Remodeling of the intestine during metamorphosis of *Xenopus laevis*

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Thyroid hormone controls remodeling of the tadpole intestine during the climax of amphibian metamorphosis. In 8 days, the *Xenopus laevis* tadpole intestine shortens in length by 75%. Simultaneously, the longitudinal muscle fibers contract by about the same extent. The radial muscle fibers also shorten as the diameter narrows. Many radial fibers undergo programmed cell death. We conclude that muscle remodeling and contraction play key roles in the shortening process. Shortening is accompanied by a temporary “heaping” of the epithelial cells into many layers at climax. Cells that face the lumen undergo apoptosis. By the end of metamorphosis, when the epithelium is folded into crypts and villi, the epithelium is a single-cell layer once again. Throughout this remodeling, DNA replication occurs uniformly throughout the epithelium, as do changes in gene expression. The larval epithelial cells as a whole, rather than a subpopulation of stem cells, are the progenitors of the adult epithelial cells.

BrdUrd | caspase-3 | smooth muscle | epithelium | thyroid hormone

The tadpole amphibian small intestine is a long simple tube with a single layer of cuboidal epithelial cells. In *Xenopus laevis*, the duodenum contains one involution called the typhlosole (1). Single-cell layers of radial and longitudinal muscle surround a thin mesenchyme. In ~1 week, at the climax of metamorphosis, the intestine shortens ~58–90% depending on the anuran species. The shortening occurs uniformly along the intestine’s length (2). The mesenchyme and muscle layers become thicker, and the epithelium folds into the typical crypts and villi that characterize all adult vertebrate intestines. All of these changes in the anuran intestine are controlled by thyroid hormone (TH).

There have been numerous studies on the cellular mechanism for this remodeling. Several researchers have described nests of adult cells in the tadpole epithelium that proliferate and expand at climax to replace the dying larval epithelium (1, 3–5). This scheme for remodeling of the epithelium requires two distinct populations of epithelial cells, larval and adult. One study considered that at least some adult cells are derived from larval epithelial cells (6).

Subtractive hybridization identified a number of genes whose expression in the intestinal epithelium is altered by TH (7). Their expression patterns fall into different developmental profiles as measured by Northern blotting. One class of mRNAs rises in expression to a peak at climax and then falls, and the expression pattern parallels the concentration of endogenous TH. A second class is the inverse of the first; gene expression is high before climax, then drops at climax (“eclipses”) and rises again at the end of metamorphosis. This eclipse has also been described for genes expressed specifically in the epithelium of the posterior intestine (6) and in the epithelium of the stomach (8). To date, no gene that is expressed exclusively in the larval intestine has been identified.

This paper addresses two features of intestinal remodeling: the phenomenon of shortening and narrowing, and the remodeling of the intestinal epithelium. We describe the TH-induced contraction of longitudinal muscle fibers that accompanies intestinal shortening and the contraction and death of radial muscles as the diameter narrows. During this time, epithelial cells facing the lumen undergo cell death. A net effect of these size changes is transient “heaping” of the epithelium into many layers. In situ hybridization of genes that are expressed in the epithelium reveals a uniform change in all of the epithelial cells as the intestine transforms into its convoluted state of crypts and villi. Similarly, DNA replication occurs uniformly throughout the larval epithelium until days after metamorphosis is completed, when it localizes to the intestinal crypts.

