Targeted mutations in the *Caenorhabditis elegans* POU homeo box gene *ceh-18* cause defects in oocyte cell cycle arrest, gonad migration, and epidermal differentiation

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We used targeted gene inactivation to analyze the function of a *Caenorhabditis elegans* POU gene, *ceh-18*, and to dissect its functional domains in vivo. In *ceh-18* mutants, oocytes exhibit an incompletely penetrant failure to arrest in diakinesis of meiotic prophase I and instead undergo multiple rounds of DNA replication without cytokinesis. *ceh-18* is expressed in the gonadal sheath cells that signal the oocyte, but not in the oocyte. This suggests that *ceh-18* affects, directly or indirectly, a sheath cell signal that causes oocytes to maintain diakinesis arrest. *ceh-18* also participates in directing gonad migration and in specifying the differentiated phenotypes of epidermal cells during postembryonic development. Analysis of targeted deletions that disrupt half of the POU domain selectively by deleting either the POUhd or the POUsp alone, indicates that each CEH-18 POU subdomain is sufficient for partial activity in vivo.

[Key Words: POU domain; germ-line development; meiotic arrest; oocyte; DNA endoreduplication; homeo domain]

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We have analyzed the in vivo role of a new member of the POU class of homeo box-containing genes, *ceh-18*, using a targeted gene knockout strategy in *Caenorhabditis elegans* (Zwaal et al. 1993). Transcription factors of the POU class of homeo box-containing genes have been implicated in the specification and proliferation of particular cell types (Rosenfeld 1991; Ruvkun and Finney 1991). Here we report an unexpected connection between the *C. elegans* POU gene *ceh-18* and cell cycle control in oogenesis.

Oocyte arrest in meiosis is a universal feature of reproduction in both invertebrates and vertebrates (Masui and Clarke 1979). Meiotic arrest is normally relieved with ovulation and fertilization. Biochemical and molecular analysis in vertebrates has established that release from meiotic arrest in oocytes is under hormonal control that triggers a kinase cascade including the Mos kinase (Sagata et al. 1988, 1989) followed by activation of the maturation promoting factor (MPF) (Smith 1989). In con-

trast, less is known about the molecular mechanisms by which oocyte arrest in meiotic prophase I is established and maintained. Here we report that targeted *ceh-18* mutations lead to a cell cycle defect in oocytes such that this meiotic arrest point can be bypassed without fertilization. We determined that oocytes in *ceh-18* mutants variably undergo multiple rounds of DNA replication (DNA endoreduplication) without karyokinesis or cytokinesis and become polyploid. We also show that *ceh-18* mutants exhibit an incompletely penetrant maternal effect lethality. Thus, *ceh-18* affects two disparate aspects of oocyte differentiation: regulating both the oocyte cell cycle and the subsequent embryogenesis. Surprisingly, *ceh-18* is not expressed in the oocyte but, rather in the gonadal sheath cells that encircle the oocyte. Recently, laser ablation of sheath cells or sheath cell precursors has been shown to result in an oocyte DNA endoreduplication phenotype (J. McCarter, R. Francis, and T. Schedl, pers. comm.). Taken together, these results suggest that *ceh-18* affects sheath cell to oocyte signals that regulate the oocyte cell cycle as well as early developmental events of the embryo.

Targeted *ceh-18* mutations also allowed us to dissect

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functionally the *ceh-18* POU domain in vivo. The POU domain is a bipartite DNA recognition domain that consists of a POU-specific region (POUsp) and a POU homeo domain (POUhd) separated by a linker region (Rosenfeld 1991; Ruvkun and Finney 1991). Analysis of targeted deletions that disrupt selectively either the POUhd or the POUsp alone suggest that each subdomain is sufficient for partial activity in vivo.

Results

ceh-18 defines a new POU class gene

We isolated a novel member of the POU class of homeo

box genes using degenerate oligonucleotide probes (Bürglin et al. 1989) from the helix-3 region of the POUhd to screen a *C. elegans* cDNA library. Several independent clones were isolated that correspond to a single locus and encode a new type of POU gene as revealed by DNA sequence analysis (Fig. 1A). We have named this gene *ceh-18* (for *C. elegans* homeo box). An alignment of the encoded protein CEH-18 with other POU proteins indicates that the CEH-18 POUsp is most closely related to the vertebrate genes Oct-1,2 and the related Skn-1, sharing 78% amino acid identity over 75 amino acids (Fig. 1B). In this domain, CEH-18 is less related to the other *C. elegans* POU proteins, UNC-86 and CEH-6, than it is to

Figure 1. Molecular structure of *ceh-18*. (A) Sequence analysis of *ceh-18*. A composite of *ceh-18* cDNA sequence, genomic sequence, and the deduced CEH-18 protein sequence is shown with key elements highlighted. Amino acids in the POU domain are in bold with the exception of amino acids in the linker region between the POUsp and the POUhd. The SL1 trans-spliced leader sequence found at the 5' end of the largest *ceh-18* cDNA is overlined. An acidic stretch of amino acids is underlined. The numbering refers to the cDNA sequence and excludes the introns. The TA dinucleotides at which *pk37::Tc1* (intron 6) and *ms2::Tc1* (penultimate codon) inserted are boxed. The deletion endpoints are indicated for the respective alleles. The novel splice acceptor sequence in *ceh-18(mg61)* is indicated (▼). Three clustered point mutations in intron 5 found adjacent to the deletion junction in *mg61* are indicated. (B) Comparison of the amino acid sequence of the CEH-18 POU domain with other POU proteins. The POUsp and the POUhd are aligned separately. The POUsp A region, POUsp B region, and helices 1–3 in the POUhd are represented by the identified bars. Amino acid positions are numbered to facilitate comparison with the Oct-1–octamer cocrystal structure (Klemm et al. 1994). Amino acid residues that make major groove contacts in the Oct–octamer cocrystal structure are in bold, and amino acid residues that make phosphate contacts are underlined. Positions with identical amino acids to CEH-18 are represented by dots. The percentage amino acid identity to CEH-18 is listed next to each sequence. References: Oct-1 (Scheidereit et al. 1988; Sturm et al. 1988), Oct-2 (Clerc et al. 1988; Ko et al. 1988; Müller et al. 1988), Skn-1 (Andersen et al. 1993), SCIP (Monuki et al. 1990), CEH-6 (Bürglin et al. 1989), UNC-86 (Finney et al. 1988), Oct-3/4 (Okamoto et al. 1990; Rosner et al. 1990; Scholer et al. 1990), Pit-1 (Bodner et al. 1988; Ingraham et al. 1988). (C) Physical and genetic maps in the *ceh-18* region. The top line is the gene structure with exons as boxes separated by introns. The direction of transcription is from right to left. The second line is the restriction map of the genomic cosmid clone C50A9. (B) *Bam*HI; (H) *Hind*III; (S) *Sal*I; (X) *Xba*I; (Sc) *Sac*I. The third line is the physical map showing the positions of *mgP40* and *mgP39*. The fourth line is the genetic map of a portion of the left end of the X chromosome.

these vertebrate proteins. The nonconserved linker between the CEH-18 POUsp and the POUhd is 57 amino acids, the longest reported linker between the two independent domains. In addition, the CEH-18 POUhd is more divergent than the POUsp, sharing only 40–50% amino acid identity with other POUhds. Thus, CEH-18 defines a new POU class. The crystal structure of the Oct-1 POU domain bound to the octamer site has been determined recently (Klemm et al. 1994). All of the amino acids in both the POUsp and the POUhd of Oct-1 that were observed to make major groove base contacts are identical in CEH-18 (Fig. 1B). Amino acid side chains that were observed to make phosphate contacts are either identical in CEH-18 or replaced by conservative amino acid substitutions. In contrast, helices 1 and 2 of the POUhd are divergent.

