sur-2, a novel gene, functions late in the *let-60 ras*-mediated signaling pathway during *Caenorhabditis elegans* vulval induction

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We describe here a new gene acting downstream of *let-60 ras* in the vulval signaling pathway of *Caenorhabditis elegans*. The *sur-2* (suppressor of *ras*) gene is defined by eight mutations identified in a genetic screen for suppressors of the Multivulva phenotype of *let-60(n1046)*, an activated *let-60 ras* mutation. *sur-2* mutations result in pleiotropic, incompletely penetrant phenotypes that include a Vulvaless phenotype in hermaphrodites, defects in development of the male tail, gonadal abnormalities, and larval lethality, indicating a role for the *sur-2* gene product in multiple developmental events. Genetic epistasis analyses suggest that *sur-2* is required late in the vulval signaling pathway, downstream of *let-60 Ras*, and is likely to act downstream of the Raf/MAP Kinase cascade. We cloned the *sur-2* gene by DNA-mediated transformation and have shown that it encodes a novel protein. We also show that a *sur-2::lacZ* transgene is expressed in the vulval precursor cells at the time of vulval determination.

[Key Words: *sur-2*; *ras*; *Caenorhabditis elegans*; vulval development; signal transduction]

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Genetic studies in *Caenorhabditis elegans* and *Drosophila*, as well as biochemical studies in several eukaryotic systems, have implicated the small GTPase Ras and its regulators and effectors as key factors in signaling cascades that control developmental pattern formation (for review, see Sternberg 1993; Brand and Perrimon 1994; Zipursky and Rubin 1994). A Ras-mediated signal transduction pathway is required for the specification of vulval cell fates as well as many other aspects of *C. elegans* development (Sternberg 1993). *C. elegans* vulval development provides a model system that enables us to dissect genetically the Ras-mediated vulval signaling pathway to test the functions of known signaling molecules in animal development, and more importantly, to identify and characterize the role of components of the Ras pathway that have eluded biochemical detection.

Multiple cell-cell interactions control cell fate specification during development of the *C. elegans* hermaphrodite vulva (for review, see Horvitz and Sternberg 1991; Sternberg 1993). The vulval cells differentiate from three of six vulval precursor cells (VPCs, P3.p–P8.p) that have equal developmental potential prior to vulval induction (Horvitz and Sternberg 1991). According to current models, an inhibitory signal from the hypodermal tissue surrounding the VPCs prevents them from adopting a vulval cell fate (Ferguson et al. 1987; Herman and Hedgecock

1990). During vulval induction, a signal from the anchor cell in the somatic gonad is proposed to alleviate the hypodermal inhibition of the three VPCs (P5.p–P7.p) that are closest to it (Fig. 1). The induced VPCs undergo further rounds of division, and their progeny generate vulval tissue. The other three VPCs adopt a nonvulval (epidermal) or tertiary fate (3°), dividing once and then fusing with the surrounding hypodermal tissue. Thus, the wild-type pattern of vulval and nonvulval cell fates is the result of integrating input from several different intercellular signaling events (Horvitz and Sternberg 1991; Katz et al. 1995; Simske and Kim 1995). Many mutants aberrant in vulval development have been isolated and have one of two phenotypic abnormalities in vulval morphology (Horvitz and Sternberg 1991): In Multivulva (Muv) mutants, more than three of the six VPCs adopt a vulval cell fate, and in Vulvaless (Vul) mutants less than three of the six VPCs adopting a vulval cell fate.

Genetic analyses of Muv and Vul mutants have ordered these genes in a pathway. The molecular characterization of several of these genes has demonstrated that components of the vulval signaling pathway are very similar to those in signaling pathways that control the proliferation and differentiation of mammalian cells. This information has led to the proposal of a genetic/molecular pathway depicted in Figure 7 (below). Activa-

Figure 1. Effects of *sur-2* mutations on cell lineages of the vulval precursor cells. (A) Vulval cell fate specification in wild-type animals. The adult hermaphrodite vulva is formed from an equivalence group of six VPCs, P[3–8].p, represented as ovals. The current model of vulval development suggests that the six VPCs are inhibited from adopting vulval cell fates by a signal from the surrounding syncytial hypodermal tissue (Herman and Hedgecock 1990). This inhibitory signal requires the function of the *lin-15* locus and likely a number of other genes (Clark et al. 1994; Huang et al. 1994). During vulval induction, an inductive signal encoded by the *lin-3* gene from the anchor cell of the somatic gonad induces three VPCs (P5.p–P7.p) to adopt vulval cell fates, represented as solid ovals (Horvitz and Sternberg 1991; Hill and Sternberg 1992). These induced cells undergo three rounds of cell division and their progeny generate vulval tissue. A lateral signal between the induced VPCs P[5–7].p ensures that P6.p adopts a primary (1°) fate while P5.p and P7.p adopt secondary (2°) fates. The *lin-12* gene product has been proposed to be the receptor for the lateral signal (Seydoux and Greenwald 1989). The other three VPCs (P3,4,8.p) divide once and fuse with the syncytial hypodermis. This 3° fate is represented here as shaded ovals. (B) Examples of vulval lineages in *sur-2* mutant animals. A range of 0%–100% vulval cell differentiation is seen in *sur-2* mutant animals. (1) 0% vulval induction, where all the VPCs adopt a nonvulval fate. The frequency of this phenotype varies among mutant alleles from 0% [*ku3* allele] to 64% [*ku49* at 15°C]. (2) P6.p is induced to adopt a primary vulval cell fate but P5.p and P7.p adopt nonvulval cell fates. This is seen in up to 72% of animals scored for the *ku9* allele. (3) Hybrid lineages are also observed when either Pn.pa or Pn.pp divides to generate vulval cells but the other does not. This is observed in 11% of *ku9* animals, when either P5.pa or P5.pp divides or when P5.pa or P5.pp and P6.pa or P6.pp divides. (C) Isolation of *sur-2* alleles as *let-60(n1046gf)* suppressors. A *let-60(n1046gf)* strain was mutagenized with EMS and the F₂ progeny were screened for non-Muv revertants. In most cases, candidates picked were Egl. Over 60 revertants were isolated after screening >50,000 mutagenized haploid genomes. Eight *sur-2* alleles were obtained from this screen.

tion of *let-60 ras* stimulates a kinase cascade that includes a series of serine threonine kinases including *lin-45* Raf (Han et al. 1993), *mek-2/let-537* MAP kinase kinase (Church et al. 1995; Kornfeld et al. 1995; Wu et al. 1995), and *sur-1/mpk-1* MAP kinase (Lackner et al. 1994; Wu and Han 1994).

Activation of the Sur-1/Mpk-1 MAP kinase is expected to result in the phosphorylation of downstream factors that leads to changes in gene expression specifying vulval differentiation (O'Neill et al. 1994; for review, see Karin 1994; Treisman 1994). In *C. elegans*, three genes, *lin-1*, *lin-31*, and *lin-25*, have been shown to function downstream of *sur-1/mpk-1* in the vulval signaling pathway. The *lin-1* gene is proposed to act downstream of Sur-1/Mpk-1 MAP kinase to negatively regulate the vulval signaling pathway (Ferguson et al. 1987; Han et al. 1990, 1993; Wu and Han 1994). The *lin-31* gene encodes an HNF-3/*fork head* transcription factor homolog and is proposed to act late in the vulval signaling pathway to regulate both positive and negative factors required during vulval development (Miller et al. 1993; Lackner et al.

1994). Recently, the *lin-25* gene has been proposed to act downstream of Sur-1/Mpk-1 MAP kinase (Tuck and Greenwald 1995). Although the kinase cascade of Raf, Mek, and MAP kinase has been well documented in several systems, including *C. elegans*, the paramount question that remains is What positive effectors act after Sur-1/Mpk-1 during vulval development?