Materials and Methods

3,5,3′-Triiodothyronine (T3) and BrdUrd Treatment, Immunohistochemistry, and in Situ Hybridization. Tadpoles were staged by the tables of Nieuwkoop and Faber (NF) (9). Gastrointestinal tracts of tadpoles at different stages were fixed in 4% paraformaldehyde, embedded in OCT compound, and cryosectioned as described (10, 11). Sections were also processed for *in situ* hybridization (10, 11) by using digoxigenin-labeled antisense probes against *Musashi-1* (GenBank accession no. BI447711), intestinal fatty-acid binding protein (a gift of Y. Bo Shi, National Institute of Child Health and Human Development, Bethesda), and TH-controlled basic leucine zipper (12). Sections were also processed by using immunohistochemistry (10, 11) with monoclonal antibodies against smooth muscle actin (Sigma), active caspase-3 (Pharmingen), and epithelial cadherin (product number 5D3, University of Iowa Developmental Studies Hybridoma Bank, Iowa City), or a polyclonal antibody against fibronectin (a gift of D. W. DeSimone, University of Virginia, Charlottesville) by using methods previously described (13–15). Antibodies were detected by using Alexa Fluor 488- and 568-conjugated secondary antibodies (Molecular Probes). Intestines of some NF stage 50 (NF 50) and NF 54 tadpoles were processed in whole-mount by immunohistochemistry as described (13, 14). These intestines were sliced open longitudinally and flat-mounted onto slides with either the serosa or mucosa side facing the coverslip.

To identify proliferating cells, live tadpoles were injected i.p. with 10 μl of BrdUrd (10 mM) 12 h before fixation with 2% trichloroacetic acid for 2 h at room temperature. Intestines were rinsed in PBS three times for 15 min, embedded in OCT compound, cryosectioned, and then counterimmunostained with a primary antibody against fibronectin (a mesenchyme-specific marker) and Alexa Fluor 568-conjugated secondary antibody. Slides were then incubated in 4 M HCl for 1 h at room temperature, rinsed three times for 5 min in PBS, incubated in ice-cold ethanol/acetic acid (2:1) for 8 min, rinsed three times for 5 min in PBS, blocked for 30 min in PBS with 10% normal goat serum, incubated for 1 h at room temperature with Alexa Fluor 488-conjugated-anti-BrdUrd (Molecular Probes) (1:30) in PBS with 10% NGS, rinsed in PBS three times for 15 min, and mounted with a coverslip.

Abbreviations: TH, thyroid hormone; T3, 3,5,3′-triiodothyronine; NF n, Nieuwkoop and Faber stage n.

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NF 50 and 54 tadpoles were induced to metamorphose with 10 nM T3 as described (13, 14).

Statistics and Experimental Design. To measure intestine shortening during spontaneous metamorphosis, the gastrointestinal tracts of sibling tadpoles at various stages (*n* = 8–10) were excised, and lengths were measured for the anterior intestine (duodenum + anterior ileum) and the posterior intestine (posterior ileum + colon + rectum). The junction between the anterior and posterior ileum is the ileum’s switchback point (see Fig. 1). Differences in mean length with developmental stage were assessed statistically by one-factor ANOVA (SuperANOVA, Abacus Concepts, Berkeley, CA), followed by Fisher’s pairwise comparisons. *P* values of <0.01 were considered significant.

To measure precocious intestinal shortening with T3 treatment, NF 54 tadpoles were induced with 10 nM T3 for 7 days. Tadpoles were sampled every 24 h (*n* = 6), and total intestine lengths (duodenum to rectum) were measured. Changes in length with time of T3 treatment were assessed statistically by one-factor ANOVA. *P* values of <0.01 were considered significant.

