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Developmental Biology 292 (2006) 244–252
DEVELOPMENTAL BIOLOGY
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Translational repression restricts expression of the *C. elegans* Nanos homolog NOS-2 to the embryonic germline

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Received for publication 27 July 2005; revised 23 September 2005; accepted 29 November 2005

Abstract

Members of the *nanos* gene family are evolutionarily conserved regulators of germ cell development. In several organisms, Nanos protein expression is restricted to the primordial germ cells (PGCs) during early embryogenesis. Here, we investigate the regulation of the *Caenorhabditis elegans nanos* homolog *nos-2*. We find that the *nos-2* RNA is translationally repressed. In the adult germline, translation of the *nos-2* RNA is inhibited in growing oocytes, and this inhibition depends on a short stem loop in the *nos-2* 3′UTR. In embryos, *nos-2* translation is repressed in early blastomeres, and this inhibition depends on a second region in the *nos-2* 3′UTR. *nos-2* RNA is also degraded in somatic blastomeres by a process that is independent of translational repression and requires the CCCH finger proteins MEX-5 and MEX-6. Finally, the germ plasm component POS-1 activates *nos-2* translation in the PGCs. A combination of translational repression, RNA degradation, and activation by germ plasm has also been implicated in the regulation of *nanos* homologs in *Drosophila* and zebrafish, suggesting the existence of conserved mechanisms to restrict Nanos expression to the germline.

Keywords: Germ cells; Nanos; Translational control; RNA degradation; CCCH finger; *Caenorhabditis elegans*

Introduction

Members of the *nanos* gene family are essential regulators of germ cell development in both invertebrates and vertebrates. Nanos was first identified in *Drosophila* for its role in embryonic patterning (Wang and Lehmann, 1991). Later studies revealed that *nanos* also regulates germ cell development: *nanos* is required maternally for primordial germ cell (PGC) migration during embryonic development, and zygotically for the differentiation of germline stem cells in the adult gonad (Kobayashi et al., 1996; Forbes and Lehmann, 1998). In *Caenorhabditis elegans*, three *nanos*-related genes, *nos-1*, *nos-2*, and *nos-3*, have been identified. *nos-2* is required maternally for efficient incorporation of PGCs into the somatic gonad and functions redundantly with *nos-1* to regulate survival and proliferation of PGC descendents during larval development (Subramaniam and Seydoux, 1999). *nos-3* functions in the sperm-to-oocyte switch in hermaphrodites (Kraemer et al., 1999). *nanos* homologs have also been identified in vertebrates. Zebrafish *nanos1* regulates PGC migration and survival during embryonic development (Koprunner et al., 2001). In mice, *nanos2* is required to form spermatogonia, and *nanos3* is required for PGC survival (Tsuda et al., 2003). Together, these studies have established *nanos* family members as critical regulators of PGC and germ cell development.

In *Drosophila*, *C. elegans* and zebrafish embryos, maternal expression of *nanos* family members is restricted to PGCs and their precursors (Wang et al., 1994; Subramaniam and Seydoux, 1999; Koprunner et al., 2001). *nanos* regulation has been investigated in greatest detail in *Drosophila*. *nanos* RNA is transcribed during oogenesis and enriched to the posterior of the oocyte with other components of the germ plasm, a specialized cytoplasm required for germ cell formation. In oocytes and embryos, *nanos* RNA is translated only in the germ plasm, and Nanos protein is eventually incorporated into pole cells, the precursors to the *Drosophila* PGCs (Wang et al., 1994). Unlocalized *nanos* RNA is translationally repressed and degraded during early embryogenesis. This behavior depends

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0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.11.046

on the *nanos* 3′UTR, which contains elements that regulate the localization, degradation, and translation of the *nanos* RNA (Dahanukar and Wharton, 1996; Gavis et al., 1996a,b; Smibert et al., 1996; Bashirullah et al., 1999). In particular, a translational repression control element termed TCE contains two stem loop structures (II and III), which mediate translation repression in oocytes (Stem loop III) and in embryos (Stem loop II) (Forrest et al., 2004). Stem loop II contains a binding site for the Smaug protein, which is required for translational repression in embryos (Dahanukar et al., 1999; Smibert et al., 1999). Translational derepression of *nanos* in the posterior depends on the germ plasm components Oskar and Vasa, but the mechanisms involved are not known (Gavis and Lehmann, 1994).

We described previously the expression pattern of the *C. elegans nos-2* gene, which, like *Drosophila nanos*, is expressed maternally and associates with the germ plasm. *nos-2* RNA is synthesized during oogenesis and enriched on germ plasm organelles called P granules (Subramaniam and Seydoux, 1999; Schisa et al., 2001;). In embryos, *nos-2* RNA is rapidly degraded in somatic blastomeres and maintained only in germline blastomeres (Fig. 1A). NOS-2 protein is first detected in P_4, the immediate precursor to the PGCs, Z2 and Z3 (Subramaniam and Seydoux, 1999). In this study, we have investigated how NOS-2 expression is restricted to the PGCs. We find that the PGC-specific expression of NOS-2 is primarily due to negative regulation at the translational level. In addition, *nos-2* RNA is also degraded in somatic blastomeres. Our findings suggest parallels between the regulation of *Drosophila nanos* and *C. elegans nos-2*.

Materials and methods

C. elegans strains

Worms were maintained as described (Brenner, 1974), with the exception that all strains were kept at 25°C to avoid silencing of transgenes in the germline (Strome et al., 2001). Transgenic lines were generated by the complex array methods [(Kraemer et al., 1999); transgenes described in Fig. 1] or by biolistic transformation [(Praitis et al., 2001); transgenes described in Tables 1 and 2].

