed in the base of the nascent primitive streak at stage (st.) 1 (arrow, **D**) and in the streak itself as it elongates (arrows, **E,F**). The potassium channel K(v)LQT-1 is also expressed in the primitive streak in the chick (arrow, **G**) and then in most tissues at st. 8 (arrow, **H**). **I:** In contrast, in *Xenopus*, we detect no expression by in situ hybridization at gastrulation stages (data not shown), but at neurulation, it is expressed in a horse-shoe pattern very similar to the location of the neural crest (arrows). Maternal mRNA for K(v)LQT-1 is located in the animal halves of cells at early cleavage stages (**J**, showing the inside surface of half of a four-cell embryo manually split down the cleavage plane after in situ hybridization; Ja: section of 1-cell embryo, Jb: inside surface of wholemount 2-cell embryo split down the cleavage plane). **K:** In chick embryos, the 16-kDa proteolipid subunit of the vacuolar ATPase is expressed in the head-folds of the closing neural tube (red arrows); it can also be seen in the regressing primitive streak (yellow arrow). In *Xenopus*, maternal mRNA for the 16-kDa proteolipid subunit can be detected as maternal mRNA in animal cells during cleavage (arrows, **L**), and similarly to the chick, is localized to the neural tissues in the dorsal aspect of the embryo at somite stages (arrows, **M**). Red arrows indicate regions of expression.

lation (Fig. 4A–C). Similarly, in chick, cKv2.2 was expressed in the base of the nascent primitive streak at st. 1 (Fig. 4D) and in the streak itself as it elongates (Fig. 4E,F).

K(v)LQT-1 (KCNQ1). The potassium channel thought to be involved in long QT syndrome in human heart pathology (Wang et al., 1999), K(v)LQT-1, is also expressed in the primitive streak in the chick (Fig. 4G) and then in most tissues at early somite stages (Fig. 4H). In contrast, we detect no expression by in situ hybridization in *Xenopus* at gastrulation stages, but at neurulation, it is expressed in a horse-shoe pattern most likely representing neural crest (Fig. 4I). Maternal mRNA for K(v)LQT-1 is located in the animal pole of unfertilized embryos and of cells at early cleavage stages (Fig. 4Ja,b).

Ductin. The 16-kDa proteolipid subunit of the vacuolar ATPase, known as ductin (Finbow et al., 1992), has also been suggested to be able to form functional gap junctions between cells (Finbow and Pitts, 1993). Because of the important physiological and regulatory roles for this subunit, we examined its expression pattern in both chick and frog embryogenesis. In chick embryos, the 16-kDa proteolipid subunit of the vacuolar ATPase is expressed in the head-folds of the closing neural tube (Fig. 4K, red arrows); it can also be seen in the regressing primitive streak (Fig. 4K, yellow arrow). In *Xenopus*, maternal mRNA for ductin can be detected in animal cells during cleavage (seen in section, Fig. 4L) and, similarly to the chick, is later localized to the neural tissues in the dorsal aspect of the embryo at somite stages (Fig. 4M).

Expression of Functionally Related Genes

We also examined the expression of several genes that are not strictly speaking ion channels or pumps, but are relevant to establishing and maintaining the voltage potential of cells.

The 14-3-3 family of regulatory proteins. The 14-3-3 family of genes encode proteins that are involved in cell cycle control and are implicated in prion diseases (Kumagai et al., 1998; Satoh et al., 1999). Additionally, they have been shown to interact with the H^+ ATPase and regulate the ion pump's activity (Baunsgaard et al., 1998; Chelysheva et al., 1999; Camoni et al., 2000; Morsomme and Boutry, 2000). Because we detected mRNA of several subunits of the proton pump in embryos (Figs. 1, 2), we next characterized the expression of several regulators of H^+ ATPase ion pumps: the 14-3-3 proteins ϵ and β/α. The 14-3-3 member ϵ is weakly expressed in the primitive streak in chick embryos at st. 3 (Fig. 5A), but by stage 4, its expression is extremely strong in all embryonic tissues except for the posterior-most margin of the area opaca (Fig. 5B, yellow arrow). The 14-3-3 β/α is detected in chick embryos in the primitive streak at st. 3 and is specifically absent from the primitive pit in Hensen's node (Fig. 5C).

