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Mesoderm Formation in *Eleutherodactylus coqui*: Body Patterning in a Frog with a Large Egg

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The direct developing frog, *Eleutherodactylus coqui*, develops from a large egg (diameter 3.5 mm). To investigate the effect of egg size on germ-layer formation, we studied mesoderm formation in *E. coqui* and compared it to that of *Xenopus laevis* (diameter 1.3 mm). First, we identified the position of prospective mesoderm in the 16-cell *E. coqui* embryo by cell-lineage tracing. Although the animal blastomeres are small, they form most of the blastocoel roof and make extensive contributions to some mesodermal tissues. Second, we performed recombinant analysis with *X. laevis* animal caps to define the distribution of mesoderm-inducing activity. Mesoderm-inducing activity in *E. coqui* was restricted around the marginal zone with strong activity in the superficial cells. Neither the vegetal pole nor the blastocoel floor had activity, although these same regions from *X. laevis* induced mesoderm. Third, we cloned *Ecbra*, a homologue of *Xbra*, an early mesoderm marker in *X. laevis*. Ecbra was expressed in the marginal ring close to the surface, similar to *X. laevis*, but *E. coqui* had weaker expression on the dorsal side. Our results suggest that mesoderm formation is shifted more animally and superficially in *E. coqui* compared to *X. laevis*.

Key Words: **Anuran; *Brachyury*; cell lineage; *Eleutherodactylus coqui*; induction; large egg; mesoderm; *Xenopus laevis*; yolk.**

INTRODUCTION

Early development is similar for different groups of vertebrates. Embryos of teleosts, amphibians, reptiles/birds, marsupials, or placental mammals can be easily distinguished given just a few characters (Gilbert and Raunio, 1997). The group similarities justify in part the model systems approach, where development of one species represents development of all members of the group. The zebrafish, *Danio rerio*, is the teleost representative; *Xenopus laevis* is the amphibian representative, and so on. The huge developmental data sets, generated by the model systems approach, now provide many parameters to determine how much variation actually exists in the early development of different species within the group.

Variation is indeed found when embryos from nonmodel species are compared to the model species. Among amphibians, fertilization is monospermic in *X. laevis*, but polyspermic in many urodeles and in some anurans (Elinson, 1986, 1987; Iwao, 2000). RNAs, localized to the vegetal pole of the *X. laevis* oocyte, are involved in formation of primordial germ cells, but primordial germ cells arise by induction in the lateral-plate mesoderm in some urodeles (Nieuwkoop and Sutasurya, 1976; Wakahara, 1996; Houston and King, 2000). A factor that initiates dorsal axial development in *X. laevis* is located close to the vegetal pole of eggs just after fertilization, but the dorsal activity extends up toward the equator in the newt *Cynops pyrrhogaster* (Doi *et al.*, 2000). Mesoderm originates primarily from deep cells in *X. laevis*, but surface cells provide varying amounts of mesoderm in other species (Smith and Malacinski, 1983; Hanken, 1986; Minsuk and Keller, 1996, 1997). The difference in mesoderm origin affects cell movements during gastrulation, as the prospective mesoderm must end up between the other two germ layers. These findings suggest that there may be profound differences in the organization of amphibian eggs, despite morphological similarities of the early embryos.

In order to explore variation in early development further, we have chosen the frog *Eleutherodactylus coqui*. Its embryos develop directly to a frog without a tadpole, and this mode of development is derived (Callery *et al.*, 2001). Coincident with the absence of a feeding tadpole is the presence of a large yolk-filled egg, which is 3.5 mm in

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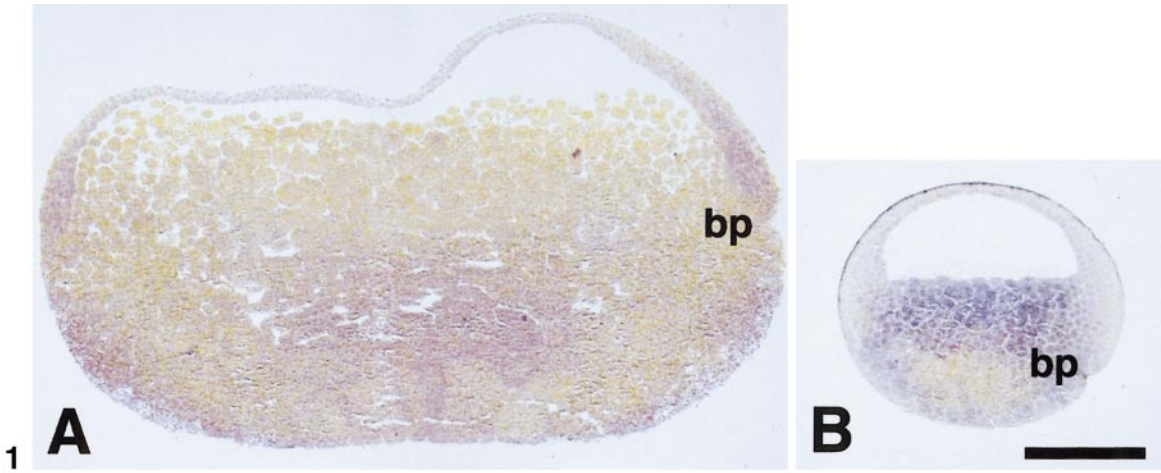

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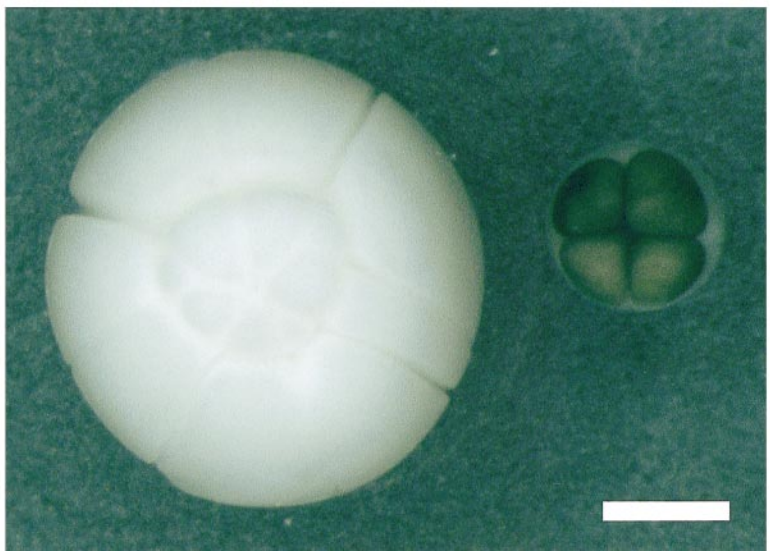

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FIG. 1. Comparison of blastopore formation in *E. coqui* and *X. laevis*. Sections through the midsagittal plane in early gastrulae stained with Azan stain (0.5% phosphotungstic acid, 1% orange G, 0.5% aniline blue, 1.5% acid fuchsin, 0.2 M Na_2HPO_4, 0.1 M citric acid). The blastopore (bp) formed equatorially in *E. coqui* (A) and subequatorially in *X. laevis* (B). Bar, 500 μm.

FIG. 2. Comparison of the early cleavage pattern in *E. coqui* and *X. laevis*. The first horizontal cleavage occurs at fourth cleavage (16-cell stage) in *E. coqui* (left) and at third cleavage (8-cell stage) in *X. laevis* (right). Animal view. Bar, 1 mm.

diameter. The egg of *E. coqui* is about 20 times the volume of the 1.3-mm egg of *X. laevis*. Previous observations indicated that sperm entry occurred relatively closer to the animal pole in *E. coqui* compared to *X. laevis* (Elinson, 1987), as did the initiation of gastrulation (Elinson and Fang, 1998). The difference in proportions between early gastrulae of these two species is readily apparent (Fig. 1). This situation provides the opportunity to examine the impact of increased egg size on early patterning of the embryo.

The body plan of *X. laevis* originates with two localized maternal determinants: a dorsal determinant and a mesoderm/endoderm determinant (Kimelman *et al.*, 1992; Harland and Gerhart, 1997; Heasman, 1997). The dorsal determinant signals through a pathway involving β-catenin, while the mesoderm/endoderm determinant appears to be the transcription factor, VegT, whose RNA is localized to the vegetal cortex of the oocyte (Zhang and King, 1996; Zhang *et al.*, 1998; Kofron *et al.*, 1999). VegT leads to the expression of mesoderm inducers, and mesoderm forms in the equatorial region of the *X. laevis* embryo. The induced cells express mesoderm-specific genes such as *Brachyury* (*Xbra*), and *Xbra* itself can cause mesoderm formation (Smith *et al.*, 1991; Cunliffe and Smith, 1992).

