DEVELOPMENTAL DYNAMICS 219:96–101 (2000)

## BRIEF COMMUNICATION

# Expression of Connexin 30 in *Xenopus* Embryos and Its Involvement in Hatching Gland Function

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***ABSTRACT*** **Connexins are a family of proteins that assemble to form gap junction channels. Cell-cell communication through gap junctions mediates many important events in embryogenesis, including limb patterning, lens physiology, neuronal function, left-right asymmetry, and secretion from gland tissue. We studied the expression of connexin 30 (*Cx30*) in the *Xenopus* embryo and find that it is expressed in the developing hatching gland and pronephros. To determine whether its expression plays a functional role in the activity of the hatching gland, we exposed pre-hatching embryos to drugs that block gap junctional communication. This resulted in a continuation of normal growth and development but specifically abolished hatching. The treatment did not affect *Cx30* or *Xenopus* hatching enzyme transcription, suggesting a post-transcriptional effect on Cx30 gap junctions. We conclude that junctional communication, possibly mediated by *Cx30*, is involved in secretion of hatching enzyme in *Xenopus*. Dev Dyn 2000;219:96–101.** © 2000 Wiley-Liss, Inc.

**Key words: connexins; gap junctions; hatching; secretion; *Xenopus***

## INTRODUCTION

Gap junctional channels formed by oligomers of proteins from the connexin family are pores between cells that allow the conduction of low-molecular-weight molecules (< 1 kd) (Bruzzone et al., 1996; Goodenough et al., 1996). Gap-junctional communication (GJC) of important regulatory signals underlies many important physiological phenomena, such as left-right asymmetry (Levin and Mercola, 1998; Levin and Mercola, 1999), carcinogenesis (Yamasaki et al., 1995; Krutovskikh and Yamasaki, 1997), and neuronal function (Bruzzone and Ressot, 1997; Dermietzel, 1998). GJC is also an obligatory feature of most gland tissues, playing a role in regulating key secretory events (Meda, 1996a, 1996b). For example, in the mammalian pancreas, abolishing GJC renders cells unable to secrete insulin (Meda et al., 1990). Despite the importance of gap junctions in many physiological events, few studies have examined the spatial patterns of expression of connexin proteins in early chick or frog embryos. In this study we characterized the expression of *Xenopus* Cx30 in embryogenesis and examined the possible role of GJC in the function of the hatching gland.

## RESULTS AND DISCUSSION

We examined the expression pattern of connexin 30 (Gimlich et al., 1988) in *Xenopus* embryos by whole-mount in situ hybridization. *Xenopus* connexin 30 is most similar to mouse connexin 32 (58.7% identity). We first detect expression of *Cx30* in ectodermal tissue at the anterior end of the closing neural tube at stage 17 (Fig. 1A). The dorso-anterior ectodermal domain of expression enlarges at stage 18; at that time, *Cx30* also is detected in endodermal tissue lining the archenteron (Fig. 1B). *Cx30* becomes specifically expressed in the hatching gland (Fig. 1C) and is visible as a stripe on the anterior dorsal ectoderm covering the head and in two semicircular stripes more anteriorly. Sectioning through the face confirms the location of the transcript in superficial ectoderm of the hatching gland (Fig 1D). At stages 26–31, *Cx30* is detected (Fig. 1E,F) in bilateral domains very similar to the pronephros expression seen with *Xwnt-4* (Carroll et al., 1999), presaging expression in adult kidney tissue as detected by Northern analysis (Gimlich et al., 1990). Although kidney tissue on both sides expresses *Cx30*, we often observed one-sided expression among younger embryos within a population examined by in situ hybridization, perhaps indicating differences in the timing of development of kidney tissue between the left and right sides. Interestingly, we do not detect expression of *Cx30* in later tadpole stages, including the brain (data not shown); in contrast, mammalian *Cx32*, which is homologous to *Xenopus Cx30*, is widely expressed in the brain (Na-

Grant sponsor: Helen Hay Whitney Foundation; Grant number: F773; Grant sponsor: National Institutes of Health; Grant number: R01HL63271; Grant sponsor: American Heart Association; Grant number: 9740005N.

