In *Caenorhabditis elegans*, the RNA-Binding Domains of the Cytoplasmic Polyadenylation Element Binding Protein FOG-1 Are Needed to Regulate Germ Cell Fates

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ABSTRACT

FOG-1 controls germ cell fates in the nematode *Caenorhabditis elegans*. Sequence analyses revealed that FOG-1 is a cytoplasmic polyadenylation element binding (CPEB) protein; similar proteins from other species have been shown to bind messenger RNAs and regulate their translation. Our analyses of *fog-1* mutations indicate that each of the three RNA-binding domains of FOG-1 is essential for activity. In addition, biochemical tests show that FOG-1 is capable of binding RNA sequences in the 3′-untranslated region of its own message. Finally, genetic assays reveal that *fog-1* functions zygotically, that the small *fog-1* transcript has no detectable function, and that missense mutations in *fog-1* cause a dominant negative phenotype. This last observation suggests that FOG-1 acts in a complex, or as a multimer, to regulate translation. On the basis of these data, we propose that FOG-1 binds RNA to regulate germ cell fates and that it does so by controlling the translation of its targets. One of these targets might be the *fog-1* transcript itself.

STUDIES of gene regulation during development often focus on transcriptional controls. However, it has long been clear that many processes are controlled by a complicated mix of transcriptional and post-transcriptional regulators. The germ line has provided fertile ground for exploring how such regulatory interactions work. In particular, the syncytial structure of the germ line, its unique role in reproduction, and the large number of target genes that must be regulated place demanding constraints on the regulatory molecules that control germ cell fates.

Several factors make the nematode *Caenorhabditis elegans* a leading model for studying these regulatory networks (reviewed by Schedl 1997; Ellis 1998). First, mutations that prevent spermatogenesis in *C. elegans* transform *XX* animals from self-fertile hermaphrodites into females, which are easy to identify and maintain. Second, most mutations that alter germ cell fates in *C. elegans* do not cause cell death, so genetic analysis of germ cell fates is simpler than in many animals. Third, these nematodes are transparent, so germ cells can be observed inside living worms.

Two decades of genetic and molecular analyses of *C. elegans* have revealed that post-transcriptional regulation plays a central role in the control of germ cell development and differentiation. For example, the *tra-2A* message is translationally repressed by GLD-1 in hermaphrodites to allow them to produce sperm during larval development (Goodwin *et al.* 1993; Jan *et al.* 1999). In addition, the *fem-3* message is repressed by the FBF proteins and NOS-3 to prevent spermatogenesis from continuing into adulthood (Ahringer *et al.* 1992; Zhang *et al.* 1997; Kraemer *et al.* 1999). Additional regulatory interactions might be required in hermaphrodites to repress other maternal messages whose products would affect the maturing germ cells (Jones and Schedl 1995). Some translational regulators appear to function in the male germ line as well. The best candidates are FOG-1, which directly controls germ cell fates (Luitjens *et al.* 2000; Jin *et al.* 2001), and CPB-1 and the FBF proteins, which regulate early spermatogenesis (Luitjens *et al.* 2000). Both FOG-1 and CPB-1 are cytoplasmic polyadenylation element binding (CPEB) proteins. This family of proteins is ancient, since other members have been identified in vertebrates (Hake and Richter 1994; Gebauer and Richter 1996; Bally-Cuif *et al.* 1998), insects (Lantz *et al.* 1992; Chang *et al.* 1999), and mollusks (Walker *et al.* 1999). The CPEB proteins have several features that make them exciting subjects for investigating how RNA-binding proteins regulate the translation of messenger RNAs.

First, all CPEB proteins share two structural features: double RNA recognition motifs (RRMs) and a C-H domain rich in cysteines and histidines (Hake and Richter 1994). Biochemical studies in Xenopus have shown that the RRM domains and the C-H domain are both needed for CPEB proteins to bind target messenger RNAs (Hake *et al.* 1998). RNA recognition motifs are found in a large number of RNA-binding proteins (Query *et al.* 1989; Kenan *et al.* 1991); thus, a deeper understand-

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ing of CPEB proteins could elucidate how and why they have diverged from other proteins that contain RRM domains. The C-H domain is likely to chelate zinc and form a zinc finger (Hake *et al.* 1998), one of the most common motifs known for interacting with nucleic acids. Thus, analysis of CPEB proteins could also elucidate how zinc fingers bind RNA.

Second, CPEB proteins appear to function by binding messenger RNAs and regulating their translation (Hake and Richter 1994; Chang *et al.* 1999; Walker *et al.* 1999). The target sequences are found in the 3′-untranslated region (3′-UTR), are rich in uridines, and are usually variants of UUUUUAU or UUUUAAU (Fox *et al.* 1989; Hake and Richter 1994; Sheets *et al.* 1994; Walker *et al.* 1999). Other proteins also regulate the translation of messenger RNAs by binding to the 3′-UTR of messages (Gray and Wickens 1998), so the question of how 3′ sequences affect the initiation of translation at the 5′ end of the transcript has become one of the most pressing in the field.

Third, CPEB proteins appear to regulate translation by a variety of mechanisms. In many instances, they promote the cytoplasmic polyadenylation of specific targets, and this activity appears to increase the rate at which the targets are translated (Hake and Richter 1994; Sheets *et al.* 1994; Chang *et al.* 1999; Minshall *et al.* 1999). However, under some circumstances they block the translation of specific messages (de Moor and Richter 1999; Walker *et al.* 1999). These disparate activities might depend, in part, on interacting proteins, such as maskin (Stebbins-Boaz *et al.* 1999), the cleavage and polyadenylation specificity factor (CPSF; Mendez *et al.* 2000), and the Eg2 kinase (Mendez *et al.* 2000). Thus, analysis of how CPEB proteins work and of the partners that mediate these different activities should clarify both positive and negative mechanisms for regulating translation.

Because there are no mutations in most known CPEB proteins, analyses of how they function have been limited to biochemical tests and studies of mutations produced *in vitro*. For example, Hake *et al.* (1998) created mutant forms of Xenopus CPEB *in vitro* and used them to show that both RRM domains and the C-H domain are required for RNA binding. Fortunately, CPEB genes have also been identified in genetic model organisms, and genetic analyses have been carried out for two of these genes: *orb* in Drosophila (Lantz *et al.* 1992) and *fog-1* in *C. elegans* (Barton and Kimble 1990). Although there are only four natural mutations in the *orb* gene, >60 alleles of *fog-1* are available for analysis, including temperature-sensitive (ts) alleles (Barton and Kimble 1990; Ellis and Kimble 1995; Jin *et al.* 2001). Furthermore, *fog-1* acts in the context of the sex-determination process in *C. elegans,* which is controlled by one of the best-characterized regulatory hierarchies found in any animal (Figure 1).

Thus, to elucidate how CPEB proteins function, we used genetic and molecular assays to characterize 33 *fog-1* mutations. Our results suggest that three domains of FOG-1 are essential for activity: RRM1, RRM2, and the C-H domain. These results imply, and biochemical tests confirm, that FOG-1 is capable of binding to RNA. In fact, it interacts with sequences in the 3′-UTR of its own message, which suggests that *fog-1* might be subject to autoregulation. Finally, we show that most missense mutations in the RNA-binding domains have dominant negative activities, which implies that FOG-1 functions in a complex, or as a multimer, to regulate germ cell fates.