We determined the genetic and physical map positions of *ceh-18* to analyze whether *ceh-18* was defined by a previously isolated mutation. Genomic cosmid clones bearing *ceh-18* (C50A9 and LLC5) were isolated. *ceh-18* has 9 exons, 8 introns, and an outtron for SL1 trans-splicing that together span 13 kb, and is contained within the cosmid C50A9 (Fig. 1C). The cosmids C50A9 and LLC5 were fingerprinted and physically located to a region on the left end of the X chromosome (A. Coulson, pers. comm.). To determine the genetic map position of *ceh-18*, we correlated the genetic and physical maps within this region. We detected two restriction fragment length polymorphisms (RFLPs) between the interbreeding strains Bristol and Bergerac using *ceh-18* flanking cosmids as probes. The genetic map position of *ceh-18* was ascertained by genetically mapping these flanking RFLPs to the *unc-78–lin-18* interval (Fig. 1C) using five-

factor crosses. Because no candidate genes have been reported in this interval (J. Hodgkin et al., pers. comm.), *ceh-18* must define a new gene.

Targeted deletions in *ceh-18*

We sought targeted deletions in *ceh-18* to elucidate its function in vivo. Three transposable element Tc1 insertions in *ceh-18* were identified by PCR and recovered by sib-selection (see Materials and methods). These Tc1 insertions were positioned molecularly in intron 4 [*ceh-18(ms1)::Tc1*], intron 6 [*ceh-18(pk37)::Tc1*], and the penultimate codon [*ceh-18(ms2)::Tc1*] of *ceh-18* (Fig. 2). These Tc1 insertions did not lead to a mutant phenotype and do not disrupt the function of *ceh-18*. Tc1 insertions have been observed to be excised from the primary transcript by mRNA splicing, sometimes resulting in a mature transcript that confers gene activity (Rushforth et al. 1993); however, Tc1 excision can result in deletions (Kiff et al. 1988; Zwaal et al. 1993). Therefore, we used the Tc1 insertions as starting points to isolate deletions in *ceh-18*-coding sequences. The transposable element insertion in *ceh-18(pk37)::Tc1* is in the 59-bp intron that lies between the exons that encode the POUsp and the POUhd, respectively, such that deletions resulting from imprecise loss of this transposable element would be likely to remove important coding sequences. We isolated four *ceh-18* deletion alleles (*mg57*, *mg58*, *mg61*, and *mg62*) using multiple rounds of PCR and subsequent sib-selection (Fig. 2). All of the deletions were isolated under conditions in which the particular *ceh-18* deletion was heterozygous to an undeleted chromosome and thus

Figure 2. Targeted deletions in *ceh-18*. (A) Molecular analysis of Tc1 insertions and targeted deletions. The positions of the Tc1 insertions in *ceh-18* are indicated above the genomic structure. All three Tc1 inserts are in the same orientation relative to the exons (denoted by boxes separated by introns), with the first base of Tc1 closest to the 5' end of the gene. (Bottom) A close-up of the portion of the gene encoding the POUsp (shaded portion) and the POUhd (solid portion). The nucleotide sequences deleted in the various deletion alleles are indicated by shaded bars above the genomic structure. The precise limits of the deletions are shown in Fig. 1. The positions of the PCR primers c18-39 and c18-26, which were used to isolate the deletion alleles, are indicated. (B) PCR analysis of *ceh-18* deletions. Single-worm PCR reactions were carried out with c18-39 and c18-26 primers and analyzed on an ethidium-stained agarose gel. Each lane is labeled with the genotype of the worm analyzed. Lane M contains the indicated DNA molecular mass markers. (C) Western blot analysis of targeted deletions. Each lane is labeled with the genotype of the stain tested. The positions of molecular mass markers (kD) are indicated at right.

could be isolated without regard to the resulting phenotype.

Each of the mutations was outcrossed to wild type at least seven times to remove any unlinked mutations. In addition, any linked mutations were crossed off the arms of the X chromosome by selecting two recombination events each between *ceh-18* and the flanking genetic markers *unc-78* or *lon-2*, respectively. The resulting *ceh-18* mutant strains are homozygous viable, yet exhibit incompletely penetrant sterile and lethal phenotypes, and are sluggish (Table 1). These phenotypes include hermaphrodite sterility, maternal effect embryonic lethality, and larval arrest. Each of these phenotypes is recessive to wild type. Several lines of evidence suggest that these phenotypes are caused by the deletions in *ceh-18*: (1) Multiple independently isolated alleles exhibit the same set of phenotypes, although they vary in severity depending on how much of the POU domain is deleted (see below); (2) the mutant phenotypes are genetically inseparable from the deletions; (3) independent *ceh-18* alleles fail to complement; thus, these phenotypes are unlikely to be caused by adventitious linked mutations; (4) the *ceh-18* mutant phenotypes are rescued by extra-chromosomal arrays of the cosmid C50A9 that contains *ceh-18* (Table 1); and (5) molecular analysis of the dele-

tion mutations indicated that the most severe allele, *mg57*, is a 1.1-kb deletion that removes the exons that encode the POUsp and the POUhd. This deletion is out of frame and is confined to *ceh-18*-coding sequences and the small introns 4–8. Because the mutation in *ceh-18(mg57)* deletes the exons that encode the entire conserved POU domain, it is a good candidate for a null allele. Consistent with this idea, *ceh-18(mg57)* in *trans* to the deficiency *syDf1* that uncovers *ceh-18* flanking genetic markers shows the same set of phenotypes as *ceh-18(mg57)* homozygotes (data not shown). However, we cannot exclude the possibility that *ceh-18(mg57)* retains residual function in vivo in the absence of the POUsp and the POUhd.

ceh-18 regulates both the oocyte cell cycle and oocyte function

We analyzed germ-line development by Nomarski microscopy of live animals and DAPI staining of fixed preparations to investigate the basis for the hermaphrodite sterility and the reduced and highly variable brood sizes in *ceh-18* mutants. In wild type, the adult hermaphrodite gonad consists of two symmetric ovotestes, or gonad arms (Hirsh et al. 1976; Kimble and Hirsh 1979) (see Fig.

