**Caenorhabditis elegans** homologue of the human azoospermia factor **DAZ** is required for oogenesis but not for spermatogenesis

Takeshi Karashima¹, Asako Sugimoto¹,² and Masayuki Yamamoto¹,*

1Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Hongo, Tokyo 113-0033, Japan
2PRESTO, Japan Science and Technology Corporation

*Author for correspondence (e-mail: myamamoto@ims.u-tokyo.ac.jp)

Accepted 30 November 1999; published on WWW 8 February 2000

**SUMMARY**

**DAZ** (Deleted in Azoospermia), the putative azoospermia factor gene in human, encodes a ribonucleoprotein-type RNA-binding protein required for spermatogenesis. A *Drosophila* homologue of **DAZ**, called **boule**, is also essential for spermatogenesis. A mouse homologue, **Dazla**, is implicated in both spermatogenesis and oogenesis. Here, we report the identification and characterization of **daz-1**, the single **DAZ** homologue in the nematode *Caenorhabditis elegans*. Loss of **daz-1** function caused sterility in hermaphrodites, by blocking oogenesis at the pachytene stage of meiosis I. Epistasis analysis suggested that this gene executes its function succeeding **gld-1**, which governs the early pachytene stage in the oogenic pathway. Spermatogenesis did not appear to be affected in **daz-1** hermaphrodites. Males defective in **daz-1** produced sperm fully competent in fertilization. Analysis employing sex-determination mutants indicated that the **daz-1** function was required for meiosis of female germline regardless of the sex of the soma. Transcription of **daz-1** was restricted to the germline, starting prior to the onset of meiosis and was most conspicuous in cells undergoing oogenesis. Thus, **daz-1** in *C. elegans* is an essential factor for female meiosis but, unlike other **DAZ** family members so far reported, it is dispensable for male meiosis.

Key words: *Caenorhabditis elegans*, Germline, **DAZ**, Meiosis, Oogenesis, Pachytene

**INTRODUCTION**

Mechanisms underlying gametogenesis are complex and apparently divergent among metazoans. The **DAZ** (Deleted in Azoospermia) gene family provides one of the few lines of evidence that argue for evolutionary conservation of gametogenesis at the molecular level. **DAZ** family proteins carry two conserved domains, namely the ribonucleoprotein (RNP)-type RNA recognition motif (RRM) and the **DAZ** motif, and have been found in both vertebrates and invertebrates (Cooke et al., 1996; Eberhart et al., 1996; Reijo et al., 1995). In humans, multiple copies of **DAZ** genes cluster in a small region on the Y chromosome (Reijo et al., 1995; Saxena et al., 1996). Deletion of the **DAZ** cluster has been correlated with azoospermia and oligospermia, which makes **DAZ** a strong candidate for the Azoospermia factor (AZF). In addition, an autosomal **DAZ**-like gene, termed **DAZLA/DAZH**, has been found on human chromosome 3 (Reijo et al., 1996; Seboun et al., 1997). Mouse carries **Dazla**, a homologue of **DAZLA/DAZH**, on an autosome, but has no **DAZ** cluster on the sex chromosome (Cooke et al., 1996). Hence, the human autosomal gene has been proposed to be the ancestor of the **DAZ** cluster, with its transposition to the Y chromosome during the evolution of primates (Saxena et al., 1996). Knock-out of mouse **Dazla** resulted in the complete absence of gamete production in both sexes (Ruggiu et al., 1997). In contrast, a homologue of **DAZ** in *Drosophila*, named **boule**, is essential for the meiotic progression in spermatogenesis but not in oogenesis (Eberhart et al., 1996). Expression of these **DAZ** family genes appears to be confined to the germline. Human **DAZ** and *Drosophila** **boule** are transcribed specifically in the male germline (Eberhart et al., 1996; Reijo et al., 1995; Saxena et al., 1996), while human **DAZLA/DAZH** and its mouse, zebrafish, and *Xenopus* homologues are expressed in the germline of both sexes (Houston et al., 1998; Maegawa et al., 1999; Ruggiu et al., 1997; Seligman and Page, 1998).

The role of the **DAZ** family genes in the production of gametes is still largely unknown, partly due to the apparent phenotypic diversity caused by their defects. A human male missing the **DAZ** cluster shows a varying range of defects in spermatogenesis, from no germ cells at all to less severe spermatogenic arrest generating some mature spermatids (Reijo et al., 1995), indicating that the **DAZ** cluster is not absolutely necessary for the entry to meiosis and sperm differentiation. In the **Dazla**-defective mouse, female germ cells arrest at prophase of meiosis I, whereas male germ cells are affected at the proliferating stage (Ruggiu et al., 1997). In the *Drosophila** **boule** mutant, male germ cells arrest at the G2/M transition in meiosis I, exhibiting limited postmeiotic differentiation (Eberhart et al., 1996). This has been attributed...
to the lowered level of translation of twine mRNA encoding a Cdc25-type phosphatase (Maines and Wasserman, 1999).