In this paper we report the genetic and molecular characterization of the *sur-2* gene, a novel component of the Ras pathway that is required for normal vulval development. The *sur-2* gene product is likely to be a commonly used factor that plays an important role in numerous developmental events. Our genetic analysis of the *sur-2* gene suggests that it acts at a late step in the vulval signaling pathway. We have cloned and sequenced the *sur-2* gene and shown that it encodes a novel protein that is likely to act downstream of Sur-1/Mpk-1 MAP kinase. We have studied the expression pattern of a *sur-2::lacZ* transgene and shown that it is expressed in the VPCs at the time of vulval induction.

Results

Suppression of the Muv phenotype of activated LET-60 Ras defines sur-2

In a genetic screen aimed at identification of genes that act downstream of *let-60 ras* in the vulval signaling pathway, we isolated suppressors of the Muv phenotype of *let-60(n1046)*, a gain-of-function (*gf*) mutation that results in a constitutively active Ras protein. *let-60(n1046)* is a point mutation that changes Gly to Glu at residue 13 (Beitel et al. 1990), a substitution that is equivalent to certain oncogenic Ras mutations in mammalian systems (for review, see Barbacid 1987). We screened >50,000 mutagenized chromosome sets and isolated >60 suppressors that map to several different complementation groups (Y. Han, D. Green, G. Herrmann, M. Hara, and M. Han, unpubl.). We have obtained mutations in the *lin-45 raf*, *mek-2*, *sur-1/mpk-1* genes, indicating that our screen is successful in identifying genes known to act downstream of Ras in Ras-mediated signaling pathways (Wu and Han 1994; Wu et al. 1995).

Eight independently isolated mutations that displayed similar mutant phenotypes were mapped to the right arm of chromosome 1 and, by complementation tests, were shown to comprise a single complementation group that defines the *sur-2* locus. Mutations in *sur-2* are completely recessive and are able to suppress the Muv phenotype of *let-60(n1046 gf)* to a Vul phenotype. In addition, in the absence of the *let-60(n1046)* mutation, seven of the eight *sur-2* mutations also cause a Vul phenotype. One mutation, *sur-2(ku60)* has a wild-type vulval lineage but is egg-laying defective (Egl), presumably because of disruption of some other component of the egg-laying system that we have not identified (Table 1).

sur-2 mutations cause defects in vulval induction

The Vul defect of *sur-2* mutant hermaphrodites is vari-

able among animals; however, examples of each of the following three types of defects can be seen for all *sur-2* mutant alleles (Figs. 1B and 2). (1) In many mutants, there is no vulval induction at all. In this case, P5.p, P6.p, and P7.p adopt a tertiary fate (3°) instead of adopting vulval cell fates, indicating that wild-type *sur-2* is required for both primary (1°) and secondary (2°) vulval cell fates (Fig. 2c). (2) The most common defect is the generation of a normal primary lineage by P6.p, but P5.p and P7.p remain completely uninduced, indicating that the secondary cell fate is more sensitive than the primary cell fate to changes in gene activity of *sur-2* (Fig. 2d). (3) There is a class of animals where hybrid lineages are observed. In these cases, a Pn.p cell divides once to generate two daughters; one of these daughters divides further and generates vulval cells, whereas the other daughter fails to divide (Fig. 2d). Examples of hybrid lineages have been reported previously (Aroian and Sternberg 1991; Wu and Han 1994) and suggest that VPCs are not irreversibly committed to vulval cell fates before the first round of cell division.

sur-2 loss-of-function mutations cause pleiotropic defects in multiple developmental events

Like loss-of-function (*lf*) mutants in many other genes acting in the vulval signaling pathway (e.g., *lin-3*, *let-23*, *sem-5*, *let-60*, *lin-45*, *sur-1/mpk-1*; for review, see Sternberg 1993; Wu and Han 1994), *sur-2* mutants display a number of pleiotropic defects. All *sur-2* mutants cause incompletely penetrant larval lethality; the dying worms arrest with a rod-like morphology in the first through fourth stages of larval development as judged by the size of their gonads and L1-specific alae. This larval lethality suggests that the *sur-2* gene has an essential function during or before larval growth. The role of *sur-2* in early *C. elegans* development is also consistent with our observation that the *sur-2* mRNA is enriched in embryonic stages of development (see Results below). The larval lethality is suppressed by the presence of *let-60(n1046 gf)* in the *sur-2(ku9)*; *let-60(n1046 gf)* double mutant. Whereas a population of *sur-2(ku9)* hermaphrodites display 4% larval lethality ($n=100$), no dead larvae have been observed in a *sur-2(ku9)*; *let-60(n1046)* double mutant strain ($n>500$). We have also tested *sur-2(ku9)* and *sur-2(ku42)* alleles for male mating ability and have found these mutant males unable to mate, suggesting that *sur-2* function is required for the establishment of cell fates in the male tail. Male tail defects in *sur-2* mutant alleles were also observed by H. Chamberlin and P. Sternberg (pers. comm.). Genes in the Ras-mediated vulval signaling pathway have been shown recently to be required for inductive signaling during male spicule development (Chamberlin and Sternberg 1994). The pleiotropies of *sur-2* mutants suggest that the *sur-2* gene encodes a factor required in numerous developmental decisions that are likely to use a Ras-mediated signaling pathway.

Table 1. Phenotypes of mutations in the *sur-2* gene

<i>sur-2</i> allele	Percent Egl (n) ^a	Percent vulval induction (n) ^b	Percent lethality (n) ^c
Wild type	0 (300)	100 (20)	0 (300)
<i>ku9</i>	100 (596)	30 (18)	4 (100)
<i>ku3</i>	100 (100)	41 (14)	7 (243)
<i>ku31</i>	100 (279)	74 (8)	4 (166)
<i>ku42</i>	100 (100)	19 (16)	2 (271)
<i>ku115</i>	100 (100)	32 (11)	3 (170)
<i>ku49</i>	100 (783)	12 (14)	2 (683)
<i>ku116</i>	100 (100)	37 (19)	7 (112)
<i>ku60</i>	45 (55)	99 (27)	<1 (326)

(n) Number of animals scored.

^aPercentage of animals that are Egl. This number does not include a small percentage of sterile animals (<5%). For example, 4% of *ku31* and 5% of *ku49* homozygotes are sterile.

^bPercentage of vulval precursor cells (P3.p–P8.p) that generate vulval cells relative to wild type as determined by Nomarski microscopy (Han et al. 1990).

^cPercentage of animals that die at larval stages (L1–L4).

Figure 2. Vulval lineage defects of *sur-2* mutants under Nomarski optics. Nomarski photomicrographs showing vulval cell differentiation in wild-type and mutant *C. elegans* hermaphrodites. All animals with vulval lineages depicted are in the early L4 larval stage. Original magnification, 1000 $\times$. (a) N2 hermaphrodite. A bracket indicates a wild-type vulva derived from the progeny of P5.p, P6.p, and P7.p. (b) *let-60(n1046 gf)*. A wild-type vulva formed from the daughters of P5.p, P6.p, and P7.p is marked by a bracket. The *let-60 gf* mutation causes extra vulval cell differentiation: P3.p, P4.p, and P8.p form pseudovulvae on the ventral surface of the animal (arrows). The photomicrograph is from Wu and Han (1994). (c) *sur-2(ku9)*. Lines from left to right indicate that P5.p, P6.p, and P7.p behave as hypodermal cells, dividing only once and adopting a nonvulval fate. (d) *sur-2(ku9)* hermaphrodite. The arrow marks the invagination formed by P6.p, which divided to form a normal primary lineage. Lines indicate that P5.p and P7.p were not induced. (e) *sur-2(ku9)* hermaphrodite. One arrow indicates an invagination formed by the descendants of P5.p and one daughter of P6.p, P6.pa; the other arrow indicates that P7.p divided. The line indicates that the other daughter of P6.p, P6.pp is undivided. This represents a hybrid lineage. (f) A *sur-2(ku9)* vacuolarized dead larvae arrested at L1, with the arrowhead indicating the gonad.

