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Translational control of maternal *glp-1* mRNA by POS-1 and its interacting protein SPN-4 in *Caenorhabditis elegans*

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SUMMARY

The translation of maternal *glp-1* mRNAs is regulated temporally and spatially in *C. elegans* embryos. The 3′ UTR (untranslated region) of the maternal *glp-1* mRNA is important for both kinds of regulation. The spatial control region is required to suppress translation in the posterior blastomeres. The temporal one is required to suppress translation in oocytes and one-cell stage embryos. We show that a CCCH zinc-finger protein, POS-1, represses *glp-1* mRNA translation by binding to the spatial control region. We identified an RNP-type RNA-binding protein, SPN-4, as a POS-1-interacting protein. SPN-4 is present developmentally from the oocyte to the early embryo and its distribution overlaps with that of POS-1 in the cytoplasm and P granules of the posterior blastomeres. SPN-4 binds to a subregion of the temporal control region in the 3′ UTR and is required for the translation of *glp-1* mRNA in the anterior blastomeres. We propose that the balance between POS-1 and SPN-4 controls the translation of maternal *glp-1* mRNA.

Key words: *Caenorhabditis elegans*, Maternal mRNA, Translational control, RNA-binding protein, 3′ UTR

INTRODUCTION

The accumulation of maternal mRNAs in oocytes is widely observed in many species, and the temporal and spatial control of their translation in early embryos is crucial for development (Seydoux, 1996; Richter, 1999; Wickens et al., 2000). Extensive studies of various maternal mRNAs have shown that the 3′ UTR (untranslated region) is important for translational control. For example, some mRNAs have a cytoplasmic polyadenylation element (CPE) in their 3′ UTR that is involved in temporally changing their poly A tail length. In these cases, the elongation of the poly A chain is thought to cause translational activation. CPE-binding proteins (CPEB) bind the CPE and are thought to regulate polyadenylation and translation. Although there is an intense research focus on this type of translational control of the maternal mRNAs, the molecular mechanism that underlies it remains largely unknown.

The embryogenesis of *C. elegans* is highly stereotyped (Sulston et al., 1983), and many maternal genes play important roles in the process (Kemphues and Strome, 1997; Schnabel and Priess, 1997). A fertilized egg, P_0, produces a large anterior blastomere, AB, and a small posterior blastomere, P_1. *par* genes determine the anterior-posterior polarity of the one-cell stage embryos. The AB blastomere produces the ABa and ABp blastomeres, and the P_1 blastomere produces the EMS and P_2 blastomeres. The early ABa and ABp blastomeres are identical and exchangeable (Priess and Thomson, 1987); however, in the late four-cell stage, the ABp has a different cell fate determination from that of ABa. For this determination, a Notch-like receptor, GLP-1, and a Delta-like ligand, APX-1, are required (Mello et al., 1994).

GLP-1 translation is regulated temporally and spatially. In spite of the presence of abundant levels of the maternal *glp-1* mRNA in oocytes and all blastomeres up to the eight-cell stage of *C. elegans* embryos, GLP-1 is first detected in the anterior AB blastomere (Evans et al., 1994). At the four-cell stage, GLP-1 is detected only in the anterior blastomeres ABa and ABp, and not in the posterior EMS and P_2. A study using a reporter RNA revealed that the 3′ UTR of *glp-1* mRNA (369 bases) is important for the appropriate temporal and spatial translation of GLP-1 (Evans et al., 1994): a 125-base temporal control region (TCR) located at the 3′ end of the 3′ UTR is required for the suppression of *glp-1* translation in oocytes and one-cell stage embryos. A 66-base spatial control region (SCR) located in the middle of the UTR is required for the suppression of *glp-1* translation in the posterior blastomeres. Moreover, the *glp-1* SCR is highly conserved in related species (Rudel and Kimble, 2001). Taken together, these findings suggest that unknown regulators bind the TCR or SCR to control *glp-1* mRNA translation.

APX-1 also seems to be translationally regulated. The *apx-1* mRNA is provided maternally and is present in every early blastomere. APX-1 is first detectable in the posterior P_1 blastomere at the two-cell stage (Mickey et al., 1996). At the four-cell stage, APX-1 is expressed in the EMS and P_2

blastomeres, mainly at the membrane of P_2, but never in the anterior blastomeres, ABa and ABp. The molecular mechanism that underlies the regulation of *apx-1* mRNA translation is completely unknown.

A maternal gene product, POS-1, is involved in the translational regulation of APX-1, because APX-1 is not detected in *pos-1* embryos in spite of the abundant *apx-1* mRNA (Tabara et al., 1999). Mutations in the *pos-1* gene result in maternal-effect embryonic lethality with abnormal cell-fate determination and cell divisions. *pos-1* embryos have little pharyngeal tissue, no intestine, no germ cells and extra hypodermis. In addition, the P_2, P_3, and P_4 germline blastomeres have abnormally short cell-cycle periods with little asymmetry. The POS-1 protein has two TIS11-type CCCH zinc motifs and is observed in the cytoplasm of the posterior blastomeres, including the germline blastomeres, as a temporary component of the P granules (Tabara et al., 1999). However, the molecular function of POS-1 is largely unknown.

In this paper, we report that POS-1 binds the 3′ UTR of *glp-1* mRNA and negatively regulates the translation of the maternal *glp-1* mRNA in the posterior blastomeres. In addition, a newly identified POS-1-interacting protein, SPN-4, which has an RNP-type RNA-binding domain, also binds the 3′ UTR of *glp-1* mRNA, and is required for the translation of *glp-1* mRNA in the anterior blastomeres. We propose that POS-1 cooperates with SPN-4 to control the maternal *glp-1* mRNA translation in early embryos.

MATERIALS AND METHODS

Worms

Bristol strain N2 was used as the standard wild-type strain. Mutant strains used in this work were JJ462 [+/*nT1* IV; *pos-1(zu148) unc-42(e270)* /*nT1* V], FX291 [+/*eT1* III; *spn-4 (tm291)/eT1* V], WM63 [+/*nT1* IV; *pos-1(ne51)/nT1* V] and YC1 [+/*nT1* IV; *spn-4 (tm291) pos-1(zu148) unc-42(e270)* /*nT1* V]. *Tm291* was isolated using a UV-TMP mutagenesis (Gengyo-Ando and Mitani, 2000). The worms were handled as described by Brenner (Brenner, 1974).