Under these circumstances, it is interesting to see whether or not the DAZ family genes perform essentially the same molecular function in gametogenesis. Here, we report analysis of a DAZ family gene in the nematode Caenorhabditis elegans at the cellular and organisinal levels. C. elegans is a favorable metazoan to investigate the process of gametogenesis, because of its genetic accessibility, the completed genome project facilitating reverse genetics, and the ease of observation of germline development (Schedl, 1997). We cloned the single DAZ family gene in C. elegans, daz-1, and isolated daz-1 loss-of-function mutants. The daz-1-defective hermaphrodite turned out to be completely sterile due to arrest at meiotic prophase in oogenesis. Unexpectedly, however, daz-1-defective males showed no significant defect and were fully fertile. Thus, although C. elegans daz-1 is a regulator of gametogenesis, its sex specificity is distinct from that of the human, mouse and Drosophila counterparts.

### MATERIALS AND METHODS

#### General methods and strains

Maintenance and genetic manipulation of C. elegans were carried out as described (Brenner, 1974). Strains used were: wild-type C. elegans var. Bristol, strain N2 (Brenner, 1974); (LGI) unc-13(e51, e1091), lin-1(n566), fog-3(q470), glp-4(bn2); (LGI) daz-1(tj3, tj7) (this study), unc-4(e120), mnl1(dpy-10(e128)) (generously provided by M. Edgley and D. L. Riddle, University of Missouri, Columbia); (LGIV) fem-3(q20gf, e2006f), ced-3(n717). Strains were maintained at 20°C, except for the temperature-sensitive mutants fem-3(q20gf, e2006f) and glp-4(bn2), which were maintained at 15°C and analyzed at 25°C.

#### Identification and cloning of daz-1

The C. elegans DAZ (ORF F56D1.5; DDBJ/EMBL/GenBank accession number U39997). A corresponding cDNA clone yk62b4, which lacked part of the 5' region, was kindly supplied by Y. Kohara. The missing 5'-terminal sequence was cloned by PCR, as described (Salles et al., 1992). Poly(A)+ RNA (3 μg) prepared from N2 strain was reverse-transcribed using a daz-1-specific primer RACE1 (5'-CGATGTGCTCTCAATGTGACCTCC-3'). Produced cDNA was amplified by two rounds of PCR, first with a SL1-specific primer (5'-GGTTAATTACCAAGTTTG-3') and a daz-1-specific primer RACE2 (5'-GTAAGCAAGCTTGTGATATGG-3'), and then with the same SL1 primer and another daz-1-specific primer RACE3 (5'-CCTGCTTTGATCTGACACC-3'). The isolated RT-PCR product contained additional 22 bases that were absent in yk62b4 (excluding the SL1 sequence). The sequence of the deduced full-length daz-1 cDNA was registered to DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB025253.

#### Isolation of daz-1 deletion mutants using UV/TMP population screening

A worm library mutagenized with a combination of trimethylpsoralen and UV irradiation (Yandell et al., 1994) was screened for a deletion in the daz-1 locus according to the protocol distributed by Moulder and Barstead (http://smcn01.omrf.uokhsc.edu/revgen/RevGen.html), essentially as we described before (Ohmachi et al., 1999). PCR primers used were as follows (the numbers given in parentheses represent the 5' end point of each primer on the cosmid F56D1). The outer primer set: b1', 5'-CCACAAAATGACCTACTAC (9914), and b4', 5'-GAACCTTAAACCTTGGTTG-3' (14153); the inner primer set: n1, 5'-TGGATTTGAGAAGAAGACT (10050), and n2, 5'-TCGCTATTCTCTCCTTTG-3' (13471).

Two independent daz-1 deletion mutants (tj3 and tj7) were isolated. To eliminate possible additional mutations, backcrosses were performed five times for each strain. Sequence analysis indicated that the tj3 allele carried a 2073 bp deletion corresponding to nucleotides 11115 to 13187 of the cosmid F56D1, whereas tj7 carried a 1609 bp deletion corresponding to nucleotides 10484 to 12092 of the cosmid.

#### RNA interference experiment

Antisense RNA molecules for daz-1 were prepared by in vitro transcription of the daz-1 cDNA fragment (nucleotides (nt) 27-1450) cloned on the pBluescript SK(--) vector, as described (Guo and Kemphues, 1995). They were microinjected into the distal arm of the gonad in wild-type young adult hermaphrodites. F1 hermaphrodites from these injected worms were examined for phenotypes. The RNA-injected hermaphrodites were also crossed with N2 males, and F1 male progeny were examined. For the RNAi experiment to interfere with gld-1, the cDNA clone yk30e10 (provided by Y. Kohara) was used as the template to prepare antisense and sense gld-1 RNA (Fire et al., 1998). Worms were soaked in the concentrated gld-1 RNA solution containing both sense and antisense strands for 24 hours before phenotypic analysis (Tabara et al., 1998).