sur-2 alleles are likely to be reduction-of-function mutations

The suppression of the Muv phenotype of an activated *let-60 ras* mutation, the Vul phenotype, the larval lethal phenotype, and the male abnormal phenotype of the *sur-2* alleles are most likely the result of reduction of *sur-2* gene activity. Mutations in the *sur-2* locus have been isolated at a high frequency, ~ 1 in 5000 mutagenized chromosome sets. When the *sur-2(ku9)* allele is placed in *trans* to *eDf14*, a deficiency of the region, there is a reduction in vulval induction from 33% for *sur-2* homozygotes to 13% for *sur-2/eDf14* (Table 2). Finally, molecular data show that five mutant alleles contain nonsense mutations that result in truncation of as much as three-quarters of the gene product (Figs. 3 and 4). The molecular nature of these mutations is consistent with these mutations causing a reduction-of-function in the gene.

sur-2 may act at a late step in the vulval signal transduction pathway

To determine where in the vulval development pathway *sur-2* acts, we examined the phenotype of hermaphrodite worms doubly mutant for *sur-2(ku9)* and either of several Muv mutations known previously to act in vulval induction. Specifically, we asked whether *sur-2* mutations could suppress the Muv phenotype caused by loss-

of-function mutations in *lin-15*, *lin-1*, *lin-31*, or a gain-of-function mutation in *lin-12*.

The *lin-15* locus is thought to function in the generation of the inhibitory signal from the hypodermis (Ferguson et al. 1987; Clark et al. 1994; Huang et al. 1994; Herman and Hedgecock 1990). In *lin-15(n765)* hermaphrodites all six VPCs adopt a vulval fate, even in the absence of the anchor cell (Ferguson et al. 1987). Genetic epistasis analysis has been used to place *lin-15* upstream of *let-23 RTK* and *let-60 ras* (Han et al. 1990; Beitel et al. 1990). The Muv phenotype of *lin-15(n765)* is suppressed by the *sur-2(ku9)* allele (Table 2), consistent with *sur-2* function being required downstream of *let-60 ras* in the signaling pathway. Interestingly, *sur-2(ku9)* does not suppress the *lin-15(n765)* Muv phenotype as well as the *let-60(n1046)* Muv phenotype (Table 2).

sur-2(ku9) is able to partially suppress the Muv phenotype of a hypomorphic *lin-1* allele, *lin-1(e1275)*. The double mutant retains characteristics of both single mutants: Adjacent primary lineages are observed in both *lin-1(e1275)* and the double mutant but not in *sur-2(ku9)* alone. However, P3.p, P4.p, and P8.p, which are usually induced in *lin-1(e1275)*, remain uninduced in the double mutant as they do in *sur-2(ku9)*. It has been observed previously that the Muv phenotype of *lin-1(e1275)* is epistatic to Vul mutations in *let-60 ras*, *lin-45 raf*, *mek-2*, and *sur-1/mpk-1* (Han et al. 1990, 1993; Wu and Han 1994; Church et al. 1995; Kornfeld et al. 1995). We have also constructed a double mutant between *sur-2(ku9)*

Table 2. Genetic interactions of *sur-2* with *let-60*, *lin-15*, *lin-1*, *lin-12*, or *lin-31*

Genotype ^a		Percent Muv (n) ^b	Percent vulval differentiation (n) ^c	Percent egg-laying ^d competent (n)
<i>sur-2</i>	others			
+/+		0	100 (20)	100 (300)
<i>ku9/eDf14</i>		N.D.	13 (9)	N.D.
<i>ku9</i> /+		0 (>500)	100 (10)	100 (150)
<i>ku9/ku9</i>		0 (200)	30 (18)	0 (200)
<i>ku60/ku60</i>		0 (>500)	99 (27)	56 (55)
+	<i>let-60</i> (n1046 <i>gf</i>)	95 (150)	158 (20)	N.D.
<i>ku9</i>	<i>let-60</i> (n1046 <i>gf</i>)	3 (200)	59 (18)	0 (200)
<i>ku60</i>	<i>let-60</i> (n1046 <i>gf</i>)	4 (150)	100 (10)	50 (100)
+	<i>lin-15</i> (n765 <i>lf</i>)	99 (100)	197 (19)	N.D.
<i>ku9</i>	<i>lin-15</i> (n765 <i>lf</i>)	8 (200)	90 (19)	sterile
+	<i>lin-1</i> (e1275 <i>lf</i>)	100 (200)	165 (20)	N.D.
<i>ku9</i>	<i>lin-1</i> (e1275 <i>lf</i>)	40 (200)	95.3 (11)	sterile
+	<i>lin-1</i> (ar147 <i>lf</i>)	100 (100)	N.D.	N.D.
<i>ku9</i>	<i>lin-1</i> (ar147 <i>lf</i>)	100 (100)	N.D.	sterile/lethal
+	<i>lin-12</i> (n137 <i>gf</i>)	100 (>200)	200 (20)	N.D.
<i>ku9</i>	<i>lin-12</i> (n137 <i>gf</i>)	100 (>200)	200 (26)	sterile
+	<i>lin-31</i> (n301 <i>lf</i>)	77 (200)	165 (14)	N.D.
<i>ku9</i>	<i>lin-31</i> (n301 <i>lf</i>)	2 (132)	92 (18)	0 (200)

^a(+) Wild-type copy of *sur-2*. (*gf*) Gain-of-function mutation; (*lf*) loss-of-function mutation. The complete genotype for the *sur-2/eDf14* strain is *lev11(x12) sur-2(ku9)/eDf14*, for the *sur-2*/+ strain is *sur-2(ku9)/unc-54(e190)*, for the *sur-2/sur-2* strain is *lev-11(x12) sur-2(ku9)*, for the *lin-1*(e1275) strain is *lin-1(e1275);lon-2(e678)*, for the *lin-12* strain is *dpy-19(e1259) lin-12(n137)*, and for the *sur-2;lin-12* strain is *sur-2(ku9);dpy19(e1259) lin-12(n137)*.

^bPercentage of animals displaying a Multivulva (Muv) phenotype observed as multiple ventral protrusions under a dissecting microscope (Ferguson and Horvitz 1985). The Muv phenotypes of *let-60*(n1046 *gf*) and *lin-15*(n765 *lf*) have been described previously (Ferguson and Horvitz 1985; Beitel et al. 1990; Han et al. 1990).

^cPercentage of vulval precursor cells (P3.p–P8.p) that generate vulval cells relative to wild type (Han et al. 1990).

^d*sur-2* mutant egg-laying-competent animals do not include a small percentage of animals (<5%) that appear to be sterile. This is based on between 100 and 683 animals, depending on the allele scored. The sterility scored was completely penetrant, and the sterile worms failed to produce progeny. Double mutants between *sur-2(ku9)* and *lin-15*(n765), *lin-1*(e1275), *lin-1*(ar147) or *lin-12*(n137) have gross gonadal abnormalities that result in a large number of sterile worms. The partial larval lethality of *ku9/ku9* homozygotes (Table 1) is suppressed by the *let60(gf)* allele but not by the *lin-15(lf)* allele. (N.D.) not determined; (n) Number of animals scored.

and an apparent null allele of *lin-1*, *ar147* (G.J. Beitel, S. Tuck, I. Greenwald, and H.R. Horvitz, in prep.). The double mutants display a strong Muv phenotype but are completely sterile (*n*>50); the majority of them die with ruptured vulvae. This indicates that the *lin-1* null allele is epistatic to *sur-2* loss of function in the vulva; *sur-2* wild-type activity is not required for the Muv phenotype of a *lin-1* null allele. However, these two genes display synthetic lethality, suggesting that they may not act in a simple linear pathway.

