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The Expression of Brachyury (T) during Gastrulation in the Marsupial Frog *Gastrotheca riobambae*

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Gastrulation in the marsupial frog *Gastrotheca riobambae* has been analyzed by the distribution of the Brachyury (T) protein. Comparison with other amphibians provides mechanistic insights, since *G. riobambae* develops slowly and has the most divergent mode of amphibian gastrulation, producing an embryonic disk. The T pattern indicates that the prospective mesoderm is superficial, as in many amphibians. The dorsal blastopore lip could not be identified by the expression of *T*, or by morphological criteria, thus it is unknown whether *Gastrotheca* embryos have a dorsal organizer before or after blastopore closure. The circumblastoporal and notochordal expression of *T*, which are temporally contiguous in *Xenopus*, are separated in *Gastrotheca*, implying that distinct regulatory mechanisms may control the expression of *T* in its two domains. The separation of the T pattern also indicates that involution at the blastopore is separate from notochord formation. In addition, extension of the archenteron and notochord occurs after blastopore closure, suggesting that dorsal convergence and extension have been delayed until after blastopore closure. Therefore, dorsal convergence and extension need not be the cause of blastopore closure in *Gastrotheca*. The separation of gastrulation events in embryos that have not been experimentally manipulated, such as those of *Gastrotheca*, helps in understanding the distinct nature of gastrulation processes.

INTRODUCTION

In which way and to what extent the insights into developmental mechanisms gained in one vertebrate animal are applicable to other vertebrates are questions of central importance, because much recent progress comes from the study of development in a rather small number of vertebrates, such as the frog *Xenopus laevis*, and the mouse (reviewed in Bolker, 1995). Although the morphology of early development differs among animals, all vertebrates reach a stage of similarity (reviewed in Elinson, 1987; Duboule, 1994). This stage, illustrated by Haeckel (1877), was named the pharyngula by Ballard (1976, 1981). It is characterized by bilateral symmetry, pharyngeal segments, and an arrangement of organ primordia typical of the vertebrates (Ballard, 1976). The pharyngula is the phylotypic stage (as defined by Sander, 1983) of vertebrates. Molecular studies have disclosed further developmental similarities among different animals (reviewed in Beddington and Smith, 1993; De Robertis *et al.*, 1994; Fietz *et al.*, 1994; François and Bier, 1995; Hinchliffe, 1994; Hogan, 1995; Krumlauf, 1994; Morgan and Tabin, 1994; and Slack *et al.*, 1993).

Although at the pharyngula stage the marsupial frog *Gastrotheca riobambae* resembles the basic vertebrate body plan, there are significant differences with *Xenopus* in reproductive strategies, oogenesis, early development, gastrulation, and somitogenesis (reviewed in del Pino, 1989; Gatherer and del Pino, 1992). *Gastrotheca* reproduces on land, and its large eggs (3–4 mm in diameter) develop inside a dorsal pouch of the mother, requiring 16 hr for first cleavage and 14 days to complete gastrulation (del Pino and Loor-Vela, 1990; del Pino, 1989). In contrast, *Xenopus* has aquatic reproduction with eggs and tadpoles developing rapidly in the water.

The slowly developing embryos of *Gastrotheca* may allow identification of gastrulation processes more easily than in the rapidly developing embryos of *Xenopus*, providing insights into the mechanisms of amphibian gastrulation. *Gastrotheca* has the most divergent mode of gastrulation among amphibians, producing an embryonic disk (del Pino and Elinson, 1983; Elinson and del Pino, 1985). This gastrulation pattern was analyzed further by whole mount immunostaining with an antibody against the protein T (Brachyury). T acts as a transcription factor (Cunliffe and Smith, 1994; Holland *et al.*, 1995; Schulte-Merker *et al.*, 1992; Kispert and Herrmann, 1993, 1994), expressed in the