Here, we report on the origin of mesoderm in *E. coqui* and compare it to that in *X. laevis*. Our results suggest that the

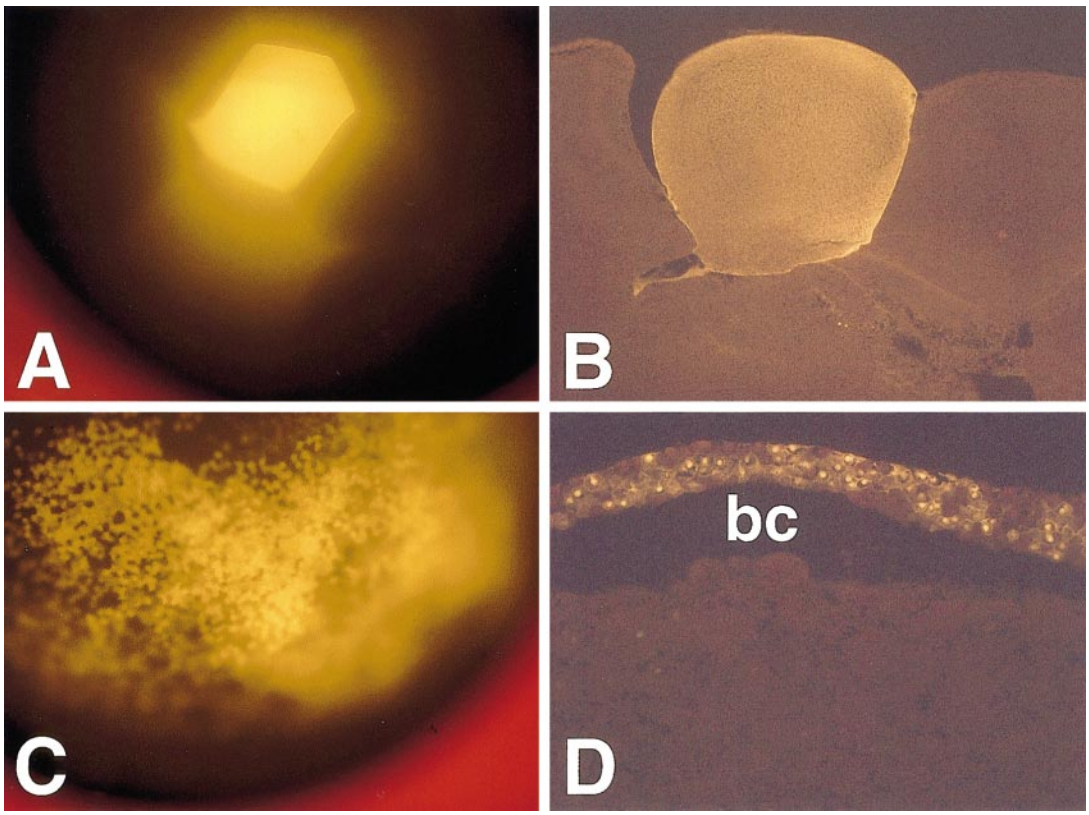


FIG. 3. Lineage tracing of *E. coqui* animal cells. A single animal blastomere in *E. coqui* at the 16-cell stage was microinjected with Rhodamine dextran amine (RDA), and the fluorescent label was observed in intact (A, C) or sectioned (B, D) embryos. (A, B) The fluorescent label was confined primarily within the blastomere, immediately after injection. (C, D) By early gastrula, labeled cells derived from the injected blastomere were well mixed with unlabeled cells. Blastocoel (bc).

increased yolk has shifted the location of mesoderm formation more peripherally and relatively closer to the animal pole of the embryo.

MATERIALS AND METHODS

Embryos

Adult *E. coqui* were collected in Puerto Rico under permits issued by the Departmento de Recursos Naturales. Embryos were obtained from natural matings in our laboratory colony as described previously (Elinson *et al.*, 1990) and staged according to Townsend and Stewart (1985) (TS). Embryos at stages earlier than TS4 were staged as follows:

Early blastula: thick animal cap, small blastocoel, approximately 10 h before blastopore formation at 23°C (TS1)

Late blastula: thin animal cap, large blastocoel, approximately 4 h before blastopore formation (TS1)

Early gastrula: dorsal blastopore formation (TS2)

Mid gastrula: yolk plug formation (TS2)

Late gastrula: small yolk plug (TS2)

Neurula: neural plate stage (TS2)

Embryos with intact jelly layers were raised in a moist chamber, and those without the outer layers of jelly were raised in 20% Steinberg's solution (Elinson, 1987).

X. laevis embryos were obtained by *in vitro* fertilization, according to standard methods, and staged according to Nieuwkoop and Faber (1994).

Cell-Lineage Tracing

For cell-lineage tracing of 16-cell *E. coqui* embryos, embryos were selected which showed a regular cleavage pattern, including a latitudinal fourth cleavage furrow (Fig. 2). Embryos were placed in 20% Steinberg's solution, and the outer layers of jelly were removed with forceps. The blastomeres were labeled by microinjecting 4.6 nl per animal blastomere or 9.2–13.8 nl per vegetal blastomere of rhodamine dextran amine (RDA; 20 mg/ml in water; Molecular Probes) with a Nanoject injector (Drummond). Label was observed by using a fluorescence microscope on living or sectioned embryos. For sectioning, embryos were fixed in 4% formaldehyde in 100% Steinberg's solution overnight, kept in 70% ethanol up to 1 week, dehydrated through ethanol, cleared in xylene, and embedded in paraplast. Embryos were sectioned at 10 μm, and the sections were rehydrated and mounted in PermaFluor aqueous mounting media (Shandon). The approximate contribu-

tions of labeled cells to each tissue were judged visually from sectioned samples.

Recombinant Analysis

To obtain tissues for recombinant analysis, *E. coqui* embryos were dejellied with forceps for the outer layers and 3% cysteine, pH 8.0, for the inner layer. *X. laevis* embryos were dejellied with 3% cysteine. After removing the fertilization membrane, the tissues were dissected in 100% Steinberg's solution with a tungsten needle. To assay mesoderm-inducing activity, each tissue was combined with the test tissue, which was animal cap from a *X. laevis* stage-9 blastula. The recombinants were cultured in 100% Steinberg's solution supplemented with 0.1% BSA and 50 μg/ml gentamicin. They were fixed in Smith's fixative when *X. laevis* sibling embryos had reached stage 37/38. *E. coqui* sibling embryos were neurula or TS4 at that time. The specimens were embedded in paraplast, sectioned at 10 μm, and stained with hematoxylin and eosin. Histological differentiation of tissues derived from the *X. laevis* animal caps was scored. Data are calculated as the number of recombinants with a particular tissue differentiation $\times$ 100/Total number of recombinants and expressed as a percentage.

To test the mesoderm-inducing activity of the superficial region, we isolated the outer layer of cells. To prepare the outer cells of the animal cap (OAC) and dorsal marginal zone (ODM) in *E. coqui*, the tissues containing both inner and outer cells were isolated in 100% Steinberg's solution. They were immediately transfered to Ca^{2+}–Mg^{2+}-free solution (50% PBS; 68 mM NaCl, 1.34 mM KCl, 4.05 mM Na_2HPO_4, 0.73 mM KH_2PO_4, pH 7.4), which dissociates the inner cells from an intact layer of outer cells. The intact outer layer was returned to 100% Steinberg's solution. Outer and inner cells of dorsal submarginal zone (DSM) and ventral marginal/submarginal zone (VM/SM) did not require Ca^{2+}–Mg^{2+}-free treatment, so both the outer and inner tissues could be isolated manually in 100% Steinberg's solution.

For comparative purposes, recombinants were made between animal cap from stage 9 *X. laevis* and tissues from *X. laevis* blastula (stage 9) or early gastrula (stage 10). Embryos for animal cap dissection were placed in 5% Ficoll in 100% Steinberg's solution and labeled by microinjecting 18.4 nl RDA (20 mg/ml) per embryo. All of outer cells were isolated manually in 100% Steinberg's solution. The recombinants were fixed in 4% formaldehyde in 100% Steinberg's solution. Every 10–15 sections were divided onto two slides, one of which was stained with hematoxylin and eosin for histological analysis and the other of which was judged for lineage under epifluorescence.

