The Caenorhabditis elegans EGL-26 Protein Mediates Vulval Cell Morphogenesis

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In screens for Caenorhabditis elegans mutants defective in vulval morphogenesis, we isolated multiple mutants in which the uterus and the vulva fail to make a functional connection, resulting in an egg-laying defective phenotype. Two of these so-called gonad defective (Cog) mutants carry alleles of the egl-26 gene. We demonstrate that vulval lineages in egl-26 mutant animals are normal, but one vulval cell, vulF, adopts an abnormal morphology. This results in formation of an abnormally thick layer of vulval tissue at the apex of the vulva and a physical blockage of the exit to the vulva from the uterus. egl-26 was cloned and is predicted to encode a novel protein. Mosaic analysis indicates that egl-26 activity is required in the primary vulval lineage for vulF morphogenesis. Expression of a functional translational fusion of EGL-26 to GFP was observed within the primary vulval lineage only in vulE, which neighbors vulF. EGL-26 is localized at the apical edge of the vulE cell. It is thus possible that vulE acts to instruct morphological changes in the neighboring cell, vulF, in an interaction mediated by EGL-26.

Key Words: Caenorhabditis elegans; vulva; morphogenesis; egl-26; organogenesis.

INTRODUCTION

Organogenesis, the formation of functional organs, requires complex developmental coordination among groups of cells of various fates as they remodel themselves into functional structures. Additionally, in order to ensure proper function, there must be strict coordination of the spatial development of groups of organs that require mutual physical interaction for function. An elucidation of the molecular mechanisms controlling cellular remodeling during organogenesis and the coordination of distinct organogenesis events will contribute to our understanding of organ development and pathological processes.

Progress has been made investigating organogenesis in a number of systems, including kidney (Cantley, 1996), tooth (Peters and Balling, 1999), lung and Drosophila trachea (Metzger and Krasnow, 1999), and flower morphogenesis (Lord et al., 1994). Primarily, this research has led to a deeper understanding of how cell fates are specified within different tissues. However, the genetic control of the later remodeling processes that these specified cells undergo is still largely unexplored. We are using development of the vulva in Caenorhabditis elegans as a model system to address questions of how organogenesis is genetically controlled and affected. Additionally, we are studying the formation of the vulval-uterine connection to understand how organogenesis of two organs can be coordinated. In C. elegans hermaphrodites, the tubular vulva, which arises from only 22 cells, connects the uterus to the outside of the body allowing passage of eggs and sperm during egg-laying and mating, respectively. The simplicity of the structure, the well-elucidated control of vulval cell-fate determination decisions, and the fact that the organ is nonessential for viability, allowing it to be easily genetically manipulated, make vulval development a powerful model system to study organogenesis.

Three precursor cells, P5.p, P6.p, and P7.p, adopt vulval cell fates in response to an inductive signal that activates a conserved Ras-mediated signaling pathway in the precursor cells (reviewed in Greenwald, 1997; Kornfield, 1997; Sternberg and Han, 1998). The inductive signal originates from the anchor cell (AC), a specialized cell of the somatic gonad. Appropriate fate determination also requires lateral signaling between the vulval precursor cells, involving activation of a conserved Notch-mediated signaling pathway (reviewed in Greenwald, 1997; Kornfield, 1997; Sternberg and Han, 1998).
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Drastic morphological changes in vulval cells begin to occur following the terminal divisions. Most strikingly, cells from the anterior and posterior sides of the vulva send out processes proximally and laterally. These processes pass...
taken in the Z plane at 0.5 μm intervals was deconvolved and analyzed by using Openlab 2.0.7 (Improvision, Lexington, MA) software. Adobe Photoshop 6.0 and Canvas 7 were used to prepare images.