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Received 17 February 2000; Accepted 30 May 2000

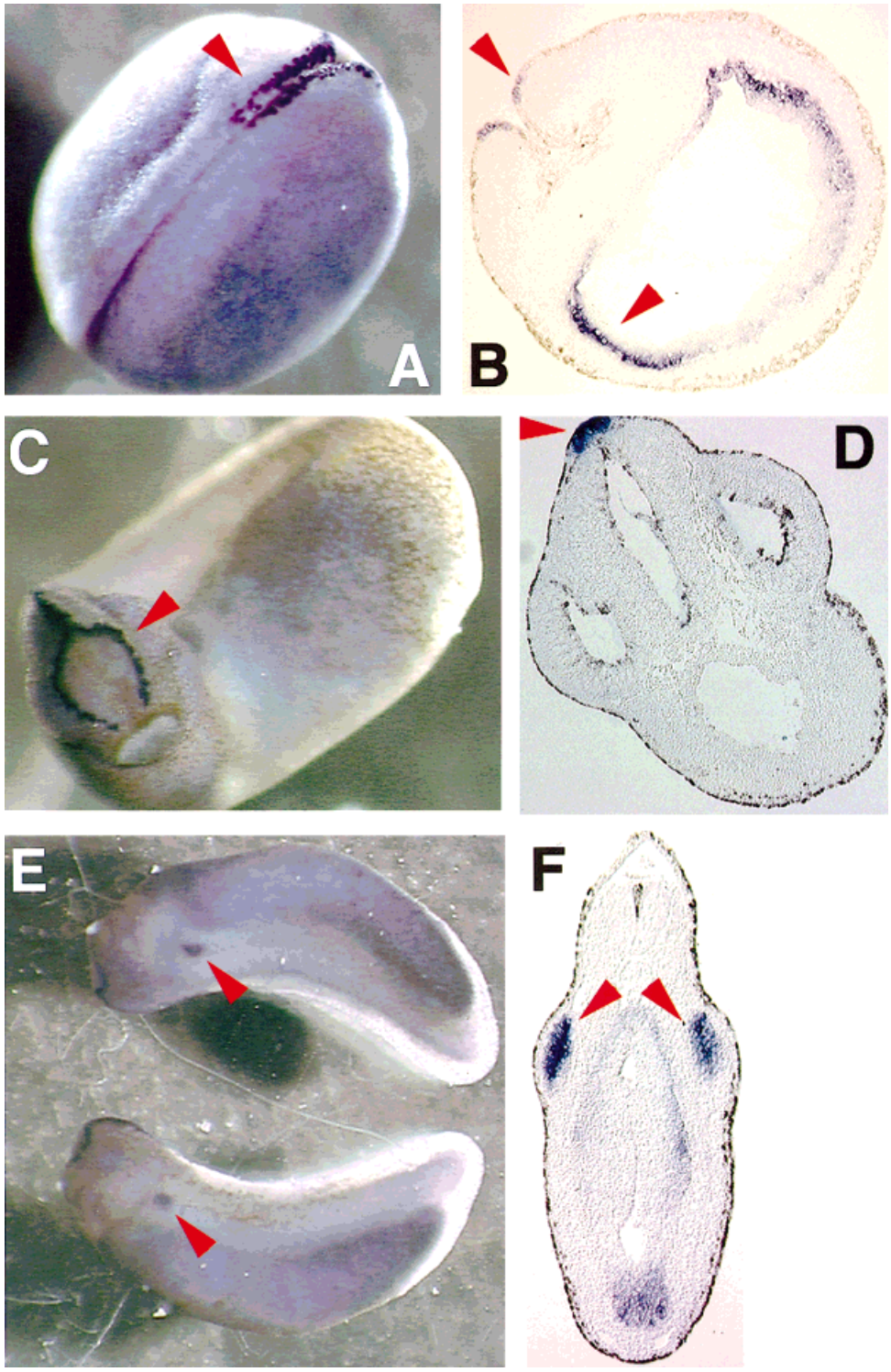


Fig. 1. *Cx30* is expressed in the *Xenopus* embryo. **A:** At stage 17, the *Cx30* signal is detected in the anterior dorsal portion of the closing neural tube. **B:** Sectioning reveals expression in the endoderm. **C:** *Cx30* is expressed in a stripe on the anterior most dorsal part of the neural tube and in two semicircular stripes on the most anterior part of the embryo. **D:** Sectioning confirms the ectodermal localization of *Cx30* mRNA in the hatching gland. **E:** Signal is also detected at stage 26 in a small spot near pronephros precursor cells. **F:** The signal remains at stage 31, as seen in section. In all figure parts, red arrowheads indicate expression.

darajah et al., 1996; Dermietzel et al., 1997; Nagy et al., 1997). This finding may indicate divergent function of connexin family members in different species.

In order to investigate the hatching gland expression more closely, we compared *Cx30* staining to the pattern of expression of the gene encoding the *Xenopus* hatching enzyme (Katagiri et al., 1997). *XHE* encodes a metalloprotease secreted by hatching gland cells to allow the embryo to escape from the vitelline membrane and is expressed in the ectoderm on the anterior dorsal portion of the head and face (Fig. 2A,B). Similarly, *Cx30* is expressed in identical locations during