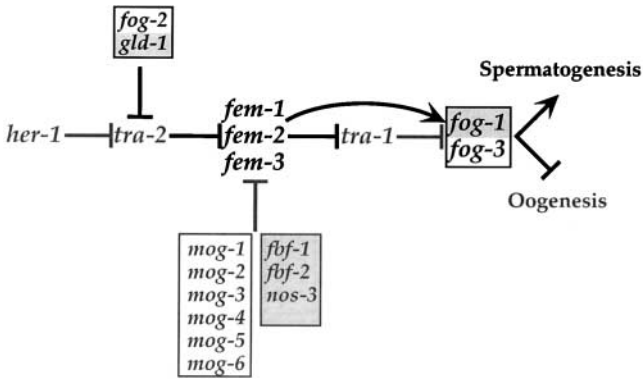


Figure 1.—*fog-1* acts at the end of the sex-determination pathway for germ cells. Sex determination in the germ line of an L4 hermaphrodite. Only genes that act downstream of the HER-1 signal are shown (for a review, see Ellis 1998). Genes that are likely to be active are shown in boldface type and those likely to be inactive in regular type. Positive regulation is indicated with an arrow, and negative regulation with a line ending in a bar. Genes that act only in germ cells are shown in boxes. Translational regulatory genes that are discussed in the Introduction are shaded. In addition, the *tra-1* product functions in part to regulate localization of RNA targets (Graves *et al.* 1999), and the products of *mog-1*, *mog-4*, and *mog-5* are DEAH box proteins that are likely to bind to messages and perhaps regulate their translation (Puoti and Kimble 1999, 2000).

MATERIALS AND METHODS

Genetic nomenclature: The genetic nomenclature for *C. elegans* is as described by Horvitz *et al.* (1979), with two exceptions. First, we use "female" to designate a hermaphrodite that makes oocytes but no sperm; by definition, female worms cannot self-fertilize. Second, we use capital letters and plain font to indicate the protein encoded by a gene. Thus, the protein produced by the *fog-1* gene is FOG-1.

Genetic analyses: We cultured *C. elegans* as described by Brenner (1974) and raised strains at 20° unless indicated otherwise. All strains were derived from the Bristol strain N2 (Brenner 1974). The *fog-1* mutations we characterized are described by Barton and Kimble (1990), Ellis and Kimble (1995), and Jin *et al.* (2001).

Cloning and sequencing mutant *fog-1* cDNAs: The *fog-1* gene was amplified in three sections, using the RNA from five pooled mutant animals as a template for RT-PCR (Mullis *et al.* 1986; Saiki *et al.* 1988). The primer pairs were RE340 and

TABLE 1

Primers used in these experiments

Name	Sequence
RE87	GACGAGAAGAACAACTCCG
RE96	CGTTGGTGGAATCTCGC
RE108	ACAGTCCTGCAGATGTTTCCCAGTGGTCA CAATG
RE109	ACAGTCGTCGACCTACTTTCCCATATTAACAAG GTACATAT
RE117	GCCCGTCTTGCAGCTGCC
RE118	ACGGGAAATTGTGGCCGCAC
RE119	GAACTCCATCCGGAGCACTG
RE120	GCCTGGAACATCATCCTCATC
RE340	GGCCAATCGACGAAAACCG
RE341	AGCTTAAAACCAATGTTTCCCAGTGGT
RE342	CGTTGGTGGAATCTCGC
RE343	CACATCATATTTCGGAGCAGG
RE344	TGATGGAAGATGTTGGATGTG
RE345	CCGGCGGTGGGAATTGTG
RE346	GACGAGAAGAACAACTCCG
RE347	GGCGTGCACCTACTCAAACCGGGA
RE348	TTCAAATCCGTCCCTGG
RE349	GCCAAGAGCTCGAGTTGGAG
RE350	GCCGGATTCCACGAAGC

RE341, RE342 and RE343, and RE344 and RE345 (Table 1). For each mutant, we determined the sequence of the entire cDNA on one strand, using the dideoxy nucleotide method (Sanger *et al.* 1977) with fluorescently labeled terminators (Halloran *et al.* 1993). The primers used for these sequencing reactions were RE346, RE347, RE348, RE349, and RE350 (Table 1).

Constructing plasmids for transient transfection: To construct amino-terminal FLAG-tagged and myc-tagged FOG-1 plasmids, we amplified the *fog-1* cDNA using *Pwo* DNA polymerase (Boehringer Mannheim, Indianapolis) and primers RE108 and RE109, digested the fragments with *Pst*I and *Sal*I, and then ligated these fragments into either the pCMV2B or the pCMV3B vectors (Stratagene, La Jolla, CA). The internal deletion constructs were derived from these plasmids by digestion with *Eco*RI, followed by self-ligation, which removed amino acids 20–243 of FOG-1, but left the rest of the sequence in frame.

Production of extracts containing FLAG-tagged or myc-tagged FOG-1: HEK293 cells that had been transferred to new medium on the previous day were transfected by conventional $CaCl_2$-phosphate methods, using 24 μg of the FLAG-tagged FOG-1 construct and 6 μg of green fluorescent protein (GFP) under control of the epidermal growth factor promoter. (The GFP plasmid was used to determine the percentage of transfected cells, which was usually over two-thirds.) Then, 4–6 hr after transfection, the cells were washed, fed with new medium, and allowed to grow for 36 hr. Finally, the cells were washed three times with polysome buffer [10 mm Na phosphate, 1 mm EDTA, 1 mm dithiothreitol (DTT), and 250 mm sucrose] and either sonicated in polysome buffer containing protein inhibitor cocktail with EDTA (Roche) or frozen for further use. Protein concentrations were determined by the protein assay system (Bio-Rad, Hercules, CA). The production of FLAG-tagged and myc-tagged FOG-1 proteins was tested by Western analysis, using α-FLAG antibody (Stratagene) or α-myc antibody (Invitrogen, San Diego).

Western analysis: Western analyses were done as described by Ausabel *et al.* (1998).

Northwestern analysis: Northwestern analysis was done as described by Daros and Carrington (1997) with minor modifications. Briefly, 50 μg of cell extracts were separated by SDS-PAGE and transferred to either polyvinylidene fluoride membrane (Millipore, Bedford, MA) or nitrocellulose membrane (Bio-Rad). The blots were blocked for 3 hr with RNA-binding buffer (10 mm HEPES pH 7.5, 25 mm NaCl, 1 mm EDTA, 1 mm DTT, and 5% glycerol) containing 10% nonfat dairy milk. After blocking, the proteins were denatured in RNA-binding buffer containing 6 m urea and gradually renatured in RNA-binding buffers containing 3 m urea, then 1.6 m urea, and finally 0.7 m urea. The blot was then washed three times with RNA-binding buffer. Finally, the blot was incubated for 2 hr with RNA-binding buffer at room temperature, radiolabeled probe was added, and hybridization was allowed to continue overnight. The blot was washed three times with RNA-binding buffer at room temperature, dried, and exposed on film.

Immunoprecipitation of RNA bound to FLAG-tagged FOG-1: Immunoprecipitation of RNA was performed as described by Chang *et al.* (1999) and Copeland *et al.* (2000) with the following modifications. We incubated 10 ng of mRNA extracted from fem-3(q96gf) adult animals with 50 μg of HEK293 cell extract for 2 hr in RNA-binding buffer. This and all subsequent incubations were done at room temperature. Next, the mixture was incubated with α-FLAG antibody-agarose beads (Sigma, St. Louis) for 2 hr in antibody binding buffer (150 mm NaCl and 1 mm Tris-HCl pH 7.5) with rotation. Then the mix was centrifuged and the pellet washed three times with antibody binding buffer. Each wash was followed by a 30-min incubation during which the tube was rotated. Finally, the pellet was dissolved in Trizol reagent and RNA was purified as described (Chen *et al.* 2000) and then reverse transcribed with MMLV-RT (Promega, Madison, WI). We used the following primer pairs to test for the presence of specific genes by the PCR: RE96 and RE87 for *fog-1*, RE117 and RE118 for *unc-37*, and RE119 and RE120 for *tra-1*.

RESULTS

The RNA-binding domains of FOG-1 are essential for activity: In nematodes, the *fog-1* gene plays a crucial role in determining whether germ cells differentiate as sperm or as oocytes (Barton and Kimble 1990). This gene produces two transcripts: a large one known as *fog-1L* that is essential for activity and a small one known as *fog-1S* that has no known function (Luitjens *et al.* 2000; Jin *et al.* 2001). The large transcript encodes FOG-1, a member of the CPEB family of proteins.

