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Role of the *C. elegans* U2 snRNP protein MOG-2 in sex determination, meiosis, and splice site selection

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ABSTRACT

In *Caenorhabditis elegans*, germ cells develop as spermatids in the larva and as oocytes in the adult. Such fundamentally different gametes are produced through a fine-tuned balance between feminizing and masculinizing genes. For example, the switch to oogenesis requires repression of the *fem-3* mRNA through the *mog* genes. Here we report on the cloning and characterization of the sex determination gene *mog-2*. MOG-2 is the worm homolog of spliceosomal protein U2A′. We found that MOG-2 is expressed in most nuclei of somatic and germ cells. In addition to its role in sex determination, *mog-2* is required for meiosis. Moreover, MOG-2 binds to U2B″/RNP-3 in the absence of RNA. We also show that MOG-2 associates with the U2 snRNA in the absence of RNP-3. Therefore, we propose that MOG-2 is a bona fide component of the U2 snRNP. Albeit not being required for general pre-mRNA splicing, MOG-2 increases the splicing efficiency to a cryptic splice site that is located at the 5′ end of the exon.

Introduction

How does a germ cell decide between mitotic proliferation and entry into meiosis? What are the factors that are necessary to differentiate a germ cell as a sperm or an oocyte? Such fundamental questions are addressed in the protandric nematode *Caenorhabditis elegans*. In this species, X0 males produce sperm for their whole adult life, while XX hermaphrodites make sperm only during a short period of their larval development and then switch to oogenesis (Hirsh et al., 1976). Genes involved in germline sex determination have been identified through genetic screens for animals that were defective in gamete production. For example, *fem-3 loss-of-function (lf)* alleles lead to feminized hermaphrodites that omit spermatogenesis (Hodgkin, 1986). In contrast, *fem-3 gain-of-function (gf)* mutant hermaphrodites continue spermatogenesis throughout their entire lives (Barton et al., 1987). In the latter, post-transcriptional repression of the *fem-3* mRNA is abolished through mutations in the 3′untranslated region (3′UTR; Ahringer and Kimble, 1991). The *fem-3* mRNA is repressed through a number of regulatory proteins, among which the FBF and the MOG proteins are the most prominent examples (Graham and Kimble, 1993; Graham et al., 1993; Zhang et al., 1997). The Puf proteins FBF-1 and FBF-2 bind directly to a regulatory region of the *fem-3* 3′UTR (Zhang et al., 1997). Although all *mog* genes cloned to date code for proteins that are homologous to splicing factors, splicing defects have not been found in the respective mutants (Belfiore et al., 2004; Kasturi et al., 2010; Puoti and Kimble, 1999, 2000).

RNA splicing is a key event in the processing of the primary transcript to mRNA. Splicing takes place in the spliceosome, a large dynamic nuclear complex consisting of five snRNAs, snRNP proteins, and non-snRNP proteins (Moore et al., 1993). U1, U2, U4/U6 and U5 snRNAs play crucial roles in RNA–RNA interactions with the pre-mRNA (Konarska and Sharp, 1987). While U1 snRNP binds to the 5′ splice site of the intron, the U2 snRNP associates with the branchpoint (Black et al., 1985). In humans, U1 snRNA binds to the snRNP protein U1A, while U2 snRNA interacts with the snRNP protein U2B″ (Scherly et al., 1990b). In vertebrates, specific binding of U2B″ to U2 snRNA requires yet another RNA-binding protein, U2A′ (Mattaj et al., 1986; Scherly et al., 1990b). Splicing is essentially identical in *C. elegans* and in vertebrates, but several differences need to be mentioned. First, as in other free-living nematodes, *C. elegans* introns are much shorter than in vertebrates, with a median length between 48 and 52 nucleotides (Blumenthal and Steward, 1997). Second, although *C. elegans* introns almost exclusively start with a GU and end with an AG dinucleotide, no branchpoint consensus and no obvious polypyrimidine tract have been identified (Blumenthal and Steward, 1997). Finally, in contrast to human U2B″, the worm homolog RNP-3 binds specifically to U2 snRNA without requiring the *C. elegans* ortholog of U2A′, also known as SAP-1 (Spliceosomal-Associated Protein 1; Saldi et al., 2007).