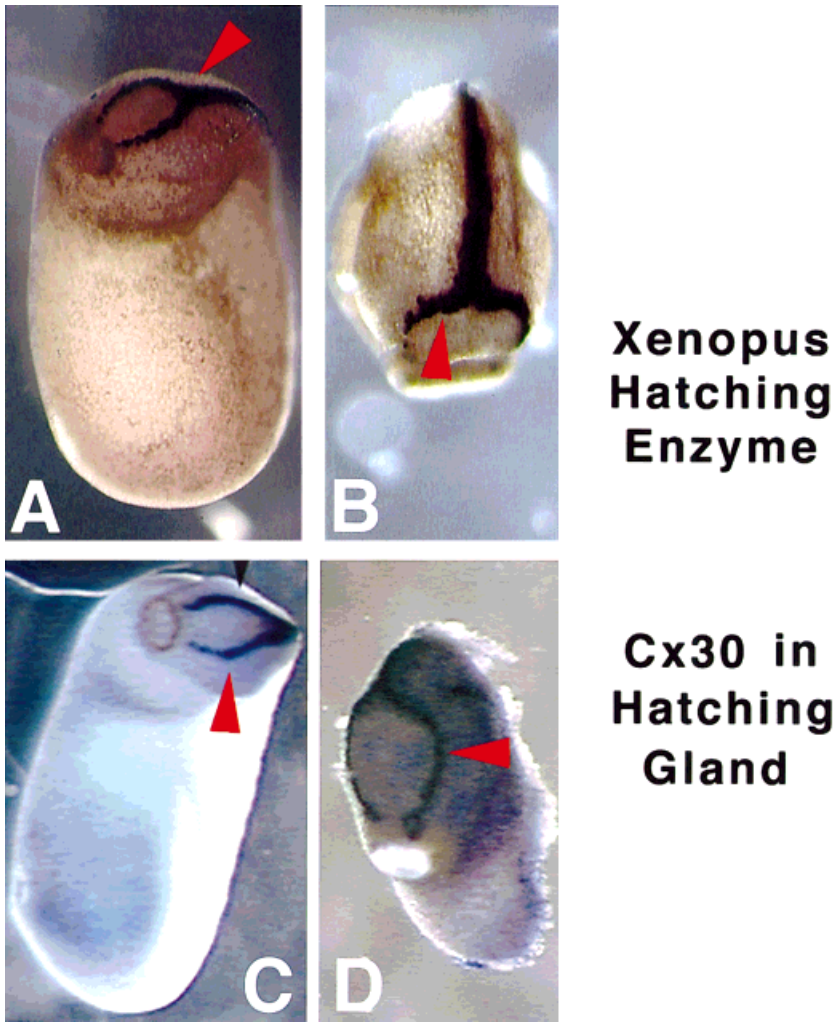


Fig. 2. *Cx30* is expressed in the hatching gland. **A,B:** *Xenopus* hatching enzyme (XHE) is expressed in a vertical stripe on the anterior dorsal aspect of the neural tube and in semicircular stripes on the face (hatching gland cells). **C,D:** *Cx30* is expressed in an identical pattern at these stages. In all figure parts, red arrowheads indicate expression.

stages 19–25 (Fig. 2C,D). We conclude that *Cx30* is expressed in tissue that secretes XHE. *Cx30* is a marker specific to the hatching gland (see also Drysdale and Elinson [1991]), in contrast to other available markers of the hatching gland, XA-1 (Hemmati-Brivanlou et al., 1990; Sive and Bradley, 1996) and XAG (Sive et al., 1989), which stain both the hatching gland and the cement gland.

We then tested the functional role of GJC in the activity of the hatching gland by inhibiting the function of endogenous gap junctions. This was done by exposing pre-hatching embryos to drugs (heptanol, glycyrrhetinic acid, and anandamide) that have been shown to close gap junctions rapidly in mammalian and *Xenopus* systems and by targeting injections of a dominant-negative connexin (H7) to the hatching gland (Davidson and Baumgarten, 1988; Chanson et al., 1989; Takens-Kwak et al., 1992; Venance et al., 1995; Levin and Mercola, 1998). In previous studies, we have shown that injection of H7 and exposure to several GJC drugs rapidly ($< 1.5$ hr) decrease GJC in *Xenopus* embryos (Levin and Mercola, 1998).

Fig. 3. *Cx30* expression in hatching gland cells is necessary for hatching gland function. **A:** Control embryos all hatch from the vitelline membrane by stages 26–27. **B:** In contrast, embryos exposed from stage 22 to drugs that inhibit the action of connexins are unable to hatch at the normal time. **C:** The same effect is observed when embryos are injected with H7, a dominant-negative construct that interferes with the function of endogenous connexins. **D:** At stages 37–38, control embryos are always hatched and develop normally. **E:** In contrast, embryos exposed to the GJC-reducing drug anandamide remain in the vitelline membrane through this very late stage; this panel shows a close-up view of a stage 41 embryo trapped within the vitelline membrane because of continued exposure to anandamide. **F:** Embryo from panel E manually freed with forceps to allow clearer staging.

Fig. 4. Reduction of hatching gland function by inhibition of GJC takes place post-transcriptionally with respect to *Cx30* and *XHE*. **A:** Control embryos show normal expression of *Cx30*. **B:** Embryos exposed to glycyrrhetinic acid do not show detectable differences in the expression of *Cx30* mRNA. **C:** Control embryos show normal expression of *XHE*. **D:** Embryos exposed to glycyrrhetinic acid do not show detectable differences in the expression of *XHE* mRNA.

