Genetic Analysis of the *Caenorhabditis elegans* MAP Kinase Gene *mpk-1*

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ABSTRACT

The *Caenorhabditis elegans mpk-1* gene encodes a MAP kinase protein that plays an important role in Ras-mediated induction of vulval cell fates. We show that mutations that eliminate *mpk-1* activity result in a highly penetrant, vulvaless phenotype. A double mutant containing a gain-of-function *mpk-1* mutation and a gain-of-function *mek* mutation (MEK phosphorylates and activates MPK-1) exhibits a multivulva phenotype. These results suggest that *mpk-1* may transduce most or all of the anchor cell signal. Epistasis analysis suggests that *mpk-1* acts downstream of *mek-2* (encodes a MEK homolog) and upstream of *lin-1* (encodes an Ets transcription factor) in the anchor cell signaling pathway. Finally, *mpk-1* may act together with *let-60 ras* in multiple developmental processes, as *mpk-1* mutants exhibit nearly the same range of developmental phenotypes as *let-60 ras* mutants.

MAP kinases are key mediators of cellular differentiation and proliferation in all animals, and they function in receptor tyrosine kinase/Ras signaling pathways (reviewed in Marshall 1994). MAP kinase plays an important role in the Ras signaling pathway because it can activate downstream substrates that directly mediate the cellular response to growth factors, suggesting that MAP kinase acts near or at the end of this signaling pathway (reviewed in Treisman 1996).

MAP kinases are activated when they become phosphorylated by the protein kinase MEK (*M*AP or *E*RK *k*inase; Adams and Parker 1992; Crews *et al.* 1992b). The major known substrate for MEK is currently MAP kinase, suggesting that the predominant function of MEK may be to activate MAP kinase (Seger *et al.* 1992). Once activated, a significant fraction of MAP kinase molecules translocate to the nucleus, and many important MAP kinase substrates are localized in the nucleus (*e.g.*, the mammalian transcription factors Elk-1 and Ets-1 (reviewed in Treisman 1996). In addition to nuclear targets, some MAP kinase substrates are located in the cytoplasm, *e.g.*, the guanine nucleotide exchange factor SOS and the protein kinase pp90Rsk (reviewed in Ferrell 1996).

Vertebrates have two MAP kinases (Erk1 and Erk2) that have overlapping substrates (reviewed in Crews *et al.* 1992a and Marshall 1994). *Saccharomyces cerevisiae* has two MAP kinase genes, *fus3* and *kss1*, that act in the pheromone and invasive growth signaling pathways, respectively (Herskowitz 1995; Madhani *et al.* 1997). In Drosophila, there is currently a single known MAP kinase, which is encoded by the *rolled* gene (Biggs *et al.* 1994). A mutation (termed *Sevenmaker*) that activates *rolled* MAP kinase results in the formation of extra R7 cells, similar to the phenotype caused by constitutively activated *Ras1* (Brunner *et al.* 1994).

In *Caenorhabditis elegans*, there is a single known MAP kinase that is encoded by the *mpk-1*/*sur-1* gene (Lackner *et al.* 1994; Wu and Han 1994). Previous studies have shown that *mpk-1* plays a role in vulval induction in the *C. elegans* hermaphrodite. The vulva is induced from a set of six ectodermal blast cells, referred to as Pn.p cells (P3.p to P8.p), that lie in a row along the ventral midline of the developing larva (reviewed in Eisenmann and Kim 1994). All six Pn.p cells are capable of adopting any one of three potential cell fates (1°, 2°, or 3°), and these fates are predominantly determined by the sequential action of two signaling pathways. First, a signal from the anchor cell induces the nearest Pn.p cell (P6.p) to adopt the 1° cell fate, which is to divide three times and generate eight descendant cells that form the inner portion of the vulva. Then, a lateral signal from P6.p induces P5.p and P7.p to adopt the 2° cell fate (Sternberg 1988; Koga and Ohshima 1995b; Simske and Kim 1995), which is to divide three times to give seven descendant cells that form the outer portions of the vulva. In addition to the lateral signal, some experiments suggest that a graded signal from the anchor cell can sometimes induce Pn.p cells to adopt the 2° cell fate (Sternberg and Horvitz 1986; Katz *et al.* 1995). The Pn.p cells furthest from the anchor cell (P3.p, P4.p, and P8.p) adopt uninduced (3°) cell fates, which is to divide once and then fuse with the hypodermal syncytium.

Genetic studies have shown that the anchor cell signal activates a receptor tyrosine kinase/Ras pathway (re-

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viewed in Kornfeld 1997). The *lin-3* gene encodes a protein similar to epidermal growth factor (EGF) that is the inductive signal secreted by the anchor cell (Hill and Sternberg 1992). The *let-23* gene encodes a receptor tyrosine kinase similar to EGF receptor that is thought to be the receptor for LIN-3 signal (Aroian *et al.* 1990). Downstream of LIN-3 and LET-23 are a Grb2-related adapter protein (encoded by *sem-5*; Clark *et al.* 1992), a Ras protein (encoded by *let-60*; Han and Sternberg 1990), a Raf homolog (encoded by *lin-45*; Han *et al.* 1993), and a protein similar to the serine/threonine protein kinase MEK (encoded by *mek-2*; Kornfeld *et al.* 1995; Wu *et al.* 1995). Loss-of-function mutations in any of these genes prevent P6.p from expressing the 1° cell fate and the lateral signal, so that all of the Pn.p cells express 3° cell fates, resulting in a vulvaless phenotype. Conversely, gain-of-function mutations in *let-23* or *let-60* cause the vulval precursor cells to adopt the 1° cell fate and to presumably express the lateral signal, resulting in a multivulva phenotype.

Previous studies have implicated *mpk-1* in vulval induction. These studies showed that two *mpk-1* alleles (*n2521* and *ku8*) suppress the multivulva phenotype of the activated *ras* allele *let-60(n1046)* (Lackner *et al.* 1994; Wu and Han 1994). This result indicates that *mpk-1* most likely acts downstream of *let-60 ras* in the vulval signaling pathway. However, *mpk-1(n2521)* mutants did not exhibit any defect in vulval induction, and *mpk-1(ku8)* exhibited only a weak defect in vulval induction. Thus, these *mpk-1* alleles do not result in a strong reduction of the anchor cell signaling pathway in P6.p. It is not clear whether these *mpk-1* alleles eliminate or might only partially reduce *mpk-1* activity, since both alleles are missense mutations and gene dosage studies have not been possible because of the lack of a deficiency that deletes the *mpk-1* locus.

Because the *mpk-1*–null phenotype has not been established, an important but unanswered question is whether the vulval induction pathway is linear between *let-23 RTK* and *mpk-1*, such that *mpk-1* transduces all of the signal from activated *let-23*. If the pathway is linear, then null mutations in *mpk-1* should prevent vulval induction and result in a Vul phenotype. Alternatively, the signaling pathway might be branched in a way that other signaling molecules might act in parallel with *mpk-1*. For instance, mammalian EGF receptor and Ras are thought to transduce signals by activating targets in completely separate signaling branches (such as phospholipase C gamma or PI3 kinase; Roche *et al.* 1996) or by activating two MAP kinases (Erk1 and Erk2). If the *C. elegans* vulval signaling pathway is similarly branched, then *mpk-1* null mutations should result in only a partial reduction in vulval induction because the *mpk-1* mutations should eliminate one but not all of the signaling branches.

In this article, we have further characterized the role of *mpk-1* in *let-60 ras* signaling pathways during *C. elegans* development. First, we present evidence suggesting that *mpk-1* transduces most if not all of the signal from *let-23 receptor* and *let-60 ras*. We used three genetic screens to recover new *mpk-1* alleles (including three putative null alleles) and found that *mpk-1*–null mutants exhibit a strong vulvaless phenotype. We also showed that activation of both MEK and MAP kinase activates the vulval signaling pathway, resulting in a multivulva phenotype. Thus, *mpk-1* is necessary for vulval induction, and *mek* and *mpk-1* together are sufficient to activate vulval induction. Second, genetic and molecular analyses suggest that *mpk-1* acts downstream of all the signal transduction genes in the *let-23 RTK* vulval signaling pathway, but upstream of *lin-1 Ets* (a vulval repressor gene encoding a putative transcription factor). Finally, our results suggest that *mpk-1* may act together with *let-60* in many, but perhaps not all, of the tissues whose fates are determined by *let-60 ras* signaling.

MATERIALS AND METHODS

General methods: Strain maintenance, genetic manipulation, and ethyl methanesulfonate (EMS) mutagenesis were performed as described by Brenner (1974). Animals were cultivated at 20° unless otherwise noted. The animals described as wild type were *C. elegans*, variety Bristol, strain N2. The following genes and alleles were used in this work [unless otherwise noted, alleles used are described by Wood (1988)]:

LGI: *mek-2(n2678)* (Kornfeld *et al.* 1995), *sup-11(n403)*, *dpy-5(e61)*, *sur-2(ku9)* (Singh and Han 1996), *smg-1(r861)* (Pulak and Anderson 1993), *unc-54(r293)* (Pulak and Anderson 1993).

LGIII: *unc-79(e1068)*, *mpk-1(ku8)* (Wu and Han 1994), *mpk-1 (oz140)* (Church *et al.* 1995), *dpy-17(e164)*, *lin-12(n137sd)*, *sDp3* (Rosenbluth *et al.* 1985), *unc-119(ed3op)* (Maduro and Pilgrim 1995).

LGIV: *lin-1(sy254)* (Beitel *et al.* 1995), *lin-45(sy96)* (Han *et al.* 1993), *let-60(n1046gf)* (Beitel *et al.* 1990), *dpy-20(e1282ts)*, *unc-30(e191)*.

LGV: *him-5(e1490)*, *lin-25(e1446)* (Tuck and Greenwald 1995).

LGX: *lin-15(n309)*, *lin-3(syIs1)* (gift from R. Hill, Cal Tech), *unc-7(e5)*.

Transgenic arrays (transgenes; cotransformation markers): *gaEx36 {hs-mpk-1(gf); hs-D-mek(gf); rol-6(d)}*, *gaEx72 {lin-31-mpk-1(gf); lin-31-D-mek(gf); unc-119(+)}*, *gaIs17{hs-mpk-1(gf); hs-D-mek(gf); unc-30(+)}*; *gaIs36 {hs-mpk-1(+); EF1alpha-D-mek(gf); unc-30(+)}*.

