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A *Xenopus DAZ*-like gene encodes an RNA component of germ plasm and is a functional homologue of *Drosophila boule*

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SUMMARY

We have identified a localized RNA component of *Xenopus* germ plasm. This RNA, *Xdazl* (*Xenopus DAZ*-like), encodes a protein homologous to human DAZ (Deleted in Azoospermia), vertebrate DAZL and *Drosophila* Boule proteins. Human males deficient in *DAZ* have few or no sperm and *boule* mutant flies exhibit complete azoospermia and male sterility. *Xdazl* RNA was detected in the mitochondrial cloud and vegetal cortex of oocytes. In early embryos, the RNA was localized exclusively in the germ plasm. Consistent with other organisms, *Xdazl* RNA was also expressed in the spermatogonia and spermatocytes of frog testis. Proteins in the DAZ-family contain a conserved RNP domain implying an RNA-binding function. We have shown that Xdazl can function in vitro as an RNA-binding protein. To determine if the function of *Xdazl* in spermatogenesis was conserved, we introduced the *Xdazl* cDNA into *boule* flies. This resulted in rescue of the *boule* meiotic entry phenotype, including formation of spindles, phosphorylation of histone H3 and completion of meiotic cell division. Overall, these results suggest that Xdazl may be important for primordial germ cell specification in the early embryo and may play a role analogous to Boule in promoting meiotic cell division.

Key words: *Xenopus*, Germ plasm, Localized RNA, Spermatogenesis, RNA-binding protein, *boule*, *DAZ*, *DAZL*

INTRODUCTION

The primordial germ cells in *Xenopus* are thought to be specified by the inheritance of germ plasm. The coincidence of germ plasm with the germline in Anurans has long been noted (Bounoure, 1934; Blackler, 1958) but distinct biochemical functions have yet to be assigned to any of its components. Ultrastructurally, germ plasm appears as an electron-dense cytoplasmic aggregation of RNP-like particles (or germinal granules), containing ribosomes, RNA, cytoskeleton and clusters of mitochondria (Czolowska, 1972). Similar structures appear in *Drosophila* pole plasm (Mahowald, 1968) and *C. elegans* P-granules (Strome and Wood, 1982), suggesting an evolutionary conservation of cytoplasmic germ cell specification between invertebrates and vertebrates.

In *Xenopus*, the germ plasm initially accumulates within the mitochondrial cloud of stage I oocytes (Heasman et al., 1984) and persists in the mature oocyte as yolk-free islands distributed in the vegetal cortex. Following fertilization, these islands aggregate to form large masses at the vegetal pole (Ressom and Dixon, 1988), a process that requires a *Xenopus* kinesin-like protein, Xklp-1 (Robb et al., 1996). During the first cell divisions, the germ plasm becomes associated with cleavage furrows (Whitington and Dixon, 1975). In the blastula stage embryo, the germ plasm remains associated with the plasma membrane and localizes to only one spindle pole at mitosis, resulting in unequal segregation. During early gastrulation, the germ plasm shifts to a perinuclear location. One result of this shift is the subsequent equal segregation of germ plasm at mitosis and an increase in the number of pPGCs (Whitington and Dixon, 1975). The germ plasm persists in postgastrula embryos, in migrating PGCs, as well as in early stages of spermatogenesis (Kerr and Dixon, 1974) and oogenesis (al-Mukhtar and Webb, 1971).

Embryological experiments support a role for germ plasm in PGC specification. Removal of vegetal pole egg cytoplasm (Buehr and Blackler, 1970), treatment of eggs with low doses of ultraviolet (u.v.) light (Züst and Dixon, 1975) and displacement of germ plasm relative to the embryonic axes by sustained egg rotation (Cleine and Dixon, 1985) all result in a reduction in numbers of PGCs or delayed PGC migration to the gonadal ridge. Additionally, injection of vegetal pole cytoplasm into normal eggs increases PGC numbers (Wakahara, 1978) and can rescue PGC formation in u.v. irradiated embryos (Smith, 1966, Wakahara, 1977). In some experiments, cells derived from explant culture of single pPGCs from cleavage stages retained the ability to form migrating PGCs and functional gametes when implanted into posterior endoderm at stage 22 (tailbud) (Ikenishi et al., 1984). In other experiments, however, explanted pPGCs and isolated

migrating PGCs could not form germ cells when placed into an ectopic location (blastocoel) in host embryos (Wylie et al., 1984). Importantly, the implanted PGCs differentiated as somatic cells and contributed to all three germ layers. These experiments indicate that germ plasm specifies, rather than irreversibly determines, the germline in *Xenopus*.

In contrast, *Drosophila* pole plasm appears to act as an irreversible determinant of germ cell fate. Injection of pole plasm at ectopic locations induces the formation of functional germ cells (Illmensee and Mahowald, 1974). Indeed, mis-expression at the anterior pole of a single mRNA, *oskar* (*osk*), is sufficient to organize pole plasm and create ectopic pole cells (Ephrussi and Lehmann, 1992). Once formed, pole cells lose their ability to contribute to somatic cell lineages (Technau, 1987).

One important similarity between the two species is the presence of localized RNA molecules in their germ plasm. Genetic studies in *Drosophila* have revealed an ordered assembly of RNA and protein components into pole plasm (reviewed in Rongo and Lehmann, 1996). Although no protein components of *Xenopus* germ plasm have been identified, several RNAs have been found with localization patterns similar to germ plasm (Forristall et al., 1995; Kloc et al., 1993; reviewed in King, 1995). Initially, all these RNAs accumulate in the mitochondrial cloud of stage I oocytes before translocating to the vegetal pole and becoming concentrated within the cortex. This pattern is temporally and spatially distinct from that of *Vg1* RNA (Melton, 1987; Forristall et al., 1995), and may represent a unique pathway for localization of germ-plasm-specific transcripts (Zhou and King, 1996). One of these RNAs, *Xcat-2*, is related to a *Drosophila* pole plasm component, *nanos* (Mosquera et al., 1993), which is essential for germline formation (Lehmann and Nüsslein-Volhard, 1991; Kobayashi et al., 1996).

Zhang and King (1996) have identified RNAs localized to the vegetal cortex by differential screening of an oocyte cDNA library. One of these cDNAs showed an expression pattern during oogenesis similar to that of *Xcat-2*. We chose this cDNA for further characterization in order to study the nature and function of germ plasm components. In this paper, we identify the cDNA as a frog homologue of the human *Deleted in Azoospermia (DAZ)*, the vertebrate *DAZ*-like (*DAZL*) and the *Drosophila boule(bol)* genes.

DAZ is present as a multicopy gene cluster on the human Y chromosome (Reijo et al., 1995; Saxena et al., 1996). Deletion of this locus correlates with azoospermia (Reijo et al., 1995) or severe oligozoospermia (Reijo et al., 1996a), with phenotypes varying from 'Sertoli cell only syndrome' to testicular maturation arrest of spermatocytes at meiotic prophase. *DAZL*, a human autosomal *DAZ* gene on chromosome 3p24, has been described by several groups (Saxena et al., 1996; Yen et al., 1996; Shan et al., 1996), as have homologues in other organisms, including mouse (*Dazl*, Cooke et al., 1996; Reijo et al., 1996b) crab-eating macaque (Carani et al., 1997) and *Drosophila (boule*, Eberhart et al., 1996). The *boule* mutant phenotype is complete azoospermia resulting from spermatocyte arrest at the G_2/M transition (Eberhart et al., 1996). In contrast, *DAZ* deletions are compatible with a range of phenotypes including very limited spermiogenesis (Reijo et al., 1996a), suggesting that *DAZ* may not be strictly required for completion of meiosis.

