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 [3º], 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]. Activat-
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A. wild type

B. examples of *sur-2* mutants

C. Isolation of *sur-2* mutants

**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* (G13E gf) 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.
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

| sur-2 allele | Percent Egl [n] | Percent vulval induction [n] | Percent lethality [n] |
|--------------|-----------------|-----------------------------|----------------------|
| Wild type    | 0 (300)         | 100 (20)                    | 0 (300)              |
| ku9          | 100 (596)       | 70 (18)                     | 0 (100)              |
| ku3          | 100 (100)       | 41 (14)                     | 7 (243)              |
| ku31         | 100 (279)       | 74 (8)                      | 4 (166)              |
| ku42         | 100 (100)       | 19 (16)                     | 2 (271)              |
| ku115        | 100 (100)       | 32 (11)                     | 3 (170)              |
| ku49         | 100 (783)       | 12 (14)                     | 2 (683)              |
| ku16         | 100 (100)       | 37 (19)                     | 7 (112)              |
| ku60         | 45 (55)         | 99 (27)                     | <1 (326)             |

[n] Number of animals scored.

*Percentage 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.

*Percentage 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].

*Percentage of animals that die at larval stages [L1–L4].

Role of sur-2 in vulval induction

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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 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)
The role of \textit{sur-2} in vulval induction

| Genotype\(^a\) | Percent Muv \([n]^b\) | Percent vulval differentiation \([n]^c\) | Percent egg-laying\(^d\) competent \([n]\) |
|----------------|------------------|-------------------------------|-----------------|
| +/+            | 0                | 100 (20)                      | 100 (300)       |
| \(ku9/eDfl4\)  | N.D.             | 13 (9)                        | N.D.            |
| \(cu9/cu9\)    | 0 (>500)         | 100 (10)                      | 100 (150)       |
| \(ku60/cu60\)  | 0 (>500)         | 99 (27)                       | 56 (55)         |
| +              | \(let-60(n1046\,gf)\) | 95 (150)                      | 158 (20)        | N.D.                        |
| \(ku9\)        | \(let-60(n1046\,gf)\) | 3 (200)                        | 59 (18)         | 0 (200)                     |
| \(ku60\)       | \(let-60(n1046\,gf)\) | 4 (150)                        | 100 (10)        | 50 (100)                    |
| +              | \(lin-15(n765\,lf)\) | 99 (100)                      | 197 (19)        | N.D.                        |
| \(ku9\)        | \(lin-15(n765\,lf)\) | 8 (200)                        | 90 (19)         | sterile                     |
| +              | \(lin-1(e1275\,lf)\) | 100 (200)                      | 165 (20)        | N.D.                        |
| \(ku9\)        | \(lin-1(e1275\,lf)\) | 40 (200)                       | 95.3 (11)       | sterile                     |
| +              | \(lin-1(ar147\,lf)\) | 100 (100)                      | N.D.            | N.D.                        |
| \(ku9\)        | \(lin-1(ar147\,lf)\) | 100 (100)                      | N.D.            | sterile/lethal              |
| +              | \(lin-12(n137\,gf)\) | 100 (>200)                     | 200 (20)        | N.D.                        |
| \(ku9\)        | \(lin-12(n137\,gf)\) | 100 (>200)                     | 200 (26)        | sterile                     |
| +              | \(lin-31(n301\,lf)\) | 77 (200)                       | 165 (14)        | N.D.                        |
| \(ku9\)        | \(lin-31(n301\,lf)\) | 2 (132)                        | 92 (18)         | 0 (200)                     |

\(^a\) Wild-type copy of \textit{sur-2} (\textit{gf}) Gain-of-function mutation; \([^i]\) loss-of-function mutation. The complete genotype for the \textit{sur-2/eDfl4} strain is \textit{lev11(x12);sur-2(ku9)/eDfl4}, for the \textit{sur-2/+} strain is \textit{sur-2(ku9)/unc-54(e190)}, for the \textit{sur-2/sur-2} strain is \textit{lev11(x12);sur-2(ku9)}, for the \textit{lin-1(e1275);lon-2(e678)}, for the \textit{lin-12} strain is \textit{dpy-19(e1259);lin-12/n137}. The \textit{sur-2;lin-31\,(n301\,If)} strain is epistatic to \textit{sur-2} (\textit{gf}). The complete genotype for the \textit{sur-2;lin-31\,(n301\,lf)} strain is \textit{sur-2/sur-2;lev-11(xl2)sur-2(ku9)/eDfl4}.

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

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

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

\(^e\) \textit{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 \textit{sur-2(ku9)} and \textit{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 \textit{ku9/ku9} homozygotes (Table 1) is suppressed by the \textit{let-60(gf)} allele but not by the \textit{lin-15(\textit{lf})} allele. N.D. not determined; \([n]\) Number of animals scored.

and an apparent null allele of \textit{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 \textit{lin-1} null allele is epistatic to \textit{sur-2} loss of function in the vulva, \textit{sur-2} wild-type activity is not required for the Muv phenotype of a \textit{lin-1} null allele. However, these two genes display synthetic lethality, suggesting that they may not act in a simple linear pathway.