Aquaporin: A water and ion channel. Aquaporin forms a pore in cell membranes that is used for regulation of the cell's water content (Parisi and Ibarra, 1996; Verkman et al., 1996). Of interest, it can also serve as an ion channel (Agre et al., 1997; Anthony et al., 2000). Thus, we examined the expression of two members of the Aquaporin family, AQP-4 and AQP-7, in *Xenopus* embryos. Aquaporin 7 mRNA is present around the circumference of the animal cap at st. 7, but is not detected in the vegetal cells (Fig. 5D). At somite stages, Aquaporin 7 is detected in the brain, eye, branchial arches, and somites (Fig. 5E). Aquaporin 4 is detected in the cortex of the animal portion of the embryo at the two-cell stage (Fig. 5F). By the four-cell stage, the mRNA is detected only in the nucleus (Fig. 5G), suggesting a dynamic regulation of mRNA localization at these stages. By somite stages, Aquaporin 4 mRNA can be detected in the brain, somites, and tail bud (Fig. 5H).

Hensin: Linking the electrical and morphologic polarity of the cell. Hensin is a protein found in the extracellular matrix of many cell types (Al-Awqati et al., 1998). It controls the polarity of cells by determining whether the apical or basal surface of cells contain the H^+ ATPase, or an anion channel (Alpern, 1996; Takito et al., 1999; Vijayakumar et al., 1999). In early chick embryos, hensin is expressed in the base of the primitive streak at stage 3 (Fig. 5I) and then in stripes in the lateral plate of head-fold stage embryos (Fig. 5J). The stripes do not seem to correspond to any previously characterized developmental compartment. Because we detected the presence of mRNA for the Kv family of ion channels (Fig. 1K,L), we next examined the presence of KCHIP genes, proteins that directly interact with voltage-gated K^+ channels (Sanguinetti, 2002). Although KCHIP4.2 was not detected (Fig. 5K), KCHIP2 mRNA was present in the primitive streak (Fig. 5L).

Gap junction genes. The voltage potentials achieved in any cell can be dissipated or spread to neighboring cells through the presence of gap junctions, i.e., direct channels between apposing cell membranes that can permit the passage of small molecules, subject to a large number of regulatory factors (Goodenough et al., 1996). Because gap junctions can be formed by oligomers of proteins from the connexin family, we characterized the expression of several connexin genes in both chick and frog to begin to understand the basis for ion flux between cells.

Cx31, which is involved in the function of the ear (Liu et al., 2000), is expressed in the posterior margin of the area pellucida in the chick embryo at st. 4^+ (Fig. 6A), similar to some of the BMP genes. In contrast, Cx47 is expressed within the posterior third of the primitive streak at st. 4 (Fig. 6B). In *Xenopus*, maternal Cx40 mRNA can be found in the anterior pole of the two-cell embryo (Fig. 6C) and then in a band perpendicular to the animal–vegetal axis in the four-cell embryo (Fig. 6D). During blastula stages, it is expressed in the cells of the animal cap (Fig. 6E). We used radioactive detection on sections to analyze the presence of