Construction of transgenes

To facilitate the analysis of 3′ UTR sequences, we constructed two new vectors. The first vector, pKS111-His, was made starting with pJH4.52, which contains the *pie-1* promoter, a fusion between GFP and Histone H2B (GFP:H2B), and 3.2 kb downstream of the *pie-1* STOP codon including the *pie-1* 3′ UTR (Reese et al., 2000). pJH4.52 was modified to remove the *pie-1* 3′UTR (bases 5535 to 5615 in pJH4.52) and add an Apa I site immediately downstream of GFP:H2B and the *pie-1* STOP codon. A 930-bp fragment, containing the longest *nos-2* 3′UTR plus an additional 520 bp of *nos-2* downstream sequence, was PCR amplified and inserted at the Apa I site to produce pKS111-His. Mutant versions of the 3′UTR were created by PCR and inserted using the Apa I site. The *unc-119* rescuing sequence from pAZ132 was added to the vector using NgoM IV and Sac II sites before biolistic transformation (Praitis et al., 2001).

The second vector, pPC2.02, was made starting with pID2.02, a GATEWAY destination vector which contains *unc-119* rescuing sequences, the *pie-1* promoter, Gateway Cassette B, and 3179 bp downstream of the *pie-1* STOP codon including the *pie-1* 3′UTR. pID2.02 was modified to replace the *pie-1* 3′ UTR sequences with "buffer" sequences taken from the *nos-2* 3′ region (bases 407–926 downstream of the *nos-2* STOP codon) to create gateway destination vector pPC2.02. GFP:H2B:*nos-2* 3′UTR (1–199 bp/331–406 bp) was PCR amplified from pKS111HisΔ5 (last deletion construct in Fig. 1), recombined with pDONR201 (GATEWAY BP reaction) and recombined with pPC2.02 (GATEWAY LR reaction) to create pCM1.01. pCM1.02-4 were constructed in the same way except that point mutations were introduced in the *nos-2* 3′UTR during PCR amplification of pKS111HisΔ5. Sequence of all constructs was verified by sequencing.

Molecular biology, immunofluorescence, and RNA-mediated interference

Northern hybridization and other cloning techniques were carried out following standard protocols (Sambrook et al., 1989). 3′RACE-PCR was carried out using a kit from Clontech Laboratories, Palo Alto, CA. NOS-2 protein distribution in embryos was determined by immunofluorescence as described (Subramaniam and Seydoux, 1999) except that polyclonal anti-NOS-2 antibody was affinity purified by column chromatography. By comparing wild-type hermaphrodites and *nos-2(ok230)*; kpIs[pRD5] hermaphrodites lacking the *nos-2* gene, we discovered that the anti-NOS-2 antibody is specific for NOS-2 in zygotes and embryos, but not in the adult germline where it reacts with another epitope.

In situ hybridization and microscopy were performed as described (Seydoux and Fire, 1995) with the following modifications: hybridization was carried out at 48°C, and incubation with the alkaline phosphatase-conjugated anti-DIG antibody was extended to 15 h at 4°C.

For RNA-mediated interference experiments, target coding sequences were amplified by RT-PCR from total RNA extracted from mixed-stage worms. PCR fragments were cloned into the RNAi feeding vector, L4440 and introduced into *E. coli* HT115. Transformants were used for feeding experiments as described (Timmons et al., 2001). Embryos were examined 24 or more hours after the start of feeding.

Results

The nos-2 3′UTR is sufficient to restrict expression of a heterologous transgene to P_4 and Z2 and Z3

Northern analyses and 3′RACE-PCR experiments revealed the presence of 4 alternative cleavage sites for the *nos-2* mRNA (Figs. 1B and C and data not shown). A region comprising the longest 3′UTR was fused downstream of a GFP:Histone H2B reporter driven by the *pie-1* promoter and introduced into worms (Materials and methods). Embryos derived from mothers carrying the GFP:H2B:*nos-2* 3′*UTR* transgene expressed GFP:H2B only in P_4 and its daughters Z2 and Z3, in a pattern identical to that observed for endogenous NOS-2 protein (Subramaniam and Seydoux, 1999). In contrast, a control transgene containing the *pie-1* 3′UTR expressed GFP:H2B in all early blastomeres (Fig. 1D). Deletion analysis revealed that a 200-bp region immediately downstream of the *nos-2* STOP codon is essential to restrict GFP:H2B expression to the PGCs.

Expression of the GFP:H2B:*nos-2 3′UTR* transgene could also be detected in the maternal germline. Highest levels were detected in the mitotic (distal) region of the gonad (data not shown). Progressively lower levels were detected as germ cells progressed through pachytene with only very low levels detected in oocytes (Figs. 1D, 2B and data not shown). A control transgene containing the *pie-1* 3′UTR was expressed at higher levels in oocytes (Fig. 1D), suggesting that the *nos-2* 3′ UTR contains sequences that inhibit translation during