Tri-hybrid analysis

A tri-hybrid analysis was performed essentially according to Putz et al. (Putz et al., 2000). pRevRX was used for the expression of the hybrid RNA. For the expression of the GAL4 activation domain (AD) fusion proteins, pACT2 (for AD-POS-1), pGADT7 [for AD-POS-1(C147Y)] and pACT (for AD-SPN-4) were used. POS-1(C147Y) has a mutation at codon 147 C (TGC) to Y (TAC) in the *pos-1* ORF. This mutation was originally found in *pos-1(ne51)*, and disrupts the second zinc finger (CCCH to YCCH) (Tabara et al., 1999). The mutation did not affect protein-protein interactions between POS-1 and POS-1 or SPN-4 in our two-hybrid analysis. Yeast strain CG-1945 was co-transformed with hybrid RNA and AD fusion protein expression plasmids. Hybrid proteins were expressed as GAL4 activation-domain fusion proteins. RevM10 was fused to the GAL4 DNA-binding domain as a fusion protein to trap the hybrid RNA. If the hybrid protein bound the hybrid RNA, GAL4-mediated transcription activation of the HIS3 reporter gene occurred, which was detected by the extent of the growth of the transformants on a selection medium containing appropriate concentrations of 3-amino 1,2,4-triazole (3-AT). To analyze their interactions, transformants were plated on a non-selection medium (SD-LW) and a selection medium (SD-LWH+3-AT) lacking histidine and including 3-aminotriazole (3-AT; 50 mM for POS-1, 10 mM for SPN-4) to select for a higher level of HIS3 activation.

Identification of POS-1-interacting proteins

Approximately 1,000,000 clones of a two-hybrid cDNA library that was kindly provided by R. Barstead were screened. Y190 was used as the host strain. For the expression of the full-length POS-1 bait, pAS2-1 (Clontech) was used. Library screening was performed as described by the manufacturer (Clontech Catalog Number K1604-1). The open reading frames of the *pip* genes were determined by sequencing the positive cDNA clones from the two-hybrid screen. The *pip-1* ORF is identical to the predicted gene ZC404.8 (The *C. elegans* Sequencing Consortium, 1998). The results of RT-PCR indicated the presence of an SL1 splice leader sequence in the transcripts. The cDNA sequence of *spn-4* has been deposited in the DDBJ under the Accession Number AB052819.

In vitro binding

Each PCR-amplified cDNA fragment was subcloned into the pGEX-KG, pGEX-5X-2 or pMAL-c2 vector. Fusion proteins were induced in *E. coli* with 1 mM IPTG at 37°C for 3 hours. GST fusion proteins were bound with Glutathione Sepharose 4B beads (Pharmacia Biotech) in cold 25 mM Tris HCl (pH 7.5), 150 mM NaCl, 5 mM DTT, 1 mM $MgCl_2$, 0.2% NP-40 (Simske et al., 1996). The beads were washed with the same cold buffer, and mixed with extracts of the *E. coli* that produced the MBP fusion proteins. The beads were washed several times with the same cold buffer. Each sample was fractionated by 8% SDS-PAGE and analyzed by western blotting using ECL (Pharmacia Biotech). We used an anti-MBP antiserum (New England Biolabs) for the primary antibody, and HRP-conjugated anti-rabbit IgG (Pharmacia Biotech) for the secondary antibody.

RNA-mediated interference

EST clones yk458c6 (GenBank Accession Number, C48581 for *spn-4*), yk61h1 (GenBank Accession Number, AB006208 for *pos-1*) and yk320h8 (GenBank Accession Number, C63438 for *glp-1*) were used as templates for double-stranded RNA synthesis. Using the PCR-amplified insert, both RNA strands were simultaneously synthesized with T3 and T7 RNA polymerase (Promega), and the RNA mixture was heat-denatured and annealed to form double-stranded RNA. Microinjection of the RNA was performed as described (Mello et al., 1991; Fire et al., 1998) at a concentration of 3~4 mg/ml in TE.

Antibodies and immunostaining

To raise anti-SPN-4 antibodies, the region corresponding to the C-terminal 111 amino acids of SPN-4 was amplified from the cDNA clone and subcloned into a His tag vector, pET15b. The fusion protein produced in *E. coli* was purified with Ni-NTA Agarose (Qiagen). Two rabbits were immunized with the fusion protein. Essentially the same staining pattern was observed with the two sera.

The anti-GLP-1 antibody was a gift from J. Kimble. The anti-POS-1 antibody was from our stock (Tabara et al., 1999). mabK76 (Strome and Wood, 1982) was from DSHB (Developmental Studies Hybridoma Bank, University of Iowa, USA).

For all the antibodies, fixation and staining were performed essentially as described by Zwaal et al. (Zwaal et al., 1996), using Cy3- or Cy5-conjugated secondary antibodies (Pharmacia Biotech). Specimens were observed on a confocal laser-scanning microscope (Zeiss, LSM510).

RESULTS

POS-1 negatively regulates the translation of maternal *glp-1* mRNA in posterior blastomeres

POS-1 has been suggested to be involved in the translational control of some maternal mRNAs, based on the observation that APX-1 (a Delta-like ligand) is not detected in *pos-1*

embryos (Tabara et al., 1999). GLP-1, a Notch-like receptor for APX-1, is also known to be regulated translationally, and the regulatory sequences in its 3′ UTR have been extensively studied (Evans et al., 1994). Therefore, we focused on the effect of the *pos-1* mutation on the expression and localization of GLP-1. GLP-1 is first detected in the anterior blastomere AB, and is localized at the membrane of its daughters, ABa and ABp, at the four-cell stage (Evans et al., 1994) (Fig. 1A,B).

We examined the localization of GLP-1 in *pos-1* embryos, and found that the embryonic expression of GLP-1 was abnormal. GLP-1 was ectopically expressed in the posterior blastomeres of *pos-1 (zu148*, a null allele). At the four-cell stage, for example, GLP-1 was detected in all blastomeres, including the posterior EMS and P_2 blastomeres (Fig. 1C) in which GLP-1 is normally not detectable (Fig. 1A). The same distribution was observed in a weak mutant of *pos-1(ne51)* (Fig. 1D) and in *pos-1(RNAi)* (RNAi; RNA-mediated interference) (Fire et al., 1998) (data not shown). GLP-1 was not observed in one-cell stage embryos (Fig. 1G) or oocytes (data not shown) of the *pos-1* mutant, suggesting that its temporal regulation was unaffected. These results suggest that the maternal *glp-1* mRNA translation is negatively regulated by POS-1 in the posterior half of early embryos.

POS-1 specifically interacts with the spatial control region (SCR) in the *glp-1* mRNA 3′ UTR

The effect of the *pos-1* mutation on GLP-1 expression led us to examine the interaction between POS-1 and the *glp-1* mRNA 3′ UTR. For this purpose, we used a yeast tri-hybrid system (Putz et al., 2000), which is a modification of the two-hybrid system. In this system, a target RNA is expressed as a hybrid RNA with the RRE (Rev responsive element). A target protein is fused to the activation domain of GAL4, and the DNA-binding domain is fused with HIV-1 RevM10, which binds RRE. If the target protein binds the target RNA, transcriptional activation occurs and is detected by the same procedure as in the two-hybrid system.