Analyses of artificially designed mutations in Xenopus CPEB protein showed that the two RRM domains and the C-H domain are required for it to bind messenger RNAs (Hake *et al.* 1998). However, a broad survey of essential residues is difficult to carry out in Xenopus. Since *C. elegans* is well suited for such studies, we used the CPEB protein FOG-1 to ask two questions. First, are the three RNA-binding domains identified in Xenopus CPEB protein also essential for other CPEB proteins to function? Second, can a broad mutational analysis identify additional domains that are required for CPEB activity? To answer these questions, we identified the

TABLE 2

Molecular lesions associated with *fog-1* mutations

Allele	Mutagen	Molecular lesion	Effect on FOG-1
		A. Missense mutations and in-frame deletions	
e1959	EMS	C645 → T	Ser204 → Phe
q181	EMS	C645 → T	Ser204 → Phe
q380	EMS	G748 → T	Lys238 → Asn
q229	EMS	G748 → T	Lys238 → Asn
q311	EMS	C1014 → T	Pro327 → Leu
q382	EMS	G1062 → A	Gly343 → Glu
oz95	Spontaneous	G1062 → A	Gly343 → Glu
q180	EMS	G1062 → A	Gly343 → Glu
e2122	EMS	G1115 → A	Gly361 → Arg
q253ts	EMS	C1131 → T	Thr366 → Ile
q254	EMS	G1256 → A	Glu408 → Lys
q255	EMS	G1260 → A	Cys409 → Tyr
q493	UV/TMP	T1769 → G	Tyr579 → Asp
q187	EMS	G1773 → A	Cys580 → Tyr
q507	UV	Delete (633 to 659)	(Gln200—Val209) → Leu
q492	UV/TMP	Delete (844 to 933)	(Met270—Tyr270) → Ile
q182	EMS	Delete (1160 to 1210)	(Ala376—Thr392) deleted
q379	EMS	G748 → T	Lys238 → Asn
		G1062 → A	Gly343 → Glu
q248	EMS	G748 → T	Lys238 → Asn
		G1061 → A	Gly343 → Arg
oz15ts	Spontaneous	G748 → T	Lys238 → Asn
		C1131 → T	Thr366 → Ile
		B. Nonsense mutations and frameshifting mutations	
q250	EMS	G63 → A	Trp10 → STOP
		G748 → T	
q372	EMS	C281 → T	Gln83 → STOP
q273	TR403	C400 → A	Cys122 → STOP
q242	Gamma ray	G738 → A	Trp235 → STOP
		G748 → T	
q219	TR403	G738 → A	Trp235 → STOP
q155	EMS	C1094 → T	Gln354 → STOP
e2121	EMS	C1688 → T	Glu552 → STOP
q218	TR403	C inserted at 618	Frameshift of +1
q272	TR403	G inserted at 1063	Frameshift of +1
q325	Gamma ray	Delete (1210 to 1216)	Frameshift of +2
q243	TR403	G inserted at 1646	Frameshift of +1
q271	TR403	G inserted at 1646	Frameshift of +1

molecular lesions associated with 33 independent *fog-1* mutations. These mutations had each been identified because they eliminate *fog-1* function in homozogous animals (Barton and Kimble 1990; Ellis and Kimble 1995; Jin *et al.* 2001). In these experiments, we amplified *fog-1L* from homozygous mutants by RT-PCR and directly sequenced the products. We confirmed each potential lesion by reamplifying and resequencing the appropriate regions of the transcript.

Of these 33 *fog-1* alleles, we identified 14 that were caused by a single missense mutation. These 14 alleles represent 10 distinct lesions (Table 2A). All of the single missense mutations altered one of three putative RNA-binding domains within FOG-1. Two lesions affected RNA recognition motif 1, four lesions altered RRM 2, and four lesions altered the C-H domain. Three additional *fog-1* alleles were caused by pairs of linked mutations; in each case, both mutations were located in the RRM domains (Table 2A). Since all three pairs include the mutation G748 → T, it is possible that this lesion is present in the background of some *C. elegans* strains. Finally, we identified three in-frame deletions; each of these also affected the RRM domains (Table 2A; Figure 2A).

Although these results suggested that the RNA-binding domains were essential for *fog-1* activity, it remained possible that the clustering of mutations was caused by the presence of mutational hot spots within the gene. This alternative seems unlikely for two reasons. First, although most of these mutations were induced by ethyl methanesulfonate (EMS), some were spontaneous and

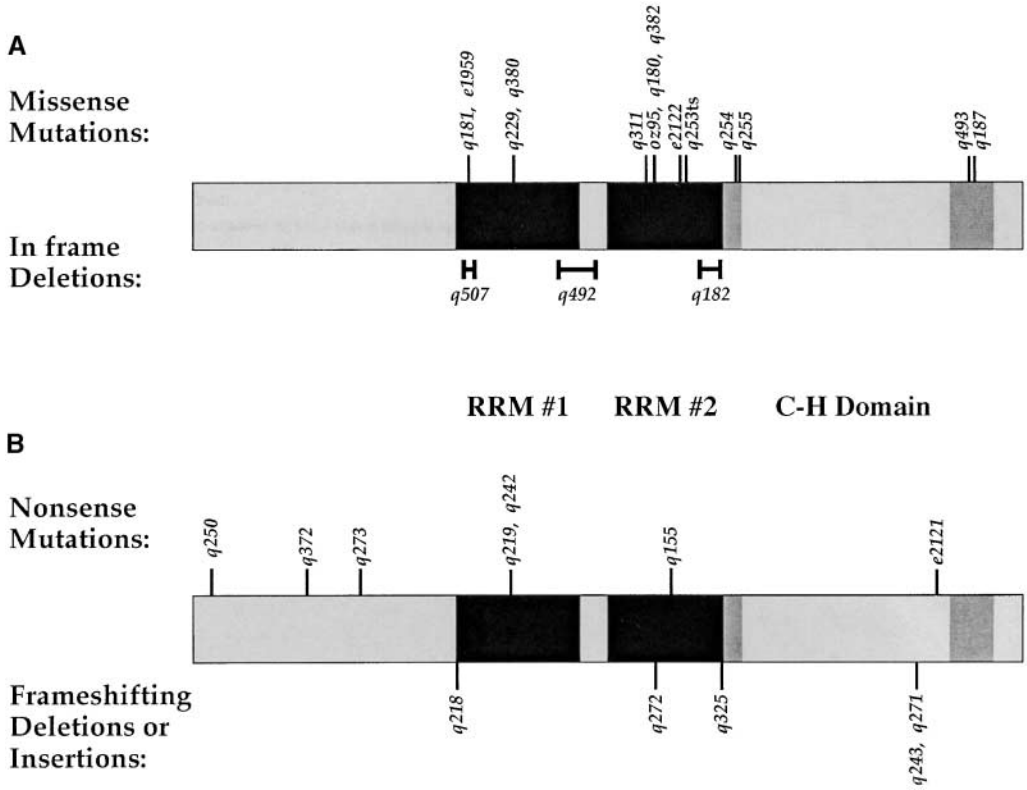


FIGURE 2.—Location of *fog-1* mutations. (A) Location of missense mutations and in-frame deletions within FOG-1, the product of the large *fog-1* transcript. FOG-1 is depicted as a bar, with the amino terminus at the left. Solid rectangles mark the two RRM domains, and shaded rectangles mark the two halves of the C-H domain. The location of each *fog-1* allele is marked with a line above the bar, and the extent of each deletion is indicated by a line below it. (B) Location of nonsense mutations (shown above the bar) and frame-shifting deletions (below it).

others were caused by ultraviolet light and trimethyl psoralen (Table 2A). It seems unlikely that each of these treatments would be biased toward inducing mutations in the same region of *fog-1*. Second, nonsense mutations and mutations that shift the reading frame were found throughout the gene (Table 3B; Figure 2B), so mutations in other regions of *fog-1* are possible and, in fact, frequent. Since mutations do occur randomly in the *fog-1* gene, we conclude that the clustering of missense mutations in the two RRM domains and the C-H domain reflects the biological importance of these three domains.