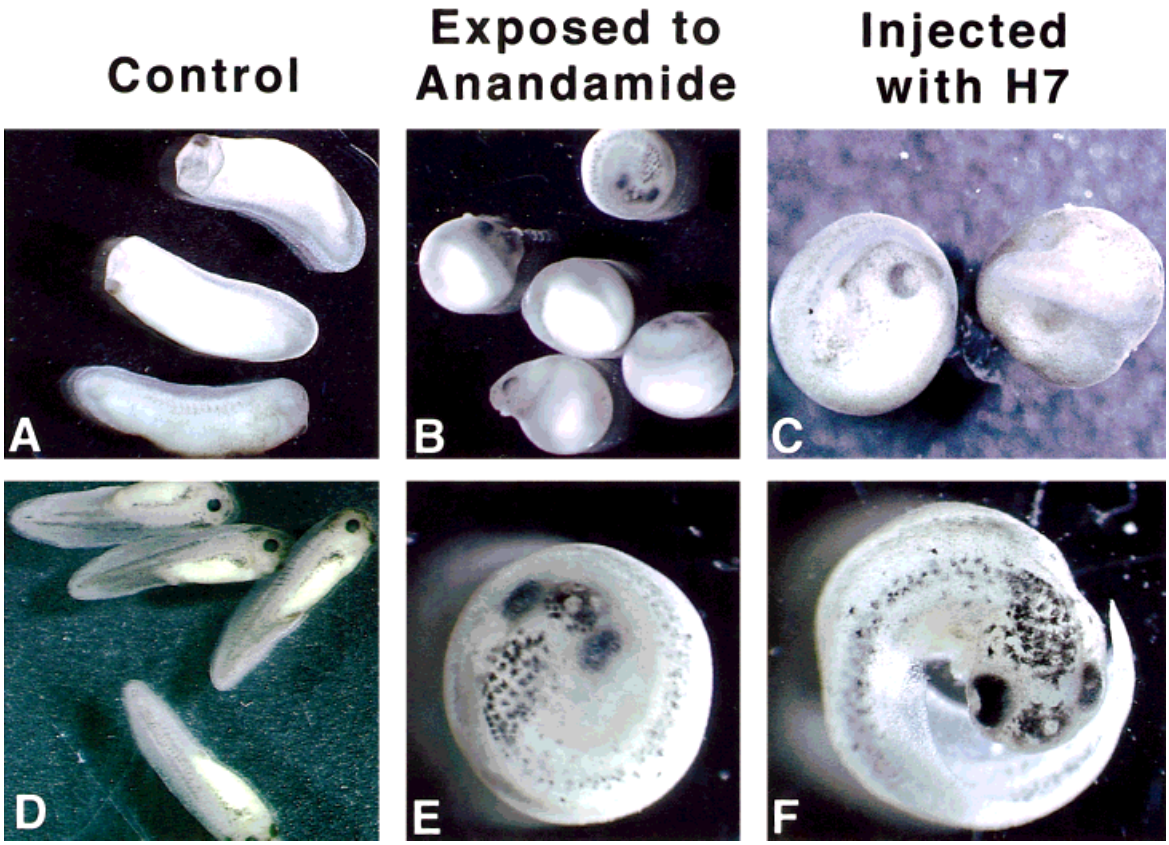


Figure 3.

