

# Evolutionary relationships between the amphibian, avian, and mammalian stomachs

Devyn M. Smith,<sup>a</sup> Rayetta C. Grasty,<sup>b</sup> Nicole A. Theodosiou,<sup>a</sup> Clifford J. Tabin,<sup>\*\*</sup> and Nanette M. Nascone-Yoder<sup>b</sup>

<sup>a</sup>Department of Genetics, Harvard Medical School, Boston, MA 02115, USA; and <sup>b</sup>Natural Science and Biology, Eckerd College, 4200 54th Avenue South, St. Petersburg, FL 33711, USA

\*Author for correspondence (email: tabin@rascal.med.harvard.edu)

**SUMMARY** Although the gut is homologous among different vertebrates, morphological differences exist between different species. The most obvious variation in the guts of extant vertebrates appears in the stomach. To investigate the evolution of this structure, we compared the histology of the stomach and gastrointestinal tract in amphibian (*Xenopus laevis*), avian (*Gallus gallus*), and mammalian (*Mus musculus*) organisms, and defined the expression patterns of several genes within the developing guts of these lineages. In all three groups, we find that the anterior portion of the stomach has a similar glandular histology as well as a common embryonic expression of the secreted factors *Wnt5a* and *BMP-4*. Likewise, within the amniote lineages, the posterior nonglandular stomach and pyloric sphincter regions are also comparable in both

histological and molecular phenotypes. The posterior stomach expresses *Six2*, *BMPR1B*, and *Barx1*, whereas the pyloric sphincter expresses *Nkx2.5*. Although the adult *Xenopus* stomach exhibits both glandular and aglandular regions and a distinct pyloric sphincter similar to that of the amniotic vertebrates, the histology of the *Xenopus* tadpole gut shows less distinct variation in differentiation in this region, which is most likely a derived condition. The molecular signature of the embryonic *Xenopus* gut correlates with the more derived morphology of the larval phase. We conclude that the global patterning of the gut is remarkably similar among the different vertebrate lineages. The distinct compartments of gene expression that we find in the gut be necessary for the unique morphological specializations that distinguish the stomachs from terrestrial vertebrates.

## INTRODUCTION

The vertebrate gut is a highly specialized structure that brings food into an organism, digests the food, absorbs nutrients, and expels waste products. The vertebrate gut originates from splanchnic mesoderm and visceral endoderm, with the mesoderm initially encircling the underlying endoderm to form a simple tubular structure. From this tube, organ primordia bud off and begin to differentiate, each organ characterized by a unique mesodermal and endodermal morphology. Organs derived from the gut tube include all of the digestive tract and parts of the respiratory tract. Most organs are conserved among existing vertebrates, although the size and shape of the individual structures derived from the gut tube differ greatly among different species.

Epithelial-mesenchymal signaling plays an important role in the patterning of the gut tube into distinct foregut, midgut, and hindgut organs (Montgomery et al. 1999). The foregut gives rise to the esophagus, lungs, and stomach, whereas the midgut gives rise to the small intestine, and the hindgut gives rise to the large intestine. Endodermally derived signals regionally specify the mesoderm, which in turn patterns the pheno-

type of the underlying endoderm (Kedinger et al. 1986, 1990). Many molecules have been implicated in this reciprocal patterning process, including the secreted protein Sonic Hedgehog (Shh), the secreted Bone Morphogenetic Proteins (BMPs), as well as the Hox family of transcription factors (Roberts et al. 1995, 1998; Smith and Tabin 1999; Grapin-Botton and Melton, 2000; Narita et al. 2000).

The stomach is located in the posterior foregut, developing as a thickening of the undifferentiated gut tube. Stomach morphology is generally characterized by thickened muscle arising from the mesoderm and by unique glands derived from the endoderm. The thickened muscle allows for elastic distension of the stomach when a large quantity of food is ingested, and for subsequent peristaltic movements required for the mechanical mixing of food and secreted products.

Although the mesoderm contributes a characteristic uniform layer of connective tissue and muscle to the stomach, the endoderm-derived epithelial layer of the stomach is regionalized. The stomach can be divided into two sections in most vertebrates. The anterior portion of the stomach is called the fundus, which is characterized by gastric glands. These glands secrete the pepsinogen and hydrochloric acid

that are critical for hydrolysis of proteins. The posterior, or pylorus, portion of the stomach also features specialized glands, which secrete mucus into the lumen of the stomach. The controlled passage of food from the stomach into the small intestine is regulated by the pyloric sphincter, an organ derived from the foregut (Smith and Tabin 1999).

The pyloric sphincter is distinguished by its thickened mesodermal layer, whereas the epithelial layer has the same morphology as the pylorus region of the stomach. The transition into small intestinal epithelium occurs at the posterior boundary of this structure. The small intestine is lined with a simple columnar layer of epithelium with large villus structures. It is divided into three regions based upon the cell types located within the epithelial layer, beginning with the duodenum, followed by the jejunum and the ileum. In contrast to the large differences between the epithelium of the anterior and posterior stomach, the epithelium in all three segments of the intestine is lined with villi composed of a simple columnar epithelium.

Although most of the variation in stomach morphology among different species can be correlated with their diverse diets, the evolutionary origin of the vertebrate stomach is unknown. The primitive chordate *Amphioxus* does not have a stomach (Romer 1962; Walker and Liem 1994). Within the fish class, there are four orders without a stomach. The primitive hagfish and cyclostomes compose two of the orders that lack a stomach (Fig. 1A, Romer 1962; Walker and Liem, 1994). In these organisms, the esophagus empties its contents directly into the small intestine where nutrients from small food particles are absorbed. Because these animals do not have a storage receptacle for ingested food, they must continuously feed. Within the other two classes, the chondrichthyes and osteichthyes, most of the members do possess a stomach within the foregut (Figs. 1B and 1C, Stevens and Hume 1995); however, a few members of these classes also do not have definitive stomach compartments. This secondary loss of the stomach can be correlated with the particulate diet of those species in which the stomach is absent (Walker and Liem 1994). In the posterior foregut, the fish contain variations of a pyloric sphincter—either a true sphincter, a mucous membrane fold, or both (Stevens and Hume 1995).