Although we identified 14 missense mutations and three in-frame deletions, none of these lesions affected residues outside of the three RNA-binding domains. Furthermore, phylogenetic analyses show that these other regions of FOG-1 are poorly conserved with respect to other CPEB proteins (JIN *et al.* 2001). Two alternatives can explain these results. First, these other regions of FOG-1 might have no detectable function. Second, these regions might have important functions that cannot be abolished by single missense mutations.

Mutations within the RRM domains are not restricted to the crucial RNP β-sheets: The core of each RRM domain is formed from two conserved β-sheets, known as the RNP1 and RNP2 sequences (BURD and DREYFUSS 1994). Some of the residues within these β-sheets are required to form the proper tertiary structure, whereas others mediate interactions with RNA. Two of the six missense mutations that affect the RNA recognition motifs of FOG-1 are located within one of these β-sheets (Figure 3). One is *e2122*, which causes a change of Gly361 (which is conserved in almost all RRM domains) to arginine; this mutation is likely to disrupt the structure of RNP1 in the second RRM domain. The other is *q253*, which causes a change of Thr366 to isoleucine and also affects this RNP1 sequence. This mutation is strongly temperature sensitive, and the affected threonine is conserved among all CPEB proteins, but not among other RRM domains. Perhaps this threonine plays a role stabilizing the structure of CPEB proteins. Two additional mutations each flank one of the RNP2 sequences—*e1959* is located next to the RNP2 sequence of the first RRM domain, and *q311* is located next to the RNP2 sequence of the second RRM domain.

By contrast, the remaining two missense mutations, *q229* and *q180*, are located far from the RNP regions (Figure 3). The conservation of the two affected residues during evolution suggests that they are important for function; our identification of these lesions confirms this hypothesis. Both mutations are located in the region linking an RNP2 β-sheet to an RNP1 β-sheet, one in the first RNA recognition motif and the other in the second.

The sequences at the carboxyl end of an RRM domain often play an important role in determining the specificity of interaction with target RNAs (BURD and DREYFUSS 1994). In CPEB proteins, the region adjacent to RRM 2 has a special structure featuring conserved cysteines and histidines that are likely to form a zinc finger

TABLE 3

Activity of *fog-1* mutations *in trans* to *fog-1(q253)*

Allele	Fog at 15° (%)	No. scored
A. Activity of missense mutations in *fog-1(x)/fog-1(q253s)* animals		
e1959	ND	ND
q181	68	108
q380	ND	ND
q229	14	108
q311	68	103
q382	ND	ND
oz95	60	121
q180	64	105
e2122	32	95
q253ts	0	105
q254	92	145
q255	96	107
q493	99	124
q187	99	201
q507	62	102
q492	78	102
q182	39	110
q379	ND	ND
q248	81	103
oz15ts	0	101
B. Activity of nonsense and frameshifting mutations in *fog-1(x)/fog-1(q253s)* animals		
qDf4	10	50
q241	17	105
q250	25	106
q372	13	124
q273	25	169
q242	19	104
q219	23	107
q155	25	101
e2121	8	178
q218	32	102
q272	34	100
q325	12	106
q243	38	97
q271	55	124

Mutant alleles are listed in the order they appear in the *fog-1* transcript, as tabulated in Table 2. Pairs of alleles caused by identical lesions are underlined. The data were gathered from crosses described in Figure 4, A and B. ND, not done.

and is therefore known as a C-H domain. Analyses of point mutants in Xenopus CPEB showed that this domain was required for RNA binding (Hake *et al.* 1998). Surprisingly, the C-H domain of FOG-1 contains a large and unique insertion (Figure 3; Jin *et al.* 2001) and thus had changed so much during evolution that it was possible that it no longer formed a functional part of the protein. However, we found that four of the missense mutations were located in the C-H domain (Table 2A, Figures 2 and 3). Each of these mutations affects either a conserved cysteine or an adjacent residue. Thus, the conserved cysteines, which are suspected to bind zinc (Hake *et al.* 1998), are crucial for FOG-1 activity, just as those in the C-H domain of the Xenopus CPEB protein are crucial for its activity.

FOG-1 can bind its own messenger RNA: Our sequence analyses suggested that FOG-1 might control cell fate by binding to specific messenger RNAs. To test this hypothesis, we needed to identify likely targets. Xenopus CPEB protein binds targets that contain CPE sites (Fox *et al.* 1989; Paris and Richter 1990). We observed a perfect CPE site in the 3′-UTR of *fog-1*, just 32 nucleotides upstream of the AAUAAA hexanucleotide, which controls the site at which the message is cleaved and polyadenylated (Figure 4A). The sequence and location of this potential CPE site are both typical of those found in Xenopus. Thus, one potential target of FOG-1 is the *fog-1* message itself. To see if FOG-1 could bind its own 3′-UTR, we carried out the following two experiments.

First, we prepared cell extracts from HEK293 cells that had been transiently transfected with a plasmid encoding myc-tagged FOG-1. After separating the proteins on an SDS-PAGE gel, we observed two myc-labeled bands, one at the 71-kD position expected for FOG-1 and a larger one of unknown composition (Figure 4B). When we prepared a Northwestern blot and probed it with a labeled fragment of the *fog-1* 3′-UTR, we observed an intense band at the position that myc-tagged FOG-1 is detected on Western blots (Figure 4B). We obtained similar results using FLAG-tagged FOG-1 (data not shown). However, we never observed binding with extract from cells producing a mutant of FOG-1 in which the amino-terminal half of the protein had been deleted (Figure 4B, FOG-1Δ lane), nor did we observe binding when the blot was probed with a fragment of the *unc-37* 3′-UTR (data not shown). We used *unc-37* as a control for these experiments because it does not contain any CPE sites. Since FOG-1 binding depends on sequences found in the *fog-1* 3′-UTR, but not in that of *unc-37*, we conclude that FOG-1 shows specificity in its selection of targets. This conclusion is supported by the observation that, in UV-crosslinking assays, cold *unc-37* RNA does not compete with radiolabeled *fog-1* probe for binding FOG-1 (data not shown). We have not defined the sequence elements that confer the ability to bind FOG-1, but it seems likely that CPE sites will be an important feature of these elements.

We wanted to determine if the specificity of this interaction was sufficient for FOG-1 to recognize and bind the *fog-1* 3′-UTR amid the complex pool of messages present in living animals. We did this by mixing total RNA extracted from *fem-3(q96*gf*)* animals with a cell extract containing FLAG-tagged FOG-1. After immunoprecipitation using anti-FLAG antibodies, we extracted RNA from the pellet and reverse transcribed it. Finally, we used the PCR to test for the presence of specific messages. We found the *fog-1* transcript in cDNA transcribed from the immunoprecipitate, but could not de-

tect *unc-37* messages (Figure 5). Does this difference show that FOG-1 binds preferentially to the *fog-1* message? There should be lots of *unc-37* mRNA in the mixture, since *unc-37* is a common transcript that is ubiquitously expressed (Pflugrad *et al.* 1997) and is easily detectable by our PCR assay (Figure 5, "all transcripts" lane). Furthermore, *unc-37* is represented more frequently in cDNA libraries than is *fog-1* (37 *unc-37* cDNAs have been deposited in the database *vs.* none for *fog-1*; http://www.wormbase.org). Thus, our results suggest that FOG-1 binds the *fog-1* message but not that of *unc-37*. In control reactions using our deletion construct of FOG-1, we could not detect any *fog-1* messages in the immunoprecipitate. These results show that FOG-1 can recognize its own message with some specificity and that this recognition depends on sequences removed in the deletion mutant. These deleted sequences include the amino terminus and the RNP2 element and surrounding sequences from the first RNA recognition motif.