We show that the *Xenopus* gene, *Xdazl* (*Xenopus DAZ*-like), is expressed as an RNA localized to the mitochondrial cloud and vegetal cortex of oocytes, and to the germ plasm of early embryos. *Xdazl* transcripts were also detected in premeiotic spermatogonia and spermatocytes in the testis. We further show that Xdazl can function as an RNA-binding protein in vitro and can rescue meiotic entry of spermatocytes in *Drosophila boule* mutants.

MATERIALS AND METHODS

Xenopus tissues, oocytes and embryos

Tissues were dissected from MS222 anesthetized frogs and frozen in liquid nitrogen. Postmetamorphic froglets (0.5-2 cm from nose to anus) were raised in the laboratory. Juvenile frogs were purchased from Xenopus Express (1-3 cm nose to anus). Ovary and testis pieces were cut into clumps prior to processing for histology. Individual oocytes were obtained either by collagenase treatment of ovaries or by manual defolliculation. Ovulated eggs from hCG-induced females were fertilized in vitro and placed in 0.1× MBS (Gurdon, 1977). Embryos were subsequently dejellied and staged according to the normal table of Nieuwkoop and Faber (1967).

DNA sequencing, analysis and 5′RACE

The original 1.9 kilobase (kb) *Xdazl* cDNA clone was obtained from a differential screening of a cDNA library enriched in localized RNAs as described in Zhang and King (1996). The insert of this clone, in the vector pSPORT1 (Life Technologies), was sequenced on both strands using the Sequenase v2.0 kit as recommended by the supplier (Amersham/USB). To obtain full-length sequence, 5′RACE was performed on stage I oocyte total RNA using the Marathon RACE kit (Clontech) according to the manufacturer's instructions. Four independent RACE cDNA clones were cloned into pSPORT1 and sequenced. The longest RACE clone (0.4 kb) and the original *Xdazl* cDNA were simultaneously subcloned into pCS2+ (Turner and Weintraub, 1994) via an overlapping *Nco*I site in the coding region to generate a full-length clone 2.3 kb in length (pXDZ). To create a plasmid containing only the *Xdazl* coding region, primers engineered with *Eco*RI sites were used to amplify this fragment by PCR. Following restriction enzyme digestion, the piece was subcloned into the *Eco*RI site of pCS2+. Plasmids with the fragment cloned into both sense and antisense orientations were obtained (pXDZs and pXDZas). Sequence analyses and database searches were performed using the GCG Wisconsin Package.

Northern blot and RNase protection

Total RNA from embryos was isolated by proteinase K extraction. RNA from various frozen tissues was isolated by guanidinium thiocyanate-acid phenol extraction (Chomczynski and Sacchi, 1987). RNAs were precipitated with LiCl prior to use. For northern blots, 15 μg total RNA was fractionated on a formaldehyde/agarose gel and transferred to nylon membranes by standard capillary blotting. The blots were processed and hybridized according to the methods of Sambrook et al. (1989). Radiolabeled *Xdazl* (*Not*I/*Sal*I fragment of the original clone) or *EF1-α* cDNA probes were generated by random primer labeling. Membranes were washed twice in 2× SSPE/0.1% SDS at room temperature and exposed to Phosphorimager screens (Molecular Dynamics).

RNase protection was performed essentially as previously described (Forristall et al., 1995) with the following modifications. The *ODC2* probe was synthesized at a low specific activity by reducing the amount of [^{32}P]CTP (800 Ci/mmol; 10 μCi/μl) in the transcription reaction fivefold and supplementing with 200 μM cold CTP. All probes were gel purified and each added to RNA samples at

an activity of 10,000 cts/minute. Hybridized samples were cooled to room temperature prior to addition of 300 μl RNase digestion buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 300 mM NaCl, 10 U/ml RNase T1 and 2.5 μg/ml RNase A). Digestion proceeded for 1 hour at room temperature. RNAs were precipitated, denatured and resolved on 6% polyacrylamide/7 M urea/1× TBE minigels. The gels were then fixed for 5 minutes in 10% methanol/10% acetic acid, dried and exposed to Phosphorimager screens. To generate an antisense *Xdazl* probe, the pXDZas plasmid was digested with *Xmn*I and transcribed with SP6 (Promega) to give a probe 262 nucleotides (nt) in length and a subsequent protected fragment of 217 nt. The *ODC* probe was used as a control for RNA loading (Bassez et al., 1990) and gave a 90 nt protected fragment.

In situ hybridization, histology and immunostaining

Tissue, oocyte and embryo samples were fixed in MEMFA (100 mM MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for 1 hour at room temperature and then embedded in paraffin. Sections were cut on a rotary microtome and adhered to SilanePrep slides (Sigma). Wax was removed in xylene and the sections were rehydrated in an ethanol series. Samples were then postfixed in 4% paraformaldehyde/PBS, washed in 2× SSPE, treated with 0.2 N HCl, 100 mM triethanolamine and 0.25% acetic anhydride. Hybridization was performed with 1.0 μg/ml digoxygenin-labeled antisense RNA probe overnight at 65°C in a humidified chamber. Slides were then treated with 20 μg/ml RNase A in 2× SSPE and washed in 2× SSPE/50% formamide at 50°C for 5 minutes. Detection with anti-digoxigenin alkaline phosphatase was performed by washing in malate buffer (MAB; 150 mM malate pH 7.4, 100 mM NaCl, 0.1% Tween 20), blocking in 2% blocking reagent (BMB) with 20% lamb serum in MAB for 1 hour, and incubation with a 1:500 dilution of anti-digoxigenin alkaline phosphatase-Fab fragments (BMB). Slides were washed twice for 20 minutes in MAB followed by alkaline phosphatase buffer. The chromagenic reaction was performed using 75 μl BM Purple (BMB) containing 5 mM levamisole. Sections were then dehydrated in an ethanol series, cleared in xylene and mounted in AquaMount (Baxter). Unhybridized sections were stained with hematoxylin and eosin. *Xdazl* antisense digoxigenin-labeled RNA probes were generated by linearizing pXDZ plasmid with *Sma*I and transcribing with T7 (Promega). Sense probes were linearized with *Asp*718 and transcribed with SP6 (Promega). In some experiments, an antisense mitochondrial large ribosomal RNA (mtLrRNA) was also used as described (Zhou and King, 1996). Whole-mount in situ hybridization was performed as described in Harland (1991).

For immunostaining of sections, stage 7 and 10 embryos were fixed for 2 hours at room temperature in 2% (w/w) trichloroacetic acid and then embedded in paraffin. Sections were cut at 7 μm and adhered to SectionLock slides (Polysciences Inc.). Following dewaxing and rehydration as above, the sections were stained with anti-vimentin antibody Z10 (a gift from Drs. J. Heasman and C. C. Wylie; Torpey et al., 1992) to identify the germ plasm.