**Table 1. Intestine lengths at different stages of tadpole metamorphosis**

<table>
<thead>
<tr>
<th>Stage</th>
<th>n</th>
<th>Total length, mm</th>
<th>Anterior length, mm</th>
<th>Posterior length, mm</th>
<th>Total % shortening from NF 60</th>
<th>Anterior % shortening from NF 60</th>
<th>Posterior % shortening from NF 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>57</td>
<td>9</td>
<td>59.8 (8.3) a</td>
<td>38.7 (5.6) a</td>
<td>21.1 (3.4) a</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>60</td>
<td>10</td>
<td>65.0 (6.5) b</td>
<td>41.9 (3.9) b</td>
<td>23.1 (3.4) ab</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>61</td>
<td>10</td>
<td>47.0 (7.2) c</td>
<td>29.2 (4.9) c</td>
<td>17.8 (2.7) c</td>
<td>27.7</td>
<td>30.3</td>
<td>22.9</td>
</tr>
<tr>
<td>61/62</td>
<td>8</td>
<td>33.0 (8.8) d</td>
<td>19.5 (0.6) d</td>
<td>13.5 (1.0) d</td>
<td>49.2</td>
<td>53.5</td>
<td>41.6</td>
</tr>
<tr>
<td>62</td>
<td>10</td>
<td>25.0 (3.1) e</td>
<td>14.9 (2.0) e</td>
<td>10.1 (1.4) e</td>
<td>61.5</td>
<td>64.4</td>
<td>56.3</td>
</tr>
<tr>
<td>63</td>
<td>8</td>
<td>16.9 (1.5) f</td>
<td>10.6 (0.9) f</td>
<td>6.3 (1.0) f</td>
<td>74.0</td>
<td>74.7</td>
<td>72.7</td>
</tr>
<tr>
<td>64</td>
<td>10</td>
<td>16.3 (2.0) f</td>
<td>10.8 (1.4) f</td>
<td>5.5 (0.7) f</td>
<td>74.9</td>
<td>74.2</td>
<td>76.2</td>
</tr>
<tr>
<td>66</td>
<td>10</td>
<td>16.2 (0.8) f</td>
<td>9.0 (1.1) f</td>
<td>6.5 (0.9) f</td>
<td>75.1</td>
<td>78.5</td>
<td>71.9</td>
</tr>
</tbody>
</table>

Lengths are expressed as mean (SD). Mean lengths with different letter symbols within columns are significantly different from each other (*P* < 0.0001), as determined by ANOVA. Anterior, duodenum + anterior ileum; posterior, posterior ileum + colon + rectum; total, anterior + posterior.

**Results**

Before metamorphosis in *X. laevis*, the tadpole intestine consists of two spiral coils: the outer coil (duodenum and anterior ileum) reverses direction at the switchback point and is followed by the inner coil (posterior ileum and colon), which terminates at the rectum (Fig. 1 *A* and *B*). The climax of metamorphosis (NF 59–65) is the period of maximal change when the endogenous TH is at its highest concentration (16). During climax, which lasts ~8 days, the intestine shortens by ~75% (Fig. 1 *C* and *D* and Table 1). From the start (NF 59) to the end (NF 66) of metamorphic climax, the percentage of shortening of the anterior and posterior intestine is similar (78.5% and 71.9%, respectively) (Table 1), supporting the findings of Pretty et al. (2) that the intestine shortens equally along its entire length. The premetamorphic tadpole gut (NF 46–54) can be induced to shorten precociously by treatment with 10 nM T3 for 3–7 days (Table 2).

Shortening of the intestine during spontaneous metamorphosis is accompanied by a change in cross-sectional morphology. The premetamorphic tadpole duodenum (NF 54) is a simple thin tube with one large involution (the typhlosole) in the anterior intestine (Fig. 2 *A*). At the end of metamorphosis...
by ANOVA. For all groups, \( n \) corresponds to the decrease in total intestine length after 72 h.

NF 54 tadpole are intestinal villi (Fig. 5), confined primarily to epithelial cells located at the tips of the villi (Fig. 5). By the end of metamorphosis (NF 66), apoptosis is measured by caspase-3, but fewer are labeled in the duodenum (data not shown). In a sagittal view of similarly treated tadpole intestine, the abrupt shortening of the anuran gut during spontaneous metamorphosis, although muscle fiber lengths become difficult to measure in vitro beyond NF 61, as individual fiber ends are not easily distinguished (data not shown). In a sagittal view of similarly treated tadpole intestine, the duodenal diameter decreases by one-third, and the epithelium collapses into folds (presumably because of longitudinal compression by the muscle fibers), but remains as a single-cell layer (Fig. 6 C and D).