Genetic mapping: The *oz140* allele was initially mapped to the left arm of chromosome *III* (T. Schedl, personal communication). To determine if it mapped to the same interval as the *mpk-1* locus, we mapped it relative to *unc-79* and *dpy-17*. Briefly, we picked Dpy non-Unc and Unc non-Dpy recombinants from a strain of the genotype *oz140/unc-79(e1068) dpy-17(e164)*. The progeny of these recombinant animals were screened for sterile (Ste) animals (the *oz140* mutant phenotype). Eighteen out of 19 Dpy non-Unc animals segregated Ste animals, whereas 1 out of 17 Unc non-Dpy animals segregated Ste animals. These data suggest that *oz140* maps to the left of *dpy-17* and very close or to the right of *unc-79*. This is the approximate map position of the *mpk-1* locus. The SD344 strain *[+ mpk-1(oz140) dpy-17(e164)/unc-79(e1068) + dpy-17*

TABLE 1

Oligonucleotide primers used in this study

Primer name	Sequence (5′ to 3′)[a]
mpk-1f	GGTAGCTTACAGTAGCCGTAC
mpk-2f	GAGAAGCGGTTATCTCGACGGTC
mpk-3r	CATCCCGTAAGCACCTTCTCC
mpk-4r	CGAAGTGTCCGTTGACAGAATG
mpk-5r	TATCGACAGTCTCCGAACGGATG
mpk-6f	CTGACACCCGTAGTTCAATTC
mpk-7f	CTCTATCCAGGAGCTGATCCA
mpk-8r	TCACAAACTGGCTCATCTCCTGG
mpk-9f	CATTCTGTCAACGGACACTTCG
mpk-10f	GTCTGGTCTGTCGGATGTATTC
mpk-11f	CAAGCATTGGCTCACCCATAC
mpk-12r	GGAGGAGGTGCATTCACATCAC
mpk-13f	GTTCAATGCCTGATCGAGACTG
mpk14r	GAATACATCCGACAGACCAGAC
mpk-15r	TGGATCAGCTCCTGGATAGAG
mpk-20f	GAGCTAGCATGGCCGACGGAGAAGCGGTT
mpk-21r	GCGGTACCTCTAAACAGGATTCTGCCCTC
xba-1	CACTAGTTCTAGTGCGGCCG
gof-1	CTACGATCCAGGAAATGAGCCAGTTTG
mpk-27f	GCGTCGACATGGCCGACGGAGAAGCGGTTATC
mpk-28r	GAGCGGCCGCCTAAACAGGATTCTGCCCTCC
mek-1f	GCGTCGACATGTCGAAGAACAAGCTGAATCTG
mek-2r	GAGCGGCCGCCTAGTTGGGCGACGTATTACGC

[a] Underlined sequences denote 8 to 12 nucleotide sequences used for subsequent cloning steps.

(e164)] was constructed by picking Dpy non-Unc recombinant progeny of hermaphrodites of genotype *mpk-1(oz140)/unc-79(e1068) dpy-17(e164).* All other newly isolated *mpk-1* alleles were shown to be linked to *unc-79* and *dpy-17* by allowing hermaphrodites of genotype *mpk-1(rf)/unc-79 dpy-17* to self, picking 20 Unc Dpy progeny and confirming that these hermaphrodites did not segregate sterile *mpk-1(rf)* animals (this experiment was performed at 25° for *ga111*).

Three mutations isolated in a clonal screen for sterile and vulvaless animals were mapped using STS polymorphisms (data not shown; Williams *et al.* 1992). STS mapping and two-factor mapping with *dpy-5* and *sup-11* indicated that *ga180* and *ga181* are linked to the left arm of chromosome *I*, close to *mek-2*. We next showed that both *ga180* and *ga181* fail to complement *mek-2(n2678)* for the Ste phenotype. STS mapping suggested that *ga119* is linked to chromosome *III*. Next, we showed that *ga119* fails to complement *mpk-1(oz140)* for the sterile phenotype.

Strain construction: Double mutants containing *mpk-1 (ga117)* and a Muv mutation (see Table 6) were all constructed using a common strategy, which is illustrated here for *lin-15(n309).* First, *dpy-17(e164); lin-15(n309)* was built. These *dpy-17(e164); lin-15(n309)* hermaphrodites were then mated with males of genotype *mpk-1(ga117) dpy-17(e164); sDp3*. *sDp3* is a chromosomal duplication that complements mutations in *mpk-1* and *dpy-17*. Non-Dpy cross-progeny of genotype *ga117 e164/+ e164; sDp3; n309/+* were then allowed to self-fertilize. Muv non-Dpy progeny of this cross were then placed on individual plates and allowed to self-fertilize. Muv non-Dpy animals in which all the Dpy progeny were Ste were taken to be of genotype *mpk-1(ga117) dpy-17(e164); lin-15(n309); sDp3*.

The *lin-45; gaEx36* strain was constructed by mating *lin-45/+* males with *gaEx36* (Rol) hermaphrodites. Cross-progeny hermaphrodites were cloned, and their self-progeny were screened for Vul Rol animals. Vul Rol animals were assumed to be of genotype *lin-45; gaEx36*. *lin-25; gaEx36* and *sur-2; gaEx36* strains were constructed similarly.

Gonad ablations: Somatic gonad precursors Z1.aaa and Z4.ppp were ablated with a laser microbeam (Laser Science, Newton, MA) as described in Avery and Horvitz (1989). Animals were then subjected to a 30-min heat shock of 33° as early L3 larvae and then scored as Muv or non-Muv.

Mutant sequence determination: We used oligonucleotide primers (mpk-1f and mpk-12r in Table 1) in polymerase chain reactions to amplify a 3.2-kb genomic DNA fragment containing the entire *mpk-1*-coding region for the SLX2 form from wild-type strains and each mutant strain. The DNA sequence of the *mpk-1* gene (exons 1–6 and the exon/intron borders) from each mutant was then determined by direct sequencing of this fragment in low-melting-temperature agarose (Kretz *et al.* 1989). DNA from two separate single-worm PCR reactions was sequenced using the following primers: mpk-1f, mpk-2f, mpk-3r, mpk-4r, mpk-5r, mpk-6f, mpk-7f, mpk-8r, mpk-9f, mpk-10f, mpk-11f, mpk-12r, mpk-13f, mpk-14r, and mpk-15r (see Figure 1 and Table 1).

Germ line transformation experiments: Germ line transformation experiments were done according to the method of Fire and Waterston (1989), as modified by Mello *et al.* (1991). DNAs were injected at concentrations of 100 μg/ml. Cotransformation markers were *rol-6(su1006)*, an *unc-30(+)* plasmid and an *unc-119(+)* plasmid injected into N2, *unc-30(e191)*, and *unc-119(ed3op)* hermaphrodites, respectively. For each type of array, at least two transgenic lines were obtained, and data from a representative line are shown. To integrate extrachromosomal arrays, L4 larvae containing the array of interest were subjected to gamma irradiation from a Cesium 137 source (4000 rad). Strains with integrated arrays were backcrossed at least once to *dpy-20; him-5*.

Manipulation of DNA and RNA: Molecular biological techniques were done essentially as described by Sambrook *et al.*

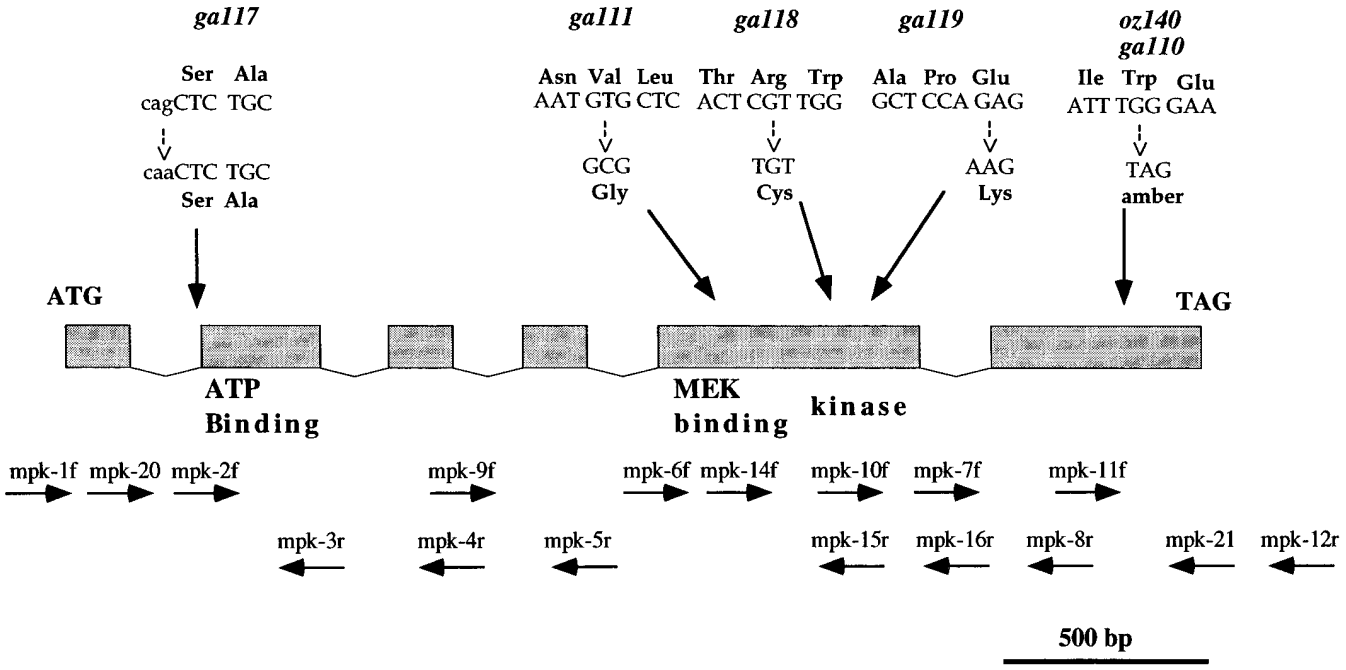

Figure 1.—Sequence alterations in *mpk-1* mutants. The boxes represent exons 2–7 of the *mpk-1* gene, slanted lines indicate introns, and arrows indicate locations in the coding sequence of the *mpk-1* mutations isolated in this study. *mpk-1* expresses two mRNAs, which differ in that one mRNA (1 × 2) includes exon 1 while the other mRNA (SL × 2) contains the SL1 *trans*-spliced leader joined to exon 2 (Lackner *et al.* 1994). The DNA sequence alteration and predicted protein coding change of each mutation is shown. Relative locations and orientations of all primers used to amplify and sequence the *mpk-1*-coding region from mutants are indicated below the boxes (Table 1). Arrows pointing to the right indicate that a given primer anneals to the antisense strand, while arrows pointing to the left indicate that a given primer anneals to the sense strand. Primers mpk-1f and mpk-12r were used in PCR experiments to amplify a 3.2-kb genomic fragment from single worms containing 292 bp of sequence 5′ to the coding region, the complete coding region, and 259 bp of 3′ untranslated region sequence. The 3.2-kb fragments from the wild type and each mutant were then sequenced using the internal primers shown, which span the entire coding region and all the intron-exon boundaries.

(1989), with minor modifications. Heat shock *mpk-1(+)* was constructed by PCR amplifying the *mpk-1*-coding region contained in cDNA pML1 using primers mpk-20f and mpk-21r. These primers were used to amplify a 1.2-kb fragment starting at the *mpk-1* start codon and ending at the stop codon. Amplified fragments were cloned into the heat shock promoter-containing vector JK465 (Roehl and Kimble 1993) to make plasmid pML54. To construct *hs-mpk-1(gf)*, we used the unique site elimination method to engineer the D324N substitution in pML1 using primers xba-1 and gain-of-function-1 (Deng and Nickoloff 1992). We then PCR amplified the insert of pML14 using primers mpk-20 and mpk-21 and ligated this fragment in the heat shock promoter vector JK465 to create construct pML40. *hs-D-mek* constructs were kindly provided by M. Koga (Koga and Ohshima 1995a). *lin-31-mpk-1(gf)* was constructed by amplifying the *mpk-1*-coding region from pML14 with primers mpk-27 and mpk-28 (Table 1) and then ligating the resulting fragment into the *lin-31* promoter-containing vector pB255. *lin-31-D-mek(gf)* was constructed by amplifying the *D-mek*-coding region from construct Dsor1(su1) (M. Koga, personal communication) with primers mek-1 and mek-2 (Table 1) and then ligating the resulting fragment into pB255.

