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Three genes of the MAP kinase cascade, *mek-2*, *mpk-1/sur-1* and *let-60 ras*, are required for meiotic cell cycle progression in *Caenorhabditis elegans*

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SUMMARY

In the germline of *Caenorhabditis elegans* hermaphrodites, meiotic cell cycle progression occurs in spatially restricted regions. Immediately after leaving the distal mitotic region, germ cells enter meiosis and thereafter remain in the pachytene stage of first meiotic prophase for an extended period. At the dorsoventral gonadal flexure, germ cells exit pachytene and subsequently become arrested in diakinesis. We have found that exit from pachytene is dependent on the function of three members of the MAP kinase signaling cascade. One of these genes, *mek-2*, is a newly identified *C. elegans* MEK (MAP kinase kinase). The other two genes, *mpk-1*/*sur-1* (MAP kinase) and *let-60 ras*, were previously identified based on their roles in vulval induction and are shown here to act in combination with *mek-2* to permit exit from pachytene. Through genetic mosaic analysis, we demonstrate that the expression of *mpk-1*/*sur-1* is required within the germline to permit exit from pachytene.

Key words: meiosis, cell cycle, MAP kinase, *ras*, MEK, *Caenorhabditis elegans*

INTRODUCTION

The growth, division and differentiation of eukaryotic cells are influenced by a wide range of extracellular signals. Perception of many of these signals is mediated by the activation of MAPK (mitogen activated protein kinase) (reviewed in Blenis, 1993; Davis, 1993; Nishida and Gotoh, 1993; Cooper, 1994; Johnson and Vaillancourt, 1994). Two major routes have been described that lead to the activation of MAPK. One is initiated by the activation of receptor tyrosine kinases (RTKs), while the other begins with the activation of G-protein-coupled heptahelical receptors. In the case of RTKs, intermediary proteins then relay the signal, resulting in the activation of ras. Activated ras then promotes the activation/phosphorylation of MEK (MAPK kinase) and, consequently, MAPK. Either raf or MEKK (MEK kinase) is capable of serving as the intermediary between ras and MEK. Once MAPK is activated, it is competent to phosphorylate a variety of substrates, including transcription factors, transmembrane receptors and elements of the cytoskeleton. Since there is divergence in the signaling pathways both upstream and downstream of MAPK, it is probably more accurate to think of the MAPK signaling system as a network rather than a linear pathway. Cellular responses to the activation of MAPK vary depending on the type of cell and the stage of the cell cycle. For example, rat PC12 cells respond by undergoing differentiation (Gomez and Cohen, 1991); *Xenopus* oocytes are triggered to complete meiosis I and arrest in metaphase II (Posada and Cooper, 1992); mouse NIH3T3 cells undergo mitotic proliferation (Seger et al., 1994).

Genetic systems (yeast, *Drosophila*, *C. elegans*) have been very useful for identifying components of the MAPK network and for understanding their in vivo functions during development. In *C. elegans*, MAPK-mediated signaling is probably required at multiple stages of development. Mutation of either *let-60 ras* (Moerman and Baillie, 1981; Beitel et al., 1990; Han and Sternberg, 1990) or *let-23* (a putative RTK; Herman, 1978; Aroian et al., 1990) results in first larval stage lethality. Analyses of hypomorphic and conditional alleles of these genes indicate that they are required postembryonically for the induction of specific cell fates in the hermaphrodite vulva and for the coordination of fates in the developing male tail (Ferguson and Horvitz, 1985; Aroian et al., 1990; Beitel et al., 1990; Han and Sternberg, 1990). Mutant alleles of *raf* (*lin-45*) (Han et al., 1993) and MAPK (*mpk-1*/*sur-1*) (Lackner et al., 1994; Wu and Han, 1994) have also been identified, based on their effects on vulval induction. The roles of MAPK-mediated signaling in other tissues within the nematode have not been extensively characterized.

Because mutations in several of the above genes result in sterility (Aroian et al., 1990; Beitel et al., 1990; Han et al., 1990), it seems possible that MAPK is also important in one or more types of intercellular signaling that regulate cell cycle progression within the *C. elegans* germline (Lambie and Kimble, 1991a; Schedl, 1991). One potential role for MAPK-mediated signaling involves the regulation of meiotic cell cycle progression that occurs after germ cells have left the mitotic distal tip region (Nigon and Brun, 1955; Hirsh et al., 1976; Klass et al., 1976). In the adult gonads of both males and hermaphrodites, germ cells that have entered meiosis remain in the pachytene stage of meiosis I for an extended period. Germ

cells do not ordinarily exit pachytene until they arrive at the flexure that connects the dorsal and ventral portions of the proximal gonad. At this point, there is a dramatic condensation of the chromosomes and a rapid increase in cytoplasmic volume. This transition, which we refer to as 'exit from pachytene', bears similarities to both the growth and maturation stages of vertebrate oocytes (Masui and Clarke, 1979). In hermaphrodites, the germ cells (primary oocytes) that have exited pachytene become arrested once again in meiosis I prophase, this time in diakinesis. Diakinesis arrest is maintained until immediately prior to fertilization (Ward and Carrel, 1979), and may be influenced by the gonadal sheath cells (Greenstein et al., 1994). In males, exit from pachytene leads directly to the completion of both meiotic divisions.

In this paper, we report that mutations in *mek-2*(MEK), *mpk-1*/*sur-1*(MAPK) and *let-60 ras* prevent meiotic prophase progression in *C. elegans*. Our results suggest that MAPK-mediated signaling is required within the germline for the execution of exit from pachytene.

MATERIALS AND METHODS

General methods

Nematodes were cultured on MYOB plates inoculated with *Escherichia coli* strain OP50, using methods similar to those described by Brenner (1974). Unless stated otherwise, all chemicals were from Sigma (St Louis, MO). MYOB plates were prepared as follows: A powdered mixture was made by combining 55 g Tris-Cl (reagent grade), 24 g Tris-OH (reagent grade), 460 g Bacto Tryptone (Difco), 800 mg cholesterol and 200 g NaCl. 7.4 g of this mix was dissolved in 1 liter of water, 20 g of agar was added and the mixture autoclaved. Stocks were maintained at 15°C, 20°C or 25°C, depending on the requirements of individual experiments.

Microscopy and imaging

Living nematodes were mounted on thin pads of 3% agarose, essentially as described by Sulston and Horvitz (1977), except that pads were made by cutting off pieces of previously prepared strips of agarose stored in 15 mM EDTA. In some cases, nematodes were anesthetized by adding 50 mM sodium azide to the mounting medium.

Microscopy was performed using a Nikon Microphot-SA fitted with standard epifluorescence filters and Nomarski differential interference contrast optics. Photographs were taken with a Nikon N6000 SLR camera connected to a Nikon PFX photomicroscopy unit. Fig. 5C was assembled in Canvas (Deneba) using images acquired with a Sony AVC-D7 CCD camera that was serially connected to a ComputerEyes R/T Framegrabber board and a Macintosh LCIII computer.

DAPI staining

Chromosomes in intact animals were stained as follows. Nematodes were picked into a small drop of water (≤ 10 μl) in a well slide. Next, 300 μl of 200 ng/ml 4′,6-diamidino-2-phenylindole (DAPI; dissolved in 95% ethanol) was pipetted into the well and the well was covered to prevent evaporation. After ≥30 minutes at room temperature, animals were mounted on agarose pads (as for Nomarski microscopy) and either viewed immediately or stored at −20°C.

Nematode strains

All nematodes used were derived from wild-type strain N2 (*Caenorhabditis elegans* var. Bristol). Standard genetic methods were used for strain constructions and genetic analysis (Sulston and Hodgkin, 1988).

