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ELT-5 and ELT-6 are required continuously to regulate epidermal seam cell differentiation and cell fusion in *C. elegans*

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SUMMARY

The *C. elegans* epidermis is a simple epithelium comprised of three major cell types, the seam, syncytial and P cells. While specification of all major epidermal cells is known to require the ELT-1 GATA transcription factor, little is known about how the individual epidermal cell types are specified. We report that *elt-5* and *-6*, adjacent genes encoding GATA factors, are essential for the development of the lateral epidermal cells, the seam cells. Inhibition of *elt-5* and *-6* function by RNA-mediated interference results in penetrant late embryonic and early larval lethality. Seam cells in affected animals do not differentiate properly: the alae, seam-specific cuticular structures, are generally absent and expression of several seam-specific markers is blocked. In addition, *elt-3*, which encodes another GATA factor normally expressed in non-seam epidermis, is often ectopically expressed in the seam cells of affected animals, demonstrating that ELT-5 and -6 repress *elt-3* expression in wild-type seam cells. Seam cells in affected animals often undergo inappropriate fusion with the epidermal syncytia. Interference of *elt-5* and *-6* function during larval development can cause fusion of all seam cells with the surrounding syncytia and pronounced defects in molting. *elt-5* and *-6* are both expressed in seam cells and many other cells, and are apparently functionally interchangeable. Their expression is controlled by separable tissue-specific regulatory elements and the apportionment of monocistronic versus dicistronic transcription of both genes appears to be subject to cell-type-specific regulation. Collectively, these findings indicate that *elt-5* and *-6* function continuously throughout *C. elegans* development to regulate seam cell differentiation and cell fusion.

Key words: Cell fusion, Alae, Molt, Epidermis, GATA factor, *C. elegans*

INTRODUCTION

The epidermis (or hypodermis) of the nematode *Caenorhabditis elegans* performs critical functions during development, including establishing the basic body form, providing the substrate for cell and axon migrations, secreting the cuticle, and producing most of the additional cells that arise during post-embryonic development (Hedgecock et al., 1987; Priess and Hirsh, 1986; Sulston and Horvitz, 1977; White, 1988). Most of the body is covered by the 'major' epidermal cells (Gendreau et al., 1994), which are invariably produced by fewer cell divisions than the 'minor' epidermal cells, the small syncytial cells at the extreme ends of the worm. Three major epidermal cell types, the seam, syncytial and P cells comprise the epidermal epithelium in the embryo.

When epidermal cells are born, they assemble into three rows on either side of the worm, corresponding to dorsal, lateral and ventral positions (Sulston et al., 1983). The dorsal, and the anterior and posterior ventral cells fuse to form several syncytia (Podbilewicz and White, 1994). Neither the P cells, the central cells in the ventral row, nor seam cells, the lateral cells, fuse during embryogenesis. The seam cells are particularly critical: they play an organizing role in embryonic and larval morphogenesis, contribute to larval growth by generating additional epidermal and nervous tissue post-embryonically, and regulate the body form during formation of the alternative larval form, the dauer larva (Singh and Sulston, 1978; Sulston and Horvitz, 1977; Wissmann et al., 1999; Wissmann et al., 1997). At hatching, there are ten bilateral pairs of seam cells, nearly all of which are blast cells that, during post-embryonic development, produce many progeny, including more seam cells, neurons and syncytial epidermis (Sulston and Horvitz, 1977). Late in the final (L4) larval stage, the seam cells undergo homotypic fusion to form the bilateral seam syncytia of adults (Podbilewicz and White, 1994).