Phenotypic characterization: When examined with a dissecting microscope, worms homozygous for strong *mpk-1* alleles have a characteristic appearance in which the gonad appears mostly clear and devoid of embryos, with several clumps of dark granular material present in the gonad. They do not produce progeny.

Pn.p cell lineages shown in Tables 2 and 3 were determined by direct observation of cells or cell divisions using Nomarski optics, as described by Sulston and Horvitz (1977).

Male mating assays were conducting by mating 20 individual *mpk-1(ga117) dpy-17* males with two *unc-119* hermaphrodites each. None produced non-Unc cross-progeny. Nine out of 10 control matings of *dpy-17* males with *unc-119* hermaphrodites gave cross-progeny.

Western blotting: Western blots were prepared essentially as described in Hoskins *et al.* (1996), with minor modifications. For each lane, 50 adult worms were picked into Laemmli buffer and boiled for 5 min before loading. Filters were immunoblotted overnight at 4° in anti-Erk2 peptide antibody K-23 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500.

RESULTS

Isolation of new *mpk-1* alleles: We wanted to determine whether all of the anchor cell signal is transduced by *mpk-1* (in which case elimination of *mpk-1* activity should prevent all vulval induction caused by the anchor cell) or whether the anchor cell signaling pathway is branched (in which case elimination of *mpk-1* activity should cause defects only in the *mpk-1* branch of the signaling pathway). To accomplish this, we isolated new *mpk-1* alleles in genetic screens, used molecular charac-

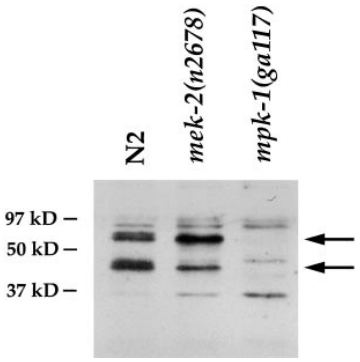

Figure 2.—Western blot analysis of MPK-1. Lysates were prepared from 50 adult worms from wild-type (N2), *mpk-1(ga117),* and *mek-2(n2678)* strains. The extracts were analyzed by Western blotting using 10% SDS-PAGE and anti–MAP kinase antibodies (Santa Cruz Biochemical). The positions and sizes (in $M_r \times 10^{-3}$) of prestained molecular weight standards (Bio-Rad, Richmond, CA) are indicated on the left. The positions of the two MPK-1 species predicted from analysis of cDNAs and Northern blots (of M_r 45 and 55 kD, respectively) are indicated by arrows.

terization to determine if any of them were likely to be null alleles, and then determined whether elimination of *mpk-1* activity completely prevented the expression of vulval cell fates.

We used three approaches to isolate additional alleles of *mpk-1*. The first approach was to screen for mutations that failed to complement the egg-laying defective (Egl) phenotype of *mpk-1(ku8).* This screen should identify *mpk-1* alleles that predominantly affect vulval development, but would miss null alleles if these alleles cause sterile or lethal phenotypes when heterozygous with *ku8*. Mutagenized N2 (wild-type) males were mated with *mpk-1(ku8) dpy-17; unc-7* hermaphrodites, and the cross-progeny hermaphrodites were screened for an Egl phenotype. From a screen involving 7500 mutagenized haploid genomes, we recovered two mutations (*ga110* and *ga111*) that mapped near to *mpk-1* in the central region of chromosome *III* and failed to complement the *mpk-1(ku8)* Egl phenotype (data not shown). An additional allele, *mpk-1(oz140),* was provided to us by T. Shedl and we subsequently showed that this allele results in the same DNA sequence change as *mpk-1(ga110)* (see Figure 1). Based on the molecular analyses described below, neither *ga110*, *ga111*, nor *oz140* are likely to be null alleles.

Our second approach was to screen for mutations that failed to complement the sterile phenotype of *mpk-1(oz140)* (Church *et al.* 1995). This screen could identify *mpk-1* null alleles if they resulted in sterility, but would miss null alleles if they cause a lethal phenotype when heterozygous with *oz140*. We screened individual males for those that might be heterozygous for a new *mpk-1* allele and, hence, sire sterile cross-progeny when mated to *oz140*/+ hermaphrodites. *him-5* (*him-5* mutations result in a *h*igh *i*ncidence of *m*ale progeny) hermaphrodites were mutagenized and allowed to self-fertilize. F_1 male progeny (potentially heterozygous for a new *mpk-1* mutation, *new*/+) were mated individually to two hermaphrodites of genotype *mpk-1(oz140) dpy-17*/*unc-79 dpy-17*. Non-Dpy cross-progeny on each plate were then screened for the presence of approximately one-quarter sterile animals, and the new *mpk-1* alleles were recovered from these plates by picking the wild-type siblings (genotype *mpk-1(new)*/+) of the sterile mutants (genotype *mpk-1(new)*/*mpk-1(oz140)*). From a screen involving 6008 haploid genomes, two mutations (*ga117* and *ga118*) were isolated that mapped near to *mpk-1* in the central region of chromosome *III* and failed to complement the sterile phenotype of *mpk-1(oz140).* As described below, our molecular analyses indicate that both *ga117* and *ga118* may severely reduce and possibly eliminate *mpk-1* activity.

The third approach used to isolate new alleles was to perform a general screen to identify heterozygous animals that segregated approximately one-quarter sterile, vulvaless self-progeny, and then to determine which were new *mpk-1* alleles. This screen could identify mutations that would be missed in the first screen (because it could identify mutations in any gene that cause a sterile, vulvaless phenotype), and it is simpler to perform than the second screen (because it does not require individual matings). Individual F_1 progeny of mutagenized hermaphrodites were placed on plates and allowed to self-fertilize, and then these plates were screened for the presence of one-quarter sterile F_2 progeny. We recovered three mutations from a screen involving 4020 mutagenized genomes. Two of these mutations are alleles of *mek-2* because they map near to *mek-2* on the left region of chromosome *I* and fail to complement the sterile phenotype caused by *mek-2(n2678).* One mutation (*ga119*) is a new allele of *mpk-1* because it maps to the central region of chromosome *III*, fails to complement the sterile phenotype of *mpk-1(oz140),* and contains a DNA sequence change in the *mpk-1* gene (see below).

Molecular characterization of *mpk-1* alleles: We used molecular analysis of the new *mpk-1* alleles to determine if any were likely to be null mutations. (Figure 1). DNA from *mpk-1(ga117)* mutants had an AG to AA nucleotide substitution in the splice acceptor site upstream of exon 3 (Figure 1), and this G nucleotide is present in all known splice acceptor sites in *C. elegans* (Blumenthal and Steward 1997). This result suggests that *ga117* results in aberrant mRNA splicing and would be predicted to eliminate expression of MPK-1 sequences after amino acid 24 of the 376-amino-acid protein. We used anti-MAP kinase antibodies in Western blotting experiments to examine MPK-1 expression levels in extracts from adult wild-type worms and *mpk-1(ga117)* mutants (Figure 2). The antibodies detect one pair of bands near 45 kD and another pair near 55 kD in protein extracts from wild-type worms. As shown below, each

TABLE 2

Phenotypes caused by *mpk-1* alleles

Genotype[a]	Wild-type vulva[b]	Abnormal vulva[b]	Vulvaless[b]	Sterile[b]	*N*[c]
Wild type	100	0	0	0	Many
ga117	0	0	100	100	40
ga118	0	0	100	100	20
ga119	0	0	100	100	20
ga118/ga117	0	0	100	ND	20
ga119/ga117	0	0	100	ND	20
oz140	40 ± 21	60 ± 21	0	100	20
oz140 at 25°	0	16 ± 16	84 ± 16	100	20
oz140/ga117	0	25 ± 19	75 ± 19	ND	20
oz140; smg-1	100	0	0	0	20
ga111	100	0	0	0	100
ga111 at 25°	100	0	0	100	20

[a] *ga111* was scored from homozygous *ga111* hermaphrodites. In the case of all other alleles shown, animals are homozygous *mpk-1(rf)* progeny segregated from hermaphrodites of genotype *mpk-1(rf) dpy-17; sDp3*. In the case of transheterozygous strains, the genotypes are *mpk-1(ga117)/mpk-1(rf) dpy-17(e164). sDp3* is a chromosomal duplication that complements *mpk-1* and *dpy-17*. The complete genotype of the *oz140; smg-1* strain is *smg-1(r861) unc-54; mpk-1(oz140) dpy-17*. All strains were examined at 20° unless otherwise indicated.

[b] Wild type indicates that vulval development appeared normal at the L4 larval stage. Abnormal vulva indicates that some vulval induction was observed, but less than in wild type. Vulvaless indicates that no vulval induction was observed. Sterile indicates that the hermaphrodite failed to produce progeny after 3 days. Numbers indicate the percentage ± the 95% confidence interval (95% confidence interval = $1.96\sqrt{(x(1-x)/N)}$, where x is the percentage of animals of a given phenotype and N is the total number of animals).

[c] Number of animals scored.

pair of bands corresponds to phosphorylated and unphosphorylated forms of one MPK-1 isoform. The sizes of the lower band in each pair are the same as those predicted to result from translation of the two types of *mpk-1* RNA (SLX2 and 1X2, respectively; Lackner *et al.* 1994). Neither pair of bands is present in extracts from *mpk-1(ga117)* mutants, suggesting that *mpk-1(ga117)* reduces expression of wild-type MPK-1 to undetectable levels as a result of improper RNA splicing.

DNA from *mpk-1(ga118)* mutants had a T to C transition that would change an arginine residue to a cysteine residue (R197C; Figure 1). This residue is conserved in all known MAP kinases and is in a region referred to as the phosphorylation loop, which contains the MEK phosphorylation site (TEY) and the kinase catalytic site (APE; Zhang *et al.* 1995). An arginine to alanine substitution at the analogous residue in human ERK1 (R208A) showed increased tyrosine phosphorylation but decreased threonine phosphorylation by MEK, and it completely abolished kinase activity of Erk1 toward its substrate, myelin basic protein (Butch and Guan 1996). Thus, the R197C substitution is likely to eliminate MPK-1 kinase activity.

DNA from *mpk-1(ga119)* mutants had a G to A transition that would change the glutamic acid in the kinase catalytic site (APE) to a lysine (APK) (Figure 1). A glutamic acid at this site is conserved in all known protein kinases (Hanks *et al.* 1988). An alanine substitution at the analogous residue in yeast cyclic AMP-dependent protein kinase resulted in a mutant protein with <0.9% of the activity of the wild-type protein (Gibbs and Zoller 1991). Similarly, the analogous mutation (E238K) in the *C. elegans mek-2* gene results in a severe loss-of-function or null phenotype (Kornfeld *et al.* 1995). Thus, the *mpk-1(ga119)* mutation may nearly eliminate MPK-1 kinase activity.

