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Expression of Axolotl DAZL RNA, a Marker of Germ Plasm: Widespread Maternal RNA and Onset of Expression in Germ Cells Approaching the Gonad

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How germ cell specification occurs remains a fundamental question in embryogenesis. The embryos of several model organisms contain germ cell determinants (germ plasm) that segregate to germ cell precursors. In other animals, including mice, germ cells form in response to regulative mechanisms during development. To investigate germ cell determination in urodeles, where germ plasm has never been conclusively identified, we cloned a *DAZ*-like sequence from axolotls, *Axdazl*. *Axdazl* is homologous to *Xdazl*, a component of *Xenopus* germ plasm found in the vegetal pole of oocytes and eggs. *Axdazl* RNA is not localized in axolotl oocytes, and, furthermore, these oocytes do not contain the mitochondrial cloud that localizes *Xdazl* and other germ plasm components in *Xenopus*. Maternal *Axdazl* RNA is inherited in the animal cap and equatorial region of early embryos. At gastrula, neurula, and tailbud stages, *Axdazl* RNA is widely distributed. *Axdazl* first shows cell-specific expression in primordial germ cells (PGCs) approaching the gonad at stage 40, when nuage (germ plasm) appears in PGCs. These results suggest that, in axolotls, germ plasm components are insufficient to specify germ cells. © 2001 Academic Press

Key Words: **urodele; axolotl; *Xenopus*; germ plasm; primordial germ cell (PGC); *DAZ*-like; nuage.**

INTRODUCTION

A fundamental problem in metazoan development is how germ cells are segregated from somatic cells. Work with model systems has shown that at least two different basic strategies have evolved in animals to regulate germ cell determination in embryos. In *Drosophila*, *Caenorhabditis elegans*, and *Xenopus* embryos, the germ line is predetermined by the differential segregation of maternal germ cell determinants into specific blastomeres during early development (Rongo *et al.*, 1997; Seydoux and Strome, 1999; Houston and King, 2000). In *Drosophila* and *Xenopus*, germ cell determinants are aggregated into a composite structure called germ plasm (or pole plasm in *Drosophila*), which is localized to a specific region in the posterior or vegetal pole of eggs. Ultrastructural studies have shown that germ plasm in *Drosophila* and *Xenopus* is very similar, containing large numbers of mitochondria, ribosomes, and structures called germinal granules that are exclusively found in germ cells (Mahowald and Hennen, 1971). During the cleavage stages of *Xenopus* embryos, germ plasm is segregated to specific blastomeres in the presumptive endoderm, and those cells that inherit germ plasm ultimately give rise to primordial germ cells (PGCs), the founder cells of the germ line (see Davidson, 1986; Wylie, 1999). By gastrulation, the germ plasm has transformed into fibrillar–granular material called nuage, positioned adjacent to the nucleus. More recent work shows that *Xenopus* germ plasm is partly composed of specific, highly conserved messenger RNAs that become associated with the mitochondrial cloud early in oogenesis, and as a result are transported to the vegetal cortex of growing oocytes (Mosquera *et al.*, 1993; Zhou and King, 1996; Houston *et al.*, 1998; Hudson and Woodland, 1998). Little is known about how these mRNAs contribute to the role of germ plasm as a determinant of the germ cell lineage.

The alternative mode of germ cell determination does not

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involve inheritance of maternal factors; instead, in embryos such as sea urchins (Ransick *et al.,* 1996) and mice, germ cell specification is controlled by regulative mechanisms acting on cells that are not predisposed to a germ cell fate. In mouse embryos, cells from ectopic locations, which normally would contribute to other lineages, can be made to enter the germ line if transplanted to the normal site of germ cell precursors (Tam and Zhou, 1996), indicating that germ cell specification is regulated by extracellular signaling. BMP-4 induces an extraembryonic mesoderm-germ cell lineage, and it is hypothesized that a second signal controls the final segregation of germ cells (Lawson *et al.,* 1999; see McLaren, 1999). Germ plasm has not been observed in rodent eggs or preimplantation embryos; however, material resembling germ plasm, or nuage, is present in the PGCs in the hindgut of early somite embryos (Eddy, 1975) about a day after germ cell specification takes place (Lawson and Hage, 1994).

In most anurans, germ cells are apparently determined by a mechanism similar to that of *Xenopus* (Blackler, 1970). However, in the embryos of urodeles (salamanders), the other major branch of the amphibian lineage, germ cells develop from cells in the marginal zone apparently by the regulative mode. A combination of cell fate, deletion, and transplantation studies demonstrates that the PGCs of several urodele species derive from cells located in the ventral marginal zone (VMZ) of gastrula stage embryos; these cells later migrate over the ventral blastopore lip in association with presumptive posterior lateral plate mesoderm (Humphrey, 1925, 1929; Nieuwkoop, 1947; Smith, 1964; Maufroid and Capuron, 1972; Ikenishi and Nieuwkoop, 1978). Furthermore, Nieuwkoop (1969) observed in his original studies on the induction of mesoderm that PGCs could be induced in animal caps of axolotl embryos by signals from the vegetal region. In further work, it was shown that the ventral vegetal region is capable of inducing PGCs, along with somatic ventral mesoderm such as blood and mesenchyme; moreover, germ cells could be induced in the animal pole, or the dorsal side of the cap (sites that would not normally produce germ cells), although at a lower frequency than in the ventral marginal zone (Boterenbrood and Nieuwkoop, 1973; Sutasurja and Nieuwkoop, 1974; Michael, 1984; Maufroid and Capuron, 1985). Thus, as in mammals, the urodele germ line apparently is produced from a pool of totipotent precursors capable of commitment to somatic development or germ cell development, depending on the signaling environment. How germ cells are specified within the milieu of other ventral mesoderm derivatives is unclear.

In spite of the evidence for induction of germ cells in urodeles, material resembling components of germ plasm has been found in the equatorial region of axolotl (*Ambystoma mexicanum*) eggs (Williams and Smith, 1971) and in the VMZ of axolotl early gastrulae (Smith *et al.,* 1983), suggesting that germ plasm could confer competence for induction (see Michael, 1984). However, Ikenishi and Nieuwkoop (1978) examined PGCs from tailbud stage 23 to larval stage 45 and did not detect nuage until stage 40, when PGCs are in the vicinity of the gonads. The appearance of nuage long after the PGCs can be identified by other histological criteria suggests that germ plasm is not present earlier and does not play a determinative role in germ cell development.

To address whether in urodele embryos the PGCs develop from predetermined germ cell precursors (as they do in frogs) or from unspecified embryonic cells (as they do in mice), we isolated a cDNA clone encoding the axolotl *DAZ*-like gene, *Axdazl*, to use as a molecular marker for germ cells. The *DAZ-like* genes encode an RNA binding protein and members of the gene family have been cloned from *C. elegans* (Karashima *et al.,* 2000), *Drosophila* (Eberhart *et al.,* 1996), zebrafish (Maegawa *et al.,* 1999), *Xenopus* (Houston *et al.,* 1998), mouse (Cooke *et al.,* 1996), and humans (Saxena *et al.,* 1996), representing animals with predetermined and regulative germ lines. Gene knockout and function-blocking experiments demonstrate that the *DAZ*-like gene is essential for meiosis or gametogenesis in several systems (Eberhart *et al.,* 1996; Ruggiu *et al.,* 1997; Karashima *et al.,* 2000). In *Xenopus*, its product is required for early germ cell development during migration toward the gonad (Houston and King, 2000). Significantly, RNA from the *Xenopus* DAZ-like gene, *Xdazl*, is a component of germ plasm at all stages of oogenesis and embryogenesis (Houston *et al.,* 1998), and therefore provides a useful molecular marker for germ plasm during development. Here, we show that maternal *Axdazl* RNA is present through most of embryogenesis. But, in contrast to *Xdazl* RNA in *Xenopus*, *Axdazl* RNA is not localized in oocytes, or in embryos through the tailbud stages. Cell-specific *Axdazl* expression is first observed in PGCs as they approach and enter the gonad. These results argue against the possibility that germ plasm components directly determine germ cells in urodele embryos.