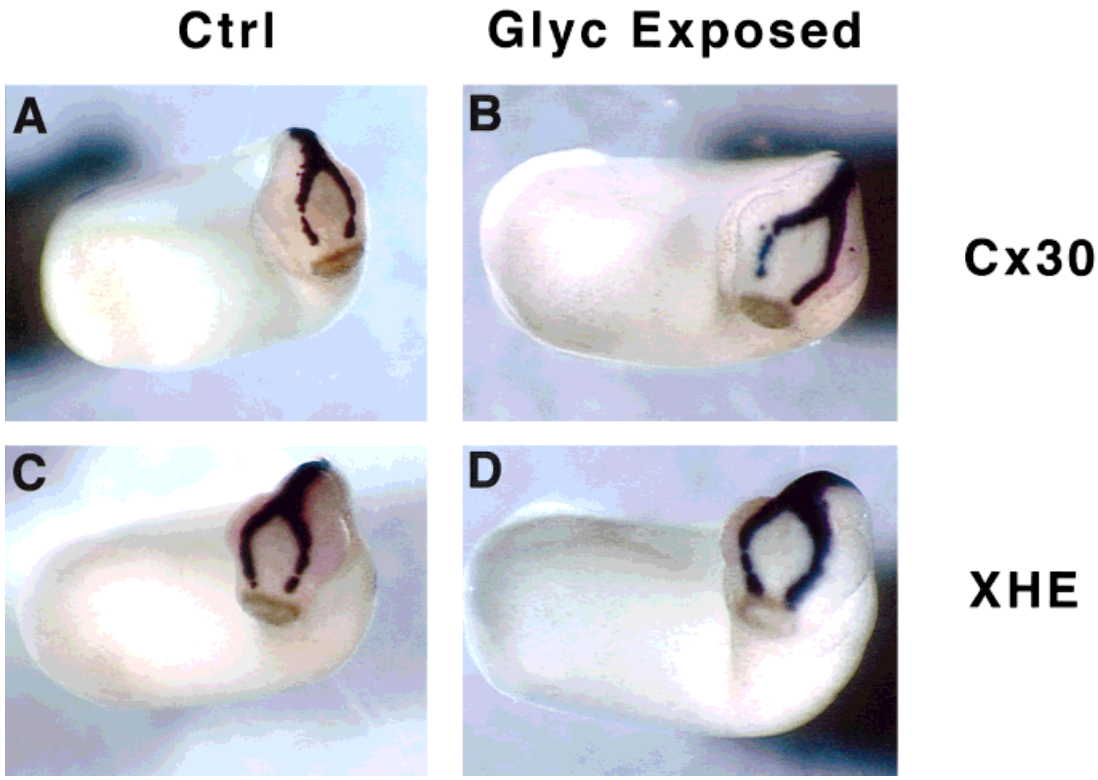


Figure 4.

Thus, we monitored hatching in batches of embryos whose medium contained heptanol, glycyrrhetinic acid, or anandamide. These batches exhibited far fewer hatched embryos when examined at a time point during the hatching process. Control embryos were all hatched from the vitelline membrane by stage 29 (Fig. 3A). In contrast, embryos exposed from stage 22 to drugs that inhibit the action of connexins were unable to hatch at the normal time (Fig. 3B). Only 52% of embryos (n = 27) exposed to glycyrrhetinic acid were hatched at a point when 100% of the control embryos (n = 30) had escaped the vitelline membrane ($\chi^2 = 16.08$; $P = 6 \times 10^{-5}$). A similar result was observed with anandamide and heptanol (two other GJC-reducing drugs; data not shown). By stage 37, control embryos are always hatched and develop normally (Fig. 3D), and by these stages most of the embryos exposed to glycyrrhetinic acid have also hatched. Prolonged exposure to anandamide, however, caused half the embryos (n = 26) to remain trapped in the vitelline membrane as late as stage 41 (Fig. 2E,F). Similar results were observed using heptanol (data not shown).

We also microinjected mRNA encoding a hybrid connexin construct, H7, which acts as a dominant negative to block GJC. Embryos injected with H7, which showed targeting to the hatching gland, also failed to hatch (Fig. 3C). A similar hatching phenotype has been described (Elinson, 1974) in embryos resulting from cross-species fertilization in frogs. However, based on the specific