Ecbra Cloning

Total RNA was extracted from *E. coqui* neurulae using the Trizol method (BRL). cDNAs were synthesized from the RNA samples using random hexamers and M-MLV reverse transcriptase (BRL) and were used as templates for PCR. Degenerate primers were designed based on the Brachyury sequences from *X. laevis*, chick, and mouse. The upstream primer (Ecbra–DLF) was 5′-TAC CGR GTG GAY CAY CTK-3′, and the downstream primer (Ecbra–DLR) was 5′-GGY NCY RTT NCT CAC ARG ACC A-3′.

PCR amplification was carried out with 1 min denaturation at 95°C, 1 min annealing at 55°C, and 1 min extension at 72°C for 39 cycles. The final extension was carried out for 5 min at 72°C. The 1,032-bp PCR product was gel purified and subcloned into pGEM-T Easy vector (Promega). Sequencing indicated that cloned product was 75% identical to *X. laevis Brachyury* (*Xbra*), and we named it *Ecbra*.

Northern Blotting

Total RNA was isolated from ovary and staged embryos by phenol extraction using Trizol (BRL) and LiCl precipitation. Equal amounts of total RNA were electrophoresed on a 1.2% agarose/formaldehyde/Mops gel and transferred onto Hybond N+ membranes (Amersham). The blot was hybridized with a gel-purified Ecbra DNA fragment, labeled with ^{32}P by random priming (BRL).

In Situ Hybridization

Dejellied embryos were fixed in MEMFA for 2 h. The fertilization envelope was dissected off, followed by an additional 1-h fixation. The Ecbra DNA inserted into the pGEM-T Easy vector was used as a template for the synthesis of Dig-11-UTP-labeled sense and antisense riboprobes (Boerhinger–Mannheim). Whole-mount *in situ* hybridization was carried out as described by Harland (1991). Solutions were changed and stirred as gently as possible to minimize breakage of embryos. The embryos were treated with 5 μg/ml proteinase K for 30 min. For *in situ* on sections, embryos were embedded in Paraplast and sectioned at 20 μm. Hybridization was performed on sections as described elsewhere (Belo *et al.*, 1997; Lemaire and Gurdon, 1994; http://www.lifesci.ucla.edu/hhmi/derobertis). Following the color reaction using NBT and BCIP, the sections were fixed in MEMFA for 5 minutes, dehydrated through an ethanol series, cleared in xylene, and mounted in Permount.

RT-PCR

E. coqui early or early/mid gastrulae were collected and dissected in 100% Steinberg's solution with tungsten needles. Each embryo was divided into four parts: dorsal marginal zone, ventral marginal zone, blastocoel floor/vegetal pole, and animal cap. RNA was isolated from each part by using Trizol. Reverse transcription (RT) and PCR were carried out following Hemmati-Brivanlou and Melton (1994). RT was performed using random hexamers with 1 μg total RNA from each part as template. PCR conditions were 39 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were analyzed on a 1.2% TBE agarose gel. The oligos used for PCR amplification of EcBra were: 5′ upstream primer (Ecbra-FL): 5′-GAG GTT CAA GGA GCT TAC CA, and 3′ downstream primer (Ecbra-RL): 5′-GGC ATT TGA AGA CCA GAC CA-3′. The molecular size of the amplified product was 736 bp. As an internal control, EcL8 was detected with its primers at the same time (Callery and Elinson, 2000). We also performed amplifications from samples in which the reverse transcriptase had been omitted from the RT reaction. No signal was obtained under these conditions.

RESULTS

Lineage of Animal Blastomeres at the 16-Cell Stage

To determine the location of the prospective mesoderm area, we first performed blastomere-lineage tracing. The first latitudinal cleavage, which divides animal and vegetal blastomeres, occurs at the 16-cell stage in *E. coqui* embryos, compared to the 8-cell in *X. laevis* embryos (Fig. 2). As

TABLE 1
Cell Lineages of *E. coqui* 16-Cell Embryos

Animal blastomere contribution to each tissue	More than 90%	50–90%	10–50%	Less than 10%
Ectoderm	Eyes Ear vesicles	Epidermis Forebrain Hindbrain Trunk spinal cord	Tail spinal cord	
Mesoderm		Tail notochord Tail somites Tail mesenchyme	Head mesenchyme Trunk notochord Trunk somites Trunk mesenchyme Hind limbs Mesothelium	Fore limbs Heart Pronephros Blood
Endoderm				Foregut Midut Hindgut Yolky endoderm

Note. All eight animal or all eight vegetal blastomeres of *E. coqui* 16-cell embryos were labeled with RDA, and the animal contribution to each tissue was estimated from sectioned TS6 embryos.

cleavage patterns to this point were relatively reproducible, we chose this stage for cell-lineage tracing. In *X. laevis*, cells mix during early stages of development, indicating that there is no strict germ-layer boundary in the early cleavage stages (Bauer *et al.*, 1994). To visualize whether cell mixing occurred in *E. coqui*, we labeled single blastomeres. Although the cleavage furrows grew slowly in *E. coqui*, the label remained properly restricted within the injected blastomeres (Figs. 3A and 3B). Labeled animal cells were mixed with unlabeled cells in the early gastrula (Figs. 3C and 3D). Cell mixing was not extensive among vegetal cells at this stage (data not shown), the same as *X. laevis* (Bauer *et al.*, 1994).

The first latitudinal cleavage at the 16-cell stage in *E. coqui* occurs close to the animal pole, so that the eight animal cells represent roughly 1% of the volume of the embryo (five embryos from two clutches). Given this relatively small volume, we were interested in determining which tissues were derived from the animal blastomeres. To address this question, we investigated the cell lineage of *E. coqui* 16-cell-stage embryo. As *E. coqui* embryos are not pigmented, we could not tell the dorsal vs. ventral sides before gastrulation. Accordingly, we labeled all eight animal or all eight vegetal blastomeres.

In the early gastrula, labeled animal cells occupied most of blastocoel roof (Fig. 4A). No animal cells were seen in the blastocoel floor. Some labeled animal cells were located close to the blastopore, and these might move inside the embryo through the blastopore to form mesoderm. To determine the tissue differentiation of the animal cells, embryos were cultured until TS6. The TS6 *E. coqui* embryo has a well-developed body attached to large yolk mass. The large eyes have a slightly pigmented iris, the limbs have elongated, and blood is circulating (Townsend and Stewart, 1985). We judged the lineage of animal blastomeres in each tissue from seven animal-labeled embryos and six vegetal-labeled embryos, derived from five clutches. Animal blastomeres contributed to both ectoderm and mesoderm (Fig. 4B; Table 1). Eyes and ear vesicles were derived from animal blastomeres, as was much of the epidermis and neural tissue (Table 1). Among mesodermal tissues, dorsal mesoderm, such as notochord and somites, contained a relatively high proportion of the animal cells (Fig. 4B; Table 1). The contributions from the animal cell lineage were higher in posterior mesoderm (tail notochord, tail somites, hind limb) than anterior mesoderm (trunk notochord, trunk somites, fore limb). Animal-lineage cells were rarely seen in endodermal tissues, forelimb, heart, pronephros, and blood (Fig. 4B). Conversely, most of these tissues were derived from the vegetal cell lineages (Fig. 4C).