For the target RNA, we used a 294 bp region from the 3′ UTR (Fig. 2A). This region includes the SCR and part of the TCR. The very 3′-end region has several motifs for the processing of mRNA including polyA addition. These motifs could cause disturbance in the assay through some complex formation; therefore, we removed the very 3′-part of TCR. The tri-hybrid analysis showed that POS-1 specifically interacted with the 294-base RNA region, as shown in rows 1 and 5 in the 'WT, S column' of Fig. 2C. To identify the interacting region within the 294-base RNA region, we subdivided the 294-base region into three parts, 5′ to 3′: the 138-base proximal region (non-SCR and non-TCR region), the 66-base SCR and the 78-base subregion of the TCR (Fig. 2A). As shown in the 'POS-1 WT' column of Fig. 2C, we found that POS-1 preferentially interacted with the 66-base SCR. We found that POS-1 also interacted with the 78-base RNA region within the TCR, but much more weakly than with the SCR. The SCR is essential for the suppression of *glp-1* translation in the posterior blastomeres (Evans et al., 1994); thus, the physical interaction between POS-1 and SCR is consistent with the idea that POS-1 negatively regulates its translation.

We next examined which part of POS-1 is important for the interaction. For this purpose, we used the *pos-1(ne-51)* mutant, which is a missense mutation in the second zinc finger of POS-1 (2nd CCCH to YCCH) (Tabara et al., 1999) (Fig. 2B). We made this choice because the *pos-1(zu148)* mutant, which is a strong allele, and therefore the most widely used, is a nonsense mutation in the first zinc finger. The results of the tri-hybrid analysis were striking: the mutation disrupted the interactions completely, as shown in the 'POS-1 ne51' column of Fig. 2C. We confirmed that the mutant POS-1 protein was expressed properly in the yeast strain (Fig. 2D). Although *pos-1(ne51)* is a weak allele, we confirmed the ectopic expression of GLP-1 in *pos-1(ne51)* embryos (Fig. 1D). These results indicate that POS-1 directly binds to the SCR, and that the second CCCH zinc finger is important for the binding and for suppressing the translation of the *glp-1* mRNA.

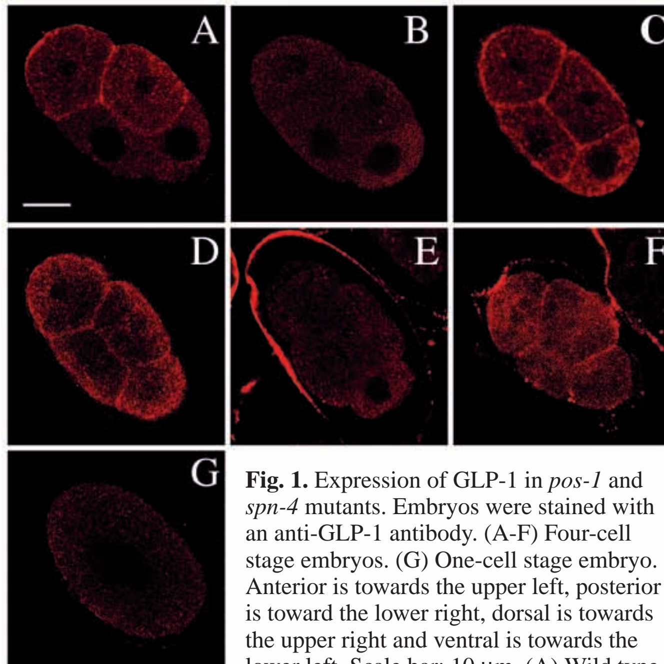

Fig. 1. Expression of GLP-1 in *pos-1* and *spn-4* mutants. Embryos were stained with an anti-GLP-1 antibody. (A-F) Four-cell stage embryos. (G) One-cell stage embryo. Anterior is towards the upper left, posterior is toward the lower right, dorsal is towards the upper right and ventral is towards the lower left. Scale bar: 10 µm. (A) Wild type. GLP-1 was detected only in the ABa and ABp blastomeres, mainly at the membrane. (B) *glp-1(RNAi)* embryo. In this embryo, there was no expression of GLP-1. Thus, the staining shown here gives the level of the background signals. (C) *pos-1(zu148)* and (D) *pos-1(ne51)* embryos. GLP-1 was detected at the membrane in all four blastomeres. (E) *spn-4(tm291).* GLP-1 was not detectable. (F) *pos-1(zu148); spn-4(tm291)* double mutant embryo. GLP-1 was detected in all four blastomeres. (G) One-cell stage *pos-1(zu148)* embryo. GLP-1 is not detectable.

A POS-1 interacting protein, PIP-1, is a RNP-type RNA-binding protein and identical with SPN-4

To analyze the POS-1 function in more detail, we identified POS-1-interacting proteins from a *C. elegans* yeast two-hybrid cDNA library using the full-length POS-1 as bait. As positive cDNA clones, we identified POS-1, MEX-3 (Draper et al., 1996), F38H4.9, which encodes a catalytic subunit of protein phosphatase 2A (PP2A) and ZC404.8, which encodes an RNP-type RNA-binding protein. We confirmed that these proteins bound directly to POS-1, using an in vitro GST pull-down assay (Fig. 3A). The fact that POS-1 was recognized in our two-hybrid screen suggested that POS-1 forms a homodimer or a homomultimer. MEX-3 is a KH-type RNA-binding

protein, and is required for the control of maternal *pal-1* mRNA translation (Draper et al., 1996; Hunter and Kenyon, 1996); therefore, POS-1 may also be required for the control of the *pal-1* mRNA. We found that the catalytic subunit of PP2A, F38H4.9, corresponds to the *let-92* gene, and that *let-92* is required for embryonic cell divisions rather than for translational regulation (K.-i.O. and Y. K., unpublished). Here, we focused on a novel protein, ZC404.8, and named it PIP-1, for POS-1 interacting protein. This gene was independently identified and named *spn-4* by Gomes et al. (Gomes et al., 2001) and Huang et al. (Huang et al., 2002). Therefore, we call it *spn-4* in this paper. Diagrams of the protein and gene are shown in Fig. 3B,C.