### Discussion

#### Intestinal Shortening.

The abrupt shortening of the anuran gut during metamorphosis has been well documented. The amount of shortening varies among species, but reports range from 58% for *Rana temporaria* (21) to as high as 90% in *Alytes obstetricans* (22). In our experiments, the *X. laevis* intestine shortens by 76% from the beginning of metamorphosis at NF 60 through its completion by NF 66, and most of the shortening is completed during spontaneous metamorphosis, although muscle fiber lengths become difficult to measure in vitro beyond NF 61, as individual fiber ends are not easily distinguished (data not shown). In a sagittal view of similarly treated tadpole intestine, the duodenal diameter decreases by one-third, and the epithelium collapses into folds (presumably because of longitudinal compression by the muscle fibers), but remains as a single-cell layer (Fig. 6 C and D).

#### Table 2. Whole intestine lengths of NF 54 tadpoles after treatment with 10 nM T3 for different time

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>Intestine length, mm</th>
<th>% Shortening from day 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>55.0 (8.7) a</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>44.7 (1.5) b</td>
<td>18.7</td>
</tr>
<tr>
<td>3</td>
<td>29.3 (6.5) c</td>
<td>46.7</td>
</tr>
<tr>
<td>4</td>
<td>21.7 (1.5) d</td>
<td>60.5</td>
</tr>
<tr>
<td>5</td>
<td>23.3 (0.6) d</td>
<td>57.6</td>
</tr>
<tr>
<td>6</td>
<td>17.0 (2.0) e</td>
<td>69.1</td>
</tr>
<tr>
<td>7</td>
<td>13.7 (2.5) f</td>
<td>75.1</td>
</tr>
</tbody>
</table>

Length is expressed as mean (SD). Mean lengths with different letter symbols are significantly different from each other \((P < 0.0001)\) as determined by ANOVA. For all groups, \( n = 6 \).

(NF 66), the epithelium is structured into crypts and villi (Fig. 2D). As the mesenchyme and muscle layers thicken at metamorphic climax (NF 62 and NF 63, Fig. 2 B and C, respectively), the intestinal epithelium temporarily thickens to five- to eight-cell layers (Figs. 2 B’ and C’), compared with a thickness of one to two cells before (NF 54, Fig. 2A’) and after (NF 66, Fig. 2D’) metamorphosis.

The expression of genes with different developmental profiles in the anterior intestinal (duodenal) epithelium has been assessed (Fig. 3). We chose the neural stem cell marker Musashi (17) because nests of adult epithelial precursor cells in the intestine were reported to express specifically this gene (18). Musashi mRNA is expressed constitutively in all cells of the epithelium at every developmental stage from NF 54–66 (Fig. 3A). Intestinal fatty acid binding protein is expressed in the anterior tadpole intestinal epithelium, shut off (eclipsed) during climax, and then expressed again after climax (5, 19). Every epithelial cell follows this gene expression pattern (Fig. 3). TH/basic leucine zipper (12) is a direct response gene expressed in the intestinal epithelium and the mesenchyme. The expression of this gene rises and falls with the endogenous TH concentration (Fig. 3C). Our *in situ* hybridization experiments demonstrate the uniformity of these gene expression profiles in the intestinal epithelium at each stage.

Intestinal epithelial cell proliferation is very low before climax (20) (Fig. 4A), but increases greatly in all layers of the heaped epithelium at climax and throughout the epithelium at a high level even late in metamorphosis after the crypts and villi have been formed (NF 65, Fig. 4E). Proliferation in the mesenchyme increases during climax, reaches a peak at NF 61, and drops by NF 66. Because BrdUrd is a nuclear marker, and mesenchyme cells are mixed with muscle cells, we could not reliably evaluate muscle proliferation.

Apoptosis within the anterior intestine, as measured by active caspase-3 immunoreactivity, is present in a small number of epithelial cells before climax (20) (Fig. 5). By NF 61, when the epithelial cells are heaped, apoptosis occurs mainly in the cells that line the lumen (Fig. 5B). Many radial, but not longitudinal, muscle fibers in the ileum are immunoreactive for active caspase-3, but fewer are labeled in the duodenum (data not shown). By the end of metamorphosis (NF 66), apoptosis is confined primarily to epithelial cells located at the tips of the intestinal villi (Fig. 5C).

