A critical role for Xdazl, a germ plasm-localized RNA, in the differentiation of primordial germ cells in Xenopus

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SUMMARY

Xdazl is an RNA component of Xenopus germ plasm and encodes an RNA-binding protein that can act as a functional homologue of Drosophila boule. boule is required for entry into meiotic cell division during fly spermatogenesis. Both Xdazl and boule are related to the human DAZ and DAZL, and murine Dazl genes, which are also involved in gamete differentiation. As suggested from its germ plasm localization, we show here that Xdazl is critically involved in PGC development in Xenopus. Xdazl protein is expressed in the cytoplasm, specifically in the germ plasm, from blastula to early tailbud stages. Specific depletion of maternal Xdazl RNA results in tadpoles lacking, or severely deficient in, primordial germ cells (PGCs). In the absence of Xdazl, PGCs do not successfully migrate from the ventral to the dorsal endoderm and do not reach the dorsal mesentery. Germ plasm aggregation and intracellular movements are normal indicating that the defect occurs after PGC formation. We propose that Xdazl is required for early PGC differentiation and is indirectly necessary for the migration of PGCs through the endoderm. As an RNA-binding protein, Xdazl may regulate translation or expression of factors that mediate migration of PGCs.

Key words: Xenopus, Germ plasm, Localized RNA, Primordial germ cell, RNA-binding protein

INTRODUCTION

In most sexually reproducing organisms, the gametes are derived from a precursor stem cell population, the primordial germ cells (PGCs). These cells originate extragonadally early in development and migrate through somatic tissues to reach the developing gonads. Once ensheathed by somatic gonad cells, PGCs divide and differentiate into definitive gametes. Discovering how the PGCs are initially specified and develop separate from the somatic lineage is a fundamental problem in developmental biology. In many organisms, germ plasm material, present in the egg, can be found in PGCs and germ cells throughout the life of the organism and is thought to act as a determinant of germ cell fate. The asymmetric inheritance of maternal factors is a common strategy used by diverse organisms such as nematodes, insects, crustaceans and Anuran amphibians to specify PGCs (Nieuwkoop and Sutasurya, 1979, 1981). Germ plasm is morphologically similar in all organisms where it is found and is typically composed of a fibrillar ‘germinal cytoplasm,’ electron-dense germinal granules, mitochondria and ribosomes (Czolowska, 1972).

In Xenopus, the germ plasm is present in eggs as numerous discrete islands at the vegetal pole. These islands aggregate after fertilization, a process that requires a kinesin-like protein, Xkpl-1 (Robb et al., 1996). Germ plasm is segregated unequally during cleavage stages until gastrulation, at which time the germ plasm becomes perinuclear and is divided equally among daughter cells (Whittington and Dixon, 1975). During subsequent embryogenesis, PGCs remain in the endoderm and are thought to undergo 2-3 cell divisions. Around stage 32/33 (late tailbud stage), the PGCs begin to migrate dorsally through the lateral endoderm. By early tadpole (stage 40), the PGCs accumulate in the dorsal crest of the posterior endoderm and are subsequently incorporated into the lateral plate mesoderm that forms the dorsal mesentery. In later stages, PGC migration continues to the dorsal body wall and then laterally to the forming genital ridges (Wylie and Heasman, 1976).

Exactly how the germ plasm functions in PGC specification remains largely unknown. Removal of vegetal pole cytoplasm (Buehr and Blackler, 1970) or low dose ultraviolet (u.v.) irradiation of the vegetal pole (Züst and Dixon, 1975; Holwill et al., 1987) cause sterility or delayed migration of PGCs. Importantly, introduction of vegetal pole cytoplasm into irradiated eggs can restore fertility (Smith, 1966; Wakahara, 1977). This observation led Smith (1966) to conclude that indeed germ plasm contained germ cell determinants. Other evidence, however, suggests that Xenopus germ plasm does not act as a ‘true determinant’ as is thought for Drosophila pole plasm. Mis-localization of pole plasm, either through cytoplasmic transfer (Illmensee and Maholwald, 1974) or mis-expression of oskar RNA, a key pole plasm component, at the anterior pole (Ephrussi and Lehmann, 1992) can cause the formation of ectopic, functional pole cells. Similar experiments
have not been successful in *Xenopus*, although transferred vegetal pole cytoplasm can cause the formation of supernumerary PGCs when injected into the vegetal pole (Wakahara, 1978), the location of endogenous germ plasm. Furthermore, ectopic *Drosophila* pole cells do not differentiate into other cell types (Underwood et al., 1980) while, in *Xenopus*, transplanted germ plasm-containing blastomeres and migrating PGCs can contribute to tissues derived from all three germ layers (Wylie et al., 1985). Thus, *Drosophila* pole plasm appears to function as an instructive determinant while Anuran germ plasm may act only in a permissive role or require the correct endodermal environment. One explanation for the apparent differences in function of germ plasm, despite the high degree of ultrastructural similarity, may be differences in the specific molecular components of germ plasm, such as RNA or proteins, between the two organisms.