The *lin-31* gene is required for 1°, 2°, and 3° cell fate specification. Loss-of-function mutations in *lin-31* result in a deregulated expression of vulval cell fates. In a *lin-31* mutant animal, P3.p, P4.p, or P8.p, VPCs that normally express a nonvulval cell fate can adopt vulval cell fates, whereas P5.p, P6.p, or P7.p, which normally adopt vulval cell fates, can remain uninduced. Thus, a single *lin-31* mutant animal can display both Muv and Egl phenotypes. A population of *lin-31*(n301) hermaphrodites is 77% Muv (Miller et al. 1993). *sur-2(ku9)* is able to sup-

press the *lin-31* Muv phenotype as observed under the dissecting microscope (Table 2). We followed the vulval cell fates of *sur-2(ku9); lin-31*(n301) hermaphrodites and observed that the percentage of vulval induction significantly decreased in the double mutant (92%) as compared to that of the *lin-31*(n301) single mutant (165%) (Table 2). Specifically, only 3 of the 18 double mutants show a half cell induction in either P4.p or P8.p (one of the daughters of P4.p or P8.p is induced), whereas in *lin-31*(n301) single mutant animals, at least one cell among P3.p, P4.p, and P8.p is induced to a vulval cell fate (*n*=14) (Miller et al. 1993). Thus, in a *sur-2(ku9); lin-31*(n301) double mutant, the *lin-31* mutant phenotype in P3.p, P4.p, and P8.p is significantly suppressed, suggesting that *sur-2* may function downstream of *lin-31* in these cells. In P(5–7).p, however, vulval induction is significantly higher in the double mutant than in the *sur-2(ku9)* single mutant (Table 2), suggesting that the *lin-31* mutant phenotype continues to be expressed in these cells.

Double mutants constructed between *sur-2(ku9)* and weak loss-of-function mutations in several genes acting in the Vulval pathway display a synthetic lethal phenotype. *let-60(n1046 ku75)*, which was isolated as an intra-genic revertant of *let-60(n1046 gf)*, is phenotypically wild type in vulval lineage and viability ($n > 500$; M. Sundaram and M. Han, in prep.). The *sur-2(ku9); let-60(n1046ku75)* double mutant is almost completely inviable, with $>90\%$ of the animals arresting as rod-like dead larvae ($n > 500$). A weak *lin-45* allele, *lin-45(ku51)*, is also phenotypically wild-type ($n > 500$; M. Han, unpubl.) but, in combination with *sur-2(ku9)*, is $>90\%$ inviable ($n > 500$). The synthetic lethal phenotype could be explained by loss-of-function in *sur-2* further reducing the activity of the *ras* pathway in weak mutants or alternatively by turning off a redundant parallel pathway.

sur-2 acts before *lin-12* in determining 2° vulval cell fate

The *lin-12* gene encodes a Notch-like transmembrane protein that is proposed to function as a receptor to me-

diate a lateral signal that specifies 2° vulval cell fates during vulval development (Greenwald et al. 1983; Sternberg 1988). Loss-of-function mutations in *lin-12* result in no 2° cell lineages, whereas gain-of-function mutations in *lin-12* cause all six vulval precursor cells to adopt 2° vulval cell fates. Previously, it was shown that *lin-12(gf)* mutations are epistatic to loss-of-function mutations in *let-23* RTK and *let-60 ras* (Sternberg and Horvitz 1989; Han et al. 1990), suggesting that *let-23* and *let-60* act before *lin-12* to specify the 2° cell fate. Mutations in the *sur-2* gene severely reduce the number of 2° vulval cells, indicating an essential role for the *sur-2* gene in specifying 2° vulval cell fates. The lack of 2° vulval cells in *sur-2* mutants could arise if *sur-2* were required for vulval induction or for production of the lateral signal. Alternatively, *sur-2* may be required in P5.p and P7.p to respond to the lateral signal. To distinguish between these possibilities, we constructed and examined a *sur-2(ku9); lin-12(n137)* double mutant. Although the *sur-2(ku9)* mutation alone causes a drastic decrease in 2° vulval cell fates, *lin-12(n137)*, a dominant hypermorphic mutation, causes all six vulval precursor

Figure 4. The *sur-2* gene structure. Seventeen exons (open rectangles) and 16 introns (open arrowheads) are depicted with their corresponding sizes in base pairs. The introns have not been drawn to scale. Two classes of cDNAs were isolated that differ in their 3' end and are depicted below the gene structure. Sequenced cDNAs are marked by stippled boxes; unsequenced DNA is marked by open boxes. The longest cDNA obtained is likely to contain the 5' end of the gene because it corresponds in size to an mRNA species identified by Northern blot. Additionally, no further open reading frames can be identified in genomic sequences 5' upstream of the known cDNA sequence (within the rescuing fragment). There are two putative translational start codons (ATG) in exon 1. The location of five of the eight *sur-2* mutations is indicated. A map of the corresponding genomic DNA region is depicted below the cDNAs. The *HindIII* site in exon 4 was used to construct the *sur-2::lacZ* transgene.

cells to adopt a 2° vulval cell fate. As indicated in Table 2, the double mutants display the phenotype of *lin-12(n137)* rather than that of *sur-2(ku9)*, indicating that the wild-type *sur-2* gene is likely to act upstream of *lin-12* to specify 2° cell fates.

The *sur-2* gene encodes a novel protein

We genetically mapped the *sur-2* gene to the right arm of chromosome 1 between *let-206* and *let-49* (see Material and methods). *sur-2* is estimated to be ~0.6 map unit to the left of *unc-54*. *sur-2* was also genetically mapped with respect to deficiencies in the region and was shown to lie between the left breakpoints of *eDf13* and *eDf14* (Fig. 3).

The physical map corresponding to the genetic interval between the left breakpoints of *eDf13* and *eDf14* is covered by cosmid clones. Candidate cosmid fragments were used to probe DNA isolated from deficiency heterozygotes in Southern blotting experiments. Cosmids were identified that showed a twofold decrease in intensity, compared with a control cosmid (one that does not lie within the region), in lanes where DNA was prepared from deficiencies that were shown genetically to uncover the *sur-2* locus (Fig. 3B, Material and methods). We then identified cosmids containing the *sur-2* gene by microinjection transformation with cosmids lying within this interval. Plasmid pRF4, containing the dominant *rol-6* mutant gene was coinjected as a marker to select transformants (Mello et al. 1991). We assayed rescue of *sur-2* with cosmids in this interval by scoring for rescue of the *sur-2(ku3)* Egl phenotype after microinjection. We used *sur-2(ku3) + / + unc-54(e190)* as a host strain for the microinjection and identified Roller lines that no longer segregated *unc-54*, in which the non-Rollers were Egl

but the Rollers were egg-laying competent. We determined that three overlapping cosmids, F39B2, W05F9, and K05C4, were each able to rescue the *sur-2* Egl phenotype.

The region of overlap among the three cosmids that conferred rescue of the *sur-2* Egl phenotype was characterized further by restriction enzyme digests, Southern analysis, subcloning, and microinjection. We defined a minimal rescuing fragment of 13.6 kb, which is composed of three overlapping plasmids. Each plasmid individually failed to rescue, whereas the combination of all three was able to confer efficient rescue. We obtained three independent transgenic stable lines, each completely rescue the *sur-2(ku3)* Egl phenotype. Such combination rescue has been documented previously and is explained by obtaining a functional gene by homologous recombination of truncated fragments after microinjection transformation (e.g., Aroian et al. 1990; Mello et al. 1991; Wu and Han 1994). The 13.6 kb of genomic DNA was used initially to probe a Northern blot of different developmentally staged RNAs and identified a 5-kb RNA species that is enriched in early embryonic and larval stages of development but is rare in adult stages of development. Expression of the *sur-2* gene during these stages is consistent with the *sur-2* mutant phenotype. We used the 13.6-kb genomic fragment to screen the appropriate stages of a staged cDNA library (gift from J. Ahringer and J. Kimble, University of Wisconsin, Madison) and an embryonic library (gift from P. Okkema and A. Fire, Carnegie Institute, Baltimore, MD) and identified several cDNA clones. We were unable to identify positive clones from three other commonly used cDNA libraries, perhaps because the *sur-2* message is rare in RNAs prepared from mixed stage populations of worms.