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prospective mesoderm and in the notochord of vertebrates (reviewed in Beddington and Smith, 1993; and Yamada, 1994) and amphioxus (Holland *et al.*, 1995). *T* expression is required for gastrulation movements in the mouse (Wilson *et al.*, 1995), and it is believed to play a role in convergence and extension movements of gastrulation in *Xenopus* (reviewed in Yamada, 1994). In *Xenopus, Xbra* expression is an immediate early response to mesoderm induction by FGF, giving a transient signal in the prospective mesoderm of the early gastrula (Smith *et al.*, 1991). At midgastrula *Xbra* is expressed also in the elongating dorsal region. *Xbra* expression becomes restricted to the notochord and posterior mesoderm at later stages (Smith *et al.*, 1991; Gont *et al.*, 1993). *Xbra* is expressed in response to activin or FGF (reviewed in Smith, 1995), but only activin induces the notochord, while FGF has no such effect (Green *et al.*, 1990; Cunliffe and Smith, 1994; O'Reilly *et al.*, 1995). To facilitate comparison of the T-protein expression patterns, the process of gastrulation in *Gastrotheca* will be briefly characterized.

MATERIALS AND METHODS

Embryos and Fixation Procedures

Embryos of *Gastrotheca riobambae* were obtained from spontaneous matings in captivity (Elinson *et al.*, 1990). Embryos were withdrawn from the maternal pouch and were observed in *G. riobambae* saline solution with 30 m*M* urea (GRS) (del Pino *et al.*, 1994). Egg jelly was removed with 2% cysteine hydrochloride, pH 7.8, in GRS for 10 min followed by several washes in GRS. The vitelline envelope was removed with forceps. Embryos were fixed in Dent's fixative (Dent *et al.*, 1989) for 2 hr at room temperature and overnight at −20°C, followed by a 20-hr wash in methanol at −20°C. Embryos were warmed to room temperature, incubated in 50% methanol/50% xylene for 10 min and for 30 min in 100% xylene (Coutinho *et al.*, 1992), followed by 4 washes of 5 min each in methanol, at room temperature. Removal of lipids by xylene enhances the immunostaining signal of *Gastrotheca* embryos. After lipid extraction, embryos were stored in methanol at −20°C. Staging was according to del Pino and Escobar (1981). Embryos of *Xenopus* were used as control.

Whole Mount Immunocytochemistry

Embryos of *Gastrotheca* were immunostained according to Hemmati-Brivanlou and Harland (1989) with the following modifications: embryos were incubated for 2 to 3 days at 4°C with a rabbit polyclonal antibody against the T-protein, diluted 1:1,000 (Kispert and Herrmann, 1994, kindly donated by B. Herrmann). As secondary antibody, sheep anti-rabbit IgG conjugated to alkaline phosphatase (Boehringer Mannheim) was used. Embryos were incubated overnight with the secondary antibody, preabsorbed previously with tadpole powder, and diluted 1:500. Embryos were washed extensively over a period of about 12 to 24 hr at 4°C, after incubation with each antibody. A strong signal was visible after a few minutes to 1 hr of incubation in the color reagent, prepared according to instructions of the manufacturer (Protoblot NBT and BCIP color development system, Promega). Embryos were washed in PBS and fixed in 4% formalin in PBS. The entire gastrula, of 3 to 4 mm in diameter, was cleared in benzyl benzoate:benzyl alcohol (2:1), while at more advanced stages, the embryonic disk was dissected from the segmented yolk. The embryonic disk was maintained flat during clearing by slight pressure with a hair loop. Embryos were observed with an Axiophot (Zeiss) and photographed with Ektapress Gold 100 professional film (Kodak). Afterward, embryos were rinsed with methanol and with PBS–Tween and stored in glycerol at −20°C.

Tadpole powder was prepared by homogenizing *Gastrotheca* tadpoles in PBS, after removal of the intestine. The homogenate was extracted twice for 10 min with 4 volumes of cold acetone at −20°C, followed by centrifugation at 10,000*g* for 10 min. The pellet was dried and the powder was stored at 4°C. A small amount of tadpole powder was inactivated by incubation at 70°C for 30 min in PBS, with 2 mg/ml of bovine serum albumin and 0.1% Triton X-100. Sheep serum (20%) was added before dilution of the secondary antibody. Tadpole powder was removed by centrifugation.

Histological Method

Anti-T immunostained embryos were sectioned with an Oxford Vibratome according to Ding *et al.* (1993), with modification. Embryos were postfixed in 10% formalin and 0.2% glutaraldehyde in PBS for 3–12 hr at 4°C, followed by extensive washes in PBS. Embryos were embedded in 3% low melting agarose in PBS. A block of agarose, containing the specimen, was affixed with instant glue to a specimen holder, taking care that the embryo becomes glued to the holder. Sections of 20–30 μm in thickness were produced at a speed of 2.5 and an amplitude of 6. The instrument tank was filled with distilled water for collecting sections. Sections were transferred to microscope slides and dried in air. Coverslips were attached to slides with immersion oil. The slides were analyzed and photographed with an Axiophot (Zeiss).