Individual longitudinal smooth muscle fibers in the ileum of a NF 54 tadpole are \( \approx 160 \mu m \) in length (Fig. 6A). After 72 h of T3 treatment, muscle fibers are much shorter (\( \approx 90 \mu m \) in length) than untreated fibers (Fig. 6B). This decrease in length by \( \approx 44\% \) corresponds to the decrease in total intestine length after 72 h of T3 treatment (Table 2). Intestinal muscle fibers also shorten during spontaneous metamorphosis, although muscle fiber lengths become difficult to measure in vitro beyond NF 61, as individual fiber ends are not easily distinguished (data not shown). In a sagittal view of similarly treated tadpole intestine, the duodenal diameter decreases by one-third, and the epithelium collapses into folds (presumably because of longitudinal compression by the muscle fibers), but remains as a single-cell layer (Fig. 6 C and D).
by the start of NF 62 (Table 1). The premetamorphic tadpole gut (NF 50–54) can be induced to shorten precociously by the same amount after treatment with TH for 7 days (Table 2), the approximate amount of time it takes for a tadpole to undergo spontaneous metamorphosis (23). By the time the gut has completed shortening, the cross-sectional diameter has decreased by about one-third. The total reduction in gut length after 3 days of treatment with TH (47%) corresponds with the shortening of individual muscle fibers by approximately the same amount (43%). This finding is similar to the observations of Bataillon (24), who reported that dissociated muscle fiber lengths after anuran gut involution were shorter than before involution, and that the relative shortening of the entire intestine corresponded to the contraction of the individual fibers. Curiously, shortening of gut length in the ileum is accompanied by death of some of the radial (but never longitudinal) muscle fibers (data not shown).

This period of abrupt shortening in length and constriction of diameter in the duodenum during spontaneous metamorphosis is accompanied by cross-sectional changes that include a dramatic heaping of the epithelium to as many as eight cells thick by NF 62, compared with only one to two cells thick before and after climax. Using *Rana clamitans*, Janes (21) observed a similar thickening of the epithelium at the start of metamorphic climax. He attributed this epithelial thickening to muscular contraction, the result of which “some of the fragmented cells are simply forced together in the mucosa region, while others are sloughed into the lumen.” The increased cell proliferation undoubtedly contributes to the temporary thickening of the epithelium at metamorphic climax. We have shown that increased epithelial cell death during metamorphosis as measured by active caspase-3 immunoreactivity takes place during heaping, but predominantly in the apical cells that line the lumen (Fig. 5). We have also observed the sloughing of epithelial cells into the lumen when the intestine is shortening. By the end of metamorphosis, after involution is complete, epithelial cell death becomes localized at the tips of the villi (Fig. 5), as has been reported (1, 5, 20). Interestingly, when metamorphosis is induced prematurely with TH treatment, the epithelium collapses into folds rather than heaping up on itself as the intestine shortens (Fig. 6). Presumably the synchrony of interacting events is lost, as it is in other prematurely induced metamorphic programs. We have also observed heaping of epithelial cells in the skin of the tadpole tail as the tail shortens during metamorphic climax (unpublished observation). Shortening of the tadpole tail is enhanced by contraction of longitudinally oriented slow muscle fibers (“cords”) (25).

**Remodeling of the Intestinal Epithelium Occurs by Transition of Larval Cells.** Changes in the intestinal epithelium at metamorphosis have been studied in detail for 40 years. Bonneville (3)
described the degeneration of larval epithelial cells in the bull frog intestinal epithelium and their replacement by a “new layer” of cells that formed the adult epithelium. Her assay for this change was phase contrast and electron microscopy. The use of methyl green-pyronin to identify “nests” of adult progenitor cells in *X. laevis* was introduced in 1977 (1). These workers measured mitotic figures at the climax of metamorphosis and also in adult frog intestine. They reported extensive mitosis throughout the epithelium of the tadpoles that became restricted to the troughs in the adult. Marshall and Dixon (26) concluded that growth replacement in the epithelium is due to the mitotic activity of what they called “specialized” cells rather than the unspecialized precursor cells that replicate in the troughs of vertebrate intestine. Ishizuya-Oka and her colleagues (5) have relied extensively on the methyl green-pyronin staining of presumptive adult cells in the *X. laevis* intestinal epithelium. She has described their replication at climax and the concomitant death of the larval cells as measured by TUNEL assay (20). More recently, they have reported that a stem cell marker, Musashi-1, is expressed specifically in these nests of adult cells (18). Amano *et al.* (6) described the eclipse of calbindin gene expression at climax in the posterior *X. laevis* intestinal epithelium and its reexpression in islet cells of the frog. They concluded that “at least some islet cells are derived from differentiated larval absorptive cells.”