Isolation, Mapping, and Rescue of egl-26 Mutants

We isolated two recessive alleles, ku211 and ku228, in screens for abnormal vulval morphology mutants (Hanna-Rose and Han, 1999). In brief, egg-laying defective (Egl) hermaphrodites were identified in the F2 generation of animals treated with 50 mM ethylmethane sulfonate (EMS) and were subjected to a secondary screen in which the morphology of the vulva was observed by using DIC microscopy. We outcrossed ku211 and ku228 at least six times. ku211 was mapped to the left end of chromosome II; e.g., 0/11 Dpy non-Unc and 10/10 Unc non-Dpy recombinants from dpy-10 unc-4/ku211 heterozygotes segregated ku211. We further refined the gene position using sequence-tagged site mapping (Williams, 1995). ku211 heterozygous males were crossed to RW7000 (Bergerac) hermaphrodites. ku211 homozygotes were recovered from the F2 generation and tested individually by PCR for the presence of sequence-tagged sites stP100 and stP196. Both markers were found in 3/70 mutants and 11/70 had stP196 only, placing ku211 to the left of stP100. ku211 and ku228 mapped to the same location, were rescued by the same cosmid (data not shown), and were found to be alleles of egl-26 as ku211 fails to complement egl-26(n481) (data not shown).

We generated transgenic strains by germline transformation (Mello et al., 1991). Cosmid (5–20 μg/ml) obtained from A. Coulson (Sanger Center) or plasmid was coinjected with the transformation markers pG696 (sur-5::gfp) (Gu et al., 1998) at 100 μg/ml or pRF4 (rol-6) (Mello et al., 1991) at 70 μg/ml into ku211, ku228, or ku211/dpy-10 unc-4 hermaphrodites. We scored restoration of egg-laying ability as positive rescuing activity. Cosmid C36F12 and the subclones pWH8 and pWH11 (see below) rescued (data not shown).

Plasmid Construction and cDNA Cloning

A 13-kbp BamHI fragment from C36F12 was ligated to pbblue-1 script to create pWH8, which was digested with SalI and religated to make pWH11. To make pWH15, the GFP translational fusion, the region between the SalI site in the gene and the last codon was amplified by using primers that introduce the HI fragment from pWH11. The resulting mosaics were identified by examining a specified set of animals with an uncoordinated phenotype in screens for C. elegans (Cog) phenotype in screens for C. elegans.
fied alleles, ku211 and ku228, are allelic to n481 and e1952, two alleles of a previously uncharacterized gene called egl-26 (under Materials and Methods) (Trent et al., 1983).

egl-26 mutant hermaphrodites display a varying penetrant egg-laying defective (Egl) phenotype (Table 2). Progeny of egl-26 Egl hermaphrodites hatch in the uterus and kill the mother. As a result of the shortened reproductive life, egl-26 hermaphrodites have diminished brood sizes relative to a wild-type brood size of approximately 300 (Table 2). However, brood sizes of egl-26 mutant animals that are able to lay eggs can approach wild-type levels (Table 2). Two egl-26 alleles are associated with a low percentage of sterile animals (Table 2), but this low penetrance phenotype has not been further investigated. Although egl-26 mutant phenotypes are not completely penetrant, when egl-26(ku211) is placed in trans to a deficiency (Table 2) but remains qualitatively the same, suggesting that ku211 and the similar allele ku228 are hypomorphic mutations.

The egl-26 Egl phenotype is correlated with a specific vulval morphology defect. Eighty-three percent of ku228 hermaphrodites are Egl (Table 2) and 87% (n = 68) display a Cog phenotype caused by thick disorganized tissue at the apex of the vulva (Fig. 2). This thick tissue blocks the exit to the vulva from the uterus and most likely results in the Egl phenotype. The thickened tissue at the apex of the vulva obscures the observation of the uterine seam cell (utse) that normally separates the vulval and uterine lumens (Fig. 2A; Newman et al., 1996). A failure to form the utse (Newman et al., 1996) or a failure of the uterus and anchor cell (AC) to fuse during the fourth larval stage (Hanna-Rose and Han, 1999) could account for a Cog phenotype. Although the utse is difficult to see in egl-26 mutants (Fig. 2B), we confirmed its presence and normal utse-AC fusion by microscopic observation of AC behavior and expression of cdh-3::gfp and cog-2::gfp marker proteins (data not shown).