DNA from *mpk-1(ga110)* and *mpk-1(oz140)* mutants had the identical DNA sequence alteration, a G to A transition predicted to change tryptophan at amino acid 351 to an amber stop codon (Figure 1). This substitution would result in truncation of the C-terminal 25 amino acids of the MPK-1 protein. These amino acids are not conserved in other MAP kinase proteins, suggesting that these mutations may reduce but not eliminate gene activity. Premature termination of translation often results in mRNA instability, so the *oz140* phenotype might result from lower mRNA levels, decreased protein kinase activity, or both. *smg-1* mutations increase the stability of nonsense messages (Pulak and Anderson 1993), and we found that the phenotypes caused by *mpk-1(oz140)* are suppressible by a *smg-1* mutation (Table 2). Thus, C-terminal-truncated MPK-1 has MAP kinase activity when the levels of *mpk-1(oz140)* mRNA are increased by the *smg-1* mutation. This result indicates that *ga110* and *oz140* partially reduce *mpk-1* activity by decreasing mRNA levels rather than by affecting protein activity.

DNA from *mpk-1(ga111)* mutants has a T to C transi-

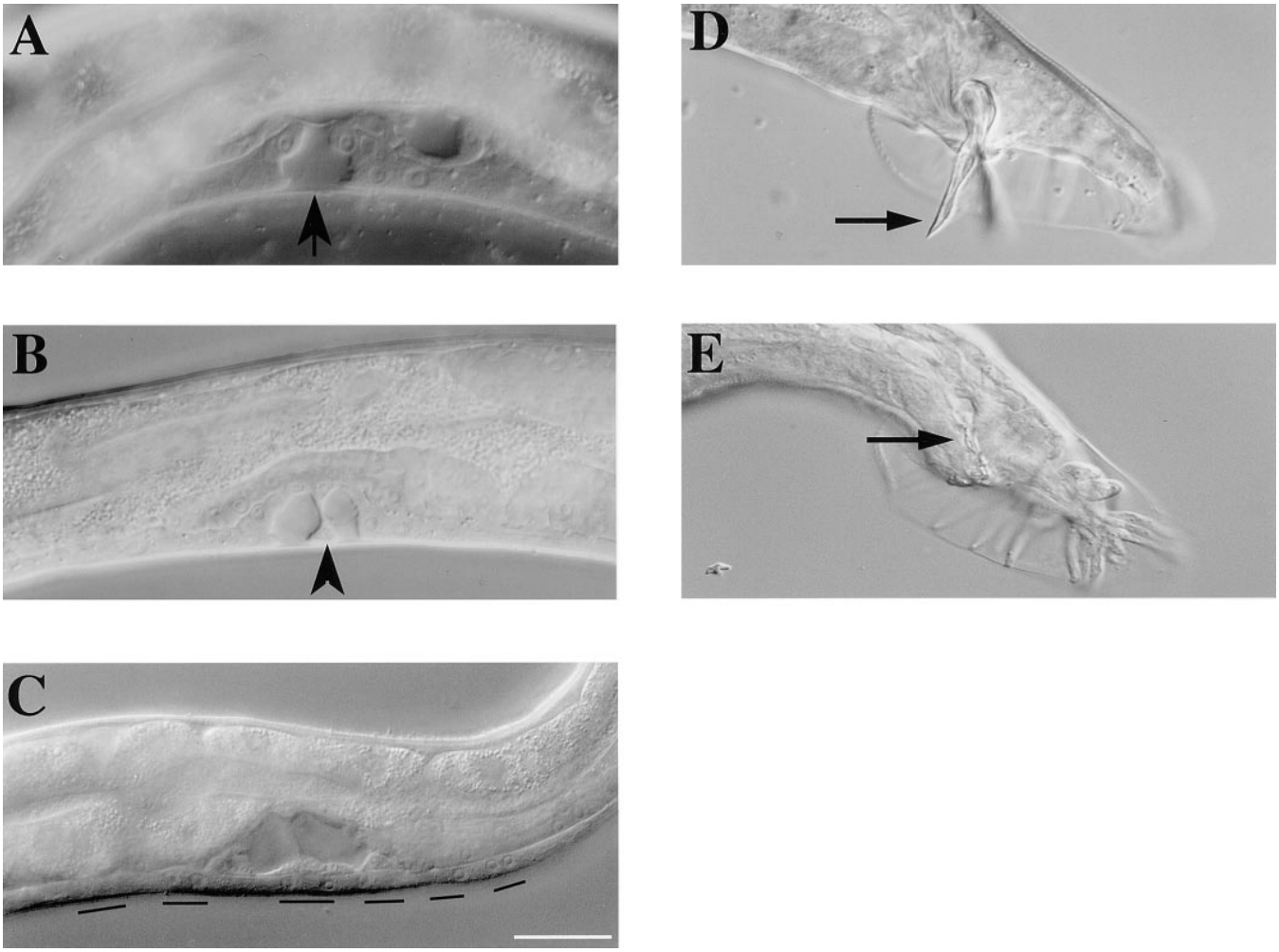

Figure 3.—Phenotypes caused by *mpk-1* alleles. (A) Wild-type hermaphrodite in the middle of the L4 larval stage. The 22 descendants of P5.p, P6.p, and P7.p have been generated and are beginning vulval morphogenesis (arrow). (B) *mpk-1(ga110)* hermaphrodite in the middle of the L4 larval stage. The posterior daughter of P6.p has failed to divide, and the undivided cell is seen adhering to the cuticle (arrowhead). The 2° cell lineages occurred normally in this animal. (C) *mpk-1(ga117)* hermaphrodite in the middle of the L4 larval stage. All the Pn.p cells have expressed the 3° cell fate (indicated by lines), and no vulval tissue has been formed. (D) Tail of a wild-type adult male. The arrow indicates a spicule, the male copulatory organ. (E) Tail of an *mpk-1(ga117)* adult male. The arrow indicates a crumpled spicule. *mpk-1(ga117)* males are unable to mate (see materials and methods). All animals were viewed with Nomarski optics. Scale bar is 10 μm.

tion that would be predicted to alter amino acid 148 from valine to glycine (Figure 1). This valine is conserved in other MAP kinase proteins but not in other types of protein kinases (Hanks *et al.* 1988). This site is near the predicted MEK-binding site (Brunet and Pouyssegur 1996; Zhang *et al.* 1995), suggesting that MPK-1(ga111) may be defective in activation by MEK.

Vulval phenotypes of *mpk-1* alleles: Our first finding is that *mpk-1* is required for vulval induction (Table 2). Using the putative null mutation *ga117*, we determined the cell lineages of the Pn.p cells in 20 mutant hermaphrodites and found that P5.p, P6.p, and P7.p expressed the uninduced 3° cell fate rather than induced 1° or 2° cell fates in every animal (Figure 3, Tables 2 and 3). Next, we determined the number and types of cells that were present at the L4 stage of development in 20 homozygous *mpk-1(ga117)*, *mpk-1(ga118)*, and *mpk-1(ga119)* animals and found that all the vulval precursor cells had expressed uninduced 3° cell fates in each case (Table 2). These results show that *mpk-1* null mutations cause a highly penetrant vulvaless phenotype.

mpk-1(ga110) and *mpk-1(oz140)* have the same DNA sequence alteration that results in a weak vulvaless phenotype that is partially temperature sensitive. At 25°, 84% of *mpk-1(oz140)* animals exhibited a Vul phenotype in which all six vulval precursor cells expressed 3° cell fates, and the remaining 16% showed a partial Vul phenotype (Table 2). At 20°, none of *mpk-1(oz140)* mutants showed a complete Vul phenotype, 60% showed partial vulval defects, and the remaining 40% had a wild-type pattern of vulval cell lineages (Figure 3 and Table 2). We determined the vulval cell lineage pattern of nine

TABLE 3

Pn.p cell lineages in *mpk-1* mutants

Genotype[a]	Pn.p cell lineage[b]						
	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	*N*[c]
Wild type	SS or U	SS	LLTN	TTTT	NTLL	SS	Many
mpk-1(ga117)	SS or U	SS	SS	SS	SS	SS	20
mpk-1(ga110) 20°	SS or U	SS	LLTN	TTTT	NTLL	SS	5
	SS or U	SS	LLTN	TTN	NTLL	SS	4
hs-mpk-(gf); hs-D-mek(gf)	TTOO	SS	LLTN	TOTT	TLLL	LTTT	1
1-hr heat shock 33°	TTTO	TTTT	LTTT	TOTT	TTLL	OOTT	1
	SS	TOTT	LOTN	TTTT	NTLL	SS	1
	TOOT	SS	LTTN	TTOO	NOLL	S OO	1
	OOOT	S TT	LLON	TOTT	NTLL	SS	1
	TOOT	TOTT	LTON	TTTT	NTLL	SS	1
	SS	TT S	LLON	TOTT	NTLL	SS	1
lin-31-mpk-(gf); lin-31-D-mek(gf)	OOTT	LL	LLTT	OTTT	NTLL	OTTT	1
	TTOT	OOOO	LLTN	OTTO	TOOT	TTTO	1
	LLTT	OOOT	LLON	TTTT	TOOT	OOTT	1
	SS	TTTO	LLON	TTTT	TOOT	OOTT	1
	TTTT	TOOO	TOTT	TTTT	OOTT	OOOL	1

[a] Strains containing *mpk-1(ga117) and mpk-1(ga110)* also contain the *cis*-linked mutation *dpy-17(e164). hs-mpk-1(gf); hs-D-mek(gf)* refers to the strain *gaIs17. lin-31-mpk-1(gf); lin-31-D-mek(gf)* refers to the strain *gaEx72* (see materials and methods).

[b] Cell fates are described in detail in the text. S, L, T, O, and N refer to the Pn.p cell that fused with the hypodermal syncytium, a longitudinal cell division, a transverse cell division, an oblique cell division, or no cell division, respectively. Underlined letters indicate cells that adhered to the ventral hypodermis. Cell fates were determined by direct observation using Nomarski optics, using the criteria of Sulston and Horvitz (1977). The 1° cell fate is TTTT, the 2° cell fate is LLTN or NTLL, and the 3° cell fate is SS. Cell fates in wild-type animals are from Sulston and Horvitz (1977). P3.p often fails to divide (U) and, hence, does not undergo a 3° cell fate (SS; Sulston and Horvitz 1977).

[c] Number of animals observed with this pattern of cell fates.

mpk-1(ga110) animals at 20°. Four animals showed defects in the expression of the 1° cell fate, and the remaining five animals showed wild-type cell lineage patterns (Table 3). Gene dosage experiments suggest that *oz140* is a partial reduction-of-function mutation because *oz140* homozygotes have a weaker phenotype than heterozygotes carrying *oz140* in *trans* to a null allele, *ga117* (Table 2).

mpk-1(ga111) results in vulval phenotypes similar to the phenotypes previously observed for *mpk-1(n2521)* (Table 2). Homozygous *mpk-1(ga111)* animals exhibit wild-type vulval development at all temperatures (Table 2). However, *mpk-1(ga111)* is a recessive suppressor of the multivulva phenotype caused by the gain-of-function *ras* allele *let-60(n1046).* Specifically, *let-60(n1046)* single mutants have a Muv phenotype, but *mpk-1(ga111); let-60(n1046)* double mutants have a wild-type vulval phenotype (data not shown).