Table 1. Phenotypes of *ceh-18(lf)* mutants

Genotype	+	+/ <i>mg57</i>	<i>mg57</i>	<i>mg57</i> · <i>mgEx92</i>	<i>mg58</i>	<i>mg61</i>
Eggs laid/hermaphrodite	245 ± 33	258 ± 17	76 ± 44 ^a	174 ± 28 ^b	225 ± 98 ^b	222 ± 48 ^b
Embryonic lethality	0.4 ± 0.4%	0.7 ± 0.6%	15 ± 10% ^a	2 ± 1%	6 ± 5%	6 ± 2%
Sterility	0.2 ± 0.4%	0%	11 ± 8% ^a	0% ^b	5 ± 9%	2 ± 2%
Larval arrest	0.1 ± 0.2%	N.D.	15 ± 14% ^a	N.D.	1 ± 1%	1 ± 1%
Gonad arms with polyploid oocytes ^c	0% ^d	0% ^d	41%	0% ^d	12% ^d	7% ^d
Migrations defective gonad arms ^c	0% ^d	0% ^d	27%	8% ^{d,e}	6% ^d	2% ^d

Numbers of eggs laid by wild-type ($n = 3$), *ceh-18(mg57) lon-2(e678)/+* ($n = 9$), *ceh-18(mg57)* ($n = 10$), *ceh-18(mg57); mgEx92* ($n = 2$), *ceh-18(mg58)* ($n = 10$), and *ceh-18(mg61)* ($n = 5$) hermaphrodites and the phenotypes exhibited by these progeny. Hermaphrodites were picked as L2–L3 animals and transferred to fresh plates at 12-hr intervals and grown at 21°C in a constant temperature room, and the numbers of eggs laid were counted. *ceh-18(mg57) lon-2(e678)/+* hermaphrodites were the F1 cross progeny of wild-type males and *ceh-18(mg57) lon-2(e678)* hermaphrodites. (+) The wild-type N2 strain received from the MRC–LMB (gift of T. Barnes). Plates were checked for hatching 24 and 48 hr after removing the parent. All the hatching embryos hatched within the first 24 hr of removing the parent. Embryonic lethality was measured as the percentage of embryos that failed to hatch by 24 hr after removing the parent. Hatching larvae were picked to individual plates. Larval arrest was measured as the percentage of hatching L1 larvae that died at the L1 stage or were younger than L4 animals 1 week after removing the parent. Sterility was measured as the percentage of animals that yielded no viable progeny by 1 week after removing the parent. For +/*mg57*, the sterility refers to 150 hermaphrodites of genotype *ceh-18(mg57) lon-2(e678)/+*. An extrachromosomal array (*mgEx92*) of the *ceh-18*-containing cosmid C50A9 was constructed by coinjection of C50A9 (10 µg/ml) and the *rol-6* transformation marker pRF4 (50 µg/ml). Control experiments indicated that the *rol-6* transformation marker alone did not rescue the *ceh-18* mutant phenotype (data not shown). For *ceh-18(mg57); mgEx92*, the sterility value refers to the *mgEx92* bearing progeny only ($n = 144$). *mg57; mgEx92* segregated ~50% *mgEx92* bearing progeny in this experiment. (N.D.) these larval arrest values were not determined quantitatively. The percentage of gonad arms that contained polyploid oocytes and the percentage of gonad arms that were defective in gonad migration were determined by microscopic examination of adult animals from mixed stage populations that were fixed and stained with DAPI. The percentage of gonad arms with polyploid oocytes was scored in adults containing at least three oocytes per gonad arm.

^a $P < 0.01$ compared with wild type.

^b $P < 0.01$ when compared with *mg57*. P values were determined using Student's t -test with appropriate modifications for proportional data.

^cGreater than 94 gonad arms were scored in each case.

^d $P < 0.01$ compared with the value for *mg57* using the χ^2 test.

^eThe gonad migration defects seen in *mg57; mgEx92* were also qualitatively less severe than those seen in *mg57* alone. *ceh-18(mg57)* males mate at 100% efficiency when quantitatively compared (Hodgkin 1983) with wild-type males.

3 for a representation of the gonad of the adult hermaphrodite). The gonad arms originate near the center of the worm along the anterior–posterior axis. One arm grows in an anterior direction, the other in a posterior direction (Fig. 3; proximal arm). Both arms then migrate dorsally, bend 90°C, and grow back toward the mid-body region in the L4 larval stage (Fig. 3; distal arm). Germ-line stem cells proliferate mitotically and then enter meiosis, giving rise to sperm in the L4 larval stage. Oocytes are then formed in the adult stage from the next series of germ-line stem cells. There is a spatial and temporal gradient of meiosis in the wild-type gonad such that germ-line stem cells enter meiosis in the distal arm of the gonad, pass into the pachytene stage more proximally, and are arrested in the final stage of meiotic prophase I (diakinesis) in the proximal arm of the gonad, just distal to the spermatheca. Oocytes are fertilized by moving through the spermatheca that contains sperm (Hirsh et al. 1976; Ward and Carrel 1979). The passage into the spermatheca relieves meiosis I arrest and allows fertilization. There is no arrest in meiosis II in *C. elegans*.

In *ceh-18* mutants, sperm differentiate as in wild type during the L4 larval stage. The sperm-to-oocyte switch in adulthood is unaffected. However, the terminal differentiation of the oocyte is severely affected. The proximal region of the gonad is often swollen and enlarged with misshapen oocytes. Staining of fixed preparations of *ceh-18* mutants with the DNA-specific fluorochrome DAPI indicated that 41% of mature adult hermaphrodite gonad arms contained polyploid oocytes (Tables 1 and 2). The fact that *ceh-18* mutant polyploid oocytes are observed only in the proximal and not in the distal arm of the gonad suggests that they have entered meiosis but are defective in maintaining diakinesis stage arrest (Fig. 4 and Table 1). In addition, the polyploid germ-line nuclei are only associated with maturing oocytes that have cellularized, suggesting that syncytial mitotic germ nuclei are not the source of polyploid nuclei. These defective oocytes appear not to complete meiosis because they do

Figure 3. Representation of one of the two gonad arms of the adult hermaphrodite. Germ-line stem cells proliferate mitotically in the distal arm of the gonad near the dtc and enter meiosis more proximally. Oocytes arrested in the diakinesis stage of meiotic prophase I are found in the proximal arm of the gonad, just next to but outside of (distal to) the spermatheca that contains the sperm. The position of one of each pair of somatic gonadal sheath cell nuclei [numbered 1–5 (as in Kimble and Hirsh 1979)] is indicated. The sheath cells and the dtc express *ceh-18*, but oocytes do not (see text and Fig. 5).

Table 2. Phenotypic analysis of gonadal sheath cells

A. Analysis of sheath cell defects in *ceh-18(mg57)*

Genotype	Total gonad arms	Arms		Not scorable ^c
		with only normal sheath nuclei ^a	with abnormal sheath nuclei ^b	
+	51	49 (96%)	2 (4%) ^d	0
<i>ceh-18(mg57)</i>	93	64 (69%)	15 (16%) ^d	14 (15%)

B. Breakdown of sheath cell defects in *ceh-18(mg57)* according to polyploid oocyte phenotype

	Gonad arms	
	with polyploid oocytes	without polyploid oocytes
Arms with only normal sheath nuclei ^a	20 (51%)	44 (81%)
Arms with abnormal sheath nuclei ^b	5 (13%) ^c	10 (19%) ^c
Not scorable ^c	14 (36%)	0
Total gonad arms ^f	39 (100%)	54 (100%)

Sheath cell nuclei were microscopically examined in DAPI-stained fixed preparations of dissected gonads from adult hermaphrodites.