The \textit{lin-31} gene is required for \(^1\text{a}, 2\text{a},\) and \(^3\text{a}\) cell fate specification. Loss-of-function mutations in \textit{lin-31} result in a deregulated expression of vulval cell fates. In a \textit{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 \textit{lin-31} mutant animal can display both Muv and Egl phenotypes. A population of \textit{lin-31(n301)} hermaphrodites is 77% Muv [Miller et al. 1993]. \textit{sur-2(ku9)} is able to suppress the \textit{lin-31} Muv phenotype as observed under the dissecting microscope (Table 2). We followed the vulval cell fates of \textit{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 \textit{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 \textit{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 \textit{sur-2(ku9), lin-31(n301)} double mutant, the \textit{lin-31} mutant phenotype in P3.p, P4.p, and P8.p is significantly suppressed, suggesting that \textit{sur-2} may function downstream of \textit{lin-31} in these cells. In P5–7.p, however, vulval induction is significantly higher in the double mutant than in the \textit{sur-2(ku9)} single mutant [Table 2], suggesting that the \textit{lin-31} mutant phenotype continues to be expressed in these cells.
Figure 3. Genetic and molecular characterization of the sur-2 locus. (A) Genetic map position of the sur-2 gene. The sur-2 gene was mapped ~0.6 map unit to the left of unc-54 between the left breakpoints of deficiencies eDf13 and eDf14 on linkage group I [see Material and methods]. (B) Identification of the region on the physical map containing the sur-2 gene. Cosmid mapping relative to deficiencies was performed to identify cosmids that are removed by eDf14 but not by eDf13. All cosmids shown on the map were tested for sur-2-rescuing activity by DNA-mediated transformation. (+) The clones that were able to rescue the sur-2 Egl phenotype; (−) remaining clones that failed to give a positive result. (C) A restriction map of the DNA within the overlap region of the rescuing cosmids. The combination of three plasmids, pNS1, pNS4, and pNS7, was able to confer almost complete rescue of the sur-2 Egl phenotype, indicating that the rescuing activity lies within this 13.6-kb piece of genomic DNA. Deletion of 1.6 kb from the left end or deletion of ~4 kb from the right end of the 13.6-kb region failed to rescue the sur-2 Egl phenotype. Restriction enzymes sites: (B) BamHI; (C) ClaI; (H) HindIII; and (S) SstII.

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 intragenic 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(n1046 ku75) 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 mediate 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 [Steinberg 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
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...
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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 initiation codon. The genomic and cDNA sequences the open reading frame and is in a good context for an predicted protein sequence to protein sequences in Gen- 

boundaries, is depicted in Figure 4. A comparison of the closely and was assigned because it is the earliest ATG in 

significant similarity to known proteins in the data base. 

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 sub-
strate. 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 im-
portant 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 activ-
ity 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

Figure 5. Nucleotide sequence of a sur-2 cDNA clone and predicted amino acid sequence. The MAP kinase substrate consensus site is boxed (PXS/TP). The nucleotide changes for five mu-

tant alleles are marked by arrows. A putative polyadenylation signal sequence (T. Blumenthal, pers. comm.) for the longer cDNA is underlined. A highly charged acidic region of sequence is overlined. The carboxy-terminal end of the predicted protein 

signal sequence (T. Blumenthal, pers. comm.) for the longer
divisions. The staining observed in the daughters of P3.p, first four and then eight daughters for P.6.p and seven for mid-L1 through L3 stages of development (Fig. 6a). During mid-L1, the P cells divide to produce Pn.a and Pn.p daughters that both stain positive for the β-galactosidase activity (Fig. 6b). These two daughters continue to generate Pn.pa and Pn.pp daughters that stain. From 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-λlacZ fusion gene [J. Dent, pers. comm.]. Following vulval differentiation, the sur-2::lacZ transgene 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-λlacZ 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 in 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

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-λlacZ fusion gene [J. Dent, pers. comm.]. Following vulval differentiation, the sur-2::lacZ transgene is no longer expressed in vulval cells [Fig. 6d].

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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.p.xx 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(DJ) 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 rafl 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, mekl, 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 mekl. 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.I. 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 deregulation could be determined, suggesting that 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 vulpal 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 vulpal 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 database. 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
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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 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(x147) (Tuck and Greenwald 1995), lin-31(n301) (Miller et al. 1993), lin-12(n137) (Greenwald et al. 1983), lin-31(n137n720) (Greenwald et al. 1983); unc-54(e190) (Waterston et al. 1980), let-49(ea44) (Waterston et al. 1982), him-8(e1489) (Hodgkin et al. 1979); lev-11(x12) (Lewis et al. 1980), let-206(e1721), let-207(e1723), edf-5, edf-6, edf-7, edf-10, edf-11, edf-13, edf-14, edf-15, and edf-24 (Anderson and Brenner 1984); hml, 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 I 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/edfX 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/hml unc-54, him-5 males were then crossed with the edfX/unc-54 hermaphrodites. Will-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]/hml 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-11 ku9-9 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[ka9]/DF animals by use of the same maternal genetic background.

Table 3. Genetic three-point mapping of sur-2[ka9] on chromosome I

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

Two-factor mapping was performed for all eight alleles using standard methods (Brenner 1974). Three-factor mapping was performed only for the sur-2[ka9] allele.

a The specific alleles of the marker genes are listed in Materials and methods.

b [Unc] Uncordinated movement; [Dpy] dumby body shape; [Lev] levamisole resistant; [let] lethal.

*Number of recombinant animals that retain the sur-2[ka9] 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 HindIII. Equal quantities of the digested DNA were run on an agarose gel and blotted to nylon filters by Southern blot procedure. DNA fragments [HindIII fragments] isolated from cosmids in the region as well as controls 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 cytial 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 Pfr4 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(ku23)/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(+). 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 differ-
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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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