In this study, we report the cloning and the initial characterization of the *mog-2* gene. *mog-2* encodes SAP-1, the worm homolog of U2A′ (Caspary and Seraphin, 1998; MacMorris et al., 2003; Polycarpou-Schwarz et al., 1996). We show that MOG-2/SAP-1 functions in splicing and that it is synthetically required for meiosis. Furthermore, we show that MOG-2

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binds to RNP-3 and to U2 snRNA in vivo. Finally, we identify a motif in MOG-2 that is crucial for RNP-3 binding and possibly for U2 snRNA binding and sex determination.

Materials and Methods

Strains

All strains were maintained by standard procedures at 20 °C unless specified. Deletion alleles *sap-1(ok1221)/mIn1[dpy-10(e128)mIs14)II* and *rnp-3(ok1424)IV* are from the CGC. *gld-2(q497)*;*mog-2*(*ok1221*) double mutants are from strain *gld-2(q497)/hT2[qIs48](I;III)*;*mog-2 (ok1221)II* and *mog-2(q75) gld-3(q730)* double mutant came from *lin-31(n301) mog-2(q75) gld-3(q730)/mIn1[dpy-10(e128)mIs14]II.* Pre-mRNA processing and decay were analyzed in *smg-1(r861)I*, *mog-2 (q75)II, mog-2(ok1221)II, smg-1(r861)I*;*mog-2(q75)II*, and *mog-2(q75) II*;*rnp-3(ok1424)/nT1g[qIs51](IV;V)* animals. *mIn1, hT2* and *nT1g* are semi-dominant fluorescent markers. Other genotypes used in 3′ splice site recognition: *dpy-10(e128)II*, *smg-1(r861)I*;*dpy-10(e128)II, mog-2 (q75)dpy-10(e128)II, unc-4(e120)dpy-10(e128)II, smg-1(r861)I*;*mog-2(q75)dpy-10(e128)II, dpy-10(e128)II*;*fem-3(q95)IV. mog-6(q465) dpy-10(e128)II* was obtained from *mog-6(q465) dpy-10(e128)II/mIn1* and *mog-5(q449)dpy-10(e128)II* is from *mog-5(q449) dpy-10(e128)/ unc-85(e1414)II.*

Cloning of mog-2 and accession number

mog-2 has been mapped on chromosome *II*, between *lin-31* and *unc-85* (Graham et al., 1993). We further mapped *mog-2* by analyzing recombinants between *mog-2* and two single nucleotide polymorphisms in cosmids R03H10 (position 9489) and ZK430 (position 13198). By sequencing candidate genes, we found that ORF H20J04.8 contained a 5-nucleotide deletion, which was not present in wild type animals. The deletion is located at the end of the first exon and removes a *Hinf*I restriction site. The accession number HM852593 has been assigned to *mog-2* cDNA sequence data.

Phenotype analyses

Fertile hermaphrodites were allowed to lay eggs at the appropriate temperature for 4 h. Developmental stages were determined by the size of the germ line. Plates were scored for dead eggs 30 h after being laid and sterile adults were observed 24 h after the vulva had formed. For sperm counting, worms were grown at 15 °C until L4 and then switched to 25 °C. To avoid larval arrest at 25 °C, we used homozygous siblings of *mog-2/mIn1* heterozygotes. Sperms were identified by their nuclear morphology upon staining with DAPI (4,6-diamino-2-phenylindole).

RNA interference

Sense and antisense RNA corresponding to the entire coding region of *mog-2* were generated using T3 and T7 RNA polymerases (Stratagene). RNA interference was performed by injecting dsRNA (0.5 μg/μl) into either young N2 or *gld-3(q730)/mIn1* adults. Worms were shifted from 20 °C to 25 °C 12 h post injection. *mog-2(q75)* and *mog-2(q75)*;*rnp-3(ok1424)* hermaphrodites were grown at 20 °C on HT115 bacteria producing *gld-1, nos-3 or rnp-2* double-stranded RNA. L3-L4 larvae were then switched to 25 °C. Adults were scored for germline defects by DAPI staining.