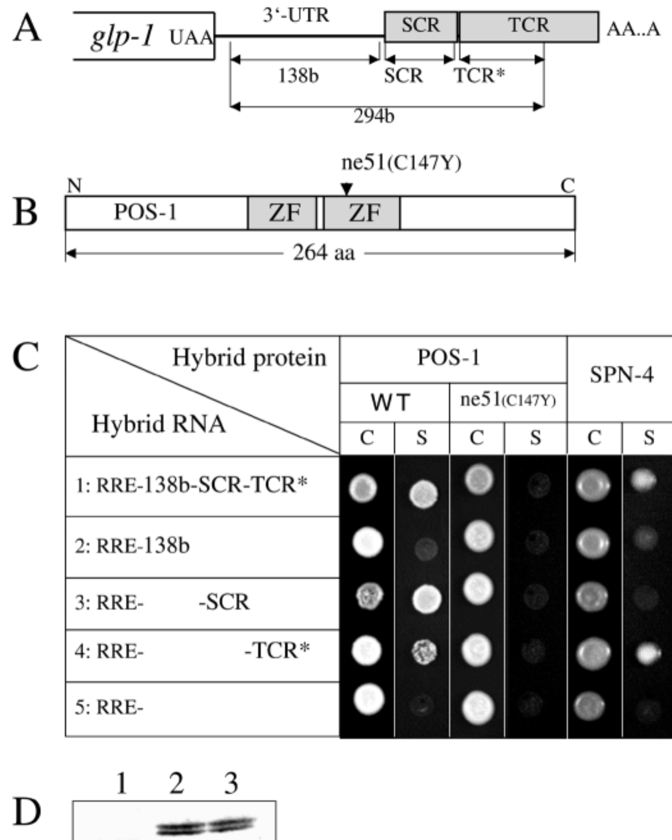

Fig. 2. Interaction of POS-1 and SPN-4 with *glp-1* 3′ UTR. (A) The *glp-1* mRNA 3′ UTR. The entire 3′ UTR is 369 bases long from the stop codon UAA to the poly A addition site. The SCR was originally defined as lying between bases 180 and 240, and the TCR between bases 245 and 369 (Evans et al., 1994). The comparison of the 3′ UTRs of closely related species (Rudel and Kimble, 2001) suggested some minor modifications on the regions as follows: modified SCR was between bases 172 and 237, and modified TCR was from base 242. Thus, the regions are as follows: the 294-base (bases 26 to 319), the 138-base (bases 26 to 163, SCR (172 to 237) and the TCR*(bases 242 to 319). TCR* indicates a subregion of TCR. (B) Outline of the structure of POS-1. Two CCCH zinc fingers are located in the middle of the 264 amino acid POS-1 protein. The allele *ne51* is a missense mutation at codon 147, C(TGC) to Y(TAC), in the second zinc finger. (C) Yeast tri-hybrid analysis. Based on the reporter RNA study (Evans et al., 1994) and the comparative study (Rudel and Kimble, 2001), the following regions were taken from the 369-base *glp-1* mRNA 3′ UTR for the construction of hybrid RNA with RRE, a Rev responsive element recognized by HIV-1 RevM10 (Putz et al., 2000): row 1, bases 26-319 for 138-base SCR-TCR* (the entire region); row 2, bases 26-163 for 138-base (non SCR, non TCR region); row 3, bases 172-237 for the SCR; and row 4, bases 242-319 for the TCR* (TCR subregion). Row 5 is a negative control RNA. C indicates growth on a non-selection medium without 3AT; S indicates growth on a selection medium containing 50 mM (for POS-1) or 10 mM (for SPN-4) 3-AT. (D) Western blot analysis of the tri-hybrid strains by using an anti-POS-1 antibody: (1) GAL4 AD, (2) GAL4 AD::POS-1 and (3) GAL4 AD::POS-1(C147Y) were expressed in yeast. In all the yeast strains, RRE-138-base SCR-TCR* was co-expressed. POS-1(C147Y) was expressed at the same level as POS-1.

Spn-4 positively regulates the translation of maternal *glp-1* mRNA in anterior blastomeres

RNP-type RNA-binding proteins, which bind RNA with a wide range of affinities and specificities, are widely present across the phylogenetic scale, from bacteria to animals (Burd and Dreyfuss, 1994). Because SPN-4 has an RNP-type RNA-

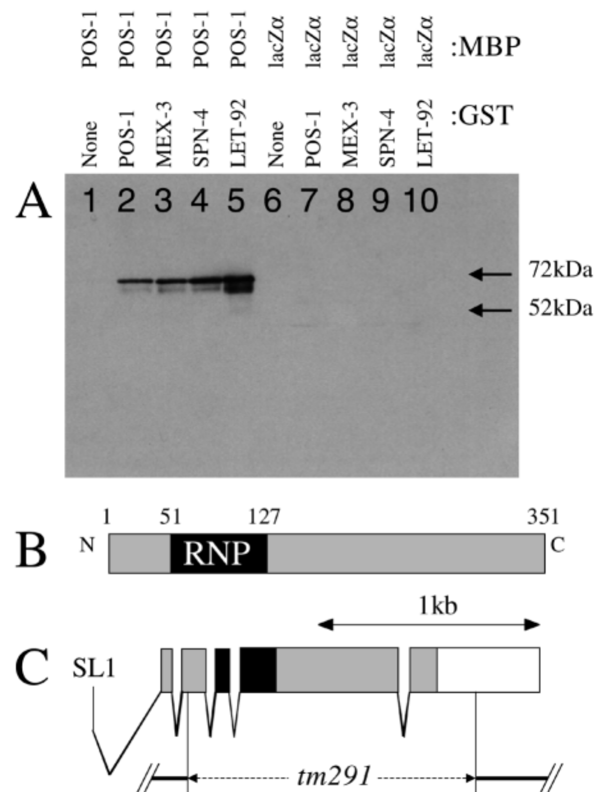

Fig. 3. Identification of POS-1-interacting proteins. (A) In vitro binding of recombinant proteins. (1) GST and MBP::POS-1, (2) GST::POS-1 and MBP::POS-1, (3) GST::MEX-3 and MBP::POS-1, (4) GST::SPN-4 and MBP::POS-1, (5) GST::LET-92 and MBP::POS-1, (6) GST and MBP::lacZα, (7) GST::POS-1 and MBP::lacZα, (8) GST::MEX-3 and MBP::lacZα, (9) GST::SPN-4 and MBP::lacZα, (10) GST::LET-92 and MBP::lacZα. Positive signals were detected only in lanes 2~5. (B) The SPN-4 protein. The RNP-type RNA-binding domain (RNP) is shown in black. The cDNA sequence of *spn-4* has been deposited with the DDBJ (DNA Data Bank of Japan) under the Accession Number AB052819.
(C) Genomic structure of the *spn-4* gene. Boxes represent exons. The ORF is shown in gray with the RNP region in black. The trans-splice leader SL1 was detected just one base upstream of the first ATG. The genomic region that was deleted in the *tm291* mutant is shown.

binding domain and binds to POS-1, it seemed likely that SPN-4 also regulates the translation of maternal *glp-1* mRNA. To analyze the functions of *spn-4* function, we isolated a deletion mutation, *tm291*, that produced a null allele (Fig. 3C). We found that the *tm291* mutant showed strict maternal-effect embryonic lethality, indicating that *spn-4* is essential only for embryogenesis. We found that the phenotypes of *tm291* deletion mutation and its RNAi were identical.