#### Mating test of daz-1 male

Males with the genotype either daz-1(tj3) unc-4(e120) II or unc-4(e120)II as a positive control were test-mated to an indicator Dpy strain, mnl1[dpy-10(e128)]. On each mating plate, one male and four mnl1 hermaphrodites were allowed to mate.

#### Observation of sperm

Both hermaphrodite and male adult worms were dissected at the position of spermatheca in 5 ml of sperm medium (50 mM Hepes (pH 7.0), 50 mM NaCl, 1 mM MgSO4, 5 mM CaCl2, 1 mg/ml BSA) (Nelson and Ward, 1980). Monensin was added to 0.5 mM to the resultant sperm suspension and activated sperm were observed under Nomarski microscopy.

#### DAPI staining and immunofluorescence

DAPI staining was performed as described (Kadyk and Kimble, 1998). Worms were fixed in –20°C methanol and stained with 0.1 μg/ml DAPI in M9. Immunostaining was performed essentially as described (Crittenden et al., 1994). Dissected gonads were fixed with 1% paraformaldehyde in PBS and permeabilized in TBS/0.5% BSA supplemented with 0.1% Triton X-100. The sample was subsequently incubated with anti-phosphohistone H3 antibody (06-570, Upstate Biotechnology), diluted 1/300 in TBS/0.5% BSA, at 4°C overnight. The sample was subsequently incubated with anti-phosphohistone H3 antibody (06-570, Upstate Biotechnology), diluted 1/300 in TBS/0.5% BSA, at 4°C overnight. The sample was subsequently incubated with anti-phosphohistone H3 antibody (06-570, Upstate Biotechnology), diluted 1/300 in TBS/0.5% BSA, at 4°C overnight.

#### Dead cell staining with SYTO12

Apoptotic germ cells were stained with SYTO12 (Molecular Probes, Eugene, OR), as described (Gumienny et al., 1999). Adult worms were incubated in 33 μM SYTO12 in M9 for 4.5 hours at room temperature. Worms were then fed on seeded plates for an hour and observed microscopically with a FITC filter.

#### Northern analysis

Total RNA was isolated using TRIZOL reagent (Gibco BRL) from a culture of L4 to adult worms grown at 25°C, for the wild-type (N2),...
Identification of a DAZ homologue in *C. elegans*

*C. elegans* has a single predicted gene that apparently belongs to the DAZ family, namely F56D1.5 on chromosome II (mapping position -1.94) (The *C. elegans* Sequencing Consortium, 1998). We call it *daz-1* hereafter. We assembled a full-length *daz-1* cDNA as described in Materials and methods. The putative initiation codon was located 27 nucleotides downstream of the SL1 trans-spliced leader sequence (Krause and Hirsh, 1987). The full-length *daz-1* cDNA was about 2.0 kb in length, consisting of the 5'-UTR of 48 nt (including SL1), the coding region of 1500 nt (including the termination codon), and the 3'-UTR of 461 nt (Fig. 1A).
Comparison of the cDNA sequence with the genomic sequence revealed that daz-1 has six exons (Fig. 2A). The predicted exons for F56D1.5 based solely on the genomic sequence was slightly incorrect.

The deduced daz-1 gene product consists of 499 amino acid residues. It has a single RNA-recognition motif (RRM) of the ribonucleoprotein (RNP) type (Burd and Dreyfuss, 1994; Nagai et al., 1995), which includes the RNPI octamer and the RNPII hexamer motifs (Fig. 1). The RRM of the C. elegans DAZ-1 is most similar to that of Drosophila Boule (41% identity, 49% similarity), and slightly less similar to the vertebrate DAZ family proteins (e.g., 35% identity to human DAZ) (Fig. 1B). The C-terminal 12 amino acids of the RRM domain, which have been thought to determine the RNA-binding specificity (Nagai et al., 1995), are highly conserved among all DAZ family members, suggesting that DAZ proteins may bind to a specific RNA sequence.

Besides the RRM, the DAZ family proteins carry another conserved domain of unknown function in their C-terminal region. This domain, called the DAZ motif, consists of approximately 24 amino acid residues rich in glutamine, proline and tyrosine (Reijo et al., 1995). Each DAZ family protein contains one DAZ motif, except for human DAZ, which has seven tandem repeats of the motif. C. elegans DAZ-1 has a single apparent DAZ motif, which is divergent from ones in mammals (Fig. 1B). The entire DAZ-1 polypeptide except the RRM domain was rich in glutamine (65/410), proline (48/410), and tyrosine (29/410), as is often the case with RRM proteins, including DAZ family members.