In wild-type animals, the nuclei within vulE and vulF are located on the lateral sides of the animal as a result of the transverse, terminal divisions in the 1° lineage. In egl-26 mutants, vulF nuclei are often abnormally located in the midline plane of the animal (e.g., Fig. 2C). Abnormal placement of the vulF nuclei is not a result of an incorrect division plane during the terminal division of the 1° lineage. egl-26 mutants are indistinguishable from wild-type animals by Nomarski microscopy observations until the middle of the fourth larval (L4) stage of vulval development, hours after the terminal divisions are complete (data not shown). However, during L4, the nuclei take up abnormal positions and the apex adopts a disorganized appearance (Fig. 2).

**vulF Adopts an Abnormal Morphology in egl-26 Mutants**

Because the apex of the vulva appears disorganized and vulF nuclei sometimes occupy aberrant positions in egl-26 mutants, we made a closer examination of the vulval toroid structure for any abnormalities. Similar to visualization of the toroid structure using MH27 indirect immunofluorescence, expression of a jam-1::gfp fusion protein permits observation of the vulval toroid structure in transgenic animals (Sharma-Kishore et al., 1999; Shemer and Podbilewicz, 2000). The epitope for the monoclonal antibody MH27, encoded by the jam-1 gene, is localized to adherens junctions between epithelial cells including the specialized epithelium of the vulva (Raich et al., 1999). By performing deconvolution and 3-D reconstruction on a series of fluorescent images through multiple planes of the vulva in an animal carrying jam-1::gfp on the integrated array, jcl1, we can visualize the toroidal structure as the space between the rings that separate each toroid (Fig. 3). In wild-type animals at the mid-L4 stage, vulE and vulF have not yet fused. Thus, lateral junctions are visible between the anterior and posterior halves of vulE and vulF (Figs. 3A and 3C).

### TABLE 2

<table>
<thead>
<tr>
<th>Allele</th>
<th>n*</th>
<th>% Egl</th>
<th>% Sterile</th>
<th>% nonEgl</th>
<th>Brood size of:</th>
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<td></td>
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<td>Egl (n = 12)</td>
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<td>nonEgl (n = 5)</td>
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<tr>
<td>ku228</td>
<td>53</td>
<td>83</td>
<td>4</td>
<td>13</td>
<td>52.5 ± 8</td>
</tr>
<tr>
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<td>52</td>
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<td>0</td>
<td>25</td>
<td>60.7 ± 11</td>
</tr>
<tr>
<td>e1952</td>
<td>53</td>
<td>66</td>
<td>9</td>
<td>25</td>
<td>55.1 ± 11</td>
</tr>
<tr>
<td>n481</td>
<td>53</td>
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<td>45</td>
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<tr>
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<td>52</td>
<td>63</td>
<td>0</td>
<td>37</td>
<td>nd</td>
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<tr>
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<td>88</td>
<td>9</td>
<td>3</td>
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* n is the number of animals scored.

$^b$ Percentage of animals with egg-laying defect. A few animals laid less than 20 eggs before becoming Egl.

$^c$ Percentage of animals that produced no progeny.

$^d$ Percentage of animals that laid more than 20 eggs.

$^e$ “nd” is not determined.
At this stage, the junctions between vulE and its neighbors form the narrowest rings in the vulva, making vulE appear to form a narrow collar near the top of the vulva (Figs. 3A and 3C). Although vulF is narrow on the ventral side adjacent to vulE, it flares above vulE to form the wider apex of the vulva (Figs. 3A and 3C). The junctions between vulF and uterine cells at the apex of the vulva form a ring just as wide as the widest vulval toroids (brackets in Figs. 3A and 3C).