The *fog-1* gene acts zygotically to control the sexual fates of germ cells: To study how different mutations alter the function of FOG-1 in living animals, we developed a genetic assay to measure the activity of each allele. Since almost all *fog-1* alleles show complete penetrance and expressivity (Barton and Kimble 1990; Ellis and Kimble 1995), we could not distinguish differences in their activities in homozygous worms. However, previous studies had shown that *fog-1* mutations show weak haplo-insufficiency (Barton and Kimble 1990; Ellis and Kimble 1995). Thus, we decided to study the effects of each mutation in heterozygotes. To do this, we analyzed *fog-1(x)/fog-1(q253*ts*) XX* animals at the permissive temperature of 15° (Figure 6, A and B). Under these conditions, the animals were poised on the borderline between making sperm or becoming Fog mutants. We found that the nature of the *fog-1* allele we used had a strong influence on this decision; the outcome varied from 0% Fog mutants for *q253* itself to 99% Fog mutants for *q187* and *q493* (Table 3). The reliability of our assay was confirmed by the results we obtained from two pairs of alleles that were each caused by identical lesions (*q219* and *q242* and *oz95* and *q180*). In both cases, similar results were obtained for each member of the pair (Table 3).

So that we could work with the large deficiency *qDf4*, the mutant chromosome we studied was sometimes derived from the father (Figure 6A), although in most cases it passed through the mother (Figure 6B). The assay yielded similar results in either case (Table 4), which implies that the ability of *fog-1* to specify germ cell fates is not affected by maternal inheritance. By contrast, an important maternal component is known for the three *fem* genes, which help regulate *fog-1* activity in the germ line (reviewed by Ellis 1998).

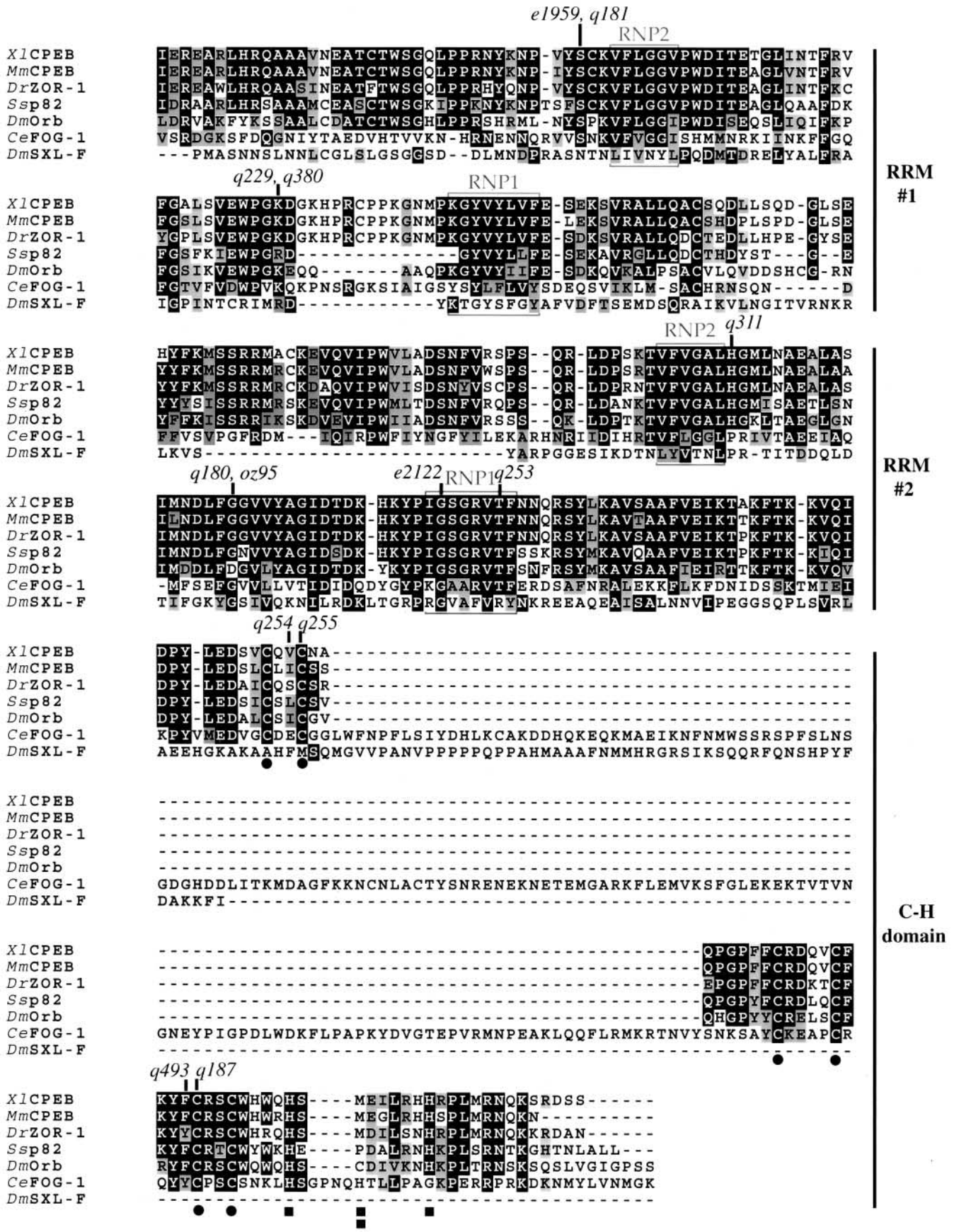
The small *fog-1* transcript plays no detectable role in controlling germ cell fate: The large *fog-1* transcript, which encodes FOG-1, is necessary and sufficient to specify that germ cells differentiate as sperm (Jin *et al.* 2001). The small transcript might have no function, might also promote spermatogenesis, or might even counteract the activity of the large transcript. To distinguish among these possibilities, we compared the activity of three different classes of *fog-1* mutations: null mutants, mutants that contain a nonsense mutation specific to the large transcript, and mutants that contain a nonsense mutation in both transcripts.

Both the large deficiency *qDf4* and the small deletion *q241* eliminate *fog-1* activity (Jin *et al.* 2001). We find that Δ*/fog-1(q253*ts*)* animals become female 15% ± 3% of the time at the permissive temperature (n = 155). The nonsense mutations *q372* and *q273* are single mutations that affect the large transcript, but not the small one. Pooling results for these alleles, we find that *fog-1L (nonsense)/fog-1(q253*ts*)* animals become female 20% ± 2% of the time (n = 293). Finally, the three nonsense mutations *q219*, *q155*, and *e2121* affect both *fog-1* transcripts. Pooling results for these alleles, we find that *fog-1L+S(nonsense)/fog-1(q253*ts*)* animals become female 17% ± 2% of the time (n = 386). When we consider the size of systematic errors, which, from analysis of mutations caused by identical lesions, we estimated to be at least 2% (Table 3), the differences between these classes of nonsense mutants seem insignificant. Thus, either the small transcript does not play an important role in *fog-1* activity or nonsense mutations do not change the function of the small transcript.

The FOG-1 protein acts in a complex or as a multimer: To determine how mutations that alter the RRM domains affect the function of FOG-1, we compared the activities of missense alleles with those of *fog-1* deletions or nonsense mutations (Table 3). We found that the missense mutations fell into three classes. First, the temperature-sensitive allele *q253* had very little effect on *fog-1* activity in this assay, which suggests that the mutant protein functions well at 15°. It is not completely wild type, however, because *fog-1* mutations *in trans* to *q253* often cause germ cells to become oocytes rather than sperm (Table 3), even though *fog-1* mutations *in trans* to a wild-type allele do not cause feminization (Barton and Kimble 1990).

Second, two missense mutations and one in-frame deletion caused phenotypes similar to those produced by null alleles. One of these, the *e2122* mutation, alters a conserved glycine in RNP1 of the second RNA recognition motif, replacing it with an arginine. Perhaps this lesion disrupts the activity of FOG-1 by preventing the proper folding of this crucial β-sheet.