Germ plasm components have been studied primarily in *C. elegans*, *Drosophila* and *Xenopus*. Proteins localized to the germ plasm of *C. elegans*, in the P-granules, appear to repress transcription and prevent somatic differentiation (reviewed in Seydoux and Strome, 1999). Transcriptional repression also occurs in *Drosophila* pole cells (Seydoux and Dunn, 1997; Van Doren et al., 1998), although the factors responsible for this have not yet been identified. *Drosophila* mutants have proved useful in identifying genes involved in the assembly of pole plasm components and genes required in somatic cells for pole cell migration. Of components localized to the pole plasm, only a few genes are known to be required for pole cell formation or migration. *nanos*, a zinc-finger gene (Kobayashi et al., 1996; Forbes and Lehmann, 1998), and *polar granule component* (pgc), an untranslated RNA (Nakamura et al., 1996), are required in the pole cells for proper migration out of the developing gut and into the somatic gonad. The genes *germ cell-less* (gcl), which encodes a nuclear pore complex protein (Jongens et al., 1994), and mitochondrial large ribosomal RNA (mtlRNA, Kobayashi and Okada, 1989) are involved in the initial formation of pole cells by still unknown mechanisms.

In *Xenopus*, several RNAs have been found localized to the germ plasm (reviewed in King et al., 1999) but none of these have previously been tested for roles in PGC specification. Several of these RNAs are similar to important *Drosophila* pole plasm components; *Xcat2* encodes a *nanos* homologue (Mosquera et al., 1993) and *Xsirts* encodes an untranslated RNA (Kloc et al., 1993), although not related to *pgc*. In this study, we focus on *Xdazl*, an RNA that we previously identified as a germ plasm component in *Xenopus* (Houston et al., 1998). *Xdazl* is expressed in the mitochondrial cloud of stage I oocytes, the source of germ plasm material (Heasman et al., 1984), and remains expressed in the germ plasm until the neurula stage. *Xdazl* RNA is also abundantly expressed in germ cells of the testis, but not in any of the somatic tissues. *Xdazl* encodes an RNA-binding protein and is highly related to genes of the *Deleted in Azoosperma* (*DAZ*, Reijo et al., 1995) family, which are involved in germ cell development in a variety of organisms ranging from *C. elegans* to humans.

Loss-of-function studies have identified roles for *DAZ* genes in germ cell development in worms, flies, mice and men. It is interesting to note, however, that the specific phenotypes differ among the organisms. In *C. elegans*, the gene *daz-1* is required for oogenesis in hermaphrodites, but is not required for spermatogenesis (Karashima et al., 2000). The *Drosophila boule* gene, on the contrary, is required for entry into meiosis during spermatogenesis and male fertility, but is not expressed in females (Eberhart et al., 1996). Male and female mice deficient in *Dazl* are sterile and exhibit defects in germ cell development and survival (Ruggiu et al., 1997). The phenotypes of human males with deletions of the *DAZ* gene cluster on the Y chromosome range from an absence of germ cells (Sertoli cell only syndrome) to severe oligospermia (Reijo et al., 1995). Humans also have an autosomal homologue, *DAZL* (Saxena et al., 1996; Shan et al., 1996; Yen et al., 1996), which may account for the variability in *DAZ* phenotypes. A role for *DAZL* in gametogenesis has not been demonstrated. Evidence suggests that the DAZ proteins are functionally homologous even though the exact roles may differ. Expression of *Xdazl* in *boule* flies results in rescue of the meiotic entry phenotype (Houston et al., 1998). Additionally, the mouse *Dazl* phenotype is partially rescued by expression of a human DAZ transgene (Slee et al., 1999). These observations suggest that the DAZ genes play highly adaptable roles in many contexts of germ cell development.

In this work, we asked whether maternal *Xdazl* RNA is necessary for PGC formation in *Xenopus*. We show that Xdazl protein is expressed in the germ plasm from the blastula stage to early tailbud stages. In embryos derived from oocytes depleted of *Xdazl* RNA, we find a severe reduction or a complete loss of PGCs by stage 42. Additionally, we found that *Xdazl* is required for migration from the ventral endoderm. These findings are the first to provide evidence that a specific molecular component of germ plasm, *Xdazl*, is required for PGC differentiation in *Xenopus*.

**MATERIALS AND METHODS**

**Recombinant Xdazl protein and antibodies**

The *Xdazl* coding region (*EcoRI* insert of pXDXz, Houston et al., 1998) was subcloned into the *NdeI* site of pET-16b (*Novagen*), or the *BamHI* site of pGEX-5X-1 (*Pharmacia*) by blunt end ligation. This produced decahistidine or GST tagged Xdazl protein, respectively. The plasmid pET-16b-XdzHT was transformed into *E. coli* strain BL21(DE3); pGEX-5X-1-XdzGST was transformed into BL21. Protein expression was induced in large-scale cultures by addition of IPTG to 1 mM for 3 hours at 37°C. Decahistidine and GST-tagged Xdazl (*XdzHT* and *XdzGST*) were both recovered in the insoluble inclusion body pellet. To purify *XdzHT*, inclusion bodies were solubilized in guanidine HCl and then dialyzed against buffer containing 1 M NDSB-201 (non-detergent sulfobetaine, Calbiochem) to maintain proteins in a soluble state. Buffer was exchanged for phosphate buffer (20 mM phosphate, pH 7.4, 0.5 M NaCl, 10% glycerol) and *XdzHT* was purified by chromatography on a HisTrap column (*Pharmacia*). The isolated protein was approximately 90% pure and was used to generate polyclonal antisera to Xdazl in rabbits (*Zymed*).