The following mutations and rearrangements were used (reviewed and summarized by Hodgkin et al. (1988), unless indicated otherwise): Linkage group (LG) I: *sup-11(n403n682)*; *fog-1(q253)* (Barton and Kimble, 1990), *unc-11(e47)*; *dpy-5(e61)*; *unc-13(e51)*, *tDf3* (R. Feichtinger, personal communication), *qDf4* (Ellis and Kimble, 1995), *sDp2* (Rose et al., 1984). LG III: *ncl-1(e1865)*, *unc-36(e251)*, *unc-32(e189)*, *glp-1 (q231)* (Austin and Kimble, 1987), *glp-1 (q175)* (Austin and Kimble, 1987), *lin-1(e1026)*, *lin-1(e1275)*, *sDp3* (Rosenbluth et al., 1985). LG IV: *fem-3(q20)*, *dpy-20(e1282)*. LG V: *let-60(n1046)* (a.k.a. *lin-34*), *let-60(s1124)*, *let-60(s1155)*, *let-60(n2031dn)* (Beitel et al., 1990), *let-60(n2021)* (Beitel et al., 1990), *unc-22(s7)*, *unc-31(e169)*, *lin-8(n111)*, *lin-9(n112)*. LG X: *lin-15(n309)*.

Isolation of *mek-2* alleles

mek-2(q425) and *mek-2(q484)* were identified during clonal mutant screens (using EMS and 310 nm UV, respectively, as mutagens) that were designed to isolate recessive larval lethal and/or sterile mutants (Lambie and Kimble, 1991b; Henderson et al., 1994). During this screen, *mek-2(q425)* homozygotes were initially identified by dissecting microscope based on their sterile (Ste) vulvaless (Vul) phenotype. *q425* was retained for further study after inspection by Nomarski and DAPI staining revealed that the proximal germ cells were arrested in pachytene. *mek-2(q484)* was recognized in subsequent screening based on its resemblance to *mek-2(q425)*. *mek-2(h294)* (a.k.a. *let-537(h294)*) was generated by Kim McKim in Ann Rose's lab (University of British Columbia) during a screen for EMS-induced lethal/sterile mutations on chromosome I (McKim, 1990).

Isolation of *let-60(n1046dx1)*

let-60(n1046dx1) was isolated in a pilot screen for dominant suppressors of the multivulva (Muv) phenotype of *let-60(n1046)*. *let-60(n1046)* hermaphrodites were mutagenized with 50 mM EMS (Brenner, 1974) and non-Muv F_1s cloned at 25°C. Of approximately 100 non-Muv clones examined, one segregated sterile vulvaless progeny (of genotype *n1046dx1/n1046dx1*). Outcrossing of the *n1046/n1046dx1* strain revealed that *n1046dx1* causes recessive L1 lethality when it is derived from an *n1046dx1/+* mother. *n1046dx1* fails to complement the loss-of-function mutation, *let-60(s1124*).

Mapping of *mek-2*

mek-2(q425) was initially assigned to LGI based on moderate linkage to *dpy-5(e61)*. Subsequent three factor mapping placed *mek-2* near and to the left of *fog-1*. To localize *mek-2* more precisely, we mapped it relative to *sup-11* by picking Unc non-Sup recombinants from *sup-11(n403n682) unc-11(e47)/mek-2(q425)* hermaphrodites. Since 20/24 recombinants segregated *mek-2(q425)*, we conclude that *mek-2* is to the right of *sup-11*.

mek-2 was further localized by its inability to complement either of two deficiencies in the region, *tDf3* and *qDf4*. Since no other genes are known to be deleted by both *tDf3* and *qDf4*, we made the *tDf3/qDf4 trans*-heterozygote to assess its phenotype. *tDf3/qDf4* animals (identity confirmed by PCR; data not shown) exhibit first larval stage lethality, similar to that of *let-60 ras* mutants. This is consistent with the null phenotype of *mek-2* being a first larval stage lethal; however, we cannot exclude the possibility that this phenotype is due to the disruption in both deficiencies of a gene distinct from *mek-2*.

RESULTS

Identification of mutations that prevent exit from pachytene

In the germline of *C. elegans* hermaphrodites, the transition from pachytene to diakinesis is accompanied by increases in cytoplasmic and nuclear volume and a dramatic condensation of the chromosomes (Fig. 1A,C). To understand how this transition is regulated, we undertook to study two mutations, *q425*