Isolation of daz-1 deletion alleles

To see the function of the daz-1 gene product in C. elegans, we isolated two daz-1 deletion mutants. One allele, termed tj3, apparently carried a 2.1 kb-long deletion, whereas the other, termed tj7, carried a 1.6 kb-long deletion (Fig. 2B). Sequence analysis confirmed that the daz-1(tj3) allele lacked exons 2 through 6, hence missing most of the ORF including the DAZ motif and part of the RRM, and that daz-1(tj7) lacked the first four exons, missing both the DAZ motif and the RRM (Fig. 2A). Thus, these alleles appeared unlikely to produce functional DAZ-1 protein. The trans-heterozygotes daz-1(tj3)/ccDf5, where ccDf5 represents a chromosomal deletion that encompasses the daz-1 locus, were phenotypically indistinguishable from either the daz-1(tj3) or the daz-1(tj7) homozygotes (data not shown; see below). This finding supported the contention that these daz-1 alleles were null rather than reduction-of-function. Because daz-1(tj3) and daz-1(tj7) were phenotypically indistinguishable in the initial characterization, we used daz-1(tj3) mainly in subsequent analyses.

Loss of daz-1 function causes sterility in hermaphrodites but not in males

Hermaphrodites homozygous for daz-1(tj3), born of heterozygous mothers, grew to adulthood without showing any phenotypic abnormality in somatic tissues. However, daz-1(tj3) adult hermaphrodites were found to be sterile with complete penetrance (Table 1). In wild-type adult hermaphrodites, each gonad exhibited distal-to-proximal polarity, in which distal germ cells proliferated whereas proximal cells entered meiosis and underwent oogenesis (Fig. 3A). Sperm were produced just before reaching adulthood and were stored in the spermatheca located proximal to the gonadal arm. In contrast, the gonad of daz-1 adult hermaphrodites contained no oocytes, but was filled mostly with small nuclei. None of the cells therein displayed morphological characteristics of the developing or mature oocytes, such as granular cytoplasm and enlarged nuclei. Apparently normal sperm were observed in the spermatheca of daz-1 hermaphrodites, although the mutant spermatheca was typically elongated compared with the wild-type (Fig. 3B). Spermatids dissected from daz-1 hermaphrodites were morphologically normal and could be properly activated by monensin in vitro (Fig. 3G,H), suggesting that the sperm produced by daz-1 hermaphrodites were not defective.

Males homozygous for daz-1(tj3) appeared to have no obvious defect in either formation of sperm or their activation (Fig. 3J). We attempted crosses between daz-1 males and mlh1[dpupal-10] hermaphrodites, the latter of which have the Dpy phenotype. The mutant male could give rise to non-Dpy

Table 1. Fertility of the daz-1 mutant

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex</th>
<th>Number of fertile adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>unc-4</td>
<td>hermaphrodite</td>
<td>30/30</td>
</tr>
<tr>
<td>unc-4 daz-1</td>
<td>male</td>
<td>26/26</td>
</tr>
<tr>
<td>unc-4</td>
<td>hermaphrodite</td>
<td>0/30</td>
</tr>
<tr>
<td>unc-4 daz-1</td>
<td>male</td>
<td>23/23</td>
</tr>
</tbody>
</table>

Hermaphrodites were tested for self fertility. Male animals were test-mated to a hermaphrodite indicator strain.
progeny as efficiently as the wild-type male (Table 1), indicating that \textit{daz-1} sperm were functionally normal. These results lead to the conclusion that \textit{daz-1} is dispensable for spermatogenesis, which makes a striking contrast to the other DAZ family members previously analyzed.

To confirm that the observed phenotype of the \textit{daz-1} mutant was caused by the deletion of \textit{daz-1} but not any other mutation, we performed an RNA interference (RNAi) experiment (Fire et al., 1998; Tabara et al., 1998). The phenotype of \textit{daz-1(RNAi)} hermaphrodites and that of the \textit{daz-1} mutant were indistinguishable from each other, supporting strongly that the deletion of the \textit{daz-1} gene was indeed responsible for the mutant phenotype (data not shown).

**Germ nuclei show pachytene arrest during oogenesis in the \textit{daz-1} mutant**

To determine which stage of oogenesis is defective in \textit{daz-1} hermaphrodites, they were stained with DAPI, and morphology of their germ nuclei was compared with that of the wild-type. In wild-type worms, transition from mitosis to meiosis occurs at the middle of the distal arm and pachytene nuclei with distinct thread-like chromosomes become visible beyond this transition zone (Schedl, 1997; Fig. 3C). Prophase of meiosis I continues as nuclei travel to the proximal arm, where late stage oocytes show highly condensed chromosomes of the diakinesis stage (Fig. 3C). DAPI-stained sperm nuclei were seen as small dots in the spermatheca in wild-type hermaphrodites (Fig. 3C).