RESULTS

The T Pattern in the Early Gastrula

Figure 1 summarizes the process of gastrulation in *Gastrotheca* and the results of immunocytochemical determination of the *T* expression pattern, some aspects of which are further illustrated in Figs. 2 and 3. The T signal was not detected in the cleavage stage or blastula of *Gastrotheca* (stages 5 and 6), but could be recognized in the gastrula and subsequent stages by strong nuclear staining. In the faint blastopore of the early gastrula (stage 7), there is a ring of T-positive nuclei of several cell diameters in width, suggesting that the marginal zone contains the prospective mesoderm (Figs. 1A, 1A′, and 2A). A cross section of embryos at this stage indicate that T-positive cells are located in the surface layer (Fig. 2B), but some T-positive cells are internal (not shown). A similar pattern was detected in embryos with a smaller blastopore (Figs. 1B, 1B′, and 2C), and is maintained until blastopore closure. The region that surrounds the blastopore contains only T-positive cells, as indicated by the spacing of T-positive nuclei and by the cell outlines (Fig. 2C). The large amount of yolk and the autofluorescence of

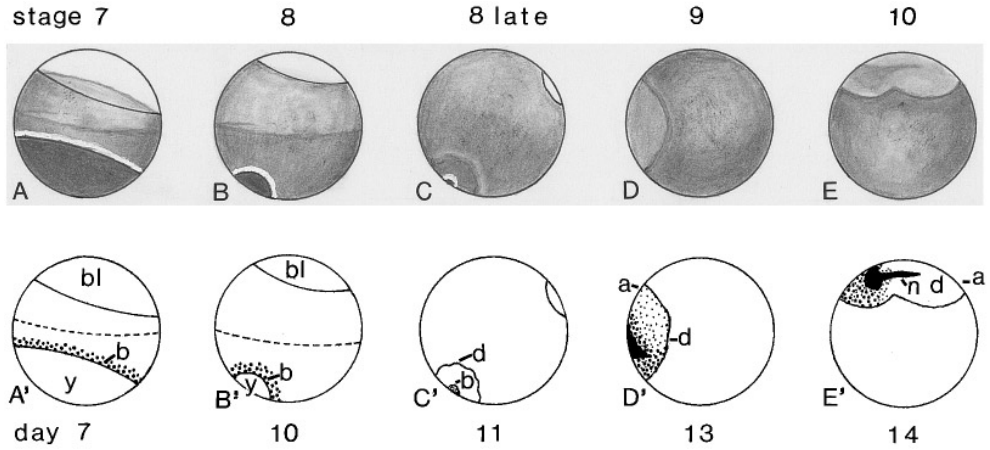


FIG. 1. The process of gastrulation in *Gastrotheca*. Top row (A–E): Drawings of the external morphology of embryos. Bottom row (A′–E′): Schematic drawings of the T-pattern in the *Gastrotheca* gastrula (indicated by stippling and solid black regions). Stages and age (in days from fertilization) are given. Embryos are oriented with the animal pole upward. (A, A′) The stage 7 embryo, early gastrula. (A) A faint blastopore (underlined in white) forms in the marginal zone of the embryo at a slight angle from the animal–vegetal axis. The blastocoele roof consists of one layer of cells, stretched and transparent due to extensive epiboly. Later, these cells cover the entire embryo. The first indication of gastrulation is the invasion of the transparent blastocoele roof by yolky cells that slide along the inner surface of the roof. Invasion of the blastocoele roof is more pronounced on one side. The dorsal blastopore lip and the future dorsal side of the embryo cannot be identified in living embryos of this stage. (A′) T-positive cell nuclei occur in the region of the blastopore lip. The blastocoele floor is signaled by a broken line in A′ and B′. (B, B′) The early stage 8 embryo. (B) The blastopore and yolk plug are smaller than at stage 7 (see A) and the roof of the blastocoele is almost completely invaded by yolky cells. (B′) T-positive cell nuclei are found around the blastopore lip. (C, C′) The late stage 8 embryo. (C) The blastopore is closed and the yolk plug has been retracted. An embryonic disk of small cells develops around the blastopore. Internally, there is a small archenteron, symmetric around the blastopore. The remnant of the blastocoele is visible. (C′) Faintly T-positive cell nuclei occur in deep regions of the closed blastopore. The borders of the embryonic disk are indicated by a solid line. (D, D′) The stage 9 embryo. (D) The archenteron and embryonic disk enlarge and the embryo slowly undergoes an upward rotation. With enlargement of the archenteron, the embryonic disk becomes thinner and somewhat translucent. The thickness of the embryonic disk changes from 10–15 cells when the disk is small to 4 cells in thickness once the archenteron expands (Elinson and del Pino, 1985). The blastocoele is totally obliterated by yolky cells. (D′) The embryonic disk is T-positive. The T signal is stronger (black region) in the area of the closed blastopore and dorsal side. The elongated T-positive