construct injections as well as the drug exposure data, we conclude that functional gap junctions are required for hatching gland function and that *Cx30* is a likely candidate for this role. We cannot rule out the presence and involvement of other connexins in the hatching gland, because known pharmacological and dominant-negative inhibitors of GJC affect multiple members of the connexin family.

In order to determine whether the effect of GJC-reducing drugs on the function of the hatching gland occurs at the mRNA or protein level, we examined the expression of the *Cx30* and *XHE* genes in embryos treated with glycyrrhetinic acid. Embryos were exposed to glycyrrhetinic acid at stage 18 and fixed at stages 24–29. Compared with control embryos (Fig. 4A,C), no differences in the expression pattern of *Cx30* (Fig. 4B; n = 25) and *XHE* (Fig. 4D; n = 27) were detected in embryos whose GJC was inhibited by glycyrrhetinic acid. We conclude that, in agreement with models for GJC function in other gland tissue, inhibition of hatching enzyme secretion by GJC-reducing agents occurs at the level of connexin protein regulation, not through down-regulation of connexin mRNA expression.

GJC is known to be involved in embryonic muscle development (Armstrong et al., 1983; Mege et al., 1994; Todman et al., 1999). However, the hatching defect is unlikely to be due to the drugs' inhibition of muscle activity, because embryos cultured in these drugs are able to move normally and because embryos cultured in tricaine, which paralyzes the embryos, are still able to hatch (data not shown).