MOG-2 antibodies and immunostaining

Polyclonal antibodies were produced against Glutathione-S-Transferase (GST)-tagged MOG-2. For Western blotting, approximately 100 μg of protein extracted from adult worms were loaded. Equal loading was verified by staining with Ponceau S or with α-Tubulin antibodies (1/2000, Sigma). Blots were incubated in Blotto/Tween with anti-MOG-2 antibodies (1/800). Secondary HRP IgG conjugates (Sigma) were used at a dilution of 1/25,000.

For immunostaining, anti-MOG-2 (1/100), anti-GLD-1 (1/100), anti-UAF-1 (1/75) and anti-GLP-1(1/8) antibodies were diluted in PBT, 5% BSA. Worms were fixed with 1.5% formaldehyde (Sigma) in 100 mM phosphate buffer at pH 7.5 followed by cold methanol for 5′ at RT. FITC or Cy-3-conjugated secondary antibodies were used at a dilution of 1/1000 (Jackson Immunoresearch). Immunostaining with anti-PGL-1 (1/500) or anti-MOG-2 (1/100) was performed on entire larvae as described (Bettinger et al., 1996). All incubations were performed overnight at 4 °C, followed by several washes. Stained worms were mounted with Vectashield containing 2 μg/ml DAPI and observed under fluorescence.

MOG-2 interaction assays and screen

mog-2, rnp-3 and rnp-2 full length cDNAs were either introduced into pBTM116 (Bartel and Fields, 1995) to create LexA protein fusions, or into pACTII to make GAL4 activation domain fusion proteins. The plasmid form of a random-primed cDNA library (λACT-RB2) was screened for MOG-2 binding proteins using a MOG-2::LexA fusion construct on minimal medium lacking leucine, tryptophan, and histidine and supplemented with 2.5 mM of 3-aminotriazole. Two-hybrid assays were performed in strain L40. All constructs were sequenced and tested for the presence of the fusion protein by Western blotting using either anti-LexA or anti-GAL4 activation domain antibodies (Upstate Biotechnology).

For in-vitro protein–protein binding assays, full-length *rnp-3* cDNA was cloned into pCITE (Novagen). [^{35}S]Met-labeled RNP-3 protein was produced using TNT-coupled reticulocyte lysate (Promega). *mog-2 (+)* and *mog-2(q75)* cDNAs were introduced into pGEX. Binding assays were performed as described (Belfiore et al., 2004).

For immunoprecipitations, mixed-stage worms were lysed in a mortar in liquid nitrogen, collected with homogenization buffer (10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 10 mM Tris–HCl pH 8.0, 50 mM sucrose, 0.05% Nonidet P-40) and treated with RNasin (0.025 U/μl). Protein G Dynabeads (Invitrogen) were coupled with anti-MOG-2 antibodies and incubated 1 h at 4 °C with worm extract in IP Buffer (100 mM KCl, 0.1 mM DTT, 20 mM Tris–HCl pH 8.0, 0.1% Nonidet P-40, 0.2 mM EDTA). Beads were washed with 1× PBS and eluted as indicated in the manufacturer's instructions with 1× NuPAGE LDS sample buffer. Mock and IP were treated with RNase-free DNase (0.05 U/μl), with Proteinase K, and finally incubated 5′ at 95 °C. Phenol/chloroform precipitates were analyzed by Northern blotting with a U2 snRNA full-length probe.

Northern analysis and RT-PCR

Poly(A)$^+$ RNA and total RNA were extracted and analyzed as described (Puoti and Kimble, 1999). RT-PCR was performed on total RNA from adult worms (Puoti and Kimble, 1999). For RT-PCR, 1 μg of DNase-treated total RNA and 100 ng of random primers were processed as described (Belfiore et al., 2004). As a positive control we used genomic DNA and random-primed single-stranded cDNAs from wild type animals. Sequences of primers are available on request. After 21 cycles of PCR, *dpy-10(e128)* cDNAs were separated on a 2% agarose gel and probed with a template that equally recognizes both spliced and unspliced variants. Quantification was performed with an Amersham Biosciences Phosphoimager.

Results and discussion

The MOG-2 protein and the Mog-2 mutant phenotype

The *mog-2(q75)* allele was isolated in a screen for recessive sterile mutants (Graham et al., 1993). *mog-2* has been mapped on chromosome