We examined the expression of GLP-1 in *spn-4* early embryos. The result was the inverse of the expression in *pos-1* mutants. There was no GLP-1 expression in any of the blastomeres in the *spn-4* mutant (Fig. 1E) and *spn-4(RNAi)* (data not shown). The GLP-1 expression in the distal region of the adult gonads (Crittenden et al., 1994) was not affected in the mutants (data not shown). These results strongly suggest that SPN-4 positively regulates the translation of maternal *glp-1* mRNA in the anterior half of early embryos.

To analyze the relationship between *pos-1* and *spn-4*, we tested the expression of GLP-1 in double mutant embryos for these genes. The pattern of GLP-1 expression in the double mutants was essentially the same as in *pos-1* embryos (Fig. 1F). The same results were obtained with the double RNAi embryos (data not shown). These results indicate that *pos-1* is genetically epistatic to *spn-4* with respect to GLP-1 expression.

SPN-4 specifically interacts with a subregion of the TCR in the *glp-1* mRNA 3′ UTR

We analyzed whether there was an interaction between SPN-4 and the *glp-1* mRNA 3′ UTR using the yeast tri-hybrid system. We found that SPN-4 specifically interacted with the same 294-base RNA within the *glp-1* 3′UTR as did POS-1 (rows 1 and 5, column 'SPN-4, S' in Fig. 2C). We found that SPN-4 interacted with the 78-base RNA region within the TCR but not with the 66-base SCR, as shown in the 'SPN-4' column of Fig. 2C. These results suggest that SPN-4 directly binds the 78-base region within the 125-base TCR, and activates the translation. The 125-base TCR was originally identified as a temporal control region for translational repression; however, deletion of the TCR disrupts the spatial regulation of translation as well as the temporal regulation (Evans et al., 1994). Therefore, we think that the 125-base TCR contains both a temporal inhibition region and a spatial activation region (probably within the 78-base region).

Localization of mRNA and protein of *spn-4*

In situ hybridization analysis revealed that *spn-4* mRNA was abundant in early embryos (Fig. 4). The mRNA was present at the same level in all blastomeres up to the 4-cell stage (Fig. 4A-C). Afterwards, it persists in the P blastomere and its sister, and then just the germ lineage (Fig. 4D-H). The mRNA was also present in the adult gonads (data not shown).

Immunostaining revealed that the SPN-4 protein was already present in oocytes (Fig. 5A). The protein was abundant in one-cell-stage embryos (Fig. 5B). At the two-cell stage, it was present with equal abundance in both blastomeres AB and P_1 (Fig. 5E). At the four-cell stage, the protein distribution became different among the blastomeres; strongly in the posterior blastomeres, EMS and P_2, and weakly in the anterior blastomeres, ABa and ABp (Fig. 5H). This could be due to stronger expression in the posterior blastomeres P2 and EMS or different stability of the protein in the anterior blastomeres compared to the posterior ones. After the four-cell stage, SPN-4 was localized to the posterior blastomeres, including the germline precursors, and became undetectable by mid-embryogenesis (data not shown). The specificity of our antibody was confirmed by the fact that SPN-4 was detected neither in the deletion mutant *tm291* nor in *spn-4(RNAi)* embryos (Fig. 5). The distribution of SPN-4 overlapped with that of POS-1 in the posterior blastomeres (Fig. 5D,G,J, yellow regions). We found a granular staining pattern for SPN-4 in the germline blastomeres (Fig. 5B,E,H). This pattern exactly matched the granular staining pattern for POS-1 (Fig. 5D,G,J), suggesting that these structures corresponded to P granules. Indeed, the granular patterns matched exactly those observed with a P-granule-specific antibody (Fig. 6A-C), indicating that SPN-4 is a component of P granules. We examined the distribution of POS-1 in *spn-4* embryos and SPN-4 in *pos-1* embryos, and found that the protein distributions were the same as in wild type (data not shown).

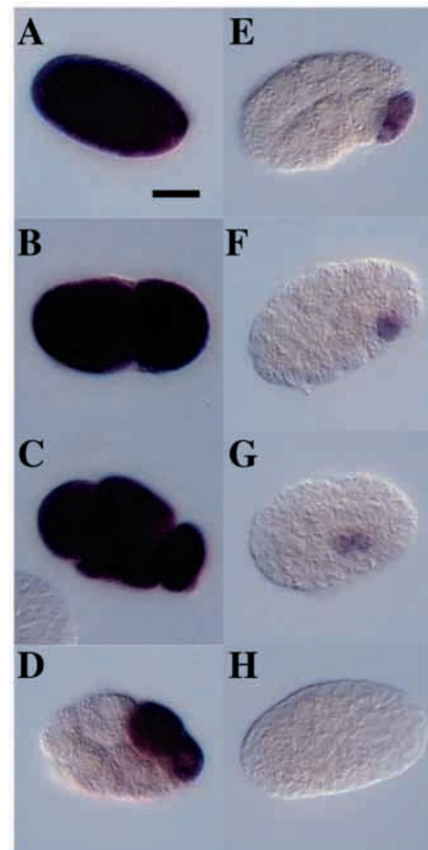

Fig. 4. Localization of *spn-4* mRNA. Wild-type embryos were analyzed by in situ hybridization with a *spn-4* cDNA probe. Anterior is leftwards, posterior is rightwards, dorsal is upwards and ventral is downwards. Scale bar: 10 μm. (A) One-cell embryo. (B) Two-cell embryo. (C) Four-cell embryo. (D) Eight-cell embryo. P3 and C are stained. (E) An embryo just before gastrulation. P4 and D are stained. (F) An embryo beginning gastrulation. Only P4 is stained. (G) An embryo during gastrulation. P4 has divided to produce Z2 and Z3, which are stained. (H) A mid-stage embryo. No staining is observed. The *spn-4* mRNA was also detected in adult gonads and in oocytes (data not shown).