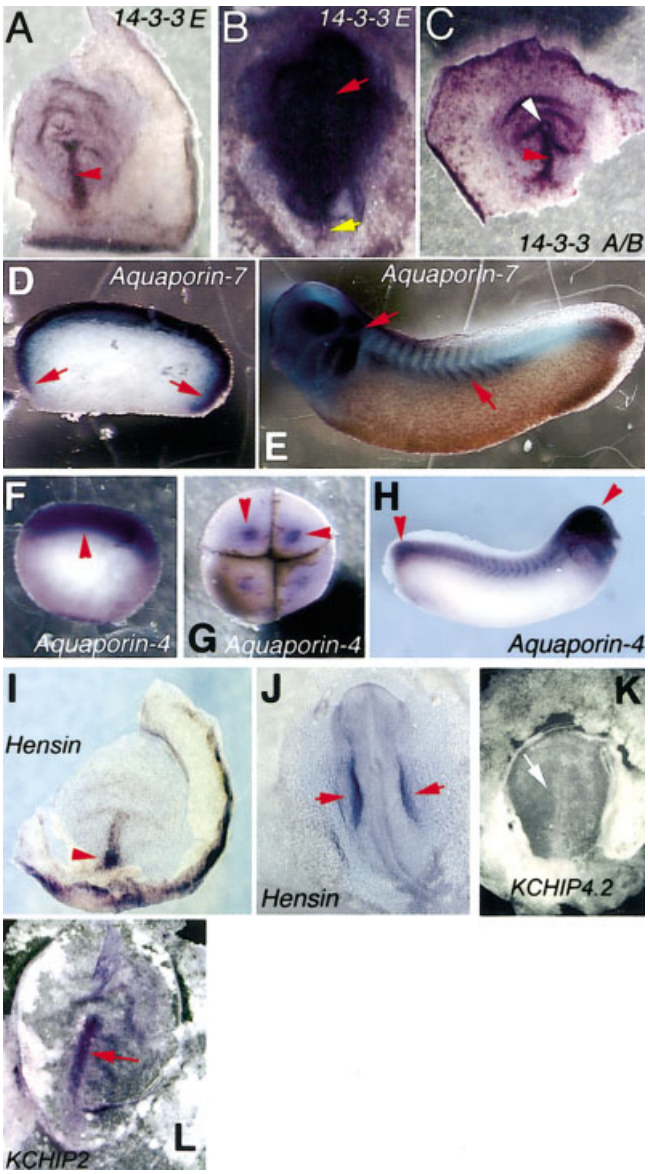

Fig. 5. Expression of accessory genes in chick and *Xenopus*. We examined the expression of several genes that, although not strictly ion pumps or channels, are relevant to maintaining a cell's membrane potential. The 14-3-3 family member ϵ is weakly expressed in the primitive streak in chick embryos at stage (st.) 3 (arrow, **A**), but by st. 4, its expression is extremely strong in all embryonic tissues (red arrow, **B**), except for the posterior-most margin of the area opaca (yellow arrow, **B**). **C:** The 14-3-3 α/β is expressed in the primitive streak at st. 3 (red arrowhead) but is specifically excluded from Hensen's node (white arrowhead). D,E: Aquaporin 7 mRNA is present around the circumference of the embryo at st. 7 (red arrows) but is not detected in the yolky vegetal cells. **D:** The inside view of a st. 7 embryo split in half parallel to the animal–vegetal axis after in situ hybridization. **E:** At somite stages, Aquaporin 7 is detected (red arrowheads) in the brain, eye, branchial arches, and somites. F–H: Aquaporin 4 is detected (red arrowheads) in the cortex of the animal portion of the embryo at the two-cell stage. **F:** The interior view of half of a two-cell embryo; the blastomeres were separated manually after in situ hybridization. **G:** By the four-cell stage, the mRNA is detected only in the nucleus. **H:** By somite stages, Aquaporin 4 can be detected in the brain, somites, and tail bud. In early chick embryos, hensin is expressed in the base of the primitive streak at st. 3 (arrowhead, **I**) and then in stripes in the lateral plate of head-fold stage embryos (arrows, **J**). KCHIP4.2 is not detected in the early chick (arrow, **K**), but KCHIP2 is expressed in the primitive streak (arrow, **L**). Red arrows indicate regions of expression.