MATERIALS AND METHODS

Adults and Embryos

Adult animals from our colony at FSU were anesthetized in MS222. Ovarian material for *in situ* hybridization experiments was removed from a fully grown adult female axolotl. Embryos were obtained from the Axolotl Colony at Indiana University, or they were obtained from spawnings performed with animals from our colony. Embryos were maintained in 20% Holtfreter's until desired stages. For embryo dissections, embryos were maintained in NAM (Peng, 1991), and dissected parts were collected on dry ice. All staging was done according to Bordzilovskaya *et al.* (1989). This staging system is similar to that used for *Xenopus* embryos (Nieuwkoop and Faber, 1967).

RNA Extraction

RNA was extracted from adult tissues by the LiCl/Urea method (Auffray and Rougeon, 1980), with further purification by overnight precipitation of RNA in 4 M LiCl. RNA was quantified by absorp-

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tion at 260 nm. In all experiments to detect embryonic expression, RNA was extracted from groups of 5–10 embryos of each stage by using Trizol Reagent (Life Technologies), according to manufacturer's suggestions. Further purification from contaminating polysaccharides was achieved by overnight precipitation in 4 M LiCl.

cDNA Preparation

From adult tissues, 5 μg of total RNA was reverse transcribed using an oligo(dT) primer and MuLV reverse transcriptase (New England BioLabs) under standard conditions (Sambrook *et al.,* 1989). Single embryo equivalents of purified embryo RNA were used in equivalent reactions to detect embryonic expression.

Cloning of Axdazl cDNA

In order to recognize conserved amino acid sequences [GVSKGYGF and P(V/I)TQ(H/Y)VQA] present in the products of *Xdazl* (Houston *et al.,* 1998), *dazla* (Cooke *et al.,* 1996), and *boule* (Eberhart, 1996), degenerate oligodeoxynucleotide primers were designed with the following sequence: forward primer, GGIGTI(A/T)(G/C)IAA(G/A)GGITA(T/C)GGITT; reverse primer, GC(C/T)TGIAC(A/G)T(A/G)(C/T)TGIGTIA(T/C)IGG. These were reacted with cDNA produced from the reverse transcription of 5 μg of total RNA isolated from adult axolotl testis in a PCR performed according to a Touchdown protocol (Roux and Hecker, 1997) using 68°C and 43°C as the extreme high and low annealing temperatures, respectively. A DNA fragment of the expected 220-bp size was eluted from an acrylamide gel, reamplified by PCR, and cloned. After sequence verification, this fragment was used to screen 10^6 plaques from a phage lambda gt11 stage 18 embryo cDNA library (Busse and Seguin, 1993) at high stringency. Inserts from purified plaques were amplified by PCR and cloned into the *Eco*RI site of pBluescript. Full-length sequencing was by primer extension. Identity of *Axdazl* cDNA was verified by sequence comparison with the database.

Expression Analysis by RT-PCR

RNA detection by RT-PCR was carried out using 1/20th of a reverse transcription reaction from either embryo or adult material as described above. PCR was performed by using an empirically determined number of cycles. In all cases, PCR products were separated on 1% agarose gels containing ethidium bromide. Reactions whose products were not visible under UV illumination were considered to be linear and were then Southern blotted to nylon filters and hybridized with random-primed probe prepared from cloned sequences. Cloned fragements encoding *Axgsc* (orthologous to *goosecoid*; Blumberg *et al.,* 1991), *Axbrn-1* (homologous to *brn-1.1*; Sampath, and Stuart, 1996), *Axszl* (orthologous to *sizzled*; Salic *et al.,* 1997), and *Axvg1* (orthologous to *Vg1*; Weeks and Melton, 1987) were cloned by RT-PCR using degenerate primers. The specific expression profile of these sequences, used as controls in these experiments, will be reported elsewhere. The primers and annealing temperatures used for PCR amplification in the experiments reported below were as follows: *Axdazl* For., GGAACTCTTTTGTATCTCAGGAGG; *Axdazl* Rev., GGAGACAGGCACATACAATCAACC, 64°C; *Axgsc* For., CTATATGAATGTGGGCACGC; *Axgsc* Rev., TCGGAGTTGCACCCTGCGGG, 55°C; *Axbrn-1* For., GCACTCCGACGAAGACACC; *Axbrn-1* Rev., CAACCGGCGACAGAAGGAG, 57°C; *Axszl* For., AAGATGACGCGCAGCAACC; *Axszl* Rev., GCACATTGGAAGGCAGCAC, 55°C; *AxEF-1α* For., GGTGTTGGACAAGCTGAAGG; *AxEF-1α* Rev., CGTGCCAGCCAGAGATTGG, 57°C; *Axvg1* For., AACAGCTGCGTTCCTTTTGC; *Axvg1* Rev., GGTCAGTCCAATCTTTTCACG, 55°C.

All annealing, melting, and extension reactions were for 1 min each. Melting was performed at 94°C, and extension was performed at 72°C.

In Situ Hybridizations

Digoxigenin-labeled RNA probes were transcribed from linearized cloned templates according to standard methods (Harland, 1991). Transcripts were purified over ProbeQuant columns (Pharmacia), and quantified by absorption at 260 nm. *Axdazl* probe represents a full-length antisense transcript of about 2100 bases, and the sense strand *Axwnt-8* control probe is approximately 1750 bases.

In situ hybridization to sections was used instead of whole-mount *in situ* hybridization, since it allows better penetration of the probe into the relatively large axolotl oocytes and embryos, and since cleavage and gastrula axolotl embryos are fragile and often break up during the whole-mount procedure. Material was fixed in MEMFA and dehydrated in MeOH as described by Harland (1991). Material was embedded in paraffin plus 2% beeswax and cut into 15-μm sections. *In situ* hybridization to sections was performed by using digoxigenin-labeled RNA probes according to the method of Henrique *et al.* (1995), except that colorimetric detection was with BM-purple (Boehringer–Mannheim).

Collection of Amphibian Oocytes

Large adult axolotl or *Xenopus laevis* females were immersed in 0.1% (w/v) aqueous solution of tricaine sulphonate (MS 222) and were sacrificed by rapid decapitation. Ovaries were minced and digested in 0.1% collagenase. Liberated oocytes were washed extensively in OR2. *Xenopus* oocytes were staged according to Dumont (1972), and axolotl oocytes were staged according to Beetschen and Gautier (1989). It is necessary to use both staging systems because axolotl oocytes are much larger than *Xenopus* oocytes. In both systems, vitellogenesis begins at stage II.

Fluorescence and Confocal Studies

Individual axolotl and *Xenopus* oocytes were incubated in 500 nM MitoTracker Red (Molecular Probes, Eugene, OR) dissolved in OR2 for 0.5 h. Labeled oocytes were washed with PBS and fixed in MEMFA. Whole oocytes were washed in PBS and examined with a Zeiss 1045 quantitative fluorescence microscope. Confocal images were obtained with a Zeiss LSM 410 laser scanning microscope. All images were acquired by using a 25× oil immersion objective and z-scan intervals were at 3.5 μm.