Mesoderm-Inducing Activity in E. coqui

The results of the cell-lineage analysis indicated that the prospective mesoderm area in *E. coqui* embryos extended relatively close to the animal pole. The mixing of cells in the blastocoel roof (Figs. 3C and 3D) suggests that there are not strict germ-layer boundaries in early cleavage-stage *E. coqui* embryos, so that inductions are involved in germ-layer formation and tissue differentiation. Accordingly, we examined mesoderm-inducing activity by combining various parts of *E. coqui* embryos with animal cap from *X. laevis* stage-9 embryos. We used animal cap from *X. laevis* instead of *E. coqui* as responders to induction for several reasons. *E. coqui* embryos are not readily available and develop slowly, reducing the ease of their use as an assay for mesoderm induction. More importantly, we have not succeeded in obtaining

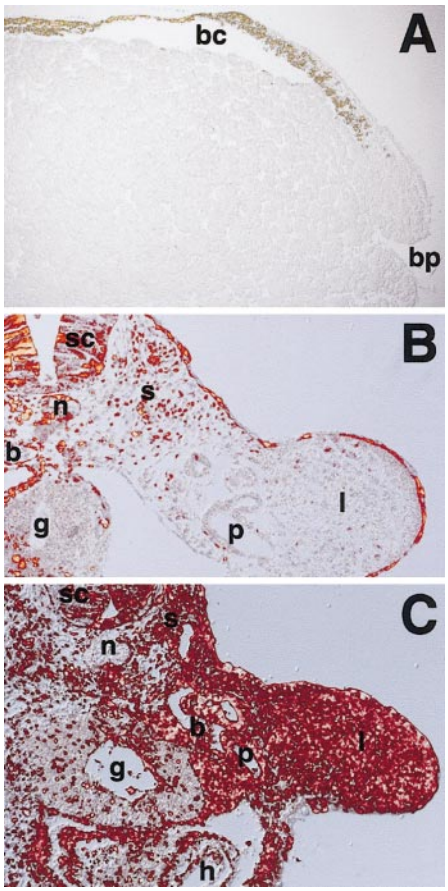


FIG. 4. Fate of *E. coqui* 16-cell stage blastomeres. All eight animal or all eight vegetal blastomeres in *E. coqui* 16-cell embryos were labeled by microinjection of RDA. (A) A midsagittal section of an early gastrula that had its animal blastomeres labeled. Labeled cells occupied most of the blastocoel roof and extended close to the blastopore (bp). Labeled cells were never seen in the blastocoel (bc) floor. (B) A trunk-level section of a TS6 embryo that had its animal blastomeres labeled. Label is present in cells of epidermis, spinal cord (sc), dorsal mesoderm [notochord (n), somites (s)] but not of lateral/ventral mesoderm [blood (b), limb bud (l), pronephric tubules (p)]. (C) A trunk-level section of a TS6 embryo that had its vegetal blastomeres labeled. Labeled cells predominated in most tissues but not in the notochord in this case. Heart (h).

dorsal mesoderm differentiation from *E. coqui* explants, for reasons that are yet unknown. The *E. coqui–X. laevis* recombinants were cultured until *X. laevis* siblings reached stage 37/38, at which time the *E. coqui* embryos were neurula or TS4. After sectioning and staining, *E. coqui* cells were easily distinguishable from *X. laevis* cells, since they lacked pigment, were lightly stained, and had not yet differentiated histologically (Fig. 5). The results of the recombinant experiments are summarized in Fig. 6.

Blastula Stages

In recombinants with tissues from *E. coqui* early blastula, *X. laevis* cells rarely differentiated into mesodermal tissues (Fig. 6; Table 2). Weak mesoderm differentiation was seen at a low frequency in recombinants with marginal zone (M), but neural differentiation was more frequently found than mesoderm differentiation (Table 2). This result could be due to a longer competence period of animal cap for neural induction (Servetnick and Grainger, 1991).

With late blastula, on the other hand, mesoderm differentiation was often seen in recombinants with marginal zone (M) and submarginal zone (SM; Fig. 6; Table 2). Mesoderm differentiation was rarely seen in recombinants with other parts of the late blastula. Several different mesodermal and neural tissue were differentiated in this stage of recombinants (Table 2).

Recent studies suggest that mesoderm induction occurs after the midblastula transition (MBT) in *X. laevis* (Wylie *et al.*, 1996; Kofron *et al.*, 1999). The difference in inducing activity in the *E. coqui* early and late blastula suggests that inducing activity in *E. coqui* forms later as well. Competence of *X. laevis* animal cap to respond to mesoderm inducers is present at stage 9 but disappears by stage 11 (Jones and Woodland, 1987). The period between stages 9 and 11 is 4.75 h at 22–24°C (Nieuwkoop and Faber, 1994), which is within one stage of the *E. coqui* embryo. Therefore, the mesoderm induction detected in our experiments must occur close to the *E. coqui* stage at the time of recombination, and mesoderm-inducing activity is not present in the *E. coqui* early blastula.

Early Gastrula Stage

The induction analysis was extended to the early gastrula stage in order to see whether inducing activity persisted. In addition, the presence of a dorsal lip allowed us to identify the dorsal side, which is not possible at the blastula stage. Recombinants with tissues from the dorsal marginal zone (DM) or dorsal submarginal zone (DSM) showed dorsal types of differentiation (Fig. 5A; Table 2). These include notochord and muscle, along with neural tissue, identified as round aggregates of densely packed cells with narrow ellipsoid nuclei (Tiedemann *et al.*, 1994; Uchiyama and Otsuka, 1995). On the other hand, recombinants with tissues from the ventral marginal zone (VM) or ventral submarginal zone (VSM) showed ventral types of differentiation (Fig. 5B; Table 2). These include ventral mesoderm, such as mesothelium and blood. The frequencies of induction in the early gastrula recombinants were higher than in the late blastula (Fig. 6; Table 2). Recombinants with other parts of the early gastrula rarely formed mesodermal tissues (Figs. 5C and 5D), although neural tissue was often formed in recombinants with the dorsal blastocoel floor (DBF) without any accompanying dorsal mesodermal tissues (Fig. 5C).

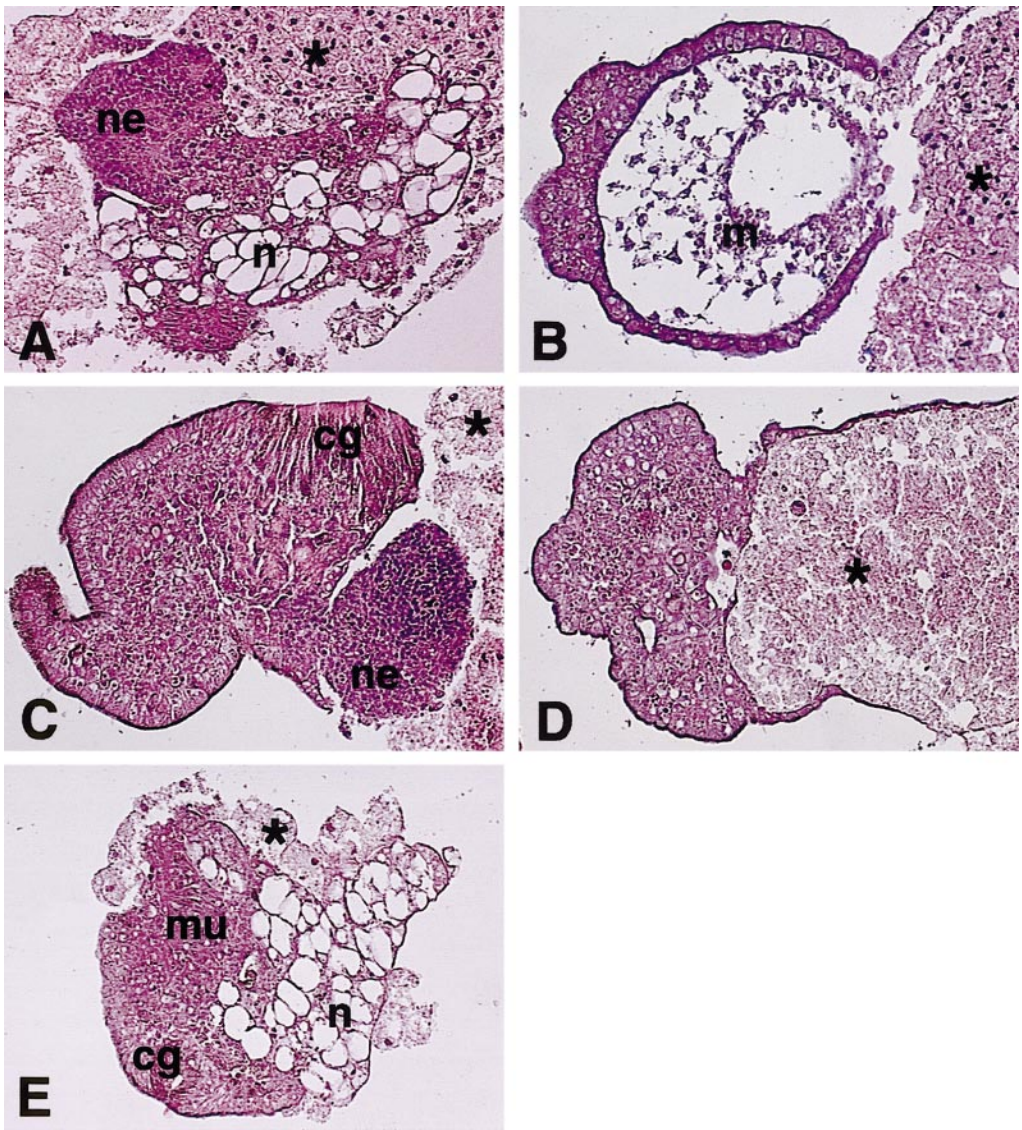


FIG. 5. Histology of *X. laevis* animal cap and *E. coqui* early gastrula (*) recombinants. (A) Recombinant with *E. coqui* dorsal marginal zone (DM) showed dorsal type differentiation, including notochord (n) and neural tissue (ne). (B) Recombinant with *E. coqui* ventral marginal zone (VM) showed ventral type differentiation such as mesothelium (m). (C) Recombinant with *E. coqui* dorsal blastocoel floor (DBF) formed neural tissue (ne) and cement gland (cg) without any dorsal mesodermal tissues. (D) Recombinant with *E. coqui* dorsal vegetal pole (DVP) differentiated only atypical epidermis. (E) Recombinant with *E. coqui* outer dorsal marginal zone (ODM) showed prominent dorsal mesoderm differentiation, including notochord (n) and muscle (mu), despite the small amount of *E. coqui* tissue (*).

