Tail formation as a continuation of gastrulation: the multiple cell populations of the *Xenopus* tailbud derive from the late blastopore lip

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SUMMARY

Three lines of evidence suggest that tail formation in *Xenopus* is a direct continuation of events initiated during gastrulation. First, the expression of two gene markers, *Xbra* and *Xnot2*, can be followed from the blastopore lip into distinct cell populations of the developing tailbud. Second, the tip of the tail retains Spemann’s tail organizer activity until late stages of development. Third, lineage studies with the tracer DiI indicate that the cells of the late blastopore are fated to form specific tissues of the tailbud, and that intercalation of dorsal cells continues during tail elongation. In particular, the fate map shows that the tip of the tail is a direct descendant of the late dorsal blastopore lip. Thus, the tailbud is not an undifferentiated blastema as previously thought, but rather consists of distinct cell populations which arise during gastrulation.

Key words: tailbud, Spemann’s organizer, chordoneural hinge, homeobox, *Xnot2*, *Brachyury*

INTRODUCTION

The fertilized *Xenopus* egg is a sphere 1.2 mm in diameter. 4 days later the resulting larva, which has not fed, reaches a length of 9 mm. When neurulation begins at the end of gastrulation, 14 hours after fertilization, the embryo is still roughly spherical; it is the formation of the tail that accounts for most of the subsequent elongation of the embryo. While much progress has been made recently in understanding the mechanism of *Xenopus* gastrulation (see reviews in Keller et al., 1991; Stern and Ingham, 1992), the process by which the tail is formed has received much less attention.

The vertebrate tail develops from the tailbud, an apparently homogenous mass of cells at the posterior of the embryo. An unresolved question is whether the tailbud is composed of truly undifferentiated pluripotential stem cells, i.e., a ‘blastema’, or whether it consists of several cell populations with differing cell fates despite its histologically homogenous appearance. The concept of a tailbud blastema was proposed by Holmdahl (1925) who distinguished the ‘primary body development’ in which the three germ layers are formed by involution movements during gastrulation, from the ‘secondary body development’ in which an undifferentiated blastema directly gives rise to all tissues of the tail. Pasteels (1943) favored a different view, in which the different tissues of the tail would derive from distinct cell populations. Recently, the mechanism of tail development has received little attention, but there seems to be general agreement that the tailbud is a blastema (Griffith et al., 1992). The question of whether or not the tailbud is homogenous can now be directly addressed using appropriate molecular markers.

In this study, we compare two gene markers that are expressed in distinct regions of the blastopore of the early gastrula and whose expression can be followed continuously as they become localized to distinct cell populations in the tailbud in the course of development. The markers used were *Xnot2*, a homeobox gene that is expressed in the dorsal lip of the blastopore, and *Brachyury* (short-tail), a gene required for tail development and expressed in the entire blastoporal ring (see description below). Having found that the tailbud consists of distinct cell populations, we then asked whether they were related by lineage to the different regions of the blastopore. This was addressed by determining the fate map of the late blastopore lip and by transplantation studies. We conclude that tail formation in *Xenopus* is a direct continuation of gastrulation movements and that the tip of the tail, which retains potent tail organizer activity, is a direct descendant of the dorsal blastopore lip.

MATERIALS AND METHODS

cDNA cloning and characterization

Approximately 1.5×10⁵ plaques of an unamplified *Xenopus* egg cDNA library made as described (Blumberg et al., 1992) were screened in duplicate on nitrocellulose filters with a 1024X degenerate mixture of ³²P end-labelled oligonucleotides: 

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corresponding to the sequence KIWFQ/KNRR of
helix 3 of the homeodomain. Hybridization was at low stringency conditions in a solution containing 1.0 M NaCl/0.1 M Tris-HCl (pH 8.3)/6.6 mM EDTA/5x. Denhardt’s 0.1% SDS/0.5% NaPP/1 25 units per ml of heparin/1 mg per ml yeast RNA, and washed at high stringency (58°C) in 3 M TMAC/0.05 M Tris-HCl (pH 8.0)/0.2 mM EDTA (Burglin et al., 1989). The longest cDNA (E-9) was subcloned and sequenced by the dideoxy method on both strands using T7 DNA polymerase (Pharmacia). DNA sequences were analyzed using the University of Wisconsin Genetics Group (Devereaux et al., 1984). The Xnot2 cDNA E-9 is 2097 bp, encodes a predicted 233 amino acid protein and contains a poly(A) tail. The homeodomain is most similar (62.7%) to the Drosophila empty spiracles homeodomain protein, making this a novel type of homeobox. A comparison of the predicted amino acid sequence reported here with that reported by von Dassow et al. (1993) shows that the predicted proteins are 90.1% identical overall and 96.6% identical in the homeodomains, with 91% of the amino acid changes being conservative (Dayhoff, 1972). The two probes would be expected to cross-hybridize, and expression patterns of the two genes appear identical at the level of resolution of whole-mount in situ hybridization. Because Xenopus is a pseudotetraploid organism, many genes are duplicated. It is possible that Xnot (von Dassow et al., 1993) and Xnot2 represent duplicated forms of the same gene.