In glands such as the pancreas and thyroid, GJC is thought to play a role in secretion by synchronizing $Ca^{2+}$ oscillations and equalizing voltage between groups of cells (Meda, 1996a; Bertuzzi et al., 1999; Hofer, 1999). Significant GJC between groups of cells results in a syncytium and allows diffusion of small molecules through a tissue. This can result in the equilibration of ionic and molecular gradients and ensures spread of signals controlling secretion. We suggest that inhibition of cell-cell communication disrupts the hatching process by interfering with hatching gland function. Our study of the expression of *Cx30* suggests that it as a likely candidate to mediate GJC in the hatching gland. Expression of *Cx30* in the lining of the archenteron and in pronephros suggests possible other roles for this gap junction protein in several organ systems in *Xenopus* embryogenesis.

## EXPERIMENTAL PROCEDURES

### In Situ Hybridization

In situ hybridization was performed as previously described. Antisense probe labeled with digoxygenin was generated from the Cx30 (Gimlich et al., 1988) and XHE (Katagiri et al., 1997) clones.

### Drug Exposure

Embryos were transferred to 0.1× MMR medium containing anandamide, 18α-glycyrrhetinic acid, or heptanol (prepared as described by Levin and Mercola, 1998) at stage 22.

### Dominant-Negative Construct Injection

Synthetic mRNA was transcribed by the SP6 polymerase from linearized SP64T plasmids containing the individual cDNAs. About 50 pg of H7 mRNA was mixed with 50 ng of RLD and 250 pg of mRNA encoding β-galactosidase (as lineage labels) and injected into the very top of the animal pole of both cells in two-cell-stage embryos.

### Statistical Analysis

Significance of numerical data was computed by the χ-square test with Pearson correction (a more stringent version of the χ-square test).

## ACKNOWLEDGMENTS

We thank David Paul and Dan Goodenough for the clone of xCx30 and H7 and Chiaki Katagiri for the XHE clone. This work was supported by Helen Hay Whitney Foundation fellowship F773 to M.L., NIH R01HL63271 to M.M., and an American Heart Association Established Investigator Award 9740005N to M.M.

## REFERENCES

Armstrong D, Turin L, Warner A. 1983. Muscle activity and the loss of electrical coupling between striated cells in *Xenopus* embryos. J Neurosci 3:1414–1421.

Bertuzzi F, Davalli A, Nano R, Socci C, Codazzi F, Fesce R, Carlo V, Pozza G, Grohovaz F. 1999. Mechanisms of coordination of $Ca^{2+}$ signals in pancreatic islet cells. Diabetes 48:1971–1978.

Bruzzone R, Ressot C. 1997. Connexins, gap junctions and cell-cell signalling in the nervous system. Eur J Neurosci 9:1–6.

Bruzzone R., White T, Goodenough D. 1996. The cellular Internet: on-line with connexins. Bioessays 18:709–718.

Carroll T, Wallingford J, Vize P. 1999. Dynamic patterns of gene expression in the developing pronephros of *Xenopus laevis*. Dev Genet 24:199–207.

Chanson M, Bruzzone R, Bosco D, Meda P. 1989. Effects of n-alcohols on junctional coupling and amylase secretion of pancreatic acinar cells. J Cell Physiol 139:147–156.

Davidson J, Baumgarten I. 1988. Glycyrrhetinic acid derivatives: a novel class of inhibitors of gap-junctional intercellular communication. Structure-activity relationships. J Pharmacol Exp Ther 246: 1104–1107.

Dermietzel R. 1998. Gap junction wiring: a "new" principle in cell-to-cell communication in the nervous system? Brain Res Rev 26:176–183.

Dermietzel R, Farooq M, Kessler J, Althaus H, Hertzberg E, Spray D. 1997. Oligodendrocytes express gap junction proteins connexin32 and connexin45. Glia 20:101–114.