In egl-26 mutants, the structure of each toroid, except vulF, resembles wild type (Figs. 3B and 3D). vulE again forms a narrow collar near the top of the vulva. However, vulF fails to flare above vulE and, instead, directs itself to the apex, such that the apex is narrow, only 1/3–1/2 the width of the widest jam-1::gfp rings (brackets in Figs. 3B and 3D). The abnormal morphology of the vulval apex can also be observed by examining jam-1::gfp rings from a ventral perspective. In wild-type animals, the apex forms a large diamond-shaped ring that is as wide as the other rings and that is largely open in the center (Fig. 3E). In contrast, in egl-26 mutants, the diamond-shaped apical ring is much smaller relative to the other rings (Fig. 3F). Additionally, the apex in the mutants appears obstructed by tight junctions between cells (perhaps vulF precursors) within the diamond-shaped ring (see arrow in Fig. 3F).

The disorganized tissue in the region of vulF, abnormal placement of vulF nuclei at the apex of the vulva, and abnormal jam-1::gfp fluorescence at the sides and the apex of the vulF toroid all support the hypothesis that the primary defect in egl-26 mutants is an abnormal vulF morphology. Abnormal morphology of vulF then results in a failure to make a functional connection between the vulval and uterine lumens and an Egl phenotype.

**egl-26 Encodes a Novel Protein**

We cloned egl-26 using microinjection transformation to rescue the Egl phenotype of ku211 and ku228. ku211 and ku228 map to the left of the physical marker stP100 on chromosome II (under Materials and Methods). We tested cosmids covering this small physical region for rescue. Cosmid C36F12 can efficiently restore the ability of ku211 and ku228 hermaphrodites to lay eggs when introduced into mutant animals on an extrachromosomal array (3/4 transgenic lines were rescued). The portion of C36F12 that overlaps cosmid C23H3 and a subclone, pWH11, which encoded only the predicted gene C23H3.1, contains the rescuing activity (under Materials and Methods, and data not shown). A cDNA clone corresponding to C23H3.1 was obtained from a Stratagene mixed-stage library and was sequenced (Fig. 4A). The sequence confirmed the structure predicted by Genefinder for C23H3.1. egl-26 is predicted to encode a novel 317-amino-acid protein (Fig. 4A). Two lesions, ku228 and e1952, are identical and, along with ku211, encode proteins with glycine to glutamic acid substitutions in the central region of EGL-26. n481 encodes a protein with a serine to phenylalanine substitution nearer the C terminus. Although EGL-26 is a novel protein with no striking homology to other proteins in the database, it is highly conserved in the closely related species Caenorhab-
vulF adopts an abnormal morphology in egl-26 mutants. Three-dimensional reconstructions of jam-1::gfp rings in (A, C, E) jcIs1[jam-1::gfp] and (B, D, F) egl-26( ku211); jcIs1 mid-L4-stage vulvae were created from a z-series of deconvoluted fluorescence photomicrographs through vulvae from a (A–D) lateral or (E, F) ventral view. Letters with arrows indicate specific vulval toroids. (A, C) The sides of vulF flare out to the apex (arrowheads), and the apex is as wide (brackets) as the widest jam-1::gfp ring. (B, D) In contrast, in mutant animals, vulF extends straight up (arrowheads) to the apex, and the apex is approximately half as wide as the widest jam-1::gfp ring. In the ventral views, (E) a large, diamond-shaped ring that is open in the center forms the wild-type apex. In contrast, (F) the mutant apical ring adopts a small diamond shape with junctions inside. (G) Wild-type and (H) mutant schematic representations of the shape of the vulval toroids as determined by jam-1::gfp ring visualization. Note the flared shape of vulF and the large size of the lumenal opening in wild type as compared to the mutant. Bars, 5 µm.
ditis briggsae (Butler et al., 1981). An EST from a C. briggsae mixed-stage library encodes an incomplete protein that is 86% identical to the C-terminal 79 amino acids of EGL-26. (Fig. 4B).