RNA homopolymer binding assay

In vitro RNA homopolymer binding assays were performed as previously described by Swanson and Dreyfuss (1988). Uncapped RNAs were translated in reticulocyte lysate (Promega) in the presence of [^{35}S]methionine and added to Sepharose-conjugated RNA homopolymers (Pharmacia or Sigma) in binding buffer. Following binding and washing, the beads were resuspended in 50 μl SDS-PAGE buffer, boiled and 25 μl was run on 12.5% SDS-PAGE gels (29:1 acrylamide:bis). The gels were dried and exposed to Phosphorimager screens. *Xdazl* transcript was synthesized from pXDZ linearized with *Spe*I. hnRNPC1 was transcribed from pHC12 (Burd et al., 1989; a gift from Dr G. Dreyfuss) linearized with *Nsi*I. Transcription reactions were performed using the MegaScript kit with SP6 polymerase (Ambion) according to the manufacturer's instructions.

Drosophila germline transformation

For *boule* rescue experiments, the *Xdazl* coding region cDNA was cloned into a P element testis expression vector (Hoyle and Raff, 1990) and co-injected with a plasmid containing P[ry+,Δ2-3] into w^{1118} embryos using standard protocols (Spradling, 1986). The testis expression vector is a pCaSper4 based plasmid that contains the β2-tubulin promoter and regulatory sequences, including the 5′ and 3′ untranslated regions of the β2-tubulin message. Genetic crosses were carried out to generate three independent lines of *boule* homozygotes carrying one or two copies of the P[w+, *Xdazl*] on the second chromosome.

Analysis of *Drosophila* testis contents

Testis contents from transgenic males less than 24 hours old were examined by phase-contrast and indirect immunofluorescence microscopy. Fixation and immunostaining followed the method of Cenci et al. (1994). Tubulin was detected using monoclonal T9026 clone DM1 (Sigma) at a 1:500 dilution. Phospho histone H3 was detected using anti-phospho histone H3 rabbit polyclonal IgG (Upstate Biotechnology) at 1:200. Cy2-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG (Jackson Laboratories) were used at 1:500. Hoechst 88842 (Sigma) was added at 0.5 μg/ml. Fluorescent images were recorded from a Leica DMRXE microscope equipped with a cool charge-coupled (CCD) digital imaging camera (Hamamatsu). Digital images were coverted to Photoshop (Adobe) and merged.

RESULTS

A *Xenopus* vegetally localized RNA encodes a DAZ-like protein

DNA sequencing of the original *Xdazl* cDNA cloned by Zhang and King (1996) revealed a 5′ open reading frame of about 200 amino acids, a stretch of 15 adenines at the 3′ end and a consensus poly(A) site AATAAA 12 nucleotides upstream from the start of the adenines. In order to obtain the rest of the 5′ sequence and open reading frame, we performed 5′ RACE on stage I oocyte cDNA using the Marathon RACE kit (Clontech). One of the RACE clones was joined to the original cDNA to generate a full-length clone. The full-length *Xdazl* cDNA was approximately 2300 bp in length, consisting of 60 nt of 5′ UTR, roughly 900 nt of coding region and a 1300 nt long 3′ UTR. A Southern blot of genomic DNA detected a single band, indicating the presence of a single *Xdazl* gene (data not shown). The translated sequence of the cDNA contains a methionine in a favorable initiation context and can encode a putative protein of 286 amino acids (Fig. 1A). Protein sequence analysis revealed a conserved RNP domain (Burd and Dreyfuss, 1994), including consensus RNP-1 octamer and RNP-2 hexamer sequence motifs, as well as a C-terminal domain containing numerous proline residues (22/175 amino acids). Database comparisons with the conceptual protein indicated strong homology to several proteins related to and including the human DAZ (Deleted in Azoospermia) protein and the *Drosophila* Boule protein. *DAZ* was identified as a candidate male sterility gene (Reijo et al., 1995) and *boule* (Eberhart et al., 1996) is required for meiotic cell division in spermatogenesis.

Similarity among the DAZ homologues is high. Xdazl and DAZ have 42% identical residues overall; while Xdazl shares approximately 60% identical residues with mouse and human Dazl proteins. Similarity to the Boule protein is less, with 27%

of the residues remaining conserved from flies to frogs. Xdazl also contains some residues corresponding to the 24 amino acid DAZ repeat (Fig. 1B), although the Xdazl protein has a three amino acid insertion, AIQ, in the middle of the motif. In general, the proteins appear well conserved within the RNP domain and more divergent at the amino terminus and in the C-terminal domain (Fig. 1B,C).

At the nucleotide level, *Xdazl* is most closely related to the murine *Dazl* gene. In a stretch of 800 nucleotides constituting most of the coding region, the two genes are approximately 70% identical overall. Also, the sequences of many inferred exon boundaries (Saxena et al., 1996) are highly conserved from frog to man. The high amino acid and nucleotide sequence conservation among the *DAZ* members suggests that the genes have evolved from a common ancestor and may be involved in similar functions in all organisms.

Xdazl mRNA is expressed in germ cells and is localized to germ plasm

Since *Xdazl* was originally cloned from an oocyte-specific library, we next determined whether the RNA was expressed in any other adult tissues. Hybridization of a radiolabeled *Xdazl* cDNA probe to total RNA from various adult tissues detected a 2.4 kb transcript in testis as well as in the ovary (Fig. 2A). Overexposure (3 days) did not reveal any *Xdazl* expression in any of the somatic tissues tested. Temporal expression during early development was examined by RNase protection (Fig. 2B). *Xdazl* RNA abundance was decreased by half from the egg to stage 10 (initial blastopore lip formation), after which the RNA was undetectable. Temporally, *Xdazl* RNA was first detected in the gonads of postmetamorphic froglets (Fig. 2C). Thus, *Xdazl* RNA is present in the embryo at the time when germ plasm moves to its perinuclear location (stage 10) and then decreases in stages immediately following.

From preliminary data, we knew that *Xdazl* RNA localization in the oocyte was restricted first to the mitochondrial cloud and then to the vegetal cortex in a manner similar to that of *Xcat-2* RNA. Also, *Xdazl* appears to be unique among the *DAZ* homologues because the transcript is abundant during both oogenesis and spermatogenesis. To examine RNA expression in oogenesis more closely, we performed in situ hybridization on sections of ovaries from juvenile and adult females. In ovaries from newly metamorphosed froglets, we detected *Xdazl* RNA uniformly distributed in the cytoplasm of early diplotene oocytes (~100 μm; Fig. 3A). Interestingly, some oocytes expressed *Xdazl* while adjacent oocytes, independent of size, failed to stain for *Xdazl* RNA, indicating variability of expression at this early stage. For all these experiments, sections hybridized with the *Xdazl* sense probe showed no visible signal. In the stage I oocytes from older juvenile frogs and from adult females, the *Xdazl* RNA was concentrated primarily in the mitochondrial cloud (Fig. 3B,D). Hybridization with a probe for the mitochondrial large subunit rRNA (mtLrRNA; Fig. 3C) was