We sequenced the longest cDNA as well as the entire

13.6 kb of genomic DNA that was able to rescue the *sur-2* mutant phenotype. The predicted open reading frame encodes a protein of 1586 amino acids (Fig. 5). The putative initiation codon has not been determined precisely and was assigned because it is the earliest ATG in the open reading frame and is in a good context for an initiation codon. The genomic and cDNA sequences were compared to define intron/exon boundaries. The gene structure, including all exons and intron/exon boundaries, is depicted in Figure 4. A comparison of the predicted protein sequence to protein sequences in GenBank revealed that *sur-2* encodes a novel protein with no significant similarity to known proteins in the data base. The protein sequence, however, possesses some interesting features. For example, the deduced protein sequence has a highly acidic domain (pI of 4.3, the overlined stretch of amino acids in Fig. 5). The carboxyl terminus of the *sur-2* gene product contains a domain that is very glutamine rich; 28% of the residues in a 190-amino-acid region are glutamine. This domain bears sequence similarity to *opa* repeats in proteins commonly found in many developmental control genes such as *Notch* and *mastermind* (Wharton et al. 1985).

The Sur-2 protein is also predicted to contain a con-

sensus MAP kinase substrate site (PXS/TP) and eight additional proline-directed kinase sites (S/TP), suggesting that *sur-2* could potentially be a Sur-1/Mpk-1 substrate. To test the importance of the consensus MAP kinase substrate site in vivo, we mutated the serine in the consensus site to an alanine and tested whether this mutant construct could rescue the Egl phenotype of *sur-2(ku9)*. The mutant construct rescued the *sur-2(ku9)* Egl defect (data not shown), suggesting that the consensus MAP kinase site is unlikely to be a physiologically important site. This experiment, however, does not address the importance of the several other proline-directed kinase sites.

sur-2 mutant alleles are nonsense mutations

To demonstrate that the gene-conferring rescuing activity corresponds to *sur-2*, we have sequenced five of the mutant alleles. DNA from mutant homozygous worms was PCR amplified by use of multiple sets of oligonucleotide primers. The PCR products were sequenced directly. All five *sur-2* alleles were determined to contain nonsense mutations that result in truncation of the gene product (Figs. 4 and 5). A single nucleotide change

(C → T) was observed in exon 5 in the *ku31* allele, changing a Trp to an ochre stop. The same change was seen in exon 6 for the *ku49* allele. A single nucleotide change (G → A) was detected for the *ku115* allele, in exon 9 of the predicted nucleotide sequence, changing a Trp to an amber stop. The identical single nucleotide change was observed for both *ku9* and *ku3* alleles in exon 14 (C → T), resulting in a change from Arg to an opal stop. The *ku31* mutation is the earliest truncation and removes the acidic and glutamine-rich domains as well as most of the proline-directed kinase sites of the protein.

A sur-2::lacZ reporter gene is expressed in the vulval precursor cells during vulval development

To determine where *sur-2* RNA is transcribed, the *lacZ* gene of *Escherichia coli* was inserted in-frame into the fourth exon of the *sur-2* genomic rescuing fragment. This construct includes 5.5 kb of regulatory sequence upstream of the initiating Met and includes the first large intron, which might also contain promoter activity. The transgenic construct was coinjected with pRF4 as a marker for transformation, into the syncytial gonad of wild-type adult hermaphrodites. A stably transmitting extrachromosomal array was integrated chromosomally, and the resulting animals were stained with X-gal.

Following their migration to the ventral cord during mid-L1, the P cells divide to produce Pn.a and Pn.p daughters. All Pn.p cells express the fusion gene in the mid-L1 through L3 stages of development (Fig. 6a). During L3, the VPCs (P3.p–P8.p) divide to form two daughters that both stain positive for the β -galactosidase activity (Fig. 6b). These two daughters continue to generate first four and then eight daughters for P.6.p and seven for P5.p and P7.p. All of these cells stain throughout these divisions. The staining observed in the daughters of P3.p,

P4.p, and P8.p is significantly weaker than that observed in P[5–7].p (Fig. 6c). Similar staining patterns in the Pn.p cells and their progeny cells are observed in strains containing a *let-60 ras-lacZ* fusion gene (J. Dent, pers. comm.). Following vulval differentiation, the *sur-2::lacZ* trans gene is no longer expressed in vulval cells (Fig. 6d).

In addition to the vulva, numerous other cells also stained positively for *sur-2::lacZ* activity, consistent with the pleiotropic defects displayed by *sur-2* mutant animals. For example, the *sur-2-lacZ* fusion gene is expressed in embryos and early larval stages consistent with a potential role for *sur-2* in embryogenesis and larval growth. The distal tip cells in the gonad also stain positive for the fusion gene expression, which may suggest a role for *sur-2* in these cells. Such a function in the distal tip cells may explain the gonad abnormalities and sterility associated with *sur-2* mutant animals. The *sur-2-lacZ* construct also appears to be expressed in cells that do not stain positive for the *let-60-lacZ* fusion gene (J. Dent, pers. comm.). Further detailed analysis of the pattern of *sur-2* expression during *C. elegans* development may help us to understand its function in Ras-dependent processes as well as Ras-independent processes.

Discussion

By use of a genetic suppressor screen, we have identified a new gene that appears to play a key role downstream of Ras in the vulval induction pathway as well as in several other developmental processes in *C. elegans*. Mutations in the *sur-2* gene result in a severe reduction of vulval induction, indicating its essential role in specifying vulval cell fates. Our genetic analyses of the eight recessive *sur-2* mutations indicate that they are loss-of-function mutations. A deficiency in *trans* to the *sur-2(ku9)* allele

Figure 6. Vulval expression of a *sur-2::lacZ* transgene. N2 hermaphrodites carrying an integrated *sur-2::lacZ* translational fusion (*ku1s15*) were fixed and stained with X-gal. (a) Late L1 larva. Many neurons stain in the ventral cord. These include the Pn.a and presumably the DB and DA neurons. The P[1–12].p cells also stain. (b) L3 larva. The Pn.p cells have divided to generate Pn.pa and Pn.pp daughters that stain. From left to right, brackets indicate P5.pa and P5.pp, P6.pa and P6.pp, P7.pa and P7.pp, and P8.pa and P8.pp. The daughters of P3.p and P4.p also stain but are not within the field of the photomicrograph. (c) L3 larva; the Pn.pxx descendants of P5.p, P6.p, and P7.p stain. These cells will generate the normal vulva. The staining in the descendants of P3.p, P4.p, and P8.p at this stage is greatly reduced. (d) Late L3 larva. The arrow depicts the vulval invagination formed from the descendants of P5.p, P6.p, and P7.p. They no longer express the *sur-2::lacZ* transgene. The strongly staining cell above the invagination is the anchor cell.

causes a small reduction in vulval induction. We cannot conclude whether the phenotype of *sur-2/Df* is more severe or equal to that of *sur-2* alone, so we cannot distinguish whether the *sur-2* alleles are partial or complete loss-of-function mutations. The molecular lesions of several *sur-2* alleles, which are nonsense mutations that result in severe truncations of the protein (deleting as much as two-thirds of the protein), may suggest that the *sur-2* mutations are null alleles; however, small amounts of readthrough product could allow for partial function of the *sur-2* gene product. If the *sur-2* null mutations do result in an incompletely penetrant Vul and Lethal phenotype, then we can hypothesize another gene that acts in concert with *sur-2*, late in the vulval signaling pathway. Alternatively, because our suppressor screen would not recover mutations in the gene that cause complete lethality, it is possible that null mutations in *sur-2* are completely lethal at either embryonic or larval stages of development like the null phenotypes of several other genes acting in the vulval development pathway (e.g., *let-60* and *let-23*; Han et al. 1990; Arioan and Sternberg 1991).