RESULTS

Axdazl Encodes a Protein That Is Highly Related to the Product of the Mouse DAZ-like Gene

Sequences encoding members of the *DAZ*-like family of gene products have now been cloned from several species and these encode proteins with highly conserved motifs. To amplify a DNA fragment encoding the axolotl *DAZ*-like

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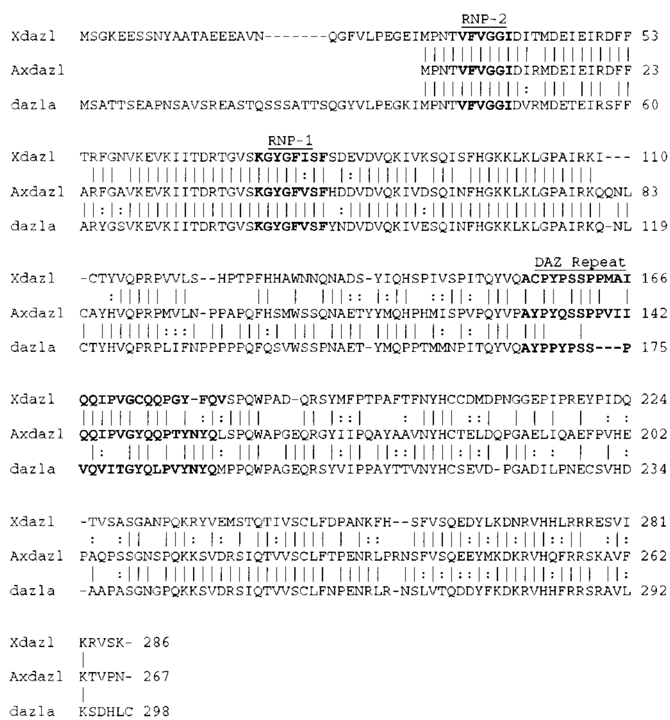

FIG. 1. Amino acid sequence comparison of the products of *Axdazl* (axolotl) with *Xdazl* (*Xenopus*) and *dazla* (mouse). The deduced amino acid sequences of the axolotl (*Axdazl*), *Xenopus* (*Xdazl*), and mouse (*dazla*) *DAZ*-like gene products are aligned. *Axdazl* encodes a protein that is 70% identical and 81% similar to the product of *dazla*, and 60% identical, 71% similar, to the product of *Xdazl*. *Xdazl* is 59% identical, 77% similar to *dazla*. RNP-1 and RNP-2 binding domains conserved in other *DAZ*-like proteins, and a region homologous to the *DAZ* repeat found in the human Y-linked *DAZ* gene are indicated by bold lettering. Identical amino acids are indicated by vertical lines. Conservative substitutions are indicated by dotted lines.

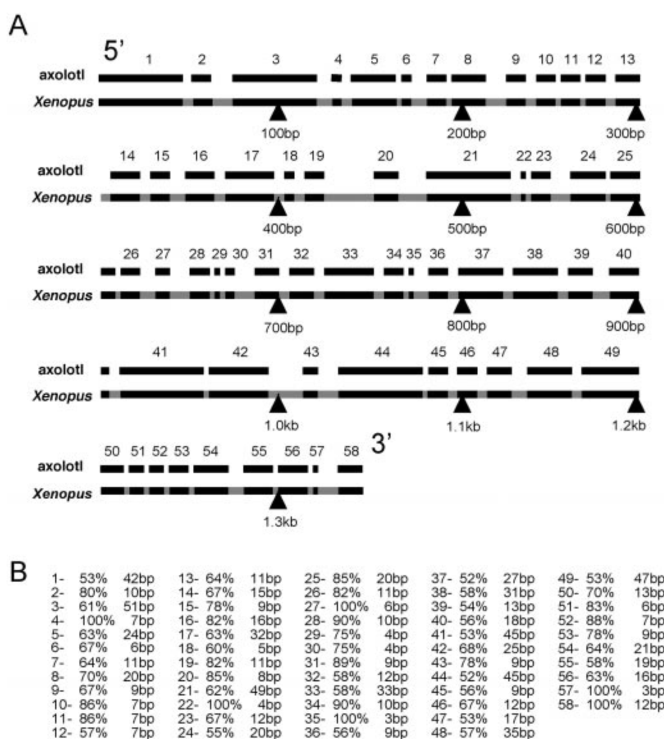

FIG. 2. Sequence organization within the 3′ UTR of *Axdazl* and *Xdazl*. (A) The 3′ untranslated regions (3′ UTR) within the mRNAs encoding *Axdazl* and *Xdazl* are aligned. The entire *Axdazl* 3′ UTR is represented on the top line. Spaces indicate positions that do not align with the *Xenopus* sequence. On the bottom line is the *Xdazl* 3′ UTR. Black segments indicate regions with homology to the axolotl sequence. Gray segments indicate unique regions of sequence that do not align with the axolotl 3′ UTR. (B) The length of each segment of conserved sequence and the percentage of identical bases.

sequence, we designed degenerate PCR primers to recognize all possible codons encoding the conserved peptide sequences GVSKGYGF and P(V/I)TQ(H/Y)VQA found in each of the reported *DAZ*-like homologs. PCR was performed by using a "touchdown" protocol on cDNA prepared from RNA of adult testis. These reactions produced a smear with a single band of the expected 220-bp size, which was cloned and showed strong homology to other *DAZ*-like sequences. The cloned DNA fragment was used to screen 10^6 plaques from a stage 18 cDNA library (Busse and Seguin, 1993). We identified two clones that reacted at high stringency. One of these, which we call *Axdazl*, was subcloned and sequenced to completion. The *Axdazl* cDNA insert is 2.1 kb in length and closely approximates the reactive RNA species on Northern blots (data not shown), so we assume that the insert is full length. Members of the *DAZ* gene family encode RNA binding proteins. The RNP-1 and RNP-2 RNA recognition motifs (Burd and Dreyfuss, 1994), which are

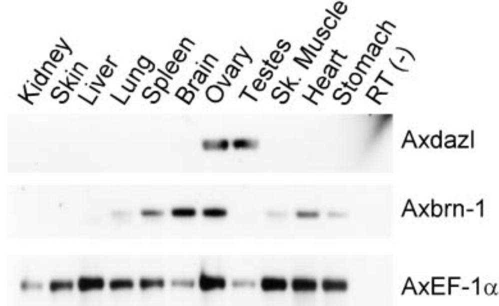

FIG. 3. *Axdazl* is expressed only in gonadally derived tissue in adults. Equal amounts of total RNA from the indicated tissues of an adult axolotl were reverse transcribed and used as a template to amplify the indicated sequences. RT-PCR was performed under identical conditions, within the linear range of amplification. Products were detected as described in Materials and Methods. *Axdazl* RNA is detected only in material from testis and ovary. *Axbrn-1* and *AxEF-1α* show a broader distribution of expression.