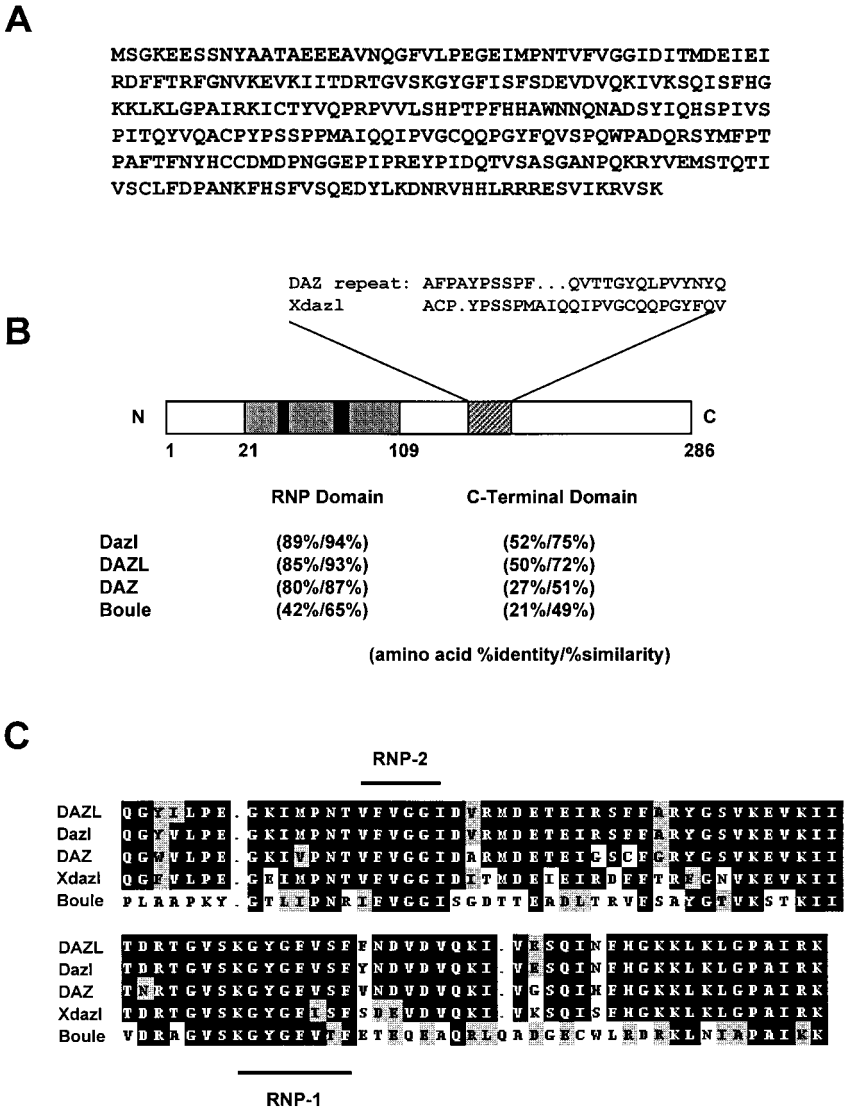

Fig. 1. Analysis of the predicted Xdazl amino acid sequence. (A) Amino acid sequence of Xdazl derived from the full-length cDNA. (B) Diagram showing domains and features of the Xdazl protein. N and C indicate the amino and carboxyl termini respectively, numbers show positions of amino acids beginning with the first in frame methionine. The gray box delineates the RNP domain, while the black stripes show the positions of the RNP-2 hexamer and RNP-1 octamer amino acid motifs respectively. The striped region represents the position of the putative DAZ repeat. Expanded above is the Xdazl sequence in that region compared to a consensus DAZ repeat. Periods indicate gaps in the sequence alignment. The table below the diagram shows the homology of Xdazl to other DAZ-family proteins in percent amino acid identity and similarity. (C) Sequence alignment of the Xdazl RNP domain. Identical residues are shaded black; similar residues are shaded gray. Periods indicate gaps in the alignment. The RNP-1 and RNP-2 motifs are indicated below and above the alignment, respectively. The GenBank accession number for the sequence reported in this paper is AF017778.

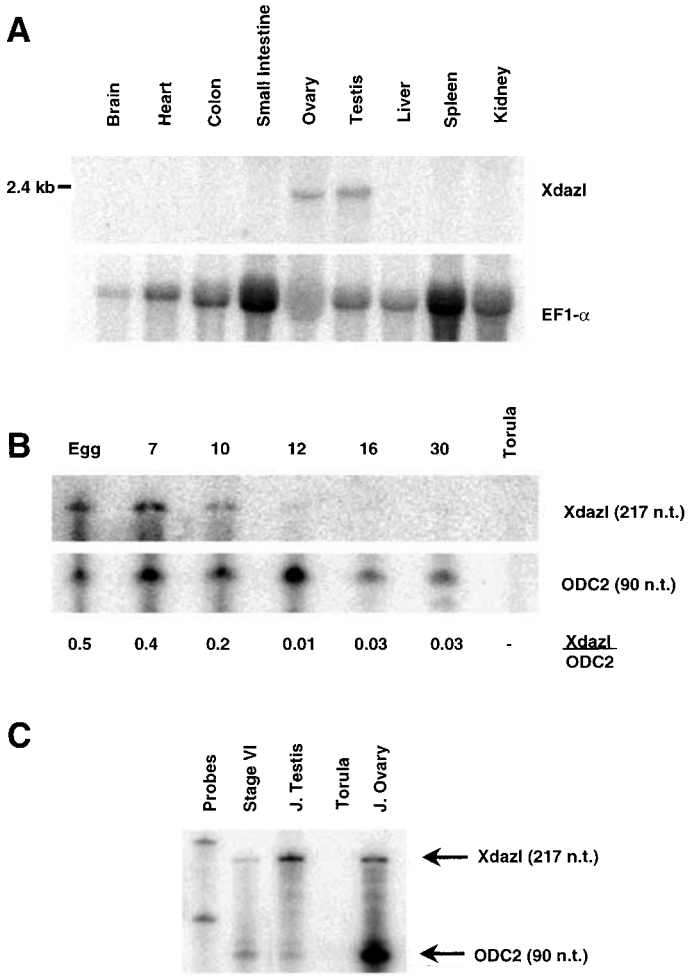

Fig. 2. Molecular analysis of *Xdazl* mRNA expression. (A) Northern blot of *Xdazl* RNA expression in adult tissues. *Xdazl* is shown in the top panel, below is the same membrane stripped and reprobed with EF1-α as a loading control. In the top panel, the size of the *Xdazl* band is shown at the left. The tissues tested are labeled above each lane. (B) RNase protection assay of *Xdazl* expression during embryogenesis. RNA from four embryo equivalents of the indicated stages was extracted and analyzed. Stages tested are indicated at the top of each lane. Torula (yeast) total RNA was included as a negative control. A probe for *ODC2* was included as a loading control. The ratio of *Xdazl:ODC2* relative band intensities as determined by Phosphorimager quantitation is shown below each lane. (C) RNase protection assay of *Xdazl* mRNA expression in juvenile testis (J. Testis) and ovary (J. Ovary). Positions of the *Xdazl* and *ODC2* protected fragments are indicated by arrows. Included here are examples of undigested probes (Probes) and two equivalents of positive control stage VI oocytes. Arrows indicate the positions of the protected fragments.

used to show that mitochondrial clouds had indeed formed by the stage analyzed. The RNA localization pattern in later stage oocytes generally resembled the *Xcat-2* pattern. *Xdazl* RNA was found in a wedge-like pattern extending to the cortex in stage II-III oocytes (Fig. 3E) and by stage VI was seen only near the vegetal cortex (Fig. 3F). This same pattern has been described for the localization of germ plasm in the oocyte (Heasman et al., 1984).