To purify antibodies to Xdazl, XdzGST was prepared as above except that the final purification was done in PBS over glutathione-Sepharose beads (*Pharmacia*). Antibodies were purified over an *Xdazl* antigen column according to the method of Koff et al. (1992). Antibodies to Xdazl were eluted with 100 mM glycine, pH 2.5. The unbound antibodies were then incubated with *XdzHT* protein bound to a nitrocellulose membrane to further deplete the antisera of antibodies to *Xdazl*. 
Whole-mount immunostaining
Embryos were fixed in MEMFA and stored in methanol. Samples were rehydrated to PBS and some were bisected along the midline with a scalpel. The bisected and intact embryos were then incubated in PBT (PBS, 2 mg/ml BSA, 0.1% Triton X-100) for 15 minutes with rocking. Non-specific binding sites were blocked in PBT containing 10% goat serum for 1 hour at room temperature. Primary antibody incubation (affinity-purified antibodies to Xdazl, 1:100 dilution) was done in blocking solution for 4 hours at room temperature followed by an overnight wash in PBT at 4°C. The following day, the embryos were washed an additional 4x1 hour each with rocking. This was followed by a 4 hour incubation in a 1:2000 dilution of goat anti-rabbit HRP conjugate secondary antibodies (Pierce). Embryos were washed as above. Detection was done with a DAB HRP substrate (Roche Molecular Biochemicals).

Oligo injection and host-transfer
The oligos used, XDZ2 and XDZ4, were 18mers with the following sequences; XDZ2: A*+T*G*AAGCCATAAACC+T*T*G, XDZ4:T*A*A+AAACCACAACC+*G*A (Genosys, * indicates a phosphorothioate bond, the remainder were phosphodiester linkages). Manually defolliculated stage VI oocytes were injected with the vegetal pole with 3·5 ng of oligo and maintained at 18°C in oocyte culture medium [OCM; 60% L-15, 0.04% BSA, 1 mM L-glutamine, 1 μg/ml gentamicin]. After 24 hours, capped Xdazl RNA was injected into some of the oocytes to rescue the depletion of endogenous Xdazl. Oocytes were then stimulated to mature and subsequently fertilized by the host-transfer technique as described (Heasman et al., 1991). Resultant embryos were sorted and maintained in 0.1 mM MMR at 18°C. In rescue experiments, translation of injected Xdazl was verified by immunoblotting of blastula and gastrula stage embryos with Xdazl antisera.

Northern blot analysis
Prior to host-transfer, eggs were frozen in dry ice and stored at −80°C. RNA was isolated and 4 egg equivalents were analyzed by northern blotting for Xdazl as described (Houston et al., 1998). The blot was then stripped and reprobed for Xcat2 (NotI-Sall insert), another germ plasm localized RNA (Mosquera et al., 1993), as a control for specific depletion of Xdazl.

Whole-mount in situ hybridization
Whole-mount in situ hybridization was performed essentially as described in Harland (1991). Embryos were fixed in MEMFA and stored in methanol. The probe for Xpat was prepared as described (Husdon and Woodland, 1998). Staining was done overnight at room temperature in BM Purple (Roche Molecular Biochemicals). Embryos were postfixed 1 hour in MEMFA, then bleached overnight in 10% H2O2/70% methanol, dehydrated in 100% methanol and cleared in Murray’s (2:1 benzylbenzoate/benzylalcohol). For subsequent sectioning, samples were transferred from Murray’s clear to toluene and embedded in paraffin (Paraplast X-tra).

Histology
For histological analysis of PGCs, tadpoles were anesthetized in 0.2 mg/ml MS222 and fixed overnight in Bouin’s. Following dehydration to 100% ethanol, the heads and tails were cut away using a scalpel. The torsos were then transferred to toluene and embedded in paraffin. Sections (10 μm) were cut on a rotary microtome, adhered to SilanePrep slides (Sigma) and stained with Haematoxylin and Eosin. PGCs were counted as in Holwill et al. (1987). To identify germ plasm in early embryos, samples were embedded as above and sections stained with a modified Heidenhain’s Azan stain (Smith and Neff, 1985), [0·25% Aniline Blue, 0·5% Orange G, 2% glacial acetic acid] for 40 minutes at room temperature. After staining with Azan, a general histological stain, germ plasm and other basophilic areas stain dark blue, while yolk platelets are stained yellow or orange (Whittington and Dixon, 1975; Smith and Neff, 1985).

RT-PCR
RT-PCR analysis of embryos was carried out as previously described (Zhang et al., 1998). The primers used were Xpat (Husdon and Woodland, 1998) and EF1-α (Wilson and Melton, 1994). Xpat primers were used at an annealing temperature of 58°C for 22-25 cycles while 22 cycles were used for EF1-α. Amplified products were analyzed on 6% polyacrylamide/1% TBE gels and exposed to PhosphorImager screens (Molecular Dynamics). Quantitation was performed with ImageQuant software (Molecular Dynamics).