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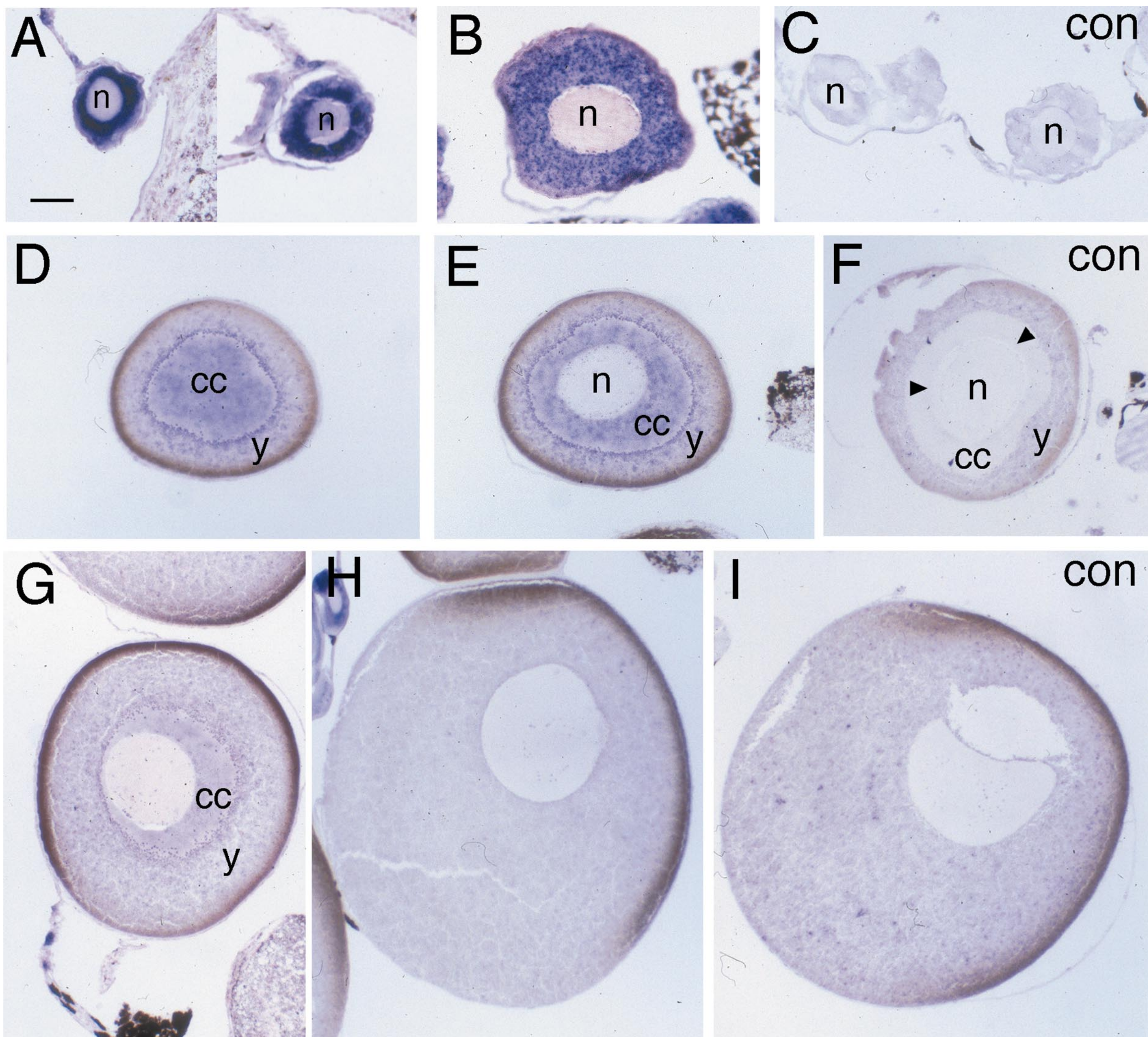

FIG. 4. *Axdazl* RNA is not localized in the cytoplasm of oocytes. Sections of fragments of adult ovaries were hybridized to an antisense *Axdazl* digoxigenin-labeled probe except where indicated. (A) Two different, small stage I oocytes showing a ring of dark staining cytoplasm surrounding the large pale nucleus. (B) A larger stage I oocyte showing stained cytoplasm surrounding the pale nucleus. This section was counterstained with safranin. (C) Section hybridized to a control sense *Axwnt-8* probe, showing two small stage I oocytes (arrows) with little cytoplasmic stain. (D, E) Sections of the same stage III oocyte. (D) The section passes through the central blue stained cytoplasm, but not the nucleus. (E) The section shows the central blue stained cytoplasm as a ring around the pale staining nucleus. Comparison with (F) shows that the *Axdazl* staining is primarily in the central layer of cytoplasm surrounding the nucleus. (F) A stage III oocyte hybridized to the sense probe. The nucleus (arrowheads) is surrounded by a strip of pale central cytoplasm and an outer strip of darker yolky cytoplasm. (G) A stage IV oocyte. The yolky layer takes up most of the cytoplasm. Light staining is still seen in the central cytoplasm surrounding the nucleus. (H) A stage V oocyte 1650 μm in diameter, showing little staining above background. Fully grown oocytes at stage VI are about 2000 μm in diameter. (I) A stage V oocyte hybridized to the sense probe. Scale bar: 100 μm (A–C); 200 μm (D–I). cc, central cytoplasm; n, nucleus; y, yolky layer of cytoplasm.

conserved in all *DAZ* family members, are indicated in bold (Fig. 1). The conserved *DAZ* motif, which is encoded seven times in the human *DAZ* gene (Reijo *et al.,* 1995), but is present only once in the *DAZ*-like genes, is also indicated.

The amino acid sequence encoded by *Axdazl* is aligned in Fig. 1 with the protein sequence of other *DAZ*-like related genes. Surprisingly, *Axdazl* encodes a protein that shows significantly greater relatedness to the *dazla* gene product from mice (Cooke *et al.,* 1996) than it does with *Xdazl*, the corresponding *DAZ-like* gene product from *Xenopus* (Hous-

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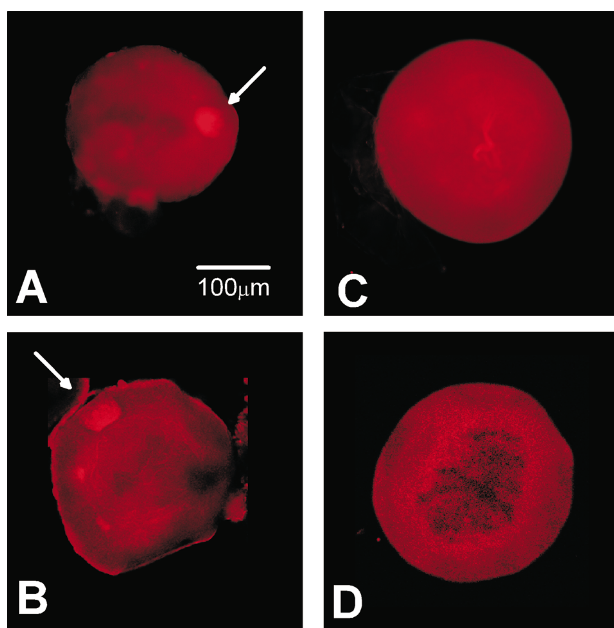

FIG. 5. Previtellogenic axolotl oocytes do not have a mitochondrial cloud. Previtellogenic axolotl oocytes ranging from 170 to 390 μm in diameter (Stage 1, Beetschen and Gautier, 1989), and *Xenopus* oocytes ranging from 140 to 290 μm (Stage 1–3, Dumont, 1972) were incubated in MitoTracker Red and examined either by Quantitative Fluorescence Microscopy (A, C) or as optical sections with a confocal microscope (B, D). (A, B) Examples of *Xenopus* oocytes showing large aggregates of mitochondria corresponding to the mitochondrial cloud (arrow). (C, D) Examples of axolotl oocytes lacking a mitochondrial cloud.

ton *et al.,* 1998). The axolotl and mouse proteins are identical at 70% of the amino acid positions, the axolotl and *Xenopus* proteins share 60% identity. This result was unexpected in view of the phylogenetic relationships of these animals. Typically, gene sequences can be used as direct measures of phylogenetic distance (Hillis *et al.,* 1996), and on this basis it would be assumed that the sequences from axolotl and *Xenopus*, which are both members of the order amphibia, would share a higher degree of homology.

Xdazl RNA is one of several messenger RNAs that are components of the germ plasm in *Xenopus* oocytes (Houston *et al.,* 1998; King *et al.,* 1999). Germ plasm-specific RNAs become associated with the mitochondrial cloud during the early stages of oogenesis. Mediated by sequences in the 3′ untranslated regions (3′ UTR), these RNAs are transported to the vegetal cortex via the METRO RNA localization pathway (Zhou and King, 1996; Kloc *et al.,* 1998, 2000). We compared the 3′ UTR from *Xdazl* (1351 bases) and *Axdazl* (938 bases), and the results (Fig. 2) show that the entire 3′ UTR of *Axdazl* is conserved within the larger 3′ UTR of *Xdazl*. However, the conserved sequences are interspersed with blocks of sequence unique to *Xdazl*. The significance of this sequence structure remains to be elucidated.