Interestingly, *mpk-1(ku8)* might have a neomorphic activity in addition to partially reducing *mpk-1* activity. We found that *mpk-1(ga111)/mpk-1(ga117)* heterozygotes are wild-type, but that *mpk-1(ga111)/mpk-1(ku8)* heterozygotes are Egl although they have a normal pattern of vulval cell fates (data not shown). The cause of the Egl phenotype in these heterozygotes has not yet been identified. These results indicate that *mpk-1(ku8)* can have a more severe phenotype than putative *mpk-1(null)* alleles in *trans* to *mpk-1(ga111).*

Activation of the vulval pathway with MEK and MAP kinase is sufficient to cause vulval differentiation: Because MAP kinase is the primary substrate for MEK in vertebrate cells (Seger *et al.* 1992), and because *mek-2* and *mpk-1* act at adjacent points in the vulval genetic pathway (see below), we have made the assumption that activating MEK results specifically in an increase in MPK-1 activity. If the vulval induction pathway is linear, then activating MEK and MAP kinase should give full vulval induction. Alternatively, if the pathway is branched, activating only MEK and MAP kinase should give rise to only partial induction because other branches should not be activated. We have addressed this issue by observing the effects of activated *D-mek* and *mpk-1* on vulval development (Table 4).

All experiments with MEK were performed using a Drosophila MEK cDNA containing the gain-of-function *Dsor1* mutation (referred to as *D-mek(gf)*) (Tsuda *et al.* 1993). Drosophila *mek* and *C. elegans mek-2* are 55% identical (Wu *et al.* 1995), suggesting that *D-mek* may be functionally homologous to *mek-2.* We expressed *D-mek(gf)* under the control of a *C. elegans* heat shock

TABLE 4

***mpk-1(gf)* and *D-mek(gf)* expression causes a multivulva phenotype**

Transgenic constructs[a]	% Muv (*N*)[b]	Lines generated[c]
hs-mpk-1(+)	0 (200)	5
hs-D-mek(gf)	0 (100)	4
hs-mpk-1(gf)	0 (100)	4
hs-mpk-1(+); hs-D-mek(+)	9 ± 5 (154)	3
hs-mpk-1(gf); hs-D-mek(+)	59 ± 9 (120)	4
hs-mpk-1(+); hs-D-mek(gf)	64 ± 9 (100)	5
hs-mpk-1(gf); hs-D-mek(gf)	90 ± 4 (212)	7
lin-31-mpk-1(gf); lin-31-D-mek(gf)	90 ± 5 (151)	4

[a] *mpk-1(+)* refers to a wild-type *mpk-1* cDNA (derived from the SLX2 mRNA form). *D-mek(+)* refers to a wild-type Drosophila *mek* cDNA. *mpk-1(gf)* contains a single base pair change predicted to cause a D324N substitution, which is analogous to the Drosophila Sevenmaker gain-of-function MAP kinase mutation. *D-mek(gf)* (also known as Dsor1) is a gain-of-function mutation in the *D-mek* cDNA.

[b] Transgenic worms from a single representative transgenic line were heat shocked at 33° for 30 min as L2 larvae, and the Muv phenotype (percentage ± 95% confidence interval) was scored in adult animals. Numbers in parentheses indicate the number of transgenic worms scored for the Muv phenotype.

[c] Number of independent transgenic lines obtained.

promoter (*hs-D-mek(gf)*) or under the control of the *lin-31* promoter, which predominantly drives expression in the vulval precursor cells (*lin-31-D-mek(gf)*) (P. Tan, unpublished results). Expression of *hs-D-mek(gf)* in transgenic worms does not result in a multivulva phenotype (Table 4).

The amino acid substitution D334N is a gain-of-function mutation of Drosophila MAP kinase (Brunner *et al.* 1994). We used site-directed mutagenesis to engineer the analogous substitution (D324N) into an *mpk-1* cDNA clone. This cDNA construct was then placed under control of a *C. elegans* heat shock promoter (*hs-mpk-1(gf)*) or the *lin-31* promoter (*lin-31-mpk-1(gf)*). We found that expression of *hs-mpk-1(gf)* in transgenic worms did not cause a multivulva phenotype (Table 4).

We next constructed transgenic lines containing both *hs-mpk-1(gf)* and *hs-D-mek(gf)* and found that heat shock expression of *mpk-1(gf)* and *D-mek(gf)* resulted in 90% of animals exhibiting a multivulva phenotype (Table 4). We followed the Pn.p cell lineages of seven animals transgenic for *hs-mpk-1(gf)* and *hs-D-mek(gf)* to determine the pattern of cell fates expressed by the Pn.p cells (Table 3). In these seven animals, there were 21 vulval precursor cells that would normally express the 3° cell fate (P3.p, P4.p, or P8.p in each animal), and we found that these cells expressed either the 1° cell fate or a hybrid 1°/2° cell fate in 13 cases.

We also constructed transgenic animals that express *mpk-1(gf)* and *mek(gf)* from the *lin-31* promoter and found that 90% of these animals exhibited a Muv phenotype (Table 4). We then followed the Pn.p cell lineages of five such animals (Table 3). In four animals, all six vulval precursor cells expressed induced (1° and 2°) cell fates. In one animal, five out of six Pn.p cells expressed induced cell fates. We observed 12 cases in which adjacent Pn.p cells both expressed 1° cell fates (out of 25 possible cases). These results suggest that expression of *mek(gf)* and *mpk-1(gf)* is sufficient to induce vulval precursor cells to express the 1° cell fate in most cases.

***mpk-1* acts downstream of signal transduction genes but upstream of transcription factor genes in the vulval signaling pathway:** We have used several complementary approaches to determine the position of *mpk-1* in the genetic pathway specifying vulval development. Our results suggest that MPK-1 acts downstream of other conserved signal transduction molecules and upstream of transcription factors. The experiments are presented in the order in which the tested gene (or signaling event) is thought to act in the anchor cell signaling pathway.

To determine whether MEK and MAP kinase act downstream of the anchor cell signal, we used laser microsurgery to ablate the entire somatic gonad, including the anchor cell, and then induced expression of *hs-D-mek(gf)* and *hs-mpk-1(gf)*. We observed that P3.p, P4.p, or P8.p expressed vulval cell fates leading to ectopic vulval tissue in L4 stage animals and a Muv phenotype in adults (Figure 4, Table 5). The vulval phenotype observed in these gonad-ablated animals was similar to that of mock-operated control animals. This result indicates that the Muv phenotype caused by activation of MEK and MAP kinase is independent of the anchor cell signal.

lin-3 encodes a protein similar to EGF that is likely to be the inductive signal produced by the anchor cell, and overexpression of *lin-3* results in a Muv phenotype (Hill and Sternberg 1992). The vulvaless phenotype of *mpk-1(ga117)* was found to be epistatic to the Muv phenotype caused by overexpression of *lin-3* because *mpk-1(ga117); lin-3(d)* double mutants have a vulvaless phenotype (Tables 6 and 7). Furthermore, *lin-3* is expressed in the anchor cell, and a genetic mosaic analysis shows that *mpk-1* acts in the vulval precursor cells, showing that *mpk-1(+)* does not regulate *lin-3* transcription (Hill and Sternberg 1992; Lackner *et al.* 1994). Thus, the Vul phenotype of *mpk-1(ga117); lin-3(d)* mutants does not result from loss of *lin-3* expression, but it indicates that *mpk-1* activity is necessary for the action of the *lin-3* gene and suggests that *mpk-1* functions downstream of *lin-3*.

Gain-of-function *let-60 ras* mutations result in a Muv phenotype. Previous work has shown that partial reduction-of-function alleles of *mpk-1* suppress the Muv phenotype caused by a gain-of-function mutation of *let-60*. However, partial reduction-of-function alleles of *mpk-1* do not block expression of the 1° cell fate in P6.p (Lackner *et al.* 1994; Wu and Han 1994), so it was not clear

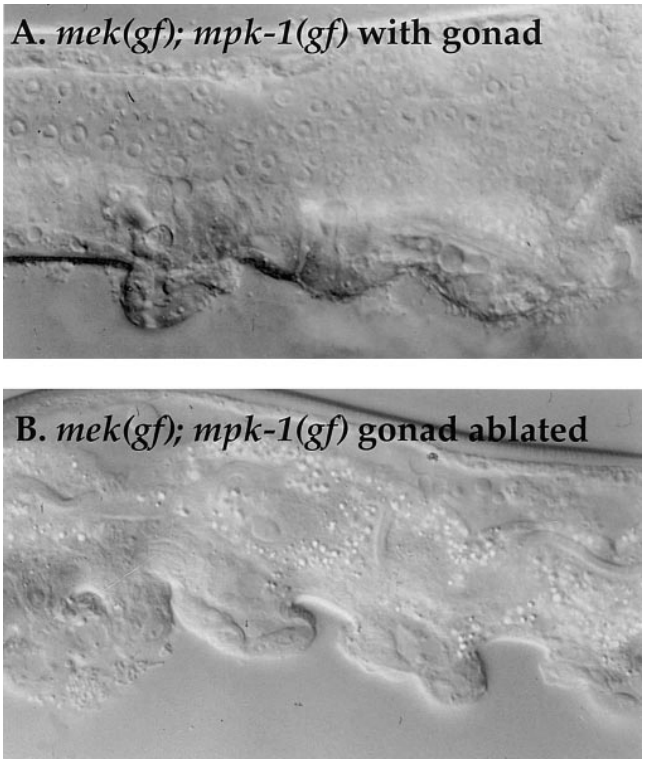

Figure 4.—The *mpk-1(gf)-D-mek(gf)* Muv phenotype is gonad independent. (A) Nomarski photomicrograph of the midbody region of an *hs-D-mek(gf) hs-mpk-1(gf)* (expressed from the integrated array *gaIs17*) adult hermaphrodite that was subjected to a heat shock (33° for 30 min) as an L2 larva. Syncytial gonad nuclei are apparent, and the animal has a multivulva phenotype. (B) Nomarski photomicrograph of the midbody region of a *gaIs17* adult hermaphrodite in which the somatic gonad precursor cells (Z1.aaa and Z4.ppp) were ablated at the L1 larval stage with a laser microbeam and that was subjected to a heat shock (33° for 30 min) as an L2 larvae. No traces of the gonad are visible, and the animal has a multivulva phenotype.

whether activation of *let-60(+)* in P6.p required *mpk-1* activity. We found that *mpk-1(ga117); let-60(gf)* double mutants are Vul, indicating that *mpk-1* activity is necessary for *let-60*–mediated expression of vulval cell fates by any of the Pn.p cells (Tables 6 and 7). This result confirms and extends previous work indicating that *mpk-1* functions downstream of *let-60*.

TABLE 5

The *mpk-1(gf); D-mek(gf)* Muv phenotype is gonad independent

Genotype	Gonad[a]	% Muv[b] (*N*)[c]
mpk-1(gf); D-mek(gf)	+	88 ± 15 (18)
mpk-1(gf); D-mek(gf)	−	100 (17)

[a] −, animals in which a laser was used to ablate the somatic gonad precursor cells in L1 larvae; +, mock-operated animals.
[b] The Muv phenotype was scored in adult *gaIs17* animals that had been heat shocked at 33° for 30 min as L2 larvae, indicated as percentage ± 95% confidence interval.
[c] Number of transgenic worms analyzed.