RESULTS
Expression of Xdazl protein in early embryos
Our previous work showed that the mRNA for Xdazl was expressed in the germ plasm from early oocytes until the neurula stage (Houston et al., 1998). To determine when the protein product of this RNA was expressed, we immunostained embryos with affinity-purified antibodies to recombinant Xdazl protein. Cleavage stage (4-cell) embryos failed to give specific Xdazl immunoreactivity (Fig. 1A) and the first detectable staining was seen in blastulae (stage 7, Fig. 1F). The stain was faint and restricted to the germ plasm. Stronger immunoreactivity was detected in the germ plasm of gastrula (stage 10.5, Fig. 1B,G) and neurula (stage 13, Fig. 1D,J) embryos. Note that, in the latter two stages, the germ plasm has become juxtanuclear, as expected. Xdazl staining was also seen in PGCs in the endoderm of stage 18 (neural groove stage, Fig. 1E) and stage 22 (early tailbud, not shown) embryos and was undetectable in later stages. We stained both intact embryos and embryos bisected along the midline and results were similar with both methods. The staining was more readily visible in bisected embryos and clearing was not necessary to see the stain, especially in the neurula and later stages.

To demonstrate the specificity of this staining, we immunostained mid-gastrula stage embryos with affinity-purified antibodies to Xdazl or antiserum depleted of antibodies to Xdazl. While strong signal was seen in the germ plasm of Xdazl-stained embryos (Fig. 1G), the germ plasm was unstained or very faintly stained in embryos incubated with the Xdazl-depleted antiserum (Fig. 1H). Subsequent Heidenhain’s Azan staining of the sections verified that this yolk-free region was indeed germ plasm (Fig. 1I). These results show that endogenous Xdazl protein is expressed in Xenopus embryos from the blastula to the early tailbud stage. This is the period of time when the population of PGCs is initially established and suggests that Xdazl might function in the early development of PGCs.

Depletion of Xdazl RNA with antisense oligodeoxynucleotides
To determine if Xdazl was important for PGC differentiation, we used injection of antisense oligodeoxynucleotides (oligos) to deplete Xdazl mRNA from stage VI Xenopus oocytes. Two oligos were found that substantially depleted Xdazl RNA (XDZ2 and XDZ4). A sense oligo complementary to XDZ2 did not affect Xdazl RNA levels and gave results similar to uninjected controls (not shown). The XDZ2 oligo provided more consistent results and was used in all experiments unless

Xdazl is required for PGC formation

449
otherwise stated. Northern blot analysis of uninjected and oligo-injected oocytes showed that Xdazl RNA was specifically eliminated while Xcat2 RNA, which co-localizes with Xdazl in the germ plasm (Mosquera et al., 1993; Forristall et al., 1995), was unaffected (Fig. 2).

Tadpoles depleted of maternal Xdazl RNA are deficient in PGCs

In Xenopus, PGCs are readily identifiable in the dorsal mesentery of swimming tadpole stage embryos (stage 43/44). Our initial studies focused on whether depletion of maternal Xdazl could cause a deficiency in histologically identifiable PGCs in tadpoles. To this end, we fertilized uninjected control and Xdazl-depleted oocytes by the host-transfer technique (Heasman et al., 1991) and raised the embryos to the tadpole stage (stage 43/44). In a typical experiment, 20-40% of transferred eggs developed to the desired stage. Overall growth and morphogenesis were similar in control and Xdazl-depleted embryos, suggesting that oligo injection did not affect these processes. Importantly, we saw normal gut coiling in over 80% of depleted embryos. Because abnormal gut coiling could affect PGC migration, we excluded these from analysis. To analyze the numbers of PGCs in control and Xdazl-depleted tadpoles, we fixed the embryos and stained serial sections with haematoxylin and eosin. PGCs were clearly visible within the mesentery and total numbers per embryo were counted. The number of PGCs in control embryos was variable (means from 12-16.6, n=35; Table 1), but similar to previously reported numbers (Holwill et al., 1987). In contrast, PGC numbers in Xdazl-depleted embryos were greatly reduced (means from 2.7-5.7, n=52; Table 1). These observations were consistent in at least four independent experiments using different donor and host females and two different oligos. Note that, in many cases, PGCs were completely eliminated (3/26 for XDZ4 and 10/26 for XDZ2).

It was reported by Züst and Dixon (1977) that PGCs could populate the gonads of u.v.-irradiated embryos at stage 48, even though PGCs were absent from the dorsal mesentery of earlier stages. To determine if this was also the case for Xdazl-depleted embryos, we examined the gonads of control and Xdazl-depleted tadpoles at stage 58 (n=3 for each). In the controls, well-differentiated gonads had formed (ovary in all three cases) with clearly developed early stage oocytes (Fig. 3A). In 2/3 Xdazl-depleted tadpoles, the gonads were devoid of germ cells and contained only masses of somatic gonadal cells (Fig. 3B). The third Xdazl-depleted tadpole had a
451

is required for PGC formation

normally formed ovary (not shown). These results suggest that PGCs in Xdazl-depleted embryos either do not form or cannot migrate properly to the dorsal mesentery.