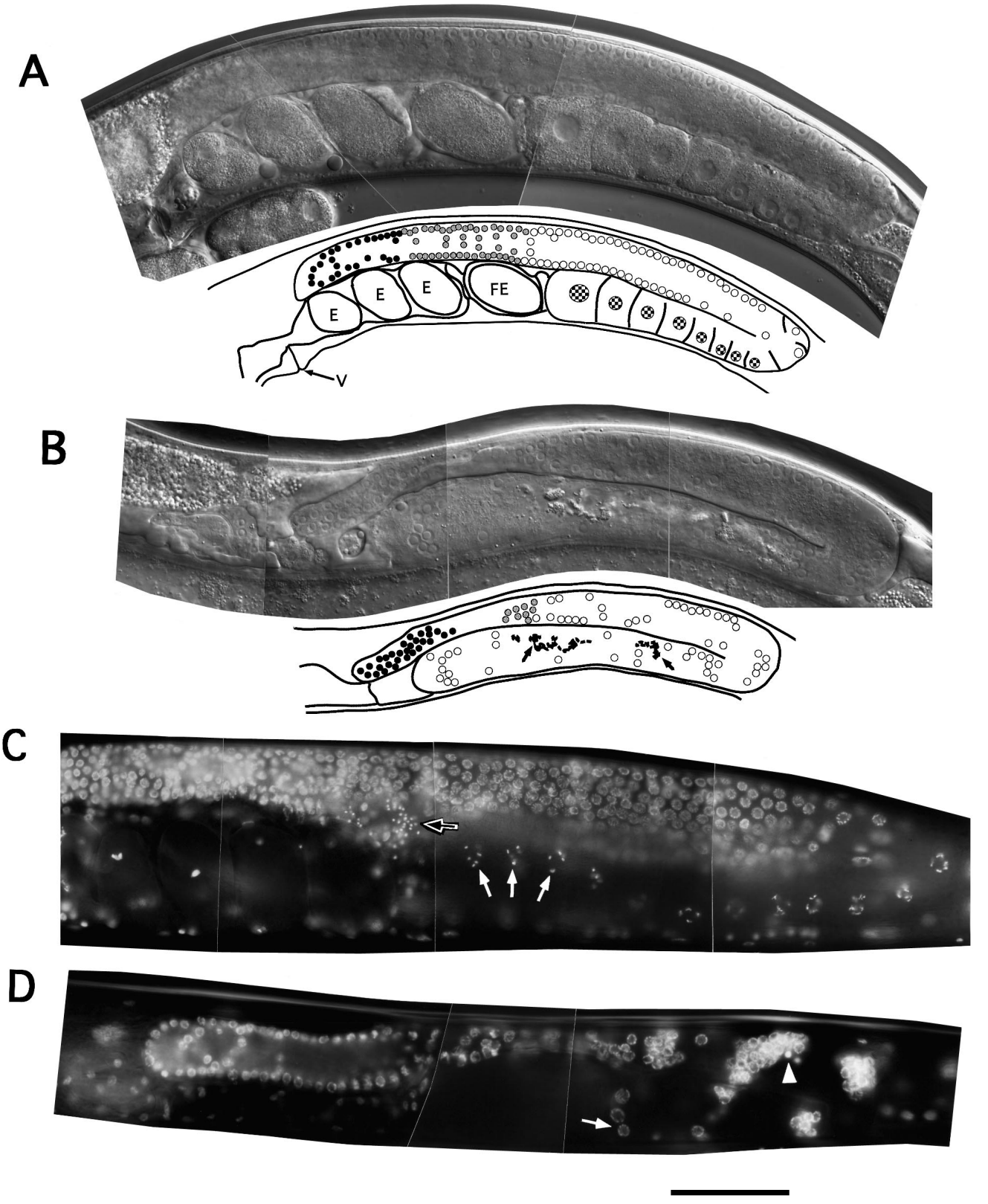

Fig. 1. Adult hermaphrodites. (A) N2 (wild type), Nomarski. Line drawing indicates relative locations of germline nuclei in mitotically active region (black), transition (gray), pachytene (open) and diakinesis (checkered). FE, fertilized egg in spermatheca; E, embryo in uterus; V, vulva. (B) *mek-2(q425)*, Nomarski. Germline nuclei indicated as in A. Arrows, clumps of granular material. (C) N2, DAPI. Solid arrows, representative oocyte nuclei in diakinesis. Open arrow, sperm nuclei. (D) *mek-2(q425)* DAPI. Arrow, representative pachytene nuclei in proximal arm of gonad. Arrowhead, condensed (degenerating?) germline nucleus among clumped pachytene nuclei. Dorsal is up and anterior to the left in all panels. Scale bar 50 μm.

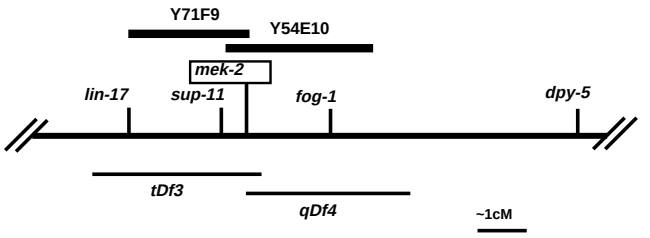

Fig. 2. The *mek-2* region of chromosome I. Map positions of genetic loci are according to the 1993 Genetic Map of *C. elegans* (J. Hodgkin, R. Durbin and M. O'Callaghan, personal communication). The location of *qDf4* is based on Ellis and Kimble (1995). PCR primers specific to the left end of YAC clone Y54E10 (gift of R. Ellis) amplify DNA from *qDf4* homozygotes, but not *tDf3* homozygotes. PCR primers specific to the right end of Y71F9 (gift of K. Kornfeld) fail to amplify DNA from either *tDf3* or *qDf4* homozygotes.

and *q484*, that prevent germline nuclei from exiting pachytene (Fig. 1B,D; for isolation, see Materials and Methods). In addition to perturbing germline development, each of these mutations also prevents the induction of the vulva. All other somatic tissues appear relatively normal in these animals; however, they are slightly sluggish and have a tendency to wander away from the *E. coli* lawn (possibly indicative of a defect in chemotaxis).

Through complementation testing, we determined that *q425* and *q484* are allelic to each other and to *let-537(h294)*, a previously described mutation that maps to the same region of chromosome I and also produces a sterile (Ste) vulvaless (Vul) phenotype (McKim, 1990; this paper). Kornfeld et al. (1995) and Wu et al. (1995) have independently identified this locus, based on mutant alleles that affect vulval induction. Because molecular analyses (Kornfeld et al., 1995; Wu et al., 1995) indicate that all of these mutations are associated with alterations in a gene encoding a MEK that is located on chromosome IL, we henceforth refer to this gene as *mek-2*.

Molecular analysis of *mek-2* mutations

Genetic mapping of *q425* placed it between *sup-11* and *fog-1*, within a region deleted by both *tDf3* and *qDf4* (Fig. 2). Alignment with the physical map indicated that this locus is near the region of overlap of YAC clones Y71F9 and Y54E10. One of us (K. L. G.) isolated a cDNA clone that maps to this region and contains most of the *mek-2* coding sequence (Wu et al., 1995). Since *mek-2* was a good candidate for the gene that we had identified by mutational analysis, we sequenced the *mek-2* coding portions of genomic DNA isolated from *q425, q484* and *h294* (Table 1). In the case of each allele, we found a single base pair change predicted to result in the alteration of an amino acid highly conserved within the protein kinase family (Hanks et al, 1988; Taylor et al., 1993). *q425* has a C→T transition predicted to substitute Leu for a conserved Ser residue. The homologous Ser residue is a primary site for activation phosphorylation of human MEK1 and is required for biological activity of the yeast *STE7* gene product, a MEK homolog involved in mating pheromone response (Seger et al., 1994; Zheng and Guan, 1994). *h294* has a G→A transition predicted to substitute Lys for a conserved Glu residue. The homologous Glu in yeast cAMP-dependent protein kinase (cAPK) appears to convey structural stability at the enzyme's active site by ion-pairing with an arginine residue; Glu-to-Ala mutation at this site results in a 1600-fold decrease in the cAPK's k_{cat}/K_m (Taylor et al., 1993). Alteration of this Glu in the Rous sarcoma virus *src* gene product pp60^{src} has been shown to reduce its protein kinase activity (Bryant and Parsons, 1984). *q484* has a T→A transversion predicted to substitute Glu for a conserved Asp residue. The homologous Asp in yeast cAPK is located in the catalytic loop and is positioned to serve as a catalytic base in phosphotransfer; Asp-to-Ala mutation at this site results in a 350-fold decrease in the cAPK's k_{cat} (Taylor et al., 1993). Thus, each of the three *mek-2* alleles, *q425*, *q484* and *h294*, involves the alteration of a highly conserved (or invariant) amino acid important in the activation, structure or catalytic function of MEK.

mek-2 acts downstream of *let-60 ras* and upstream of *lin-1* in vulval induction

Epistasis analysis was performed to establish the position of *mek-2* in the vulval induction pathway (Table 2). The Vul phenotype of *mek-2* is epistatic to the multivulva (Muv) phenotypes of *let-60gf, lin-15*, and the *lin-8; lin-9* double mutant. The Muv phenotypes of *lin-1* and *lin-12gf* are epistatic to the Vul phenotype of *mek-2*. These results are consistent with the established vulval induction pathway, wherein *let-60 ras* activates *mpk-1*/*sur-1* (MAP kinase), which then (directly or indirectly) inactivates *lin-1* and activates *lin-12* (for review, see Horvitz and Sternberg, 1991; Lambie and Kimble, 1991a; Hill and Sternberg, 1993; Sternberg, 1993). Since in vitro experiments have shown that MEK can directly activate MAPK (Crews and Erikson, 1992; Zheng and Guan, 1993), it is reasonable to conclude that *mek-2* acts as an intermediary between *let-60 ras* and *mpk-1/sur-1*.

Genetic characterization of *mek-2* alleles

q425, q484 and *h294* all appear to be loss-of-function mutations, since each is completely recessive and fully penetrant for the sterile phenotype and is also sterile when placed in *trans* to a deficiency (Table 3). This is consistent with

Table 1. DNA and predicted protein alterations of *mek-2* alleles

Allele	Codon number	Wild-type codon	Mutant codon	Wild-type amino acid	Mutant amino acid	Function of wild-type amino acid
q484	195	GAU	GAA	D	E	Catalysis/phosphotransfer
q425	227	UCA	UUA	S	L	Activation/phosphorylation site
h294	238	GAA	AAA	E	K	Maintain structure at active site

The sequencing strategy used was essentially that described by Kornfeld et al. (1995). Since the 5′ end of *mek-2* is refractory to conventional cloning methods, we were unable to design primers that would enable us to amplify the first 27 nucleotides of coding sequence from genomic DNA. See Wu et al. (1995) for the complete sequence of *mek-2*.