area signals the central part of the embryonic disk as the dorsal side. Its tip, opposite the blastopore, marks the anterior region. (E, E′) The stage 10 embryo. (E) Rotation has been completed, and the archenteron and embryonic disk face upward. The embryonic disk has become translucent and the archenteron has enlarged considerably. The notochord can be observed as an elongated opaque region. With development of the notochord, the dorsal and anterior regions can be identified in living embryos. (E′) The T signal is strong and restricted to the notochord and tailbud (shown in black) and the surrounding area. Abbreviations: a, anterior; bl, blastocoele; b, blastopore; d, disk; n, notochord; y, yolk plug.

yolk platelets precluded confirmation of this observation by double staining with DAPI, a fluorescent DNA marker. The T pattern in the blastopore is transient, since after involution, the T signal disappears.

T Pattern in the Late Gastrula

When the blastopore closes, the superficial T pattern of the marginal zone disappears completely (advanced stage 8). Instead, nuclei of deep cells in the region of the closed blastopore become faintly stained with anti-T (Figs. 1C, 1C′, and 2D). This weak pattern could not be seen in embryo sections. However, when expansion of the archenteron takes place (stage 9), the T signal of cells located deeply in the embryonic disk becomes strong (Figs. 2E and 2F). There is a graded distribution of the T-protein, being strongest in the dorsal and closed blastopore regions and weakest toward the periphery of the embryonic disk (Fig. 2E). The region of the closed blastopore generates an elongated triangular T-positive region (Figs. 1D′ and 2E). The central T-positive portion of the embryonic disk marks the dorsal side. The tip of this T-positive region, opposite the blastopore, marks the anterior region of the embryo. In living embryos of *Gastrotheca*, the dorsal and anterior regions are indistinguishable at stage 9 (Fig. 1D), while in *Xenopus*, the dorsal side is marked by the dorsal blastopore lip at the onset of gastrulation. Since the dorsal side of *Gastrotheca* embryos could not be detected before blastopore closure, and the dorsal blastopore lip has not been identified by morphological criteria (reviewed in del Pino, 1989), it remains unresolved whether *Gastrotheca* embryos have a dorsal organizer before blastopore closure which has yet to be identified, or whether dorsalization in this species occurs at an unusually late stage in embryogenesis.

T Pattern in the Neurula and Later Stages

At stage 10, when the archenteron expands, the prospective notochord becomes visible in living embryos (Fig. 1E). The nascent notochord, the tailbud, and scattered cells of the tailbud are T-positive (Figs. 1E′ and 3A). In subsequent stages of *Gastrotheca* development, the growing notochord and the tailbud are T-positive (Figs. 3B and 3C). The notochord of a 4-somite embryo (stage 12) (Fig. 3B) is shorter than in a 7-somite embryo (stage 13) (Fig. 3C). In both cases, the entire length of the notochord and the tailbud are T-positive. At stage 15 (with 15 somites), only the caudal region of the notochord and the tailbud are T-positive (Fig. 3D). The T pattern in the notochord is equivalent to that of *Xenopus* (reviewed in De Robertis *et al.*, 1994). The region of the anus is outlined by T-positive cells (stage 13), (Fig. 3C). Sections of stage 10 embryos reveal T-positive cells mainly in the mesoderm, although T-positive cells occur in the ectoderm as well (Figs. 3E and 3F). The lining of the archenteron roof also contains T-positive cells in the region of the notochord (Fig. 3F).