TABLE 6

Genetic interactions between *mpk-1* and other vulval signaling genes

Genotype[a]	Muv (%)[b]	(*N*)[c]
lin-3(gf)	100	100
lin-3(gf); mpk-1(gf)	0	151
let-60(gf)	93 ± 5	111
mpk-1(lf); let-60(gf)	0	178
lin-45(lf)[d]	0	32
lin-45(lf); mpk1(gf); D-mek(gf)	67 ± 13	51
mpk-1(gf); D-mek(gf)	92 ± 7	51
lin-25(lf)[e]	0	231
lin-25(lf); mpk-1(gf); D-mek(gf)	3 ± 6	29
sur-2(lf)[f]	0	596
sur-2(lf); mpk-1(gf); D-mek(gf)	0	25
lin-1(lf)	100	117
mpk-1(lf); lin-1(lf)	100	100
lin-15(lf)	100	95
lin-15(lf); mpk-1(lf)	0	124
lin-12(gf)	100	171
lin-12(gf); mpk-1(lf)	100	145

[a] Alleles used: *mpk-1*(*ga117*) (strains with this allele also contain the *cis*-linked mutation *dpy-17(e164)*), *lin-3(syIs17)* (overexpresses *lin-3(+)*), *let-60(n1046), lin45(sy96)* (a partial reduction-of-function allele), *lin-1(sy254), lin-25(e1446), sur-2(ku9), lin-15(n309), lin-12(n137sd). mpk-1(gf); D-mek(gf)* strains contain *hs-mpk-1(gf); hs-D-mek(gf)* and the cotransformation marker *rol-6(su1006sd)*.
[b] Percentage ±95% confidence interval.
[c] Number of animals analyzed.
[d] Data from Han *et al.* (1993).
[e] Data from Tuck and Greenwald (1995).
[f] Data from Singh and Han (1995).

lin-45 is a homolog of the proto-oncogene *raf*, and loss-of-function *lin-45* mutations cause a Vul phenotype (Han *et al.* 1993). We found that the Muv phenotype caused by *D-mek(gf)* and *mpk-1(gf)* is epistatic to the Vul phenotype caused by *lin-45(lf)*, suggesting that *mek* and *mpk-1* function downstream of *lin-45* (Table 6).

To order the activity of *mpk-1* relative to *mek-2*, we determined whether a *mek-2* null mutation prevents phosphorylation of MPK-1. *mpk-1* expresses two RNAs (1X2 and SLX2) predicted to encode proteins of 45 and 55 kD, respectively (Lackner *et al.* 1994). Based on studies of vertebrate MAP kinase, phosphorylation of each of the MPK-1 protein isoforms by MEK would be expected to result in the appearance of a slightly slower migrating band on Western blots. As expected, Western blots of protein extracts from wild-type worms immunoblotted with anti-MAP kinase antibodies detect two pairs of bands, with one pair migrating at ~45 kD and the other at 55 kD (Figure 2). The upper bands of both the 45- and 55-kD doublets are missing in extracts from a *mek-2* null mutant, suggesting that MPK-1 is not phosphorylated in these mutants. As phosphorylation

is required for MAP kinase activation, this result suggests that MPK-1 is not activated in a *mek-2* mutant and, hence, that *mpk-1* is likely to function downstream of *mek-2*.

Genetic epistasis experiments suggest that *lin-25* and *sur-2* may function at a step between *let-60 ras* and transcription factors such as *lin-1 Ets* (Tuck and Greewald 1995; Singh and Han 1996). Both *lin-25* and *sur-2* encode novel gene products, and animals homozygous for a null mutation in either gene exhibit decreased levels of vulval differentiation and protruding vulva (Pvl) phenotypes. We found that the Pvl phenotype caused by either a *lin-25* or a *sur-2* mutation was epistatic to the Muv phenotype caused by a *D-mek(gf); mpk-1(gf)* array. Specifically, *lin-25; hs-mek(gf); hs-mpk-1(gf)* and *sur-2; hs-mek(gf); hs-mpk-1(gf)* animals have an Egl and Pvl phenotype (indicating reduced expression of the 1° cell fate by P6.p), but not a Muv phenotype (which would indicate increased expression of vulval cell fates by P3.p, P4.p, or P8.p; Table 6 and data not shown). Thus, the activity of *lin-25* and *sur-2* are necessary for the expression of vulval cell fates caused by *mek-2(gf)* and *mpk-1(gf)*, indicating that *lin-25* and *sur-2* may act downstream of *mek-2* and *mpk-1* or in a parallel pathway.

Next, we ordered the activity of *mpk-1* relative to *lin-1*, which encodes a protein with a region of similarity to the Ets DNA-binding domain (Beitel *et al.* 1995). *lin-1* loss-of-function mutations cause a Muv phenotype. *mpk-1 (ga117); lin-1(sy254)* double-mutant worms also exhibited a Muv phenotype (Table 6), indicating that *lin-1* likely acts downstream of *mpk-1*.

In addition to the *let-23 RTK*/*let-60 ras*/*mpk-1* signaling pathway, at least two other signaling pathways are involved in specifying the wild-type pattern of vulval cell fates. The first pathway is an inhibitory pathway postulated to arise from the surrounding hypodermis. *lin-15* acts in this pathway and encodes two novel proteins that function to produce an inhibitory signal in the hypodermis (Hedgecock 1990; Clark *et al.* 1994; Herman and Huang *et al.* 1994). The Vul phenotype of *mpk-1(ga117)* was found to be epistatic to the Muv phenotype caused by a *lin-15* null mutation (Tables 6 and 7). This result suggests that *mpk-1* functions downstream of *lin-15* or in a parallel pathway.

The second pathway is a lateral signaling pathway in which a signal from the 1° cell induces adjacent Pn.p cells to adopt the 2° cell fate. *lin-12* encodes a protein with significant homology to the Notch family of transmembrane receptors (Yochem and Greenwald 1989), and is likely to be the receptor for the lateral signal. *lin-12(n137)* is a gain-of-function mutation that causes a Muv phenotype in which all six Pn.p cells express the 2° cell fate (Yochem and Greenwald 1989). We found that an *mpk-1(ga117) lin-12(n137)* double-mutant strain exhibited a Muv phenotype, suggesting that most or all of the vulval precursor cells expressed the 2° cell fate (Table 6). This result indicates that *mpk-1* does not act downstream of *lin-12* in the specification of the 2° cell fate.

Constitutive activation of the anchor cell signaling pathway does not cause precocious vulval induction: The vulval cell divisions begin ~29 hr after egg laying when wild-type animals are raised at 25°. The timing of vulval induction might be controlled by the time at which the vulval signaling pathway is activated. For example, some component of the signaling pathway, such as the anchor cell signal, might not be expressed until 29 hr after egg laying. Alternatively, the vulval precursor cells might not be competent to respond to activation of the *let-23*/*let-60*/*mpk-1* signaling pathway until 29 hr after egg laying. We have constitutively activated the anchor cell signaling pathway by using animals that ubiquitously express *D-mek(gf)* from the EF1a promoter and constitutively express *hs-mpk-1(+)* when grown at 25°. As shown above, constitutive activation of *mek* and *mpk-1* should be sufficient to induce vulval cell fates even if upstream components of the signaling pathway are not expressed before 29 hr after egg laying. In these transgenic worms, the vulval cell divisions began ~29–30

TABLE 7

Vulval cell lineages in double mutants

	Pn.p cell fate[b]					
Genotype[a]	P4.p	P5.p	P6.p	P7.p	P8.p	*N*[c]
Wild type	3°	2°	1°	2°	3°	5
mpk-1(lf)	3°	3°	3°	3°	3°	10
mpk-1(lf); lin-3(gf)	3°	3°	3°	3°	3°	10
mpk-1(lf); let-60(gf)	3°	3°	3°	3°	3°	10
lin-15(lf); mpk-1(lf)	3°	3°	3°	3°	3°	10

[a] Genotypes for *mpk-1(ga117)* double mutants were *mpk-1(ga117) dpy-17(e164); lin-3(syIs1), mpk-1(ga117) dpy-17(e164); lin-15(n309),* and *mpk-1(ga117) dpy-17(e164); let-60(n1046gf).*

[b] Cell fates were inferred by direct observation of L4 larvae with Nomarski optics. 3° cell fate is S S, 2° cell fate is LLTN or NTLL, and 1° cell fate is TTTT (abbreviations are defined in Table 3). P3.p was 3° or U in every animal.

[c] Number of L4 hermaphrodites observed.

TABLE 8

Timing of vulval precursor cell divisions in activated MAP kinase strains

Hours after egg laying[a]	Number of animals in which vulval precursor cell divisions have begun[b]	
	Wild type	*hs-mpk-1(+); EF1a-D-mek(gf)*[c]
27	0/5	0/5
28	0/5	0/5
29	0/5	1/5
30	2/5	1/5
31	4/5	3/5
32	5/5	5/5
33	5/5	5/5

[a] Twenty-five hermaphrodites were allowed to lay eggs for 1 hr on seeded plates, after which they were removed and progeny were placed in a 25° incubator.

[b] At each time point, five animals of each genotype were examined under Nomarski optics to determine if any of the Pn.p cells had divided.

[c] Thirty-three out of 35 of these animals expressed a Muv phenotype as adults.

TABLE 9

***mpk-1* null mutations result in maternal effect larval lethality**

Genotype of mother (m1/m2)[a]	F_1 lethality[b] (m1/m1)	F_1 lethality (m1/m2 and m2/m2)[b]
ga117/+	0 (120)	0 (344)
ga117/ga111	0 (458)	NA
ga117/ga111	97 (195)	4 (400)
ga119/ga111	92 (140)	3 (372)

[a] Full genotypes of animals: *ga117/unc-79 dpy-17*, *unc-79 ga111*, *ga117 dpy17/unc-79 ga111*, and *ga117 dpy-17/unc-79 ga111*.

[b] Percentage lethality is shown. Parentheses indicate the number of animals scored. Fertile non-Vul mothers were picked to individual plates, and entire F_1 broods were analyzed. Lethality was scored by counting dead Dpy larvae (m1/m1) or dead non-Dpy larvae (m1/m2 and m2/m2).

hr after the eggs were laid, as they do in wild-type worms (Table 8). The transgenes resulted in activation of the anchor cell signaling pathway because 90% of these animals exhibited a Muv phenotype. This result indicates that the vulval precursor cells are not competent to divide in response to MEK and MAP kinase signaling until at least 29 hr after egg laying.

***mpk-1* acts in the *let-60 ras* signaling pathway in multiple cell types:** *let-60 ras* functions in at least six cell fate decisions because *let-60* loss-of-function mutations affect at least six different cell types. An important question is whether *let-60* signals through *mpk-1* in all six cell types. If so, then *mpk-1* mutants should show defects in all the cell types that are affected in *let-60* mutants.

First, *let-60* acts in the germ line, as loss-of-function mutations cause a sterile phenotype by preventing oocyte nuclei from exiting the pachytene stage of meiosis (Church *et al.* 1995). The *mpk-1(oz140)* allele has previously been shown to cause similar sterility in homozygous animals (Church *et al.* 1995). We report here that the *mpk-1(ga117)* null allele also causes this sterile phenotype and that the *mpk-1(ga111)* allele causes a temperature-sensitive sterile phenotype (Table 2). These results suggest that *let-60* and *mpk-1* may act in the same signaling pathway in meiotic prophase progression.