Comprehensive genetic screens have shown that specification of the embryonic epidermis and its patterning into the three major types are genetically complex processes (Chanal and Labouesse, 1997; Terns et al., 1997). Most of the epidermis, including all of the seam and P cells, arises from the AB 'founder cell' (Sulston et al., 1983). A combination of inductive interactions and asymmetric cell divisions in the early embryo (Gendreau et al., 1994; Hutter and Schnabel, 1994; Hutter and Schnabel, 1995; Kaletta et al., 1997; Lin et al., 1998; Mango et al., 1994; Mello et al., 1994; Moskowitz et al., 1994) dictates which early AB-derived blastomeres will

ultimately produce epidermis. These early events restrict expression of the ELT-1 GATA transcription factor to precursors of the major AB-derived epidermal cells and their descendants. *elt-1* is required to specify the major epidermal fate (Page et al., 1997); in *elt-1(−)* mutants, all cells that would normally become major epidermal cells instead adopt the fates of their closest non-epidermal relatives, such as muscle and neurons. LIN-26, a transcription factor expressed in non-neuronal ectodermal cell types (Labouesse et al., 1994) is a potential target of ELT-1. Epidermal cells degenerate or become neurons in *lin-26* mutants. Another likely target of ELT-1 is ELT-3, a GATA factor expressed in all major epidermal cells except the seam cells (Gilleard et al., 1999). *elt-3* null mutants show no obvious defects, suggesting that *elt-3* may be functionally redundant with another gene (Gilleard and McGhee, 2001). The exclusion of ELT-3 specifically from seam cells suggests that it may play a role in distinguishing non-seam from seam epidermis.

Although little is known about how the epidermis is patterned into three distinct types and how fusion is regulated in the embryo, a good deal is known about the mechanisms that distinguish individual epidermal cells according to their anteroposterior position and developmental stage, particularly during post-embryonic development (for reviews, see Ambros, 1997; Kenyon et al., 1997). For example, two Hox genes, *lin-39* and *mab-5*, interact to regulate fusion of the posterior daughters of some P cells in hermaphrodite and male larvae (Salser et al., 1993). In addition, in *mab-5*(−) male larvae, two posterior seam cells, V5 and V6, which normally produce the sensory rays, instead produce alae (Salser and Kenyon, 1996). Though several other genes (Wrischnik and Kenyon, 1997) are also known to determine the precise fate of particular seam and P cells based on positional or temporal (Bettinger et al., 1996) information, genes that regulate seam cell development per se have not been reported.

In the present study, we have sought to identify genes that impart the seam fate as opposed to the P or syncytial epidermal fates. As the ELT-1 GATA factor is required to specify all major epidermal cells, and the ELT-3 GATA factor is expressed in non-seam epidermis, we hypothesized that there may be another GATA factor that functions in the seam epidermis. Indeed, we report here that two GATA factors, ELT-5 and ELT-6, function in seam cell development. Removal of *elt-5* and *-6* function results in profound defects in many aspects of seam cell development, including fusion with neighboring epidermal syncytia, failure to express several seam-specific markers, and inappropriate expression of *elt-3*. *elt-5* and *-6* are expressed in seam cells and many other cells, under the control of separable, cell type-specific enhancer regions. *elt-5* and *-6* apparently form an operon, and appear to be transcribed both monocistronically and dicistronically, depending on the specific cell expressing them. Our findings demonstrate that *elt-5* and *-6* are required continuously for embryonic and post-embryonic seam cell development and for repression of epidermal cell fusion.

MATERIALS AND METHODS

Strains and alleles

C. elegans Bristol variety N2 was used as the wild-type strain. Maintenance of strains was as described previously (Brenner, 1974).