The three amphibian orders— anurans, urodeles, and caecilians—are considered to form a monophyletic group and are characterized by the metamorphosis that occurs between the larval stage and adulthood. In many avians, the larval gut is relatively primitive with a long, coiled intestine for maximum absorption of nutrients from algae or detritus (Fig. 1D). Most anuran tadpole larvae are generally characterized as lacking a “true” stomach receptacle and proteolytic enzymes until after metamorphosis. They instead possess a specialized glandular region (manicotto glandulare) as part of a linear tube with no enlarged or extendable chamber (Burggren and Just 1992). In contrast, most urodele (salamander) and caecilian larvae are carnivorous and do have a stomach that

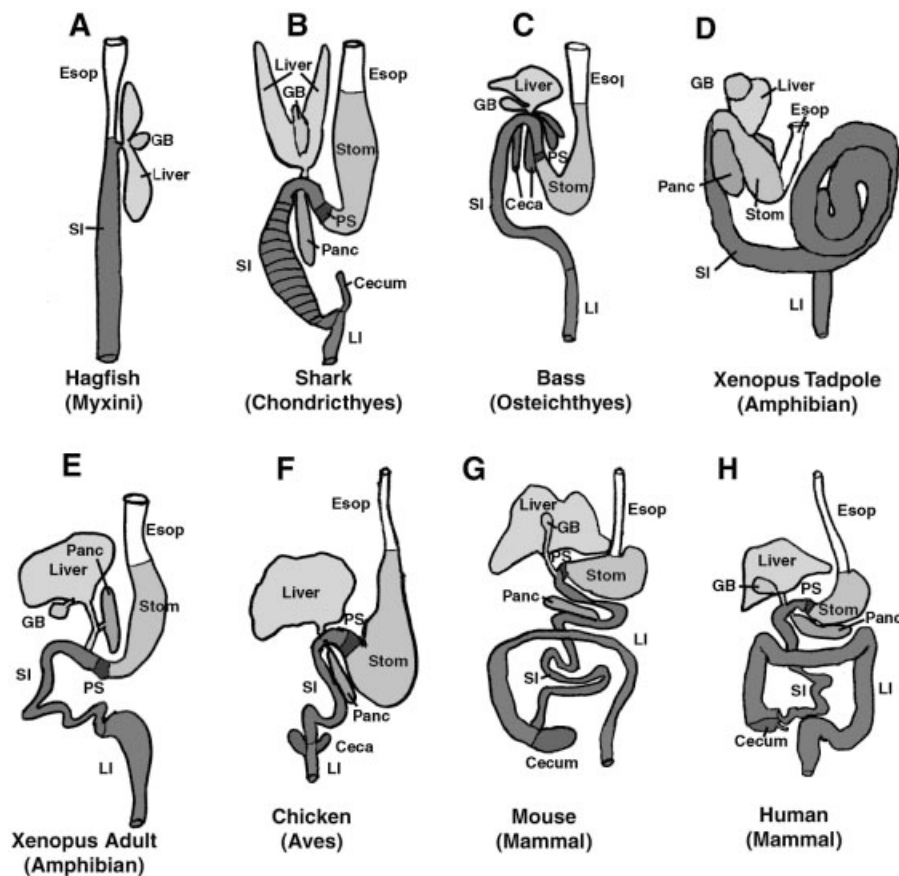
can digest proteins. During metamorphosis, extensive modifications occur within the anuran gut, with smaller relative changes in the urodeles and caecilians. In the anurans, the stomach enlarges and develops mature glands, whereas the intestine undergoes shortening and histological remodeling. (Fig. 1E, Stevens and Hume 1995; Sanderson and Kupferberg 1999). In contrast to the larva, the adult stomach develops a thick connective tissue layer and muscular pyloric sphincter. As the original amphibian assemblage was unlikely to have been herbivorous, the simple elongated gut of anuran tadpoles is a derived character, whereas the adult amphibian gut is similar to that of the higher vertebrates (Sues and Reisz 1998).

Within the reptiles, there are three orders, comprising the turtles, the snakes/lizards, and the crocodylians. The stomach of the turtle and snake/lizard orders is typically a long tube, whereas the crocodylians have some unique modifications within the stomach. The pylorus region of the crocodylian stomach is characterized by very thick musculature and is separated from the anterior region of the stomach by a constriction, similar to the gizzard found in birds (described below) (Stevens and Hume 1995).

The birds have a stomach that is composed of two very different regions separated by a distinct constriction (Fig. 1F). The anterior portion of the stomach (termed the proventriculus) contains numerous glands, whereas the posterior portion of the stomach (the gizzard) is composed of thick layers of muscle and a keratin-like covering (the koilen) over the epithelial cells (Stevens and Hume 1995). The proventriculus secretes the gastric juices that mix with the food in the gizzard. The gizzard functions to grind up the food with the aid of gastroliths, as the birds typically do not have teeth specialized for chewing and grinding (Walker and Liem 1994). Whereas the gizzard may compensate for the lack of teeth in birds, it still functions as a region of storage and digestion of proteins (Stevens and Hume 1995). Based upon histological analyses, it has been suggested that the gizzard in birds is homologous with the pylorus region of the stomach in other vertebrates (Pernkopf 1929). This idea is further supported by the similarities of the gizzard in birds to the crocodylian pylorus (Pernkopf 1929; Stevens and Hume 1995).

The mammals have perhaps the most diverse stomachs of any of the vertebrate classes. The great assortment of shapes, sizes, and types of their stomachs is due to the great variety of habitats they occupy, as well as to the wide range of food that the various species ingest (Figs. 1G and 1H). Nonetheless, the typical mammalian stomach is characterized by the distinct epithelial regions discussed above, with a homogeneous distribution of smooth muscle and connective tissue throughout the stomach mesoderm. Further, the mammals are the only group with a unique cardiac epithelium, which contains mucus secreting cardiac glands and is found in the most anterior region of the stomach (Romer 1962).

The phylogenetic relatedness of apparently homologous organs of the digestive tract, like the stomachs of different ver-



**Fig. 1.** Schematic diagram of the gastrointestinal tracts in several vertebrates. Similarly shaded regions depict regions that are functionally homologous. (A) Gut of the chordate, nonvertebrate Hagfish showing the lack of a stomach. (B) Gut of a cartilaginous fish, shark. (C) Gut of a bony fish, bass. (D) Gut of a larval frog, *Xenopus laevis*. (E) Gut of an adult frog, *Xenopus laevis*. (F) Gut of an avian, chicken (*Gallus gallus*). (G) Gut of a mammal, mouse (*Mus musculus*). (H) Gut of a mammal, human (*Homo sapiens*). Abbreviations: Esop, esophagus; GB, gall bladder; SI, small intestine; LI, large intestine; Stom, stomach; PS, pyloric sphincter; and Panc, pancreas.

tebrates, has not been demonstrated by molecular criteria. In addition, ontogenetic similarities between the guts of different vertebrates have not been identified. Further, very little is known about gut development in vertebrates and no comparisons between species have been performed (Grapin-Botton and Melton 2000). Here, we apply a combined molecular and morphological approach to the study of gut evolution. In this study, we focused on the guts of one anuran amphibian (*Xenopus laevis*), one bird (*Gallus gallus*), and one mammal (*Mus musculus*). Although these species were selected out of convenience, due to the availability of specimens as developmental model organisms and the availability of cloned genes with which to make probes for in situ hybridization, they nonetheless provide insight into the evolution of the vertebrate digestive system and suggest models that can be tested by examining phylogenetically relevant species in the future.