sur-2 may act at a late step in the vulval signaling pathway

Because all of the *sur-2* alleles suppress the Muv phenotype of *let-60(gf)*, *sur-2* likely acts downstream of *let-60 ras* in the pathway. Because Muv mutants resulting from gain-of-function mutations in the *lin-45 raf* and *sur-1/mpk-1* genes are not available at present in *C. elegans*, the position of *sur-2* relative to these two genes could not be tested directly. The *lin-1* gene appears to be a negative factor acting downstream of *sur-1/mpk-1* in the pathway because the Muv phenotype of the *lin-1* loss-of-function mutation *e1275* is epistatic to the Vul phenotype of *let-60*, *lin-45*, *mek-2*, and *sur-1/mpk-1* mutations (Han et al. 1990; 1993; Church et al. 1995; Kornfeld et al. 1995; Wu and Han 1994). In contrast, in a *sur-2(ku9)*; *lin-1(e1275)* double mutant, no clear epistatic relationship could be determined, suggesting that *sur-2* may not simply act upstream of *lin-1* as does *sur-1/mpk-1* or *mek-2*. Double mutants of the genotype *sur-2(ku9)*; *lin-1(ar147)*, however, are Muv, indicating that the Muv phenotype of the *lin-1* apparent null allele (G.J. Beitel, S. Tuck, I. Greenwald, and H.R. Horvitz, in prep.) is epistatic to the Vul phenotype of a *sur-2* loss-of-function mutation. This result suggests that *lin-1* acts downstream of *sur-2* in vulval cell fate specification. Because *sur-2(ku9)* and *lin-1(ar147)* display synthetic lethality, these two genes do not appear to act in a simple linear pathway (Fig. 7A).

lin-31 is required for 1°, 2°, and 3° cell fate specification. Because *lin-31* is required for both vulval as well as nonvulval cell fate specification, Miller et al. (1993) suggest that *lin-31* may form different complexes in VPCs that can act to repress vulval fates and activate hypodermal fates or alternatively to activate vulval fates and repress hypodermal fates. This model implies that different proteins interact with *lin-31* in the VPCs. The dereg-

Figure 7. Proposed role of the *sur-2* gene in vulval signal transduction pathway. Arrows indicate positive regulation of one gene product by another. T bars represent negative regulation of one gene product by another. Neither arrows nor T bars necessarily represent a direct interaction. (A) The *sur-2* gene is likely to act downstream of *sur-1/mpk-1* in a signal transduction pathway that induces vulval differentiation. The *lin-3* gene encodes a growth factor that has been proposed to be inductive signal from the anchor cell (Hill and Sternberg 1991). The *let-23* gene encodes a putative tyrosine kinase receptor (EGF receptor-like) for the Lin-3 signal (Arioan et al. 1990). The *lin-15* locus is a component of the inhibitory signal that acts to repress vulval induction and is composed of two novel gene products (Clark et al. 1994; Huang et al. 1994). The activated receptor tyrosine kinase is proposed to bind to an SH2-SH3 domain-containing adaptor protein encoded by the *sem-5* gene (Clark et al. 1992). The *let-60* gene encodes a Ras protein (Han and Sternberg 1990). A kinase cascade, which includes LIN-45 Raf (Han et al. 1993), MEK-2 MAP kinase kinase (Church et al. 1995; Kornfeld et al. 1995; Wu et al. 1995) and SUR-1/MPK-1 MAP kinase (Lackner et al. 1994; Wu and Han 1994), is stimulated upon RAS activation. The *lin-1* gene product acts after *sur-1/mpk-1* to negatively regulate vulval induction (Ferguson et al. 1987; Church et al. 1995; Kornfeld et al. 1995; Wu and Han 1994). *lin-31*, which encodes a Hnf-3/fork head putative transcription factor may act downstream of *sur-1/mpk-1* and appears to be required for the proper expression of both vulval and hypodermal cell fates (Miller et al. 1993; Lackner et al. 1994). Genetic interactions between *sur-2* and *lin-1* and between *sur-2* and *lin-31* (see Table 2) suggest that *sur-2* may also act downstream of *sur-1/mpk-1*. *sur-2* may negatively regulate *lin-1* activity and be negatively regulated itself by *lin-31*. *sur-2* is not likely to act in a simple linear pathway with either *lin-1* or *lin-31* (see Discussion). *lin-25*, which encodes a novel protein and displays a mutant phenotype similar to that of *sur-2* (Tuck and Greenwald 1995), may also act in parallel to *sur-2*. Models regarding the interactions among *lin-25*, *lin-1*, and *lin-31* have been proposed by Tuck and Greenwald (1995). (B) *sur-2* acts upstream of *lin-12* to specify 2° vulval cell fate. The *sur-2* gene is required for specifying both the 1° and the 2° vulval cell fates. The *lin-12* gene product is required for 2° cell fates (Greenwald et al. 1983; Sternberg 1988) and appears to act after *let-60 ras* for this function (Han et al. 1990). Our genetic epistasis tests (Table 2) indicate that *sur-2* acts before *lin-12* for 2° vulval cell fate specification.

ulated fates of P3.p, P4.p, and P8.p in *lin-31* are suppressed by *sur-2* in a *sur-2; lin-31* double mutant, suggesting that Sur-2 may be negatively regulated by Lin-31 in P3.p, P4.p, and P8.p (Fig. 7A). However, the *lin-31* mutant phenotype in P[5–7].p is not suppressed in the *sur-2; lin-31* double mutant, suggesting that *sur-2* does not act downstream of *lin-31* in these cells. Perhaps *sur-2* is regulated by other molecules in P[5–7].p to promote vulval cell fates such as *sur-1/mpk-1* MAP kinase.

Loss-of-function mutations in the *lin-25* gene, several alleles of which were also isolated in our screen for suppressors of *let-60(gf)*, cause a Vul phenotype similar to that of *sur-2* mutations, and *lin-25* is also predicted to act downstream of *sur-1/mpk-1* (Tuck and Greenwald 1995). Interestingly, both *sur-2* and *lin-25* encode novel proteins (Tuck and Greenwald 1995). Because both *sur-2* and *lin-25* loss-of-function alleles display incompletely penetrant Vul phenotypes it is possible that *lin-25* and *sur-2* act redundantly as positive effectors of the pathway. Alternatively, the *sur-2* alleles that we isolated could be partial loss-of-function mutations, and *sur-2* null alleles could display a completely penetrant Vul phenotype. It therefore appears that multiple factors are likely to act downstream of *sur-1/mpk-1* in the vulval signaling pathway. Our genetic data indicate that these genes are unlikely to act in a simple linear pathway. Use of several factors, both positive and negative, acting in parallel to one another, perhaps interacting with one another, imparts a fine tuning mechanism that can allow for the precise regulation of cell fate specification in the six VPCs.

sur-2 links the let-23 receptor-mediated inductive signaling pathway to lin-12 receptor-mediated lateral signaling pathway