The 5’ untranslated region of Xnot2 contains 7 repeats of a 28 bp sequence (consensus: GGGTGGGTGCTAGT-GATCAGGGTGCT), three repeats have this exact sequence, with the other four showing at most four mismatches. A search of Genbank did not reveal any matches that would indicate a probable function for the repeats.

In situ hybridization

To detect Xnot2 or Xbra transcripts in embryos, the whole-mount in situ hybridization protocol of Harland (1991) with minor modifications (Cho et al., 1991a) was used. Some embryos were refixed in Bouin’s, embedded in Paraplast and sectioned. Digoxigenin-labelled sense and antisense RNAs were generated by in vitro transcription of an Xnot2 full-length cDNA clone (E13) lacking the 5’ leader repeats or of the published Xbra probe (Smith et al., 1991), using the digoxigenin labelling kit (Boehringer Mannheim) following the manufacturer’s instructions.

Einstech assay

The Einstech assay was carried out as described (Ruiz i Altaba and Melton, 1989; Cho et al., 1991b) with minor modifications. Host embryos (stage 10) were transferred into 1× MBS saline (Gurdon, 1976) and dechorionated, the ventral blastocoel cavity accessed with a cut made with a tungsten wire, and the graft implanted using a blunt hair instrument. The embryos were then placed immediately in 0.1× MBS saline for heating and further culture.

The chordoneural hinge and prechordal plate were dissected from stage 25 embryos using an eyebrow hair and forceps. The hinge was obtained by removing the morphologically undifferentiated tissue just anterior to the neurenteric canal, which is visible at early tailbud stages as a black spot in the roof of the gut. The distance between the neurenteric canal opening and the anus is stage-dependent; we consider this process homologous to the regression of Hensen’s node in the chick embryo.

The tip of the tail was removed from stage 35 embryos anesthetized in 0.01% Tricaine (Sigma) by excising the tailbud with iridectomy scissors and transferring to Ca²⁺/Mg²⁺-free OR2 medium (Kay and Peng, 1991) in a 1% agarose dish for one hour. The ectoderm was removed using tungsten needles and the tailbud tissue transferred to 1× MBS for 10 minutes. The posterior-most region of the tail, approximately corresponding in size to the extent of the Xnot2-staining region shown in Fig. 1A, was then excised with tungsten needles and fine scissors. To obtain notochord and hindbrain for control transplants, the ventral endoderm and ectoderm were manually removed using a needle and forceps from anesthetized stage 35 tadpoles. The notochord and hindbrain were isolated by alternately drawing up and expelling the tissue in a drawn-out micropipet after incubation for 30 minutes in 1:1 (pan- creatin, Gibco, resuspended following the manufacturer’s suggestion: 1× MBS) and washed extensively in 1× MBS. Fragments of approximately the same size as the isolated tip of the tail were prepared for these control tissues. Animal caps were isolated from stage 7 blastulae using a tungsten needle. In order to determine which tissues were induced in the host, embryos were injected at early stages (2-4 cells) with 4 nl per blastomere of rhodamine or fluorescein dextran amine (Gimlich and Braun, 1985) at 5-10 mg/ml.

Implanted chordoneural hinge (from stage 25 embryos) induced tail structures in 13 recipients (n=14). Four of these were analyzed by histology; three contained organized notochord, muscle and neural tissue. 19 out of 27 implanted tail tips (from stage 35 embryos) induced tails; five out of eight of these analyzed by histology contained organized notochord and neural tissue and four contained muscle. Transplanted hindbrain fragments from stage 30-35 embryos (n=13) did not induce structures. Three specimens were analyzed by histology; neither induced notochord nor muscle was observed. Transplants of notochord fragments (stage 30-35) of the same size as the tailtips (n=16), obtained from the middle third of the embryo, produced small bumps on the ventral side, some of which were pigmented. Histological analysis (n=3) did not show induced differentiated tissue. When a middle third of a notochord was implanted into a host gastrula, filling the entire blastocoel, tail-like structures formed in 2 out of 5 cases; histological analysis of these structures showed organized muscle and neural tissue in one (n=2) case.

Lineage tracing with DiI

DiI labelling was performed according to the procedure of Selleck and Stern (1991) and Izipisúa Belmonte et al. (1993), adapted for Xenopus embryos. Injection capillaries were filled with DiI (1,1’-dioctadecyl-3,3,3’,3’-tetramethyl indocarbocyanine perchlorate; Molecular Probes) dissolved at 3 mg/ml in 0.2 M sucrose/30% N,N’-dimethylformamide (C. Stern, personnel communication), and using gentle air pressure from a Narashige microinjection device, a small bolus of dye was applied to the dorsal, ventral, or lateral lips of dechorionated stage 13 embryos. Because DiI precipitates upon contact with aqueous medium, the pipette is clogged easily; this can be partially prevented by expelling a constant flow of dye. When DiI precipitates clog the needle, the tip can be broken off to restore flow. The marked spot is easier to see in albino embryos. Lineage-traced embryos were incubated in the dark until stage 33 or 40, and fixed overnight in MEMFA (Harland, 1991). For sectioning, embryos were rinsed 3 times 20 minutes in 0.1 M Tris (pH 7.4) and illuminated (547 nm) for photo-oxidation in a solution of 500 µg/ml 3-3’-diaminobenzidine in 0.1 M Tris (pH 7.4) until fluorescence was no longer visible. Embryos were transferred to methanol, embedded in Paraplast, and 10 µm sections counterstained with light green for histological analysis.