Table 2. Epistasis analysis of *mek-2*

Genotype	Vulval phenotype
mek-2[1]	Vul
mek-2; lin-15[2]	Vul
mek-2; lin-8; lin-9[3]	Vul
mek-2; let-60gf[4]	Vul
mek-2; lin-1[5]	Muv
mek-2; lin-12gf[6]	Muv

Double mutants were constructed and analyzed essentially as described by Ferguson et al. (1987).

[1]Hermaphrodites homozygous for *mek-2* alleles *h294* and *q425* are 100% Vul when raised at 15°C or 25°C. *q484* homozygotes are 100% Vul at 15°C, and 90% Vul at 25°C.

[2]*lin-15(n309)* was tested with *mek-2(q425)*.

[3]*lin-8(n111)* and *lin-9(n112)* were tested in combination with *mek-2(q425)*.

[4]*let-60(n1046)* was tested in combination with *q425*, *q484* and *h294*.

[5]*lin-1(e1275)* was tested in combination with *q425*, *q484* and *h294*. *lin-1(e1026)* was tested with *mek-2(q425)*.

[6]*lin-12(n137)* was tested in combination with *q425*.

Table 3. *mek-2* homozygotes versus *trans*-heterozygotes

Genotype	Percent Vul	*n*
mek-2(q484)/mek-2(q484) [1,2]	90%	500
mek-2(q425)/mek-2(q425) [1]	100%	>500
mek-2(h294)/mek-2(h294) [3]	100%	>500
mek-2(q484)/qDf4 [2,4]	6%	302
mek-2(q484)/tDf3 dpy-5(e61) [2,4]	3%	106
mek-2(q425)/qDf4 [5]	100%	168
mek-2(h294)/qDf4 [6]	100%	219
mek-2(q484)/mek-2(h294) unc-11(e47) [2,4]	13%	201
mek-2(q484)/mek-2(q425) unc-11(e47) [4]	100%	231
mek-2(q484)/+ [7]	0%	>500
mek-2(q425)/+ [7]	0%	>500
mek-2(h294)/+ [7]	0%	>500
mek-2(q484)/mek-2(q484)/+ [8]	0%	122
mek-2(q484)/ + / + [9]	0%	≤145[10]

[1]Generated by selfing hermaphrodites of genotype *mek-2(–); sDp2*.

[2]Non-Vul animals had one or two ventral protrusions, similar to *mpk-1/sur-1(ku1)* (Wu and Han, 1994), and different from the Hin phenotype associated with weak alleles of *let-23* (Aroian and Sternberg, 1991).

[3]Generated by selfing hermaphrodites of genotype *mek-2(h294)/unc-11(e47)*.

[4]Generated by crossing *q484/+* males with hermaphrodites of genotype *qDf4; sDp2* or *tDf3 dpy-5(e61); sDp2* or *mek-2(h294) unc-11(e47)/hT2* or *mek-2(q425) unc-11(e47); sDp2*.

[5]Generated by crossing *qDf4/+* males with hermaphrodites of genotype *mek-2(q425) dpy-5(e61); sDp2*.

[6]Generated by crossing males of genotype *mek-2(h294)/+* with purged hermaphrodites of genotype *qDf4; sDp2*.

[7]Generated by selfing hermaphrodites of genotype *mek-2(-)/unc-11(e47)*.

[8]Full genotype: *mek-2(q484)/mek-2(q484); sDp2[mek-2(+)]*.

[9]Full genotype: *mek-2(q484)/mek-2(+); sDp2[mek-2(+)]*.

[10]An estimate obtained by halving the number of hermaphrodite progeny scored after crossing +/+ males with purged hermaphrodites of genotype *mek-2(q484);sDp2*.

The frequency of Vul animals of genotypes *mek-2(q484)/qDf4* and *mek-2(q484)/tDf3 dpy-5(e61)* is not significantly different ($0.5 < P < 0.1$; G-test (Sokal and Rohlf, 1981)). The frequency of Vul animals of each other genotype is significantly different from both deficiency heterozygotes ($P < 0.001$; G-test). *mek-2(–)/mek-2(–)* or *mek-2(–)/Df* animals were unambiguously scorable by dissecting scope due to the presence of large clumps of dark material within the sterile gonad. All animals were raised at 25°.

each mutation causing a decrease in *mek-2* activity, as would be predicted based on the DNA sequence alterations associated with each of these mutant alleles (see above).

Each allele is also completely recessive for the Vul phenotype (Table 3). However, when the fraction of animals that are completely Vul is used to monitor *mek-2* gene activity, it is evident that none of these alleles completely eliminates *mek-2* gene activity. *q484* is almost certainly not a null allele, because it has an incompletely penetrant, temperature-sensitive Vul phenotype (Tables 1, 3). This is useful, because a weak allele would be expected to produce the same phenotype when placed in *trans* to a deficiency as when placed in *trans* to a null allele. Therefore, we tested *q484* in *trans* to *q425*, *h294* and two different deficiencies. Surprisingly, the penetrance of the Vul phenotype is much lower in *q484/tDf3* and *q484/qDf4* animals than in *q484/q484* animals. Although unanticipated, this observation is not inconsistent with *q484* being a loss-of-function mutation (see Discussion). Moreover, this result leads to the prediction that *q484/mek-2(0)* should exhibit an equally low penetrance of the Vul phenotype. Accordingly, *h294* is not a null allele of *mek-2*, since the penetrance of the Vul phenotype among *q484/h294* animals is significantly higher than among *q484/Df* animals. Likewise, *q425* is not a null allele, since 100% of *q484/q425* animals are Vul.

mek-2 is required for meiotic progression during oogenesis and spermatogenesis

The germline phenotypes of *q425*, *q484* and *h294* are indistinguishable. Homozygous *mek-2* mutant hermaphrodites appear very similar to wild type even as late as the last larval stage. However, by young adulthood, it is obvious that the proximal germline nuclei, which undergo spermatogenesis in wild-type animals, remain arrested in pachytene in *mek-2* mutants (Fig. 1B,D). The more distal pachytene nuclei (presumptive oocytes) also remain arrested. As these animals age, the pachytene nuclei tend to clump together and appear to degenerate. At the same time, large aggregates of granular material, resembling that normally packaged into oocytes, accumulate within the syncytial core of the gonad.

Mutations in *mek-2* result in similar defects within the male germline (Fig. 3). However, a small number of sperm are usually made before the pachytene block occurs. We suspect that this apparent leakiness is the result of a sex-specific difference in the concentration of maternally contributed *mek-2(+)* gene product that is present at the onset of gametogenesis. Since spermatogenesis is initiated earlier in males than in hermaphrodites, the volume of the germline (and consequent dilution of maternally contributed gene products) is smaller. Also, if maternally contributed *mek-2* gene product were unstable, then its level would be higher if gametogenesis were initiated earlier. Consistent with either of these explanations, all of the germ cells in *mek-2; glp-1(0)* hermaphrodites (in which meiosis would be expected to be initiated significantly earlier than usual (Austin and Kimble, 1987)) differentiate into sperm (Fig. 4). The sex of the germline per se does not appear to be critical for determining the effects of *mek-2* mutations, since neither the masculinizing mutation, *fem-3(q20gf)*, nor the feminizing mutation, *fog-1(q253)*, alters the *mek-2* mutant phenotype of XX animals (E. J. L., unpublished).