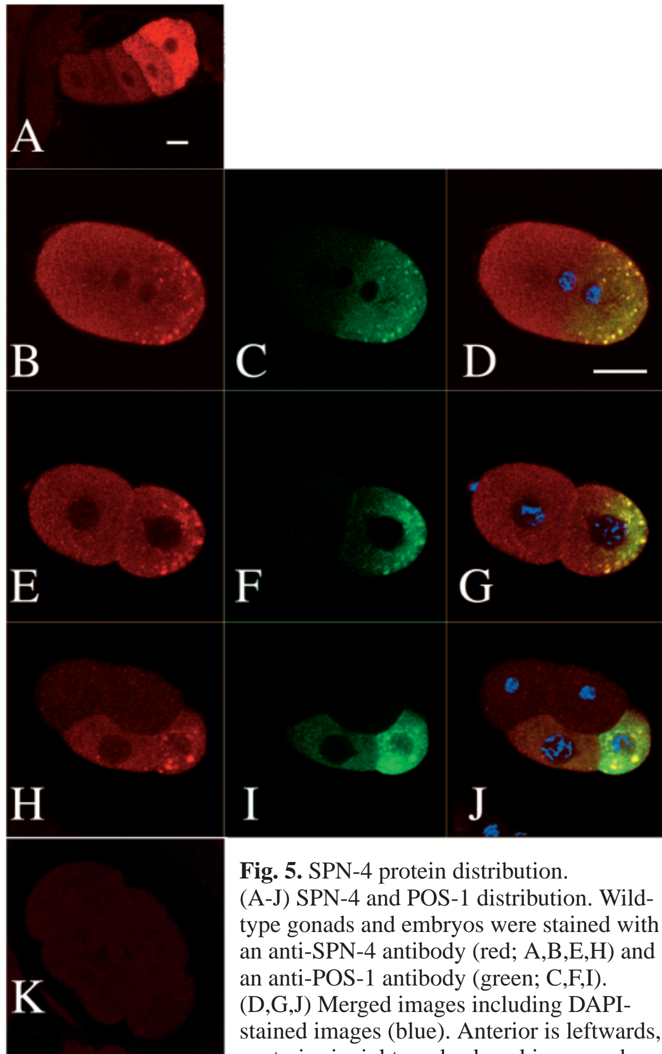

Fig. 5. SPN-4 protein distribution. (A-J) SPN-4 and POS-1 distribution. Wild-type gonads and embryos were stained with an anti-SPN-4 antibody (red; A,B,E,H) and an anti-POS-1 antibody (green; C,F,I). (D,G,J) Merged images including DAPI-stained images (blue). Anterior is leftwards, posterior is rightwards, dorsal is upwards and ventral is downwards. Scale bars: 10 μm. (A) Oocytes. SPN-4 was detected in oocytes from adult animals. (B-D) One-cell embryo just before the first cleavage. (E-G) Two-cell stage embryo. (H-J) Four-cell stage embryo. SPN-4 was uniformly present in the cytoplasm up to the two-cell stage, then began to localize to the posterior blastomeres P_2 and EMS. Co-localization of SPN-4 and POS-1 was mainly observed in the cytoplasm of the posterior blastomeres, including the germline (D,G,J; yellow regions) and P granules (yellow dots, see Fig. 6). (K) SPN-4 in *spn-4* embryo at four-cell stage.

spn-4 is required for cell differentiation in embryogenesis

The *tm291* deletion mutants lack pharyngeal tissue and contain P granules in extra cells, suggesting the production of extra germ cells and about half of the mutant embryos lacked an intestine and intestinal valve cells (data not shown). Furthermore, we found that some of the deletion mutant embryos failed to complete cytokinesis at the one-cell stage, resulting in the generation of aneuploid cells, and had an abnormal spindle orientation at least at the two-cell stage (data not shown). These phenotypes are the same as previously described for missense and deletion mutants of *spn-4* and after RNAi depletion of SPN-4 (Gomes et al., 2001). These findings suggest that SPN-4 plays a role in various processes during development.

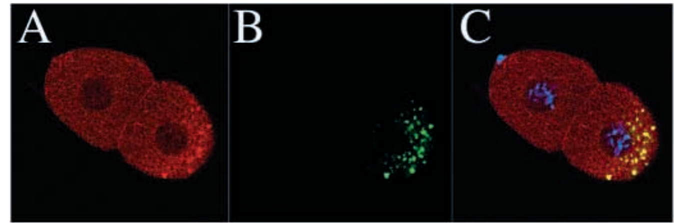

Fig. 6. Co-localization of SPN-4 and the P granules. Wild-type embryos were stained with an anti-SPN-4 antibody (red, A) and mabK76, which recognizes P granules (green, B). (C) is a merged image and includes a DAPI-stained image (blue). The granular staining of the anti-SPN-4 antibody matches the mabK76 staining pattern of the P granules (C, yellow dots).

DISCUSSION

The translation of maternal *glp-1* mRNA is temporally and spatially controlled in *C. elegans* embryos (Evans et al., 1994). Its 3′ UTR is involved in this regulation, and two subregions, named SCR (spatial control region) and TCR (temporal control region), are essential for it. We show that a maternal gene product, POS-1, worked negatively in this regulation through binding to the SCR and a protein that interacts with POS-1, SPN-4, bound to the TCR and worked positively in the regulation.

Negative regulation by POS-1 through its zinc fingers

We showed that GLP-1 was ectopically translated in the posterior blastomeres of *pos-1* embryos, although its temporal regulation did not seem to be affected. Using the yeast tri-hybrid system, we also found that POS-1 specifically interacted with the *glp-1* mRNA SCR, and that a missense mutation in the second CCCH zinc finger of POS-1 (2nd CCCH to YCCH) disrupted this interaction. These results indicate that POS-1 directly binds the *glp-1* SCR and suppresses the translation of maternal *glp-1* mRNA in the posterior blastomeres.

Our preliminary studies using the tri-hybrid system showed that POS-1 interacted with various maternal mRNAs and that a mutant version of POS-1 (2nd CCCH to YCCH) disrupted this interaction (N.K. and Y.K., unpublished). Thus, the zinc fingers of POS-1 are likely to play an important role in the translational regulation of other mRNAs, probably by binding to their 3′ UTRs. The importance of zinc fingers in translational regulation has been suggested in a study of another protein. Tenenhaus et al. (Tenenhaus et al., 2001) reported that a maternal gene product, PIE-1, which has a similar zinc-finger structure to POS-1 is required for the normal expression of NOS-2 from maternally provided mRNA. In this case as well, the second CCCH finger of PIE-1 was important for the normal expression of NOS-2.