RESULTS

We became interested in tail development after isolating a divergent homeobox cDNA whose expression is exquisitely localized to the tip of the tail (Fig. 1A). This expression can be traced continuously back through development to the forming tailbud (Fig. 1B), a narrow region of prospective notochord at late gastrula (Fig. 1C), and a wider area above the dorsal lip of the early gastrula as convergence and
extension movements begin (Fig. 1D). The expression pattern suggested the hypothesis that the tip of the tail might derive from the dorsal lip of the blastopore; this hypothesis was tested in the present investigation.

We intended to name this gene differently but were anticipated by the publication by von Dassow et al. (1993) on Xnot, a homeobox gene expressed in the dorsal lip and notochord. The predicted amino acid sequence encoded by the cDNA that we isolated (see Materials and Methods) is 90% identical to that of Xnot, and we therefore named our gene Xnot2. The full-length sequence of Xnot2 can be accessed in Genbank (#L19566). The study by von Dassow et al. (1993) investigated the regulation of Xnot by growth factors, but was not concerned with tail development, which is the subject of this study.

To determine whether different populations of cells are present in the tailbud, we compared the expression of Xnot2 to that of the Brachyury (Xbra) gene. Hemizygous Brachyury mouse mutants are characterized by a short tail (Dobrovolskaia-Zavadskaja, 1927; Gluecksohn-Schloenheimer, 1938) and the gene has been intensively investigated in a number of species. In the mouse and zebrafish, Brachyury is expressed in the notochord and tailbud (Herrmann, 1991; Schulte-Merker et al., 1992). In Xenopus, Xbra marks the entire marginal zone (Smith et al., 1991; Green et al., 1992; Fig. 2A’), while Xnot2 is expressed on the dorsal side of the blastopore (Fig. 2A). At late gastrula, Xbra is expressed in the notochord and in a thick circumblastoporal collar of cells (Green et al., 1992; Fig. 2B’), while Xnot2 is expressed in the notochord and in cells that are still converging towards the midline (Fig. 2B). Thus, Xnot2 provides a marker for dorsal blastopore cells while Xbra marks dorsal, lateral and ventral blastopore cells.

**Distinct cell populations in the developing tail**

Figs. 2 and 3 show the distribution of Xnot2 and Xbra transcripts in the developing tailbud. Xnot2 stains a U-shaped region which consists of the posterior spinal cord and notochord as well as the region of continuity between these two tissues (Figs 2C, 3A). The latter region is designated the chordoneural hinge (Pastels, 1943). Xnot is expressed in the notochord and floor plate (von Dassow et al., 1993) but, when more posterior sections are analyzed, Xnot2 transcripts are also found in the entire ventral half of the spinal cord (Fig. 4A). This suggests that the chordoneural hinge may give rise, in addition to the notochord of the tail, to a significant portion of the cells of the spinal cord. (The fact that cells of the spinal cord and of the posterior notochord are continuous with each other may be of particular interest in view of the recent demonstration of planar neural induction; Dixon and Kintner, 1989; Doniach et al., 1992; Ruiz i Altaba, 1992).

Brachyury transcripts are found in the notochord and in a mass of cells that lies posteriorly, ventrally and laterally to the chordoneural hinge (Figs 2C’, 3A’). Cells expressing Xbra are also found in the root of the spinal cord (arrowheads in Fig. 3A’,B’), which appears to be continuous with the posterior mass of cells expressing Xbra. In favorable preparations, it may be seen that the chordoneural hinge (which is Xnot2 and Xbra-positive) is separated from the posterior mass of Xbra expressing cells by a narrow canal (arrowhead in Fig. 2E’). This structure, called the neurenteric canal, connects the lumen of the spinal cord to that of the gut. The anterior wall of the neurenteric canal is formed by the chordoneural hinge (which is both Xnot2- and Xbra-positive), while the posterior wall is formed by the posterior mass of Xbra-positive, Xnot2-negative cells (henceforth referred to simply as the ‘posterior wall’ cells). In Xenopus, the neurenteric canal is very narrow and can be easily missed in sagittal sections (Fig. 3A,B). However, in frontal sections one should expect to find it every time, and this is indeed the case (Fig. 3C,C’). A favorable sagittal section spanning the Xenopus neurenteric canal is shown in Fig. 4C. The relationship of the neurenteric canal to the other elements of the tailbud is indicated in the diagrams of Fig. 3; its embryological origin is explained below.