Third, we found that most missense mutations caused a more severe mutant phenotype than did null alleles (Figure 7; Table 3A). These missense mutations fall into two groups. Those in the RRM domains cause between 60 and 80% of the *fog-1(x)/fog-1(q253*ts*)* animals to develop as females. By contrast, missense mutations in the C-H domain cause between 92 and 99% of the *trans-*


e1959, q181
RNP2
XlCPEB
MmCPEB
DrZOR-1
Ssp82
DmOrb
CeFOG-1
DmSXL-F
RRM #1
q229, q380
RNP1
RNP2 q311
RRM #2
q180, oz95
e2122
RNP1 q253
q254 q255
C-H domain
q493 q187

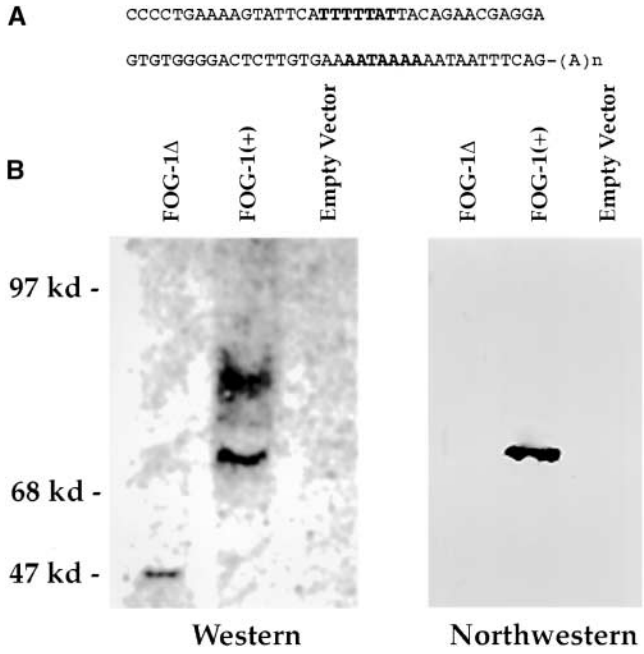


FIGURE 4.—FOG-1 can bind the *fog-1* 3′-untranslated region. (A) Sequence of the 3′ end of the *fog-1* transcript. A potential CPE site and the hexanucleotide are shown in boldface. (B) Parallel Western and Northwestern blots of FOG-1 produced during transient transfection (see MATERIALS AND METHODS). Lanes: FOG-1Δ, extract from cells producing myc-labeled FOG-1 that lacked the amino-terminal half of the protein; FOG-1(+), extract from cells producing myc-labeled wild-type FOG-1; "Empty Vector," extract from cells transfected with an empty vector. The Western blot was probed with anti-myc antibodies and the Northwestern with radiolabeled *fog-1* 3′-UTR (see MATERIALS AND METHODS).

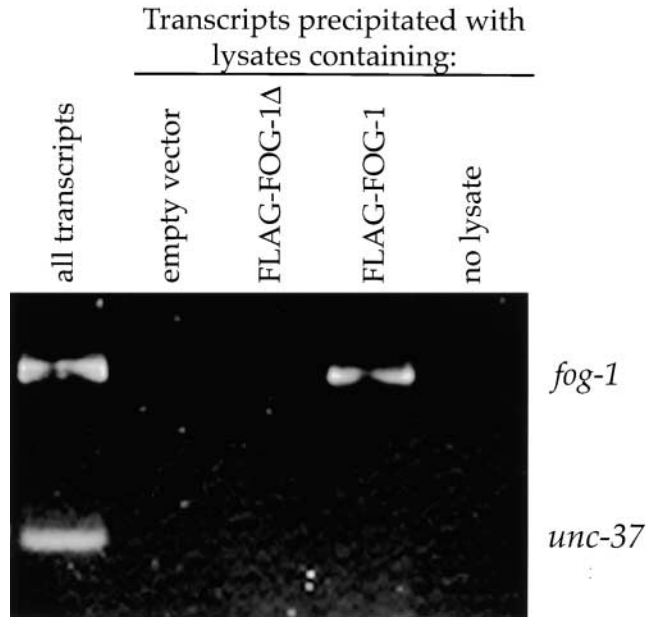


FIGURE 5.—FOG-1 specifically co-immunoprecipitates the fog-1 transcript. RT-PCR analysis of transcripts selected by co-immunoprecipitation with FLAG-FOG-1 and anti-FLAG antibodies. Lanes: "all transcripts," total mRNA from *fem-3(q96)* animals; "empty vector," extract from cells transfected with an empty vector; "FLAG-FOG-1Δ," extract from cells producing FLAG-labeled FOG-1 that lacked the amino-terminal half of the protein; "FLAG-FOG-1," extract from cells producing a FLAG-labeled wild-type FOG-1; "no lysate," no extract used in the immunoprecipitations. Primers used for RT-PCR are described in MATERIALS AND METHODS.

heterozygotes to become females. These phenotypes are more severe than the 15% rate of feminization seen for Δ/*fog-1(q253*ts*)* worms. Thus, these missense alleles exert a dominant negative effect on *fog-1* activity, which implies that the mutant FOG-1 proteins actively prevent spermatogenesis and promote oogenesis.

To see if these dominant negative effects could also be observed *in trans* to a wild-type allele of *fog-1*, we developed a method for measuring *fog-1* activity that did not involve the *q253*ts allele. In this assay, we relied on the fact that *fog-1* mutations act as semidominant suppressors of the *fem-3(q96*gf,ts*)* mutation, which causes germ cells to differentiate as sperm (Figure 6C; BARTON and KIMBLE 1990). We found that missense mutations of *fog-1* were more effective suppressors than null alleles (Table 5, A and B). These results indicate that the dominant negative effects caused by these *fog-1* missense mutations do not depend on having a *fog-1(q253*ts*)* allele *in trans* to the mutation being studied. However, this assay is less sensitive than the one presented in Table 3 and does not distinguish between the activities of missense mutations in the RRM domains and those in the C-H domain.

Although the nonsense alleles of *fog-1* are distributed throughout the gene, all appear to eliminate FOG-1 activity. One explanation for this result is that any truncation disrupts the structure of FOG-1, thereby preventing it from interacting with other proteins or RNA targets. Alternatively, *fog-1* transcripts that contain nonsense mutations might be eliminated by the Smg pro-

FIGURE 3.—Amino acid residues affected by *fog-1* missense mutations. The precise location of the 10 missense lesions of FOG-1, presented on an alignment of its sequence with those of other CPEB proteins. Conserved cysteines are marked with solid circles, conserved histidines with solid squares, and the position of the final FOG-1 histidine with two squares. *D.m.* Orb (LANTZ *et al.* 1992), *S.s. p82* (WALKER *et al.* 1999), *D.r.* ZOR-1 (BALLY-CUIF *et al.* 1998), *X.l.* CPEB (HAKE and RICHTER 1994), and *M.m.* CPEB (GEBAUER and RICHTER 1996). The sequence of the Drosophila sex lethal protein is provided for comparison, since it contains two RRMs, but is not a CPEB protein. The *X.l.* CPEB sequence begins at residue 277, *M.m.* CPEB at 274, *D.r.* ZOR-1 at 268, *S.s.* p82 at 373, *D.m.* Orb at 540, *C.e.* FOG-1 at 169, and *D.m.* SXL-F at 93. Solid boxes indicate identical amino acids in the alignment, and shaded boxes indicate similar ones.