The above results indicated that mesoderm-inducing activity in the *E. coqui* gastrula is restricted around the marginal zone (Fig. 6). Little activity was present in either the vegetal pole (DVP, VVP) or the inner blastocoel floor cells (DBF, VBF). Shih and Keller (1992) reported that in *X. laevis*, the epithelium cells of the dorsal lip had stronger inducing activity than the inner cells of the dorsal lip. Consequently, we examined whether the most outer cells in *E. coqui* have mesoderm-inducing activity or not. Outer animal cap (OAC) and outer dorsal marginal zone (ODM) cells were prepared by Ca^{2+}–Mg^{2+}-free treatment, which dissociates inner cells from outer layer (Fig. 7). Dorsal submarginal zone (DSM) and ventral marginal/submarginal zone (VM/SM) were manually divided into outer (ODSM, OVM/SM) and inner (IDSM, IVM/SM) cells. Although these outer tissues contained only a small number of cells, they

TABLE 2

Mesoderm and Neural Differentiation (%) of *X. laevis* Animal Cap Combined with *E. coqui* Tissues

	Early blastula					Late blastula					Early gastrula									Early gastrula, outer or inner cells					
Region of *E. coqui*	AC	M	SM	BF	VP	AC	M	SM	BF	VP	AC	DM	VM	DSM	VSM	DBF	VBF	DVP	VVP	OAC	ODM	ODSM	OVM/SM	IDSM	IVM/SM
No. of recombinants	24	36	36	32	28	21	45	40	44	33	42	38	32	36	30	47	40	30	26	21	23	36	29	36	33
Mesoderm	0	11	0	0	0	0	27	43	7	9	0	61	53	50	43	0	3	3	0	0	74	44	55	3	3
Notochord	0	3	0	0	0	0	9	10	0	0	0	61	0	28	0	0	0	0	0	0	52	19	0	0	0
Muscle	0	6	0	0	0	0	9	23	0	0	0	13	3	28	0	0	0	0	0	0	30	33	0	0	0
Mesenchyme	0	0	0	0	0	0	7	13	0	0	0	0	13	19	23	0	3	0	0	0	17	8	14	0	0
Mesothelium	0	8	0	0	0	0	13	20	7	9	0	0	41	6	10	0	0	3	0	0	0	8	38	3	3
Blood	0	0	0	0	0	0	2	5	2	0	0	0	22	0	17	0	0	0	0	0	0	0	3	0	0
Neural tissue	0	19	0	3	0	0	24	28	7	0	0	90	0	64	3	40	0	0	0	0	70	67	0	36	0

Note. Abbreviations for "region of *E. coqui*" are found in the legend to Fig. 6. The percentages are calculated as the Number of recombinants with a particular tissue differentiation × 100%/Total number of recombinants. If a recombinant had both notochord and muscle tissue, it would be counted as positive for mesoderm, notochord, and muscle. Because of the double-scoring, the sum of the percentages for individual mesodermal tissues is greater than the percentage of mesoderm.

indeed had mesoderm-inducing activity (Figs. 5E and 6, Table 2). Mesoderm tissues were frequently differentiated in recombinants with both in dorsal (ODM and ODSM) and ventral (OVM/SM) outer cells but not inner cells (IDSM and IVM/SM). Although recombinants with IDSM rarely formed mesodermal tissues, they often formed neural tissue (Fig. 6, Table 2).

Mesoderm-Inducing Activity in X. laevis

The results from the *E.* coqui–X. *laevis* recombinants indicated that mesoderm-inducing activity was restricted around the marginal zone and close to the surface in *E. coqui* late blastulae and early gastrulae. Despite the extensive analysis of mesoderm induction in *X. laevis* (Harland and Gerhart, 1997), activity in some regions of the embryo has not been reported. In order to compare the *E. coqui* pattern with that in *X. laevis*, we repeated the recombinant analysis with *X. laevis* embryos as the inducing tissue (Fig. 6; Table 3). We examined differentiation of labeled animal cap cells combined with unlabeled marginal or vegetal cells. At late blastula (stage 9), all vegetal tissues, including the blastocoel floor (BF) and the vegetal pole (VP), which consisted of less than 10 cells, had strong mesoderm-inducing activity, as did submarginal (DSM, VSM) cells. Marginal zone (DM, VM) cells had slightly weaker activity at this stage. Neural tissue rarely formed without accompanying dorsal mesodermal tissues.

At early gastrula (stage 10), most marginal or vegetal cells still had mesoderm-inducing activity (Fig. 6; Table 3). The strongest activity was present in outer cells of both dorsal (ODM) and ventral (OVM/SM) marginal zones. Inner cells had weaker inducing activities than outer cells. Strong neural and dorsal mesoderm induction was only seen in dorsal marginal zone (ODM, IDM).

TABLE 3

Mesoderm and Neural Differentiation (%) of *X. laevis* Animal Cap Combined with *X. laevis* Tissues

	Blastula							Early gastrula								
Region of *X. laevis*	AC	DM	VM	DV	VV	BF	VP	AC	ODM	OVM/SM	IDM	IVM/SM	ODSM	IDSM	BF	VP
No. of recombinants	36	20	30	34	35	34	34	33	22	29	30	29	37	38	31	34
Mesoderm	0	70	47	94	97	100	94	0	96	90	70	62	54	11	23	74
Notochord	0	25	0	12	0	0	0	0	55	0	27	0	0	0	0	0
Muscle	0	60	0	80	0	35	15	0	73	0	53	0	5	0	0	3
Mesenchyme	0	20	0	56	0	35	15	0	68	0	27	0	0	0	0	0
Mesothelium	0	10	47	24	91	62	77	0	0	86	0	55	54	11	23	74
Blood	0	0	13	6	49	24	35	0	0	24	0	14	11	0	0	18
Neural tissue	0	95	0	71	0	27	3	0	100	0	100	0	3	0	3	6

Note. Abbreviations for "region of *X. laevis*" are found in the legend to Fig. 6. Calculation of percentages is the same as Table 2 legend.

Expression of EcBra

During early cleavage, the upper limit for prospective mesoderm is close to the animal pole in *E. coqui* (Table 1). In the blastula and gastrula, tissues capable of inducing mesoderm are found at or near the surface, with activity centered in the marginal zone (Fig. 6). To follow mesoderm formation further, we examined the expression of *EcBra*, the homologue of *Brachyury*. In *X. laevis*, *Xbra* is an early marker for mesoderm and plays an important role in the formation of mesodermal tissues (Smith *et al.*, 1991; Cunliffe and Smith, 1992; Conlon *et al.*, 1996; Conlon and Smith, 1999). *Xbra* is expressed in a ring at the marginal zone and in the prospective notochord.

We cloned *EcBra* by isolating RNA from neurulae, making cDNA, and running PCR with degenerate primers based on *X. laevis*, chick, and mouse *Brachyury*. The 1,032-nt fragment (GenBank Accession No. AF350418) was 75% identical to *Xbra* and 71% identical to *Xbra3* at the nucleotide level. Expression of *Xbra3* in *X. laevis* has a slightly later onset and persists longer in the notochord than expression of *Xbra*, and it may be involved in neural development (Hayata *et al.*, 1999; Strong *et al.*, 2000). We have not made extensive searches for other forms of *Brachyury* in *E. coqui*, but a 366-nt fragment, cloned with a different set of degenerate oligos (Yasuo *et al.*, 1996) was identical in sequence to the region of overlap with the 1,032-nt fragment.