Second, *let-60* is required for larval viability, as *let-60* null alleles cause developmental arrest in the L1 stage with a characteristic rod-like lethal phenotype. We have found that *mpk-1* is also required for larval viability, but that maternal *mpk-1(+)* can rescue lethality in homozygous mutant larvae. We found that *mpk-1(ga117)* progeny segregated from *mpk-1(ga117)*/+ hermaphrodites do not exhibit lethality (Table 9). We reasoned that maternal *mpk-1(+)* might rescue larval lethality in these animals, so we asked whether larval lethality was observed in a strain with less maternal *mpk-1(+)* activity. We examined homozygous *mpk-1(ga117)* animals segregated from mothers heterozygous for a partial loss-of-function *mpk-1* allele and a null allele, *i.e.*, of genotype *ga111*/*ga117*. The heterozygous *mpk-1(ga111)*/*mpk-1(ga117)* mothers are not sterile, though they do show reduced fecundity (data not shown). We observed that 97% of *mpk-1(ga117)*/*mpk-1(ga117)* progeny segregated from *mpk-1(ga111)*/*mpk-1(ga117)* parents arrested in the L1 stage as rod-like larvae (Table 9). The remaining 3% of *mpk-1(ga117)*/*mpk-1(ga117)* animals were non-Let and grew into sterile, vulvaless adults. Similar results were obtained with another allele, *mpk-1(ga119)*, which may strongly reduce *mpk-1* function (Table 9). These results suggest that *let-60* and *mpk-1* may act in the same signaling pathway in early larval development.

Third, mutations in *let-60* have been shown to affect cell fate decisions in the male tail (Chamberlin and Sternberg 1993, 1994) that result in defective spicule structure and defective male mating. *mpk-1(ga117)* homozygous males also have crumpled spicules and are unable to mate (Figure 3, materials and methods), suggesting that *mpk-1* and *let-60* may act in the same signaling pathway during spicule development.

Fourth, *let-60* mutants have defects in the migration of the sex myoblasts; the partial loss-of-function mutation *mpk-1(ku1)* causes weak defects in the migration of the sex myoblasts (Sundaram *et al.* 1996). However, we did not observe migration defects in *mpk-1(ga117)* null mutants. One possibility is that *mpk-1* has a weak role in sex myoblast migration and that an *mpk-1* null mutation causes a defect that is too subtle for us to score. Another possibility is that *mpk-1(ku1)* may be neomorphic or anti-

morphic, such that the migration defect might reflect interference from the mutant MPK-1(ku1) protein rather than a requirement for wild-type MPK-1(+). Thus, it is not clear whether *mpk-1(+)* acts in sex myoblast migration.

Fifth, *let-60* mutants have defects in the expression of the P12.p cell fate (Sundaram *et al.* 1996). In contrast, *mpk-1(ga117)* mutants segregated from *mpk-1(ga117)*/*mpk-1(ga111)* mothers do not appear to have defects in the expression of the P12.p cell fate. One possibility is that maternal *mpk-1* might be active in P12.p, so *mpk-1* mutants would not exhibit cell fate defects in these cells because of maternal rescue of *mpk-1(+)* from their heterozygous mothers. Another possibility is that *let-60 ras* transduces signals independently of *mpk-1* in this cell, possibly involving another *C. elegans* MAP kinase homolog.

Sixth, we have not observed *mpk-1* phenotypes other than the ones mentioned above. This result suggests that *mpk-1* may be required only in *let-60 ras* signaling pathways because all the *mpk-1* phenotypes are also observed in *let-60 ras* mutants.

DISCUSSION

The anchor cell signaling pathway may be linear: Previously isolated *mpk-1* alleles have weak vulval phenotypes, whereas loss-of-function alleles in other genes in the *let-23 RTK*/*let-60 ras* signaling pathway have a strong vulvaless phenotype. These results raised the possibility that the *let-23* signaling pathway may branch in a way that another branch of the signaling pathway could transduce the anchor cell signal in *mpk-1* mutants. We have tested this possibility and have shown that *mpk-1* null mutations cause a highly penetrant, vulvaless phenotype. Thus, there is no evidence for a signaling branch acting in parallel to *mpk-1*, and our data are consistent with the idea that the anchor cell signaling pathway is linear through the *mpk-1* step. However, our results do not rule out the possibility that the pathway may be branched in a way that *mpk-1* transduces the majority of the anchor cell signal and another branch has a minor role. In the absence of *mpk-1* activity, the minor branch may not be capable of mediating observable amounts of vulval induction.

The case for a linear signaling pathway is strengthened by our finding that coexpression of *D-mek(gf)* and *mpk-1(gf)* results in a strong multivulva phenotype. Both genes act at the end of the anchor cell signal transduction pathway, and the primary known function for MEK is to activate MAP kinase. These results indicate that the pathway is not branched upstream of *mek* and *mpk-1*, as *mek-2* and *mpk-1* are both required for vulval induction and coexpression of *mek(gf)* and *mpk-1(gf)* is sufficient for vulval induction.

However, expression of either gene alone [either *mek(gf)* or *mpk-1(gf)*] did not cause a Muv phenotype. One possibility is that these two genes act in one linear pathway and that gain-of-function mutations at two sequential steps are required to fully activate the pathway. It could be that full activation of MPK-1 requires both the D324N missense mutation (analogous to Drosophila *Sevenmaker*) and expression of D-MEK(gf). One finding that supports this idea is that a strong gain-of-function allele of *mek-2* results in a Muv phenotype in single mutants (Wu *et al.* 1995), showing that *mek-2* is sufficient to activate vulval cell fates, even though the *mek(gf)* allele used in these studies does not cause full activation of *mek*. Another possibility is that these two genes act in separate signaling branches and both branches need to be activated to cause a Muv phenotype.

***mpk-1* acts downstream of signal transduction genes but upstream of transcription factor genes:** We have found that the MAP kinase homolog *mpk-1* acts downstream of the Ras homolog *let-60*, the Raf homolog *lin-45*, and the MEK homolog *mek-2*, consistent with the biochemical position of MAP kinase in vertebrate signaling pathways and the genetic position of the Drosophila *rolled* MAP kinase in the R7 photoreceptor signaling pathway (Biggs *et al.* 1994; Brunner *et al.* 1994; Treisman 1996).

Furthermore, we show that *mpk-1* acts upstream of *lin-1*, which encodes a putative DNA binding protein similar to mammalian Ets-1 and Drosophila Yan (Beitel *et al.* 1995). *mpk-1* also acts upstream of *lin-31*, which encodes a winged helix transcription factor similar to mammalian HNF-3 and Drosophila forkhead (Miller *et al.* 1993; Tan *et al.* 1998). Both LIN-1 and LIN-31 are phosphorylated by MAP kinase *in vitro* and *in vivo* (Tan *et al.* 1998). Unphosphorylated LIN-1 and LIN-31 bind to each other and form a protein complex that inhibits vulval induction. Phosphorylation of these proteins disrupts the LIN-1/LIN-31 protein complex and, thus, prevents inhibition of vulval cell fates. Phosphorylation of LIN-31 may also create a transactivation domain and permit LIN-31 to act as a transcriptional activator, thereby promoting expression of vulval cell fates.

An interesting point is that the anchor cell signaling pathway may be predominantly linear between *lin-3* and *mpk-1*, but may branch downstream of *mpk-1*. *mpk-1* null mutations have a highly penetrant vulvaless phenotype, but null mutations in genes that act downstream of *mpk-1* (*lin-1*, *lin-25*, *lin-31*, and *sur-2*) neither completely activate nor completely prevent vulval induction. Specifically, null mutations in *lin-25* and *sur-2* result in a partial vulvaless phenotype (Tuck and Greewald 1995; Singh and Han 1996), *lin-1* null mutations cause expression of partial or hybrid 1° or 2° cell fates (Beitel *et al.* 1995), and *lin-31* null mutations result in partially penetrant Muv and Vul phenotypes (Miller *et al.* 1993; Tan *et al.* 1998). These results suggest that these genes may function on separate branches downstream of MAP kinase. Null mutations in any one gene may not completely block or activate vulval induction as they may

affect only a subset of the branches downstream of MAP kinase.

***mpk-1* and *let-60 ras* may act together in many developmental processes:** We have shown that *mpk-1* may function with *let-60 ras* in one signaling pathway that regulates many developmental processes. One issue that has yet to be resolved regards signaling specificity of the *let-23 RTK*/*let-60 ras*/*mpk-1* signaling pathway. How can activation of this signaling pathway in different cell types and times lead to different developmental outcomes? One possibility is that different levels of activation might lead to different cell fates. For example, it has been proposed that different levels of MPK-1 activity might cause vulval precursor cells to adopt either the 1° or 2° vulval cell fate. This graded signal hypothesis is consistent with some experiments (Sternberg and Horvitz 1986; Thomas *et al.* 1990; Katz *et al.* 1995), but is not consistent with others (Greenwald *et al.* 1983; Sternberg and Horvitz 1989; Koga and Ohshima 1995b; Simske and Kim 1995). Another possibility is that the *mpk-1* signaling pathway might act combinatorially with other signaling pathways, such that activation of different combinations of signals could specify distinct cell fates. A third possibility is that different cell types intrinsically differ in how they respond to activation of MPK-1. MPK-1 might phosphorylate different targets in different cell types, leading to distinct cellular responses. For example, LIN-31 appears to be a target for MPK-1 in the vulval precursor cells and not in other cell types (Tan *et al.* 1998). Phosphorylation of LIN-31 in these cells may allow them to express vulval-specific target genes. In this case, signaling specificity reflects the range of MAP kinase targets expressed within the cell itself before activation of MAP kinase. Thus, MAP kinase activation may be a trigger to elicit a cellular response, but the manner in which the cell responds may be determined in a cell-intrinsic fashion.

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LITERATURE CITED

Adams, P. D., and P. J. Parker, 1992 Activation of mitogen-activated protein (MAP) kinase by a MAP kinase-kinase. J. Biol. Chem. **267:** 13135–13137.

Aroian, R. V., M. Koga, J. E. Mendel, Y. Ohshima and P. W. Sternberg, 1990 The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. Nature **348:** 693–699.

Avery, L., and H. R. Horvitz, 1989 Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of C. elegans. Neuron **4:** 473–485.

Beitel, G. J., S. G. Clark and H. R. Horvitz, 1990 *Caenorhabditis elegans ras* gene *let-60* acts as a switch in the pathway of vulval induction. Nature **348:** 503–509.

Beitel, G. J., S. Tuck, I. Greenwald and H. R. Horvitz, 1995 The *Caenorhabditis elegans* gene *lin-1* encodes an ETS-domain protein and defines a branch of the vulval induction pathway. Genes Dev. **9:** 3149–3162.

Biggs, W. H. III, K. H. Zavitz, B. Dickson, A. Van Der Straten, D. Brunner *et al.*, 1994 The *Drosophila rolled* locus encodes a MAP kinase required in the *sevenless* signal transduction pathway. EMBO J. **13:** 1628–1635.

Blumenthal, T., and K. Steward, 1997 RNA processing and gene structure, pp. 117–146 in *C. elegans II*, edited by D. Riddle, T. Blumenthal, B. Meyer and J. Priess. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Brenner, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics **77:** 71–94.