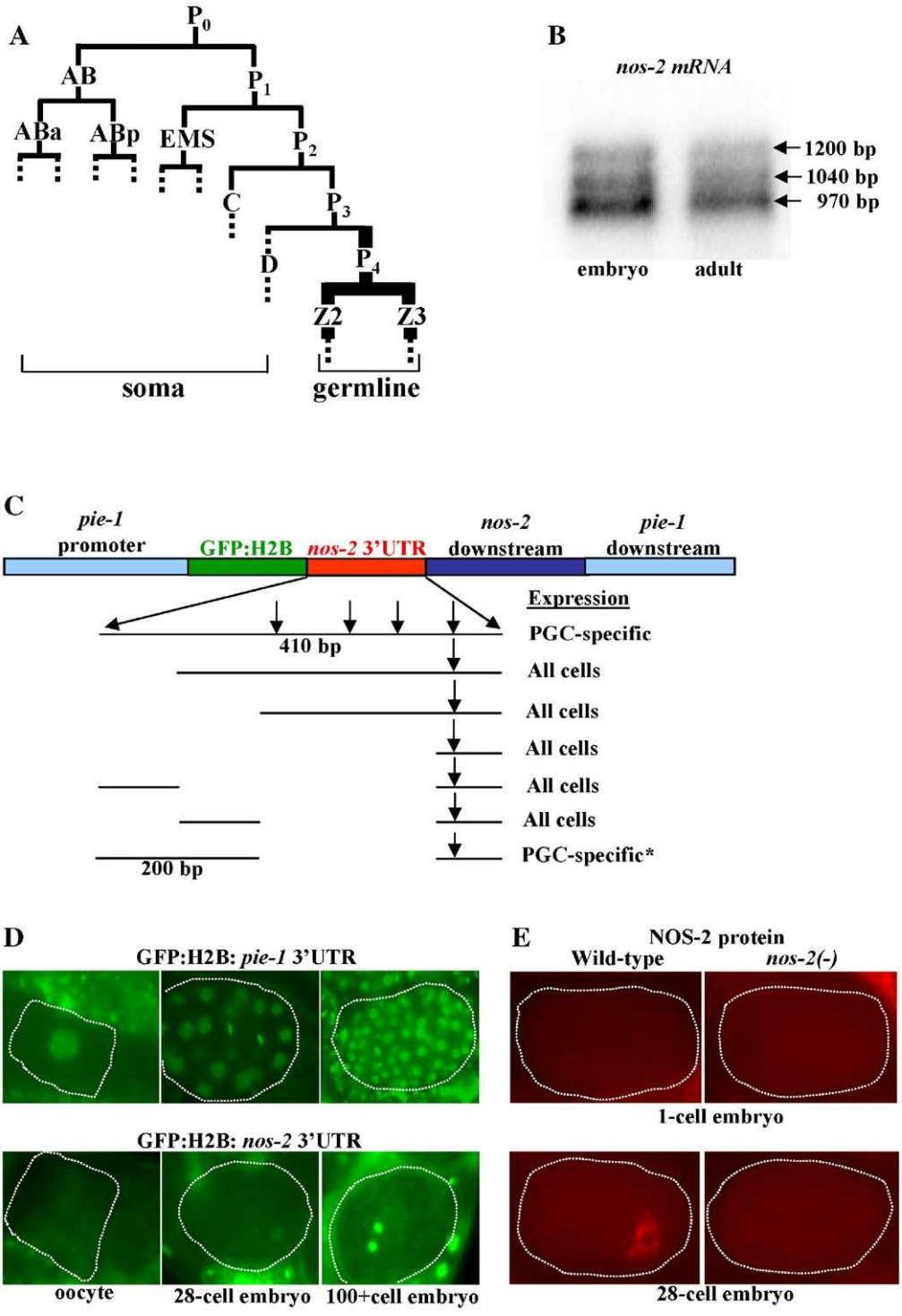

Fig. 1. The *nos-2* 3′UTR is sufficient for PGC-specific expression. (A) Distribution of *nos-2* RNA and protein in the embryo. Abbreviated embryonic lineage is shown in the form of a line diagram (Sulston et al., 1983). Solid lines indicate cells with *nos-2* RNA, broken lines indicate cells with no *nos-2* RNA, and bold lines indicate cells with both *nos-2* RNA and NOS-2 protein (Subramaniam and Seydoux, 1999). (B) Northern blot hybridized to a *nos-2* probe. Three predominant *nos-2* transcripts are detected, viz., 970 bp, 1100 bp, and 1200 bp. (C) Schematic illustration of the GFP:H2B:*nos-2* 3′UTR transgene constructs. The vertical arrows indicate the positions of the four alternative cleavage sites identified by sequencing of the 3′RACE-PCR products. To ensure proper cleavage and polyadenylation, all constructs include at a minimum the 4th cleavage polyadenylation site. GFP:H2B:*nos-2* 3′UTRmin, which was used in the mutational analysis described in Fig. 2 and Table 1, is marked with an asterisk. (D) Oocytes and embryos (outlined) expressing GFP:H2B under the control of the *pie-1* 3′UTR or the *nos-2* 3′UTR. (E) Distribution of NOS-2 protein in the wild-type and *nos-2(ok230)*;kpIs[pRD5]embryos, which, marked in the figure as *nos-2(−)*, lack the coding region of *nos-2*.

Table 1
Mutational analysis of the *nos-2* 3′UTR

	Distal germ cells	Oocytes	1- to 8-cell embryos	8- to 28-cell embryos	28- to 100-cell embryos	100+ cell embryos
Wild-type	ON (16/16)	Weak (23/24)	OFF (72/75)	OFF (54/55)	P_4 (33/34)	Z2 + Z3 (63/63)
SubA	ON (22/22)	Strong (27/27)	ON (49/54)	ON (27/34)	ON (13/34)	Z2 + Z3 (35/49)
SubB	ON (5/5)	Weak (6/6)	ON (12/30)	ON (3/8)	ON/P_4 (21/39)	ON/Z2 + Z3 (10/14)
SubC	ON (4/4)	Weak (4/4)	ON (6/11)	NA	ON (20/20)	ON/Z2 + Z3 (33/33)
SubD	ON (6/6)	Weak (6/6)	OFF (21/22)	1/2 cells (5/5)	P_4 + other (16/16)	Z2 + Z3 + other (26/43)
SubE	OFF (5/5)	OFF (6/6)	OFF (11/11)	OFF (11/11)	OFF (18/20)	OFF (18/18)
M1	ON (43/43)	Strong (42/42)	ON (60/60)	ON (63/63)	ON (30/55)	Z2 + Z3 (70/103)
M2	ON (56/56)	Strong (55/55)	ON (51/52)	ON (45/48)	ON/P_4 (19/41)	Z2 + Z3 (72/101)
M1 + M2	ON (52/52)	Weak (53/53)	OFF (33/35)	OFF (40/40)	P_4 (26/31)	Z2 + Z3 (97/97)