## MATERIALS AND METHODS

### Embryos

Chicken eggs were obtained from SPAFAS (Preston, Connecticut, USA) and incubated to the desired stages (Hamburger and Hamil-

ton 1951). Timed pregnant Swiss Webster mice were received from Taconic (Germantown, NY, USA). Mice were sacrificed by cervical dislocation and embryos removed at the desired ages. *Xenopus* embryos were derived by *in vitro* fertilizations and allowed to develop to the desired stages (Nieuwkoop and Faber 1956). All embryos were fixed in 4% paraformaldehyde for 24 h, washed with phosphate buffered saline, and dehydrated through a methanol series. Embryos were then stored at  $-20^{\circ}\text{C}$  in 100% methanol until needed for in situ hybridization.

### In situ hybridization

Whole-mount in situ hybridization was performed as described (Riddle et al. 1993; Harland 1991; Cheng et al. 2000). For section in situ hybridization, tissue was fixed in paraformaldehyde overnight, washed with phosphate buffered saline following fixation, and then dehydrated through an ethanol series up to 100% ethanol. The tissue was then placed into xylene and then embedded in paraffin. Tissue was sectioned at  $10\ \mu\text{m}$  and then rehydrated, and section in situ hybridization was performed using digoxigenin labeled probes (Roberts et al. 1998). Probes used include: *cBarx1* (Barlow et al. 1999); *mBarx1* (Tissier-Seta et al. 1995); *mWnt5a* (Yamaguchi et al. 1999); *cWnt5a* (gift of A. McMahon) and *XWnt5a* (gift of M. Mercola); *mBMPR1B* (Baur et al. 2000); *cBMPR1B* (Zou et al. 1997); *xBMPR1* (Graff et al. 1994); *mNkx 2.5* (Kasahara et al. 1998); *cNkx2.5* (Buchberger et al. 1996); *XNkx-2.5* (Tonissen et al. 1994);

*cBMP-4* and *mBMP-4* (Baur et al. 2000); *XBMP-4* (Nishimatsu et al. 1992); *cSix2* and *mSix2* (Oliver et al. 1995); *xSix2* (Seo et al. 1999); *mNkx2.3* (Pabst et al. 1999); *cNkx2.3* (Buchberger et al. 1996); and *XNkx-2.3* (Evans et al. 1995).

### Histology

Gut tissue was isolated from embryos and juvenile or adult animals and cut into small pieces. The tissue was then washed briefly with phosphate buffered saline and placed in 4% paraformaldehyde for 24 h. The tissue was then washed with phosphate buffered saline three times for 1 h each. The gut tissue was then dehydrated through an ethanol series, placed into xylene or HistoClear (Sigma, St. Louis, MO, USA), and then embedded in paraffin. The tissue was then sectioned at 4–10  $\mu\text{m}$  using a microtome. Sections were dried overnight and then stained with Hematoxylin and Eosin using standard techniques. Sections were then evaluated using a microscope with a digital camera attached.

## RESULTS

To attempt to understand the evolutionary relationship among various vertebrate gut-derived organs, we began by performing an anatomical and histological comparison of the stomach, pyloric sphincter, and the small intestinal regions of several vertebrate guts. The histology of both tadpole and adult gut organs in *Xenopus laevis* (African clawed frog, Fig. 1D), adult *Gallus gallus* (chicken, Fig. 1E), and adult *Mus musculus* (mouse, Fig. 1F) was compared.

### Histology of the stomach/small intestine

The typical histology of the gut tube is comprised of four layers. The innermost layer is the mucosa, which is composed of the epithelial lining of the gut and the adjacent overlying mesoderm, including the muscularis mucosa, a thin layer of smooth muscle. The next layer is the submucosa, characterized by undifferentiated connective tissue and vascular tissue. The next layer is the muscularis layer, composed of layers of smooth muscle and the enteric nervous system plexi. This smooth muscle is found in two layers of muscle fibers throughout most of the gut, as an inner circular layer and an outer longitudinal layer. This smooth muscle is responsible for the peristaltic action of the gut. The outermost layer is the serosal layer, composed of a thin layer of epithelial tissue.

In the *Xenopus* tadpole, the stomach region is surrounded by very thin layers of muscularis and serosa, but shows no obvious submucosa (Chalmers and Slack 1998). The surface epithelial differs markedly between the glandular anterior (Figs. 2A and 2B) and glandless posterior mucosae (Figs. 2C and 2D) of the linear *Xenopus* tadpole stomach. The anterior stomach is lined by a ciliated columnar mucus epithelium, with shallow folds and multiple underlying serous acini (Figs. 2A and 2B). The posterior stomach lining is also a single-layered columnar epithelium; however, there are no un-

derlying acinar glands as observed in the anterior region (Figs. 2C and 2D). Likewise, there is no discernible pyloric sphincter, and the transition from stomach to small intestine is almost imperceptible and indistinct (Figs. 3A and 3B). The epithelium of the small intestine consists of a single layer of taller columnar mucus cells organized into shallow folds (Figs. 3C and 3D). Thus, although there are discernable differences in the histological composition of the various segments of the *Xenopus* tadpole gut, it appears to be a simple and relatively unspecialized digestive tract.

The morphology of the adult *Xenopus* gut, however, differs significantly from that of its tadpole larva. Whereas the mesodermal layers of the *Xenopus* stomach are of uniform thickness and morphology, the epithelium has two distinct histological regions. The anterior region of the stomach has a large number of gastric glands and a thick, folded epithelium (Figs. 2E and 2F). In contrast to the anterior stomach, the posterior stomach is characterized by a loss of the gastric glands and a relatively thin endoderm, which is composed of a single layer of columnar cells (Figs. 2G and 2H). This pylorus region is arranged into villus-type structures (Fig. 2H). In contrast to the tadpole, the adult *Xenopus* also has a distinct pyloric sphincter that is characterized by a thickened musculature and, at its posterior border, by the sharp transition from pylorus epithelium to small intestinal epithelium (Figs. 3E and 3F). The adult *Xenopus* small intestine contains numerous villi (Figs. 3G and 3H). Thus, the adult *Xenopus* gut contains both morphological and histological specializations not observed in the larva.