The maternal germ-plasm-like expression pattern of *Xdazl* RNA as well as its persistence until stage 10 suggested that the transcript is also restricted to the germ plasm during early embryogenesis. Initial whole-mount in situ hybridization experiments with DIG-labeled *Xdazl* antisense RNA probes showed that, indeed, *Xdazl* RNA was localized specifically to germ-plasm patches at the vegetal pole of 2- and 4-cell embryos (Fig. 4A,B). By stage 6, the *Xdazl* staining was limited to a smaller area in the vegetal pole blastomeres (Fig. 4C). To examine *Xdazl* expression during early embryogenesis in greater detail and to confirm that staining was specific for germ plasm, we hybridized *Xdazl* probes to sections of 8-cell, stage 7 and stage 10 embryos. These three stages were selected for analysis because clear descriptions of *Xenopus* germ-plasm morphology at these stages can be found in the literature (Whitington and Dixon, 1975). In the 8-cell embryo, germ plasm is easily identified in unstained sections, or in sections hybridized with control *Xdazl* sense probes (Fig. 5A), as yolk-free areas at the vegetal poles of the four vegetal blastomeres. In sections from the same embryo hybridized with antisense *Xdazl* probes, staining can be seen exclusively in the yolk-free zone corresponding to the germ plasm (Fig. 5B).

In stage 7 embryos, *Xdazl* RNA staining is seen as streaks corresponding to the germ plasm in vegetal blastomeres (Fig. 5C,D). As a control, we immunostained sections of separate stage 7 embryos with the anti-vimentin antibody Z10 (Fig. 5E), previously shown to identify germ plasm (Robb et al., 1996). Vimentin-stained germ plasm was found in streaks of similar size and position within the embryos as the *Xdazl* RNA.

By stage 10, *Xdazl* RNA was observed in yolk-free areas surrounding nuclei in cells situated near the floor of the archenteron. In some cases, clusters of four pPGCs were seen grouped together at the archenteron floor (Fig. 5F). Consistent with published descriptions, germ plasm stained with *Xdazl* (Fig. 5G) or with Z10 antibodies (Fig. 5H) appears closely apposed to the nuclei and eccentrically positioned. The speckled areas surrounded by the *Xdazl* staining were verified as nuclei by bisbenzimide staining (not shown). The positions of *Xdazl*-positive pPGCs in the embryo as well as the morphology of perinuclear staining varied greatly both within individual embryos and between batches of embryos from different females. At least four *Xdazl*-positive pPGCs could be found in experiments where all serial sections were examined, in good agreement with the number of pPGCs previously reported (Whitington and Dixon, 1975; Cleine and Dixon, 1985). Thus, the evidence strongly suggests that *Xdazl* labels the germ plasm in all pPGCs, although we cannot formally rule out the presence of germ-plasm containing cells that do not stain for *Xdazl* RNA.

Xdazl RNA expression in spermatogenesis

In addition to oocytes and early embryos, *Xdazl* RNA was present in the testis of adult and juvenile frogs. To determine if the RNA was present during specific stages of spermatogenesis, we hybridized sections of adult and juvenile testis tissue with DIG-labeled antisense RNA probes. In both the adult (Fig. 6A) and juvenile (not shown), *Xdazl* RNA was found in discrete spermatocysts. Sections hybridized with sense probes showed no specific staining. Comparison of these images to sections from the same tissue stained with hematoxylin and eosin (Fig. 6B) identified the majority of *Xdazl*-expressing cells as spermatocytes. The observation of

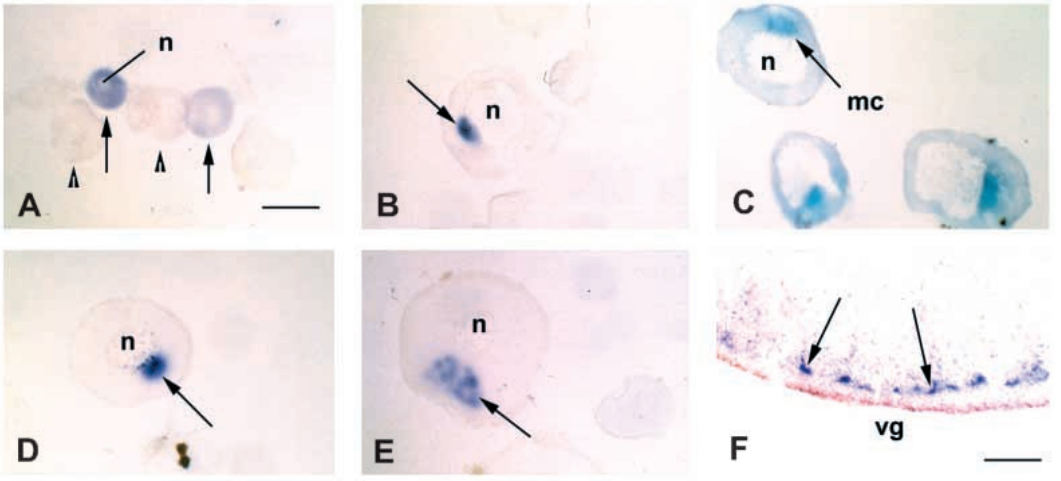

Fig. 3. Localization of *Xdazl* mRNA during oogenesis. *Xdazl* DIG-labeled antisense RNA probes were hybridized to previously sectioned material of postmetamorphic froglets (A), juvenile ovary (B), adult ovary (D,E), or defolliculated stage VI oocytes (F).
(A) Postmetamorphic froglet ovary. Arrows indicate oocytes stained for *Xdazl* mRNA, arrowheads show adjacent oocytes lacking in *Xdazl* signal. (B) Stage I oocyte from a juvenile ovary. Arrow shows *Xdazl* localization in the mitochondrial cloud. (C) Stage I oocytes from the same tissue sample as (B) hybridized with mtLrRNA to show presence of mitochondrial clouds. (D) Stage I oocyte from adult probed for *Xdazl.* (E) *Xdazl* probed stage II oocyte from adult showing *Xdazl* localization in the fragmenting mitochondrial cloud (arrow) (F) *Xdazl* probed adult stage VI oocyte. Arrows show islands of *Xdazl* staining at the vegetal pole (vg) of the oocyte. Scale bars, 200 μm (A-E), 62.5 μm (F). mc, mitochondrial cloud; n, nucleus.

metaphase plates (Fig. 6B, arrowhead) in some of the spermatocyte cysts confirms that *Xdazl* RNA is expressed in the frog testis in the cells undergoing meiotic cell division. Some staining was also detected in spermatogonia; however, *Xdazl* RNA was consistently absent in spermatids and mature sperm.