egl-26 Acts in the 1° Vulval Lineage

egl-26 plays a vital role in the morphogenesis of a vulval toroid, vulF, located where the vulva and the uterus connect, and it appears to be expressed near the lumen of both organs in the vicinity of vulF (see below). In order to determine which tissue requires egl-26 activity for functional morphogenesis to occur, we generated and analyzed egl-26 mosaic animals. Mosaic analysis is facilitated in C. elegans by the largely invariant lineage of the animals. A C. elegans zygote divides to produce two cells called AB and P₁. The vulva is derived entirely from the AB lineage and, specifically, the ABpl and ABpr sublineages (Fig. 5A). In contrast, the uterus (and the entire somatic gonad) is derived entirely from the MS sublineage of P₁ (Fig. 5A). We created animals with wild-type vulval cells and mutant uterine cells and vice versa to address the question of which organ requires egl-26 activity.

Extrachromosomal DNA arrays that are formed in C. elegans upon injection of DNA into the syncytial gonad can be utilized for mosaic analysis because they are segregated with less fidelity than normal chromosomes. During a cell division, the extrachromosomal array can be lost in one daughter and maintained in the other. When an array is lost during an early embryonic division, a mosaic animal with...
Mosaic analysis indicates that egl-26 activity is required specifically in P6.p. (A) Schematic of the early C. elegans lineage. The vulval tissue is derived from the ABp lineage, while the uterine tissue is derived from the MS lineage. Filled pie charts indicate proportion of rescued mosaics when a loss occurred in the indicated lineage. (B) Egg-laying defective phenotype (% Egl) and rescue activity (+ or −) of nonmosaic controls and mosaic animals is indicated. “n” is the number of animals of each category examined. ABp or ABpr losses result in mosaicism within the vulva, and animals with these losses were scored for maintenance of the array in each of the vulval lineages (i.e., for fluorescence in the P5.p, P6.p, and P7.p lineages).

an entire mutant lineage is generated. We introduced an extrachromosomal array carrying wild-type copies of the egl-26 gene (pWH11) and copies of a sur-5::gfp fusion construct into egl-26(ku211) mutants. pWH11 rescues the Egl phenotype (Fig. 5B), and sur-5::gfp, which causes expression of fluorescent protein in the nucleus of almost every cell in the hermaphrodite (Yochem et al., 1998), acts as a cell-autonomous marker to distinguish mutant and wild-type cells. We identified mosaics that had lost the array in some lineage or lineages by selecting animals with less intense fluorescence as observed in the dissecting microscope. We scored a large number of cells in each mosaic animal for nuclear fluorescence to deduce at which point in the lineage the array had been lost (see Materials and Methods). Once the point of loss had been determined, we recovered the animals from the slide and scored for egg-laying activity.

We identified a total of 17 mosaics with array losses in P1 and, thus, in the uterus. P1 mosaic animals were rescued for egg-laying activity, indicating that egl-26 is probably not required in the uterus or any other gonadal cell (Fig. 5B). We identified 11 animals with a loss of the array in the AB or ABp lineages and, thus, in the vulva. These losses resulted in loss of egl-26-rescuing activity consistent with a function for egl-26 in the vulva (Fig. 5B). Furthermore, we were able to determine the specific vulval lineage that required egl-26 by obtaining animals mosaic in the ABp or ABpr lineages which leads to mosaicism within the vulva. By incorporating the unc-36(e251) mutation, which results in an uncoordinated (Unc) phenotype, into the strain and an unc-36-rescuing construct on the array, we obtained mosaic animals with losses in ABp or ABpr. The focus for unc-36 activity is in the ABp lineage, but animals with a loss in only ABp or ABpr are semiUnc (Kenyon, 1986). We selected SemiUnc animals that retained some fluorescence from animals carrying the unc-36(+), egl-26(+), sur-5::gfp array, and we scored these animals as above. In this manner, we identified a total of 15 vulval mosaics. Arrays that were maintained in P6.p, regardless of whether they were maintained in the 2° vulval lineages, permitted rescue of egg-laying activity. In contrast, arrays that were lost in P6.p while being maintained in at least one of the vulval 2° lineages did not mediate rescue, indicating that egl-26 activity in P6.p is necessary and sufficient for function (Fig. 5B).