^aGonad arms were scored as normal if they contained 10 sheath cell nuclei of wild-type size in the correct positions (see Kimble and Hirsh 1979).

^bGonad arms were scored as abnormal if they contained fewer or more than 10 sheath cell nuclei or if the sheath cell nuclei were small in size or misplaced.

^cGonad arms that contained polyploid oocytes were often unscorable because they contained lysed and nonlysed polyploid oocyte nuclei which obscured visualization of sheath cell nuclei.

^d $P < 0.05$ when these values were compared using the χ^2 test.

^eThese values were not significantly different when compared using the χ^2 test.

^f42% of the *ceh-18(mg57)* adult hermaphrodite arms contained polyploid oocytes.

not generate polar bodies as assayed by DAPI staining (data not shown). The conclusion that polar bodies are not generated by polyploid oocytes in *ceh-18* mutants is provisional because additional defects in karyokinesis or cytokinesis might lead to fusion between polar bodies and the endoreduplicating nucleus, and consequently, we may have failed to observe them.

The *ceh-18* defect is specific to meiosis in oogenesis and not in spermatogenesis, as males are unaffected and mate with wild-type efficiency (see legend to Table 1). Moreover, polyploid germ-line nuclei are never observed in hermaphrodites during the L4 larval stage, the time at which spermatogenesis occurs. These observations are consistent with the idea that *ceh-18* is involved in the control of an arrest point specific to oogenesis, as in spermatogenesis there is no meiotic arrest point; unlike oocytes, sperm complete meiosis before fertilization.

The oocyte meiotic defect contributes to the small and

Figure 4. *ceh-18* promotes terminal differentiation of the oocyte. (a,b) Polyploid oocytes in *ceh-18* mutants. DAPI staining of wild type (a) and *ceh-18(mg57)* (b) adult hermaphrodite gonads. The open triangles show polyploid oocytes in *ceh-18(mg57)*. The solid arrowheads indicate diakinesis stage nuclei, and the small solid arrows indicate pachytene stage germ cell nuclei. (c–h) Maternal effect embryonic lethality in *ceh-18* mutants. Nomarski micrographs of wild-type (c,e,g) and *ceh-18(mg57)* (d,f,h) embryos at the four-cell stage (c,d), twofold stage (e,f), and threefold stage (g,h). Note the symmetry of blastomeres in the *mg57* embryos. Bar, 50 μ m. The debris outside of the embryo in h is yeast cells that were inadvertently in the culture.

variable brood sizes in *ceh-18* mutants. Polyploid oocytes were found distal to the spermatheca and never had eggshells, suggesting that they were unfertilized. When abnormal oocytes undergo DNA endoreduplication, they appear to block distal maturing oocytes from entering the spermatheca and being fertilized (Fig. 4). Eventually, the polyploid oocytes appear to lyse and DNA fills an expanded gonad. Each arm of the hermaphrodite gonad is independent and either or both can be sterile in *ceh-18* mutants. If either gonad arm is fertile the hermaphrodite can produce viable progeny. The most severely affected *ceh-18* mutant animals (~16% of adults) produce no fertilized eggs and lay no unfertilized oocytes.

In the hermaphrodite gonad, germ cells differentiate into both sperm and oocytes. Therefore, a possible explanation for the polyploid oocytes observed in *ceh-18* mutants could be that the oocytes become activated by defective sperm or sperm products. To test this possibility, we examined double mutants between *ceh-18(mg57)* and the germ-line sex determination mutants *fem-1(hc17ts)* and *fem-2(b245ts)* that produce no sperm [Nelson et al. 1978; Kimble et al. 1984]. Polyploid oocytes were seen distal to the spermatheca in both double mutants, *ceh-18(mg57); fem-1(hc17ts)* and *ceh-18(mg57); fem-2(b245ts)* at the restrictive temperature, indicating that oocyte DNA endoreduplication can occur in the absence of sperm in the gonad. Thus, the oocyte cell cycle arrest defect is not caused by abnormal fertilization.

In addition to the meiotic defect, there is a mild germ-line hypoproliferation phenotype in *ceh-18* mutants. Although germ-line stem cells proliferate, some animals have smaller gonads than wild type with fewer germ cells. The hypoproliferation phenotype seen in *ceh-18* mutants is less severe than that caused by *glp-1* or *glp-4* mutations [Austin and Kimble 1987; Beanan and Strome 1992]. This germ-line proliferation phenotype might be related to a defect in distal tip cell function (see below) or to a defect in the function of the gonadal sheath cells [J. McCarter, R. Francis, and T. Schedl, pers. comm.).

ceh-18 mutations also lead to an incompletely penetrant maternal effect embryonic lethality (Table 1). Approximately 15% of embryos from homozygous *ceh-18(mg57)* mutant mothers fail to hatch (Table 1). This embryonic lethality was not rescued zygotically (data not shown). Therefore, we studied embryonic development in *ceh-18* mutants by Nomarski microscopy. The terminal phenotype of embryonic lethal *ceh-18* mutant embryos is heterogeneous with developmental arrest occurring at a variety of embryonic stages (Fig. 4). Most of these lethal embryos have a spherical shape in contrast to wild-type embryos that are oval in shape with the long axis corresponding to the anterior–posterior axis. The embryo derives its shape from an eggshell that it synthesizes within the first 30 min after fertilization. Therefore, both the early and heterogeneous phenotypes and the abnormal shape of embryonic lethal *ceh-18* mutant embryos are consistent with a very early defect in embryogenesis and could result from an abnormal oocyte. The maternal effect embryonic lethality could be caused by aneuploidy or polyploidy as a secondary consequence of the oocyte meiotic cell cycle defect. However, we were unable to detect gross alterations in the ploidy of *ceh-18* mutant embryos by DAPI staining. In addition, *ceh-18* mutants do not have a Him (high incidence of males, karyotype XO) phenotype indicating that the frequency of nondisjunction of the X chromosome was the same as in wild type. These data suggest that the embryonic lethality is not simply attributable to random aneuploidy as a consequence of earlier cell cycle defects.