The morphology of the chicken gut is similar to the adult *Xenopus* gut in several respects, although some key differences exist in the stomach region. As observed in *Xenopus*, the chicken stomach has two distinct morphological regions: the proventriculus and the gizzard. The proventriculus is characterized by a thick glandular epithelium surrounding a central lumen (Figs. 2I and 2J). The glands contain mucous and digestive enzyme-secreting cells arranged in large glandular regions within the endoderm. The mesoderm of the proventriculus is quite thin compared to that of the gizzard (compare Fig. 2I with Fig. 2K), which is characterized by a very thick layer of musculature (Fig. 2J). Koilen, a keratin-like substance, coats the gizzard epithelium in a thick protective layer (Fig. 2L). The epithelial cells are arranged in villi (Fig. 2L), but the epithelium is much thinner than in the proventriculus (compare Fig. 2K with Fig. 2I). The pyloric sphincter in the chicken is recognized by a clear thickening of the mesoderm as well as by a distinct transition from the gizzard endoderm to the small intestinal endoderm at its posterior border (Figs. 3I and 3J). The small intestine has uniform villus structures, with a glandular crypt at the base of each villus (Figs. 3K and 3L). Thus, despite the distinct specializations of the chicken stomach, the overall morphology of the adult *Xenopus* and chicken guts is conserved.

**Fig. 2.** Histological sections of the anterior and posterior stomach regions of a larval *Xenopus*, adult *Xenopus*, adult chicken, and adult mouse. (A–B) Section through the anterior stomach of a *Xenopus* tadpole (stage 48) seen at low magnification (A) and high magnification (B). (C–D) Section through the posterior stomach of a *Xenopus* tadpole (stage 48) seen at low magnification (C) and high magnification (D). (E–F) Section through the anterior stomach of a *Xenopus* adult seen at low magnification (E) and high magnification (F). (G–H) Section through the posterior stomach of a *Xenopus* adult seen at low magnification (G) and high magnification (H). (I–J) Section through the anterior stomach of an adult chicken seen at low magnification (I) and high magnification (J). (K–L) Section through the posterior stomach of an adult chicken seen at low magnification (K) and high magnification (L). (M–N) Section through the anterior stomach of an adult mouse seen at low magnification (M) and high magnification (N). (O–P) Section through the posterior stomach of an adult mouse seen at low magnification (O) and high magnification (P). Yellow arrowheads in (B, F, J) point to glands within anterior stomach epithelium, whereas yellow arrowhead in (K) points to koilen lining the lumen of the posterior chicken stomach and green arrowhead in (H) points to simple columnar epithelium.

The mouse gut bears similarities to both the adult *Xenopus* and the chicken, with the main deviations again localized to the stomach. Based on epithelial histology, the stomach epithelium in the mouse appears to be composed of several different regions. The most anterior region, the cardiac, is not discussed in this study, as it is a feature unique to mammals and comprises a very small portion of the anterior stomach nearest the esophagus. As with the anterior stomach regions of the adult *Xenopus* and chicken, the fundus of the mouse stomach is characterized by a thick layer of glandular tissue (Figs. 2M and 2N). The posterior stomach, or pylorus region, in the mouse does not have the thickened glands seen in the fundus (Figs. 2O and 2P), but has villus-like structures very similar in shape and size to those seen in the chicken gizzard. The mesoderm of the posterior stomach in the mouse is much thinner than the gizzard mesoderm in the chicken (compare Fig. 2O with Fig. 2K). At the terminus of the pylorus region, one can see the distinct valvular flaps of the pyloric sphincter, as well as the decrease in thickness of the mesodermal layer from the stomach to the small intestine (Figs. 3M and 3N). In addition, there is an abrupt change in the histology of the epithelial lining from the pyloric epithelium to that of the columnar small intestinal epithelium (Fig. 3N). Finally, the mouse small intestine (Figs. 3O and 3P) appears very similar to the small intestines of the chicken and adult *Xenopus*, with numerous villi with crypts at the base of each villus (compare Fig. 3P with Figs. 3L and 3H). Thus, despite the primitive nature of the *Xenopus* tadpole digestive tract, the overall histological features of the adult *Xenopus*, chicken, and

mouse guts are generally conserved, with the most deviation observed in the specialized stomach regions of the chicken.

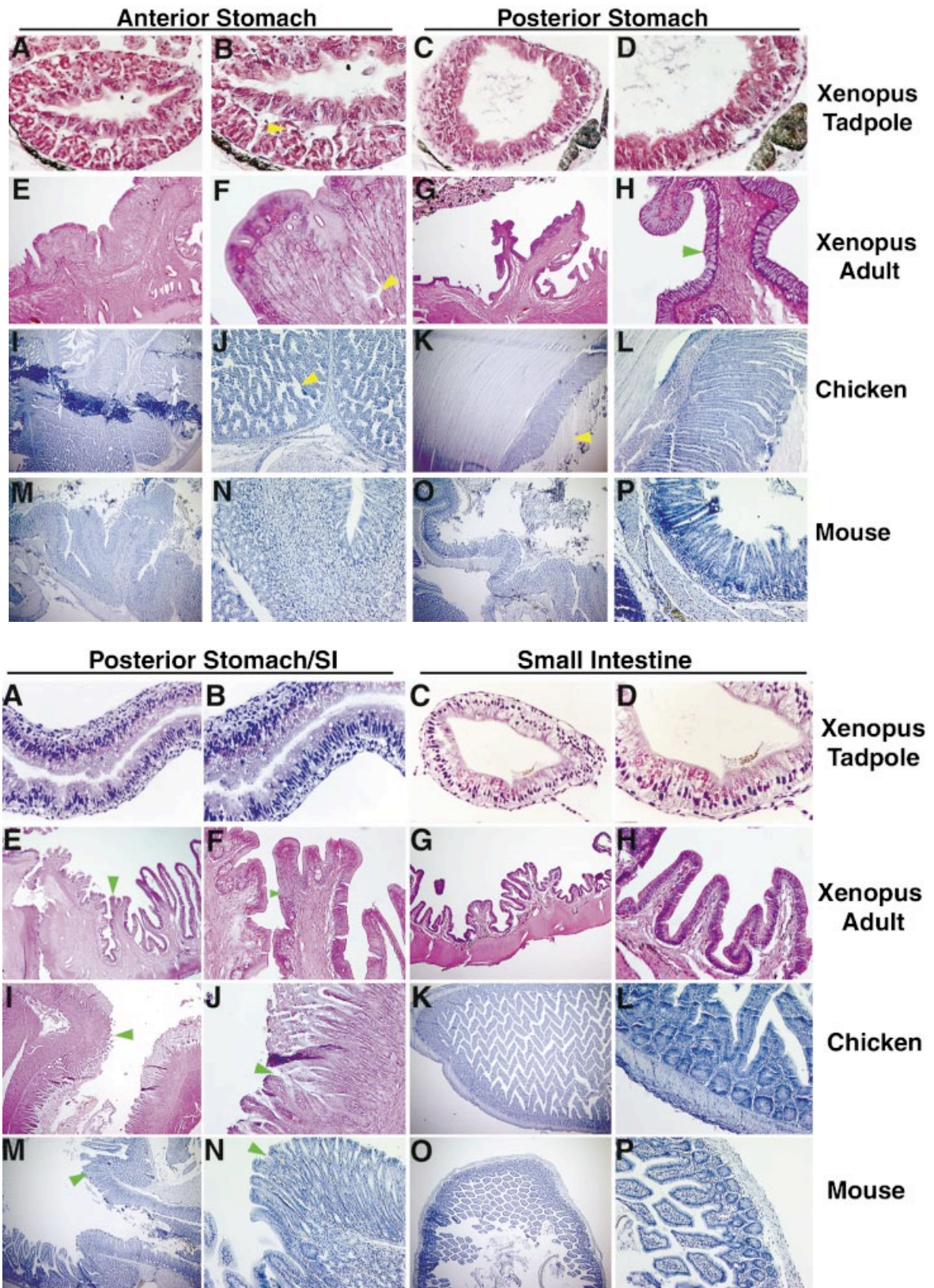
### Molecular markers of the anterior stomach