The \textit{daz-1} hermaphrodites had mitotic and transition zone nuclei at the distal end of the gonad, as in the wild-type, but the rest of the mutant gonad was filled with pachytene nuclei. Occasionally smaller nuclei were seen among pachytene nuclei, which appeared similar to mitotic germ nuclei in size (Fig. 3D). Nuclei at the diakinesis stage could never be observed in the mutant gonad. DAPI-stained sperm in the mutant spermatheca showed no obvious aberrance, confirming the results of the Nomarski microscopy. The number of sperm per gonad was about 130-150 in both the wild-type and the \textit{daz-1} mutant. DAPI-stained sperm in \textit{daz-1} males revealed no obvious defect, either (Fig. 3E,F).

**Fig. 3.** Gametogenesis in the \textit{daz-1} deletion mutant. (A,B) Images of the gonad of adult hermaphrodites by Nomarski microscopy. A wild-type hermaphrodite (A) and a \textit{daz-1} hermaphrodite (B) were examined. O, oocytes; S, sperm; E, fertilized eggs. (C-F) Images of the gonad stained by DAPI. A wild-type hermaphrodite (C), a \textit{daz-1} hermaphrodite (D), a wild-type male (E), and a \textit{daz-1} male (F) were examined. Pachytene nuclei seen in both wild-type and \textit{daz-1} hermaphrodites are enlarged in the upper insets of panels C and D, respectively. Diakinesis nuclei in the wild-type are enlarged in the lower inset of panel C. Typical smaller nuclei seen in \textit{daz-1} are evident in the lower inset of panel D. P, pachytene nuclei; D, diakinesis nuclei. (G-J) Morphology of activated sperm. Sperm from a wild-type hermaphrodite (G), a \textit{daz-1} hermaphrodite (H), a wild-type male (I), and a \textit{daz-1} male (J) were examined by Nomarski microscopy. PP, pseudopod. Scale bar: 10 \textmu m throughout.
To examine further the status of arrested germ cells in the daz-1 mutant, we asked whether dying germ cells could be visualized by a vital dye SYTO12 in daz-1 adult hermaphrodites. In wild-type animals, a small population of female germ cells undergoes programmed cell death at the late pachytene stage, which can be seen typically in the distal arm of the gonad (Gumienny et al., 1999; Fig. 4A). In daz-1 animals, the proximal arm of the gonad revealed many cell corpses (Fig. 4B). Cell corpses seen in the mutant appeared to be produced by the apoptotic pathway, because daz-1 animals exhibited no corpses if they are defective in ced-3, a gene essential for apoptosis (Ellis and Horvitz, 1986) (Fig. 4C). These results imply that the arrested daz-1 germ cells reached the cell-death sensitive, late pachytene stage in oogenesis, although we cannot completely exclude the possibility that an unusual cell death pathway might be activated by the abnormal meiotic arrest. A possible reason for the increase of cell corpses in daz-1 will be that arrested germ cells stay longer in the cell-death-sensitive period, raising the probability that they die. Alternatively, germ cells that are unable to exit pachytene in a certain period of time may be destined to cell death, in both the mutant and the wild-type.

**gld-1 is epistatic to daz-1 at the pachytene stage**

We suspected that the smaller nuclei seen in the proximal arm of the mutant gonad might be mitotic, as is the case with the gld-1 mutant (Francis et al., 1995a). To test this possibility, we stained the daz-1 hermaphrodites with anti-phosphohistone H3 antibody, which specifically stains condensed mitotic chromosomes at M-phase (Ajiro et al., 1996). We stained the gld-1(null) hermaphrodites for comparison, in which germ nuclei reach the meiotic pachytene phase and then reinitiate mitosis, giving rise to a 'tumorous' germine (Francis et al., 1995a). The results indicated that the mitotic nuclei were scattered up to the proximal arm in gld-1(null) hermaphrodites, whereas they were restricted to the distal region of the gonad in daz-1 hermaphrodites and the wild-type (Fig. 5A,C). Thus, the majority of the nuclei seen in the proximal region of the daz-1 mutant gonad are not mitotic and are likely to be arrested at an early phase of meiosis.