The strain JR672 contains an integrated array (*wIs54*, made by M. Fukuyama and J. Kasmir in our laboratory) expressing a seam-specific GFP marker (SCM), a derivative of a construct described previously (Terns et al., 1997). The strain SU93 (*jcIs1*) contains an integrated array expressing a JAM-1::GFP fusion protein in adherens junctions (Mohler et al., 1998). (We use all upper cases to refer to translational fusions, and lower-case italics followed by upper cases, as in *elt-3*::GFP, to refer to transcriptional fusions.) Both JR672 and SU93 are available from the *Caenorhabditis* Genetics Center. Strain JG5 (*vpIs1*), which carries *elt-3*::GFP on an integrated transgenic array (Gilleard et al., 1999), was obtained from J. Gilleard. The following strains carrying seam-specific reporters on extrachromosomal arrays were obtained from P. Sengupta (Miyabayashi et al., 1999): PY1215 (*nhr-72*::GFP), PY1269 (*nhr-73*::GFP), PY1267 (*nhr-74*::GFP), PY1214 (*nhr-77*::GFP), PY1282 (*nhr-81*::GFP), PY1324 (NHR-82::GFP) and PY1397 (*nhr-89*::GFP). JR1736 was created by co-injecting an *nhr-75*::GFP construct (a gift from P. Sengupta) and an *unc-119(+)* rescuing construct into *unc-119(ed4)* hermaphrodites.

Sequence analysis of *elt-5* and *-6* cDNAs and determination of the 5′ end of *elt-6* cDNA

The *elt-5* and *-6* cDNA sequences were determined by sequencing EST clones yk474 and yk113, respectively. (yk clones were generous gifts from Y. Kohara.) These sequences differ in several respects from the Worm Genome Consortium's predictions for the corresponding open reading frames (ORFs) F55A8.1 and F52C12.5 (*C. elegans* Sequencing Consortium, 1998). Most notably, the predicted ORF F55A8.1 does not include the last intron (3) and exon (4) of the *elt-5* cDNA, and the predicted ORF F52C12.1 does not include exons 1 and 2, and introns 1 and 2 of the *elt-6* cDNA. The *elt-5* and *-6* cDNA sequences have been reported to GenBank (Accession Numbers AF353302 and AF353303, respectively).

An *elt-6*-specific primer (5′ GAAGCACGGCTTTCAGTTG 3′) from Exon 3 was used to PCR-amplify the 5′ end of the *elt-6* gene from an embryonic cDNA pool prepared by J. Zhu (Zhu et al., 1997). The PCR products were cloned into vector pCR2.1 (Invitrogen). Nine clones, determined by PCR to be approximately the expected size or somewhat longer, were sequenced. Four of these were *trans*-spliced with SL1, and two carried 105-120 nucleotides of sequence upstream of the *elt-6 trans*-splice site. The remaining three sequences started a few bases after the *trans*-splice site, and did not provide useful data. A cDNA clone, yk391a6, also starts ~120 bp upstream of the *elt-6 trans*-splice site.

Reporter constructs and transgenic lines

Molecular cloning procedures were performed according to standard methods (Sambrook et al., 1989). Expression constructs were made with PCR products containing tagged restriction sites amplified from genomic DNA (fragments C, D, E and G), the cosmid GGC8 (fragments A and B), or the cDNA clone yk113 (fragment F). The PCR fragments are as follows (the numbers in parentheses correspond to the base position relative to the base A of the *elt-5* ATG; see Fig. 5 below).

A: 3.4 kb upstream of the *elt-5* ATG (−3380, +4);
B: 1.2 kb upstream of the *elt-5* ATG (−1211, +4);
C: between the *elt-5* ATG and poly(A) site (+8, +4398);
D: between the *elt-5* ATG and the *elt-6* ATG (+8, +4512);
E: between the *elt-6* ATG and poly(A) site (+4518, +8158);
F: between the *elt-6* ATG and poly(A) site (+4518, +8158);
G: 3.1 kb upstream of the *elt-6* ATG (+1425, +4512).

The coding region of GFP (fragment H) was amplified from pGFPm5 (a gift from R. Zeller).

Translational fusions were made by inserting the coding region of GFP (fragment H) into the 5′ end of *elt-5* or *elt-6* in frame shortly after the ATG. Three transcriptional fusions (pKK41, pKK44 and pKK7) and six translational fusions (pKK39, pKK52, pKK38, pKK47, pKK25 and pKK49) containing various combinations of PCR

fragments were made as follows (numbers in parentheses refer to the concentrations of injected DNA in µg/ml).

pKK39: A, H, D and E in pPD96.04 (2);
pKK52: A, H and C in pBluescript (50);
pKK38: A, D, H and E in pPD96.04 (2);
pKK41: A and D in pPD96.04 (50);
pKK44: G in pPD95.67 (50);
pKK7: A in pPD96.62 (20);
pKK47: A, H and E in pPD96.04 (50);
pKK25: B, H and E in pPD96.04 (50);
pKK49: *nhr-74* promoter, H and F in pBluescript (50).