RNA-binding properties of Xdazl

The amino acid sequence of Xdazl derived from the cDNA indicated a consensus RNP domain. Since the in vivo RNA target of Xdazl is unknown, we tested whether Xdazl could function as an RNA-binding protein in vitro by using homopolymeric RNAs bound to beads. This assay has been used to characterize the RNA-binding properties of a variety of proteins (Swanson and Dreyfuss, 1988; Siomi et al., 1993). To test the specificity of the assay, we included hnRNPC1 as a positive control. hnRNPC1 was previously shown to bind poly(G) and poly(U) in the homopolymer assay (Swanson and Dreyfuss, 1988), and behaved accordingly in experiments conducted at the same time as Xdazl (Fig. 7, upper panel).

Xdazl bound primarily to poly(G) and poly(U) RNAs in moderate salt (100 mM NaCl) and did not bind to poly(C) or ssDNA (Fig. 7, lower panel). We did observe weak binding to poly(A) RNA sequences at a level approximately 50-fold less than the poly(U) as determined by Phosphorimager analysis. Xdazl was also able to bind poly(U) sequences in high salt conditions (2 M NaCl; data not shown), suggesting that the binding is mediated partially through base-specific hydrophobic interactions. From these experiments, we conclude that Xdazl can function as an RNA-binding protein and may bind specifically to G- or U-rich RNAs in vivo.

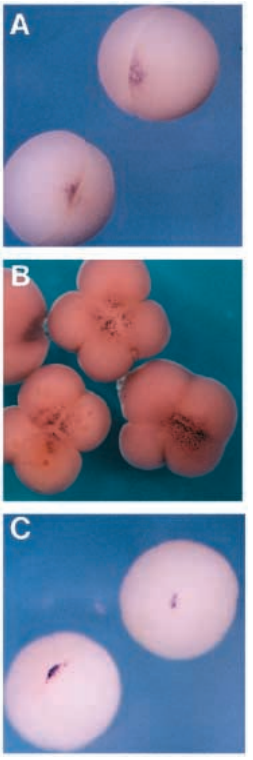

Fig. 4. Expression of *Xdazl* mRNA during early embryogenesis analyzed by whole-mount in situ hybridization. (A) Vegetal view of albino embryos at the 2-cell stage hybridized with *Xdazl* probe, showing staining in discrete patches. (B) Vegetal view of 4-cell embryos. (C) Vegetal view of stage 6 albino embryos. *Xdazl* is localized in a few cells at the extreme vegetal pole.

Xdazl can rescue meiotic entry in *boule* testes

boule is required for the entry of spermatocytes into meiosis during *Drosophila* spermatogenesis (Eberhart et al., 1996). To test the ability of *Xdazl* to substitute for *boule* in meiosis, we expressed the *Xdazl* cDNA in *boule* flies and scored for rescue of meiotic cell division. The heterologous β2-tubulin promoter and regulatory sequences were used to direct the testis-specific expression of *Xdazl* and *boule* cDNAs. As reported previously (Eberhart et al., 1996), expression of the *boule* cDNA in mutant flies results in rescue of meiotic entry and spermiogenesis in over half the germline cysts. Expression of the *Xdazl* cDNA under the same regulatory control restores meiotic entry in most cysts and corrects many postmeiotic differentiation defects in flies homozygous for *boule*. Evidence of meiotic entry included histone H3

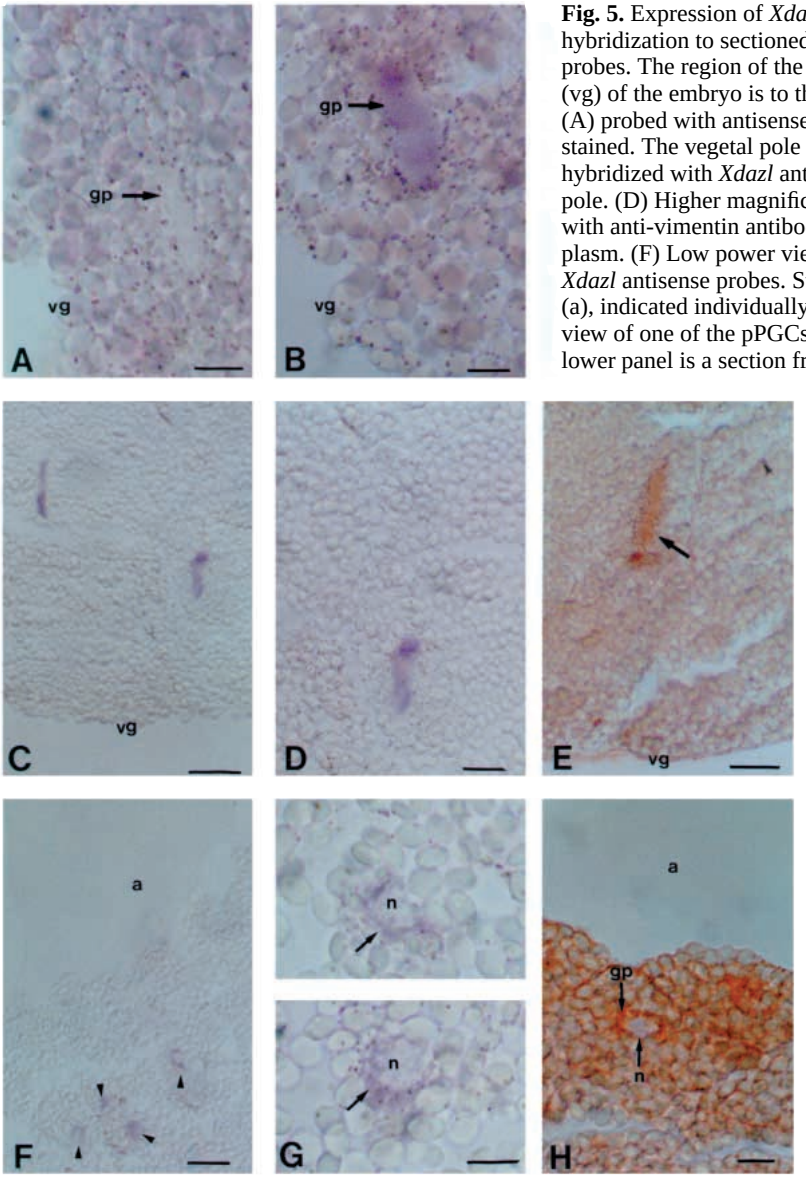

Fig. 5. Expression of *Xdazl* mRNA during embryogenesis analyzed by in situ hybridization to sectioned material. (A) 8-cell embryo hybridized with *Xdazl* sense probes. The region of the germ plasm (gp) is shown by the arrow. The vegetal pole (vg) of the embryo is to the lower left. (B) Another section from the same embryo as (A) probed with antisense *Xdazl* probes. The yolk-free region of the germ plasm is stained. The vegetal pole (vg) is to the lower left. (C) Section from a stage 7 embryo hybridized with *Xdazl* antisense probes. Staining is seen in streaks near the vegetal pole. (D) Higher magnification of (C). (E) Control stage 7 section immunostained with anti-vimentin antibody Z10. The arrow indicates the vimentin-stained germ plasm. (F) Low power view of a section from a stage 10 embryo hybridized with *Xdazl* antisense probes. Staining reveals a number of pPGCs near the archenteron (a), indicated individually by arrowheads. (G) The upper panel shows a higher power view of one of the pPGCs in (F), showing perinuclear *Xdazl* staining (arrow). The lower panel is a section from a different embryo showing a similar staining pattern (arrow). (H) Control stage 10 section immunostained with Z10. The germ plasm is indicated (arrow) and surrounds a nucleus along the floor of the archenteron. Scale bars, 250 μm (C, F), 160 μm (D), 125 μm (E), 62.5 μm (A,B,G,H). n, nucleus.

phosphorylation, chromatin condensation, spindle formation and cytokinesis (Fig. 8). Rescue data were gathered from a minimum of two independent lines per transgene. Rescue for all transgenes was dose dependent; some meiotic entry is evident with one copy of a transgene, while more cysts with meiotic products are observed with two copies. These results provide strong evidence for functional homology between *Xdazl* and *boule*.