In Adults, Axdazl Is Expressed Only in Gonadal Tissue

The founding member of the *DAZ* gene family was originally identified as the gene mutated in the condition known as Deleted in Azoospermia in humans, and is located on the Y chromosome (Reijo *et al.,* 1995). Each of the related sequences isolated from nonprimates, the *DAZ*-like genes, is expressed from autosomes in both male and female germ lines. To examine the expression profile of *Axdazl* in adult tissues, RT-PCR was performed on cDNA prepared from various organs under conditions that would amplify *Axdazl*, or the indicated control sequences. *Axdazl* RNA is found in adult testis and ovary, but not in any of the somatic tissues tested (Fig. 3). As a control, we performed reactions designed to detect *Axbrn-1*, a POU domain transcription factor expressed in the embryonic nervous system (T.M. and A.D.J., unpublished observations), as well as *AxEF-1α* which shows ubiquitous expression (Masi *et al.,* 2000). *Axbrn-1* is expressed in a much broader array of tissues than *Axdazl*, while *AxEF-1α* is found in all tissues. These results demonstrate that *Axdazl* is expressed specifically in both male and female gonads, indicating that it shows an adult pattern of expression equivalent to that of the *DAZ*-like genes from other vertebrates.

Axdazl RNA Is Not Localized in Oocytes

In *Xenopus* oocytes, the mitochondrial cloud transports germ plasm to the vegetal cortex (Heasman *et al.,* 1984). *Xdazl* RNA is uniformly distributed in prestage I (Dumont, 1972) oocytes before becoming associated with the mito-

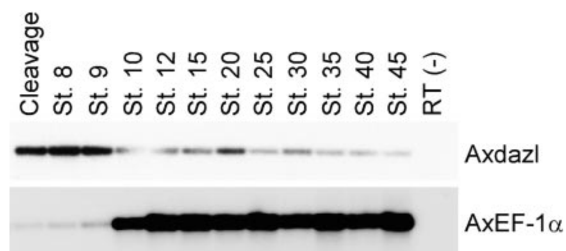

FIG. 6. *Axdazl* RNA is expressed throughout embryogenesis. RNA was extracted from groups of five embryos at each of the indicated stages. An equivalent amount of RNA from each stage was reverse transcribed. Portions of each reaction were used in PCR to amplify the indicated sequences within the linear range. Products were detected by hybridization of labeled cloned sequences to Southern blots. Maternal *Axdazl* RNA is inherited by embryos, and levels begin to decline by stage 10. Low levels of RNA are found in embryos through development to stage 45. *EF-1α*, which becomes transcriptionally active at the midblastula transition, is included as a control.

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chondrial cloud on one side of the oocyte during stage I (previtellogenesis). During vitellogenesis, it shows a wedge-shaped distribution extending from the germinal vesicle to the vegetal cortex, before becoming localized to the vegetal cortex by stage IV (Houston *et al.,* 1998; King *et al.,* 1999). Thus, beginning at stage I, *Xdazl* RNA is localized throughout the remaining stages of oogenesis.

Reasoning that if germ plasm were to exist in axolotl oocytes, it would contain *Axdazl* RNA among its components, we examined *Axdazl* RNA distribution during oogenesis. Sections from adult ovaries hybridized to an *Axdazl* probe show strong expression (purple) throughout the cytoplasm of stage I previtellogenic oocytes less than 500 μm in diameter (staging according to Beetschen and Gautier, 1989) (Figs. 4A and 4B). Little staining is seen in stage I oocytes hybridized to the sense probe (Fig. 4C). During early vitellogenesis, *Axdazl* RNA-containing cytoplasm forms a complete sphere of central cytoplasm around the nucleus, while the yolky peripheral layer shows little stain above background (for stage III oocytes, compare Figs. 4D and 4E with 4F). At early stage IV, *Axdazl* RNA staining has become less intense but is still visible in the central cytoplasm (Fig. 4G). During later vitellogenesis, yolk granules occupy the entire cytoplasm and little or no staining above background can be observed (compare Fig. 4H with 4I). The sensitivity of the method does not allow us to determine whether maternal *Axdazl* RNA is still present in the full-grown oocyte.

To more precisely exclude localization of *Axdazl* RNA, eight growing oocytes ranging in size from 200 μm to 1000 μm in diameter were examined in complete or almost-complete serial sections, and in no case (nor in any of the other less complete series of sections) was a small region of more intense staining observed. For larger oocytes, the orientation of the section could be approximated from the pigment in the animal half, allowing specific examination of the vegetal pole region. These results demonstrate that germ plasm containing *Axdazl* RNA is not present in axolotl oocytes.

Axolotl Oocytes Lack a Mitochondrial Cloud

Because the mitochondrial cloud mediates transport of germ plasm-specific mRNAs to the vegetal cortex, we next asked whether previtellogenic axolotl oocytes contain a mitochondrial cloud. We incubated previtellogenic oocytes from the ovaries of axolotl and *Xenopus* adults in a mitochondria-specific dye (Mito-tracker Red). Oocytes were viewed by fluorescence microscopy, or optical sections were examined in a confocal microscope, under conditions that would detect mitochondria-specific fluorescence. The results show a localized aggregate of mitochondria in previtellogenic stage I *Xenopus* oocytes (Figs. 5A and 5B, arrow), which corresponds to the mitochondrial cloud. An equivalent structure is not present in previtellogenic stage I axolotl oocytes (Figs. 5C and 5D). Identical results were obtained for 26 *Xenopus* oocytes and 23 axolotl oocytes. We conclude from these data that axolotl oocytes do not contain a mitochondrial cloud, consistent with a previous statement that, in contrast to *Xenopus*, the mitochondria of axolotl oocytes are dispersed in the cytoplasm (Al-Mukhtar and Webb, 1971). These results demonstrate that germ plasm of the type found in the cytoplasm of anuran oocytes is not present in axolotl oocytes.

Maternal Axdazl RNA Is Widely Distributed in the Embryo

To address whether *Axdazl* RNA is inherited as a maternal transcript, we first prepared Northern blots with RNA from early embryos, but found that *Axdazl* RNA could not be detected by this technique. Using RT-PCR, we were able to detect low levels of *Axdazl* RNA in cleavage stage embryos (stages 4 through 8) (Fig. 6). This is before the midblastula transition (MBT) [stage 9 in axolotls (Masi *et al.,* 2000)], when the zygotic genome becomes transcriptionally active (Krieg *et al.,* 1989). Thus, RNA appearing at these early stages is of maternal origin. The level of *Axdazl* RNA remains constant through stage 9, after which it begins to decline gradually (Fig. 6). Nevertheless, at no stage is *Axdazl* RNA undetectable, and, in this respect, its expression profile differs from *Xdazl* RNA which disappears after the neurula stage (Houston *et al.,* 1998, 2000). These results do not allow us to determine whether the *Axdazl* RNA present after MBT is of maternal or embryonic origin.

The presence of maternal *Axdazl* RNA presents the possibility that germ plasm becomes organized in early embryos and has the potential to function as a germ cell determinant. To examine this, we carried out *in situ* hybridization using the *Axdazl* probe on sections of fertilized eggs, cleavage, and blastula stage embryos, and found that *Axdazl* RNA is broadly distributed and difficult to distinguish from background by this method (data not shown). Embryos at gastrula, neurula, and tailbud stages also show no localized concentration of *Axdazl* RNA or evidence of new expression in individual cells (usually seen as a circle of stain around the nucleus). This suggests that the mass of maternal *Axdazl* RNA that is inherited by the embryo is not localized to cells in a specific region, but is instead distributed widely in the embryo.