From these descriptive studies using gene markers, we conclude that at least three types of cells can be distinguished in the Xenopus tailbud: (1) the chordoneural hinge and notochord, which are positive both for Xnot2 and Xbra, (2) the posterior wall cells and cells in the roof plate of the spinal cord which are Xbra-positive but Xnot2-negative, and (3) the ventral spinal cord which is Xnot2-positive but Xbra-negative. We conclude that the Xenopus tailbud is not a histologically homogenous blastema as currently thought (e.g., Fig. 36 in Hausen and Riebesell, 1991), but rather consists of distinct cell populations.

**On the formation of the neurenteric canal**

While the presence of a neurenteric canal in the tail is
Fig. 2. Expression of *Xnot2* and *Xbra* in the blastopore lip and tailbud. Top row: *Xnot2* whole-mount in situ hybridizations. Bottom row: *Xbra* hybridizations. (A, A') Stage 10; early gastrula; (B, B') stage 12, late gastrula; (C, C') stage 22, early tailbud; (D, D') stage 26; (E, E') stage 29; (F, F') stage 31 tailbud embryo. (A, A', B, B') Vegetal views of the blastopore lip; (C-F, C'-F') side views of the embryonic tailbud. Expression of *Xnot2* is restricted to the prospective notochord and dorsal lip (A, B), whereas *Xbra* is expressed in the prospective notochord and the entire circumblastoporal region (A', B'). In the tailbud, *Xbra* (C'-F') stains a larger region than does *Xnot2* (C-F). Arrow indicates dorsal lip. Arrowhead in E' indicates the neurenteric canal; note that *Xbra* marks its posterior wall.
Spemann's organizer in the *Xenopus* tailbud

Fig. 3. *Xnot2* and *Xbra* are expressed in distinct regions of the tailbud. Embryos were hybridized with *Xnot 2* (A,B,C) or *Xbra* (A’,B’,C’) antisense RNA, developed with alkaline phosphatase, embedded in paraffin wax and sectioned. (A,A’) Stage 23, sagittal section; (B,B’) stage 28, sagittal section; (C, C’) stage 31, frontal section. (A”,B”,C”) Schematic drawings indicating the anatomy of the region based on analysis of serial sections. Note that *Xnot2* is expressed in the chordoneural hinge (anterior wall of the neurenteric canal) and ventral spinal cord. *Xbra*, in addition to the chordoneural hinge and notochord, is expressed in the posterior wall of the neurenteric canal and in cells of the roof of the spinal cord (arrowheads). Note that the neurenteric canal is visible in the frontal sections (C).
mentioned briefly in the literature (Kerr, 1919; Pasteels, 1943; Nieuwkoop and Faber, 1967; Balinsky, 1981), it has not received much attention from embryologists in recent times. What is the origin of this intriguing structure? The formation of the neurenteric canal in *Xenopus* can be followed by simply observing dechorionated neurulae. During the neural plate stage, the blastopore becomes elongated forming a narrow slit. As shown in Fig. 5, cells of the circumblastoporal collar (which are *Xbra*-positive) rise up on either side of the central portion of the slit (lateral lips) and eventually fuse in the dorsal midline. As a result, a canal is formed that is open anteriorly at the neural plate and posteriorly at the future anus (Fig. 5B,C). In a second set of movements, the neural folds rise and form the neural tube, enclosing the anterior opening of the neurenteric canal, but not the posterior opening (Fig. 5A). In this way, the lumen of the spinal cord (also called the ependymal canal) becomes connected to the anus via the neurenteric canal. Thus, the posterior wall of the neurenteric canal is formed from *Xbra*-positive cells of the lateral portion of the circumblastoporal collar (Fig. 3B'). While this description does not differ significantly from those provided for *Rana* by previous workers (Kerr, 1919; Pasteels, 1943), it seemed useful to review it here as the process of formation of the
Fig. 6. Fate map of the late blastopore in *Xenopus*. (A) Diagram of morphogenetic movements at mid-gastrula (stage 11). (B) Morphogenetic movements at late gastrula/early neurula (stage 13), note that involution stops and that both ectoderm and mesoderm of the late dorsal lip move towards the posterior. (C) Experimental design of the Dil injection experiments: blastopore lips were marked at stage 13 at dorsal, lateral or ventral sites as shown, and analyzed at stage 33. (D) Dorsal injection, epifluorescence; (D') same embryo in brightfield optics. (E, E') Lateral lip injection. (F, F') Ventral lip injection. (G) Histological section of dorsally injected embryo, the notochord and spinal cord are labelled. The Dil lineage tracer was photo-oxidized before embedding and sectioning, melanin pigment is found in the skin layer. CNH (chordoneural hinge), LPM (lateral plate mesoderm), No (notochord), PAG (postanal gut), PW (posterior wall), Sc (spinal cord), So (somite).
neurenteric canal plays a central role in the development of the postanal tail.