SubA, SubB, SubC, SubD, and SubE, M1 and M2 are mutations described in Fig. 2.
ON: GFP:H2B in all cells.
OFF: GFP:H2B in no cells.
Z2 + Z3: GFP only in Z2 + Z3.
P_4: GFP only in P_4.
ON/P_4: GFP in all cells and brighter in P_4.
ON/Z2 + Z3: GFP in all cells and brighter in Z2 and Z3.
Numbers in parentheses are the number of embryos showing the indicated GFP phenotype/total number of embryos examined. For example, in the case of SubA, of the 34 8- to 28-cell stage embryos examined, 27 had GFP in all cells and 7 had GFP in no cells. For each construct, data were collected from at least two independent transgenic lines generated by the biolistic method. Expression patterns were consistent among all the transgenic lines derived from the same construct.

oogenesis (see below). Potential perdurance of GFP:H2B, however, prevents us from determining precisely when this translational inhibition first begins. The available anti-NOS-2 polyclonal antibody (Subramaniam and Seydoux, 1999) is not specific for NOS-2 in the germline, preventing us from describing the pattern of expression of endogenous NOS-2 in this tissue. Staining of embryos with affinity-purified anti-NOS-2 antibody, however, confirmed that NOS-2 is not present in the 1-cell stage and is not expressed until the 28-cell stage in P_4 (Fig. 1E and Materials and methods).

The nos-2 3′UTR functions primarily by inhibiting translation

To identify regulatory elements within the 200-bp *nos-2* 3′ UTR, we substituted 30-bp stretches with a non-specific sequence [$(TG)_{15}$] of the same length and examined the effect of such substitutions on the expression of the GFP:H2B:*nos-2 3′UTR* transgene containing the minimal 200-bp region, hereafter referred to as GFP:H2B:*nos-2 3′UTR*min (Table 1). Remarkably, we found that 4 of 5 substitutions caused GFP:H2B to be ectopically expressed.

SubA led to high levels of GFP:H2B expression in oocytes (Fig. 2 and Table 1). GFP:H2B could also be detected in embryonic blastomeres with progressively lower levels as the embryos aged, likely due to the progressive turn over, or dilution, of GFP:H2B synthesized in oocytes. By the 100-cell stage, high levels of GFP:H2B were detected primarily only in Z2 and Z3, as is seen with the wild-type *nos-2* 3′UTRmin. These results suggest that subA specifically disrupts a sequence required to inhibit expression in oocytes.

The next two substitutions (sub B and C) showed normal levels in oocytes but high levels in embryonic blastomeres starting in the 2- to 4-cell stage. Expression in somatic blastomeres began to decrease around the 100-cell stage, with high levels remaining only in the PGCs. These observations suggest that SubB and C affect sequences primarily required to inhibit expression in early embryonic blastomeres.

SubD did not affect expression in oocytes or early embryonic blastomeres but instead caused ectopic expression of GFP:H2B in a small number of cells located next to P_4. The position and number of these cells (1–4 increasing with age) indicate that they correspond to the somatic D blastomere and its descendents. These observations suggest that SubD affects a sequence required to prevent expression in the D blastomere. Alternatively, since P_4 and D are derived from the same germline blastomere P_3 (Fig. 1A), SubD could affect a sequence required to prevent premature expression in P_3. The last substitution, SubE, blocked transgene expression in all cells (including the distal gonad), suggesting that this substitution affects a sequence generally required for translation and/or

Table 2
Expression of GFP:H2B:*nos-2* 3′UTRmin in embryos depleted for *pos-1*, *mex-5*, and *mex-6* by RNAi

	Distal germ cells	Oocytes	1- to 8-cell embryos	8- to 28-cell embryos	28- to 100-cell embryos	100+ cell embryos
Wild-type	ON (16/16)	Weak (23/24)	OFF (72/75)	OFF (54/55)	P_4 (33/34)	Z2 + Z3 (63/63)
pos-1(RNAi)	ON (4/4)	Weak (1/2)	OFF (7/7)	OFF (5/5)	OFF (3/3)	OFF (47/47)
mex-5(RNAi) mex-6(RNAi)	ON (8/8)	Weak (9/10)	ON (41/53)	ON (9/9)	ON (31/31)	ON (23/23)
mex-5(RNAi) mex-6(RNAi) pos-1(RNAi)	ON (6/6)	Weak (6/6)	OFF (14/14)	OFF (3/3)	OFF (12/12)	OFF (32/32)
SubA + pos-1(RNAi)	ON (26/26)	ON (26/26)	ON (14/14)	ON (29/30)	OFF (15/19)	OFF (41/47)

See Table 1 legend for details.