To look for a local concentration of *Axdazl* RNA by a more sensitive technique, we dissected embryos from several different stages into specific regions as indicated in Fig. 7. RNA was extracted from the dissected parts and used in RT-PCR to detect *Axdazl* RNA, or RNA encoded by control markers expressed in specific regions of the embryo. Expression of *AxEF-1α* was used to standardize the amounts of input cDNA. At stage 8, *Axdazl* RNA is widely distributed in the animal half, and adjoining region of the vegetal zone, with the highest concentration in the marginal zone (Fig. 7A). Expression of *Axvg1* in the vegetal hemisphere confirms the vegetal to animal dissection. At gastrula stage 10.75, *Axdazl* RNA is present in all dissected regions, while expression of the dorsal marker *Axgsc* and the ventral

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marker *Axszl* confirms the dissection of dorsal and ventral marginal zones (Fig. 7B). At neurula stage 15, *Axdazl* RNA is again detected in every dissected region (Fig. 7C). In contrast, *Axbrn-1*, which is expressed primarily in anterior neural tissue (T.M. and A.D.J., unpublished observations), is expressed most strongly in the dorsal region. At stages 34 and 40, *Axdazl* RNA is widely distributed in the trunk and is low or absent in the head, while *Axbrn-1* RNA is detected only in the head (Figs. 7D and 7E). Finally, at stage 45, when the PGCs have colonized the gonads (Ikenishi and Nieuwkoop, 1978), *Axdazl* RNA is found only in the posterior middle fraction of the embryo, which contains the developing gonads.

These results demonstrate that *Axdazl* RNA is widely distributed throughout the embryo from cleavage to tailbud stages, and is not restricted to the posterior lateral mesoderm, where PGCs arise. By the time the PGCs are in the gonads, *Axdazl* RNA has disappeared from all regions of the embryo except the region that contains the germ cells.

Cell-Specific Axdazl Expression Is Restricted to PGCs in or Near the Gonad

By stage 45, *Axdazl* RNA is found only in the region of the embryo that harbors the PGCs. At this stage, the PGCs contain abundant material resembling the nuage found in the PGCs of frog and mouse embryos (Ikenishi and Nieuwkoop, 1978), suggesting that *Axdazl* RNA has commenced expression in the embryonic germ line. In fact, hybridization of cross sections of stage 45 embryos to the *Axdazl* probe shows strong staining in the developing gonads located in the posterior half of the trunk lateral to the dorsal mesentery of the suspended gut tube (Fig. 8A). Little background is observed in other regions of the embryo. An enlarged view of the same section (Fig. 8B) shows that staining is localized to PGCs in the gonad. A sense strand probe for *Axwnt-8* hybridized under identical conditions gives minimal staining (Fig. 8C).

Nuage first appears in PGCs of axolotl embryos at about stage 40 (Ikenishi and Nieuwkoop, 1978), which suggests that transcription of the *Axdazl* gene might be initiated in the PGCs at this time. To test this possibility, we analyzed sections from stage 20, 23, 25, 29, 33, 35, 36, 40, 42, 43, and 45 embryos with the *Axdazl* probe to detect cell-specific expression. No localized expression was detected until PGCs began to accumulate *Axdazl* transcripts at stage 40; PGCs (arrows) were identified by their location medial to mesonephroi (Figs. 8D and 8E arrowheads; also see Humphrey, 1925). By stage 42, the PGCs have begun to colonize the gonadal region in a more medial position (Figs. 8G and 8H). The level of *Axdazl* expression increases considerably between stages 40 and 45, which is coincident with the accumulation of nuage during this period (Ikenishi and Nieuwkoop, 1978), and indicates accumulation of zygotic transcripts. In some samples hybridized to either the sense strand control probe or the *Axdazl* probe, background reaction was seen within the yolk of the gut, and within the wall of the gut, presumably due to endogenous alkaline phosphatase (Fig. 8I). However, the background within the gonad was consistently low.

In conclusion, the onset of embryonic expression of the *Axdazl* gene in germ cells coincides closely with the appearance of nuage, commencing at about stage 40 of development. The diffuse RNA present in the embryo up to stage 40 is, as far as we can detect, of maternal origin.

DISCUSSION

It is widely assumed that the embryonic development of urodeles and anurans, the two major amphibian lineages, is very similar. However, urodeles and anurans diverged from a common ancestor about 250 million years ago (Cannatella and Hillis, 1993), and since then many developmental processes have diverged [e.g., position of the presumptive mesoderm, development of the notochord, somites, hind limbs, blood, and head (Nieuwkoop and Sutasurya, 1976; Hanken, 1986; Keller, 2000)], in part explaining the obvious morphological differences between frogs and salamanders. Yet, in no aspect of development is the divergence of amphibian embryos more dramatic than in the processes that govern germ line development. In this respect, experiments with urodele embryos should provide novel insights into the mechanisms underlying germ cell specification in vertebrates.

Germ Plasm Components in Axolotl Embryos

Maternal *Xdazl* RNA is a molecular marker for germ plasm in *Xenopus* (Houston *et al.,* 1998). Maternal depletion studies suggest that *Xdazl* may not play a determinative role in germ cell development in *Xenopus*; rather its role may be to support germ cell function during early germ cell migration (Houston *et al.,* 2000). Nevertheless, *Xdazl* RNA is associated with other components of germ plasm that are required for germ cell formation in *Xenopus*, and we have used it as a marker to search for germ plasm in axolotls.

We have found that *Axdazl* RNA is distributed throughout the cytoplasm of previtellogenic and early vitellogenic oocytes, rather than localized as is *Xdazl* RNA in *Xenopus*. In larger oocytes and early embryos, the sensitivity of the *in situ* hybridization method was not sufficient to detect *Axdazl* RNA, but the absence of any localized staining indicates that it is diffuse, presumably due to dilution during oocyte growth. [*Xdazl* RNA is clearly detected by similar methods in the germ plasm of large *Xenopus* oocytes (Houston *et al.,* 1998).] These results are consistent with the absence of a mitochondrial cloud, which mediates formation of the germ plasm in the vegetal region of frog oocytes. Taken together, we provide strong evidence that germ plasm of the type found in anuran eggs is not present in the vegetal pole of axolotl eggs.