Brunet, A., and J. Pouyssegur, 1996 Identification of MAP kinase domains by redirecting stress signals into growth factor responses. Science **272:** 1652–1655.

Brunner, D., N. Oellers, J. Szabad, W. H. I. Biggs, S. L. Zipursky *et al.*, 1994 A gain-of-function mutation in *Drosophila* MAP kinase activates multiple receptor tyrosine kinase signaling pathways. Cell **76:** 875–888.

Butch, E. R., and K.-L. Guan, 1996 Characterization of ERK1 activation site mutants and the effect on recognition by MEK1 and MEK2. J. Biol. Chem. **271:** 4230–4235.

Chamberlin, H. M., and P. W. Sternberg, 1993 Multiple cell interactions are required for fate specification during male spicule development in *Caenorhabditis elegans*. Development **118:** 297–324.

Chamberlin, H. M., and P. W. Sternberg, 1994 The *lin-3-let-23* pathway mediates inductive signalling during male spicule development in *Caenorhabditis elegans*. Development **120:** 2713–2721.

Church, D. L., K.-L. Guan and E. J. Lambie, 1995 Three genes of the MAP kinase cascade, *mek-2*, *mpk-1*/*sur-1* and *let-60 ras*, are required for meoitic cell cycle progression in *Caenorhabditis elegans*. Development **121:** 2525–2535.

Clark, S. G., M. J. Stern and H. R. Horvitz, 1992 *C. elegans* cell-signalling gene *sem-5* encodes a protein with SH2 and SH3 domains. Nature **356:** 340–344.

Clark, S. G., X. Lu and H. R. Horvitz, 1994 The *Caenorhabditis elegans* locus *lin-15*, a negative regulator of a tyrosine kinase signaling pathway, encodes two different proteins. Genetics **137:** 987–999.

Crews, C., A. Alessandrini and R. Erikson, 1992a Erks: their fifteen minutes has arrived. Cell Growth Differ. **3:** 135–142.

Crews, C. M., A. Alessandrini and R. L. Erikson, 1992b The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. Science **258:** 478–480.

Deng, W. P., and J. A. Nickoloff, 1992 Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. Anal. Biochem. **200:** 81–88.

Eisenmann, D. M., and S. K. Kim, 1994 Signal transduction and cell fate specification during *Caenorhabditis elegans* vulval development. Curr. Opin. Genet. Dev. **4:** 508–516.

Ferrell, J. E., Jr., 1996 MAP kinases in mitogenesis and development, pp. 1–60 in *Current Topics in Developmental Biology*, edited by R. A. Pedersen and G. P. Schatten. Academic Press, San Diego.

Fire, A., and R. H. Waterston, 1989 Proper expression of myosin genes in transgenic nematodes. EMBO J. **8:** 3419–3428.

Gibbs, C. S., and M. J. Zoller, 1991 Rational scanning mutagenesis of a protein kinase identifies functional regions involved in catalysis and substrate interactions. J. Biol. Chem. **266:** 8923–8931.

Greenwald, I. S., P. W. Sternberg and H. R. Horvitz, 1983 The *lin-12* locus specifies cell fates in *Caenorhabditis elegans*. Cell **34:** 435–444.

Han, M., A. Golden, Y. Han and P. W. Sternberg, 1993 *Caenorhabditis elegans lin-45 raf* gene participates in *let-60 ras*-stimulated vulval differentiation. Nature **363:** 133–140.

Han, M., and P. W. Sternberg, 1990 *let-60*, a gene that specifies cell fates during *C. elegans* vulval induction, encodes a *ras* protein. Cell **63:** 921–931.

Hanks, S., A. Quinn and T. Hunter, 1988 The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241:** 42–52.

Herman, R. K., and E. M. Hedgecock, 1990 Limitation of the size of the vulval primordium of *Caenorhabditis elegans* by *lin-15* expression in surrounding hypodermis. Nature **348:** 169–171.

Herskowitz, I., 1995 MAP kinase pathways in yeast: for mating and more. Cell **80:** 187–197.

Hill, R. J., and P. W. Sternberg, 1992 The gene *lin-3* encodes an inductive signal for vulval development in *C. elegans.* Nature **358:** 470–476.

Hoskins, R., A. F. Hajnal, S. A. Harp and S. K. Kim, 1996 The *C. elegans* vulval induction gene *lin-2* encodes a member of the MAGUK family of cell junction proteins. Development **122:** 97–111.

Huang, L. S., P. Tzou and P. W. Sternberg, 1994 The *lin-15* locus encodes two negative regulators of *Caenorhabditis elegans* vulval development. Mol. Biol. Cell **5:** 395–411.

Katz, W. S., R. J. Hill, T. R. Clandinin and P. W. Sternberg, 1995 Different levels of the *C. elegans* growth factor LIN-3 promote distinct vulval precursor fates. Cell **82:** 297–307.

Koga, M., and Y. Ohshima, 1995a *Drosophila* MAP kinase kinase suppresses the vulvaless phenotype of *lin-3*, *let-23* and *lin-45* mutations in *Caenorhabditis elegans.* Mech. Dev. **53:** 15–22.

Koga, M., and Y. Ohshima, 1995b Mosaic analysis of the *let-23* gene function in vulval induction of *Caenorhabditis elegans.* Development **121:** 2655–2666.

Kornfeld, K., 1997 Vulval development in *Caenorhabditis elegans.* Trends Genet. **13:** 55–61.

Kornfeld, K., K.-L. Guan and H. R. Horvitz, 1995 The *Caenorhabditis elegans* gene *mek-2* is required for vulval induction and encodes a protein similar to the protein kinase MEK. Genes Dev. **9:** 756–768.

Kretz, K. A., G. S. Carson and J. S. O'Brien, 1989 Direct sequencing from low-melt agarose with Sequenase. Nucleic Acids Res. **17:** 14.

Lackner, M. R., K. Kornfeld, L. M. Miller, H. R. Horvitz and S. K. Kim, 1994 A MAP kinase homolog, *mpk-1*, is involved in ras-mediated induction of vulval cell fates in *Caenorhabditis elegans.* Genes Dev. **8:** 160–173.

Madhani, H. D. , C. A. Styles and G. R. Fink, 1997 MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation. Cell **91:** 673–684.

Maduro, M., and D. Pilgrim, 1995 Identification and cloning of *unc-119*, a gene expressed in the *Caenorhabditis elegans* nervous system. Genetics **141:** 977–988.

Marshall, C. J., 1994 MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr. Opin. Genet. Dev. **4:** 82–89.

Mello, C. C., J. M. Kramer, D. Stinchcomb and V. Ambros, 1991 Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J. **10:** 3959–3970.

Miller, L. M., M. E. Gallegos, B. A. Morisseau and S. K. Kim, 1993 *lin-31*, a *Caenorhabditis elegans* HNF-3/*forkhead* transcription factor homolog, specifies three alternative cell fates in vulval development. Genes Dev. **7:** 933–947.

Pulak, R., and P. Anderson, 1993 mRNA surveillance by the *Caenorhabditis elegans smg* genes. Genes Dev. **7:** 1885–1897.

Roche, S., J. McGlade, M. Jones, G. Gish, T. Pawson *et al.*, 1996 Requirement of phospholipase C gamma, the tyrosine phosphatase Syp and the adaptor proteins Shc and Nck for PDGF-induced DNA synthesis: evidence for the existence of Ras-dependent and Ras-independent pathways. EMBO J **15:** 4940–4948.

Roehl, H., and J. Kimble, 1993 Control of cell fate in *Caenorhabditis elegans* by a GLP-1 peptide consisting primarily of ankyrin repeats. Nature **364:** 632–635.

Rosenbluth, R. E., C. Cuddeford and D. L. Baillie, 1985 Mutagenesis in *Caenorhabditis elegans.* II. A spectrum of mutational events induced with 1500R of gamma-irradiation. Genetics **109:** 493–511.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Seger, R., N. G. Ahn, J. Posada, E. S. Munar and A. M. Jensen, 1992 Purification and characterization of mitogen-activated protein kinase activator(s) from epidermal growth factor-stimulated A431 cells. J. Biol. Chem. **267:** 14373–14381.

Simske, J. S., and S. K. Kim, 1995 Sequential signaling during *Caenorhabditis elegans* vulval induction. Nature **375:** 142–146.

Singh, N., and M. Han, 1996 Sur-2, a novel gene, functions late in the *let-60 ras*-mediated signaling pathway during *Caenorhabditis elegans* vulval induction. Genes Dev. **9:** 2251–2265.

Sternberg, P. W., 1988 Lateral inhibition during vulval induction in *Caenorhabditis elegans.* Nature **335:** 551–554.

Sternberg, P. W., and H. R. Horvitz, 1986 Pattern formation during vulval development in *C. elegans.* Cell **44:** 761–772.

Sternberg, P. W., and H. R. Horvitz, 1989 The combined action of two intercellular signaling pathways specifies three cell fates during vulval induction in *C. elegans.* Cell **58:** 679–693.

Sulston, J. E., and H. R. Horvitz, 1977 Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans.* Dev. Biol. **56:** 110–156.

Sundaram, M., J. Yochem and M. Han, 1996 A Ras-mediated signal transduction pathway is involved in the control of sex myoblast migration in *Caenorhabditis elegans.* Development **122:** 2823–2833.

Tan, P. B., M. R. Lackner and S. K. Kim, 1998 MAP kinase signaling specificity mediated by the LIN-1 Ets/LIN-31 WH transcription factor complex during *C. elegans* vulval induction. Cell **15:** 569–580.

Thomas, J. H., M. J. Stern and H. R. Horvitz, 1990 Cell interactions coordinate the development of the *C. elegans* egg-laying system. Cell **62:** 1041–1052.

Treisman, R., 1996 Regulation of transcription by MAP kinase cascades. Curr. Opin. Cell Biol. **8:** 205–215.

Tsuda, L., Y. H. Inoue, M. A. Yoo, M. Mizuno, M. Hata *et al.*, 1993 A protein kinase similar to MAP kinase activator acts downstream of Raf kinase in *Drosophila* eye development. Cell **72:** 407–414.

Tuck, S., and I. Greenwald, 1995 *lin-25*, a gene required for vulval induction in *Caenorhabditis elegans.* Genes Dev. **9:** 341–357.

Williams, B. D., C. Schrank, R. Huynh, R. Shownkeen and R. H. Waterston, 1992 A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. Genetics **131:** 609–624.

Wood, W. B., 1988 *The Nematode Caenorhabditis elegans.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Wu, Y., and M. Han, 1994 Suppression of activated Let-60 Ras protein defines a role of *Caenorhabditis elegans* Sur-1 MAP kinase in vulval differentiation. Genes Dev. **8:** 147–159.

Wu, Y., M. Han and K.-L. Guan, 1995 MEK-2, a *Caenorhabditis elegans* MAP kinase kinase, functions in Ras-mediated vulval induction and other developmental events. Genes Dev. **9:** 742–755.

Yochem, J., and I. Greenwald, 1989 *glp-1* and *lin-12*, genes implicated in distinct cell-cell interactions in *Caenorhabditis elegans*, encode similar transmembrane proteins. Cell **58:** 553–564.

Zhang, J., F. Zhang, M. H. Cobb and E. J. Goldsmith, 1995 Activity of the MAP kinase ERK2 is controlled by a flexible surface loop. Structure **3:** 299–307.

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