DISCUSSION

Location of the Prospective Mesoderm in Gastrotheca

The similarity of *Xbra* and *T*-expression patterns in the marginal zone of *Xenopus* and *Gastrotheca* embryos, respectively, strongly suggests that the T-positive ring of the *Gastrotheca* gastrula marks the prospective mesoderm. However, definitive proof awaits cell lineage tracing studies. The *Gastrotheca* T-positive ring is superficial, indicating that the prospective mesoderm may be superficial also, in contrast with the deep location of the *Xbra* signal in *Xenopus* embryos (Smith *et al.*, 1991). The prospective mesoderm is located in the surface of the marginal zone in urodeles, cecilians, and many species of frogs (reviewed in Purcell and Keller, 1993) and even a fish, the white sturgeon (Bolker, 1993), and different from the deep prospective mesoderm of *Xenopus* (reviewed in Bolker, 1994; Keller, 1986). T-positive cells of the archenteron roof at the neurula stage of *Gastrotheca* embryos (Fig. 3F) may correspond to mesodermal cells ingressing to their destination in the notochord and other mesodermal fates, as occurs in the frog *Ceratophrys* and in the white sturgeon (Purcell and Keller, 1993; Bolker, 1993).

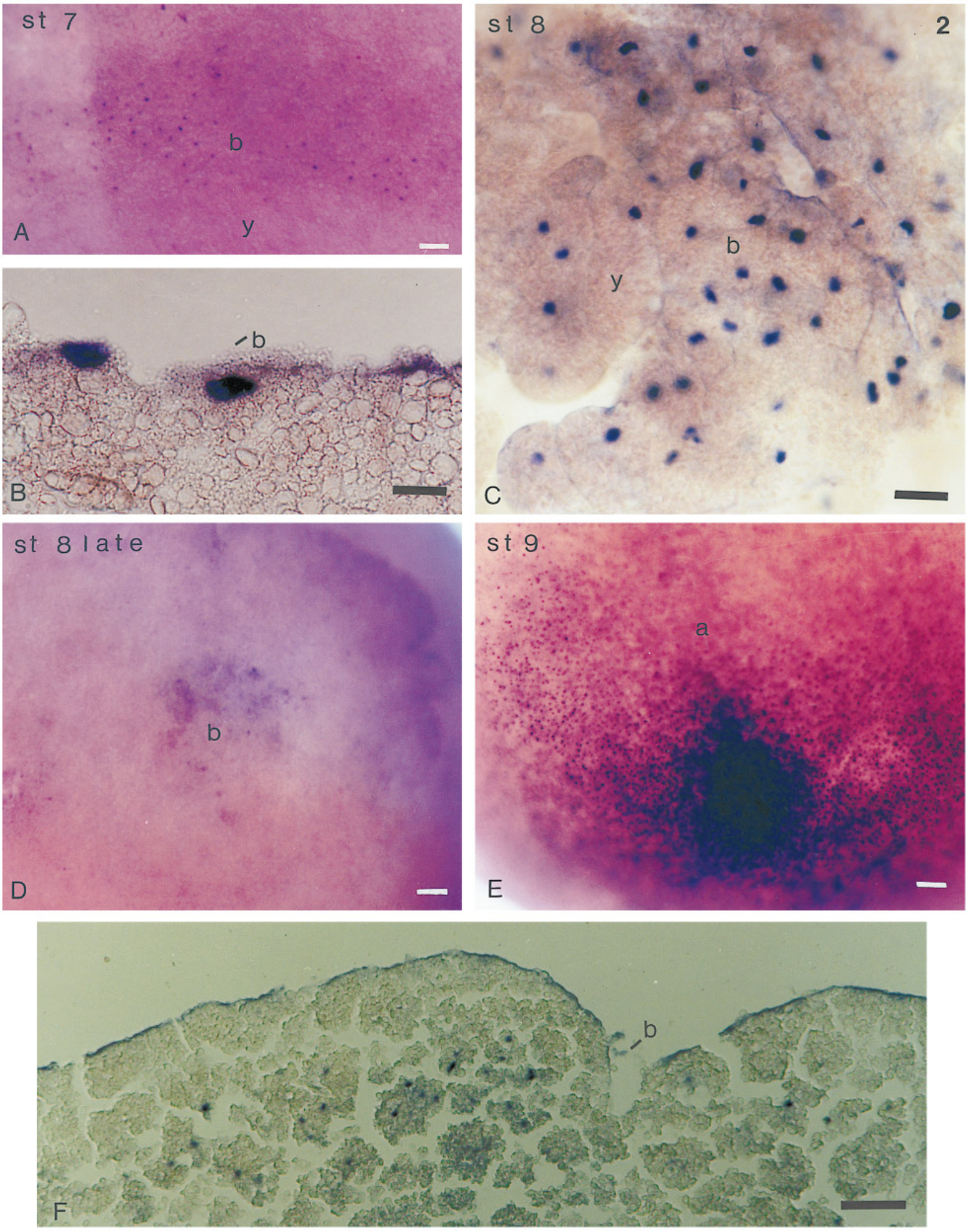
The distribution of the T-protein in embryos of *Gastrotheca* can be compared with the *Xbra* mRNA pattern of *Xenopus* embryos (Smith *et al.*, 1991; Gont *et al.*, 1993) because *Xbra* mRNA and protein have similar distribution patterns in *Xenopus* (Cunliffe and Smith, 1994). Similarly in *Gastrotheca*, the hybridization of embryos at the late gastrula stage and at more advanced stages with a *Xenopus* riboprobe against *Xbra* and the immunostaining with anti-T gave comparable patterns, the immunostaining signal being stronger (stages 10, 12, and 15; data not shown).