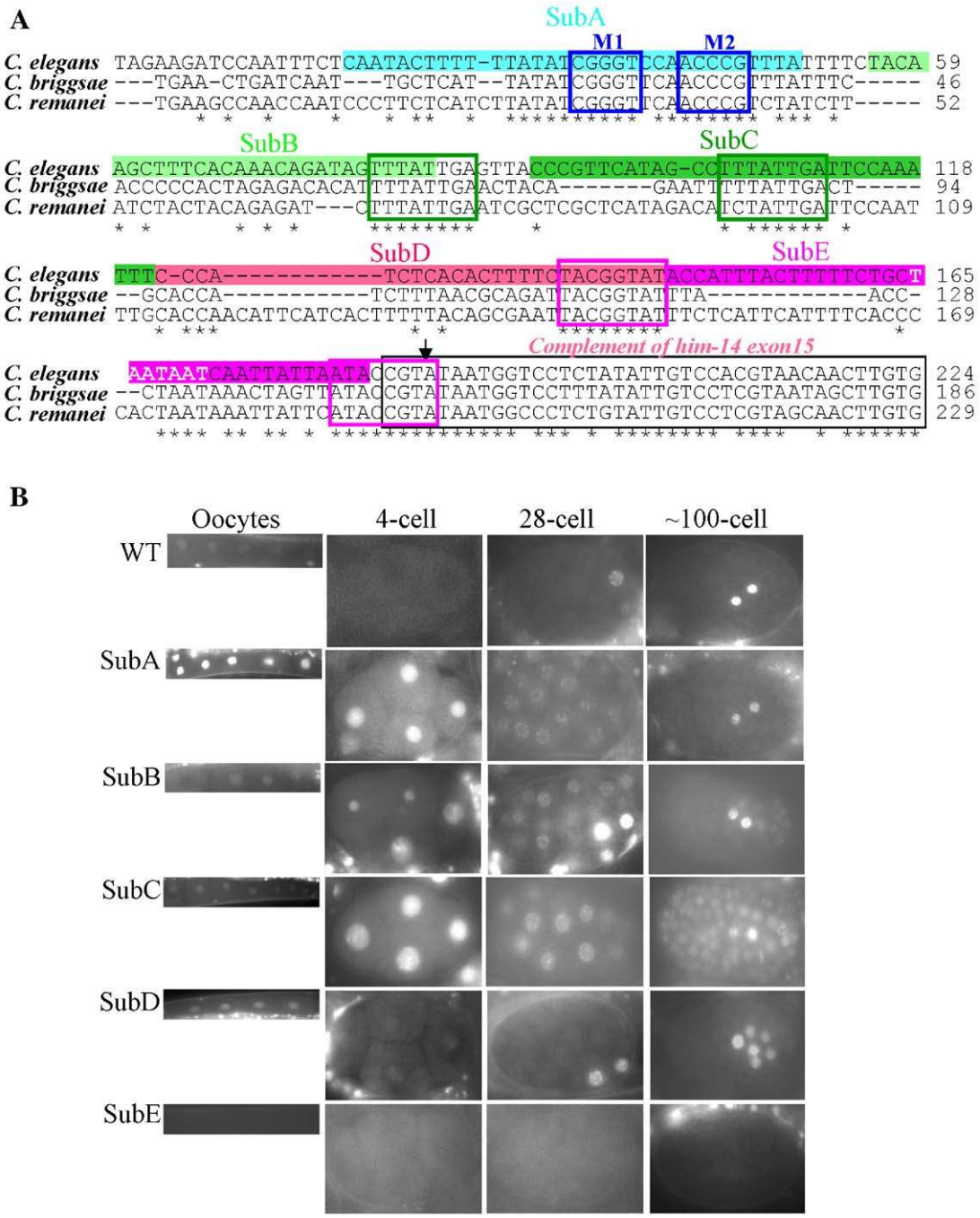

Fig. 2. Dissection of the *nos-2* 3′UTR. (A) Alignment of *nos-2* 3′UTRs from *C. elegans*, *C. briggsae*, and *C. remanei*. The arrow marks the polyA addition site in *C. elegans nos-2*, which runs into exon-15 of *him-14* on the complementary strand (boxed in black). Sequences in SubA predicted to pair are boxed with blue lines. Two 8-bp direct repeats present in the SubB and SubC regions are boxed in green. The 8-bp inverted repeats in the SubD and SubE regions are boxed in pink. The putative polyadenylation signal AATAAT is in white. (B) GFP:H2B expression in oocytes and embryos derived from mothers carrying the GFP:H2B:*nos-2* 3′UTRmin transgenes with the indicated substitutions.

RNA stability. Indeed, this substitution eliminates a polyadenylation consensus sequence (Fig. 2).

Like many maternal RNAs in *C. elegans*, *nos-2* mRNA is rapidly turned over in somatic blastomeres and maintained only in the P blastomeres (Seydoux and Fire, 1994; Subramaniam and Seydoux, 1999). To investigate whether the ectopic expression observed with SubC and SubD was due to stabilization of the GFP:H2B:*nos-2 3′UTR* RNA in somatic blastomeres, we hybridized embryos expressing wild-type, SubC, or SubD transgenes to a probe complementary to GFP. In all cases, we found that the GFP:H2B RNA could be detected in all blastomeres up to the 4-cell stage, and primarily in P blastomeres in later stages, as is the case for endogenous *nos-2* RNA (Fig. 3). We conclude that SubC and SubD do not affect RNA stability and therefore most likely interfere with translational regulation.