DISCUSSION

We have shown that *Xdazl* is a *DAZL* homologue and is expressed in *Xenopus* germ plasm as a localized mRNA. We found that *Xdazl* is also expressed during spermatogenesis in frogs and can rescue spermatocyte entry into meiosis in *boule* mutant flies, demonstrating functional homology between Xdazl and Boule. The ability of *Xdazl* to bind RNA may be necessary for its function and could involve germline-specific regulation of RNA processing, translation, storage or transport.

Xdazl is a *DAZL* homologue

Previous work identified a set of cDNAs that were expressed as localized mRNAs in *Xenopus* oocytes (Zhang and King, 1996). To study some of the functions of *Xenopus* germ plasm, we selected an uncharacterized cDNA for further analysis because it showed a germ-plasm-like expression pattern in oogenesis. Sequencing and subsequent cloning identified the cDNA as a homologue of the human *DAZ*, vertebrate *DAZL* genes and the *boule* gene in *Drosophila*. Xdazl contains an RNP domain, one copy of the DAZ repeat and a C-terminal domain. As expected, the frog sequence is most closely related to the mouse gene at both the nucleotide and protein level. The RNP domain is highly conserved in all organisms, especially within the RNP-1 and RNP-2 peptide motifs. Residues at the C-terminal end of the RNA-binding domain, implicated in RNA-binding specificity (Avis et al.,

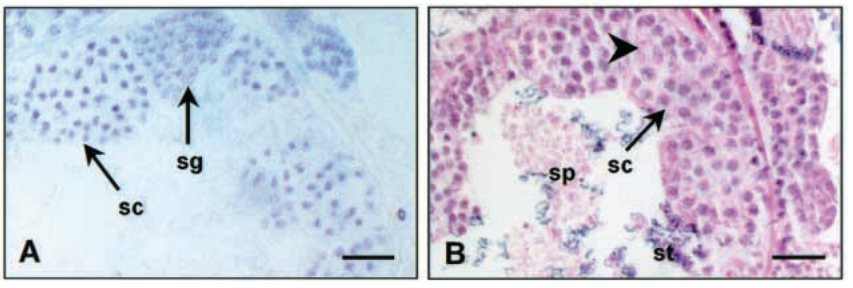

Fig. 6. *Xdazl* expression in the testis. (A) Section of an adult testis stained with antisense *Xdazl* probes. Spermatogonia (sg) and spermatocytes (sc) are shown by the arrows. (B) Section from the same testis stained with hematoxylin and eosin. Locations of the sperm (sp), spermatids (st) and spermatocytes (sc) are shown (arrows). This staining also revealed meiotic cell division in one of the spermatocyte cysts (arrowhead). This was confirmed at higher magnification (not shown). Scale bar, 160 μm.

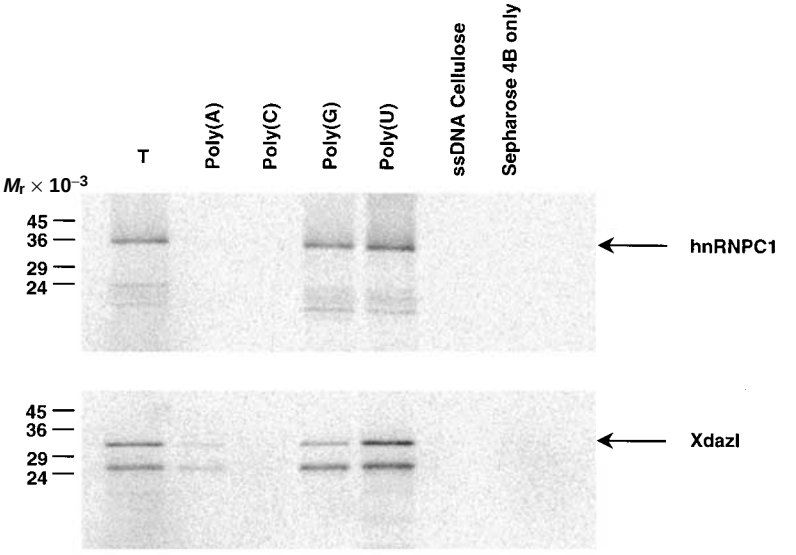

Fig. 7. Xdazl protein can bind RNA. In vitro translated hnRNPC1, a previously characterized RNA-binding protein, (top panel) or Xdazl (lower panel) were incubated with various homopolymeric RNA or control beads (ssDNA, Sepharose 4B). After washing, eluted proteins were separated by SDS-PAGE and visualized by Phosphorimager exposure. M_r is shown at the left. (T), aliquot of the initial translation corresponding to 20% of the input per binding reaction. A lower M_r Xdazl translation product was consistently generated when uncapped RNAs were used. This product was most likely the result of translation initiation at an alternate methionine and bound homopolymeric RNAs with the same specificity as the full-length protein.

1996), are well conserved. This suggests that the target of the *DAZ* genes may be the same in all organisms. The RNA-binding ability of Xdazl protein was shown in an in vitro assay. The protein bound mostly to poly(G) or poly(U) suggesting that Xdazl might also bind to G- and/or U-rich RNAs in vivo.

The DAZ repeat in the Xdazl protein is located at a conserved distance from the RNP domain but has some significant sequence differences. The presence of a three amino acid insertion that is not conserved in mouse, human or fly suggests that this event occurred within *Xenopus* evolution. Whether or not the DAZ repeat has any functional significance has yet to be demonstrated. The DAZ repeats in Boule and Xdazl are quite divergent, and the proteins have limited regions of identity overall. Despite this, the *Xenopus* cDNA is still able to rescue the *boule* defect. This suggests that the DAZ repeat is not critically important, or that a smaller motif is actually the functional unit. Indeed, within the DAZ repeat, the sequence YPXS is conserved in all known DAZ-family members and is reiterated in some form in all DAZ repeats of the DAZ protein itself (see Fig. 1). This type of motif could potentially be phosphorylated on either the tyrosine or serine residue and therefore is a candidate site for regulation.