**EGL-26 Is Expressed in vulE and Displays a Distinct Localization Pattern**

To examine the expression pattern of egl-26, we created a construct, pWH15, to encode a full-length functional EGL-26::GFP fusion protein. Because pWH15 retained egl-26 activity as assayed by ability to rescue ku211 and ku228 mutants when expressed from an extrachromosomal array (2/4 lines rescued), we assumed that the EGL-26::GFP expression pattern would accurately represent global and subcellular localization of EGL-26. In animals that carry this fusion construct on an extrachromosomal array, kuEx90, we observed expression in many regions of the animal, although not ubiquitously. Expression is strong around the cells of the spermatheca, around the mouth, and lining the pharynx, the rectum (Figs. 6C and 6D), and the excretory canals (data not shown). We also see expression in the pharyngeal intestinal junction cells, transiently during L3 in the anchor cell, in rectal epithelial cells D, VL, and VR, in B (Figs. 6C and 6D) and in Y, and in several cells with a neuronal appearance (data not shown). However, we focused our attention on the expression in and around the vulva, where the primary defect was identified and where the mosaic experiments indicate egl-26 functions.

Expression is obvious near the vulva and the uterus only during L4 (Figs. 6A and 6B). However, it is often very difficult to tell which cell is actually expressing egl-26::gfp because the fusion protein appears to line the lumen of the uterus and portions of the vulval lumen and is not obviously associated with any particular cell cytoplasm (Figs. 6A and 6B). Even in cases where a cell cytoplasm obviously contains egl-26::gfp, expression is often brighter around the apical edge of the cell (see uv3 cell in Fig. 6B).

Mosaic analysis indicates that egl-26 activity in the P6.p lineage is necessary and sufficient for function. P6.p gives
**FIG. 6.** EGL-26::GFP is expressed by vulE and has a distinct subcellular localization pattern. (A, C) Nomarski photomicrographs and (B, D) corresponding fluorescence images of lateral view of mid-L4-stage animals carrying a functional egl-26::gfp translational fusion on an extrachromosomal array, kuEx90. (A, B) EGL-26::GFP is tightly associated with the edge of the uterine lumen, u, and portions of the vulval lumen, v. uv3, ventral uterine cell. (C, D) EGL-26::GFP is expressed in the B cell and lines the distal portion of the rectum. (E, F) Fluorescence images from the left and right sides of a mid-L4-stage animal carrying an egl-26::gfp transcriptional fusion with a nuclear localization signal on an integrated array, kuIs36. Arrowheads indicate vulE nuclei and the arrow indicates the edge of the vulB2 toroid. (G) Nomarski photomicrograph of left lateral view of mid-L4-stage vulva showing two of the vulE nuclei (arrowheads). A 3-D reconstruction of the egl-26::gfp translational fusion expression pattern (I) is overlaid on Nomarski photomicrograph from the same animal (G) to create (H), demonstrating that the area of expression seen lining the central region of the vulval lumen in (B) (arrowhead) is most closely associated with vulE (arrowheads in H). Arrow indicates the edge of the vulB2 toroid. Bars, 5 μm.
rise to only vulE and vulF. Furthermore, phenotypic analysis indicates that loss-of-function mutations in egl-26 result in abnormal vulF morphology. Together, these results suggest that egl-26 acts in either vulE or vulF to promote proper morphological changes in vulF. To distinguish further between a cell-autonomous function in vulF or a non-cell-autonomous function in vulE, we examined the expression pattern of egl-26::gfp more closely. Because of the lack of nuclear or cytoplasmic expression, it was difficult to determine which cells were expressing egl-26::gfp in the animals carrying the translational fusion. Therefore, we created a second line of transgenic animals that carried an integrated array, kuls36, expressing an egl-26::gfp transcriptional fusion with a nuclear localization signal. This transcriptional fusion construct is expected to cause fluorescence in the nuclei of cells where egl-26 is normally expressed. In kuls36 animals, it was obvious that the egl-26 promoter is active in vulE nuclei (Figs. 6E and 6F). However, activity cannot be detected in vulF nuclei (Fig. 6, and data not shown). Expression was reproducibly either anterior–posterior or left–right asymmetrical in the vulE nuclei (Figs. 6E and 6F, and data not shown). Expression is also visible in the vulB nuclei but not in other vulvar toroids (Figs. 6E and 6F, and data not shown).