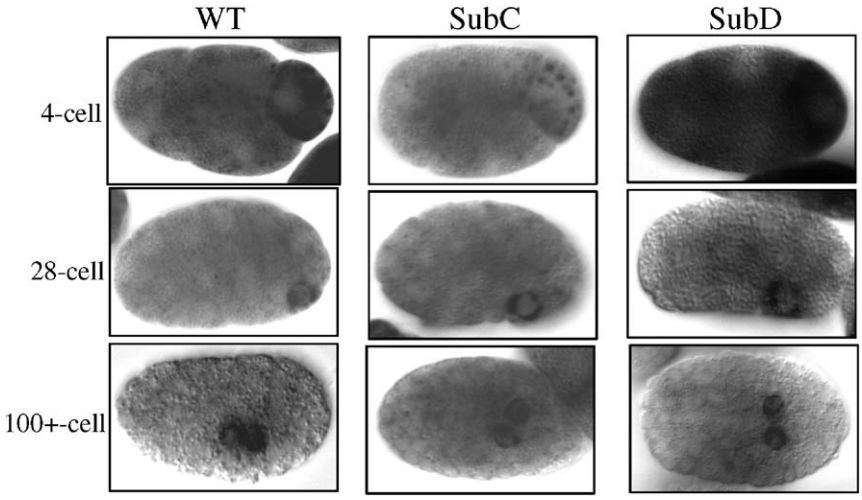

Fig. 3. In situ hybridization showing the distribution of GFP:H2B:*nos-2* 3′UTRmin RNAs in embryos. GFP:H2B:*nos-2* 3′UTRmin RNAs are detected in all cells up to the 4-cell stage and are maintained primarily only in germline blastomeres in later stages.

A stem-loop is required for translational repression in oocytes

Alignment of the *nos-2* 3′UTR from *C. elegans* and two related nematodes *C. briggsae* and *C. remanei* revealed several conserved blocks (Fig. 2A). In particular, in the SubA region, we noted a conserved inverted repeat with the potential to form a short stem loop (Fig. 2A). To test the significance of this structure, we created two mutants predicted to disrupt pairing in the stem (M1 and M2), and a double mutant (M1M2) predicted to restore pairing. We found that the M1 and M2 mutations lead to ectopic expression of GFP:H2B in oocytes (Fig. 4), as was observed for SubA. In contrast, the M1M2 double mutant was expressed in a pattern indistinguishable from wild type, consistent with restoration of the stem loop (Fig. 4). We conclude that inhibition of *nos-2* translation in oocytes depends on a conserved stem loop structure within SubA.

POS-1 is required to activate nos-2 translation in P_4

We showed previously that maintenance of the *nos-2* RNA in P blastomeres depends on the CCCH finger

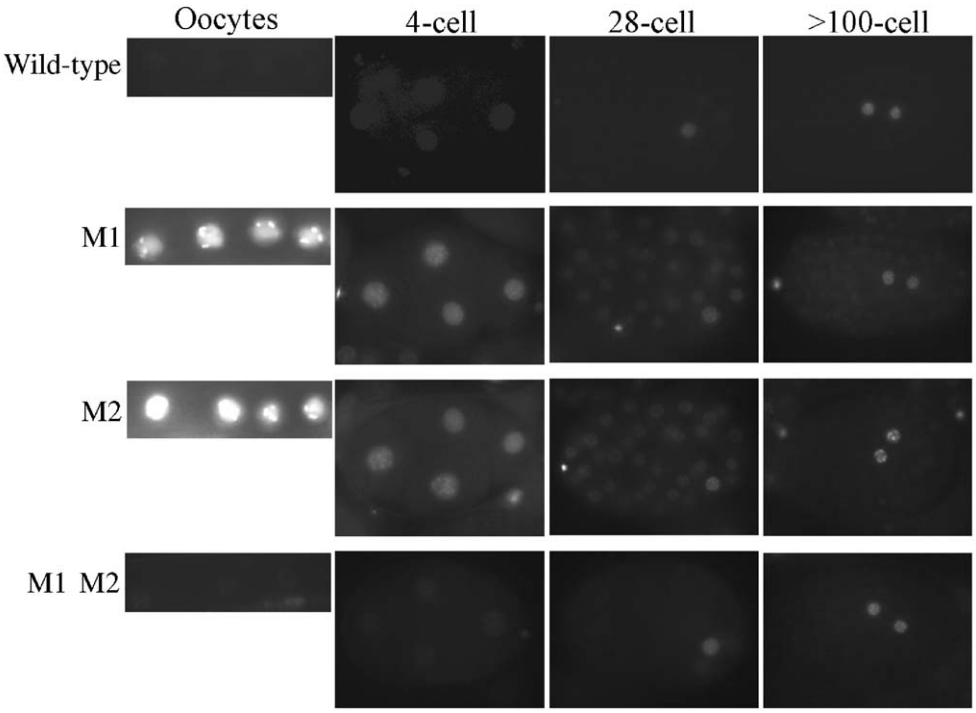

Fig. 4. GFP:H2B expression in transgenic lines carrying mutations in the stem/loop structure. M1: mutation in the first 5′ strand of the stem (CGGG to GCCC). M2: mutation in the 3′ strand of the stem (CCCG to CGGG). M1/M2: mutations in both strands. M1 and M2 individually are predicted to disrupt pairing in the stem, whereas the combined mutation M1/M2 is predicted to restore pairing.

protein PIE-1 (Tenenhaus et al., 2001). POS-1 is another CCCH finger protein, which like PIE-1 segregates with the germ lineage (Tabara et al., 1999). To investigate a possible role for POS-1 in regulating *nos-2* expression, we examined the effect of depleting POS-1 by RNAi in embryos expressing the GFP:H2B:*nos-2 3′UTR*min transgene (Materials and methods). We found that this treatment blocks GFP:H2B expression in P_4, and in Z2 and Z3 (Fig. 5; Table 2). Endogenous NOS-2 expression was also lost in *pos-1(RNAi)* embryos (data not shown). To test whether loss of NOS-2 expression was due to loss of the *nos-2* RNA, we examined the distribution of *nos-2* RNA in *pos-1 (RNAi)* embryos. We found that the distribution of the *nos-2* RNA was not affected in *pos-1(RNAi)* embryos (Fig. 5). We conclude that unlike PIE-1, POS-1 regulates NOS-2 expression primarily at the level of translation and not RNA stability.