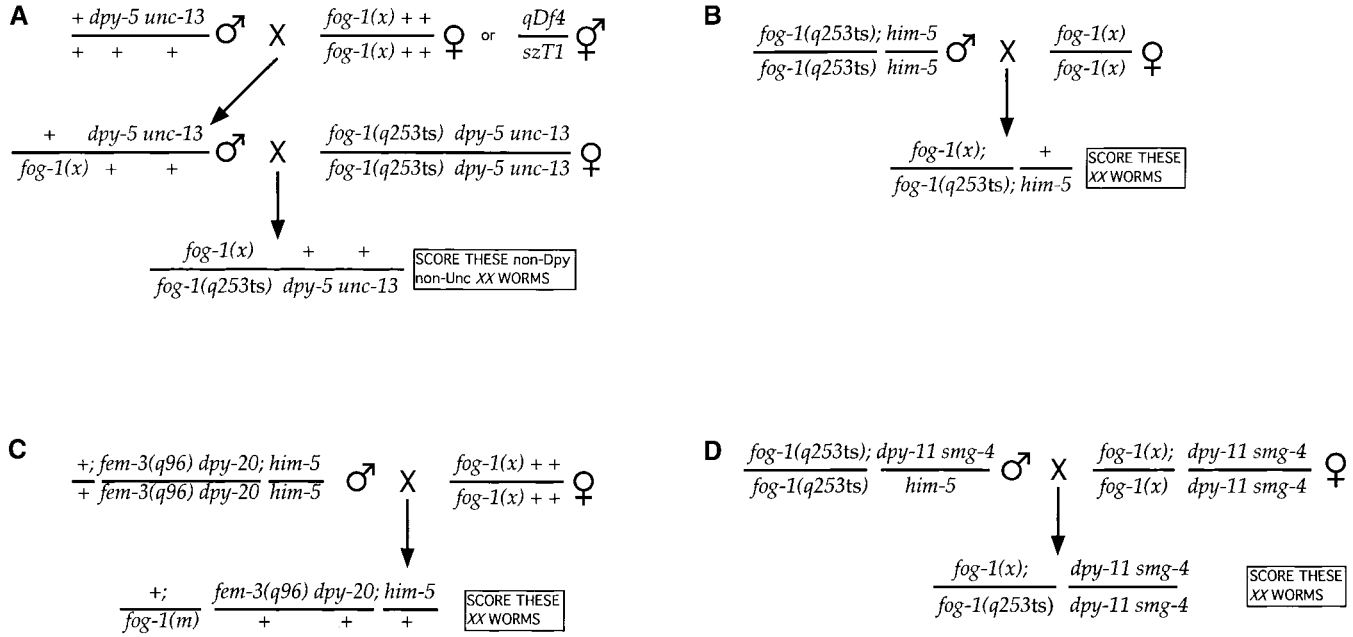


FIGURE 6.—Genetic assays for the activity of *fog-1* alleles. (A) Method for studying paternally derived *fog-1* mutations or deletions. Single F_1 males were used for each cross, and their genotype was confirmed by examination of the F_2 progeny. To confirm that self-fertile F_2 worms had actually received *fog-1(x)*, we examined their F_3 self-progeny and disregarded animals that were revealed to be recombinants. Crosses were carried out at 15°. (B) Method for studying maternally derived *fog-1* mutations. Crosses were carried out at 15°. (C) Method for scoring the semidominant suppression of *fem-3(q96*gf,ts*)* by mutations in *fog-1*. Crosses were carried out at 25°. (D) Method for studying the effects of *smg* mutations on *fog-1* activity. Homozygosity for the *smg-4(ma116)* allele was scored by assaying for the linked mutation in *dpy-11*.

teins, which recognize and degrade aberrant messenger RNAs (HODGKIN *et al.* 1989; CALI and ANDERSON 1998). To distinguish between these alternatives, we measured *fog-1* transcript levels in homozygous nonsense mutants in both wild-type and *smg* genetic backgrounds. To do this, we used quantitative RT-PCR analysis to study *fog-1* transcripts in individual L4 *XX* larvae (CHEN and ELLIS 2000). Our data show that a *smg* mutation causes only a small increase in the levels of *fog-1* transcripts. The largest increase we measured was for *fog-1(q155)*; in this case, the presence of a *smg-4(ma116)* mutation increased the level of *fog-1L* transcripts 1.7-fold. Such a small increase suggests that *fog-1* transcripts might have a rapid turnover rate. By contrast, *smg* mutations can cause a 10-fold increase in the levels of stable transcripts from genes like *unc-54* (PULAK and ANDERSON 1993). The *smg-4(ma116)* mutation did not cause any of the *fog-1* nonsense alleles to produce a dominant negative effect when they were assayed *in trans* to *fog-1(q253)* (Figure 6D; Table 6). Thus, we could not use this technique to map the region of FOG-1 required for interaction with other proteins.

DISCUSSION

FOG-1 must be able to bind RNA to regulate germ cell fates: The fact that FOG-1 is a member of the CPEB family of proteins implied that it might function by binding to messenger RNAs and regulating their translation (LUITJENS *et al.* 2000; JIN *et al.* 2001). However, the unusual structure of the C-H domain of FOG-1, which contains a large insertion, raised the possibility that it also differed from other family members in its function. For example, FOG-1 might have lost its ability to bind RNA and instead worked purely through protein-protein interactions. Two lines of evidence argue against this alternative.

First, our analyses of *fog-1* mutations indicate that all missense mutations affect one of the three RNA-binding domains. Since there are 10 distinct missense mutations, it is unlikely that each of them disrupts the entire struc-

TABLE 4

Activity of paternally and maternally derived *fog-1* mutations

Allele	Source of allele	% fog at 15°	No. scored
e2121	Father	2	72
	Mother	11	106
q507	Father	60	38
	Mother	55	69
q187	Father	100	61
	Mother	99	140

The paternal data were gathered from crosses described in Figure 6A and the maternal data from crosses described in Figure 6B.

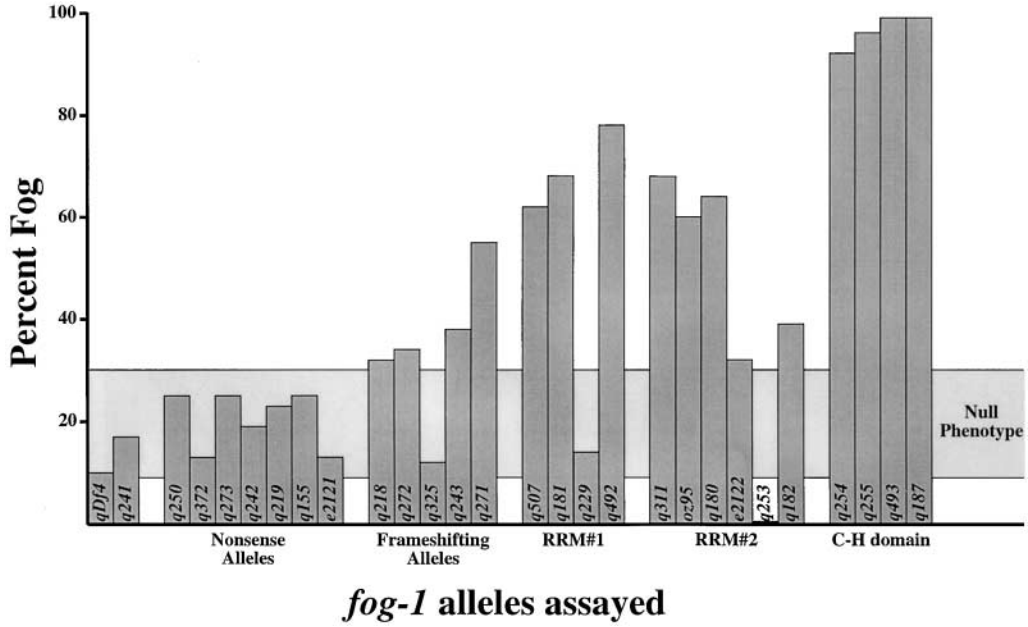


FIGURE 7.—Most missense mutations of *fog-1* have dominant negative effects. Graph of results from Table 3. *XX* animals *trans*-heterozygous for *fog-1 (q253*ts*)* were examined at 15° for their ability to produce sperm for self-fertilization. Alleles are grouped by their nature and location, with the two deletion alleles shown at the far left. The limits expected for a null allele are indicated by a shaded bar and were set arbitrarily to include results from all deletion and nonsense alleles of *fog-1*.

ture of the protein. Rather, it seems likely that some (and perhaps all) of these missense mutations abolish the ability of FOG-1 to bind RNA without otherwise altering its structure. This hypothesis is supported by the fact that most of the missense mutations have dominant negative activities, which implies that they encode FOG-1 proteins that retain some function and are thus likely to fold normally.