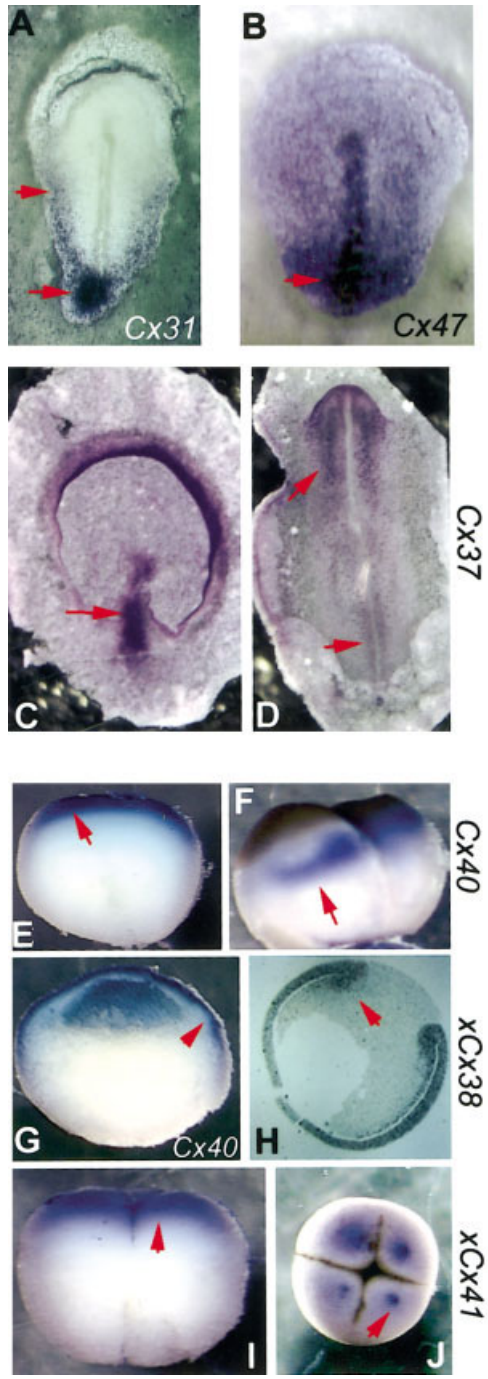

Fig. 6. Expression of connexin genes in frog and chick embryos. **A:** Cx31 is expressed in the posterior margin of the area pellucida in the chick embryo at stage (st.) 4^+ (arrows). **B:** Cx47 is expressed within the posterior third of the primitive streak at st. 4 (arrow). Cx37 is expressed in the early primitive streak (arrow, **C**), and in a punctate pattern identifying a subset of the cells of the primitive ridges and neural plate at st. 7 (arrows, **D**). In *Xenopus*, maternal Cx40 mRNA is in the anterior pole of the two-cell embryo (arrow, **E**) and then in a band perpendicular to the animal–vegetal axis in the four-cell embryo (arrow, **F**). **G:** During blastula stages, it is expressed in the cells of the animal cap (arrowhead). **H:** Radioactive in situ hybridization on sections shows that Cx38 is present in the animal cap and in gastrulating cells (arrow, radioactive section). Cx41 mRNA is spread throughout the animal pole of the two-cell embryo (arrow, **I**) but is only detected in the nucleus by the four-cell stage (arrow, **J**). Red arrows indicate domain of expression.

Cx38 in *Xenopus*; it is also present in the animal cap and in gastrulating cells (Fig. 6F). Cx41 mRNA is spread throughout the animal pole of the two-cell embryo (Fig. 6G) but is only detected in the nucleus by the four-cell (Fig. 6H), perhaps indicating a dynamic mRNA degradation event.

DISCUSSION

Fields and Currents in Embryos

Ion pumps such as Na^+/K^+-ATPases and V-ATPases build up voltage potential differences at the expense of ATP (Gluck, 1992; Marin and Redondo, 1999). A complementary role is played by ion channels, which allow the passive exit of ions from the cell and, thus, serve to shape and regulate the potential by creating a dependence of ion flux on voltage, pH, or signaling factors. Gap junctions are another important component, because they provide isopotential syncitia of cells. Epithelial cell sheets often consist of highly polarized cells that drive current through the embryo much like the plasma membrane drives ion flux through the single cell (Nuccitelli, 1986). Strong standing membrane potentials and ion currents have been found in many organisms (Nuccitelli, 1988) and have been specifically described in various tissues in chick and frog embryos (Oconnor et al., 1977; Kline et al., 1983; Hotary and Robinson, 1990; Levin et al., 2002).

Interference with endogenous fields causes specific developmental defects, suggesting that these potential differences and ion flows have roles in normal embryonic morphogenesis (e.g., Hotary and Robinson, 1992, 1994, Borgens and Shi, 1995). To enable functional analyses of developmental ion flux, we conducted a survey of the expression of genes that control ion flow and membrane voltage. Although the presence of a transcript cannot, by itself, prove that the protein has a causal role during development, expression data are a required complement to electrophysiology to focus the generation of antibodies on plausibly promising targets and to suggest candidates for further functional analysis by means of genetic deletion, morpholinos, and pharmacology.

Channel and Pump Subunits Transcribed in Chick and Frog Embryos

We detected specific expression of K^+ and anion channels, as well as the Na^+/K^+-ATPase, in the primitive streak of chick embryos and in the dorsal lip of the blastopore in *Xenopus*—two key organizing centers during early embryogenesis. The specific, strong, and spatially patterned expression of ion channels and pumps during early stages (before the appearance of neurons) is consistent with morphogenetic roles for these genes, in contrast with housekeeping functions required in all cells. Expression patterns such as Girk1 in the somites of chick (Fig. 1D) and the β subunit of the Na^+/K^+ ATPase in the *Xenopus* somite (Fig. 2E, see also Davies et al., 1996; Messenger and Warner, 2000) suggest developmental roles for ion flows and/or voltage potentials, such as in somite patterning. Kir3.4 is expressed in the developing otic vesicle (Fig. 3A,B) and may be important for the morphogenesis of the ear. Other expression patterns, such as that of the β3 Na^+/K^+-ATPase in the ventral hemisphere of the blastopore (Fig. 2C,D) identify specific novel subpopulations of cells consistent with the existence of as yet uncharacterized embryonic signaling centers (e.g., on the ventral side, in addition to the known signaling properties of the dorsal side of the blastopore). Although expression along the length of the neural tube and in the head are a common pattern for ion channels and pumps (Figs. 2H, 3A,E, 4M, 5H), it is important to note that this pattern is neither an artifact of whole-mount in situ hybridization nor obligatory, because other genes (e.g., Fig. 2J) do not exhibit the same staining.