Separation of Gastrulation Events

The observations reported in this paper on the gastrulation process and the expression of *T* in naturally developing *Gastrotheca* embryos provide a clear example for separation of blastopore closure and convergence and extension as well as notochord formation. The fact that such complete separation does occur in an embryo that has not been experimentally manipulated is significant in understanding the distinct nature of these processes. Further, the circumblastoporal expression and notochordal expression of *T* (*Xbra*), which are temporally contiguous in *Xenopus* (Smith *et al.*, 1991; Gont *et al.*, 1993) but are separated in *Gastrotheca*, imply that distinct regulatory mechanisms may control the expression of *T* in its two domains.

Gastrotheca embryos may delay dorsal convergence and extension until after blastopore closure. The delay in archenteron and notochord extension which occur after blastopore closure, and the elongation of vital dye markings placed in the embryonic disk, particularly those located in the future dorsal region (del Pino and Elinson, 1983; Elinson

FIG. 2. The T pattern in the gastrula of *Gastrotheca*. The external surface faces upward in B and F. (A) Whole mount of a stage 7 embryo; the animal pole faces upward. T-positive cell nuclei were detected in the forming blastopore. There was no indication of the dorsal blastopore lip (compare with Figs. 1A and 1A′). (B) Cross section through the marginal zone of a stage 7 embryo. The T-positive nuclei belong to yolky cells of the marginal zone and are located in the surface of the embryo. (C) Ventral view of the blastopore in a stage 8 embryo with a small yolk plug. The region of the blastopore has been dissected from the large yolk mass. The blastopore lip is surrounded by T-positive cells. Some cells of the retracting yolk plug are also T-positive, although earlier the yolk plug was T-negative (see A). The blastopore of this embryo was smaller than in Figs. 1B and 1B′. (D) Ventral view of an advanced stage 8 embryo with a closed blastopore, seen in whole mount. There is a faint T-signal located in the internal nuclei of the closed blastopore region. The embryonic disk is forming (Compare with Figs. 1C and 1C′). (E) View of the expanding embryonic disk of a stage 9 embryo, focused on deep cells. The region of the closed blastopore is strongly T-positive and has a triangular shape. This region signals the dorsal side. Its apex signals the anterior region. The T signal is strong in the central region of the disk and faint toward the periphery, suggesting that the entire mesoderm mantle is transiently T-positive in *Gastrotheca* (compare with Fig. 1D and 1D′). (F) Cross section at the level of the closed blastopore of a stage 9 embryo. T-positive nuclei are restricted to deep cells of the embryonic disk. Bars correspond to 100 μm in A and D–F, 25 μm in B, and 50 μm in C. Abbreviations: a, anterior; b, blastopore region; st, stage; y, yolk plug.