The *lin-12* gene encodes a Notch-like transmembrane protein that has been proposed to act as a receptor in a number of lateral signaling events during *C. elegans* development (Yochem et al. 1988; Seydoux and Greenwald 1989). During vulval pattern formation, *lin-12* is required for the lateral signaling that acts between vulval precursor cells to specify 2° vulval cell fates (Fig. 1). *lin-12* loss-of-function mutations disrupt the lateral signal and result in an absence of 2° vulval fates, whereas *lin-12* gain-of-function mutations cause ligand-independent lateral signaling that result in all six vulval precursor cells adopting a 2° vulval fate. Because the inductive signal from the anchor cell and its signal transduction pathway (mediated by *let-23 RTK*, *let-60 ras*, etc.) is also required for the 2° vulval cell fate, the inductive signaling and the lateral signaling pathways must interact. Previous genetic data indicate that *lin-12* acts downstream of *let-23* and *let-60* to specify the 2° fates (Sternberg and Horvitz 1989; Han et al. 1990). We show here that the All-2°-vulval cell fate phenotype of a *lin-12* gain-of-function mutant is epistatic to the Vul phenotype (no 2° or 1° cells) of *sur-2*, suggesting that *lin-12* acts downstream of *sur-2* to specify the 2° vulval cell fate. Like *let-60 ras*, *sur-2* appears to function in the determination

of all vulval cell fates (1° and 2°). Our results (above) are consistent with the model that 2° cell fate specification requires two tandemly linked intercellular cell signaling events. The Let-23 RTK/Let-60 Ras-mediated signaling pathway specifies both 1° and 2° cell fates, whereas the Lin-12 protein-mediated lateral signaling pathway acts downstream of the first pathway to specify 2° cell fates. The *sur-2* gene product appears to act late in the former Let-60 pathway (Fig. 7B). Because the Lin-12 protein is thought to act as a receptor in the presumptive 2° cells for a lateral signal from the presumptive 1° cells, *sur-2* might act to promote production of the lateral signal. The mechanism of such a relationship is an attractive problem for further investigation.

sur-2 encodes a novel protein

The *sur-2* gene encodes a putative 190-kD protein that bears no significant homology (over the entire protein) to known proteins in the data base. The *sur-2* protein does, however, possess distinctive features that are suggestive of its biochemical function. The protein contains nine proline-directed kinase substrate sites, one of which conforms to the consensus substrate site for the proline-directed kinase, MAP kinase. Because our genetic analyses suggest that *sur-2* acts downstream of *sur-1/mpk-1*, an attractive hypothesis is that *sur-2* is a substrate of *sur-1/mpk-1* MAP kinase. Because abolishing the MAP kinase consensus site had no effect in vivo, it is possible that the other substrate sites act redundantly in vivo or, alternatively, that *sur-2* is not the direct target of a proline-directed kinase.

The Sur-2 protein is predicted to contain two distinct domains, both of which have been observed for nuclear regulatory proteins: an acidic region with a pI of 4 and a carboxy-terminal domain that shows high homology to *opa* repeat-containing proteins, such as *Drosophila* Notch and Mastermind, and mouse Mopa. The *opa* repetitive element is reflected on both the nucleotide and amino acid levels, consisting predominantly of triplets CAX, which can code for the amino acids Gln and His (Wharton et al. 1985). This element is shared by many other developmentally regulated gene products; however, the function of *opa* repeats in these proteins is unclear. It is noteworthy that several *opa*-containing proteins are nuclear localized. It is possible that the *sur-2* gene encodes such a nuclear factor that acts terminally in the vulval signaling pathway to regulate the transcription of vulval specific genes.

Concluding remarks

The greatest remaining mystery in the Ras-mediated signaling pathway is how the Ras–Raf–Mek–MAP kinase cascade can signal specific developmental programs. Clearly, the answers to the specificity of this pathway will lie in its downstream targets. SUR-2 appears to be a commonly used factor acting downstream in the Ras-mediated signaling pathway to control multiple developmental decisions. *sur-2* displays pleiotropic mutant

phenotypes that appear to overlap with those of *let-60 ras* and other upstream genes in the pathway. For example, *sur-2* mutations cause defects in male tail development, sterility and larval lethality, all of which are associated with mutations in *let-23 RTK*, *let-60 ras* and other upstream genes. *sur-2* could be a worm-specific gene acting late in the Ras-mediated pathway or, alternatively, SUR-2 could be structurally or functionally conserved among different organisms. The factors acting downstream of SUR-2 could be more specific to individual functions such as vulval differentiation. Further analysis of the function of *sur-2* may be very important in understanding the mechanisms by which signal transduction pathways control specific developmental functions.

Materials and methods

Strains and genetic methods

Methods for culturing, handling, and genetic manipulation of *C. elegans* were as described previously (Brenner 1974). All genetic experiments were performed at 20°C unless specified otherwise. Methods for analysis of vulval defects under dissecting microscopes were as described previously (Ferguson and Horvitz 1985; Han et al. 1990). Nomarski microscopy and photography were performed as described previously (Avery and Horvitz 1987). The references for alleles of various mutants used in this study are *dpy-5(e61)*, *unc-32(e189)*, *dpy-19(e1259)*, *lon-2(e678)* (Brenner 1974); *let-60(n1046gf)* and *lin-15(n765)* (Ferguson and Horvitz 1985); *lin-1(e1275)* (Horvitz and Sulston 1980); *lin-1(ar147)* (Tuck and Greenwald 1995); *lin-31(n301)* (Miller et al. 1993); *lin-12(n137)* (Greenwald et al. 1983); *lin-12(n137n720)* (Greenwald et al. 1983); *unc-54(e190)* (Waterston et al. 1980); *let-49(st44)* (Waterston et al. 1982); *him-8(e1489)* (Hodgkin et al. 1979); *lev-11(x12)* (Lewis et al. 1980); *let-206(e1721)*, *let-207(e1723)*, *eDf5*, *eDf6*, *eDf7*, *eDf10*, *eDf11*, *eDf13*, *eDf14*, *eDf15*, and *eDf24* (Anderson and Brenner 1984); *hlnI*; *unc-75(n1042)* (Zetka and Rose 1992); *let-60(n1046ku75)*, *lin-45(ku51)* (M. Hara, Y. Han, and M. Han, unpubl.). Double mutant constructions and complementation tests were done following standard protocols.

Isolation and genetic characterization of *sur-2* alleles

Eight *sur-2* alleles were isolated independently as recessive suppressors of the Muv phenotype of *let-60(n1046gf)*. Animals homozygous for *let-60(n1046gf)* were mutagenized with 50 mM ethylmethane sulfonate (EMS) (Brenner 1974) and the F₂ progeny were screened for non-Muv revertants. Both wild-type and Egl candidates were picked. Candidates that continued to segregate non-Muv progeny were further genetically characterized. Eight *sur-2* alleles were obtained from screening >60,000 mutagenized haploid genomes. Three additional alleles were obtained in screens for Vul and male abnormal phenotypes (G. Jongeward, H. Chamberlin, and P. Sternberg, pers. comm.). Outcrossing and genetic mapping of all *sur-2* alleles were performed using standard genetic procedures (Brenner 1974). The percentage of larval lethality associated with *sur-2* alleles was determined by counting of the total number of progeny on a plate and removing dead larvae as they formed over the next 2 days or until progeny had reached adulthood. Because *sur-2* hermaphrodites do not form a functional vulva, they cannot lay eggs and instead their progeny hatch in utero forming a “bag” of worms.

The dead larvae associated with the “bagged” mother were not included in the lethality data. The *sur-2* mutant alleles were mapped genetically to the right arm of chromosome 1 following standard three-factor mapping methods (Table 3).