Comparison of Expression of the Same Ion Flux Genes Between Chick and Frog

We find that some ion flux genes (such as Kv2.2 and ductin) are expressed in homologous locations in both species, consistent with a conserved role in development. Others mRNAs, such as K(v)LQT-1, have differing expression patterns (Fig. 4G–J). If indeed it is the overall membrane potential/ion flow profile that controls aspects of development, evolutionary diversification may allow significant interspecies variability in expression, because a particular voltage can be achieved by the activity of any of several ion pumps and channels. This finding has implications for genetic knockdown studies (e.g., underexpression by means of morpholinos and knockout mice), because many other functionally similar channels or pumps can be expected to provide regulation or compensation of membrane voltage potential.

Expression of Regulatory Subunits

In addition to bona fide ion channels and pumps, we also describe expression of other genes that contribute to the regulation of cell membrane potential. Members of the 14-3-3 protein family, which have roles in regulating V-ATPase activity, as well as aquaporin mRNA, are specifically expressed in early chick and frog embryos. One of the accessory subunit proteins for the Kv channel family, KCHIP2, is expressed in early chick embryos (Fig. 5L) and, thus, is colocalized with Kv3.1 (Fig. 1K) and partially overlapping with Kv6.2 (Fig. 1L). Voltage-sensitive ion channels are important candidates for transducing endogenous voltage gradients to downstream targets; functional studies of the role of the Kv family of channels in the primitive streak will have to consider the effects of interaction with the KCHIP family of accessory proteins, which are known to modify the behavior of the channel (Rosati et al., 2001; Beck et al., 2002; Guo et al., 2002).

Of interest, we found expression of magainin (Cruciani et al., 1992; Baker et al., 1993; Soballe et al.,

1995, Matsuzaki, 1998) during embryonic development (Fig. 3I). This antibacterial protein produced by adult *Xenopus* skin has been found to have antitumor activity (Baker et al., 1993); taken together with the role of membrane voltage in regulating tumorigenicity of several cell types (Bianchi et al., 1998; Klimatcheva and Wonderlin, 1999), magainin and related compounds may have important roles in growth regulation during development and neoplasm through modulation of membrane voltage potential of cells.

Expression of Gap Junction Genes

Ionic events occurring in a cell as a result of channel and pump function can influence adjacent cells directly through current spread and dissipation by means of gap junctions. Analysis of gap junctional expression is particularly important, because functional fluorescent dye permeation data, often used to assess gap-junctional communication between embryonic tissues, does not necessarily reveal or mirror the ionic coupling that can exist between cells (Loewenstein, 1981). We detect several different domains of connexin mRNA in chick and frog embryos that could define regions of isopotential cell fields. In particular, the expression of Cx31, Cx37, and Cx47 in the base of the primitive streak hint at important and uncharacterized signaling roles for this embryonic region. Connexin 43 (Cx43) has already been characterized during early chick embryo development and shown to have an important role in left–right asymmetry (Levin and Mercola, 1999). Gap-junctional communication is likewise known to be important in left–right patterning in *Xenopus* (Levin and Mercola, 1998), but it is as yet unknown which connexin genes underlie the endogenous coupling. Because gap junctions are subject to numerous regulatory steps at the posttranslational level, functional electrophysiology studies are needed to determine what open junctional paths exist for various types of ions during different stages of embryogenesis. That gap-junction gating and selectivity properties depend greatly on which connexin family members comprise the junction (Elfgang et al., 1995; Bruzzone et al., 1996b; Meda, 2000) further underscores the necessity for a comprehensive understanding of which connexins are expressed in which embryonic cells during patterning. Overlapping patterns of connexin expression are consistent with the presence of heteromeric or heterotypic gap junctions, which could possess complex gating properties not present in either connexin alone. This picture is further complicated by the fact that gap junctions are sensitive to pH and voltage (Brink, 2000; Morley et al., 1996); because of this recursive control loop with proteins that produce ionic flows and modulate membrane voltage, sophisticated electrophysiological models will be needed to understand the true pattern of ion flux within and outside embryonic tissues during development.