Consistent with the results from the transcriptional fusion, we also determined that the expression observed near the top of the vulval lumen in animals carrying the translational fusion (arrowhead in Fig. 6B) corresponds most closely to the position of vulE (Figs. 6G–6I). By performing deconvolution and 3-D reconstruction on a series of fluorescent images through multiple planes of mid-L4 stage vulvae from the lateral side of kuEx90 animals, we can visualize the 3-D expression pattern of the EGL-26::GFP translational fusion protein (Fig. 6I). In this manner, expression is observed in a ring around the ventral region of the vulva (arrow in Fig. 6I), in a thicker region near the center of the vulva (arrowhead in Fig. 6I), and at the apex of the vulva corresponding to where the utse lies separating the vulval and uterine lumens. When this 3-D representation from Fig. 6I is overlaid on a DIC photomicrograph of the lateral side of the same animal where the vulE nuclei are visible (Fig. 6G), it becomes obvious that the thick region of expression in the center of the vulva closely corresponds to the vulE cells (Fig. 6H). The ring around the ventral region of the vulva most likely corresponds to expression by vulB2 as assayed by the transcriptional fusion (data not shown). The thick region in the center of the vulva when viewed from several angles indicates that expression is localized to the inside edge of the vulE cell, adjacent to the lumen, as well as between the vulE nuclei where the vulE precursor cells have not yet fused, and overlapping partially to the area on the top and the bottom of the vulE toroid (Fig. 6G, and data not shown). Thus, we conclude that EGL-26 is expressed in vulE and is not detectable in vulF. Furthermore, EGL-26 becomes localized to the apical edge of the vulE cell adjacent to the vulval lumen and perhaps to a portion of the interface between vulE and the neighboring toroids vulD and vulF. These data suggest that this novel protein with a distinct apical localization acts non-cell-autonomously in determining cell morphology of vulF. Alternatively, if EGL-26 expression level is simply too low to detect in vulF, EGL-26 could act cell-autonomously in vulF.

**DISCUSSION**

By searching for genes involved in vulval morphogenesis, we identified two alleles of egl-26. In egl-26 mutants, vulF, the most dorsal vulval cell, develops an abnormally narrow and occluded apex, preventing formation of the vulval-uterine connection and resulting in an Egl phenotype. egl-26 encodes a novel protein with a distinctive localization pattern on the apical side of some vulval and most uterine cells, adjacent to the vulval and uterine lumens. Mosaic analysis demonstrated that egl-26 functions in either vulE or vulF, but surprisingly, although the primary defect is in vulF, egl-26 expression can only be detected in vulE, suggesting that egl-26 acts non-cell-autonomously to control the morphogenesis of vulF.

In the absence or reduction of EGL-26 activity, vulF develops a narrow apex and a small or occluded opening into the vulval lumen. One proposed model for EGL-26 function suggests that vulE directs morphological changes in vulF in an interaction that is mediated by EGL-26. This model predicts that we might expect vulF to adopt a morphology similar to what is observed in egl-26 mutants when vulE is absent. We have begun to test this model by ablating the cells that give rise to vulE, P6.paa, and P6.paa. Alternatively, it remains possible that, although we cannot detect expression of egl-26 in vulF, egl-26 may function cell-autonomously. Mosaic analysis cannot distinguish between these two possibilities, but our expression analysis argues against a cell-autonomous model.