We set out to examine the epistasis between daz-1 and gld-1 using RNAi. The wild-type adult hermaphrodites treated with the gld-1 RNA solution exhibited the tumorous germine phenotype within 48 hours (Fig. 5B). The gld-1 dsRNA treatment of homozygous daz-1(tj3) adult hermaphrodites gave rise to similar tumorous germine, and its immunostaining by anti-phosphohistone H3 revealed a pattern clearly distinct from that of the untreated daz-1 germine (Fig. 5D). Analysis using the daz-1(tj3); gld-1(null) double mutant gave essentially the same results (data not shown). Thus, the defect in gld-1 is epistatic to daz-1, suggesting that daz-1 function is not necessary for gld-1 tumor formation. If one assumes that daz-1 and gld-1 act in a pathway of dependent events (Hartwell, 1974), then daz-1 normally functions later than gld-1 in the course of oogenesis. This hypothesis is consistent with the observation of cell death in the gonad of daz-1 hermaphrodites described above: germ cells defective in daz-1 can apparently proceeded to the cell-death sensitive stage (Fig. 4B), whereas ones defective in gld-1 do not reach this stage (Gumienny et al., 1999) (see Discussion for additional arguments).

**daz-1 phenotype depends on the sexual identity of the germine**

To examine how the oogenic defect in daz-1(tj3) depends on the sexual fate of the germine, we performed genetic epistasis analysis using sex determination mutants. First, we tested the effect of daz-1(tj3) in the feminization mutant, fem-3(lf). In the wild-type hermaphrodite (XX) germine, spermatogenesis takes place during late L4 stage, then the germine switches its sex in young adult so that it undergoes oogenesis (Ellis and Kimble, 1994). In the germine of XX fem-3(lf), spermatogenesis never occurs and only oocytes are produced from the start of germ cell differentiation (Hodgkin 1986; Fig. 6A). The XX adults of daz-1: fem-3(lf) that we constructed did not produce mature oocytes, and their germ nuclei stained with DAPI showed morphology characteristic to the pachytene stage (Fig. 6B), as in the daz-1 hermaphrodite germine. Thus, daz-1 is required for oogenesis, whether or not spermatogenesis precedes it.

We then examined the effect of daz-1 mutation in the masculinized germine in fem-3(gf) XX animals, which showed a Mog (masculinization of germine) phenotype (Barton et al., 1987). In fem-3(gf) hermaphrodites, switching from spermatogenesis to oogenesis does not occur, and sperm are continuously produced in the female soma. The fem-3(gf); daz-1 animals showed masculinized germines, producing apparently normal sperm (data not shown). They produced
sperm throughout adulthood, excluding the possibility that maternally contributed daz-1 mRNA permits spermatogenesis in young adults but is not sufficient to allow gametogenesis to continue as animals get older.

Finally, we asked whether somatic sex may affect the oogenic defect brought by the daz-1 mutation. fog-3(1f) (feminization of germline) XO animals are somatically male, but their germline is feminized and produces only oocytes (Ellis and Kimble 1995; Fig. 6C). The daz-1; fog-3(1f) XO animals that we constructed produced neither oocytes nor sperm. DAPI staining of these animals showed that the germ nuclei were arrested in the pachytene stage (Fig. 6D), suggesting that the meiotic defect in daz-1; fog-3(1f) animals was due to the same dysfunction as in the daz-1 hermaphrodite or the daz-1; fem-3(1f) female. Taken together, the meiotic prophase arrest caused by daz-1 loss of function is dependent on the female fate of germline, regardless of chromosome sex or sex of somatic tissues.

**The transcription of daz-1 is limited to the germline and is developmentally regulated**

Northern blot analysis using poly(A) + RNA prepared from wild-type adults revealed a single band of approximately 2.0 kb in length as the daz-1 transcript (Fig. 7), which is consistent with the size of the full-length cDNA. In the glp-4 mutant, which produces few germ cells (Beanan and Strome, 1992), the daz-1 transcript was not detectable (Fig. 7). In the fem-3(1f) mutant, in which the germline is feminized, daz-1 transcripts were slightly more abundant than in the wild-type, whereas they were less abundant in the fem-3(1f) mutant, in which the germline is masculinized (Fig. 7). These results indicate that daz-1 is expressed exclusively in the germline, more strongly during oogenesis than during spermatogenesis.

To analyze temporal and spatial distribution of daz-1 mRNA, we performed in situ hybridization against the wild-type worms at each developmental stage (Fig. 8). In hermaphrodites, the daz-1 mRNA was first detected in the germline of the L2 stage larva (Fig. 8B). Since germ cells enter the meiotic pathway at late L3, the onset of transcription of daz-1 appeared to precede the initiation of meiosis. The hybridization signal increased its intensity as the developmental stage progressed (Fig. 8C-E). During the larval stages, the daz-1 mRNA appeared more abundant at the distal region than the proximal region of the gonad. In young adult hermaphrodites, the daz-1 transcripts were detected at a very low level in the distal mitotic region of the gonad, began to accumulate in the meiotic transition zone, and reached the maximum level in the proximal pachytene region (Fig. 8E,G). The transcript level dropped sharply in mature oocytes. No hybridization signal was seen in the spermatheca. Compared to hermaphrodites, a much weaker signal, if any, was detected in the germline of adult males (Fig. 8F). In XX fem-3(1f) adults, daz-1 transcripts were detectable but restricted to the distal region of the gonad (data not shown), which may account for the low level of mRNA detected by northern blot analysis in Fig. 6. Thus, daz-1 transcription is virtually confined to the female germline and is regulated according to the developmental stages of oogenesis.