The GFP and β-galactosidase-coding regions in the vector pPD96.04 were replaced by PCR fragments in pKK39, pKK38, pKK47 and pKK25. The *nhr-74* promoter in pKK49 was subcloned from the *nhr-74*::GFP construct obtained from P. Sengupta (Miyabayashi et al., 1999). All pPD constructs were gifts of A. Fire. Each reporter construct, as well as a mixture of two constructs, pKK25 and pKK49, was co-injected with pRF4 (*rol-6^D*, 200 µg/ml) into N2 hermaphrodites or with pDP#MM016B (*unc-119(+),* 200 µg/ml) into *unc-119(ed4)* hermaphrodites. Strains containing pKK25, pKK47 or pKK49 were crossed with the *unc-119(ed4);jsIs1* strain to obtain strains carrying each of the constructs and JAM-1::GFP.

The translational fusions proved to be somewhat toxic. It was difficult to obtain lines with pKK38 and pKK39, two constructs that contain both the *elt-5* and *-6* genes, owing to embryonic or larval lethality caused by these sequences. To circumvent this problem, these constructs were injected at low concentrations. The lines obtained showed low overall levels of expression. pKK52, an *elt-5* reporter construct, and pKK41, an *elt-6* reporter construct, contained the *elt-5-* but not the *elt-6*-coding region. We were able to obtain several lines with these constructs that gave relatively high expression levels. The expression patterns of the two *elt-5* reporters containing 3.4 kb upstream and the entire *elt-5* gene, pKK39 and pKK52, appeared identical except that pKK52, but not pKK39, showed low levels of expression in the descendants of the vulval precursor cells (K. K. and J. H. R., unpublished). In addition, two *elt-6* reporters, pKK41 and pKK44, showed occasional expression in some larval and adult intestinal cells, which was not observed with any other reporter (not shown).

Antibody production and immunofluorescence

Anti-ELT-5 antibodies were raised against ELT-5-specific peptides, pep5A (EDPMDQDVKQEESERSDIPTC) and pep5B (TETRPESAEQQHHEC), and anti-ELT-6 antibodies were raised against ELT-6-specific peptides, pep6A (RKRKPTKESVNRHLEC) and pep6B (CLEQMSESGSEEKYP; custom synthesized by Sigma Genosys). The peptides were selected from regions of little or no homology to minimize potential crossreactivity. Two rabbits, UCSB47 and UCSB48, were immunized against the ELT-5-specific peptides (performed by Cocalico Biologicals). Sera from the two rabbits gave essentially the same staining pattern. Anti-ELT-5 sera were used directly, as affinity purification did not improve staining. Two additional rabbits, UCSB49 and UCSB50, were immunized against the ELT-6-specific peptides. Sera from UCSB49 were used after affinity purification using a peptide-coupled column (AminoLink Plus kit by Pierce), according to the manufacturer's protocol. Sera from UCSB50 gave similar but slightly weaker staining.

Embryos were fixed and stained for immunofluorescence as described (Sulston and Hodgkin, 1988). Anti-ELT-5 sera and affinity-purified anti-ELT-6 antibodies were used at a 1:25 to 1:100 dilution. Monoclonal antibody (mAb) MH27, which recognizes epithelial adherens junctions (Priess and Hirsh, 1986; Waterston, 1988), was a generous gift of R. Waterston.