CEH-18 is expressed by the gonadal sheath cells

To understand the basis for the gonadal defects in *ceh-18*

mutants, we prepared antibodies specific for the portion of CEH-18 deleted in *ceh-18(mg57)* (referred to as α -CEH-18 Δ mg57 antibodies) and used these antibodies to determine the *ceh-18* expression pattern. These antibodies are specific for CEH-18, and there is no immunoreactivity in *ceh-18(mg57)* at any stage of development (Fig. 5; data not shown). In addition, these antibodies

detect a protein of ~ 70 kD in Western blots of wild-type *C. elegans*, but not from *ceh-18(mg57)* (Fig. 2C); thus, this protein is encoded by *ceh-18*. In wild type, α -CEH-18 Δ mg57 antibodies detect CEH-18 in the nuclei of the sheath cells of the somatic gonad (Fig. 5). In contrast, CEH-18 is not detected in diakinesis stage oocytes, pachytene stage meiotic germ cells, mitotic germ cells,

Figure 5. Immunofluorescence detection of CEH-18-expressing cells. CEH-18-expressing cells were detected using α -CEH-18 Δ mg57 antibodies to stain wild type (*a, c, g, i*), or *ceh-18(mg57)* (*e*). Nuclei were also visualized with DAPI (*b, d, f, h, j*). The same samples are shown in *a* and *b* (dissected gonad of adult hermaphrodite), *c* and *d* (wild-type threefold-stage embryo), *e* and *f* (*ceh-18(mg57)* threefold-stage embryo), *g* and *h* (wild-type 12-cell embryo), *i* and *j* (wild-type ~ 150 -cell embryo). There are five pairs of gonadal sheath cells per gonad arm, designated 1–5 (see Fig. 3). Only one of each pair is shown; the other five nuclei are out of the plane of focus but also stain. The DAPI image in *b* is in a slightly lower focal plane to show the diakinesis stage nuclei in the proximal arm of the gonad (indicated by arrows). The arrows mark CEH-18-expressing gonadal sheath cell nuclei, which are also numbered. The distal arm of the gonad containing pachytene stage and mitotic germ cell nuclei is indicated by an arrow. Sperm nuclei are indicated. The background in the germ-line cytoplasm is attributable to the secondary antibody. No staining was seen in *ceh-18(mg57)* at any stage of development (see text). Note CEH-18 is first expressed late in embryogenesis at the threefold stage, before hatching, and is absent from early embryos. At hatching, CEH-18 is most abundant in the pharyngeal muscle nuclei, the nuclei of the hypodermal syncytia (hyp 1–11), the nuclei of the seam cells, the P hypodermal blast cells, and the body muscle nuclei. CEH-18 is notably absent from nearly all neurons at this stage, although it is expressed by the anterior touch cells ALML/R. Bars, 50 and 25 μ m, in *a* and *c*, respectively. *a* and *b* were kindly provided by R. Francis and T. Schedl (Washington University School of Medicine, St. Louis, MO).

mature sperm, or fertilized oocytes (Fig. 5; data not shown). These results suggest that *ceh-18* regulates the oocyte cell cycle by acting in cells other than the oocyte. Germ-line stem cells differentiate into oocytes during the adult stage. We determined that CEH-18 is present in sheath cell nuclei as early as the L4 stage (data not shown), before the onset of oogenesis.

Electron micrographs of gonadal sections (Hirsh et al. 1976) have shown that the gonadal sheath cells encircle developing oocytes. Laser ablation of half of the sheath cells can phenocopy the oocyte DNA endoreduplication defect seen in *ceh-18* mutants (J. McCarter, R. Francis, and T. Schedl, pers. comm.). This result suggests that the sheath cells are likely to signal the oocyte to maintain meiotic arrest before fertilization. In contrast to oocytes, sperm do not exhibit meiotic arrest and are unaffected by *ceh-18* mutations. It is interesting to note that the male gonad lacks gonadal sheath cells and is sheathed only partially by the distal cells of the seminal vesicle. This is consistent with the idea of a sex-specific function, signaling to maintain oocyte meiotic arrest, for the gonadal sheath cells.

We considered three hypotheses to explain the meiotic arrest defect in *ceh-18* mutants in the absence of *ceh-18* expression in oocytes. In the first hypothesis, *ceh-18* might affect the gonadal sheath cell lineages such that the correct number of sheath cells are not generated in *ceh-18* mutants. According to this hypothesis, the oocyte meiotic arrest defect would be a secondary consequence of the absence of sheath cells. The second hypothesis we considered was that *ceh-18* could function in the differentiation of sheath cells. According to this hypothesis, *ceh-18* might affect directly or indirectly the production or transmission of sheath cell to oocyte signals that regulate meiotic arrest. A third hypothesis is that a meiotic arrest signal to the germ-line arises from elsewhere in the animal. Besides gonadal sheath cells, many other cells, including syncytial hypodermal cells (hyp 7), located throughout the body, also express *ceh-18* (see Fig. 5 and legend). According to this hypothesis, *ceh-18* mutations would perturb the oocyte cell cycle by a different mechanism than the sheath cell ablations.

To distinguish between the first two hypotheses (e.g., lineage vs. differentiation), we analyzed the number, size, and positions of sheath cell nuclei in *ceh-18(mg57)* adult hermaphrodite gonads (Table 2). We determined that sheath cells are generated in *ceh-18(mg57)* hermaphrodites and that the majority of gonad arms contained wild-type numbers of sheath cell nuclei (10) located in the correct positions. Significantly, we observed many *ceh-18* mutant gonad arms (20/39) that contained polyploid oocytes, yet had a normal number of gonadal sheath nuclei with wild-type morphology and positions (Table 2B). Thus, we conclude that the oocyte meiotic arrest phenotype is not a secondary consequence of changes in the lineages that generate the sheath cells. We also used anti-desmosomal monoclonal antibody MH27 to analyze the generation of spermathecal cells in *ceh-18(mg57)* hermaphrodites, as the lineages that generate the sheath cells also generate spermathecal cells

(Kimble and Hirsh 1979). We determined that *ceh-18(mg57)* hermaphrodites contain the correct number of spermathecal cells, which further suggests that the sheath cell lineages are normal in *ceh-18* mutants. However, we observed a small but significant percentage of *ceh-18* mutant gonad arms (16%) that were abnormal in that one or two sheath cell nuclei were small, misplaced, or missing. We observed one gonad arm that contained an extra sheath cell nucleus. Such defects were observed at a much lower frequency in wild-type gonads (Table 2). These observed sheath cell abnormalities were independent of the oocyte meiotic arrest defect. Our observation of *ceh-18* mutant gonad arms that contained abnormal sheath cells is suggestive of a role for *ceh-18* in the differentiation of sheath cells but not in their generation.

The maternal effect embryonic lethality that we observe, in principle could be attributable to *ceh-18* action in the gonad, the embryo, or both. In wild-type embryos, CEH-18 is first detected in the nuclei of many but not all cells late in embryogenesis, at the threefold stage before hatching (Fig. 5). However, we observed no *ceh-18* expression at the early embryonic stages affected by *ceh-18* mutations (Fig. 5). Because *ceh-18* mutants often exhibit defects before the late embryonic stage when expression is first observed, *ceh-18* might act in the gonadal sheath cells to regulate embryogenesis indirectly.

ceh-18 functions in germ-line migration

In addition to a defect in oogenesis, *ceh-18* mutants exhibit an incompletely penetrant gonad migration defect. Approximately 27% of hermaphrodite mutant gonad arms migrate along abnormal trajectories, often migrating into the head or tail rather than reflexing into the mid body region as in wild type (Fig. 6 and Table 1). The gonad migration defective phenotype is not correlated with the sterile phenotype: We observed both sterile animals with normal gonad migration and fertile animals with abnormal gonad migration. Immunofluorescence detection of CEH-18 in migrating hermaphrodite gonads indicated that CEH-18 is expressed by the distal tip cell (dte) of the somatic gonad from early L2 through the adult stage (Fig. 6). The dte has been demonstrated to direct migration of the gonad (Kimble and White 1981). Therefore, a simple model is that CEH-18 expression by the dte might mediate its function in directing the migration of the gonad.