**DISCUSSION**

**Relationship between daz-1 and other meiosis/gametogenesis-related genes in C. elegans**

This study has shown that the function of C. elegans daz-1 is required for the germ cells to traverse the pachytene stage of meiosis I. So far only a limited number of genes have been shown to be relevant to the meiotic regulation in C. elegans. The glp-1 signaling pathway, involving glp-1, lag-1 and lag-2, is essential for the inhibition of precocious entry of germ nuclei into meiosis (Austin and Kimble, 1987; Christensen et al., 1996; Henderson et al., 1994). LAG-2, a ligand secreted by the distal tip cell, binds to the receptor GLP-1 located on the germ cell. NO. 5. Anti-phosphohistone H3 staining of gonads. Gonads were dissected from the wild-type (A), gld-1 (B), daz-1 (C) and gld-1; daz-1; gld-1(RNAi) (D) animals and stained with the antibody. Staining by anti-phosphohistone H3 is represented in red, while staining by DAPI is represented in green. Scale bar: 50 μm.
cell membrane (Henderson et al., 1994). The receptor then activates the transcription factor LAG-1, which promotes mitotic proliferation of germ nuclei (Christensen et al., 1996). When LAG-2 signaling is no longer effective, germ cells enter the meiotic pathway by virtue of the redundant function of gld-1 and gld-2 (Kadyk and Kimble, 1998). In addition, the RAS/MAPK signaling pathway, involving mek-2, mpk-1/sur-1 and let-60 ras, is necessary for the transition from pachytene to diplotene/diakinesis (Church et al., 1995).

Among the above genes, gld-1 appears to share several features with daz-1. First, loss of function of gld-1 results in a failure of the progression of meiotic prophase in oogenesis (Francis et al., 1995a). Second, gld-1 function is required only when the germline sex is set as female (Francis et al., 1995b). Third, gld-1 is expressed specifically in the germline, at a much higher level in hermaphrodites than males (Jones et al., 1996). In addition, GLD-1 also is an RNA-binding protein, although its RNA recognition motif is a KH domain (Jones and Schedl, 1995) and hence is different from the RNP-type RRM in DAZ-1. Germ cells in the gld-1(null) hermaphrodites enter the meiotic pathway and proceed up to the pachytene stage. However, unlike the daz-1 mutant, they do not arrest at this stage but resume aberrant mitotic growth, giving rise to a ‘tumorous’ germline (Francis et al., 1995a). Our epistasis analysis suggests that daz-1 executes its function after the meiotic progression is ensured by the function of gld-1. This is supported by the observation that germ cells do not reach the cell-death-sensitive late pachytene stage in the absence of GLD-1, whereas they do so in the absence of DAZ-1. Strictly speaking, however, the possibility remains that apoptosis may occur in the meiotic region but elude detection in the gld-1 mutant, because the vast majority of germline cells are mitotic in this mutant. Obviously more work is required to clarify the functional relationship between daz-1 and gld-1.

C. elegans mutants defective in a component of the RAS/MAP kinase pathway also show pachytene arrest but in both sexes (Church et al., 1995). The arrested nuclei in these mutants are clumped in the gonad, whereas those in the daz-1 germline are uniformly distributed. Furthermore, the RAS/MAPK mutants show virtually no germ cell death
DAZ homologue in C. elegans

1077

(Gumienny et al., 1999). These apparent differences may indicate that daz-1 and the RAS/MAPK cascade contribute differently to pachytene progression.

Target RNA of DAZ-1

DAZ-1 contains an RNP-type RNA-binding motif. This motif has been implicated in a variety of different functions, such as regulation of splicing, processing of the 3'-end of pre-mRNA, and transport and translation of mature mRNA (Nagai et al., 1995). Identification of RNA species that interact with DAZ-1 should await further work, but the following consideration may be beneficial for this purpose.

In C. elegans, RNA-protein interaction has been shown to play a pivotal role in the regulation of germline sex determination. For example, GLD-1 represses translation of tra-2, one of the sex-determination genes, by binding to the 3'-UTR of the tra-2 mRNA (Jan et al., 1999). GLD-1 is likely to regulate translation of other genes also, because loss of translational repression of tra-2 alone cannot account for all the phenotypes brought by the gld-1 deficiency. TRA-1, a member of the GLI protein family, also binds to the 3'-UTR of the tra-2 mRNA and promotes its export from the nucleus (Graves et al., 1999). Another example can be seen in the repression of translation of fem-3 mRNA by FBF-1, the latter of which is a member of a new family of RNA-binding proteins including Drosophila Pumilio (Zhang et al., 1997).