Ductin: A Component of Ion Pumps and Gap Junctions

Ductin is a particularly interesting molecule because its suggested ability to form functional gap junctions on its own (Finbow and Pitts, 1993; Bruzzone and Goodenough, 1995; Finbow et al., 1995) complements its established role as the 16-kDa proteolipid subunit of the vacuolar ATPase (H^+ pump). Genetic inhibition of ductin function leads to neoplastic transformation in cells and to major embryonic defects (Finbow et al., 1991; Bohrmann and Lammel, 1998; Saito et al., 1998; Inoue et al., 1999). The plethora of data on the role of gap junctions in development (reviewed in Lo, 1999; Levin, 2001) concern junctions composed of connexin proteins (Bruzzone et al., 1996a); ductin is important because it may comprise a little-understood alternative basis for gap-junctional communication. We detect expression of ductin in both chick and frog embryos; a comparison of roles in the two species, and an evaluation of contributions to H^+ flux or GJC, awaits functional analysis.

Of interest, Hensin has been shown to determine the complementary localization of ductin and an anion channel that are targeted to opposite poles within cells (Al-Awqati et al., 1998; Takito et al., 1999; Matsushita et al., 2000). Thus, Hensin is a good candidate for orienting the electrical polarity of cell groups relative to the developmental polarity of the rest of the tissue (Al-Awqati, 1996). The overlapping expression of anion channels and V-ATPase subunits in the chick primitive streak is consistent with a patterning role in early embryogenesis, because the streak is developmentally and electrically polarized in three dimensions (Stern, 1982; Levin et al., 2002). The *Xenopus* embryo, with large highly polarized blastomeres, will be an ideal context in which to probe the association between Ductin and Hensin and ascertain its functional significance. Hensin is also expressed in thin stripes in lateral tissue during neurulating embryos, which correspond to no known embryonic compartments. Thus, this expression domain may indicate novel regionalization events that remain to be characterized.

Subcellular Localization of Channel and Pump Subunits

In addition to examining the expression of zygotically transcribed genes in post-MBT embryos, we visualized subcellular maternal mRNA localization in *Xenopus*. Some mRNAs, such as Aquaporin 4 (Fig. 5G) and Kv2.2 (data not shown), are detected only in the nucleus at certain stages and are unlikely to be translated. In contrast, mRNA for the α subunit of the Na^+/K^+ ATPase and the 16-kDa proteolipid subunit of the vacuolar ATPase are found localized to the cortex of the animal-most region of the cleaving embryo (Fig. 2H,L), suggesting, consistently with their role as cell-membrane ion pumps, that these mRNAs may be translated and functional as plasma-membrane pro-

teins. Significantly, mRNA for AQP-4 is detected throughout the animal portion of the cortex of the one- and two-cell *Xenopus* embryo but then only seen in the nucleus of the four-cell embryo (Fig. 5F,G), consistent with active degradation or transport of mRNA, which would affect translation and, thus, the presence of Aquaporin protein.

The subcellular localization of maternal mRNA can be due to differential degradation, anchoring, or active transport (Bloom and Beach, 1999; Lipshitz and Smibert, 2000); the differential dorsoventral localization of a V-ATPase subunit (Fig. 2C) is particularly notable in this regard, because it could comprise a regulatory mechanism: this mRNA may be translated in ventral but not dorsal cells. We are currently pursuing the functional significance of this phenomenon. Protein-protein interactions (and PDZ domains in particular) are thought to be important to targeting ion channel proteins to subcellular locations in neurons (Sheng and Wyszynski, 1997). The differential localization patterns we describe for genes such as AQP-4 through early developmental stages suggest that mRNA localization may play an important regulatory role in controlling the function of ion channel and pump proteins at the translational level. These results underscore the need for analysis of maternal mRNA distribution in expression studies of developmentally important genes, because differential localization patterns can contribute to fine control over the spatial locations of ion channel and pump proteins, and thus, setting up specific patterns of ion current flow.

Future of Ion Flux Genes' Expression

A plethora of as yet uncharacterized channel and pump genes remain to be described; embryonically important ion flux proteins will continue to be identified through genome projects as well as pharmacologic screens using channel and pump blockers. A variety of accessory proteins such as gap junctions and channel/pump regulatory factors must also be considered in formulating detailed, predictive models of embryonic ion flux and cellular responses to this developmental signal. The development of pH- and voltage-sensitive fluorescent dyes and confocal detection forms an essential complement to self-referencing probe approaches in characterizing endogenous fields and potentials (Loew, 1992; Messerli et al., 1999; Smith et al., 1999; Altizer et al., 2001; Smith and Trimarchi, 2001). Pharmacologic reagents and dominant negative constructs can then be used to functionally test specific embryonic roles of ion flux. The understanding, at the molecular level, of the role of ion currents and voltage and pH gradients in embryogenesis is likely to have important repercussions for the areas of developmental biology, tumor progression, and regeneration, in both basic science and biomedicine.