ceh-18 regulates epidermal differentiation

ceh-18 mutations result in an incompletely penetrant larval arrest phenotype that is observed most frequently as an L1 lethal phenotype (see Table 1). This larval arrest phenotype is not strictly zygotic. We were able to phenocopy this L1 lethal phenotype by expression of antisense *ceh-18* mRNA from a heat shock promoter during the L1 larval stage (data not shown). Because CEH-18 is expressed widely in many epidermal cells at hatching (see Fig. 5), we analyzed the differentiation of lateral hypodermal seam cells in *ceh-18* mutants. mAb NE2/1B4-

Figure 6. *ceh-18* mutant phenotypes. Gonad migration in wild-type (a) and *ceh-18(mg57)* (b) adult hermaphrodites visualized by DAPI staining. An arrow marks the distal region of the anterior gonad arm. Note that in *ceh-18(mg57)*, the distal arm of the gonad is positioned close to neurons in the nerve ring (Δ) and an anterior intestinal cell nucleus (arrow in b), whereas in wild type the distal gonad arm is positioned posterior to the anterior intestinal nucleus (arrowhead in a). Expression of CEH-18 in the dtc was detected using α -CEH-18 Δ mg57 antibodies in the developing gonad of a wild-type L3 larva (c). (d) The DAPI stain of the same animal; the germ cell nuclei do not express CEH-18 (arrow). Lateral hypodermal seam cells in L1-stage larvae were analyzed in wild type (e) and *ceh-18(mg57)* (f) using immunofluorescence with mAb NE2/1B4-14. Bar, 50 μ m. Anterior is to the left.

14 detects an unknown antigen in a characteristic pattern that is specific for the seam cells of the lateral epidermis (J. Rothman, pers. comm.). We observe that the components of the seam cell-specific antigen detected by NE2/1B4-14 are disorganized in *ceh-18(mg57)* (Fig. 6). Moreover, ectopic staining is seen in cells of the large hypodermal syncytium hyp 7 compared with wild type. This abnormality in hypodermal cell differentiation, as detected by NE2/1B4-14, is highly penetrant; 124 of 126 L1 stage animals were scored as abnormal. This suggests that *ceh-18* is required for the structure and/or function of the L1-specific epidermis.

Function of *ceh-18* in the absence of a POUhd or POUsp

We isolated two *ceh-18* mutant alleles (*mg58* and *mg62*) in which the seventh exon encoding the POUhd was deleted, but all exons 5' to exon 7 were intact, including the one encoding the POUsp domain (Figs. 1 and 2). We find that both *ceh-18(mg58)* and *ceh-18(mg62)* are less severe than *ceh-18(mg57)* but display the same range of phenotypes (Table 1; data not shown). Specifically, *ceh-18(mg58)* and *ceh-18(mg62)* are more fertile, exhibit a reduction in the penetrance of the maternal effect embryonic lethality and larval arrest phenotypes, move better, and appear healthier upon examination under the dissecting microscope than *ceh-18(mg57)*. Moreover, the penetrance of the oocyte DNA endoreduplication defect and the gonad migration defect were also reduced in both *ceh-18(mg58)* and *ceh-18(mg62)*. Because *ceh-18(mg57)*

is likely to be null, we infer that *ceh-18(mg58)* and *ceh-18(mg62)* are both non-null alleles and, therefore, *ceh-18* can have residual in vivo function in the absence of a homeo box.

Similarly, we isolated another mutant allele, *ceh-18(mg61)*, which deletes the POUsp but retains the POUhd and is also less severe than *ceh-18(mg57)* (see Table 1). *ceh-18(mg61)* results from a deletion that removes the splice acceptor sequence of intron 5, all of exon 6 that encodes the POUsp, all of intron 6, and 9 bp of exon 7. Because *ceh-18(mg61)* is less severe than *ceh-18(mg57)* and because *ceh-18(mg61)* animals reacted with the α -CEH-18 Δ mg57 antibodies on immunostaining (Table 1; data not shown), we considered the possibility that the *ceh-18(mg61)* transcript might be spliced so as to contain the homeo box. We characterized the spliced transcripts from *ceh-18(mg61)* using reverse transcription and PCR (RT-PCR). The results indicate that the extant splice donor in intron 5 in *ceh-18(mg61)* is spliced in-frame to the C-residue at the position 14 of exon 7 corresponding to the POU domain linker (see Fig. 1). The splice acceptor is 5'-TTACAT-3' instead of the consensus 5'-TTTCAG-3'. We estimate that ~30% of the message is spliced in this manner, and the remaining 70% is not spliced from the intron 5 donor (data not shown). The protein encoded by *ceh-18(mg61)*, therefore, would contain a POUhd but not a POUsp. Western blot analysis of protein extracts prepared from the *ceh-18(mg61)* mutant strains with the α -CEH-18 Δ mg57 antibodies indicates that the encoded protein product is ~20 kD smaller in the mutant, consistent with the

results of the RT-PCR experiment (see Fig. 2C). Because *ceh-18(mg61)* results in a less severe phenotype than *ceh-18(mg57)*, we conclude that the POUhd in the absence of a POUsp is sufficient for partial *ceh-18* activity in vivo. The *mg58/mg61* trans-heterozygote exhibits a *ceh-18* mutant phenotype, indicating that the encoded proteins, each of which exhibits partial function, are unable to compensate for one another.

Discussion

Reverse genetic approaches to analyze gene function directly are now possible in *C. elegans* using a targeted gene knockout strategy (Zwaal et al. 1993). We have taken this approach because many members of developmental control gene families have been defined molecularly, but not genetically, especially by the *C. elegans* genome sequencing project (Sulston et al. 1992). For example, only one-third of the known *C. elegans* homeobox-containing genes have been defined genetically (Bürlin et al. 1989). We have used this approach both to study the in vivo role of a divergent POU gene, *ceh-18*, and to dissect its functional domains in vivo.

We find that *ceh-18* loss-of-function (*lf*) mutations are pleiotropic and incompletely penetrant, affecting a number of developmental processes. Analysis of these mutations suggests that *ceh-18* plays several roles in the development of the hermaphrodite germ line. We find that *ceh-18(lf)* mutations profoundly affect the meiotic cell cycle during oogenesis. In the absence of functional CEH-18, oocytes exhibit a meiotic cell cycle defect—they can undergo multiple rounds of DNA replication in the absence of fertilization and without completing meiosis. When oocytes undergo DNA endoreduplication in *ceh-18* mutants, it is not clear whether there are repeated rounds of S phase or rather uncontrolled DNA replication. Our data do not differentiate between removal of a block to S phase in oogenesis or ectopic production of an S phase signal.

In contrast to *ceh-18* mutants, in wild type the meiotic cell cycle is modified to allow two rounds of chromosome segregation without intervening DNA replication. This difference suggests that *ceh-18* mutations may define a meiotic cell cycle checkpoint in oogenesis in accordance with the definition of an *lf* mutation that removes a dependent event in the cell cycle (Hartwell 1991), in this case, the dependence of S phase on completion of the events of meiosis and fertilization. Another possibility is that *ceh-18* mutations disrupt a meiotic cell cycle arrest point and that there is no checkpoint in oogenesis. In fact, wild-type oocytes are released from diakinesis arrest only after they leave the proximal arm of the gonad and enter the spermatheca (Ward and Carrel 1979). Moreover, DNA endoreduplication can be observed in unfertilized oocytes of wild type that have been activated by entering a spermatheca that is depleted of sperm (Ward and Carrel 1979). However, in wild type this activation never occurs distal to the spermatheca, whereas in *ceh-18* mutants we observe polyploid oocytes

distal to the spermatheca, in the proximal arm of the gonad (Fig. 3).