Because there are clear morphological and histological differences in the stomach epithelium of these three species, we decided to analyze the expression patterns of stomach markers to determine whether a correlation exists between gene expression and morphology. Previously, it was shown that *BMP-4* is expressed in the mesoderm of the small intestine and proventriculus of the chick embryo (Roberts et al. 1995, 1998); *BMPRII* is expressed in the mesoderm of the developing chicken gizzard (Roberts et al. 1998; Smith and Tabin 1999); and the homeodomain-containing transcription factor *Nkx2.5* is an early marker of the mesoderm of the chicken pyloric sphincter (Smith and Tabin 1999). In addition, the homeodomain-containing transcription factor *Barx1* has been described as a marker of the mesoderm of the mouse stomach (Tissier-Seta et al. 1995). Further, the transcription factor *Six2* (Oliver et al. 1995) and the secreted protein *Wnt5a* (Yamaguchi et al. 1999) are both expressed in a regionally restricted manner within the mesoderm of the chicken stomach.

We analyzed each of the above six markers for their expression patterns within the developing guts of the *Xenopus* embryo, chicken embryo, and mouse embryo. We find that *Wnt5a* and *BMP-4* mark the anterior stomach in all three lineages (Fig. 4). In *Xenopus*, *Wnt5a* and *BMP-4* mark the most anterior region of the stomach (Figs. 4A and 4C); in chickens, *Wnt5a* and *BMP-4* mark the proventriculus (Figs. 4E

**Fig. 3.** Histological sections of the small intestine and pyloric sphincter regions of a *Xenopus* larvae, adult *Xenopus*, adult chicken, and adult mouse. (A–B) Section through the posterior stomach/small intestinal border region of a *Xenopus* tadpole (stage 48) at low magnification (A) and high magnification (B). (C–D) Section through the small intestine of a *Xenopus* tadpole (stage 48) seen at low magnification (C) and high magnification (D). (E–F) Section through the pyloric sphincter region of an adult *Xenopus* seen at low magnification (E) and high magnification (F). (G–H) Section through the small intestine of an adult *Xenopus* seen at low magnification (G) and high magnification (H). (I–J) Section through the pyloric sphincter region of an adult chicken seen at low magnification (I) and high magnification (J). (K–L) Section through the small intestine of an adult chicken seen at low magnification (K) and high magnification (L). (M–N) Section through the pyloric sphincter region of an adult mouse seen at low magnification (M) and high magnification (N). (O–P) Section through the small intestine of an adult mouse seen at low magnification (O) and high magnification (P). Green arrowhead points to sphincter in low magnification and abrupt transition from sphincter epithelium to small intestinal epithelium in high magnification.





and 4G); and in mice, *Wnt5a* and *BMP-4* mark the fundus region of the stomach (Figs. 4I and 4K). The only difference in expression among the three embryos examined is that *BMP-4* is also expressed in the posterior stomach of the early *Xenopus* gut; therefore, there is no clear restriction in its pattern to one region of the stomach (Fig. 4C).

### Molecular markers of the posterior stomach

Posterior markers of the stomach include *Six2*, *Nkx2.5*, *BMPR1B*, and *Barx1*. In the *Xenopus* embryo, *Six2* is expressed throughout the posterior stomach (Figs. 5A and 5B). *Nkx2.5* expression is also present in the posterior stomach region of the *Xenopus* embryo, although its expression domain is shifted slightly more posterior, closer to the intestinal region (Fig. 5C), than that of *Six2*. In contrast, *BMPR1* is expressed throughout the anterior and posterior regions of the *Xenopus* embryo stomach (Fig. 5D). The only cloned BMPR in frogs, *BMPR1*, was used in this study. Although it is not clear if the *Xenopus BMPR1* is the direct homologue of the amniotic BMPR1Bs, we do find that the expression pattern of *xBMPR1* resembles that of the amniotic *BMPR1B* rather than that of *BMPR1A* in the gut (data not shown).

In the chicken, *Six2* is expressed throughout the gizzard mesoderm, although it is absent from the most anterior portion of the gizzard (Figs. 5E and 5F). *Nkx2.5* is expressed only in the most posterior gizzard where the pyloric sphincter will form (Smith and Tabin 1999, Fig. 5G). *BMPR1B* is expressed throughout the gizzard mesoderm (Fig. 5H), whereas *Barx1* marks the entire stomach with strong expression in the gizzard and much weaker expression in the proventriculus in the chicken (Fig. 5I). In the mouse embryo, *Six2* is expressed in the posterior stomach (Figs. 5J and 5K), whereas *Nkx2.5* marks the posterior stomach/pyloric sphincter region (Fig. 5L). *BMPR1B* is expressed throughout the posterior stomach, whereas *Barx1* is expressed throughout the stomach (Fig. 5M), with a much stronger expression in the posterior stomach (Fig. 5N).

Although there is general conservation in the expression of these genes, a comparison of the expression patterns of the posterior stomach markers indicates significant deviation in the embryonic *Xenopus* gut. For example, *BMPR1B* and *Six2* are found at the posterior or pylorus stomach region of all three

embryos, and *Nkx2.5* expression at the pyloric sphincter region of each gut is also conserved. However, the expression of *Nkx2.5* is nearly as broad as that of *Six2* in the *Xenopus* embryo, in contrast to the limited expression of *Nkx2.5* in the mouse and chicken stomachs. *BMPR1* is also expressed throughout the stomach of the developing *Xenopus* gut, with stronger expression in the anterior stomach than in the posterior stomach. In general, the markers are more broadly expressed in *Xenopus* than in the amniotes, with less distinct compartments of gene expression between the anterior and posterior stomach and between the posterior stomach and small intestine.

### Molecular markers of the small intestine

To further characterize the evolutionary relationships among these lineages, we have analyzed the expression patterns of two genes that are expressed in the small intestine. *BMP-4* is a marker for the anterior small intestinal mesoderm, whereas *Nkx2.3* is a marker for the entire small intestinal mesoderm (Buchberger et al. 1996; Roberts et al. 1998; Smith and Tabin 1999).