While we found no evidence in the literature for the existence of a neurenteric canal in the chick embryo, nor is it mentioned in the most detailed mouse embryology atlas (Kaufman, 1992), the neurenteric canal probably represents an ancestral chordate feature, for it is present in Amphioxus (Hopper and Hart, 1985), shark (Kingsbury, 1932), amphibian (Nieuwkoop and Faber, 1967), gecko (Kerr, 1919), turtle (Yntema, 1968) and human embryos (Moore, 1977). The neurenteric canal is of interest in medical embryology since alterations in its development may lead to the formation of neurenteric cysts (e.g., intestinal cysts in the spinal cord sometimes accompanied by ventral spina bifida) (Rhaney and Barclay, 1959; Mackenzie and Gilbert, 1991).

The fate map of the late blastopore lip

The fact that Xnot2 and Xbra expression can be followed continuously from the blastopore to the tail raises the question of whether these regions are related by descent. To answer this, we mapped the fate of the blastopore lip by lineage tracing with the fluorescent carbocyanine dye DiI. After microinjection into embryonic tissues this lipophilic dye intercalates in the cell membrane, marking small groups of cells (Selleck and Stern, 1991; Izpisúa-Belmonte et al., 1993).

We initially marked dorsal blastoporal lips of mid to late gastrulae (stages 11-12) and found that the lineage tracer did not mark the tailbud (at stage 33), but instead marked the mesoderm of the trunk. This indicated that involution movements (Fig. 6A) are the main activity taking place at the dorsal lip even as late as stage 12. However, when we marked blastoporal lips at early neurula (stage 13), just as the blastopore starts to elongate, labelling of the tailbud proper was consistently observed. Our interpretation of these results is that at stage 13 an important change takes place in the movements of cell layers at the blastopore: the ectodermal and mesodermal cell layers stop involuting, attach to each other, and move together towards the posterior of the embryo (Fig. 6B). This is in keeping with current ideas concerning the involution of the marginal zone during gastrulation (Keller, 1991; Keller et al., 1991; Gerhart et al., 1991), except that we now determined experimentally that in Xenopus involution stops at the beginning of stage 13.

As shown in Fig. 6C, small amounts of DiI were injected in dorsal, lateral or ventral positions of the blastopore and then the embryos were analyzed at the late tailbud stage. Dorsal injections marked a resident cell population at the tip of the tailbud (74%, n=73 embryos), as well as a line of cells extending along the midline (Fig. 6D). The cells detected at the tip of the tail in these injections correspond to the chordoneural hinge and when the DiI was photo-oxidized...
Fig. 8. Tail organizer activity persists in the chordoneural hinge. All embryos shown here result from the transplantation of tissue fragments into the blastocoele of early gastrulae by the Finstock procedure. (A) Chordoneural hinge (bottom embryo) and prechordal plate (middle embryo) grafts from early tailbud (stage 25) embryos and a sham-operated embryo (top embryo) are shown. (B) Contribution of FDA-labelled chordoneural hinge to the induced secondary tail. The graft formed only a small part of the induced tail; by histological analysis the labelled cells formed chordoneural hinge, notochord and some neural tissue (not shown). (C) Low power view of the same embryo shown in B, showing the orientation of the secondary tail with respect to the host embryo. Note the presence of well-formed dorsal and ventral fins in the secondary tail. (D) Section of a secondary tail resulting from implantation of the tip of the tail of a 2-day tadpole (stage 35); note the presence of notochord (No), muscle blocks (Mu) and neural tube (NT). (E) Graft of the tip of the tail of an unlabelled donor into a host embryo whose cytoplasm was labelled with rhodamine dextran; the majority of the induced tail structures, in particular the notochord and muscle, are red and therefore derived from the host. The tip of the secondary tail (outline indicated with arrowheads), and the neural tissue are unlabelled and presumably derived from the graft. The histology of the region indicated by brackets of the same embryo is shown in D. (F) Graft of a fragment of rhodamine dextran marked neural tissue obtained from the hindbrain of a stage 30 embryo; (G) Histological section through a similar graft. Hindbrain grafts fail to induce structures. (G) Implanted rhodamine dextran-labelled notochord (stage 30), from the middle third of the embryo and of similar size to the implant in D; (G') histological section through a similar graft. Notochord does not induce significant structures in the Einstock assay. Cg, cement gland; CNH, chordoneural hinge; DF, dorsal fin; VF, ventral fin; 1°, primary tail; 2°, secondary tail; Ne, neural tissue; No, notochord.
formation, we labelled late blastoporal lips at dorsal (bracket in Fig. 6E). These lateral injections labelled the from regions of the chordoneural hinge that were not marked with DiI. This pattern of interspersed cells is diagnostic of discontinuity with the posterior wall (see diagram in Fig. 3C). This suggests that in Xenopus, the Xbra-positive cells of the posterior wall may be somitic precursors.