POS-1 could be required generally for *nos-2* translation or more specifically for activation in P_4. To distinguish between these possibilities, we examined whether expression of the SubA transgene was dependent on *pos-1*. We found that depletion of *pos-1* by RNAi did not block SubA expression in oocytes and early blastomeres but did prevent expression in Z2 and Z3 (Table 2). These observations suggest that POS-1 is required to activate *nos-2* expression specifically in PGCs. Consistent with this view, *pos-1(RNAi)* did not block expression of the GFP:H2B:*nos-2 3′UTR*min transgene in the mitotic germ cells of adult hermaphrodites (data not shown).

MEX-5 and MEX-6 are required for the degradation of nos-2 mRNA in somatic blastomeres

MEX-5 and MEX-6 are two redundant CCCH finger proteins which, unlike PIE-1 and POS-1, are enriched in somatic blastomeres and present only at low levels in germline blastomeres. Inactivation of *mex-5* and *mex-6* cause PIE-1, POS-1, and P granules to become stabilized in all cells (Schubert et al., 2000). We found that in *mex-5(RNAi) mex-6(RNAi)* embryos, the *nos-2* RNA also became stabilized in all cells up to the 20-cell stage (Fig. 5). Consistent with this general delocalization of germ plasm components, *mex-5(RNAi) mex-6(RNAi)* embryos expressed the GFP:H2B:*nos-2 3′UTR*min transgene in all blastomeres (Fig. 5). Expression of GFP:H2B in *mex-5(RNAi) mex-6 (RNAi)* was dependent on *pos-1*. In contrast, stabilization of the *nos-2* RNA in *mex-5(RNAi) mex-6(RNAi)* was not dependent on *pos-1* (Fig. 5). We conclude that *mex-5* and *mex-6* are required for degradation of the *nos-2* RNA in somatic blastomeres, whereas *pos-1* is required for translational activation.

Discussion

Our results demonstrate that the *nos-2* RNA is under extensive translational repression. First, in the adult germline, the *nos-2* RNA becomes translationally repressed as germ cells proceed through oogenesis. This repression depends on a stem loop in the *nos-2* 3′UTR. Second, after fertilization,

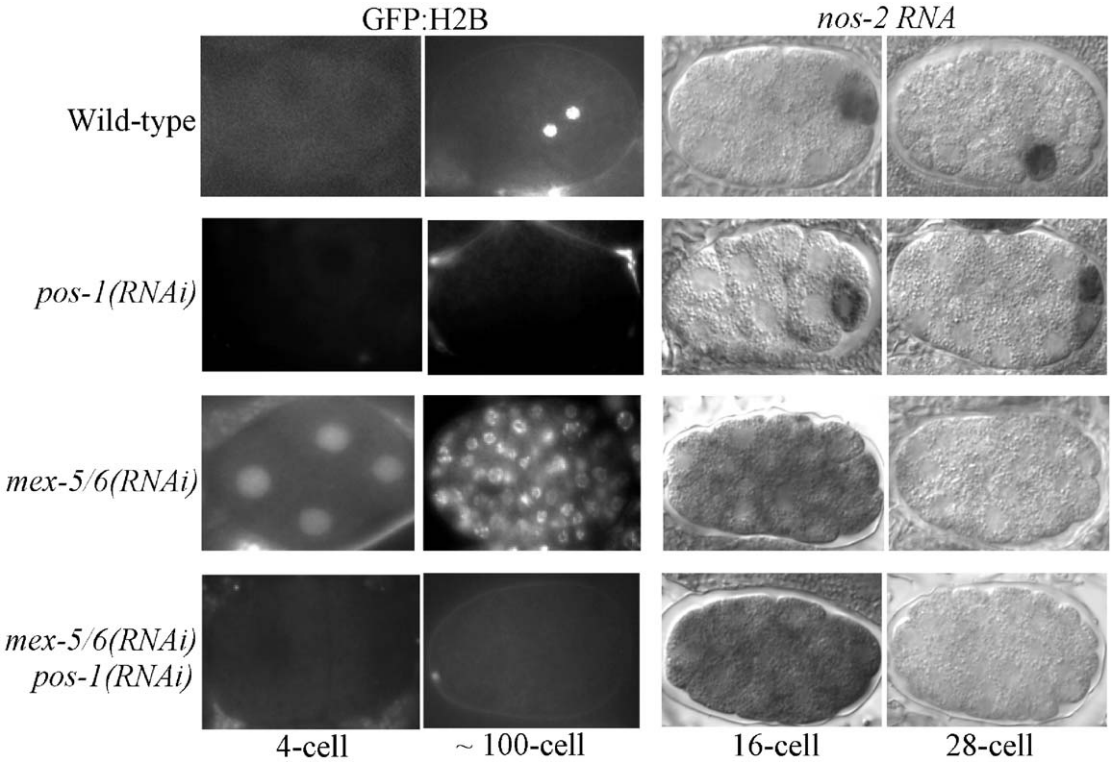

Fig. 5. Distribution of GFP:H2B:*nos-2* 3′UTRmin and *nos-2* RNA in embryos depleted for the indicated genes by RNAi. The panels marked *mex-5/6(RNAi)* indicate simultaneous depletion of both MEX-5 and MEX-6.

the *nos-2* mRNA continues to be translationally repressed, but this repression depends on a separate domain in the 3′ UTR. *nos-2* translation is derepressed only in P_4, by a mechanism requiring the germ plasm component POS-1. In addition to these translational controls, RNA degradation dependent on the CCCH finger proteins MEX-5 and MEX-6 depletes *nos-2* RNA from the somatic blastomeres.