EXPERIMENTAL PROCEDURES

Collecting Embryos

Chick embryos were collected and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992) by fixing in 4% paraformaldehyde. Before in situ hybridization, *Xenopus* embryos were collected and fixed in MEMFA (Harland, 1991). All embryos were washed in phosphate buffered saline + 0.1% Tween-20 and then transferred to 100% methanol through a 25%/50%/75% series. *Xenopus* embryos were lightly bleached for 2 hr in 30% H_2O_2/70% MeOH under a fluorescent light to enable detection of transcripts in pigmented blastomeres while maintaining difference in pigment between dorsal and ventral cells, which aids in spatial analysis of localization.

Clones and Probes

Probes for in situ hybridization were generated in vitro from linearized templates using digoxigenin (DIG) labeling mix from Roche. The references for constructs used for each ion channel and pump probe are listed in Tables 1–4. In all cases, the entire available sequence was used to generate the probe. For EST fragments, the identity was confirmed by running the sequence through the BLAST algorithm (Altschul et al., 1990) at the National Center for Biotechnology Information's Web site.

Chromogenic In Situ Hybridization

In situ hybridization was performed according to a standard protocol (Harland, 1991) using an alkaline phosphatase-coupled antibody to DIG, which produces a blue signal. In all cases, the duration of the chromogenic reaction was optimized to the probe used by determining the length of staining, which produced the best contrast between signal and background. For chick embryos, this timing varied between 3 hr for strong probes to 24 hr for weaker probes. For *Xenopus* embryos, the length was between 12 and 30 hr. *Xenopus* embryos were washed several times in methanol after the chromogenic reaction to reduce nonspecific background. Negative controls (no probe, to control for endogenous alkaline-phosphatase activity and sense probe to control for specificity) were very clean and showed no signal (Figs. 1A, 2A). Presented data are based on at least 5 chick embryos or 10–20 *Xenopus* embryos, which all showed the same signal. Signal was photographed by using darkfield, brightfield, or incident ring-light using a Nikon SMZ-1500 dissecting microscope and CoolPix digital camera using OpenLab software. Criteria for classification (Table 1 vs. other tables) was as follows: nonspecific expression was that which produced no regions of differential signal under any duration of chromogenic reaction; lack of expression was that which produced no signal after chromogenic exposure that was twice as long as the exposure needed to produce signal in the weakest probes (approximately 3 days for *Xenopus*, 2 days for chick em-

TABLE 3. Ion Pumps, Gap Junctions, and Regulatory Proteins Expressed Specifically in Chick Embryos[a]

Clone	Gene type	Reference
72.H12	Na^+/K^+ ATPase, beta subunit	Unpublished; accession no. AI982062
7.D5	→ Hensin	Unpublished; accession no. AW198392
KCHIP2	Kv channel-interacting protein	Unpublished; accession no. AF508736
29.C3	14-3-3 protein epsilon	Unpublished; accession no. AI980654
54.B7	14-3-3 protein beta/alpha	Unpublished; accession no. AI981461
NCKX rod	$Na^+/K^+/Ca^{++}$ exchanger	Prinsen et al., 2000
NCKX cone	$Na^+/K^+/Ca^{++}$ exchanger	Prinsen et al., 2000
A2_ch	→ H^+ ATPase 116 kDa subunit A2 isoform	Mattsson et al., 2000
A3_ch	→ H^+ ATPase 116 kDa subunit A3 isoform	Mattsson et al., 2000
70.G11	Vacuolar-type H^+-ATPase 115 kDa subunit	Unpublished; accession no. AI981985
5.G5	H^+-transporting ATPase	Unpublished; accession no. AI979881
cV-ATPase	Catalytic subunit of the V-ATPase	Hernando et al., 1999
Cx31	→ Connexin 31	Heller et al., 1998
11.C13	Connexin 37	Unpublished; accession no. #BI067876
5.N11	Connexin 47	Unpublished; accession no. BI391875

[a]The transcripts of these genes (which include ion pump subunits, connexins, and regulatory factors for electrogenic proteins) exhibited specific expression in chick embryos. Particularly interesting candidates are identified with an arrow; see figures and text for details of in situ analysis and significance.