Second, biochemical tests indicate that FOG-1 can bind to its own 3′-UTR *in vitro*. These assays used FLAG- or myc-tagged FOG-1 produced in transiently transfected mammalian cells. In both of our assays, wild-type FOG-1 was able to bind to the *fog-1* 3′-UTR, but not to control messages, and a FOG-1 deletion mutant was unable to bind RNA.

What function does the amino terminus of FOG-1 play? Although FOG-1 and other CPEB proteins share two RRM domains and a C-H domain, they have large amino termini that differ dramatically in sequence and structure. We did not recover any missense mutations in this amino-terminal domain, although it represents almost half of the protein. What function might it serve? One possibility is that the amino terminus contains sequences that give each CPEB protein its own specific function. For example, in *C. elegans* there are four different CPEB proteins: FOG-1, CPB-1, CPB-2, and CPB-3 (LUITJENS *et al.* 2000; JIN *et al.* 2001). FOG-1 controls whether germ cells become sperm or oocytes, CPB-1 regulates early

TABLE 5

Activity of *fog-1* mutations as suppressors of *fem3(q96*gf,ts*)*

Genotype	% producing oocytes	No. scored
A. The ability of *fog-1* null mutations to suppress *fem-3(q96*gf,ts*)*		
q241/+	17	70
q325/+	26	74[a]
e2121/+	28	53
B. The ability of *fog-1* missense mutations to suppress *fem-3(q96*gf,ts*)*		
q180/+	85	62[b]
q255/+	84	44
q493/+	88	60[c]
q187/+	65	62[c]
C. The ability of a *fog-3* control allele to suppress *fem-3(q96*gf,ts*)*		
fog-3(q469)/+	0	32

The animals were produced as described in Figure 6C and raised at the restrictive temperature of 25°.

[a] Six animals produced exclusively oocytes.

[b] One animal produced exclusively oocytes.

[c] Two animals produced exclusively oocytes.

TABLE 6

Activity of *fog-1* nonsense mutations in a *smg-4* mutant background

Allele	% fog at 15°	No. scored
q253	0.0	30
q273	4	25
q242	10	36
q155	11	38
e2121	14	14

The animals had the genotype *fog-1(x)/fog-1(q253*ts*) I; dpy-11 (e224) smg-4(ma116) IV*. Homozygosity for the *smg-4* mutation was inferred from homozygosity for the linked *dpy-11* mutation.

spermatogenesis, and the remaining two proteins have unknown functions. Since these proteins differ dramatically in their amino termini, this region might help confer their specialized activities.

Is *fog-1* autoregulatory? The fact that FOG-1 can bind its own 3′-UTR *in vitro* indicates that it might also do so *in vivo*. Recent studies have shown that the Orb protein of Drosophila regulates its own localization (Tan *et al.* 2001). Furthermore, the Zorba transcript in zebrafish contains potential CPE sites (Bally-Cuif *et al.* 1998). Might FOG-1 actually regulate its own activity? Definitive tests will require anti-FOG-1 antibodies to determine whether the levels of FOG-1 protein are altered by mutations in the *fog-1* 3′-UTR or by mutations that alter FOG-1 activity. However, some genetic data are consistent with the possibility that FOG-1 is autoregulatory. First, analyses of the temperature-sensitive mutation *fog-1(q253)* reveal that FOG-1 activity is required continuously for spermatogenesis (Barton and Kimble 1990). Furthermore, if this activity is interrupted by a shift to the restrictive temperature, the animals permanently lose the ability to produce sperm, even after the permissive temperature is restored. One explanation of this result is that FOG-1 activity is required for the continued translation of *fog-1* messages, so that if FOG-1 is inactivated, translation cannot be resumed. Second, null alleles of *fog-1* are weakly haplo-insufficient. For example, *fog-1(null)/+* males produce oocytes during adulthood, whereas wild-type males never do so (Barton and Kimble 1990; Ellis and Kimble 1995). In addition, *fog-1* null mutations are dominant suppressors of *fem-3(gf)* alleles (Barton and Kimble 1990; Ellis and Kimble 1995). Finally, null mutations in *fog-1* cause some feminization when *in trans* to *fog-1(q253*ts*)* (Table 3). These results indicate that *fog-1* activity is very sensitive to changes in gene dosage. This observation can be explained by models in which FOG-1 is autoregulatory, since small decreases in *fog-1* activity might lead to large decreases in the translation of *fog-1* messages; such a cycle could quickly shut down the activity of the *fog-1* gene.

Does the small transcript of FOG-1 have a function? Our genetic results show that nonsense mutations that affect only the large transcript behave like nonsense mutations that affect both transcripts. This result is not consistent with models in which the small transcript produces a truncated form of FOG-1 that helps to regulate germ cell fates. However, it does not rule out models in which the small transcript functions purely as an RNA molecule. For example, the 3′-UTR of the small transcript might bind to and titrate out FOG-1 protein. If this were the case, one might expect nonsense mutations that affected only the large transcript to behave differently from deletions of the *fog-1* gene, which should eliminate both transcripts. Since we do not observe such an effect, we suspect that the small transcript has no function in the control of germ cell fates. To test this hypothesis, we are cloning the *fog-1* genes from related nematodes to see if production of a small transcript has been conserved.

Do CPEB proteins require partners to regulate translation? CPEB proteins can regulate translation both positively and negatively (Gray and Wickens 1998). What proteins do they require to assist in these processes? In Xenopus, a cytoplasmic form of the CPSF is required for cytoplasmic polyadenylation (Dickson *et al.* 1999), and this factor appears to directly interact with Xenopus CPEB protein (Groisman *et al.* 2000; Mendez *et al.* 2000). Xenopus CPEB protein also interacts with maskin, possibly to repress translation of specific transcripts (Stebbins-Boaz *et al.* 1999). In addition, the CPB-1 protein of *C. elegans* interacts with the translational regulator FBF-1 (Luitjens *et al.* 2000). CPEB proteins could potentially interact with other proteins that regulate polyadenylation or that control the efficiency of translation. Finally, it is possible that some CPEB proteins function as dimers. For example, human hnRNP A1 contains two RNA recognition motifs, and the crystal structure of its amino terminus reveals that it binds its target as a dimer (Ding *et al.* 1999).

Our genetic assays show that most missense alleles of *fog-1* have dominant negative effects. This result suggests that these mutants produce FOG-1 proteins that have lost the ability to bind RNA targets, but which can bind to and titrate out other members of a translational regulatory complex. Thus, we suspect that FOG-1, much like Xenopus CPEB and *C. elegans* CPB-1, acts as part of a complex to regulate its targets. Alternatively, FOG-1 might act as a multimer. In either case, these dominant negative mutations appear to prevent FOG-1 from binding RNA without disrupting protein/protein interactions. To find proteins that interact with FOG-1 to regulate translation, we have begun a large-scale mutagenesis to find suppressors that restore self-fertility to *fog-1 (q253*ts*)* mutants. These suppressor mutations appear to be both abundant and varied in nature.

Conclusion: Our results suggest that FOG-1 binds specific RNA targets in order to regulate cell fates. Furthermore, *in vitro* studies suggest that one target might be FOG-1 itself. Finally, our results suggest that FOG-1 acts as part of a protein complex or as a multimer. We have begun an extensive screen for suppressors of the *fog-1 (q253*ts*)* mutation to identify proteins that interact with FOG-1 to control germ cell fates or that regulate FOG-1 activity. Molecular analyses of these suppressors might reveal additional factors that are needed for CPEB proteins to regulate translation of their target messages.

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