If germ plasm were to exist in axolotl eggs and early

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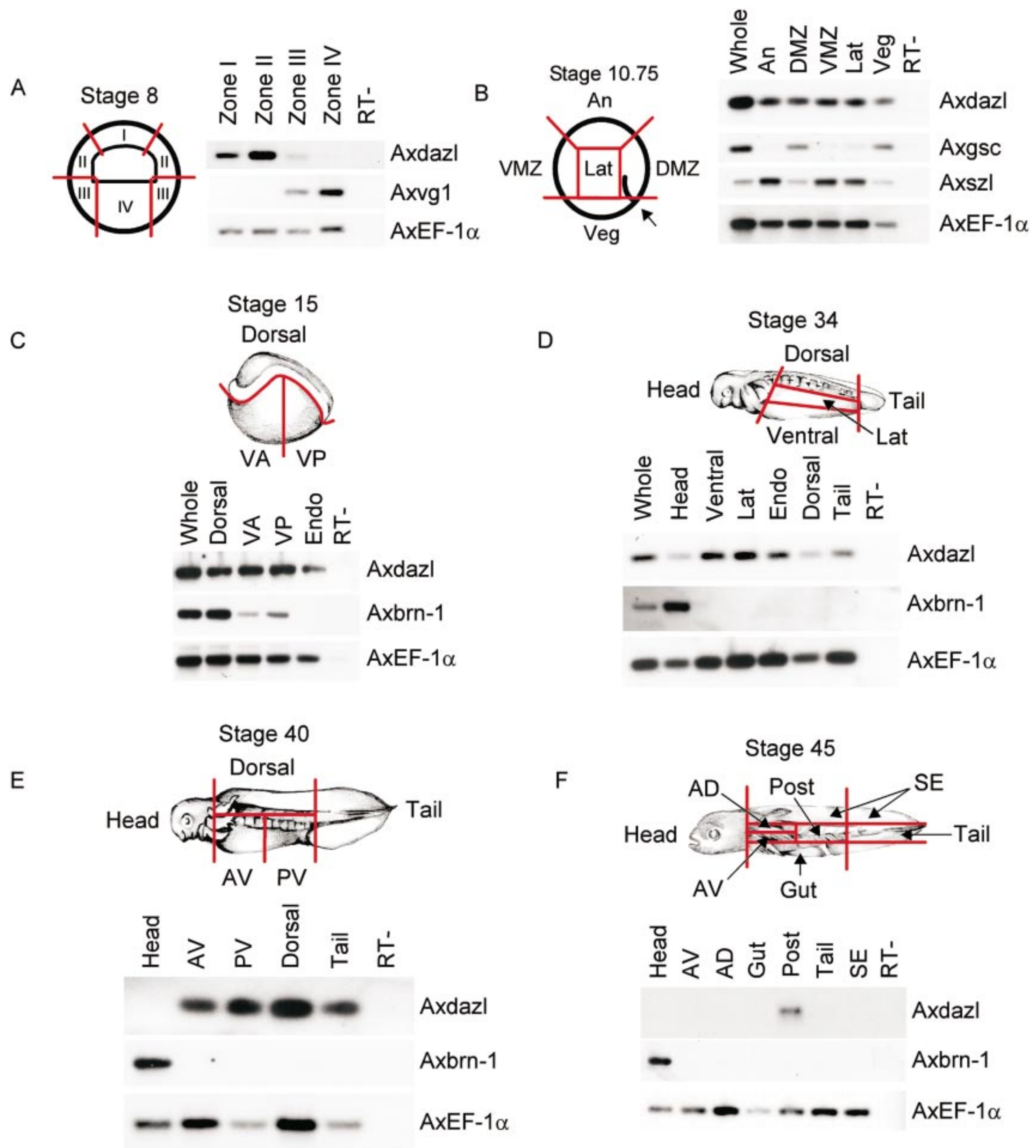

FIG. 7. *Axdazl* RNA shows widespread distribution in embryos. Ten embryos from each of the indicated stages were dissected into small parts as indicated on the figure. RNA was extracted from each region and reverse transcribed. Input cDNA was normalized by RNA input and *AxEF-1α* levels. The indicated sequences were amplified by PCR and detected as described in Materials and Methods. (A) Stage 8 embryos. Regions I, II, III, and IV corresponding to the animal pole region, the peripheral region of the animal cap including the animal portion of the marginal zone, vegetal region of the marginal zone, or vegetal pole, respectively, were prepared as described by Nieuwkoop (1969). (B) Stage 10.75 embryos. Fractions are as follows: Whole (whole embryo); An (animal pole); DMZ (dorsal marginal zone); VMZ (ventral marginal zone); Lat (lateral marginal zone); Veg (vegetal pole). (C) Stage 15 embryos. Fractions are as follows: Whole (whole embryo); Dorsal; VA (ventral anterior); VP (ventral posterior); Endo (endoderm). (D) Stage 34 embryos. Fractions are as follows: Whole (whole embryo); Head; Ventral; Lat (Lateral); Endo (Gut Endoderm); Dorsal; Tail. (E) Stage 40 embryos. Fractions are as follows: Head; AV (anterior ventral); PV (posterior ventral); Dorsal; Tail. (F) Stage 45 embryos. Fractions are as follows: Head; AV (anterior ventral mesoderm); AD (anterior dorsal mesoderm); Gut (gut tube); Post (posterior ventral mesoderm); Tail; SE (surface ectoderm). An RT (−) lane showing the product of a reaction performed with RNA from a whole embryo that was not reverse transcribed is included for each stage as a negative control. *Axdazl* RNA expression is widespread in early embryos, but is specific to the posterior ventral mesoderm, which includes the gonads, by stage 45.

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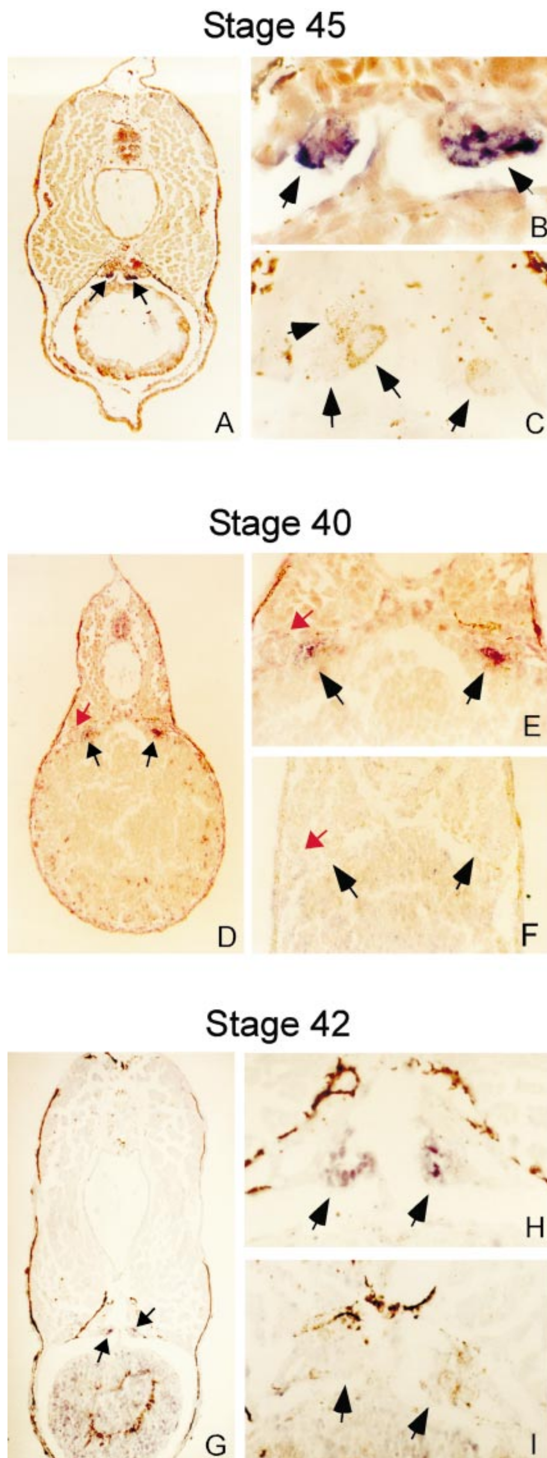