Lateral lip injections also labelled a row of cells spanning the region between the tailbud and the anus (Fig. 6E). As shown in Fig. 6F, a similar population was stained by ventral lip marks (64%, n=14). After photo-oxidation and sectioning, the label was found in cells surrounding the posterior gut (the so-called postanal gut); these cells reflect the extensive stretching that the blastopore remains undergo as the tail tip and the anus grow apart from each other (Fig. 3B’). In addition, ventral lip cells gave rise to a more diffuse population of cells (Fig. 6F) (64%, n=14) that were found to correspond to lateral plate mesoderm by histology. The ventral lip did not contribute to the tailbud proper.

From this fate map of the late blastopore we conclude that: (1) the dorsal lip becomes the chordoneural hinge and gives rise to tail notochord and ventral spinal cord, (2) the lateral lip gives rise mostly to somite precursor cells, and (3) the ventral lip gives rise to lateral plate mesoderm as well as to the postanal gut that stretches from the anus to the tailbud.

**Cell intercalation continues at late stages of tail development**

The main mechanism that drives involution of mesodermal cells through the blastopore lip (Fig. 6A) is convergence and extension (Keller and Danilchik, 1988; Keller et al., 1991; Shih and Keller, 1992). As cells converge on the dorsal midline (both in the mesodermal and the ectodermal layers) they intercalate with each other, resulting in an elongation that is the main engine of gastrulation movements. To investigate whether such movements continue during tail formation, we labelled late blastoporal lips at dorsal (n=47) and lateral (n=24) positions and allowed the embryos to develop until the swimming tadpoles had well-developed tails and were 6.5 mm long.

Fig. 7A shows a tail from a dorsal blastopore injection. Stained cells are distributed throughout the notochord, extending to the tip of the tail. In the posterior notochord, labelled cells are interspersed with unlabelled cells derived from regions of the chordoneural hinge that were not marked with Dil. This pattern of interspersed cells is diagnostic of cell intercalation in Xenopus (e.g., Niehrs and De Robertis, 1991). Thus, intercalation movements continue even at late stages of tail development. It is not known to what extent this late cell intercalation may contribute as a mechanism of tail elongation.

Fig. 7B shows a tail of an embryo that was injected in the lateral lip. A row of muscle cells, spanning the chevron-shaped somites, is labelled. The cells become increasing longer in the older, more anterior somites. This lengthening of the muscle segments could provide a second mechanism which might contribute to tail elongation in frogs, as has been proposed by others (Elsdale and Davidson, 1983).

Further studies will be required to investigate the mechanism by which the Xenopus tail elongates in the relatively short period of two or three days. However, from the results shown in Fig. 7A, we can conclude that the intercalation movements that occur at gastrulation continue during tail formation.

**The chordoneural hinge and the tip of the tail retain organizer activity**

The dorsal lip of the blastopore has potent inducing activities; the early dorsal lip acts as a ‘head organizer’ while the late dorsal lip of the gastrula behaves as a ‘trunk-tail organizer’ (Spemann, 1931; Hamburger, 1988). Since the chordoneural hinge is derived from the dorsal lip, we asked whether it retains organizer activity. The morphogenetic potential of tissue fragments can be tested by implanting them into the blastocoele of a Xenopus gastrula by the Einsteck procedure of Mangold (Ruiz i Altaba and Melton, 1989; Cho et al., 1991b).

In order to test the persistence of organizer activity through late stages of development, two types of grafts were performed. First, the inducing potential of the chordoneural hinge from tailbuds was tested. This region can be excised with some precision because we realized that the neurenteric canal is visible as a black spot at the site at which it opens into the roof of the gut cavity, about 1 mm from the anus at stage 25 (Fig. 4D). Second, to test whether the inducing activity persists at stages in which the tail is already formed, stage 35 tail tips (see Fig. 1A) were dissected and transplanted. At this late stage, it is no longer possible to dissect the hinge region from the posterior wall because the neurenteric canal, a transient structure, collapses at stage 35 of Xenopus development (Nieuwkoop and Faber, 1967).

Fig. 8A, bottom embryo, shows that when the chordoneural hinge (i.e., a small fragment of apparently undifferentiated tissue just anterior to the neurenteric canal, of approximately the same width as the notochord) was implanted into a host gastrula, tail-like structures resulted (in 13 out of 14 grafts). In contrast, implants of prechordal plate from the same stage embryos produced structures with anterior characteristics (in 7 out of 8 cases), such as cement glands, instead of tails (Fig. 8A, middle embryo). When stage 35 tail tips were tested, the embryos developed tail-like structures (in 19 out of 27 grafts), despite the very small size of the implant. The structures formed by both types of implants are typical tails, as indicated by the presence of dorsal and ventral fins (Fig. 8B,C) and of well-organized notochords flanked by paired muscle blocks and neural tissue (Fig. 8D).