PGCs in Xdazl-depleted embryos are lost prior to entering the dorsal mesentery

We next wanted to establish when PGC development was disrupted in Xdazl-depleted embryos. Xdazl could be required for any of the steps in PGC formation from germ plasm aggregation to PGC migration or survival. To identify PGCs in developing embryos, we stained control, Xdazl-depleted and rescued embryos for Xpat RNA, a PGC-specific molecular marker (Hudson and Woodland, 1998), by whole-mount in situ hybridization. Xpat, a maternal RNA, is localized in the germ plasm of oocytes and embryos and persists until stage 40. We first examined stage 39/40 embryos to discover if PGCs were lost before or after incorporation into the dorsal mesentery in Xdazl-depleted embryos.

Control embryos had numerous Xpat-stained PGCs located along the dorsal endoderm (Fig. 4B,D). Consistent with the results seen at stage 43, Xdazl-depleted stage 40 embryos showed a severe reduction in the number of PGCs. Most embryos had no detectable Xpat expression while some had a few PGCs that completed migration to the dorsal endoderm (Fig. 4C,E,F). Overall, PGCs were reduced in 6/20 embryos (30%) and absent in 13/20 cases (65%, Table 2). RT-PCR analysis showed that Xpat RNA levels in Xdazl-depleted embryos were reduced to about 40% that of controls (Fig. 4A).

To ensure that the reduction in Xpat abundance and PGC numbers were specific to depletion of Xdazl RNA, we performed rescue experiments by injecting in vitro synthesized Xdazl RNA (300 pg) into oocytes previously depleted of

Fig. 4. PGCs are reduced in number or absent in Xdazl-depleted early tadpoles (stage 39/40). (A) RT-PCR analysis of Xpat RNA levels in individual uninjected (Un), oligo injected (Oligo) or rescued (Oligo+Xdazl) stage 39 embryos. EF1-alpha was assayed as a loading control. (B-F) Whole-mount in situ hybridization for Xpat RNA in uninjected (B, D) and Xdazl-depleted (C,E,F) stage 39/40 embryos. (B) Uninjected control embryos showing numerous Xpat-labelled PGCs in the posterior dorsal endoderm (arrowheads). (C) Xdazl-depleted embryos showing examples of reduced (arrowhead) or absent Xpat-labelled PGCs. (D) Close up view of PGCs in an uninjected tadpole. (E) Close up of an oligo-injected tadpole showing only four PGCs. (F) Example of an oligo-injected tadpole with no PGCs.

Fig. 5. PGC migration from the ventral endoderm is impaired in Xdazl-depleted, late tailbud (stage 35) embryos. (A-I) Whole-mount in situ hybridization for Xpat RNA. (A) uninjected control embryo. (B) Xdazl-depleted embryo. (C) Close up view of the embryo in A showing Xpat-labelled PGCs in the process of migration. Note that many are equal or dorsal to level of the gut tube (dashes). (D) Section of the embryo in C showing position of PGCs in the lateral endoderm (arrowhead). (E) Close up of the Xdazl-depleted embryo in B. Xpat-labelled cells are located exclusively below the level of the gut tube (dashes). (F) Section through the embryo in E demonstrating the ventral location of PGCs (arrowhead). (G) Uninjected embryo from another experiment showing distribution of Xpat-labelled PGCs (arrowhead). (H) Xdazl-depleted embryo in which PGCs are absent. (I) Xdazl-depleted embryo rescued with injected Xdazl RNA. Note the restoration of migration and Xpat expression (arrowhead). (J) RT-PCR analysis of Xpat expression in stage 35 embryos (3 embryos pooled for each lane). Scale bar, 150 μm.
endogenous Xdazl. Rescued stage 40 embryos showed a restoration of both PGCs (Table 2) and Xpat RNA levels (Fig. 4A).

Although our results with stage 43/44 tadpoles suggested otherwise, we asked whether there were PGCs present in the stage 40 embryos that lacked Xpat RNA. We analyzed serial sections of three Xdazl-depleted stage 40 embryos that lacked Xpat RNA and failed to find cells with the morphological features of PGCs (not shown). This observation suggests that PGC migration did not occur in the absence of Xpat RNA. Additionally, the lack of Xpat-containing cells remaining in the gut indicates that PGC migration was not merely delayed and that residual PGCs do not persist in the endoderm of Xdazl-depleted embryos.

### Table 1. Injection of Xdazl oligos inhibits PGC formation

<table>
<thead>
<tr>
<th>Series</th>
<th>Treatment</th>
<th>n</th>
<th>Number of PGCs per embryo (stage 43/44)</th>
<th>Means±d.</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Uninjected</td>
<td>12</td>
<td>6.7,9,10,12,13,14,15,19,21</td>
<td>12.6±4.4</td>
</tr>
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<td></td>
<td>XDZ4, 3.5 ng</td>
<td>15</td>
<td>0.0,0,0,2,3,3,4,4,4,5,7,7,8,10</td>
<td>*4±3</td>
</tr>
<tr>
<td>2</td>
<td>Uninjected</td>
<td>14</td>
<td>8.8,9,10,12,13,13,14,14,15,17,19</td>
<td>12.7±3.2</td>
</tr>
<tr>
<td></td>
<td>XDZ4, 5 ng</td>
<td>11</td>
<td>2.3,3,3,4,6,8,8,8,9,9</td>
<td>*5.7±2.8</td>
</tr>
<tr>
<td>3</td>
<td>Uninjected</td>
<td>19</td>
<td>10,13,13,14,14,14,15,16,16,17,19,20,22,22,24</td>
<td>16.6±3.7</td>
</tr>
<tr>
<td></td>
<td>XDZ2, 3 ng</td>
<td>27</td>
<td>0,0,0,0,0,0,0,1,1,1,1,1,2,3,4,4,6,6,7,8,8,11</td>
<td>*2.7±3.4</td>
</tr>
</tbody>
</table>

PGCs located in the dorsal mesentery of control uninjected or oligo (XDZ2 or XDZ4)-injected embryos were counted in Haematoxylin and Eosin-stained sections. Numbers of PGCs in individual embryos from three different experiments are shown, as well as the means and standard deviations (s.d.). Asterisks (*) indicate significant differences (P<0.00001) between oligo-injected and control groups as determined by the Student’s t-test.