Translational repression

Our mutational analysis of the *nos-2* 3′UTR indicates that multiple elements mediate translational repression of the *nos-2* RNA. In particular, we have found two non-overlapping regions required for translational repression in oocytes and embryos. The SubA substitution and M1 and M2 mutations lead to ectopic expression in oocytes but do not seem to affect translational repression in the embryo, since expression of the reporter diminishes significantly after fertilization. Conversely, the SubB and SubC substitutions cause ectopic expression in embryos but not oocytes. These observations suggest that the mechanisms that mediate translation repression in oocytes and embryos are distinct and can function independently from each other. This is reminiscent of the situation with *Drosophila nanos* where distinct stem-loops were shown to mediate translational repression in oocytes and embryos (Forrest et al., 2004). Remarkably, our analysis also implicates the involvement of a stem-loop for translational repression of *nos-2* in oocytes, although this loop does not bear obvious sequence similarity to the *nanos* stem-loops. A second potential stem-loop is present in the *nos-2* 3′UTR (bases 139–188) but is outside the region required for translational repression in embryos, which instead contains a pair of direct repeats. The significance of these repeats remains to be determined. In *Drosophila*, translational repression in embryos depends on the Smaug protein, which recognizes a sequence termed SRE in Stem-loop II. This sequence is not present in the *nos-2* 3′UTR. Consistent with this, RNAi of ZC190.4, the *C. elegans* protein most similar to Smaug (37% identity in a 108-amino acid stretch), does not affect *nos-2* expression (K. Subramaniam, unpublished observations). Therefore, although the distinction between translational repression in oocytes and embryos appears conserved between *Drosophila* and *C. elegans*, mechanistic details may have diverged between the two species.

RNA degradation

In addition to being translationally repressed in somatic blastomeres, the *nos-2* RNA is also degraded in these cells starting in the 4-cell stage. Degradation of *nanos* RNA in somatic cells has also been observed in *Drosophila* and zebrafish (Wang et al., 1994; Koprunner et al., 2001). As shown in *Drosophila* (Bashirullah et al., 1999), we find that RNA degradation does not depend on translational repression, since mutations that derepress translation do not prevent RNA degradation. We do not yet know whether *nos-2* RNA degradation depends on specific sequences in the 3′UTR.

Degradation in somatic blastomeres is characteristic of many maternal RNAs in *C. elegans* and could represent a default state for maternal RNAs (Seydoux and Fire, 1994). Alternatively redundant elements in the 3′UTR could regulate RNA stability as was found for *nanos* and *Hsp83*, another *Drosophila* transcript degraded during early embryogenesis (Lipshitz and Smibert, 2000). Most recently, the Smaug protein was shown to trigger the degradation of Hsp83 through the recruitment of the CCR4/POP2/NOT deadenylase, suggesting that Smaug functions both in translational repression and RNA degradation in *Drosophila* embryos (Semotok et al., 2005). We do not yet know what triggers the degradation of *nos-2* in *C. elegans*. We have found that *nos-2* RNA degradation is dependent on MEX-5 and MEX-6, two CCCH finger proteins enriched in somatic blastomeres. MEX-5 and MEX-6 belong to the TTP family of RNA-binding proteins (Schubert et al., 2000). TTP has been shown to recognize a specific RNA sequence (ARE) and to induce the degradation of ARE-containing RNAs (Carballo et al., 1998). A potential ARE is present in the *nos-2* 3′UTR, raising the possibility that *nos-2* could be a direct target of MEX-5/6. Alternatively, since MEX-5 and MEX-6 regulate the localization of many germ plasm components, their effect on *nos-2* RNA could be indirect and simply reflect delocalization of the germ plasm. How the germ plasm protects RNAs from degradation is not known.

Translational activation in P_4 depends on the germ plasm component POS-1

We have found that derepression of *nos-2* translation in P_4 depends on the germ plasm protein POS-1. POS-1 is an RNA-binding protein previously implicated in the translational activation of the Notch ligand APX-1 and the translational repression of the Notch receptor GLP-1 in P blastomeres (Tabara et al., 1999; Ogura et al., 2003). Our evidence suggests that POS-1 is required specifically to antagonize the mechanisms that inhibit *nos-2* translation, since POS-1 is not required when translational repression is lifted. Since POS-1 is present in the P lineage continuously from the time of fertilization, it is not clear how translational activation of *nos-2* is delayed until P_4. One possibility is that a yet-to-be-identified P_4-specific protein is also required for activation of *nos-2* translation. Another possibility is that the mechanisms that repress *nos-2* translation in the embryo weaken with each P cell division, eventually reaching a level in P_4 that can be overcome by POS-1. Our finding that the SubD substitution leads to ectopic expression specifically in D, the last somatic blastomere to be born, is consistent with the idea that translational repression at this stage may be weaker and therefore more sensitive to perturbations.

It will be important to identify sequences in the *nos-2* 3′ UTR that function with POS-1 to activate translation. Activation of *nanos* in *Drosophila* also requires germ plasm

components (Gavis and Lehmann, 1994), but the mechanisms involved remain unclear. Association with the germ plasm is a conserved characteristic of *nanos* RNA in *Drosophila*, *C. elegans*, *Xenopus*, and zebrafish (Gavis and Lehmann, 1992; Mosquera et al., 1993; Subramaniam and Seydoux, 1999; Wang et al., 1994 Koprunner et al., 2001). How germ plasm components eventually allow *nanos* RNAs to escape translational inhibition will be an interesting question for future experiments.

Acknowledgments

This work was supported by NIH grant R01HD37407. KS is currently supported by a Senior Research Fellowship from the Wellcome Trust, London, UK.

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