Our data suggest that *ceh-18* does not act within the oocyte to control its cell cycle, as *ceh-18* is not expressed in the oocyte at the level of detection of immunostaining. Our results are more consistent with a nonautonomous role in oogenesis involving cell interactions between the *ceh-18*-expressing gonadal sheath cells and developing oocytes. Mosaic analysis ultimately will be important to determine whether the function of *ceh-18* is nonautonomous with respect to the oocyte cell cycle defect. On the basis of laser ablation of gonadal sheath cells and sheath cell precursors, which results in an oocyte DNA endoreduplication phenotype, J. McCarter, R. Francis, and T. Schedl (pers. comm.) have proposed that the sheath cells signal to maintain oocyte meiotic cell cycle arrest. Thus, a possible molecular mechanism for action of *ceh-18* in the gonadal sheath cells would be the control of signals for oocyte cell cycle arrest before fertilization. *ceh-18* mutations could affect the production or transmission of these signals indirectly by altering sheath cell differentiation, or these signals could be directly downstream of *ceh-18*. It is well established that growth factors, such as transforming growth factor (TGF- β), can arrest the cell cycle in G₁ (Kingsley 1994). Thus far, no growth factor has been described that confers diakinesis arrest in meiotic prophase I and prevents DNA endoreduplication. Our data imply the existence of such a sheath cell to oocyte signal, either directly or indirectly downstream of *ceh-18*.

In vertebrates, oocyte arrest in meiosis I can be released by progesterone treatment that leads to activation of PI3 kinase, Mos kinase, and ultimately MPF (Sagata et al. 1988, 1989; Smith 1989; Muslin et al. 1993b). Recent evidence has implicated an evolutionarily conserved protein kinase cascade, including Raf-1 protein kinase and MAP kinase in the progesterone-triggered signal transduction pathway in the oocyte that leads to the activation of MPF (Muslin et al. 1993a; Nebreda and Hunt 1993). Therefore, we suggest that the most likely explanation for the aberrant oocyte cell cycles in *ceh-18* mutants is that there exists a defect in the production or transmission of the sheath cell signal that normally regulates the pathway to meiotic arrest and DNA replication inhibition in oocytes. Given the conservation of the signal transduction pathway by which oocyte maturation is achieved, it is tempting to speculate that the arrest mechanisms will also be conserved and may even involve negative controls on the maturation pathway.

In *Drosophila*, the interacting maternal effect genes *gnu*, *pan gu*, and *plutonium* have been shown to regulate DNA replication subsequent to oogenesis after completion of meiosis and during early embryogenesis (Freeman and Glover 1987; Shemanski and Orr-Weaver 1991). Recently, *plutonium* has been cloned and shown to encode an ankyrin repeat protein that has been proposed to function by binding and inhibiting the function of an essential DNA replication factor (Axton et al. 1994). If analogous factors are participating in oogenesis in *C. elegans*, they could also act in a pathway with *ceh-18*.

ceh-18(lf) mutations also cause maternal effect embryonic lethality that taken together with the expression data are suggestive of somatic gonad to oocyte signaling. We observe that *ceh-18* mutant embryos exhibit defects in embryogenesis long before the point at which CEH-18 is expressed, late in embryogenesis. Therefore, the embryonic lethality is most likely caused by disruption of *ceh-18* function in the parent, as CEH-18 is not expressed early in embryogenesis and the lethality is not zygotically rescued. Furthermore, *ceh-18* mutant embryos often have an abnormal spherical shape that is indicative of a very early defect in embryogenesis. Interestingly, symmetrical embryos are observed in the *Drosophila* mutants *gurken*, *torso-like*, and *torso* that disrupt signaling between follicle cells and the oocyte (Sprenger et al. 1989; Stevens et al. 1990; Neuman-Silberberg and Schüpbach 1993; Savant-Bhonsale and Montell 1993). It is possible that like the follicle cells in the *Drosophila* mutants, *ceh-18* mutant sheath cells are defective in the production of a signal that normally induces oocyte asymmetry.

Our analysis indicates that *ceh-18* also plays a role in directing gonad migration and is expressed by the dtc. Laser ablation experiments have demonstrated that the dtc leads gonad migration (Kimble and White 1981). Other homeo proteins have been implicated in the control of cell migration by acting in migrating cells (Salser and Kenyon 1992; Niehrs et al. 1993). *ceh-18* might regulate expression of a guidance receptor in the dtc for migration into the mid-body region or production of extracellular guidance cues from the epidermis.

An important attribute of *ceh-18* mutations is that they are incompletely penetrant. Because our strongest allele, *ceh-18(mg57)*, is a good candidate for a null mutation (see Results), we suggest that incomplete penetrance is more likely to result from other genes with partially redundant activities.

We have isolated two classes of non-null *ceh-18* alleles. Two alleles, *mg58* and *mg62*, have significant in vivo activity despite lacking a POUhd. Likewise, *mg61* also retains partial in vivo function despite lacking a POUsp. In vitro binding studies have shown that both the POUsp and the POUhd make DNA contacts and are both required for high-affinity DNA binding (Sturm and Herr 1988; Ingraham et al. 1990; Kristie and Sharp 1990; Verrijzer et al. 1990a,b, 1992). Isolated POUhds and POUsp have been demonstrated to mediate low-affinity DNA binding in vitro (Sturm and Herr 1988; Ingraham et al. 1990; Kristie and Sharp 1990; Verrijzer et al. 1990b, 1992). This low-affinity in vitro DNA binding may not be sufficient to explain the partial in vivo function of mutants that lack the POUsp or the POUhd. Therefore, interactions with other proteins, possibly those postulated to have redundant activities (see above), may be important in vivo to stabilize DNA binding by these mutant CEH-18 proteins and could contribute to the normal function of the wild-type protein. In addition, other regions of CEH-18, such as the amino-terminal acidic stretch, may also contribute to in vivo function.

Recently, the three-dimensional structure of the Oct-1

POU domain bound to DNA was determined by X-ray crystallography of the cocrystal (Klemm et al. 1994). These results show that both the POUsp and the POUhd constitute independent DNA-binding domains, both making major groove contacts on opposite faces of the DNA helix without interdomain protein-protein interactions. Thus, our genetic results are consistent with the X-ray crystallographic results and show that each POU subdomain is able to function independently in the control of oocyte meiotic cell cycle arrest.