Upstream and downstream mediators of *mek-2* activity within the germline

Since *let-60 ras* is an obvious candidate for an upstream activator of *mek-2* within the germline, we examined the

effects of several *let-60 ras* alleles (Beitel et al., 1990) on meiotic progression in hermaphrodites. We were able to do this by making use of the fact that the constitutively active allele, *let-60(n1046)*, will maternally rescue progeny that are homozygous for either strong loss-of-function or dominant negative alleles of *let-60 ras* (Beitel et al., 1990; Han et al., 1990). Four of the *let-60 ras* alleles that we examined produce noticeable effects. The strong loss-of-function allele, *s1124*, completely prevents exit from pachytene during oogenesis (Fig. 5A,B). The dominant negative allele, *n2031dn*, has a phenotype nearly indistinguishable from that of *s1124*. However, in *n2031dn* hermaphrodites that have been adults for several days, one or two of the most proximal oocyte nuclei occasionally exit pachytene. The weaker loss-of-function alleles, *s1155* and *n1046dx1*, cause a significant retardation of pachytene exit during oogenesis (Fig. 5C). In these hermaphrodites, oocytes do not exit pachytene until well after they have progressed from the dorsal to the ventral arm of the gonad. The weak loss-of-function allele, *let-60(n2021)*, has no discernible effect on the germline.

The phenotype of *let-60 ras* mutants differs from that of *mek-2* mutants in several ways. First, *let-60 ras* hermaphrodites always make an approximately normal number of sperm. Given the fact that the survival of *let-60(lf)* animals past the L1 larval stage is dependent on maternally contributed gene product (Beitel et al., 1990; Han and Sternberg, 1990), this germline phenotype may also be the consequence of maternal rescue. Second, the total number of germline nuclei is much smaller in *let-60 ras* mutants. It is not clear whether this is due to a direct requirement for *let-60 ras* in germline proliferation or instead an indirect result of the general scrawniness of these animals. In the case of *s1124*, the scrawny phenotype may be partially due to the presence of the tightly linked marker mutations, *unc-22(s7)* and *unc-31(e169)*. However, *n2031dn* homozygotes have a similar appearance, despite the absence of linked markers. *let-60 ras* mutant males also make some sperm; however, because the germline is small (as in *let-60 ras* mutant hermaphrodites), it is difficult to determine whether the relatively low number of primary spermatocytes observed is due to a specific meiotic progression defect or is simply a secondary consequence of the reduced germline population.

mpk-1/sur-1 (MAPK; Lackner et al., 1994; Wu and Han, 1994) is likely to be a direct target of *mek-2* during vulval induction. We were able to examine the requirement for *mpk-1*/*sur-1* during germline development thanks to a newly identified allele, *oz140*. This mutation was generously provided by Tim Schedl (Washington University, St Louis), who initially identified it as a recessive sterile mutation that maps to the left arm of chromosome III, near the *mpk-1*/*sur-1* locus. We performed complementation testing with *mpk-1*/*sur-1(n2521)* and determined that *oz140* is an allele of *mpk-1*/*sur-1*. The male and hermaphrodite germline phenotypes of *mpk-1*/*sur-1(oz140)* are nearly indistinguishable from those caused by mutations in *mek-2* (data not shown). As in *mek-2* mutants, the development of somatic tissues (other than members of the vulval equivalence group) in *mpk-1*/*sur-1(oz140)* animals appears to be relatively normal. *mpk-1/sur-1(oz140)* is 100% penetrant for the pachytene arrest phenotype.

In principle, all of the genes that function in vulval induction could also act to regulate cell cycle progression within the

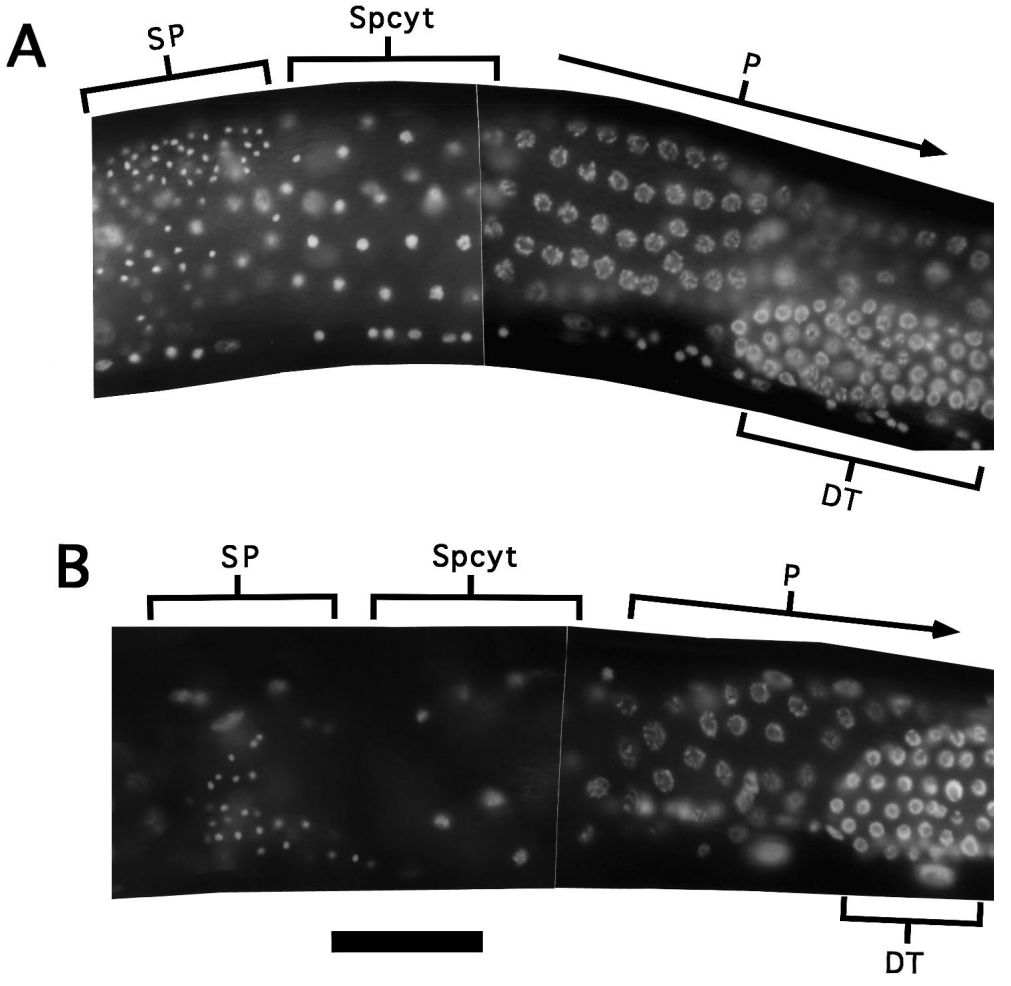

Fig. 3. Adult males. (A) N2 (wild type), DAPI. Distal tip (DT), pachytene (P), primary spermatocyte (Spcyt) and spermatid (SP) regions indicated by brackets. (B) *mek-2(q425)*, DAPI. Regions indicated as in A. Dorsal is up and anterior to the right in both panels. Scale bar 50 μm. *mek-2* mutant males are unambiguously scorable because they have crumpled spicules (data not shown).

germline. However, we found that all double mutant combinations involving *mek-2* and *lin-1* or *lin-12(gf)* have germline phenotypes identical with that of the *mek-2* single mutant (data not shown). Furthermore, *lin-12(lf)* mutations do not prevent exit from pachytene (Greenwald et al., 1983; Seydoux et al., 1990). Therefore, it is likely that genes other than *lin-1* and *lin-12* function downstream of *mek-2* and *mpk-1*/*sur-1* within the germline.