RNA-mediated interference (RNAi)

Two non-overlapping regions of *elt-5*, corresponding to amino acids 58-215 and 224-363, were amplified by PCR from the cDNA clone yk474 and served as the templates for *elt-5* dsRNAs. dsRNAs directed to both *elt-5* regions gave the same phenotypes. The region of *elt-6* corresponding to amino acids 94-286 was PCR amplified from the cDNA clone yk113 and served as the template for *elt-6* dsRNA. RNA was synthesized and purified using the MEGAscript kit (Ambion) according to the manufacturer's protocol. *elt-5* or *-6* dsRNA, or a mixture of the two, was injected into young hermaphrodites as described (Fire et al., 1998). Progeny laid at least 10 hours after injection were analyzed.

To obtain strong embryonic effects, *elt-5* RNA was injected at 2 mg/ml. To obtain weaker effects, which allowed us to observe post-embryonic phenotypes, *elt-5* RNA was injected at 0.2 mg/ml. Throughout the paper, '*elt-5(RNAi)*' animals refers to animals treated with high levels of *elt-5* dsRNA.

Microscopy and image acquisition

All Nomarski and some fluorescence images were acquired with an Optronics VI-470 camera on a Nikon Microphot SA microscope. Other fluorescence images were acquired with a BioRad 1024 Confocal Microscope.

RESULTS

elt-5 and *-6* are adjacent genes that encode similar GATA factors

In an effort to learn how the *C. elegans* epidermis becomes patterned into the three major epidermal cell types during embryogenesis, we have attempted to identify factors that impart seam cell-specific identity (Terns et al., 1997). Because GATA factors, namely ELT-1 and -3, are involved in other aspects of epidermal development, we examined several GATA factor-encoding genes predicted from the *C. elegans* genomic sequence (*C. elegans* Sequencing Consortium, 1998) for a possible role in embryonic seam cell development. We found that RNA-mediated interference (RNAi) of a GATA factor-encoding gene we have named *elt-5* (erythroid-like transcription factor 5) results in penetrant embryonic and early larval lethality and causes morphological defects that include the lack or malformation of seam-specific cuticular specializations, called alae. This observation led us to investigate the role of *elt-5* and its paralog, *elt-6* in seam cell development.

elt-5 and *elt-6* are adjacent genes encoding single-finger GATA factors. Their encoded DNA binding domains are 76% identical and are ~60% identical to those in other GATA factors (Fig. 1). As is typically the case for GATA factors, ELT-5 and -6 are not significantly similar to the other GATA factors outside the DNA-binding domains. However, the two proteins are 46% identical overall, implying that one of the genes arose by duplication of the other. The putative polyadenylation site of the upstream gene, *elt-5*, and the *trans*-splice site of the downstream gene, *elt-6*, are separated by only ~130 base pairs, characteristic of genes that reside on the same operon (Blumenthal and Steward, 1997). However, attempts to determine whether the two genes indeed form an operon have not produced conclusive evidence. As most, if not all, downstream genes are *trans*-spliced to the SL2 leader (Spieth et al., 1993; Zorio et al., 1994), we looked for, but failed to find, evidence for SL2 *trans*-splicing of transcripts from the downstream gene, *elt-6* (see Materials and Methods). All seven *elt-6* cDNA clones examined were either *trans*-spliced to the SL1 leader or were not *trans*-spliced. This suggests that *elt-6*