Materials and methods

Isolation of *ceh-18*

Standard molecular biological techniques were used. An amplified λ gt10 cDNA library from a mixed stage population of wild-type *C. elegans* (gift of S. Kim, Stanford University, CA) was screened with a mixture of two degenerate oligonucleotides POU-1 and POU-2, as described (Bürglin et al. 1989). Ten positive phage were obtained from $\sim 2 \times 10^6$ phage screened. A positive phage with the largest insert ($\phi 6$) was sequenced completely on both strands using Sequenase v. 2.0 (U.S. Biochemical). The structure of the *ceh-18* cDNA was verified further by directly sequencing DNA fragments PCR-amplified from oligo(dT)-primed first-strand cDNA. Genomic clones were isolated by screening cosmid genomic libraries using the $\phi 6$ cDNA as a probe. Two overlapping cosmids, C50A9 and LLC5, were isolated and positioned on the physical map by fingerprinting (Coulson et al. 1986). The positions of the intron/exon boundaries and the indicated intron sequences were determined by sequencing directly from cosmid DNA and DNA fragments amplified directly from genomic DNA. Intron sizes were determined exactly by sequencing or estimated by sizing restriction fragments amplified with flanking primers. The physical and genetic maps in the *ceh-18* region were correlated as follows. The RFLPs *mgP39* and *mgP40* were detected by Southern hybridization of restriction enzyme digests (*Hind*III for *mgP39* and *Cla*I for *mgP40*) of total genomic DNA from *C. elegans* var. Bristol and Bergerac, using the cosmids F14H9 and T14G12 as probes, respectively. Recombinants were isolated from the strain *unc-78(e1217) lin-18(e620) lon-2(e678)/+ + +* (Bergerac RW7000). Homozygous Non-Unc Lin Lon ($n=19$) and Unc Non-Lin Non-Lon ($n=7$) recombinants were analyzed by whole genomic Southern blots to give the genetic order *unc-78* (9/25) *mgP40* (4/25) *mgP39* (12/25) *lin-18*. In addition, 6/6 Non-Unc Non-Lin Lon recombinants had the Bergerac pattern for both *mgP39* and *mgP40*, whereas 4/4 Unc Lin Non-Lon recombinants had the Bristol pattern for both *mgP39* and *mgP40*.

Isolation of *Tc1* insertions in *ceh-18* and deletion derivatives

Transposable elements were targeted using a PCR and sib-selection scheme (Ballinger and Benzer 1989; Kaiser and Goodwin 1990) as modified for *C. elegans* and its transposable elements (Rushforth et al. 1993; Zwaal et al. 1993). The positions of the *Tc1* insertions were confirmed with multiple PCR reactions using flanking primers, followed by directly sequencing PCR products from both insertion junctions. Deletion derivatives resulting from imprecise excision of *Tc1* from *ceh-18(pk37::Tc1)* were identified by PCR using c18-39 (5'-CCCACACCAAGTTTCCACAAATGGC-3') and c18-26 (5'-AGGCTAGAAAGTTCTACGGG-3') primers. Single worm PCR reactions were carried out as described (Williams et al. 1992). (Details of the PCR and sib-selection procedure can be obtained by anonymous ftp or

gopher from weeds.mgh.harvard.edu.) The deletions were characterized by directly sequencing the PCR products shown in Figure 2. Upon isolation, the heterozygous deletion strains were outcrossed to wild type at least seven times, and the arms of the X chromosome were crossed off in genetic mapping experiments. *ceh-18(mg57)* was genetically mapped as follows. Unc Non-Lin Non-Lon recombinants and Non-Unc Lin Lon recombinants were selected from the strain + *ceh-18(mg57)* + +/*unc-78(e1217)* + *lin-18(e620)* *lon-2(e678)*; the results were *unc-78(e1217)* 19/43 *ceh-18(mg57)* 24/43 *lin-18(e620)*.

Preparation of α -CEH-18 Δ mg57 antibodies, Western blotting, immunofluorescence, and microscopy

Full-length CEH-18 was expressed in *Escherichia coli* starting with the first methionine in the cDNA. The cDNA sequence (5'-GCAAGAAATATG-3'), 5' to the ATG, was altered to (5'-GCATGCCATATG-3') using PCR. The entire restriction fragment subjected to PCR was sequenced to ensure that no errors were introduced by *Taq* polymerase. The resulting *Nde*I fragment was then cloned into pEt3a (Studier et al. 1990) to create pCEH-18T7. Antisera were produced by injecting gel-purified CEH-18 into two rabbits as described (Finney and Ruvkun 1990). Sera from both rabbits yielded identical results. Affinity-purified sera specific to the portion of CEH-18 deleted in *ceh-18(mg57)* (CEH-18 Δ mg57) were prepared by chromatographing α -CEH-18 IgG on CEH-18 Δ mg57 coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia). The plasmid (pCEH-18 Δ mg57) encoding a His-tagged derivative of CEH-18 corresponding to the portion of the protein deleted in *ceh-18(mg57)* was constructed as follows. The oligonucleotides c18-45 (5'-CGCGGATCCATCGAGGGTAGAGCAAATGGTCTGGGTGCTTCA-3') and c18-46 (5'-CGCGGATCCACTGTCATCATGTGCTGGCTTG-3') were used in PCR reactions using pCEH-18T7 as template. The resulting product was digested with *Bam*HI and cloned into pQE-8 (Qiagen) cut with *Bam*HI.

For Western blotting, protein extracts were prepared from newly hatched L1 stage animals by boiling for 30 min in 50 mM Tris-Cl (pH 6.8), 4% SDS, and β -mercaptoethanol. L1 stage animals were used because CEH-18 appears to be most stable at this stage. Similar Western blot results were obtained with other stages. The protein was transferred to nitrocellulose and analyzed with α -CEH-18 Δ mg57 antibodies (1:5000) using the ECL detection system (Amersham Inc.) CEH-18 migrates slightly slower than the predicted molecular mass of 65 kD; however, as CEH-18 from *C. elegans* extracts comigrates with both in vitro-translated CEH-18 and CEH-18 expressed in *E. coli* (data not shown), this anomaly is likely to be an intrinsic property of the protein and not caused by post-translational modifications.

Immunostaining of fixed preparations was carried out using affinity-purified antisera as described previously (Finney and Ruvkun 1990) and visualized using a Zeiss microscope equipped for fluorescence microscopy.

Embryos were mounted (Sulston et al. 1983) for microscopy and visualized using differential interference contrast microscopy. mAb NE2/1B4-14 was a gift of J. Rothman (University of Wisconsin, Madison).

RT-PCR analysis of *ceh-18(mg61)*

RNA was prepared from N2 and *ceh-18(mg61)*, and first-strand cDNA was prepared using an oligo(dT) primer and SuperScript RNase H⁻ Reverse Transcriptase (GIBCO-BRL). Nested PCR was conducted using SL1 and c18-26 primers in the first PCR reaction and c18-11 (5'-AGTCCATCTTTGTCTGGGAGCCAGC-3') and c18-40 (5'-CCGAGAAATCAAACGTGCGAAT-

CAG-3') in the second PCR. The cDNA from *ceh-18(mg61)* was a mixture of two products: 70% was spliced at all introns except the intron 5 donor; 30% was spliced at the intron 5 donor to the novel acceptor 5'-TTACAT-3' formed by the deletion junction indicated in Figure 1. The spliced and unspliced PCR products were separated on a 4% nondenaturing polyacrylamide gel and sequenced directly.

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