Although the molecular lesions that we have identified in egl-26 mutations are not obvious molecular nulls, they act as loss-of-function alleles in regard to vulval morphogenesis, allowing us to conclude that a normal function of EGL-26 is to promote proper morphology of the vulF cell. We propose that egl-26 is acting as a morphogenesis gene as opposed to a specification gene. Primary vulval cell fate appears to be properly specified in the absence or reduction of egl-26. Furthermore, the egl-26::gfp transcriptional fusion is still expressed in vulE, but not vulF, in an egl-26 mutant (data not shown), suggesting that vulE fate is properly specified in the mutant and that vulF is not simply taking on a vulE-like fate. Consistent with a role for egl-26 after specification of fates is complete, egl-26::gfp expression in the vulva is not evident until relatively late in vulval morphogenesis, during the fourth larval stage. Although egl-26 is also expressed in vulB2, no striking morphological role appears associated with this expression since no dramatic vulval morphology defects are associated with the portion of the vulval derived from the secondary lineages in egl-26 mutants (Fig. 2, and data not shown). We attempted
to use RNA inhibition (RNAi) to look for stronger phenotypes associated with an egl-26 null mutation (Fire et al., 1998; Guo and Kemphues, 1995). However, after trying various RNAi methods, including injecting, feeding, and expressing dsRNA from a tissue-specific vector, we have been unable to observe any egl-26(RNAi) phenotype, including the EGL phenotype associated with our egl-26 mutations. RNAi also failed to significantly reduce the expression of egl-26::GFP in vulval cells (data not shown), indicating that either the egl-26 gene or the vulval tissue is resistant to RNAi. We have found RNAi to be ineffective in phenocopying mutations in several genes involved in vulval morphogenesis, some of which are known nulls (W.H.-R., M.H., Z. Chen, and D. Fay, unpublished observations), suggesting that RNAi in the vulva may be inefficient at later stages.

Control of cell shape is a complex process that is typically highly regulated and often involves intricate cytoskeletal and cell membrane alterations. In light of a potential non-cell-autonomous function of egl-26 in controlling the morphology of vulF, the localization of EGL-26 is quite intriguing. EGL-26 appears to be tightly associated with the apical edge of vulval cells. Expression abuts the vulval lumen and just slightly overlaps onto the top and bottom of the vulE toroid at the junction of vulE and vulF and vulE and vulD, respectively. Thus, EGL-26 is in a location permitting a potential direct (or very nearly direct) interaction with the vulF cell. Alternatively, an EGL-26-mediated signal could be propagated across the membrane by an unknown intermediate. It is unclear from the GFP expression experiments whether EGL-26 is a cytoplasmic, extracellular, or perhaps membrane-associated protein. Furthermore, EGL-26 has no motif to explain its subcellular localization. The expression patterns of mutant egl-26::GFPs suggest that localization of EGL-26 is important for function. We created full-length functional fusions between EGL-26 proteins containing the mutant lesions and GFP and assayed their expression patterns. egl-26(ku211)::gfp is not expressed well, perhaps explaining why this lesion results in reduction of EGL-26 function (data not shown). However, egl-26(n481)::gfp is still expressed at a relatively high level, but the localization of the protein is disrupted. EGL-26(n481)::GFP protein is observed throughout the vulE cell and not just at the apical edges of the cell (data not shown). Secreted molecules such as yeast pheromone are known to affect the morphology of nearby cells. However, there is no evidence that EGL-26 acts in this manner since expression in the neighboring uterine cells and in the anchor cell is not sufficient for function and expression is required in the primary lineage of the vulva for function.

Morphogenesis of vulval cells has been an ongoing area of investigation. Other screens have turned up critical players in controlling the morphology of vulval cells (Chamberlin et al., 1997; Chang et al., 1999). The genetic control of coordination of morphogenesis of these two organs is clearly an area of study that is ripe for further investigation.

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