Mosaic analysis of *mpk-1*/*sur-1*

In order to determine whether MAPK-mediated signaling is required within the germline itself, we generated genetic mosaics of *mpk-1*/*sur-1*. This was done with a strain of genotype *mpk-1*/*sur-1(oz140) ncl-1(e1865) unc-36(e251); sDp3[mpk-1$^+$ ncl-1$^+$ unc-36$^+$]*, using methods described by Herman (1989). As can be seen in Fig. 6, expression of *mpk-1*/*sur-1* in the somatic gonad is not sufficient to rescue the *mpk-1*/*sur-1* mutant germline phenotype. Instead, our results indicate that *mpk-1*/*sur-1* function within the germline is necessary to permit exit from pachytene.

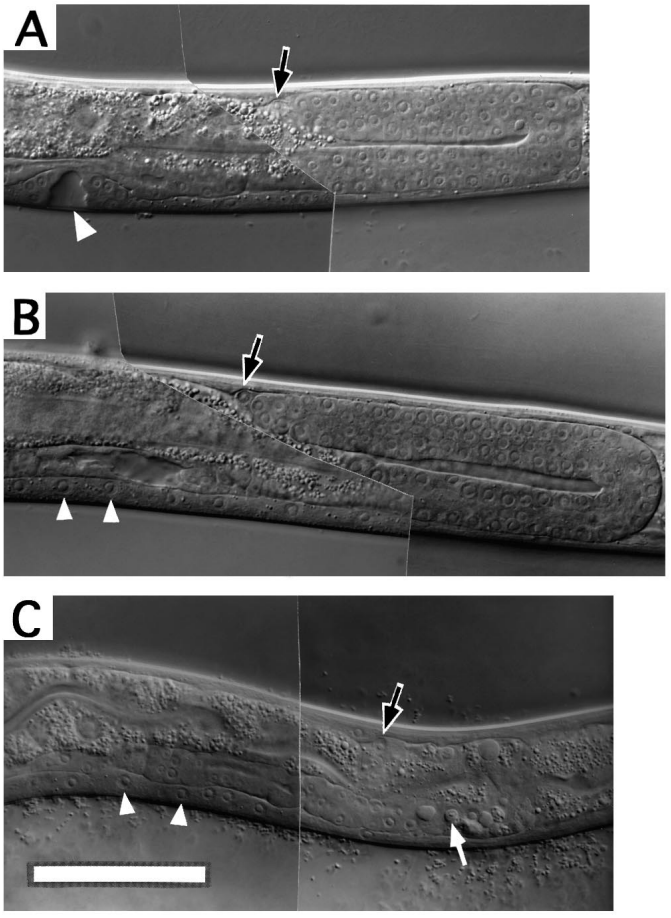

Fig. 4. L4 stage hermaphrodites. (A) N2 (wild type). Open arrow, distal tip cell. Arrowhead, vulva. (B) *mek-2(q425)*. Open arrow, distal tip cell. Arrowheads, representative Pn.p daughters. (C) *mek-2(q425); unc-32(e189) glp-1 (q175)*. Open arrow, distal tip cell. Arrowheads, representative Pn.p daughters. Solid arrow, representative sperm in proximal arm. All panels Nomarski, with dorsal up and anterior to the left. The left and right halves of each panel are from different focal planes. Scale bar 50 μm.

DISCUSSION

In this paper, we describe the identification of mutations in a *C. elegans* MEK gene, *mek-2*. The phenotype caused by mutation of *mek-2* suggests that this gene is required for the transition from pachytene to diakinesis during meiosis I prophase. In addition, we report that mutations in *let-60 ras* and *mpk-1*/*sur-1* (MAPK) result in similar defects in germline cell cycle progression. This phenotypic correspondence is not unexpected, since ras, MEK and MAPK are known to act sequentially in signal transduction (reviewed in Blenis, 1993; Davis, 1993; Nishida and Gotoh, 1993; Cooper, 1994; Johnson and Vaillancourt, 1994). Also consistent with expectation, we found that mutations in *mek-2* prevent vulval induction and are epistatic to the constitutively activated *ras* allele, *let-60(n1046)*. A comparison between the genetic regulation of vulval induction and exit from pachytene is diagrammed in Fig. 7.

Functional character of *mek-2* alleles

Several lines of evidence support the idea that the *mek-2* mutant germline phenotypes that we have observed are representative of a loss of gene activity, rather than a gain of function. First, all three of the *mek-2* alleles that we have characterized are completely recessive for the germline defects. Second, the sequence alterations associated with these alleles would be predicted to result in a reduction of *mek-2* kinase activity. Finally, since recessive mutations in both *let-60 ras* and *mpk-1*/*sur-1* produce comparable germline defects, the simplest interpretation is that a loss or decrease in MAPK-mediated signaling is responsible. A possible caveat here is that none of the mutations that we have examined are known to be protein nulls. Therefore, it is formally possible that all of these mutations are recessive antimorphs that act by interfering with a signaling mechanism for which they are not actually required.

Our analysis of *trans*-heterozygotes involving the putative hypomorphic allele, *mek-2(q484)*, suggests that neither *q425* nor *h294* is a null allele. We suspect that a *mek-2(0)* mutation might cause first larval stage lethality, since this is the consequence of strong mutations in other genes required for MAPK-mediated signaling in *C. elegans*. In addition, we found that *tDf3/qDf4* animals (which might be homozygously deleted only for *mek-2*) exhibit first larval stage lethality. However, we cannot exclude the possibility that this phenotype is due to the disruption in both deficiencies of a gene distinct from *mek-2*.

The *q484 trans*-heterozygote experiments produced two surprising results. First, the percentage of animals with overt vulval induction was found to be higher when *q484* was placed over a deficiency than when *q484* was homozygous. This appears contrary to expectation, since *q484/Df* animals should have less *mek-2* activity than *q484/q484* animals. Although counterintuitive, this result is not unprecedented. Aroian and Sternberg (1991) have provided evidence that lowering the activity level of *let-23* can produce an increase in the degree of vulval induction. Therefore, it is possible that this is a general feature of components of the vulval induction pathway, perhaps reflecting a negative feedback mechanism (Aroian and Sternberg, 1991; Nishida and Gotoh, 1993). A second surprising result was that *mek-2(q425)* and *mek-2(h294)*, which have identical phenotypes when homozygous, behave differently when placed over *mek-2(q484)*. This difference may result

from either a qualitative or quantitative difference in *mek-2* activity between these two alleles.

Anatomical localization of signaling components

Although it is reasonable to posit that *let-60 ras*, *mek-2* and *mpk-1*/*sur-1* function within the germline itself, it could be that these genes actually function within some other tissue (e.g., the somatic gonad), which then regulates germline development via some other signaling pathway. To distinguish between these possibilities, we performed a genetic mosaic analysis of *mpk-1*/*sur-1*. Our results indicate that MAPK-mediated signaling is required within the germline. We cannot absolutely exclude the possibility that *mpk-1*/*sur-1* is also required within the somatic gonad; however, if this were the case, we should have also detected phenotypically Ste mosaic animals that had lost the unstable free duplication from the somatic gonad, but not the germline.

The source of the signal or signals that activate MAPK within the germline is currently unknown. In hermaphrodites, the somatic sheath cells appear to be appropriately positioned to provide a signal that triggers exit from pachytene. However, there are no sheath cells in the male gonad. In fact, the region of the male gonad in which exit from pachytene occurs is surrounded only by a basement membrane. Therefore, either a different signaling mechanism is employed in males and hermaphrodites or signaling is mediated by a diffusible factor. In the simple case of a single diffusible factor, the best candidate somatic tissues for signal production are the seminal vesicle (in males) and the spermatheca (in hermaphrodites). In more complex scenarios, it could be that multiple, sex-specific signals are produced by overlapping sets of somatic tissues and act redundantly to trigger exit from pachytene. Furthermore, it is not inconceivable that a diffusible regulatory factor could be produced and transported within the germline itself. We are currently attempting to distinguish between these numerous possibilities through a combination of laser microsurgery and genetic experiments.