Xdazl mRNA in germ plasm

The expression pattern of maternal *Xdazl* RNA correlates with the location of the germ plasm from early oogenesis to early gastrula and suggests a function in primordial germ cell specification. Germ plasm also exists in early stages of *Xenopus* spermatogenesis. However, it is only detected at the ultrastructural level in association with nuclear pores, and it is no longer seen by pachytene (Kerr and Dixon, 1974). Since *Xdazl* staining was readily detected by light microscopy and was detected in pachytene spermatocytes, we propose that *Xdazl* RNA does not localize exclusively to germ plasm during spermatogenesis. This might be because PGC specification in early embryogenesis requires that *Xdazl* RNA be restricted to a limited number of cells while, in the spermatogonia and spermatocytes, specific localization of its RNA is not necessary for function.

Although it was surprising to discover a *DAZ* homologue in germ plasm, it was not unexpected to find localized mRNAs for RNA-binding proteins. RNAs for *nanos* and *vasa*, both implicated in RNA-binding and in establishing the germ cell lineage, are long-studied components of *Drosophila* pole plasm (reviewed in Rongo and Lehmann, 1996). Specifically in *Xenopus*, a *nanos*-like gene (*Xcat-2*; Mosquera et al., 1993) and a translation initiation factor-like putative RNA helicase (*Deadsouth*; H. MacArthur and M. L. King, unpublished observations) are also expressed in germ plasm. Thus the presence of RNAs coding for RNA-binding proteins seems to be a common theme in germ cell determination by maternal cytoplasmic localization. How the inheritance of these molecules establishes the germline is still unknown, but the mechanism may involve translational inhibition or germ-cell-specific regulation of mRNA splicing or stability. Recently, it was shown that translational suppression by *nanos* was required for entry of primordial germ cells into the *Drosophila* gonad (Kobayashi et al., 1996). The maternal RNA localization of *Xdazl* suggests that it may function to specify pPGCs. However, *Xdazl* may not be translated until after specification has occurred and may function in the later aspects of germ cell differentiation. Future studies, such as antisense oligonucleotide-mediated depletion of maternal *Xdazl* RNA, may determine whether *Xdazl* is necessary for primordial germ cell specification.

Xdazl rescues *boule*

The similarity in sequence and expression among *boule*, *DAZ* and *DAZL* suggested that their function in spermatogenesis is conserved from flies to humans. By demonstrating that *Xdazl* can substitute for *boule* in promoting meiotic entry in flies, we have obtained strong evidence for functional homology.

The primary defect in *boule* testes is azoospermia resulting from a block in cell division prior to the first meiotic metaphase (Eberhart et al., 1996). Expression of an *Xdazl* cDNA under control of a spermatocyte-specific promoter resulted in a readily detectable restoration of meiotic entry. Although the *Xdazl* transgene did not restore fertility, phenotypic rescue with the *boule* cDNA was similarly incomplete and also failed to

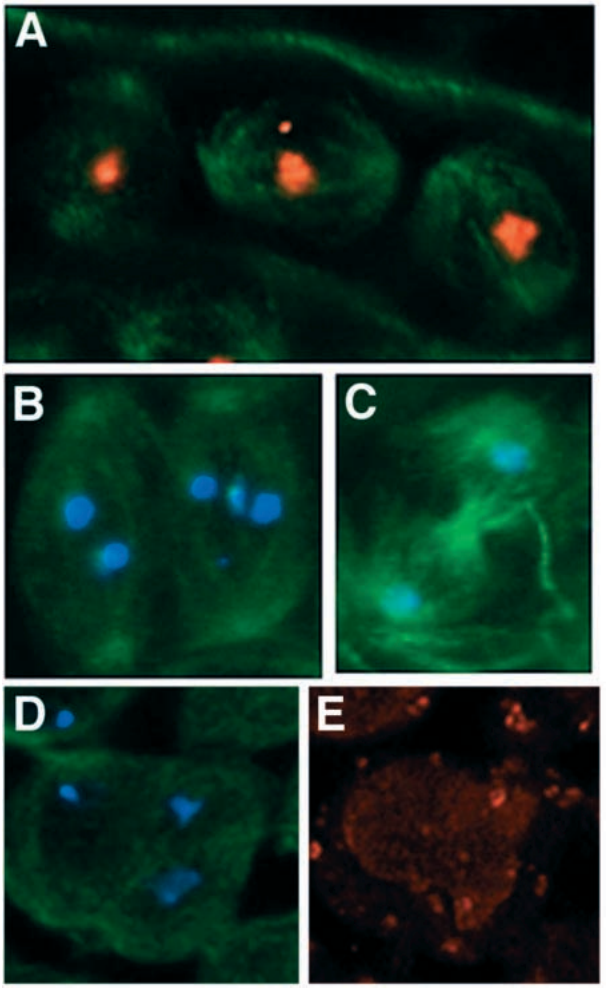

Fig. 8. *Xdazl* expression rescues meiotic entry in *boule* mutant testes. Photographs of squashed, fixed testis contents from newly eclosed *boule* homozygotes carrying the *Xdazl* transgene (A-C) or *boule* alone (D,E). (A) Metaphase of meiosis I. Appearance of the meiotic spindle (green) and phospho histone H3-positive chromosomes (orange) indicates that meiotic entry and the metaphase transition have occurred. (B) Prophase of meiosis I. Assembly of a bipolar spindle (green) and the appearance of condensed DNA labeled with Hoechst (blue) indicates that meiotic entry is beginning. (C) Cytokinesis of meiosis I. Meiotic spindle constriction is evident with daughter nuclei (DNA, blue) segregating to two new cells. (D) Late primary spermatocyte in *boule* homozygotes. Cytoplasmic array of microtubules (green) is evident while no bipolar spindles are detected. (E) Late primary spermatocyte in *boule* homozygote. Phospho histone staining coincident with the chromosomes is not evident. Rare phosphohistone-positive chromosomes have been observed in *boule* mutant spermatocytes, but bipolar spindle formation and cytokinesis has not been observed in *boule* mutants lacking the *Xdazl* transgene.

result in fertile males. It thus appears that transgene expression from the heterologous β2-tubulin promoter and flanking sequences does not recapitulate expression of *boule* from its endogenous promoter.

The loss-of-function phenotype for the *boule* locus is very similar to that for the *pelota* (Eberhart and Wasserman, 1995) and *twine* (Alphey et al., 1992; Courtot et al., 1992; White-Cooper et al., 1993) loci. Twine, the *Drosophila* germline-specific Cdc25 phosphatase, presumably acts at the G_2/M transition of meiosis I to activate the Cdc2/cyclin B complex and initiate meiotic cell division. If *boule* and *pelota* act upstream, they may be required for expression or activation of Twine. Given that *boule* encodes an RNA-binding protein, it is possible that Boule regulates the processing, stability or translation of the mRNA for Twine or a Twine regulator. By analogy, Xdazl may serve to regulate meiotic cell divisions, as well, perhaps, as the early events of germline specification in *Xenopus*.

While this manuscript was in preparation, Ruggiu et al. (1997) published their work on the disruption of the mouse *Dazl* gene. They show that *Dazl* is essential for the development and survival of germ cells in both the testis and ovary. The study of Xdazl in oocytes, which are much more abundant and accessible in frogs than in mice, may yield insights into the diverse roles of this gene family in germ cell development.

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