Drysdale T, Elinson R. 1991. Development of the *Xenopus laevis* hatching gland and its relationship to surface ectoderm patterning. Development 111:469–478.

Elinson R. 1974. A block to cross-fertilization located in the egg jelly of the frog *Rana clamitans*. J Embryol Exp Morphol 32:325–335.

Gimlich R, Kumar N, Gilula N. 1988. Sequence and developmental expression of mRNA coding for a gap junction protein in *Xenopus.* J Cell Biol 107:1065–1073.

Gimlich R, Kumar N, Gilula N. 1990. Differential regulation of the levels of three gap junction mRNAs in *Xenopus* embryos. J Cell Biol 110:597–605.

Goodenough DA, Goliger JA, Paul DL. 1996. Connexins, connexons, and intercellular communication. Ann Rev Biochem 65:475–502.

Hemmati-Brivanlou A, Frank D, Bolce M, Brown B, Sive H, Harland R. 1990. Localization of specific mRNAs in *Xenopus* embryos by whole-mount in situ hybridization. Development 110:325–330.

Hofer T. 1999. Model of intercellular calcium oscillations in hepatocytes: synchronization of heterogeneous cells. Biophys J 77:1244–1256.

Katagiri C, Maeda R, Yamashika C, Mita K, Sargent T, Yasumasu S. 1997. Molecular cloning of *Xenopus* hatching enzyme and its specific expression in hatching gland cells. Int J Dev Biol 41:19–25.

Krutovskikh V, Yamasaki H. 1997. The role of gap junctional intercellular communication (GJIC) disorders in experimental and human carcinogenesis. Histol Histopathol 12:761–768.

Levin M, Mercola M. 1998. Gap junctions are involved in the early generation of left-right asymmetry. Dev Biol 203:90–105.

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

Meda P. 1996a. Gap junction involvement in secretion. Clin Exp Pharmacol Physiol 23:1053–1057.

Meda P. 1996b. The role of gap junction membrane channels in secretion and hormonal action. J Bioenerg Biomembr 28:369–377.

Meda P, Bosco D, Chanson M, Giordano E, Vallar L, Wollheim C, Orci L. 1990. Rapid and reversible secretion changes during uncoupling of rat insulin-producing cells. J Clin Invest 86:759–768.

Mege R, Goudou D, Giaume C, Nicolet M, Rieger, F. 1994. Is intercellular communication via gap junctions required for myoblast fusion? Cell Adhes Commun 2:329–343.

Nadarajah B, Thomaidou D, Evans W, Parnavelas J. 1996. Gap junctions in the adult cerebral cortex: regional differences in their distribution and cellular expression of connexins. J Comp Neurol 376:326–342.

Nagy J, Ochalski P, Li J, Hertzberg E. 1997. Evidence for the colocalization of another connexin with connexin-43 astrocytic gap junctions in rat brain. Neuroscience 78:533–548.

Sive H, Bradley L. 1996. A sticky problem: the *Xenopus* cement gland as a paradigm for anteroposterior patterning. Dev Dyn 205:265–280.

Sive H, Hattori K, Weintraub H. 1989. Progressive determination during formation of the anteroposterior axis in *Xenopus laevis*. Cell 58:171–180.

Takens-Kwak B, Jongsma H, Rook M, VanGinneken A. 1992. Mechanism of heptanol-induced uncoupling of cardiac gap junctions: a perforated patch-clamp study. Am J Physiol 262:C1531–C1538.

Todman M, Baines R, Stebbings L, Davies J, Bacon J. 1999. Gap-junctional communication between developing *Drosophila* muscles is essential for their normal development. Dev Gen 24:57–68.

Venance L, Piomelli D, Glowinski J, Giaume C. 1995. Inhibition by anandamide of gap junctions and intercellular calcium signalling in striatal astrocytes. Nature 376:590–594.

Yamasaki H, Mesnil M, Omori Y, Mironov N, Krutovskikh V. 1995. Intercellular communication and carcinogenesis. Mutat Res 333: 181–188.