### Table 2. Summary of Xpat expression in Xdazl-depleted embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Xpat expression</th>
<th>Stage 12 %</th>
<th>Stage 26 %</th>
<th>Stage 35 %</th>
<th>Stage 39/40 %</th>
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<tr>
<td>Uninjected</td>
<td>Normal</td>
<td>5/5 (100)</td>
<td>3/3 (100)</td>
<td>11/12 (92)</td>
<td>7/7 (100)</td>
</tr>
<tr>
<td>Reduced</td>
<td>0/5 (0)</td>
<td>0/3 (0)</td>
<td>0/12 (0)</td>
<td>0/7 (0)</td>
<td>0/14 (0)</td>
</tr>
<tr>
<td>Absent</td>
<td>0/5 (0)</td>
<td>0/3 (0)</td>
<td>1/12 (8)</td>
<td>0/7 (0)</td>
<td>0/14 (0)</td>
</tr>
<tr>
<td>Oligo (3 ng XDZ2)</td>
<td>Normal</td>
<td>8/8 (100)</td>
<td>4/4 (100)</td>
<td>1/14 (7)</td>
<td>1/20 (5)</td>
</tr>
<tr>
<td>Reduced</td>
<td>0/8 (0)</td>
<td>0/4 (0)</td>
<td>5/14* (36)*</td>
<td>6/20 (30)</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0/8 (0)</td>
<td>0/4 (0)</td>
<td>8/14 (57)</td>
<td>13/20 (65)</td>
<td></td>
</tr>
<tr>
<td>Rescue (oligo +300 pg Xdazl RNA)</td>
<td>Normal</td>
<td>N.D.</td>
<td>N. D.</td>
<td>6/11 (55)</td>
<td>3/12 (25)</td>
</tr>
<tr>
<td>Reduced</td>
<td>4/11 (36)</td>
<td>6/12 (50)</td>
<td>3/12 (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>1/11 (9)</td>
<td></td>
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</table>

Embryos were analyzed for Xpat expression at the indicated stages by whole-mount in situ hybridization. The data are shown as the number of embryos with the indicated expression out of the total number tested. Values are represented as percentages in parentheses. Normal Xpat expression: ≥4 expressing cells/embryo at stage 12 and ≥10 expressing cells at later stages. Reduced expression in stage 26, 35 and 40 embryos: 1-9 expressing cells/embryo. Absent represents no detectable Xpat expression. Asterisks (*) indicate Xpat-expressing embryos with abnormal migration. N.D., not determined.

Xdazl-depleted PGCs fail to migrate from the ventral endoderm of late tailbud embryos

We next analyzed progressively earlier stages by staining for Xpat to determine if the defect in PGC formation in Xdazl-depleted embryos became apparent. PGCs in uninjected stage 35 embryos were typically found in the process of migration from the ventral and lateral endoderm with some cells reaching the dorsal endoderm (Fig. 5A,C,G). In stage 35 Xdazl-depleted embryos, Xpat-containing cells were either lost (8/14 cases, Table 2), or reduced in number compared to controls (5/14 embryos). Additionally, the PGCs that were detected in depleted embryos remained located in the ventral endoderm (Fig. 5B,E). In only one Xdazl-depleted embryo (out of 14) did we see PGCs reaching the dorsal endoderm. This impaired migration phenotype is striking in sectioned embryos (compare Fig. 5D with F). RT-PCR analysis confirmed that Xpat RNA was reduced in abundance (~60% control levels, Fig. 5J). In rescued stage 35 embryos, normal Xpat expression and PGC migration were seen in 6/11 cases (55%) compared to 1/14 (7%) in depleted embryos (Fig. 5I, Table 2). Also, the number of embryos lacking Xpat expression declined from 8/14 (57%) in oligo-injected embryos to 1/11 (9%) in rescued Xdazl-depleted embryos. The restoration of Xpat expression in rescued embryos was also confirmed by RT-PCR (Fig. 5I). These findings show that, in Xdazl-depleted embryos, PGCs are lost at the late tailbud stage, at or near the time when PGCs normally begin their dorsal migration. Migration is the first overt indication of PGC differentiation.