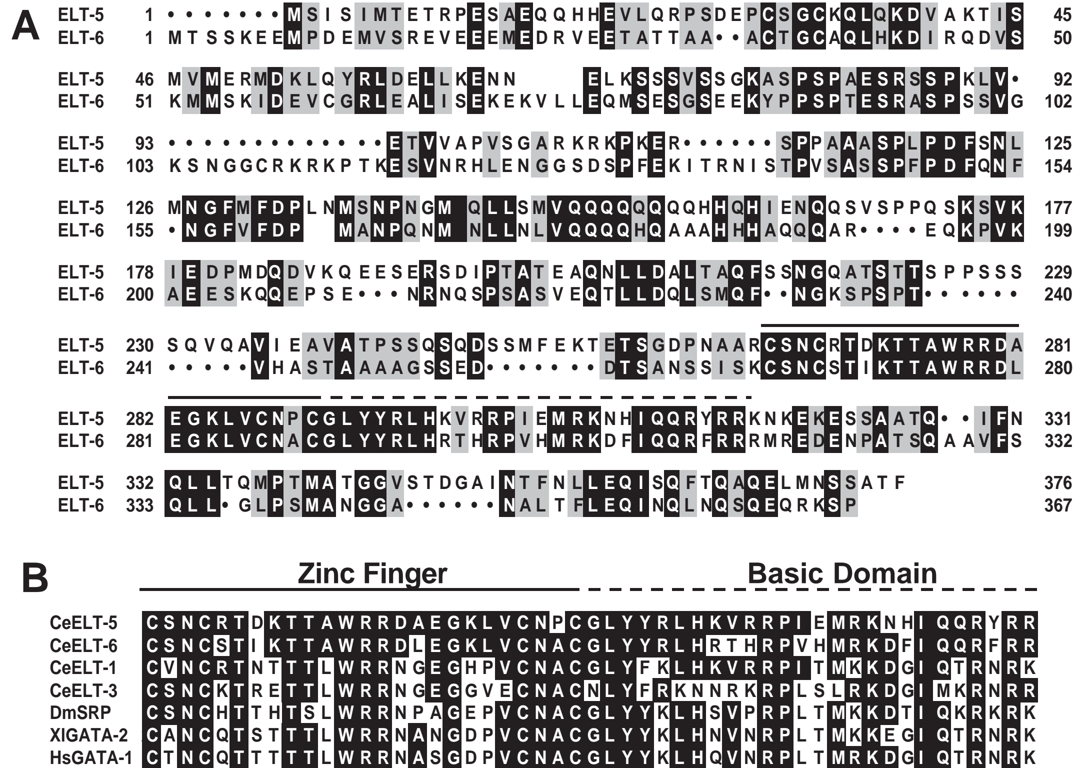

Fig. 1. Predicted ELT-5 and ELT-6 proteins. (A) Alignment of the predicted ELT-5 and -6 proteins. Identities are indicated by black background and similarities are indicated by gray background. The zinc-finger region and basic domain are indicated by solid and broken lines, respectively. (B) Comparison of the ELT-5 and -6 zinc-finger and basic domains with those of *C. elegans* ELT-1 and ELT-3, *Drosophila* SERPENT, *Xenopus* XGATA-2, and human GATA-1. The second fingers of ELT-1, XGATA-2, and GATA-1 are shown. Identities between either ELT-5 or -6 and at least one other protein are indicated by black background.

is often transcribed from its own transcription initiation site near the SL1 splice site rather than co-transcribed with *elt-5* as a dicistronic transcript. This interpretation is consistent with data obtained with a reporter construct (see below). However, based on the effects of *elt-5* dsRNA on reporter constructs, it seems likely that at least a fraction of mature *elt-6* message is generated from *elt-5/elt-6* dicistronic transcripts (see below).

Interference of *elt-5* and -*6* function leads to defects in seam cell development in embryos and larvae

We used the technique of RNAi (Fire et al., 1998; Guo and Kemphues, 1995) to assess the developmental function of *elt-5* and -*6*. We found that nearly all (90/95) progeny of hermaphrodites injected with high levels of *elt-5* dsRNA (see Materials and Methods) arrest late in embryogenesis (pretzel stage) or as early L1 larvae. The arrested L1 larvae are invariably uncoordinated (Unc phenotype), lumpy (Lpy phenotype) and slightly dumpy (Dpy phenotype), suggesting defects in epidermal development (Fig. 2B). In addition, the entire buccal capsule, the cuticular structure of the mouth (Wright and Thomson, 1981), invariably fails to attach to the anteriormost region of the head ('pharynx unattached' or Pun phenotype; Fig. 2B), suggesting defects in the buccal epidermis. A small fraction (5/95) of embryos arrest earlier, apparently with ruptures at the head or ventral midline (not shown).