The fundamental property of the organizer is that it is able to recruit cells from the host into a twinned axis (Spemann and Mangold, 1924). In order to determine whether the grafts were able to induce host tissues to form axial structures, two types of lineage-traced transplantation experiments were performed. When the donor chordoneural hinge was uniformly labelled with fluorescein dextran (FDA, Gimlich and Braun, 1985), it contributed to only a small part of the secondary tail (Fig. 8B,C), which consisted of chor-
doneural hinge, notochord and some neural tissue by histological analysis. A similar result was obtained when the late dorsal lip was traced by DiI injection (Fig. 6D,G); thus, two independent lines of evidence suggest that the chordoneural hinge forms the dorsal midline of the tail.

To perform the reciprocal experiment, host embryos were labelled with rhodamine dextran and grafted with unlabelled stage 35 tail tips. As shown in Fig. 8E, the secondary axis consists mostly of cells derived from the host which are labelled in red. Induced cells include notochord and muscle blocks (compare the region indicated in brackets in Fig. 8E with the histological section of the same embryo in 8D), while the tip of the secondary tail (arrowheads in Fig. 8E) and the neural tube are mostly unlabelled and presumably derive from the graft. It is of interest that the notochord in the secondary tails can derive from both the host (Fig. 8E) and the graft (Fig. 8B), because organizer tissue can induce the formation of notochord in neighboring cells (Stewart and Gerhart, 1991) as well as self-differentiating into notochord (Hamburger, 1988). In control experiments (see Materials and Methods), embryos that were sham operated or implanted with similarly sized fragments of hindbrain or notochord (Fig. 8F, F′, G, G′), or stage 8 animal caps (not shown) failed to induce tail structures.

We conclude from these grafting experiments that the chordoneural hinge and the tip of the tail, even at late stages of development, retain Spemann’s tail organizer activity.

DISCUSSION

The main conclusion that emerges from these studies is that tail development in Xenopus results from a continuation of cell movements initiated during gastrulation that persist until late stages of development. This conclusion rests on three lines of evidence. First, the tailbud is made up of cells that are related by lineage to specific regions of the late blastoporal lip. Second, organizer activity, present in the dorsal lip of the gastrula, is also found in the tip of the tail. Third, gene markers expressed in different regions of the blastoporal lip can be traced to specific cell populations within the apparently homogeneous tailbud. This view of tail development as a continuation of gastrulation is in stark contrast with the prevailing idea that the tailbud in the vertebrate embryo is a homogenous proliferating blastema that gives rise to all tissues of the tail (Griffith et al., 1992).

Gene markers reveal heterogeneity of the tailbud

The multiplicity of cell populations in the apparently homogeneous tailbud was revealed by the gene markers Xnot2 and Xbra. Xnot2, which is initially expressed in the dorsal lip, marks the notochord, the ventral half of the spinal cord and the region of continuity between these two tissues. This region, the chordoneural hinge, forms the anterior wall of the neurenteric canal. A population of Xbra-positive, Xnot2-negative cells forms the posterior wall of the neurenteric canal, and this mass of cells is continuous laterally and ventrally with the differentiating somites (Fig. 3C). This cell population is derived from the lateral lip of the late blastopore and may represent a reservoir of mesodermal cells that will go on to differentiate into the somites of the tail. It is possible that the transcription factor encoded by Xbra participates directly in this process: Xbra is expressed in the entire prospective mesoderm and then is turned off as the somites are formed (Smith et al., 1991), and microinjection of Xbra mRNA can switch on muscle differentiation in Xenopus animal caps (Cunliffe and Smith, 1992).

The presence of a population of somitic precursor cells in the posterior of the neurenteric canal might help explain one of the old riddles of amphibian embryology. It has long been known that the posterior part of the neural plate gives rise to tail somites, rather than to spinal cord as might have been expected (Bijtel, 1936; Spofford, 1948; Woodland and Jones, 1988). As can be seen in the embryo shown in Fig. 5C, the dorsal opening (indicated by an arrow) of the neurenteric canal is more anterior than the anal opening (arrowhead). The chordoneural hinge extends only to the anterior limit of the dorsal opening of the neurenteric canal at this stage (E. D. R., unpublished), while all tissues posterior to this are derived from the lateral lips (Fig. 5B). Thus, although the posterior part of the neurula appears to be part of the neural plate, this region is in fact posterior to the chordoneural hinge and consists of posterior wall cells, derived from the lateral lips, destined to form predominantly somites.

The fate map of the late blastoporal lip

It may seem curious that the lineage of the late blastopore lip has not been previously determined, after so many years of intensive research in amphibians. In order to trace this lineage, we had to first determine the stage at which invo- lution of the marginal zone is completed. Not until the blastopore starts to elongate at stage 13, can one mark cells that will remain as resident cell populations in the developing tailbud. By labelling with DiI we showed that: (1) the dorsal lip gives rise to the chordoneural hinge, the ventral spinal cord, and notochord, (2) the lateral lip gives rise to the posterior wall and somites, and (3) the ventral lip gives rise to the lateral plate mesoderm and the stretch of postanal gut spanning the region between the anus and the tailbud. Thus, different parts of the blastoporal lip give rise to different parts of the tail. The interspersion of labelled and unlabelled cells in the notochord region (Fig. 7A) indicates that cell intercalation continues at least until the swimming tadpole stage. This is of interest because cell intercalation along the dorsal midline starts much earlier in development and is considered one of the main driving forces of gastrulation movements (Shih and Keller, 1992; Keller et al., 1991).