Germ plasm aggregation and early PGC formation are normal in Xdazl-depleted embryos

Embryos at stage 12 (gastrula), and 26 (tailbud) were next examined to determine if initial events in PGC specification had occurred properly in Xdazl-depleted embryos (Figs 6; Table 2). Analysis of Xpat expression by RT-PCR in both stages confirmed that the abundance of Xpat RNA was similar in control and depleted embryos (Fig. 6G). Azan staining (Fig. 6A-D) or Xpat in situ hybridization (Fig. 6E,F; Table 2) of control and Xdazl-depleted gastrulas showed a similar number and distribution of PGCs located in the presumptive endoderm. Germ plasm aggregation occurred in both controls and Xdazl-depleted embryos, as judged by the presence of large pools of germ plasm positively stained with Azan. Importantly, the
germ plasm was perinuclear at this stage, indicating that re-localization was not affected by depletion of Xdazl (Fig. 6C,D). Sectioning confirmed that Xpat expression was restricted to the germ plasm (Fig. 6E,F).

At stage 26, Xdazl-depleted embryos and uninjected controls had similar numbers of PGCs, as judged by Xpat staining (Fig. 7A,B). Additionally, these numbers were increased from gastrula embryos, suggesting that the germ plasm in Xdazl-depleted embryos was segregated equally into dividing daughter cells. Although the PGCs were situated in similar positions within the endoderm, PGCs in oligo-injected embryos seemed to cluster together more than in the control embryos (Fig. 7B, arrows). Sectioning also confirmed that PGCs were located in similar positions within the endoderm. These results show that the reduction in PGC numbers in Xdazl-depleted tadpoles is not caused by defects in the physical integrity or movements of the germ plasm, nor in the initial expansion of the PGCs.

**DISCUSSION**

Numerous genes are known to be required for PGC formation in Drosophila and C. elegans (reviewed in Wylie, 1999). In contrast, until this study, no germ plasm-localized molecules have been shown to be necessary for PGC formation in Xenopus. This is mainly because the genetic screens used to identify genes in flies and worms are unavailable for frogs. The results presented here show for the first time that a specific molecule in frog germ plasm, Xdazl, has a role in PGC development in Xenopus. Xdazl is an RNA-binding protein homologous to the human Deleted in Azoospermia (DAZ) and related proteins in many species (Houston et al., 1998). Both DNA and protein sequences are highly similar in DAZ-family genes and functional conservation has been demonstrated in some cases (Houston et al., 1998; Slee et al., 1999). Despite this conservation, roles of DAZ genes in germ cells appear to differ significantly between organisms. This work shows that Xdazl is involved in still another aspect of germ cell development, PGC migration in Xenopus.

Previous work demonstrated that Xdazl RNA is expressed in the germ plasm until the neurula stage. The immunostaining results presented here demonstrate that Xdazl protein is also localized to the germ plasm of embryos. The protein becomes detectable in blastula stages and persists until early tailbud stages (stage 22). The increase in protein accumulation occurs during the time when the RNA levels decrease. This is consistent with degradation of the maternal transcript during translation. Within the cell, Xdazl protein is cytoplasmic and localized to the germ plasm. Other DAZ proteins are also cytoplasmic (Ruggiu et al., 1997; Cheng et al., 1998), arguing against roles in nuclear RNA metabolism such as splicing. The period of time when Xdazl protein is present spans the shift in germ plasm position from plasma membrane to perinuclear and continues until a stable PGC population is formed. The protein then becomes undetectable during the migratory phase of PGC development. These results suggest a role for Xdazl during early PGC differentiation.

Although Xdazl RNA is present from early oogenesis, the protein is not detected until the blastula stage. This implies that Xdazl RNA is under translational repression. Another germ plasm-localized RNA, Xcat2, is under similar repression (MacArthur et al., 1999). The factors that mediate this repression must also be present early in oogenesis and may be part of the RNA localization apparatus. Derepression of translation of maternal germ plasm RNAs might be one of the initial steps in PGC specification. It will be important to establish whether this occurs cell autonomously or as a result of inductive interactions.

In addition to demonstrating expression of Xdazl protein in early PGCs, these results indicate an essential role for Xdazl in PGC development. This was shown in several ways. First, tadpoles grown from oocytes that were depleted of Xdazl RNA have few PGCs or none at all. Second, gonads from metamorphosing tadpoles depleted of Xdazl are deficient in germ cells. Third, analysis of Xpat RNA levels in Xdazl-depleted embryos showed that PGCs fail to migrate to the dorsal endoderm at the correct time and are subsequently lost. These effects were all specific to depletion of Xdazl as injection of synthetic Xdazl RNA rescued PGC development to a large extent. In our experiments, we injected Xdazl RNA 24 hours after the oligos, which is sufficient time for the oligos to be degraded (Zhang et al., 1998). Thus, rescue of the phenotype occurs by supplying Xdazl protein to embryos that lack it, rather than by competition with endogenous Xdazl for oligo binding. The rescue experiments control for both degradation of RNAs other than Xdazl as well as non-specific oligo effects.

Several pieces of evidence indicate that while early PGC formation is normal, PGC migration is severely affected. Azan staining or Xpat in situ hybridization of gastrula embryos showed that germ plasm aggregation and perinuclear accumulation occur normally. These observations rule out a role for Xdazl in germ plasm movements, even though the protein is made prior to the perinuclear accumulation of the germ plasm. Evidence also argues against the involvement in PGC proliferation because PGC numbers and Xpat RNA levels appear normal in stage 26 embryos. Also, although Xdazl protein is expressed during the first wave of PGC mitoses between stages 10 and 22 (Dziadek and Dixon, 1977), Xdazl is not detectable during the time of the second wave of PGC cell division (stages 26-31).