Genetic identity and function of signaling components

We do not know which genes operate upstream of *let-60 ras* in the germline (Fig. 7). Although non-null *let-23* mutations do not perturb exit from pachytene (Aroian and Sternberg, 1991), these alleles may simply not be strong enough to produce an

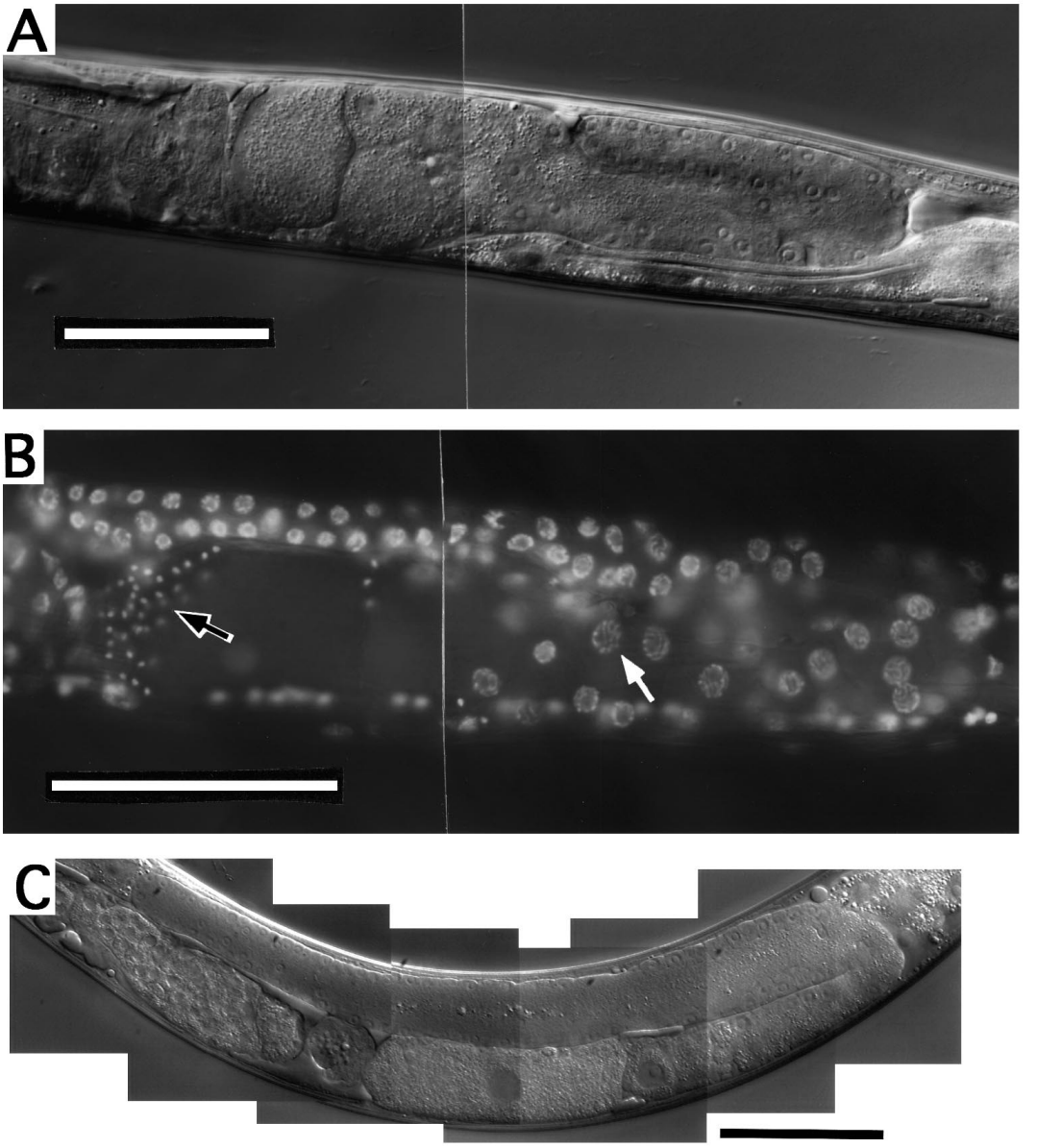

Fig. 5. Adult *let-60 ras* hermaphrodites. (A) *let-60(s1124) unc-22(s7) unc-31(e169)*, Nomarski. Enlarged nuclei and membranous folds that superficially resemble oocytes are present in the proximal region. DAPI staining of such individuals revealed that the chromosomes in these nuclei are still in the pachytene configuration. (B) *let-60(s1124) unc-22(s7) unc-31(e169)*, DAPI. Solid arrow, representative pachytene nucleus in proximal arm of gonad. Open arrow, sperm nuclei. (C) *let-60(n1046dx1)*, Nomarski. Scale bars 50 μm.