FIG. 8. *Axdazl* expression is PGC specific beginning at stage 40. Stage 40–45 embryos were sectioned and hybridized *in situ* with digoxigenin-labeled probes corresponding to either *Axdazl* (antisense probe) or *Axwnt-8* (sense strand probe). Probes were detected by standard colorimetric methods. Signal is indicated by purple stain. (A) Cross section through a stage 45 embryo, hybridized with probe to *Axdazl* RNA. Signal is detected in embryonic gonads (arrows) which protrude ventrally into the coelom on either side of the dorsal mesentery. Nuclei are lightly stained with hematoxylin. (B) High magnification of (A), showing PGC specific expression (arrows). (C) High magnification view of a section through a stage 45 embryo hybridized with a sense strand probe for *Axwnt-8,* to show background hybridization. (D) Section through a stage 40 embryo, hybridized with *Axdazl* probe. Signal indicated by black arrows. At this stage, the mesonephric ducts are located laterally, and PGCs are found within the splanchnic mesoderm, just medial to the mesonephric ducts (red arrow). Nuclei are stained red with safranin. (E) Higher magnification of (D). PGCs indicated by black arrows. (F) Stage 40 embryo reacted with sense strand control. Mesonephric ducts are indicated by red arrows. PGCs are difficult to identify conclusively in this section in the absence of hybridization signal. This section was not counterstained with safranin. (G) Stage 42 embryo, hybridized to *Axdazl* probe. Signal indicated by arrows. At this stage, the Wolffian ducts have moved toward the midline, and germ cells are located in two clusters just medial to the Wolffian ducts. The ducts are visible in this field. (H) Stage 42 embryo at higher magnification showing PGC specific hybridization (arrows). (I) Stage 42 embryo, hybridized with sense strand *Axwnt-8* probe, showing background staining in gonads (arrows). These results demonstrate expression of *Axdazl* RNA in the PGCs from stage 40 to stage 45.

embryos, it would most likely be localized to the marginal zone cytoplasm from which the PGCs are derived. In fact, in blastulae (stage 8), *Axdazl* RNA is most concentrated in the marginal zone but also extends throughout the animal hemisphere and into the vegetal hemisphere. By stage 10.75, *Axdazl* RNA is found quite equally distributed in all regions of the embryo with similar concentrations in the ventral and dorsal marginal zone, and this wide distribution continues at stage 15. It should be noted that, since we have used only *Axdazl* RNA as a marker, it is possible that other germ plasm elements are present and have a more localized distribution.

Based on the results presented here, it is unlikely that germ plasm components are sufficient for germ cell formation in axolotl, since they appear to be in low abundance and are widely distributed in the early embryo, rather than localized to the germ cell-forming region. Nevertheless, germ plasm components could promote or even be required for competence to form germ cells. In fact, the distribution of *Axdazl* RNA in the blastula correlates with the ability of different regions of the animal cap to form germ cells; that is, more germ cells can be induced from the marginal zone than from the center of the animal cap (Sutasurja and Nieuwkoop, 1974; Michael, 1984), suggesting that the concentration of germ plasm components could influence the ability to form germ cells. On the other hand, our data suggest that the higher competence of the ventral side to produce germ cells is not due to different concentrations of germ plasm elements, but may be due to other dorsal–ventral differences established during cleavage, related to embryonic patterning (Moon and Kimelman, 1998).

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It has been proposed (see Michael, 1984) that required maternal factors might be widely distributed, and germ cell formation takes place when cells containing these factors occupy appropriate sites in the embryo. Our data do not refute this hypothesis. In this scenario, germ plasm components could act according to the conventional view of germ plasm as agents that "protect" germ cells from the action of patterning signals that would specify them to a somatic fate (see Blackler, 1958; Eddy, 1975; Dixon, 1994). Alternatively, they could act positively to mediate a germ cell fate in response to signals from adjoining tissues. However, given the low abundance of *Axdazl* RNA, and potentially other germ plasm components as well, it is also possible that germ plasm/nuage has little function in the embryo, but is a remnant of a period of relatively high expression and important function in small oocytes during oogenesis. Further work testing the effect of depletion of maternal *Axdazl*, and other possible germ plasm components that we identify in axolotls, will be required to resolve these alternatives.

We were unable to detect germ cell-specific *Axdazl* expression by *in situ* hybridization until stage 40, when it appears in PGCs, and then accumulates to high levels by stage 45. This timing correlates closely with the absence of germ plasm/nuage in PGCs at tailbud stages, its appearance at stage 40, and its increase to stage 46, as reported by Ikenishi and Nieuwkoop (1978). This suggests that *Axdazl* RNA is a component of nuage in PGCs. Germ plasm or nuage components may promote activation of genes required for germ cell differentiation in the gonad. Blocking *DAZ*-like function has effects on germ cells during gametogenesis in different animals (Eberhart *et al.,* 1996; Ruggiu *et al.,* 1997; Karashima *et al.,* 2000), and it is likely that *Axdazl* plays a critical role at some point in later development of axolotl germ cells.

Continuity of the Germ Line

The concept of an uninterrupted germ line running from generation to generation is one that dates back to Weismann (1893), and has been supported by work with model systems that develop with a predetermined germ line (see discussion by Blackler, 1970). Yet, the predetermined germ line–germ plasm model does not apply to many animals such as mice and urodeles, or a variety of invertebrates (Nieuwkoop and Sutasurya, 1981) including sea urchins (Ransick *et al.,* 1996). In these animals, the germ line arises anew at each generation from a pool of totipotent precursors also capable of commitment to somatic development (see McLaren, 1981; Nieuwkoop and Sutasurya, 1981). Since axolotls are in this group, our results lay the groundwork for future experiments to determine the embryonic patterning signals required to maintain a population of totipotent cells during early embryogenesis, and whether germ plasm components play an essential role in this process.

Is Regulative Germ Cell Development a Basal Feature of the Chordate/Vertebrate Lineage?

The work that we present here supports the view that common mechanisms of germ cell development are shared by urodele and mouse embryos (McLaren, 1999). Urodeles in general represent a less derived taxon than anurans, resembling more closely the ancestral amphibian that gave rise to all terrestrial vertebrates (Duellman and Trueb, 1985). This raises the possibility that the regulative mechanism of germ cell development observed in axolotls is a basal trait, present in the amphibian ancestor, and retained and adapted during the evolution of mammals. In agreement with this, we have recently shown (M.D. and A.D.J., unpublished observations) that small ovarian oocytes from lungfish, the closest living relative of the tetrapod ancestor (Zardoya *et al.,* 1998), do not have a mitochondrial cloud, suggesting that germ plasm was indeed absent in the common ancestor of all tetrapods. In this context, a plausible scenario is that germ cell development in anurans diverged significantly toward a more determinant mechanism; supporting this possibility is the three-way amino acid sequence comparison of the *DAZ*-like gene encoded in axolotl, *Xenopus* and mouse, indicating that the frog has the more divergent sequence.

Recent evidence shows that localized germ plasm is found in eggs and early embryos of at least two other vertebrates, in addition to anurans: chicken (Tsunekawa *et al.,* 2000) and zebrafish (Knaut *et al.,* 2000), indicating a predetermined type of germ cell development. Therefore, if the regulative mechanism is basal in vertebrates, predetermined germ cells, and, indeed, germ plasm, must have evolved several times. To determine the plausibility of this model, evidence from primitive chordates and vertebrates must be considered. In their survey of germ cell development in chordates, Nieuwkoop and Sutasurya (1979) noted that germ cell determination involving a role for germ plasm is associated with an endodermal origin of the PGCs (anurans), while a regulative mode is associated with origin in posterior lateral mesoderm (urodeles), suggesting a correlation between site of origin and mode of germ cell determination. This correlation holds in mouse and chick embryos: in mammals, a posterior mesoderm site (the posterior primitive streak and allantois) is associated with the regulative mode, and, in chick, a relatively anterior endodermal site is associated with the germ plasm mode. PGCs appear at sites similar to that of mammals in turtles, believed to retain features of basal amniotes (Gauthier *et al.,* 1988), in some lizards (Nieuwkoop and Sutasurya, 1979; Hubert, 1985), and perhaps in amphioxus (a cephalochordate) and ascidians (urochordates) (Nieuwkoop and Sutasurya, 1979). According to the proposed correlation, these animals may then have the regulative form of germ cell determination. Further analysis of various chordates will be required to resolve with certainty which mode is basal to the vertebrates, as well as the mechanisms underlying the

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apparent ease with which evolution has converted one form to the other.

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