We find that *Nkx2.3* is expressed throughout the small intestine in the developing *Xenopus* embryo, but is also expressed throughout the posterior stomach (Fig. 4D). *BMP-4* is expressed in the anterior and posterior stomach, but is expressed only at low levels, if at all, in the anterior-most region of the small intestine of the *Xenopus* tadpole (Fig. 4C). Further, *BMP-4* is expressed in the anterior small intestine in the chicken and mouse (Figs. 4G and 4K). *Nkx2.3* is expressed in the small intestine in both the chicken and mouse guts (Figs. 4H and 4L). This identical expression of *BMP-4*, coupled with the conservation of expression of *Nkx2.5* and *BMPR1B* in these species, suggests that the patterning of the pyloric sphincter is the same in both the chicken and the mouse (Smith and Tabin 1999). The *Xenopus* embryonic gut expresses both *Nkx2.3* and *BMP-4*, but does so in a pattern different from that of the chicken and mouse.

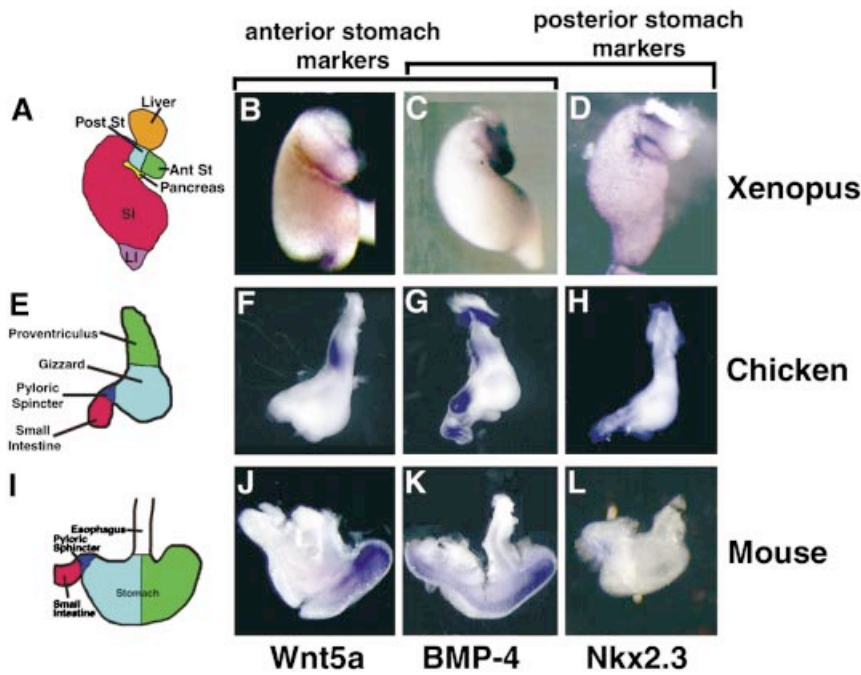
## DISCUSSION

The vertebrate gut is a highly complex structure in adults that is derived from a simple multilayered tube found in the em-

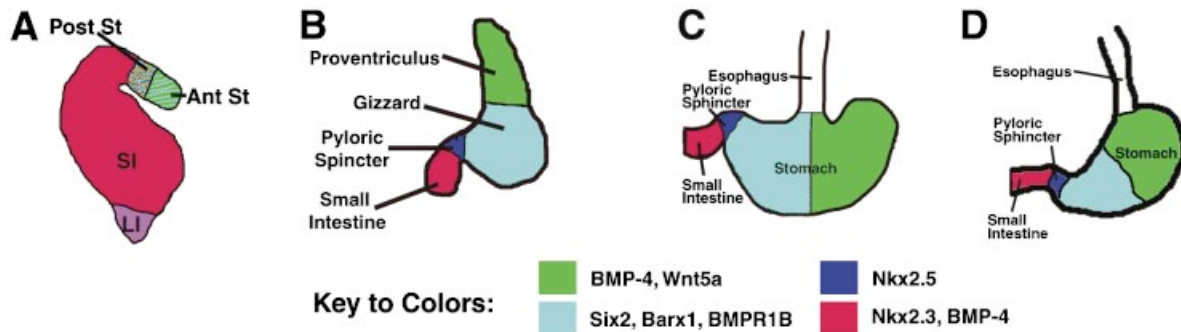
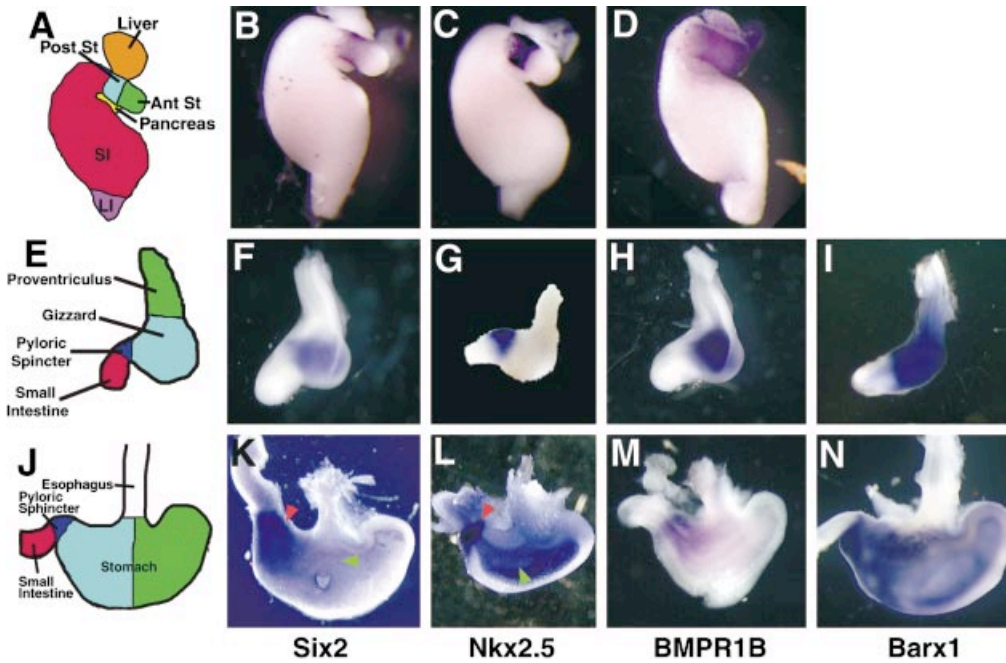
**Fig. 5.** In situ hybridization of posterior stomach markers in the *Xenopus* tadpole (A–D), chicken (E–I), and mouse embryos (J–N). (A) Schematic of the stage 42 larval *Xenopus* gut. (E) Schematic of the E4.5 embryonic chicken gut. (J) Schematic of the E11.5 embryonic mouse gut. Expression of *Six2* in the *Xenopus* tadpole (B), chicken (F), and mouse (K) guts. Expression of *Nkx2.5* within the pyloric sphincter region of *Xenopus* (C), chicken (G), and mouse (L) guts. Expression of *BMPR1B* within the tadpole (D), chicken (H), and mouse (M) guts. Expression of *Barx1* within the chicken (I) and mouse (N) guts. Luminal staining in (N) is pooling, this was confirmed via section in situ hybridization. Green arrowhead in (K) and (L) points to background staining due to pooling within the lumen of the stomach, whereas the red arrowhead points to real staining—results confirmed upon sections.