Organizing the Xenopus tail

The transplantation experiments described here showed that the chordoneural hinge and the tip of the tail, which descend from the dorsal lip of the late gastrula, retain tail-organizer activity at late stages of development. It is worth noting that these structures have the ability to induce the formation of notochord in the host, a property of organizer tissue (Stewart and Gerhart, 1991). The current working model of Xenopus mesoderm induction is that a dorsalizing signal emanates from the gastrula organizer, patterning the formation of muscle blocks in the lateral marginal zone (Smith and Slack,
1983; Slack, 1991; Kimelman et al., 1992; Sive, 1993). The presence of a cell population with strong dorsal inducing activity in the late embryo raises the possibility that dorsal signalling by the organizer might continue much later than previously thought, perhaps inducing the somites of the tail.

It should be stated that already in 1943 Pasteels suggested that the chordoneural hinge should descend from the dorsal lip. His evidence was purely histological, but based on detailed analyses of mitotic indices and anatomy, he argued that the tailbud was not an undifferentiated blastema (Holmdahl, 1925; Griffith et al., 1992) and our results vindicate his view.

It could be argued that the Xenopus tailbud still might contain a small population of undifferentiated cells that are not stained by Noto2 and Xbra and which by proliferation might give rise to the tail. This view is contradicted by the lineage tracing results, which show that the different components of the tail in fact derive from different regions of the late blastopore lip, which is composed of the Xbra-positive population of cells forming the circumblastoporal collar. Thus, in the course of normal development, the bulk of the tail results from the continuation of the morphogenetic movements of the late blastopore.

However, this can not be the only way of building a tail, since a new tail can be formed by transplantation of the chordoneural hinge (Fig. 8) or by regeneration. If the tail of a Xenopus tadpole is severed (which removes the chordoneural hinge), a fully differentiated tail will regenerate (Deuchar, 1975). It is not known whether a pluripotent blastema is formed, or whether different tissues of the tail contribute distinct precursor cells to the regenerate, but this is under investigation. Initial experiments indicate that both Noto2 and Xbra are re-expressed at the tip of regenerating tails (L. K. G. and E. D. R., unpublished results); determining whether they are expressed in different cell populations will require analysis by double-labelled in situ hybridization.

Gastrulation movements and the formation of the tail

Three different cell populations, which can be distinguished on the basis of their morphogenetic movements, are found in the Xenopus dorsal lip during development. First, a population of deep migratory cells invaginates from the dorsal lip, driven anteriorly by crawling movements (Keller, 1976; Winklbauer, 1990) and forms the prechordal plate. It is this cell population that expresses the homeobox gene goosecoid (Cho et al., 1991a; Niehrs et al., 1993) and presumably corresponds to Spemann’s head organizer (Spemann, 1931; Gerhart et al., 1991). Second, the marginal zone layer, driven by convergence-extension movements (Keller and Danilchik, 1988; Keller et al., 1991; Shih and Keller, 1992), involutes through the dorsal lip and forms the notochord and somites of the trunk. These involuting cells presumably correspond to Spemann’s trunk organizer (Spemann and Mangold, 1924; Spemann, 1931; Hamburg, 1988). Third, when involution ceases the dorsal lip becomes the chordoneural hinge which, perhaps driven by continuing cell intercalation movements, moves toward the posterior (Fig. 6B). This posteriorly moving, non-involuting hinge contains the cells that maintain organizer activity in the present set of transplantation experiments (Fig. 8), and is presumed to be responsible for the development of the tail proper. This late, posterior movement of the chordoneural hinge in Xenopus may be considered homologous to the regression of the late Hensen’s node in the chick embryo (Spratt, 1955; Bellairs, 1986).

In addition to Noto2 and Brachyury, other Xenopus genes, some with potent biological activities, are expressed in the developing tailbud: FGFRs (Isaacs et al., 1992; Tannahill et al., 1992), Xwnt3A (Wolda et al., 1992), integrin α3 (Whittaker and DeSimone, 1993), Xcad1 and 2 (H. S. and E. D. R., unpublished) and XRAR-γ2 (P. Pfeffer and E. D. R., unpublished data). The availability of these markers should permit rapid progress in the analysis of how the vertebrate tail develops.

The main conclusion from this work is that the dorsal lip of the late blastopore, Spemann’s late organizer, can be traced to the tip of the tail. Tail development might be considered unusual in Xenopus because the tadpole must swim only three days after fertilization, and this temporal constraint is not expected to apply to all vertebrate embryos. However, given that the mechanisms of development are highly conserved, it does not seems unlikely to us that the general principle that the tail is formed as a continuation of gastrulation will apply to all chordates.

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