Positive regulation by the POS-1-interacting protein, SPN-4

We showed that a POS-1-interacting protein, SPN-4, has an

RNP-type RNA-binding motif. Proteins containing the RNP-type RNA-binding motif are widely present in organisms from bacteria to animals, and bind RNA with a wide range of affinities and specificities (Burd and Dreyfuss, 1994). A protein bearing an RNP-type motif that is highly homologous to SPN-4 is *C. elegans* FOX-1 (54.5%/77 amino acids) (Hodgkin et al., 1994). FOX-1 is a nuclear protein that post-transcriptionally reduces the level of XOL-1, which acts as a sex determination switch (Nicoll et al., 1997). FOX-1 binds poly(A), poly(G) and poly(U) RNA in vitro (Skipper et al., 1999). By analogy, SPN-4 probably binds directly to RNA through its RNP motif.

Analysis of the deletion mutant, *tm291*, showed that the *spn-4* mutation caused strict maternal-effect embryonic lethality, indicating that *spn-4* is essential only for embryogenesis. In contrast to *pos-1* mutants, GLP-1 was never expressed in the *spn-4* mutant. The mutant embryos had almost no pharynx and about half had no intestinal valve cells. GLP-1 is required for the differentiation of both the ABa and ABp blastomeres. Half of the pharynx is derived from ABa, and the intestinal valve cells are derived from ABp; therefore, these phenotypes are consistent with the lack of GLP-1 expression in these embryos. We showed that SPN-4 preferentially interacted with the 78-base RNA region within the 125-base TCR of the *glp-1* mRNA 3′ UTR using the yeast tri-hybrid system. These results suggest that SPN-4 positively regulates the translation of the maternal *glp-1* mRNA in the anterior blastomeres by directly binding to the 78-base RNA region.

The 125-base TCR was originally identified as a temporal control region for translational repression (Evans et al., 1994); however, the deletion of the TCR results in the temporal misexpression of the reporter in oocytes and all cells of early embryos (Evans et al., 1994). Thus, the 125-base TCR could contain a spatial control region as well as the temporal control region. We think that the 78-base region contains a spatial control region for the expression in the anterior blastomeres that is regulated by binding with SPN-4. We also showed that POS-1 interacts with the TCR, although much more weakly than with the SCR. POS-1 may compete with SPN-4 for the same binding region, or the 78-base region may have another site for POS-1 binding.

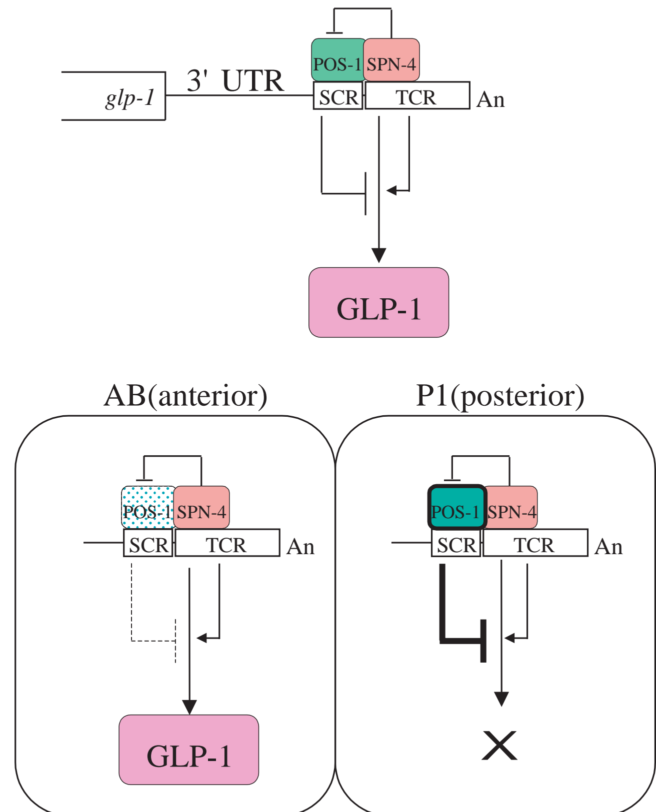

Fig. 7. A model for the roles of POS-1 and SPN-4 in the *glp-1* mRNA translation. (Top) SCR: the 66-base spatial control region within the *glp-1* mRNA 3′ UTR. TCR: the 125-base temporal control region within the *glp-1* mRNA 3′ UTR. In this model, POS-1 binds the SCR with its second CCCH finger. SPN-4 binds the TCR. POS-1 suppresses and SPN-4 activates the translation. SPN-4 may also inhibit the POS-1 function by direct binding. (Bottom) POS-1 is abundant in the posterior blastomere (right) but present at a much lower level in the anterior one (left), while SPN-4 is abundant in both. We propose that the translation of maternal *glp-1* mRNA is suppressed in the posterior half of the embryo by the POS-1 repressor activity, while it is turned on in the anterior half, where the concentration of SPN-4 and its activating function predominates.

A model for the translational regulation of maternal *glp-1* mRNA by POS-1 and SPN-4

The expression patterns of GLP-1 in *spn-4;pos-1* double mutant embryos were essentially the same as in *pos-1* embryos, indicating that *pos-1* is genetically epistatic to *spn-4* with respect to GLP-1 expression. An attractive explanation of the epistasis results would be that SPN-4 participates in restricting POS-1 to the posterior, however, this is not the case because POS-1 was distributed apparently normally in *spn-4* mutant embryos (and vice versa). Based on these experimental observations, we made a working model for the translational regulation of the maternal *glp-1* mRNA by POS-1 and SPN-4, as shown in Fig. 7.

POS-1 is abundant in the posterior blastomeres but present at much lower levels in the anterior ones (Fig. 5F), whereas SPN-4 is abundant in both (Fig. 5E). In our model, POS-1 binds to the SCR of *glp-1* mRNA through its second CCCH finger. We propose that the translation of maternal *glp-1* mRNA is suppressed in the posterior half of the embryo by the POS-1 repressor activity (Fig. 7, in the P_1 blastomere) but turned on in the anterior half, where the concentration of SPN-4 and its activating function predominates over the POS-1 activity (Fig. 7, in the AB blastomere).

In a *pos-1* mutant, the suppression of translation in the posterior embryo is released, leading to the ectopic translation of *glp-1* mRNA in the posterior embryo. In a *spn-4* mutant, the activation in the anterior end does not occur and low levels of POS-1 that may be present in the anterior cells can inhibit *glp-1* mRNA translation; thus, there is no translation of *glp-1* mRNA in the anterior embryo. In a *pos-1; spn-4* double mutant, neither suppression nor activation occurs, and the maternal *glp-1* mRNA is translated in an unregulated fashion, resulting in its expression in all blastomeres.

GLP-1 is not translated in oocytes or one-cell embryos, which have abundant SPN-4 but no POS-1. During this period, some factor(s) are expected to bind to the TCR to repress the translation. Thus, the above model explains the translational regulation after the release of the temporal control.