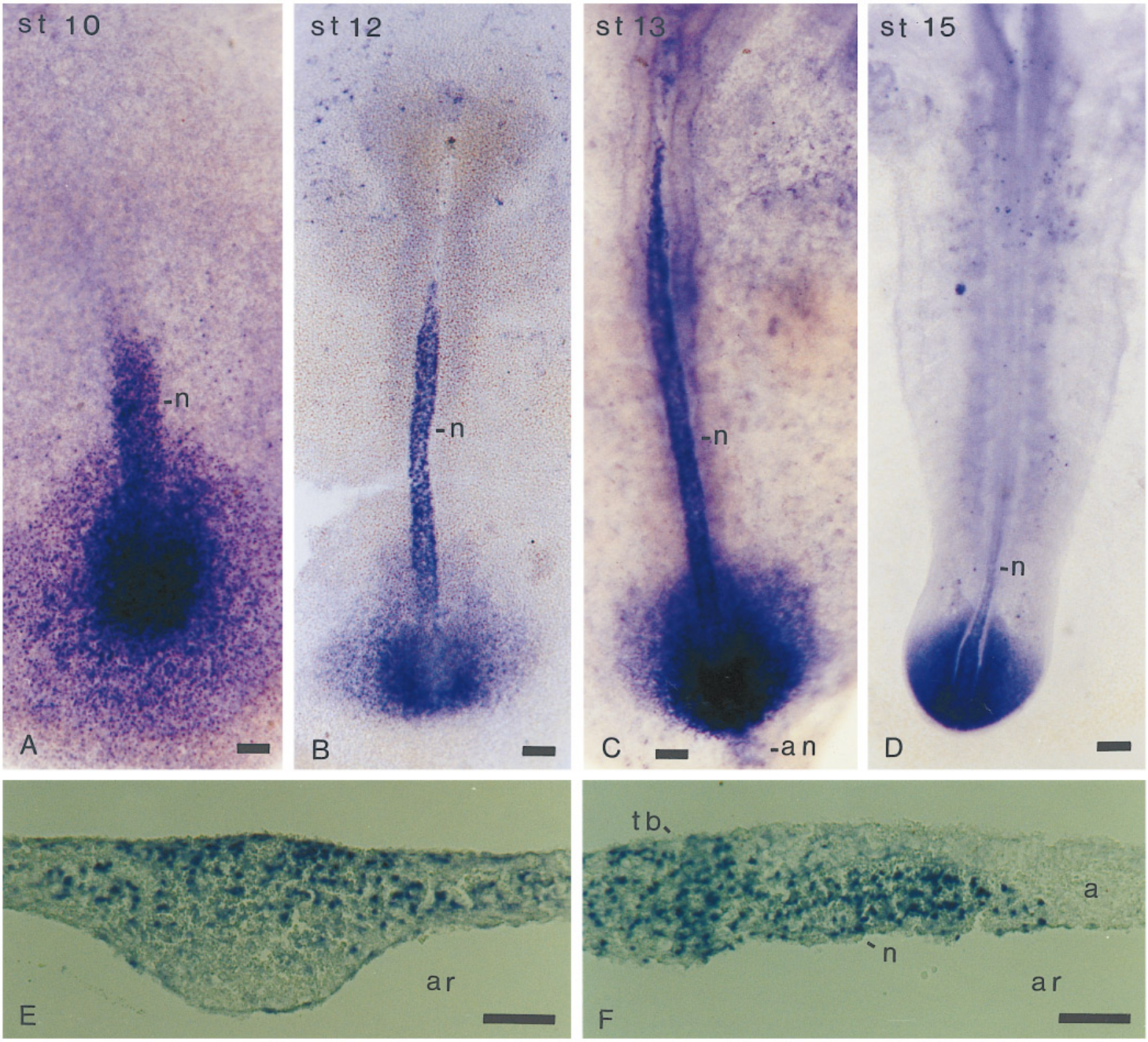


FIG. 3. The T-pattern of the neurula and later stages of *Gastrotheca*. The embryonic disks were dissected from the yolk and prepared as flat mounts. The rostral region faces upward in A–D. The external surface faces upward in E, F. (A) Stage 10 embryo. The T signal is restricted to the emerging notochord and tailbud regions. A faint T signal occurs in nuclei of other regions of the embryonic disk (compare with Figs. 1E and 1E′). (B) Stage 12 embryo. The T signal is restricted to the notochord and tailbud. The notochord is larger than in stage 10 embryos (shown in A), and somites begin to form. (C) Stage 13 embryo. The notochord and tailbud are T-positive. The notochord is longer than in the stage 12 embryo (shown in B) and it is T-positive throughout its length. The anus (an) is outlined by T-positive cells. It is caudal to the tailbud, since there is no caudal fold at this stage. A large neurenteric opening was seen in sections (not shown), indicating that *Gastrotheca* shares with *Xenopus* similar mechanisms of tail specification (Gont *et al.*, 1993). (D) Stage 15 embryo. The caudal region of the notochord and the tailbud are T-positive. (E) Cross section through the tailbud of a stage 10 embryo (comparable with A). There are T-positive nuclei in deep cells. T-positive nuclei occur also in the external surface and in the archenteron roof. (F) Sagittal section through a stage 10 embryo (comparable with the embryo shown in A). T-positive nuclei occur in all cell layers of the tailbud, in the notochord, and in the archenteron roof. Bars correspond to 100 μm. Abbreviations: a, anterior; an, anal region; ar, archenteron; n, notochord; st, stage; tb, tailbud region.

and del Pino, 1985), lend support to this hypothesis. The *Gastrotheca* gastrula exaggerates trends also observed in urodele gastrulation, since dorsal convergence and extension occur from the late gastrula onward in *Pleurodeles* (Shi *et al.*, 1987). As in *Gastrotheca*, elongation of the notochord is delayed in *Taricha* (reviewed in Jacobson, 1981, 1991) and in *Ambystoma* and *Pleurodeles* (Youn *et al.*, 1980; urodele gastrulation is reviewed in Keller and Winklbauer, 1992, and Johnson *et al.*, 1992). The situation is different in *Xenopus;* the archenteron and notochord extend immediately following their appearance. These processes are thought to be driven by convergence and extension of the dorsal marginal zone, which begins at midgastrula (reviewed in Keller, 1991; Keller and Winklbauer, 1992).