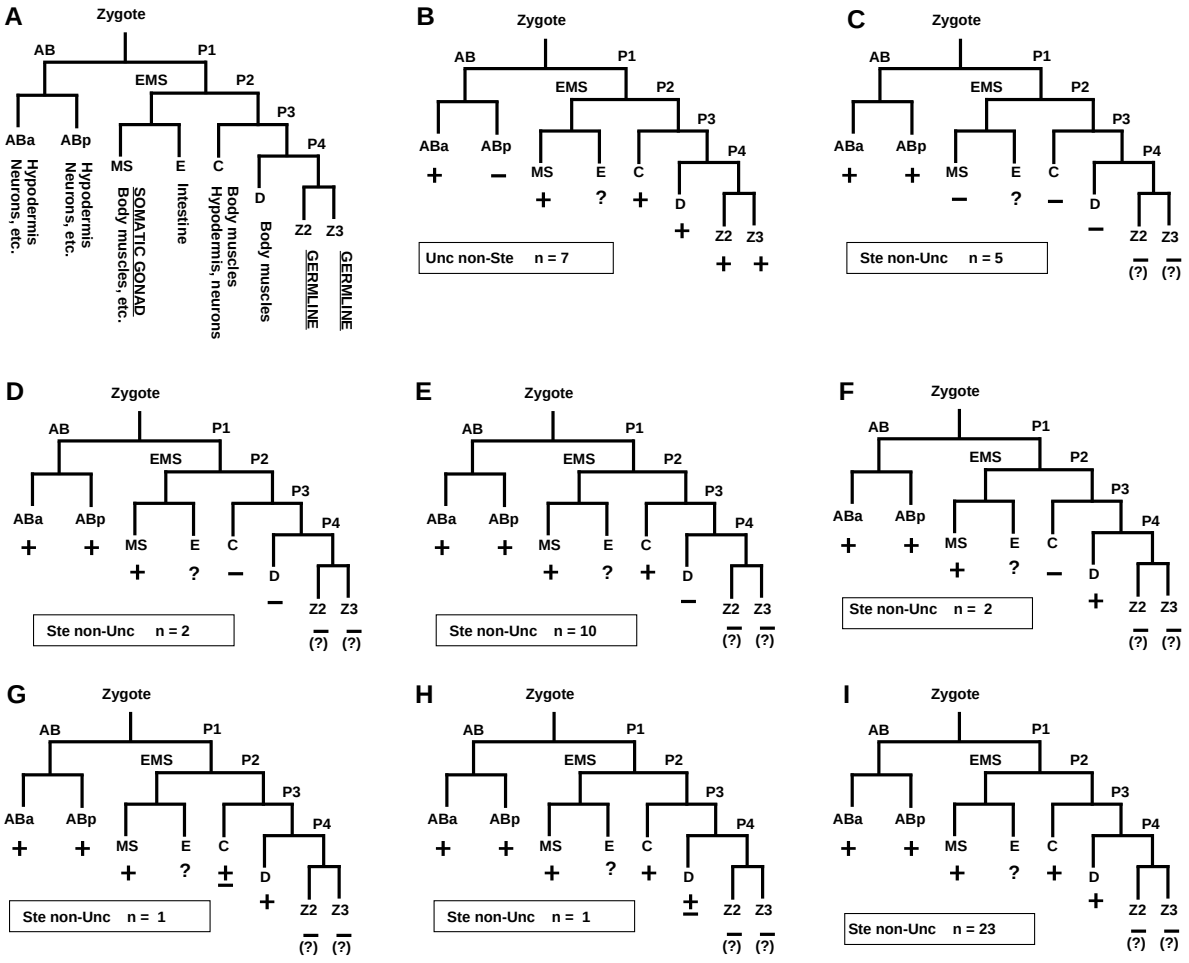

Fig. 6. Mosaic analysis of *mpk-1/sur-1*. (A) Wild-type lineage, with major derivatives of embryonic founder cells. (B-I) Different classes of mosaic animals. Mosaics from *mpk-1/sur-1(oz140) ncl-1(e1865) unc-36(e251); sDp3[mpk-1/sur-1$^+$ ncl-1$^+$ unc-36$^+$]* were initially detected by dissecting microscope based on either a sterile (Ste) non-Unc or an Unc non-Ste phenotype. The expression of *unc-36*$^+$ in descendants of ABp is sufficient to prevent the Unc phenotype (Kenyon, 1986). *ncl-1(e1865)* is a cell-autonomous mutation that affects the size of the nucleolus (Herman and Hedgecock, 1990). Putative mosaics were inspected by Nomarski microscopy to score the Ncl phenotype of representative descendants of embryonic founder cells and (in the case of Ste non-Uncs) to confirm that exit from pachytene was blocked. The Ncl phenotype cannot be scored in either the intestine or the germline. However, the loss of the duplication from the germline was deduced based on the absence of consistent duplication loss in any other lineages among the Ste non-Uncs. The indirect nature of this assessment is indicated by '(?)'. ± indicates mosaicism among the progeny of a founder cell. Independent loss of the free duplication in separate lineages (e.g., F,G,H) is not unexpected (Herman, 1989).

effect. Mosaic analysis with a null allele of *let-23* could potentially address this issue.

Because reduced *let-60 ras* activity prevents exit from pachytene, one might expect that the constitutive activation of *let-60 ras* by the *n1046* mutation would lead to premature exit from pachytene. However, germline development appears essentially normal in *let-60(n1046)* homozygotes (Ferguson and Horvitz, 1985; E. J. L., unpublished data). Several possible explanations exist for this observation. First, it may be that the level of activity provided by *let-60(n1046)* is not sufficient to induce exit from pachytene. Indeed, Lackner et al. (1994) have provided evidence that *n1046* does not fully activate *let-60 ras*. Second, it may be that activation of *let-60 ras* is necessary, but not sufficient to trigger exit from pachytene. Perhaps an additional signaling pathway must also be activated, which then either operates in parallel or merges downstream of *let-60 ras*. Finally, it is possible that germ cells are obligated to remain in pachytene for an extended period of time before they become competent to respond to the exit signal.

lin-45 raf is probably responsible for relaying the inductive signal from *let-60 ras* to *mek-2* during vulval induction (Sternberg et al., 1993). Although interference with normal *lin-45 raf* function is capable of causing sterility, the cellular basis for this phenotype has not been determined (Han et al., 1993). Mutations in a *C. elegans* MEKK, the other candidate for a *ras*-MEK intermediary, have not been reported. Therefore, we do not know whether either *raf* or MEKK operates to relay the signal between *let-60 ras* and *mek-2* in the germline (Fig. 7).

We are equally ignorant of the genes that function downstream of *mpk-1*/*sur-1* (MAPK) in the germline. In the vulval induction pathway, *lin-1* and *lin-12* are regulated (directly or

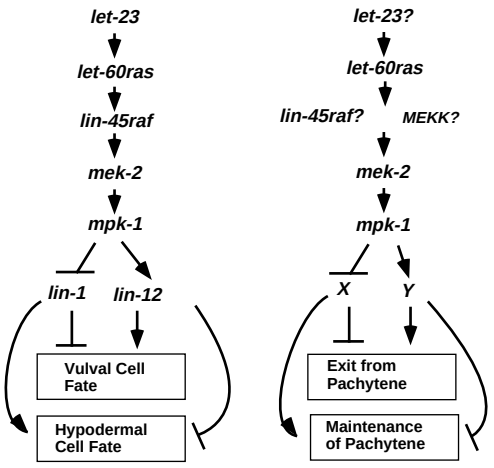

Fig. 7. Genetic regulation of vulval induction and pachytene exit. Hypothetical positive (arrowhead) and negative (crossbar) regulatory interactions are depicted. Proposed interactions are not necessarily direct. The left panel is based on Sternberg (1993), Lackner et al. (1994) and Wu and Han (1994). X and Y are hypothetical germline-specific targets of *mpk-1*/*sur-1*.

indirectly) by *mpk-1*/*sur-1* (Ferguson et al., 1987). However, mutation of either *lin-1* or *lin-12* does not appear to affect exit from pachytene. Therefore, it is likely that a distinct set of genes operate downstream of *mpk-1*/*sur-1* in the germline (Fig. 7). Mutations in germline-specific target genes could be identifiable based on their ability to block exit from pachytene without affecting vulval induction.

In *C. elegans*, as in most other organisms, there are at least two MEKs (*mek-1*, K. L. G. unpublished, and *mek-2*) and two MAPKs (*mpk-1*/*sur-1* and *mpk-2*; Wu and Han, 1994; Lackner et al., 1994). It is not known whether *mpk-2* and/or *mek-1* participate in the regulation of cell division within the germline. Perhaps they function primarily in non-gonadal tissues, in which case we would be unlikely to identify them in screens for sterile mutants.

Comparisons and prospects

The MAPK-mediated regulation of germline development that we describe here for *C. elegans* may be comparable to that which occurs in vertebrates. In mice, the *c-kit* receptor tyrosine kinase (which probably acts via MAPK) is required for the migration and proliferation of the primordial germ cells (Russell, 1979; Yarden et al., 1987; Qiu et al., 1988). c-kit is also expressed by immature diplotene stage oocytes, where it is important during the follicular growth phase of oogenesis (Manova et al., 1990; Horie et al., 1991). Later in oocyte development, MAPK is known to be activated during maturation (Verlhac et al., 1993). In male mice, the proliferation of A-type spermatogonia depends on c-kit activity (Yoshinaga et al., 1991). The fact that signaling via c-kit mediates sequential developmental decisions highlights the possibility that MAPK may also be required in *C. elegans* for the bona fide maturation of oocytes. However, we were unable to determine whether this is so, because the formation of maturation-competent oocytes is prevented by the mutations that we have examined.

In the case of *Xenopus*, there is good evidence that oocyte maturation is dependent on the activation of MAPK (Posada and Cooper, 1992). The accessibility of the *Xenopus* oocyte to in vitro manipulation and microinjection have made it an important system for studying the biochemistry and pharmacology of MAPK activation. This approach to dissecting MAPK-mediated signaling has recently been adapted by Lu et al. (1993) to the *Drosophila* embyro. Since the germline syncytium of *C. elegans* is also an easy target for microinjection, it may be feasible to apply this approach to the study of cell cycle progression in the nematode germline.

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