**Fig. 6.** Composite of in situ hybridization patterns of genes used in this study on schematic guts showing the conservation of regions of expression throughout the vertebrate lineages. (A) *Xenopus* gut. (B) Chicken gut. (C) Mouse gut. (D) Predicted expression in the Human gut. Abbreviation: LI, large intestine.





**Fig. 4.** In situ hybridization of anterior stomach makers in the *Xenopus* tadpole (A–C), chicken (E–G), and mouse embryos (I–K) and small intestinal markers in *Xenopus* (C, D), chicken (G, H) and mouse embryos (K,L). (A) Schematic of the stage 42 *Xenopus* tadpole gut with the various gut organs labeled. (B) Expression of *Wnt5a* in the stage 42 tadpole gut. (C) Expression of *BMP-4* in the stage 42 tadpole gut. (D) Expression of *Nkx2.3* in the stage 42 tadpole gut. (E) Schematic of the E4.5 embryonic chicken gut. (F) Expression of *Wnt5a* in the E4.5 embryonic chicken gut. (G) Expression of *BMP-4* in the E4.5 chicken gut. (H) Expression of *Nkx2.3* in the E4.5 chicken gut. (I) Schematic of the E11.5 mouse embryonic gut. (J) Expression of *Wnt5a* in the E11.5 mouse gut. (K) Expression of *BMP-4* in the E11.5 mouse gut. (L) Expression of *Nkx2.3* within the E11.5 mouse gut.





bryo. In this study, we attempted to establish the evolutionary relationships of the guts of different vertebrate species, including the chicken, the mouse, and both the larval and adult forms of *Xenopus laevis*. By understanding how the stomach evolved within the vertebrates, we may gain insight into the evolution of organs and what forces have led to the formation of specific organs or organ characteristics within certain lineages.

### Composite of the in situ hybridization patterns

The markers that are specific for the small intestine and the anterior and posterior stomach have conserved expression patterns in the chick and mouse (Figs. 6B and 6C). We believe that the chicken and mouse results can be extrapolated to avian and mammalian lineages, since the chicken gut is very similar to other avian guts morphologically, whereas the mouse gut is morphologically similar to most mammalian guts. On the basis of our data, we would predict that the expression patterns in the human gut would be similar to those seen in the mouse and chicken (Fig. 6D). In contrast, the *Xenopus* embryo has quite distinct expression patterns in the stomach and small intestine (Fig. 6A).

### Relationship between mouse and chicken stomachs

The mammals are thought to have evolved from a distinct primitive reptilian ancestor (Gerhart and Kirschner 1997). Hence, the mammalian and avian lines are separated in evolutionary time by many millions of years (approximately 350 Myr) (Gerhart and Kirschner 1997), yet the stomachs of these two groups are remarkably similar in molecular and morphological phenotype. Here, we show that the anterior portion of the stomach contains large numbers of glands in both the mouse and the chick, whereas the posterior stomach contains few glands and a villus-like endoderm. The major difference between the chicken and mouse stomachs is the thick mesoderm found in the chicken posterior stomach and the layer of koilen found covering the gizzard endoderm. The small intestines of both species are very similar morphologically. Thus, our histological observations support the hypothesis that the anterior stomach in the mouse is homologous to the proventriculus of the chicken, whereas the posterior stomach of the mouse is homologous with the gizzard of the chicken.

This model is supported by gene expression patterns in developing mouse and chicken guts. The expression of molecular markers of the stomach is remarkably similar in the mouse and the chick. Each specific marker examined for the anterior stomach of the chicken was also expressed in the anterior stomach of the mouse embryo (e.g., *BMP4*). The same result was seen with markers for the posterior stomach (e.g., *Six2*) as well as a marker for the entire stomach (e.g., *Barx1*). These results suggest that, although the two lineages are very far removed evolutionarily, the genes playing a role in stom-

ach development and patterning still retain conserved expression patterns. This finding suggests that the patterning of the stomach is a very ancient blueprint that was set up before the divergence of the avian and mammalian lineages. In addition, it provides further evidence that the anterior stomach in the mouse is homologous with the chicken proventriculus, whereas the posterior stomach in the mouse is homologous with the chicken gizzard (Pernkopf 1929). Indeed, we have yet to find any genes whose expression patterns are not conserved between the chicken and the mouse within the stomach and small intestinal regions.

### Relationship between frog and higher vertebrate stomachs

The relationship between the *Xenopus* gut and the chicken and mouse guts is more complex. Although the adult gut morphology of the *Xenopus*, chicken, and mouse are very similar in appearance and structure, the *Xenopus* larval gut is a relatively unspecialized linear tube. And, although there are distinct histological regions of glandular and nonglandular epithelium in the stomach area, the *Xenopus* tadpole lacks both a distinct stomach compartment and a pyloric sphincter with a recognizable transition to the intestinal epithelium.

These differences between the *Xenopus* tadpole and the mouse and chicken embryos correlate with a loss of organ-specific borders of gene expression in the embryonic *Xenopus* gut. For example, we find expression of *BMP-4* in the anterior stomach in all three species, but also in the posterior stomach of the *Xenopus* tadpole, albeit at lower levels. We also find the posterior stomach markers to be more broadly expressed in the *Xenopus* tadpole gut. For instance, the pyloric sphincter marker *Nkx2.5* is expressed from the duodenum into the posterior stomach region of the tadpole gut, and *BMPRI* is expressed in both the anterior and posterior stomach of the *Xenopus* tadpole. Even the small intestinal markers are expressed differently within the tadpole gut. *BMP-4* is seen only at very low levels, if at all, in the tadpole small intestine, whereas *Nkx2.3* is expressed throughout the small intestine as well as in the posterior stomach. These overlapping domains of marker gene expression in the *Xenopus* embryo could account for the more indistinct morphological boundaries observed in the tadpole digestive system.

This difference in embryonic expression patterns between amniotes and *Xenopus* may be better understood in the context of the life history of anuran amphibians. The tadpole represents a specialized larval phase in the anuran life history that is adapted to different habitats and resources from the adult, and consequently utilizes unique feeding strategies and, therefore, distinct gut morphology. In *Xenopus*, the aquatic tadpole is an herbivorous filter feeder, whereas the adult frog is carnivorous. Hence, one would expect the anuran tadpole gut to be different inasmuch as it is specialized for filter feeding, whereas the adult frog gut is adapted for a

terrestrial habitat and thus is morphologically similar to birds and mammals. Therefore, it is not unexpected that the expression of genes in the embryonic *Xenopus* gut would differ from that observed in the mouse and chicken, as the indirect development of *Xenopus* requires that it first pattern a morphologically and functionally distinct digestive system for the larval phase.