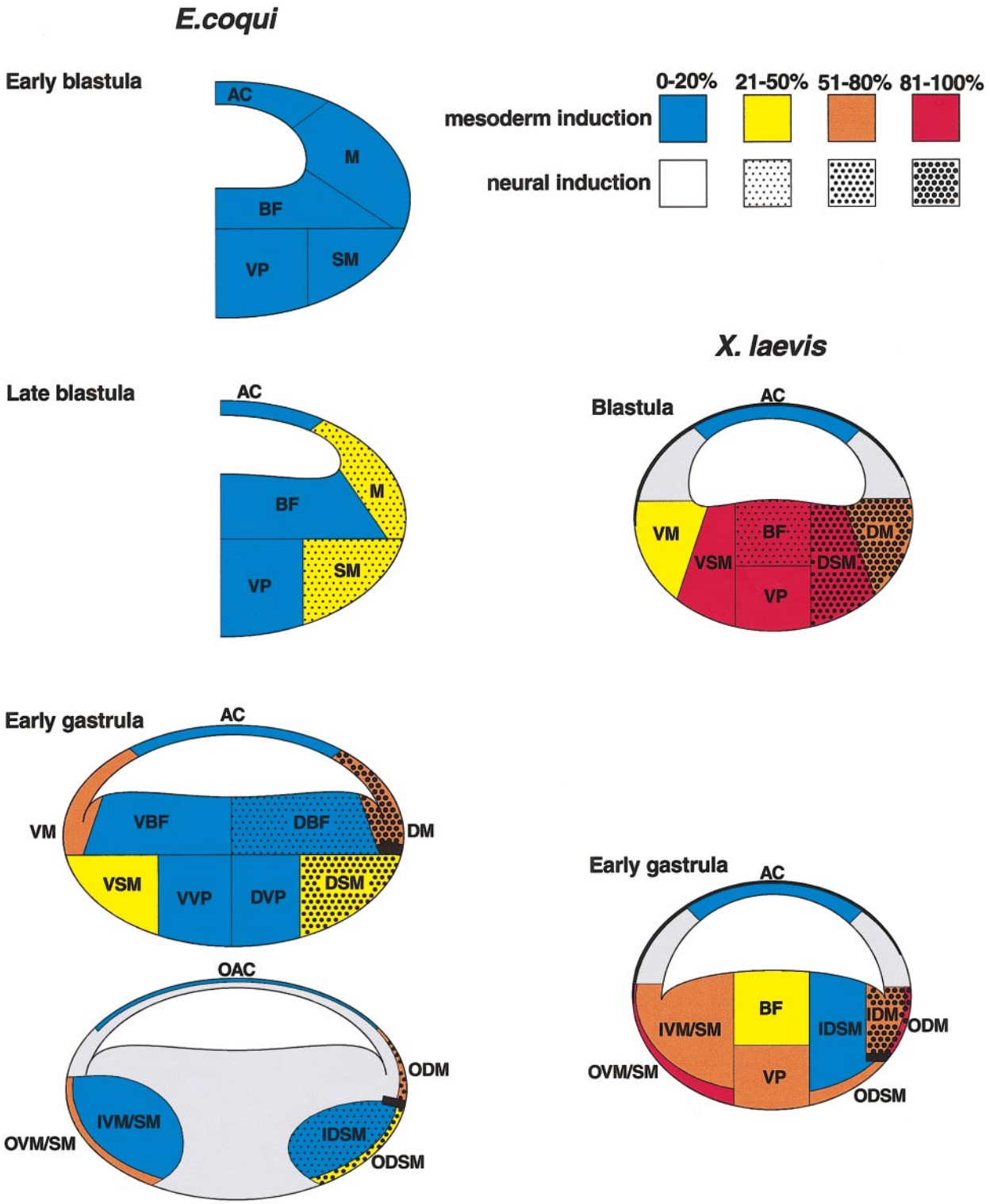
The approximate time of expression of *EcBra* was determined with a developmental Northern blot (not shown). *EcBra* was not expressed in oocytes or in early blastulae. It was strongly expressed through gastrulation and neurulation, and declined by TS4, when limb buds appear, about 1 day after neurulation. The size of the *EcBra* mRNA was about 2.3 kb. The temporal pattern of *EcBra* expression is similar to the timing of expression of *Xbra* in *X. laevis*.

The location of *EcBra* expression was examined by whole-mount *in situ* hybridization. Little staining was seen in the early gastrula when the dorsal lip first formed, but the whole marginal zone was faintly stained in the midgastrula (Fig. 8A). At late gastrula, staining occurred around the closing blastopore lip (Fig. 8E). We consistently observed less staining on the dorsal side of the ring of expression, and this pattern continued as the blastopore closed (Figs. 8A and 8B). At the neural plate stage, the region around the closed blastopore expressed *EcBra*, and there was weak expression in the notochord (Fig. 8H). This expression pattern is similar to that seen in *X. laevis* (Smith *et al.*, 1991; Gont *et al.*, 1993), although the lesser stain on the dorsal side was consistently seen in *E. coqui*.

To see how deep the expression of *EcBra* extended, we embedded embryos in paraplast, sectioned them, and carried out *in situ* hybridization on the sections. Strong expression at the marginal zone was confined to a relatively small group of cells (Figs. 8B–8D), and the ventral lip usually stained more than the dorsal lip (Figs. 8F and 8G). No expression was detected in deeper cells. At neurula, staining around the closing blastopore reached to the surface, while staining of the forming notochord was deeper (Figs. 8I and 8J). As suggested by the whole mounts, the notochordal expression was weaker than the ventral blastoporal expression.

We could not detect *EcBra* expression at the time of dorsal lip formation by *in situ* hybridization, but the recombinant experiments suggested that mesoderm induction occurred as early as late blastula (Fig. 6). To look for expression at the start of gastrulation, early gastrulae were dissected into animal cap, dorsal marginal zone, ventral marginal zone, and blastocoel floor/vegetal pole. RNA was extracted from each piece, and *EcBra* was detected by RT-PCR (Fig. 9). *EcBra* was readily detected in both the dorsal and ventral marginal zones in all three samples, two of which were early gastrula, equivalent to stage 10 in *X. laevis* and one early/midgastrula, equivalent to stage 10.5. In each of the three samples, *EcBra* expression was also found in the animal cap, although at a level lower than in the marginal zone. In two of the samples, the blastocoel floor/vegetal pole did not have *EcBra* present, while, in the third case, it was. Low levels of *Xbra* expression have occasionally been seen in *X. laevis* animal caps (Darras *et al.*, 1997; Lerchner *et al.*, 2000).

FIG. 6. Summary of mesoderm- and neural-inducing activities in *E. coqui* and *X. laevis* embryos. The percentages indicated by the different colors (mesoderm induction) and dot patterns (neural induction) are the frequencies of differentiation in *X. laevis* animal caps combined with each tissue. In *E. coqui*, there was little inducing activity in early blastula. In the late blastula, mesoderm-inducing activity was seen in the marginal and submarginal zones. Cells of neither the vegetal pole nor the blastocoel floor had activity, although these same regions from *X. laevis* blastulae induced mesoderm. In the early gastrula of *E. coqui*, mesoderm-inducing activity remained confined to the marginal and submarginal zones, with strong activity in the outer layer of cells (ODM, ODSM, OVM/SM). Mesoderm-inducing activity extended to deeper cells in the *X. laevis* early gastrula, although the activity was weaker than the outer layer of the cells. Neural-inducing activity was present on the dorsal side, including deep cells, which did not have mesoderm-inducing activity in *E. coqui*. In the *X. laevis* early gastrula, neural-inducing activity was restricted to the dorsal marginal zone. AC, animal cap; BF, blastocoel floor; DBF, dorsal blastocoel floor; DM, dorsal marginal zone; DSM, dorsal submarginal zone; DVP, dorsal vegetal pole; IDSM, inner dorsal submarginal zone; IVM/SM, inner ventral marginal zone/submarginal zone; M, marginal zone; OAC, outer animal cap; ODM, outer dorsal marginal zone; ODSM, outer dorsal submarginal zone; OVM/SM, outer ventral marginal zone/submarginal zone; SM, submarginal zone; VBF, ventral blastocoel floor; VM, ventral marginal zone; VSM, ventral submarginal zone; VP, vegetal pole; VVP, ventral vegetal pole.