Our interest in intestinal remodeling was prompted by the availability of specific promoters that have enabled us to direct transgene expression to different intestinal cell types. Our goal has been to assess the importance of cell–cell interaction in *TH*-induced remodeling. To prepare for these experiments, we have reexamined epithelial remodeling along with the principals that underlie the remarkable concurrent shortening. We

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**Fig. 4.** Cell proliferation in the intestinal epithelium during tadpole metamorphosis. Proliferating cell nuclei were labeled with a 12-h pulse of BrdUrd injected i.p. into living tadpoles. Cryosections of the duodenum were stained with antibodies against BrdUrd (e-cadherin, green) and mesenchyme (fibronectin, red). (A) BrdUrd label is very low during premetamorphosis (NF 54); increases uniformly throughout the epithelium during metamorphic climax at stages NF 60 (B), NF 61 (C), and NF 62 (D); and continues to be widespread near the end of climax at NF 65 (E). (F) By the end of climax (NF 66), BrdUrd incorporation begins to decrease at the crest regions of the intestinal involutions. (G) Localization of BrdUrd incorporation to cells in the troughs is apparent 2 weeks after completion of metamorphosis. (Scale bar: 100 μm.)

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**Fig. 5.** Active caspase-3 immunoreactivity in the tadpole intestine varies with developmental stage. Intestines (duodenum) at different stages of metamorphosis were labeled in cryosections (A–C) with antibodies against active caspase-3 (green), smooth muscle (red), and nuclei counterstained with DAPI (white). (A) NF 58 tadpole. (B) Climax at NF 61. (C) The end of climax (NF 66). (Scale bar: 100 μm.)
were struck by the uniformity of epithelial gene expression (Fig. 3) regardless of whether it was measured by a constitutive marker (Musashi-1), a known TH-induced gene (basic leucine zipper), or a gene that undergoes TH-induced eclipse (intestinal fatty acid binding protein). These gene expression patterns are not localized specifically to any nests of cells that might serve as progenitors for the adult epithelium. Furthermore, DNA replication occurs uniformly throughout the epithelium (Fig. 4) and continues to do so even after the formation of crypts and villi (NF 65, Fig. 4). Replication gradually becomes restricted to the cells in the troughs of the epithelial folds only days after metamorphosis is complete. At the climax of metamorphosis, when the epithelium is heaped into many layers, DNA replication occurs throughout the epithelium (Fig. 4), but cell death as measured by the presence of active caspase-3 is restricted primarily to the cells lining the lumen (Fig. 5). This same distribution of dying cells during and after metamorphosis was previously described by using TUNEL as an assay (20).

Remodeling by Transition of Larval Cells. Many tadpole organs remodel into frog organs that function with similar purpose. These tadpole organs are formed during embryogenesis without the input of TH and are the free-living tadpole. Examples of organs or cells that remodel include the brain, liver, blood, immune system, skin, heart, pancreas, eye, and kidney. The limb is one of the exceptions. In the absence of TH, no functional limb develops. TH does not remodel a limb; rather, it induces its growth and differentiation from a tiny limb bud. Limb formation requires several cell-autonomous, but coordinated, TH-controlled programs (27). There has been a general consensus that remodeling of organs during amphibian metamorphosis involves separate nests of adult stem cells that await the hormonal cue to replicate and differentiate. Our evidence supports the idea that the adult intestinal epithelium originates from functioning larval epithelial cells.

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