A separate patterning mechanism must exist to later specify the morphology observed in the adult *Xenopus*, perhaps utilizing the same gene set observed in the chicken and mouse embryonic gut. The tadpole gut exemplifies a highly derived version of the vertebrate gut, which is then transformed during metamorphosis to a system with the increased compartmentalization and functional morphology of carnivores. This hypothesis is supported by examples of apparently heterochronic accelerations observed in the development of the gut of rare species of anuran larvae with different habitats and feeding strategies from *Xenopus laevis*. For example, the New Mexico spadefoot toad (*Scaphiopus multiplicatus*) can develop into two environmentally induced morphs, omnivorous or carnivorous, based on the available food resources in the larval habitat. The carnivorous morph has precociously shortened intestines, a transformation normally induced during metamorphosis and the transition to adult carnivory (Pfennig 1992). Likewise, the larval phase of an arboreal tadpole, *Philautus carinensis*, utilizes unhatched, conspecific eggs as a food source, and therefore must digest animal proteins like the adult. This mode of feeding is also associated with post-metamorphic gut morphology, including shorter intestines and an expanded stomach for storage of eggs (Wassersug et al. 1981). Finally, *Lepidobatrachus laevis*, exhibits obligate carnivory in the tadpole phase. This unusual larval feeding strategy is accompanied by adult-like gut development in which the swimming tadpole acquires a distensible pepsin-producing stomach pouch with a muscular pyloric sphincter. Unlike related but non-carnivorous taxa, the simple "larval" stomach morphology is never observed in this species (Ruibal and Thomas 1988). These examples support the contention that the premetamorphic gut tube of most tadpoles is a derived structure adapted specifically for an herbivorous filter-feeding strategy that must then be transformed into the adult digestive system during metamorphosis. It will be informative to use the molecular markers employed in this study to examine the developing guts of these anuran species with different life histories.

### Metamorphosis and gene expression patterns

During metamorphosis, extensive modifications are necessary to convert the herbivorous aquatic tadpole into a carnivorous, usually terrestrial, frog (*Xenopus laevis* remains aquatic as an adult). In *Xenopus*, these changes include apoptosis of the primary tadpole epithelium; proliferation and differentiation of mesenchymal and muscle tissue; and development

of a secondary, adult epithelium, in addition to a complete remodeling of the conformation of the stomach and intestine (Hourdry et al. 1996). Thus, the cells of the primitive larval gut must be re-patterned during metamorphosis to achieve new GI tract morphology. Stolow and Shi (1995) have found that embryonic patterning genes such as *Xhh* become reactivated during natural and artificially induced metamorphosis in *Xenopus*, with particularly high levels found in the stomach region. In the chick, *Sonic Hedgehog* is expressed in the embryonic gut endoderm, and induces other patterning genes such as *Bmp-4* and the Hox genes (Roberts et al. 1995). It would, therefore, be interesting to evaluate the expression patterns of the genes described herein, especially *BMP4*, *Nkx-2.5*, *BMPRI*, and *Nkx-2.3*, in metamorphosing *Xenopus* tadpoles and in direct-developing amphibian species that lack a larval transition, such as *Eleutherodactylus coqui* (Fang and Elinson 1996). We would predict that the expression patterns of these genes in amphibians undergoing the latter developmental scenarios would be more similar to the expression patterns we observed in the chicken and mouse embryonic guts. Such studies could yield insight into the evolution of vertebrate gut patterning and show the utility of the *Xenopus* tadpole gut as a good model for evolutionary studies.

### Gene expression and organ patterning

As mentioned above, the *Xenopus* tadpole lacks the distinct gene expression boundaries found in the chicken and mouse embryonic guts. This lack of defined gene expression boundaries correlates with a lack of defined organ boundaries found within the tadpole gut, whereas the chicken and mouse guts have distinct organ boundaries. It is also interesting to note that whereas *Nkx2.5* plays a role in patterning the pyloric sphincter in the mouse and chicken, the tadpole does not have a pyloric sphincter, although *Nkx2.5* is expressed throughout the posterior stomach. These data have led us to hypothesize that distinct organ boundaries are formed by distinct gene expression boundaries. Formation of distinct boundaries at later stages of the amphibian life cycle, therefore, would require refinement of the gene expression patterns. For instance, with the refinement of the expression of *Nkx2.5* to the pyloric sphincter region and other organ-specific genes to their specific patterns, this would allow a unique molecular signature to occur within the sphincter primordium and, hence, a new organ could be formed. Conversely, the evolution of the derived *Xenopus* larval gut is likely dependent on the relaxation of the borders of region-specific gene expression, allowing the more uniform gut morphology.

### Evolution of the stomach

It appears that the stomach has an ancient origin. The stomach first appears in the fish lineage. The prevertebrate chordates do not have a true stomach, whereas the cartilaginous

and bony fish do. Although most fish do have a true stomach, some fish species appear to have lost the stomach secondarily. The remaining vertebrate lineages do have a true stomach (at least in the adult animal), although there is great variation in the size and shape of the stomach. The general conservation of molecular markers within the guts of amphibians, birds, and mammals suggests an ancient patterning event has been retained throughout the vertebrate lineages.

The great variation in stomach morphology seen among vertebrate lineages could be due to several possibilities. It could be that slight modifications in gene expression patterns (i.e., timing and levels of expression or subtle changes in cell cycling of progenitors) could lead to the vast array of shapes and sizes that are seen within the stomach of vertebrates. Future experiments could focus on identifying some of these differences, using the molecular signatures of the gut organs defined herein.

It has been hypothesized that indirect larval development in the vertebrate lineage evolved to adapt organisms to the transition from endotrophic feeding on maternal yolk reserves to exotrophic feeding strategies (Sanderson and Kupferberg 1999). A separate larval feeding stage would also facilitate a wider dispersal of the population, and a greater chance for survival of the species by adaptation to different habitats. Direct development is believed to be a secondary modification of this ancestral indirect-developing ontogeny in which metamorphic changes are retained, but somewhat concealed by a temporal compression of the developmental sequence (Rose 1999). The evolution of eggs with maternal yolk stores large enough to support all of development could have allowed the larval gut pattern to be modified in direct developers, as their life history did not necessitate a functional larval digestive system (Hart and Wray 1999). Thus, modifications of gene expression patterns could occur without detriment, allowing more specialized stomach compartments to evolve. The new localized expression patterns were subsequently kept by all stomach containing lineages. This model will be interesting to test upon many other species.

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