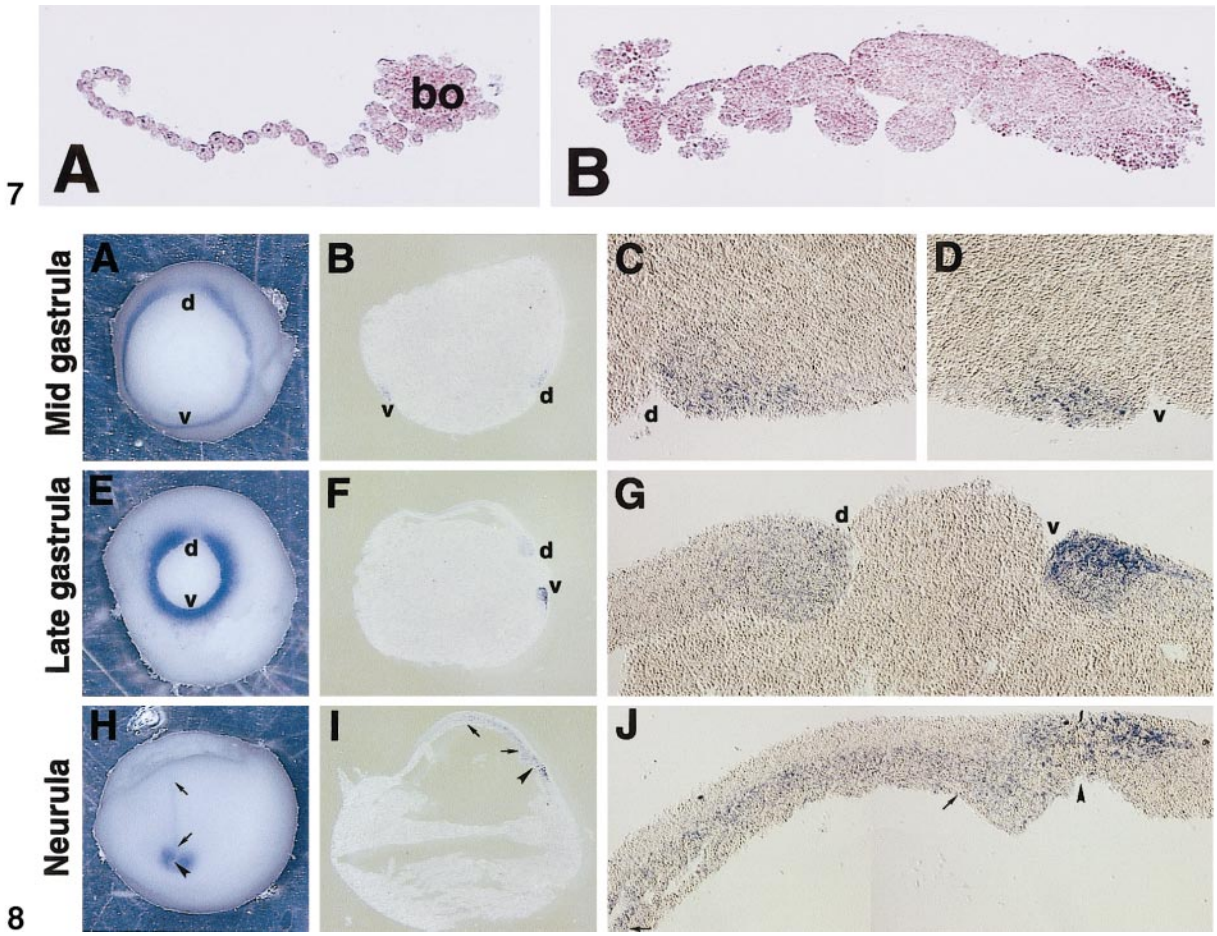


FIG. 7. Isolation of outer cells from *E. coqui* early gastrulae. (A) Outer dorsal marginal zone (ODM) cells were fixed immediately after isolation by Ca^{2+}–Mg^{2+}-free treatment. Although the bottle cells (bo) made a clump, the isolate consisted of a monolayer. (B) Outer dorsal submarginal zone (ODSM) cells were fixed immediately after manual isolation. The large cells were, for the most part, a monolayer. Animal is to the left, and the outer surface is uppermost.

FIG. 8. *In situ* hybridization of *Ecbra*. Detection of *Ecbra* expression was done on both whole mounts (A, E, H) and on sagittal sections (B–D, F, G, I, J). The stages shown are midgastrula (A–D), late gastrula (E–G), and neurula (H–J). (A) At midgastrula, *Ecbra* was expressed as a marginal ring with slightly weaker expression dorsally (d). (B–D) On sections, *Ecbra* expression was restricted to a small group of cells on the dorsal (d) and ventral (v) blastopore lips. (E) At late gastrula, the expression in the dorsal marginal zone was clearly weaker than the ventral (v)/lateral marginal zone. (F, G) The d/v difference was clearly visible in section. (H) At neurula, weak notochord expression (arrows) was detected in addition to circumblastoporal expression (arrowhead). (I, J) Sections confirm that *Ecbra* expression was weaker in the prospective notochord (arrows) than on the ventral side of the blastopore (arrowhead).

DISCUSSION

Location of Germ-Layer Formation

In *X. laevis*, the first horizontal cleavage usually occurs at the 8-cell stage and the second horizontal cleavage occurs at the 32-cell stage. In *E. coqui*, the first horizontal cleavage occurs at the 16-cell stage close to the animal pole. As a result, eight small animal blastomeres form, which contain roughly 1% of total egg volume. This relative volume is far smaller than that of the eight animal blastomeres (A tier) of the *X. laevis* 32-cell stage embryo, which contain 12–15% of total egg volume (Dale and Slack, 1987). Despite their small relative volume, most of the blastocoel roof in *E. coqui* is derived from the eight animal blastomeres of the 16-cell embryo (Fig. 4A). Conversely, cells derived from the eight animal blastomeres were never seen on the blastocoel floor. This result indicates that the first horizontal cleavage in *E. coqui* goes through the position of the blastocoel, just as the first horizontal cleavage at the 8-cell stage does in *X. laevis*.

In the TS6 embryo, significant amounts of mesodermal tissues were derived from the animal blastomeres of 16-cell

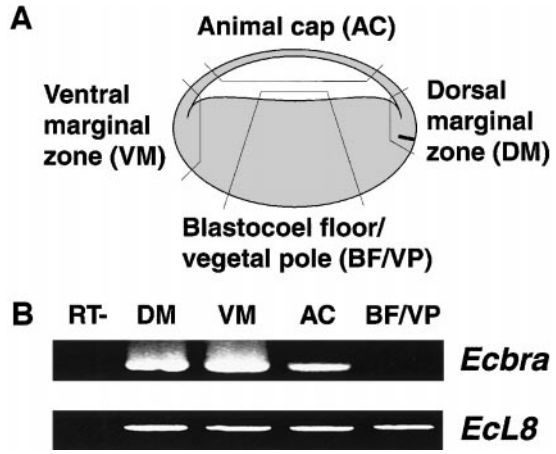


FIG. 9. RT-PCR analysis of *Ecbra* expression. (A) Schematic diagram of dissection. The early/midgastrula (stage 10.5 of *X. laevis*) was dissected into four pieces. (B) Each piece was analyzed for *Ecbra* expression by RT-PCR, using *EcL8* as an internal control. In this case, *Ecbra* was expressed strongly in both dorsal (DMZ) and ventral (VMZ) marginal zones, weakly in animal cap (AC), but not in the blastocoel floor/vegetal pole (BF/VP). Neither *Ecbra* nor *EcL8* were detected when reverse transcriptase was omitted (RT−).

stage embryo. For instance, 10–50% of trunk notochord and trunk somites was derived from the animal blastomeres. In *X. laevis*, Dale and Slack (1987) found similar amounts of these tissues from the four animal blastomeres at the 8-cell stage, with less mesoderm from the eight animal blastomeres at the 32-cell stage. Moody (1987), on the other hand, detected considerably more mesoderm from the eight animal blastomeres at the 32-cell stage. Regardless of the differences in these reports, the relative volume of the eight animal blastomeres of the *E. coqui* 16-cell embryo is far smaller than the relative volume of the eight animal blastomeres of the *X. laevis* 32-cell embryo. Based on these comparisons, the prospective mesoderm region in *E. coqui* appears to be located geometrically more animally than that in *X. laevis*. This conclusion is complicated, however, by the cell mixing that occurs among animal cells, and, as discussed in the next section, other support for this conclusion is provided by considering the location of mesoderm-inducing activity.