If this model is correct, the ectopic expression of POS-1 in anterior cells should suppress GLP-1 translation. Schubert et al. (Schubert et al., 2000) reported that GLP-1 was not detected in *mex-5;mex-6* embryos at the four-cell stage. In this mutant, POS-1 is mislocalized and present in all blastomeres. This finding supports our model, because ectopic POS-1 in the anterior blastomeres would be expected to suppress translation. However, Crittenden et al. (Crittenden et al., 1997) reported that in *par-1* embryos, GLP-1 was detected in all blastomeres, in a manner similar to *pos-1* embryos. We examined POS-1 expression in *par-1(RNAi)* embryos, and found that POS-1 was abundant in all blastomeres (data not shown). This does not appear to be consistent with our model, however, as the *par-1* mutation disrupts the anteroposterior polarity at the very beginning of embryogenesis, it might well affect the transition from temporal control to spatial control, leading to some unregulated status. Alternatively, if a post-translational modification, for example, phosphorylation, is necessary for POS-1 to be active, the *par-1* mutation might affect this process.

How does POS-1 suppress the translation of *glp-1* mRNA in posterior blastomeres? It is generally thought that the elongation of the poly A tail of mRNA leads to its active translation (Seydoux, 1996; Richter, 1999; Wickens et al., 2000). Interestingly, we have detected poly A tails of two different sizes in *glp-1* mRNAs after the two-cell stage, and found that the longer poly A tail is present only in the anterior cell AB where *glp-1* is translated, but not in the posterior P_1, where it is not (S. Onami and Y.K., unpublished). POS-1 may shorten the poly A tail of the *glp-1* mRNA in the posterior blastomeres. There are interesting parallels between POS-1 and mammalian TTP. Like POS-1, TTP has two CCCH-type zinc fingers. TTP can bind to an AU-rich element (ARE) in the 3′ UTR of the TNFα mRNA to promote deadenylation and destabilization of the mRNA (Lai et al., 1999). The TTP zinc CCCH finger is important for this binding. As to SPN-4, although there is no link between SPN-4 and poly A length regulation, an attractive hypothesis is that the poly A tail length of the maternal *glp-1* mRNA is determined by the balance between POS-1 and SPN-4 activity.

Translation of other maternal mRNAs are also regulated by POS-1 and SPN-4

In the case of *apx-1*, the *pos-1* mutation causes the disappearance of its expression; thus, POS-1 regulates it positively (Tabara et al., 1999). This is the inverse of the *glp-1* case. Interestingly, our preliminary experiments showed that SPN-4 negatively regulated the temporal and spatial translation of maternal *apx-1* mRNA (K.-i.O. and Y.K., unpublished). This is also the inverse of the *glp-1* case. Recently, Gomes et al. (Gomes et al., 2001) reported that SPN-4 negatively regulates the translation of maternal *skn-1* mRNA in the anterior blastomeres. Huang et al. (Huang et al., 2002) have reported that SPN-4 negatively regulates the translation of maternal *pal-1* mRNA in anterior blastomeres. In these cases, SPN-4 negatively controls the translation of the mRNAs. Our preliminary studies using the yeast tri-hybrid analysis showed that SPN-4 specifically interacts with the 3′ UTRs of *apx-1*, *skn-1* and *pal-1* mRNA (N.K. and Y.K., unpublished); therefore, we think that SPN-4 directly controls the translation of these maternal mRNAs. In the SCR and TCR of *glp-1* mRNA, two sets of NRE (Nanos Response Element)- like motifs (Murata and Wharton, 1995) are observed, and in the TCR, a TGE (TRA-2 and GLI Element)-like motif (Jan et al., 1997) is observed. Interestingly, the TGE-like motif is present in the 3′ UTRs of the SPN-4 regulating genes, *pal-1* and *skn-1*, as well as *apx-1*. The NRE-like motif is found in *apx-1* and *pal-1* 3′ UTRs, but not in *skn-1*. Work is in progress to examine whether these motifs are functionally important for the translational control. POS-1 and SPN-4 may change their function depending on the target mRNAs, and in combination with other factors.

POS-1 and SPN-4 have other functions in regulating embryogenesis

The phenotypes of the *pos-1* and *spn-4* mutations are rather pleiotropic, suggesting that the roles of POS-1 and SPN-4 are not restricted to regulating the translation of the maternal *glp-1* mRNA.

pos-1 embryos have little pharyngeal tissue, lack an intestine and germ cells, and the P_2, P_3, and P_4 blastomeres have abnormally short cell-cycle periods, with little asymmetry (Tabara et al., 1999). Except for the anterior pharynx, which is derived from the anterior ABa blastomere, these affected tissues and cells are all derived from the posterior blastomeres P_2 or EMS (Sulston et al., 1983), which express GLP-1 ectopically in the *pos-1* mutants. However, the ectopic expression of GLP-1 probably does not cause the phenotype, because its ligand, APX-1, is not expressed in *pos-1* embryos. Indeed, *pos-1(RNAi);glp-1(RNAi)* double RNAi embryos still lack intestine (data not shown).

On the other hand, we and Gomes et al. (Gomes et al., 2001) observed that the terminal *spn-4* embryos have almost no pharynx, extra germ cells and (for half of the embryos) no intestine. Except for the anterior pharynx, these affected tissues and cells are derived from the posterior blastomeres P_2 or EMS. Although GLP-1 is not detectable in *spn-4* mutants, its absence is unlikely to be responsible for these effects, because GLP-1 is not required for the differentiation of the posterior blastomeres (Priess et al., 1987). These results suggest that POS-1 and SPN-4 may cooperate to regulate the translation of other maternal mRNAs that are required for the differentiation of the posterior blastomeres. This regulation might occur at the P granules, where both POS-1 and SPN-4 are present.

SPN-4 but not POS-1 was present in oocytes. Many maternal mRNAs accumulate in oocytes, and the translation of some of them is temporally and spatially regulated (Kemphues and Strome, 1997; Schnabel and Priess, 1997). *spn-4* is also required for normal cytokinesis and spindle orientation in early embryos (Gomes et al., 2001) (this study). Thus, SPN-4 could regulate the translation of various maternal mRNAs in oocytes and very early embryos independent of POS-1, either by itself or in association with another factor. We also show that SPN-4 is a temporal component of the P granules. P granules are important for germline development (Kawasaki et al., 1998; Amiri et al., 2001); therefore, the extra germ cells observed in *spn-4* embryos may result from the loss of the SPN-4 function in the P granules. For example, SPN-4 may repress the activity of a determinant for germ cells in the P granules.

POS-1 and SPN-4 seem to have many functions and many targets. More factors are expected to work in translational regulation. These factors should constitute a network; therefore, the identification and characterization of other

regulators and target mRNAs is needed to understand the nature of the translational control of maternal mRNA.

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