The first visible effect on PGC development in Xdazl-depleted embryos is a clustering of PGCs during the early tailbud stages. The significance of this aggregation is unknown. Increased self-adhesion of PGCs could be due to acquisition of endodermal characteristics. Alternatively, this could be the first indication of inhibited migration. Abnormal PGC clustering is also seen in Drosophila pole cells lacking nanos activity (Forbes and Lehmann, 1998), suggesting that regulation of cell adhesion is important for PGC development in multiple species.

In normal development, PGCs begin to migrate from the ventral endoderm during late tailbud stages (stages 30-36). At first the PGCs move laterally and then dorsally until they reach the dorsal crest of the endoderm by stage 40. In Xdazl-depleted embryos, PGCs appear laterally, but do not migrate dorsally. This was seen dramatically in sections, with PGCs in Xdazl-depleted embryos restricted below the level of the gut tube. The lateral movement of PGCs in Xdazl-depleted embryos could be the result of diminished migratory activity or may be the result of passive displacement due to normal morphogenetic movements of the gut endoderm. In subsequent stages, the
majority of PGCs are lost and cannot be identified either histologically or by Xpat in situ hybridization. The few PGCs that do make it past this point migrate normally into the dorsal mesentery. The variation seen in the number of PGCs in Xdazl-depleted embryos probably results from incomplete destruction of maternal Xdazl.

The loss of PGCs could be due either to death or differentiation into other cell types. We did not see any evidence of apoptotic nuclei in DAPI-stained sections or in whole-mount TUNEL analysis (not shown) of Xdazl-depleted stage 35 embryos. These observations do not prove that the PGCs are not dying, however other evidence suggests that differentiation into other cell types is more likely. First, Wylie et al. (1985) demonstrated in PGC transplantation experiments that germ plasm-containing blastomeres and migrating PGCs could both differentiate into somatic cell types when placed ectopically. This demonstrates that transdifferentiation of PGCs is possible in Xenopus, contrary to the situation in Drosophila (Underwood et al., 1980). Also, initial stages in PGC formation are normal in Xdazl-depleted embryos and Xpat RNA persists until the migration phenotype is pronounced. This also differs from the case in Drosophila. In pole cells lacking nanos and pgc, ectopic pole cells continue to express PGC-specific markers such as Vasa (Forbes and Lehmann, 1998; Nakamura et al., 1996). Xdazl-depleted embryos do not exhibit ectopic PGCs and PGC-specific markers are lost. Although more experiments are needed to determine if Xdazl-depleted PGCs are indeed differentiating as other cell types, the above evidence taken together suggests that the PGCs lose their pluripotency and differentiate according to the surrounding environment. Also, because pluripotency seems to be linked with Xpat expression, it will be interesting to determine the role of this novel molecule in PGC development.

One plausible model for the effects seen in Xdazl-depleted embryos, is that Xdazl is required for the establishment of the migrational competence of PGCs during the formation of the initial PGC population. It is unlikely that Xdazl is directly involved in migration in later stages (stages 30-40) because the protein is only detected earlier. As migration is the first aspect of differentiation displayed by PGCs, and as Xdazl is unlikely to affect migration directly, it is more correct to state that Xdazl is necessary for PGC differentiation. Xdazl encodes an RNA-binding protein (Houston et al., 1998) and the protein is cytoplasmic, thus possible roles for Xdazl include regulation of translation, RNA storage, RNA transport or regulation of RNA stability. In establishing migrational competence, Xdazl could regulate the translation or expression of specific cell surface or cytoskeletal proteins needed to respond to...
migrational cues. Alternatively, Xdazl could be involved in repressing expression of factors that inhibit migration. PGC migration is often regulated by signals from the environment (reviewed in Wylie, 1999). In this model, PGCs lacking Xdazl are not competent either to receive or respond to such signals, resulting in the absence of migration, the same signals or competing ones may then cause differentiation into other cell types, possibly endoderm, accounting for the loss of PGCs and Xpat RNA.

Clearly, additional work is needed to determine if this model is correct. It will important to establish whether the Xdazl-depleted PGCs are indeed becoming endodermal cells. This could be accomplished by double-labeling experiments to show expression of endodermal markers in PGCs. Also, the identification of PGC-specific markers unique to different stages of development would aid in determining the exact time that PGC development is perturbed in Xdazl-depleted embryos.

The predominant view in the literature about the function of germ plasm is that it acts to ‘protect’ the pluripotent state of PGCs (Blackler, 1958; Dixon, 1994). The data presented here do not directly support or refute the idea of germ plasm protection. However, PGCs in Xdazl-depleted embryos fail to migrate while still expressing the maternal PGC-specific marker Xpat. Thus Xdazl does not appear to be involved in specifying cells as PGCs, rather it is required for a specific aspect of PGC differentiation. It is possible that some germ plasm components are critical for conferring pluripotency while others are responsible for differentiation. Alternatively, pluripotency could be a consequence of PGC differentiation, not its cause. The experiments described here demonstrate the utility of antisense oligo depletion of maternal mRNAs in studying the function of germ plasm components. By studying other maternal germ plasm components, a clearer picture of how PGCs develop in Xenopus should emerge.

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Xdazl is required for PGC formation 455