In *E. coqui*, the dorsal or posterior mesoderm tended to contain more cells from the animal blastomere lineage than the ventral or anterior mesoderm. This result coincides with the data of Dale and Slack (1987) and the recent report of Lane and Smith (1999), in which the somites come from a more animal region than the blood in *X. laevis*. The prospective regions of the various mesodermal tissues in *E. coqui* appear to be basically similar to that in *X. laevis*.

Location of Mesoderm-Inducing Activity

Extensive cell mixing occurred in the blastocoel roof of the *E. coqui* blastula as it does in *X. laevis* (Bauer *et al.*, 1994). This indicates that germ-layer formation in both species is likely the result of cell–cell interactions and that the contribution of the progeny of the animal blastomeres to the mesoderm does not reflect the existence of maternal mesoderm determinants in these cells. Consequently, the distribution of mesoderm-inducing activity would more accurately reflect the maternal contribution to germ-layer formation. In our recombinant analysis, mesoderm-inducing activity in *E. coqui* was present in the marginal zone but lacking in the cells of the blastocoel floor and the vegetal pole. Strong activity was present in these same regions in *X. laevis*. Therefore, mesoderm-inducing activity in *E. coqui* was localized relatively more animally and superficially than in *X. laevis*.

In *E. coqui*, the outermost cells around the marginal zone had strong mesoderm-inducing activity. Similarly, in *X. laevis*, Shih and Keller (1992) reported that the dorsal lip epithelium has strong organizer activity. *Xnr3* has been suggested as the source of this activity, since it expressed in dorsal lip epithelium (Smith *et al.*, 1995; Glinka *et al.*, 1996). *Xnr3* is a target gene of the dorsal determinant and is expressed only on the dorsal side (McKendry *et al.*, 1997). However, we detected strong mesoderm-inducing activity in both dorsal and ventral outer cells. This activity was also seen in *X. laevis*, although the difference between outer and inner cells was not as clear as in *E. coqui*. Therefore, the strong mesoderm-inducing activity in the outer epithelial cells must be caused by a mesoderm/endoderm determinant rather than the dorsal determinant or one of its downstream targets like *Xnr3*.

Recently, *VegT*, a T-box transcription factor, was identified as a mesoderm/endoderm determinant in *X. laevis* (Zhang *et al.*, 1998; Kofron *et al.*, 1999; Xanthos *et al.*, 2001). *VegT* RNA is localized to the vegetal pole of the *X. laevis* oocyte (Zhang and King, 1996) and is responsible for initiating mesoderm induction after the midblastula transition (MBT; Kofron *et al.*, 1999). Although an MBT has not been defined for *E. coqui*, our work indicates that mesoderm induction did not start until late blastula. This result suggests that a maternal transcription factor like *VegT* controls mesoderm induction in *E. coqui*. If this were the case, we predict that VegT in *E. coqui* is likely to be localized to the cortex of the marginal zone rather than the vegetal pole, since no inducing activity was found in the vegetal pole and the blastocoel floor in *E. coqui*. This hypothesis can be tested by cloning the *E. coqui* homologue of *VegT* and determining where its RNA is localized in the oocyte.

We had previously shown that injection of *X. laevis noggin* RNA into *E. coqui* embryos was more likely to yield complete secondary axes with heads than was found for *noggin* injections into *X. laevis* (Fang *et al.*, 2000). Fang *et al.* (2000) hypothesized that this effect may be due to a broader distribution of the dorsal determinant, but our present results suggest another hypothesis for the enhanced activity of *noggin* in *E. coqui*. Mesoderm-inducing activity is present throughout the vegetal region in *X. laevis*, but is

restricted to more superficial regions in *E. coqui* (Fig. 6). If *noggin* acts differently in cells without mesoderm-inducing activity compared to those with it, then this other action could occur in *E. coqui* but not in *X. laevis*. In some way, this action of noggin in the absence of mesoderm-inducing activity would cause head formation.

Expression of Ecbra

The gastrula of *E. coqui* is much larger than that of *X. laevis* (Fig. 1), raising the possibility that mesoderm forms mainly associated with the body axis on the dorsal side of this large embryo. Our results from the recombinants indicate that this is not the case, since mesoderm-inducing activity is found on the ventral side as well as the dorsal side. Similarly, *Brachyury* (*Ecbra*), an early mesoderm marker, is expressed around the entire marginal zone in *E. coqui*, the same as in *X. laevis* (Smith *et al.*, 1991; Gont *et al.*, 1993).

There are several differences between expression of *Ecbra* and its *X. laevis* homologue *Xbra*. First, the expression of *Ecbra* was significantly weaker in the most dorsal area of the marginal ring. This interruption in the ring has generally not been reported for *Xbra*. Second, *Ecbra* was less strongly expressed in the notochord compared to the lateral and ventral marginal zone, a pattern not noted for *Xbra*. Finally, *Xbra* is expressed in the presumptive notochord as an anteriorly extended portion of the marginal ring in the gastrula (Gont *et al.*, 1993). In *E. coqui*, however, this elongated notochord expression was only seen after blastopore closure. The same delay in *Brachyury* expression associated notochord elongation was also seen in another frog with large eggs, *Gastrotheca riobambae* (del Pino, 1996).

A potential explanation for these differences lies in the demonstration that the promoter regulating *Xbra* in the lateral and ventral marginal zone is different from the promoter regulating *Xbra* in the dorsal marginal zone and notochord (Lerchner *et al.*, 2000). When only the lateral and ventral marginal zone promoter is used to drive expression of the gene for green fluorescent protein (GFP), GFP RNA is expressed in a marginal ring with a lack of expression on the dorsal side. This pattern is similar to the endogenous expression that we found for *Ecbra*. In *X. laevis*, the timing and the strength of *Xbra* expression driven by these different promoters would be matched. This would give expression of a complete ring. Conversely, in *E. coqui*, expression from these different putative promoters would not be matched, resulting in the lower dorsal expression and the lower expression in the notochord relative to the lateral and ventral marginal ring. The later *Ecbra* expression associated with notochord elongation may be due to the promoters, but it may also be due to the later morphogenetic movement of the notochord relative to blastopore closure. Indeed, the anterior expansion of the archenteron is temporally dissociated from blastopore closure in *G. riobambae* (del Pino and Elinson, 1983; Elinson and del Pino, 1985; del Pino, 1996), showing that the relative timing of gastrulation events can be different in different species.

Evolutionary Consequences of Large Egg Size

Our study shows that mesoderm formation in *E. coqui* is biased more animally and superficially compared to *X. laevis*. This bias may reflect the asymmetric addition of yolk in the evolution of the *E. coqui* egg, leading to its increased size. The increased yolk supply is a derived condition associated with the lack of a feeding tadpole in this direct developer (Callery *et al.*, 2001). If yolk were added vegetally and centrally, the mesoderm-forming region would shift animally and superficially. Alternatively, the shift may have a phylogenetic basis, a possibility that would require determination of the location of mesoderm-inducing activities in embryos of other anurans. The animal shift of the mesoderm-forming region is not a necessary consequence of an increase in vegetal yolk. *G. riobambae* also has large eggs (3 mm). While the body of its embryo appears to form more superficially than in *X. laevis*, blastopore lip formation and mesoderm development in *G. riobambae* occur relatively closer to the vegetal pole than even *X. laevis* (del Pino and Elinson, 1983; del Pino, 1996).

Amphibians exhibit complete or holoblastic cleavage, and they gastrulate through the blastopore. Amniotes, such as birds and reptiles, exhibit incomplete or meroblastc cleavage, and they gastrulate through a primitive streak and Hensen's node. The amphibian mode of development is thought to be primitive for terrestrial vertebrates, with the evolution of the amniote egg associated with a large increase in yolk (Romer, 1957; Carroll, 1970; Elinson, 1989; Collazo *et al.*, 1994; Packard and Seymour, 1997; Arendt and Nübler-Jung, 1999). Arendt and Nübler-Jung (1999) have outlined a hypothetical series of developmental changes that may have occurred as the amniote egg evolved. Central to their hypothesis is the preferential vegetal accumulation of yolk and the shifting of the fate map animally. Our analysis of mesoderm formation in *E. coqui* suggests that alterations of early development in *E. coqui* due to large egg size may parallel events that occurred 350 million years ago when the amniote egg arose.

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