While the separation of dorsal convergence and extension from blastopore closure in *Gastrotheca* differs from the situation in normal *Xenopus* embryos, experimental interventions can create a similar separation in that species. *Xenopus* embryos made deficient in dorsal convergence and extension by the injection of suramin into the blastocoel (Gerhart *et al.*, 1989), or by the ventralization with UV light (Scharf and Gerhart, 1980), are capable of closing their blastopores. The blastopore closes in UV-ventralized embryos of *Pleurodeles* as well (Shi *et al.*, 1989). Conversely, the blastopore does not close in *Xenopus* embryos defective in gastrulation by RNA injection of a mutant platelet-derived growth factor receptor-α, although dorsal convergence and extension have not been inhibited (Ataliotis *et al.*, 1995). This evidence suggests that dorsal convergence and extension need not be the cause of blastopore closure, although it may accelerate the process in *Xenopus* embryos, since there is delay of blastopore closure in UV-ventralized embryos (Scharf and Gerhart, 1980; Scharf *et al.*, 1989). Dorsal convergence and extension are considered the main forces in closing the *Xenopus* blastopore, since blastopore closure does take place after ablation of the animal cap (Keller and Jansa, 1992). By contrast, in *Pleurodeles*, the blastopore does not close after animal cap ablation, suggesting that mesoderm cell migration is important for this process in this species (Shi *et al.*, 1987). In general, animal cap ablation experiments may not provide definitive results because besides dorsal convergence and extension, involution (reviewed in Keller, 1986), circumblastoporal convergence (Keller and Danilchik, 1988), and shrinkage of the vegetal surface remain and can provide forces that may close the blastopore. Vegetal shrinkage occurs throughout gastrulation in *Xenopus* (Keller, 1978) and *Gastrotheca*, being driven in *Gastrotheca* by pronounced elongation of vegetal cells, with the long axis perpendicular to the vegetal surface (Elinson and del Pino, 1985). Overlapping and probably redundant forces provide plasticity to the mechanisms of blastopore closure and combined with differences in timing of gastrulation events contribute to the variation in amphibian gastrulation patterns. *Gastrotheca* provides an example of the separation of gastrulation events that occurs when development slows down.

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