In contrast to their role in sex determination, the role of RNA-binding proteins in the regulation of meiosis remains obscure. Although GLD-1 is essential for completion of the pachytene stage, no RNA molecule has been identified that interacts with GLD-1 to promote meiosis. It appears interesting to see whether DAZ-1 regulates translation of specific mRNA, similarly to GLD-1. Recent evidence suggests that Drosophila Boule is involved in the control of translation of twine mRNA (Maines and Wasserman, 1999), although direct interaction between the protein and the mRNA remains to be substantiated. Twine encodes a Cdc25-type phosphatase essential for the meiotic G2/M transition (Alphey et al., 1992; Courtot et al., 1992; White-Cooper et al., 1993). Expression of fission yeast cdc25 is significantly enhanced following the completion of premeiotic DNA synthesis, and its function is essential for both meiosis I and II (Iino et al., 1995). Thus, an attracting question is whether C. elegans DAZ-1 upregulates the activity of a Cdc25-type phosphatase by interacting with its mRNA during female meiosis.

In fission yeast, an RNA-binding protein Mei2 serves as a key regulator of meiosis. Mei2 carries three RRMs and is essential for both premeiotic DNA synthesis and meiosis I.

Fig. 8. In situ hybridization of daz-1 transcripts. Whole-mount in situ hybridization was performed using the daz-1 probe against L1 (A), L2 (B), L3 (C), L4 (D) and adult (E) hermaphrodites. An adult male (F) and a dissected adult hermaphrodite gonad (G) were also examined. (H) DAPI-stained image of (G). Scale bar: 50 μm.
We thank Tim Schell for critical reading of the manuscript and valuable comments, Lisa Kadyk for helpful discussion, and Yui Kohara for providing cDNA clones. Some nematode strains were provided by the Caenorhabditis Genetics Center funded by the NIH National Center for Research Resources (NCRR). This work was supported by a Grant-in-Aid for Specially Promoted Research from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES


Evolutionary twist of the DAZ family genes

DAZ family genes are conserved from nematode to mammals. However, no DAZ homologue has been found in unicellular organisms, including not only bacteria but also meiosis-proficient eukaryotic microbes like budding and fission yeast. This may imply that the emergence of the DAZ family gene in the course of evolution was associated with the increase of complexity in the mechanisms of meiosis in multicellular organisms.

All of the DAZ family genes so far investigated are expressed exclusively in the germ line, but their sex-specificity is not identical. The DAZ family genes in Drosophila and C. elegans are required for gametogenesis in one sex: Drosophila boule for spermatogenesis (Eberhart et al., 1996) and C. elegans daz-1 for oogenesis. Murine Dazla is required for both oogenesis and spermatogenesis, but differently (Ruggiu et al., 1997): in Dazla-deficient mice, oogenesis appears to be normal until it is arrested at the pachyten stage, hence mimicking the situation in C. elegans, whereas the male germline generates fewer germ cells, which do not proceed beyond the spermatogonial stage. It is unknown whether the human counterpart of murine Dazla, namely DAZL/DAZH, is involved in gametogenesis. A deletion of the human DAZ cluster on Y chromosome results in a wide range of spermatogenic deficiency (Reijo et al., 1995). This variability may reflect the functional redundancy and divergence between DAZ and DAZL/DAZH (Agulnik et al., 1998; Saxena et al., 1996).

How have the DAZ family genes acquired such versatile sex-specificity during evolution? We speculate the following. Yeast has only one mode of meiosis, i.e., diploid cells generate four spores. In contrast, higher organisms produce asymmetrical gametes. To regulate spermatogenesis and oogenesis independently according to each program, they will require two modes of meiosis, which should be distinct from each other at least partly. Thus, we assume that multicellular organisms have developed two types of mechanisms for meiotic regulation, one of which uses the DAZ family gene at the pachyten stage and the other not. Meanwhile, strategies for sex determination are strikingly divergent among species, with no overlapping molecular mechanisms being discovered in C. elegans, Drosophila and mammals (Marin and Baker, 1998; Parkhurst and Meneely, 1994). Taken together, during the evolution from a primitive multicellular organism, some organisms may have employed the DAZ-dependent meiosis for spermatogenesis and the DAZ-independent meiosis for oogenesis, whereas others did vice versa. Hopefully more extensive analysis of the DAZ family genes and more intensive analysis of their molecular function will prove or disprove the validity of this hypothesis.
DAZ homologue in C. elegans 1079