Deficiency mapping

Several deficiencies that uncover the *unc-54* gene were isolated previously (Anderson and Brenner 1984). To facilitate genetic characterization and cloning of the *sur-2* gene, we mapped *sur-2* relative to the left breakpoint of these deficiencies (see Fig. 3). *unc-54/+* males were crossed to *eDfX/eDf24* hermaphrodites (*eDf24* is a balancer chromosome on 1R), and Unc cross progeny were picked that had a genotype of *unc-54/eDfX*. As all deficiencies of interest (*eDfX*) remove the *unc-54* gene, appearance of the Unc progeny verified the presence of the deficiencies in the strains. *lev-11 ku9/hlnI unc-54*; *him-5* males were then crossed with the *eDfX/unc-54* hermaphrodites. Wild-type L4 cross progeny were picked and screened the next day for Egl hermaphrodites. If the deficiency uncovered the *sur-2* locus, *lev-11 ku9/eDf14* animals would be Egl as was observed for several deficiencies in the region. If no Egl worms were observed, the deficiency was scored as not uncovering the *sur-2* locus.

eDf14/sur-2(ku9) animals were also scored for the extent of vulval induction using Nomarski optics. Egl progeny from a cross between *lev-11 sur-2(ku9)/hlnI unc-54* males and *eDf14/unc-54* hermaphrodites were picked individually onto plates. Their progeny were scored for vulval differentiation. Each of the scored animals was subsequently recovered to individual plates to assess whether they were homozygous or heterozygous for *lev-11*. Because *eDf14* does not remove *lev-11*, the only animals displaying a Lev-11 phenotype (uncoordinated mild twitcher in 1 mM levamisole; Lewis et al. 1980) are the *lev-11ku-9* homozygotes. The non-*lev-11* worms were *lev-11 ku9/Df*. In this way, the percent vulval induction could be compared for *sur-2(ku9)* versus *sur-2(ku9)/Df* animals by use of the same maternal genetic background.

Table 3. Genetic three-point mapping of *sur-2(ku9)* on chromosome I

Genotype of heterozygotes ^a	Phenotype of recombinants ^b	Recombinants with <i>sur-2</i> /total recombinants ^c
<i>dpy-5 unc-101</i> + + + <i>sur-2</i>	Dpy non-Unc Unc non-Dpy	6/6 0/3
<i>lev-11</i> + <i>let-49</i> + <i>sur-2</i> +	Lev non-Let	12/23
<i>let-206</i> + <i>unc-54</i> + <i>sur-2</i> +	Unc non-Let	3/6

Two-factor mapping was performed for all eight alleles using standard methods (Brenner 1974). Three-factor mapping was performed only for the *sur-2(ku9)* allele.

^aThe specific alleles of the marker genes are listed in Materials and methods.

^b[Unc] Uncoordinated movement; [Dpy] dumpy body shape; [Lev] levamisole resistant; [let] lethal.

^cNumber of recombinant animals that retain the *sur-2(ku9)* allele out of total recombinants homozygous for one marker mutation.

Mapping cosmids relative to deficiencies

Genomic DNA from deficiency heterozygotes was prepared and digested with restriction enzyme *Hind*III. Equal quantities of the digested DNA were run on an agarose gel and blotted to nylon filters by Southern blot procedure. DNA fragments (*Hind*III fragments) isolated from cosmids in the region as well as a cosmid outside the region (used as a control) were radiolabeled and used as probes. The intensity of the bands corresponding to each probe was compared between genomic DNA from various deficiency strains. A twofold decrease in band intensity was taken as an indication that a deficiency removed a particular DNA fragment within the cosmid.

Microinjection transformation

All cosmids used in this study were obtained from A. Coulson and J. Sulston (MRC Laboratory of Molecular Biology and Sanger Center, Cambridge, UK). DNA isolation, analysis, and subcloning were performed by use of standard molecular biology methods. Microinjection of the cloned DNAs into the syncytial gonad of *C. elegans* hermaphrodites was carried out as described previously (Mello et al. 1991). All cosmids that were candidates to contain the *sur-2* gene (located between the left breakpoints of *eDf13* and *eDf14*) were injected as a pool of three nonoverlapping cosmids initially at a concentration of 20–40 ng/μl with pRF4 as a marker at a concentration of 65 ng/μl (causes a dominant Roller phenotype). All cosmids from positive pools were then injected individually. A strain of genotype *sur-2(ku3)/unc-54(e190)* was used as a host for the injection. Multiple stably transmitting lines were obtained for each injection experiment and rescue of the *sur-2* Egl phenotype was assessed by scoring the egg-laying competency of the transgenic Roller and nontransgenic non-Roller animals. Because of a maternal effect, some non-Roller progeny of Roller mothers carrying positive clones appeared to be egg-laying competent. Almost complete rescue was observed in all stable lines obtained. The region of overlap of positive testing cosmids was extensively restriction mapped and assembled as subclones in the plasmid vector pBluescript SK(+). Combinations of plasmids were injected at a concentration of 10–20 ng/μl to assay for rescue.

DNA sequence analysis of the genomic and cDNA clones

The entire genomic rescuing fragment of 13.6 kb and cDNAs were sequenced in both directions by sequencing a nested set of deletions of plasmids with the Erase-a-base system (Promega). Single-stranded DNA was prepared for each deletion construct by use of standard procedures. All sequencing was performed by use of Sequenase (U.S. Biochemical) with primers that hybridize to the polylinker region of pBluescript SK(+) vector. The sequence was assembled into a single contig and analyzed by use of programs in the GCG software package from the Genetics Computer Group (Madison, WI). cDNAs in this region were obtained by the screening of a stage-specific library constructed by J. Ahringer and J. Kimble (pers. comm.) with subcloned fragments in the region of genomic DNA as a probe. Additional cDNAs were obtained by screening of the Okkema embryonic library. Five cDNAs were obtained by screening ~1.5 million phage plaques of the Okkema embryonic library. Many (>100) cDNAs were obtained from screening embryonic and early larval stages of the Ahringer and Kimble library. The 3' ends of the cDNAs were determined by identification of the poly(A) tails of the cDNA clones. Putative polyadenylation signal sequences were found 14–15 nucleotides upstream of the end of the cDNAs (T. Blumenthal, pers. comm.). Two classes of cDNAs dif-

fering in their 3' ends were obtained from the five clones from the Okkema library (Fig. 4). RNAs with these two types of 3' ends were also obtained by reverse transcription of total RNA with poly(dT) as a primer followed by PCR (data not shown). By comparison of the cDNA and genomic sequence, the intron/exon boundaries were determined.

Determination of sur-2 molecular lesions

To determine the molecular lesions of the *sur-2* mutant alleles, DNA fragments containing the exons and intron/exon boundaries were obtained by PCR amplification from DNA prepared from homozygous mutants as well as wild-type animals. The PCR fragments were gel purified in low melting point agarose and directly sequenced (Kretz et al. 1989).

Construction of sur-2::lacZ fusion and β-galactosidase histochemistry

A 7.4-kb *Hind*III genomic fragment containing ~5 kb of promoter DNA, the first exon, first large intron, and part of the fourth exon of *sur-2* genomic DNA was subcloned into the *Hind*III site of the *lacZ* expression vector pPD21.28 (Fire et al. 1990). This construct was injected into N2 hermaphrodites at a concentration of 10 ng/μl. pRF4 was used as a marker for transformation at a concentration of 65 ng/μl. A chromosomally integrated line was obtained by γ-irradiation of adult worms from a stably transmitting line. Five hundred progeny of the γ-irradiated worms were cloned individually to plates, and their progeny were screened for integrants in the next generation, expecting 75% Rollers for a heterozygous integrated line. Homozygous stable lines were obtained by cloning Rollers. Three integrated lines were obtained and one was used for staining. β-Galactosidase was detected by a standard protocol in which worms are desiccated, fixed in acetone, followed by staining overnight in X-gal at 37°C (A. Fire, pers. comm.). To obtain worms synchronized at different larval stages of development, gravid hermaphrodites were allowed to lay eggs on a single plate for 1 hr and the hermaphrodites were removed. The vulvae of a few sample worms were scored under Nomarski optics to stage the worms, and then the population of that stage was fixed and stained. Stained worms were mounted on slides and observed under Nomarski optics.

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Note added in proof

The sequence data for *C. elegans* *sur-2* have been deposited to the GenBank data library under accession number U33051.

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N Singh and M Han

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