TABLE 4. Genes Specifically Expressed in *Xenopus*[a]

Clone	Gene type	Reference
xKv1.1	Shaker-like (delayed rectifier) K^+ channel	Ribera and Nguyen, 1993
xKv2.2	Shaker-like (delayed rectifier) K^+ channel	Burger and Ribera, 1996; Gurantz et al., 1996
xKv3.1	Voltage-gated channel	Gurantz et al., 2000
p24-15	→ Neural beta3 subunit of Na^+/K^+ ATPase	Good et al., 1990; Richter et al., 1988
pGEM2-α	α1 subunit of Na^+-K^+ ATPase	Verrey et al., 1989
pGEM2-β	β1 subunit of Na^+-K^+ ATPase	Verrey et al., 1989
xH/K-ATPase	α1 subunit of gastric H^+-K^+ ATPase	Mathews et al., 1995
KVLQT1	Subunit that coassembles with minK to form I(Ks)	Sanguinetti et al., 1996
xAQP4	Aquaporin 4	Unpublished; accession no. AW199121
xAQP3	Aquaporin 7	Schreiber et al., 2000
Magainin	Ion pore	Terry et al., 1988
H^+ pump subunit	→ V-ATPase 16 kDa subunit	Unpublished; accession no. BE025931
H^+ pump subunit	→ Vacuolar H^+ synthase 16 kDa subunit	Unpublished; accession no. AW768111
DKFZp724A033	Vacuolar H^+ ATPase	Unpublished; accession no. BE025931
Cx40	→ Connexin 40	Unpublished; accession no. AW765892
Cx41	→ Connexin 41	Yoshizaki, 1995
Cx38	Connexin 38	Ebihara, 1996

[a]The transcripts of these genes exhibited specific expression in *Xenopus* embryos. Particularly interesting candidates are identified with an arrow; see figures and text for details of in situ analysis and significance.

bryos). In most cases, developmental stages that showed specific expression were preceded and followed by stages during which expression was absent. For reasons of brevity, the figures show only stages during which specific expression was detected.

Radioactive In Situ Hybridization

Radioactive in situ hybridization was performed as described in (O'Keefe, 1991). Embryos were fixed in MEMFA (Harland, 1991), embedded in paraffin, and sectioned to a thickness of 8 μm before incubation with [^{35}S]UTP-labeled cRNA probe. Serial sections were hybridized to antisense probes generated in vitro by using standard methods.

ACKNOWLEDGMENTS

The authors thank Changwan Lu for the Kir6.1 and Kir3.4 clones; Jonas Galper for the GIRK1; Benjamin Kaupp for the cCNG, pCCG6, and pCCG8B clones; Carl Oberholtzer for the cIRK1 and cSlo1 clones; Klaus Bister for the cCO6 clone; Douglas M. Fambrough for the SERCA2, SERCA1, and Na^+/K^+ ATPase β1 and α1 clones; Jim Hudspeth for the Alpha1D clone; Bjorn Vennstrom for the BIII1 clone; William Horne for the chick V-ATPase clone; Mark Bothwell for the Kv6.2-114 and Kv2.2 clones; Joan Burnside for the 72H12, 42A5, 29C3, 54B7, 5G5, 70G11, and 7D5 clones; Jan Mattsson for the A2 and A3 isoforms of the chick H^+ ATPase; Angeles Ribera for the xKv1.1 and xKv2.2 clones; Rainer Schreiber for the AQP3 clone; Bruce Blumberg for the AQP4 clone; Igor Dawid for the p24-15 clone; Kaethi Geering for the α subunit of the H^+/K^+-ATPase, and the pGEM2-α and pGEM2-β clones; Paul Schnetkamp for the NCKX clones; James L. Rae for the KCHIP2 and KCHIP4 clones; Mike Zasloff for the magainin clone; Kristin Kwan for the

Xombi clone; Nicholas Spitzer for the Kv3.1 clone; Daniel Goodenough and David Paul for the Cx38 clone; James Hudspeth for the Cx31 clone; Richard Patino for the Cx41 clone; and Igor Splawski for the xKVLQT-1 clone. EST clones were obtained from the following sources: Joan Burnside and the University of Delaware chick EST archive project (http://www.chickest.udel.edu/), Bruce Blumberg (distributed by Research Genetics), Sandy Clifton and the Washington University School of Medicine Xenopus EST project (distributed by the RZPD: http://www.rzpd.de), and Richard Harland (distributed by the IMAGE Consortium/LLNL at: http://image.llnl.gov/image/html/iresources.shtml). We also thank Mark Mercola for his generous support and many helpful discussions, as well as Richard Borgens and Ken Robinson whose studies on developmental electrophysiology motivated this work. M.L. gratefully acknowledges a Helen Hay Whitney Foundation fellowship.

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