Injection of *elt-6* dsRNA at high levels did not produce any observable phenotype, and co-injection of both *elt-5* and *elt-6* dsRNAs at high levels did not result in an enhanced phenotype compared with *elt-5* dsRNA alone. These results, however, do not necessarily indicate that the observed phenotypes are due to elimination of *elt-5* function alone. In fact, *elt-5* dsRNA affects expression of both *elt-5* and -*6* in seam cells (see below), and the observed phenotypes of *elt-5(RNAi)* animals may arise from inhibition of both genes or of *elt-5* alone. For simplicity, we will use the notation *elt-5/6* to refer to the function of either the *elt-5* gene alone or of both the *elt-5* and -*6* genes. Based on its effects on reporter gene constructs, *elt-5* dsRNA at high levels appears to abolish its function (as well as *elt-6* function in some tissue types; see below); thus, these phenotypes are likely to reflect a strong loss-of-function or null phenotype.

To investigate a possible post-embryonic role for *elt-5*, we injected hermaphrodites with lower levels of *elt-5* dsRNA. Such injections resulted in a mixture of weakly and strongly affected progeny. Weakly affected larvae appeared normal at hatching; they were neither Lpy nor Unc, and their buccal capsules were properly attached. Many of these larvae, however, became lethargic and sickly at later stages, were molting defective, and arrested at various stages of larval development. A small fraction of the weakly affected larvae also showed other gross morphological abnormalities that were suggestive of epidermal defects, including Roller (Rol) and protruding vulva (Pvl) phenotypes (not shown). The post-embryonic developmental defects were examined in more detail, as described later. All *elt-5(RNAi)* animals described in the remainder of this paper were obtained from mothers injected with high levels of *elt-5* dsRNA.

The gross phenotypes we observed suggested defects in epidermal structure and/or development. To characterize the epidermis in *elt-5(RNAi)* embryos, we visualized their epithelial adherens junctions with monoclonal antibody MH27 (Priess and Hirsh, 1986; Waterston, 1988). In a wild-type embryo, all epidermal cells, including the row of ten lateral seam cells on each side, are clearly outlined by MH27 staining (Fig. 2C). In contrast, although most of the epidermal pattern

appeared normal in *elt-5(RNAi)* embryos, the rows of seam cells often showed gaps in MH27 staining (Fig. 2D). In addition, seam cells were occasionally displaced from the linear row of lateral cells. For example, Fig. 2F shows an *elt-5(RNAi)* embryo in which a seam cell, V1 (asterisk), is ventrally misplaced such that neighboring seam cells, H2 and V2, contact each other (compare with Fig. 2E). The misalignment and gaps in the pattern were never observed in P cells.

There are at least four possible explanations for the gaps in the seam rows seen in *elt-5(RNAi)*: (1) seam cells are misspecified as non-epidermal cells at birth; (2) seam cells are misspecified as epidermal syncytial cells at birth, leading them to fuse with other syncytial epidermal cells; (3) seam cells, although correctly specified initially, lose their identity and later adopt a syncytial-type identity; or (4) seam cells retain their seam identity, but fusion is misregulated. (For simplicity, cells that normally become seam cells in wild-type will be called seam cells, irrespective of their ultimate identity in *elt-5(RNAi)* animals.) In an effort to distinguish between these possibilities, we examined the epidermal pattern over time using the JAM-1::GFP marker, which reveals the MH27 adherens junction pattern in living embryos (Mohler et al., 1998). These studies demonstrated that the gaps in the seam row probably result from fusion of existing seam cells with the surrounding epidermal syncytium. In wild-type embryos, most dorsal and ventral syncytial cells complete their fusion between the 1.5- and twofold stage of elongation (Podbilewicz and White, 1994). In *elt-5(RNAi)* embryos at the same stages, only occasional lateral cells lacked JAM-1::GFP expression. By hatching, however, many lateral cells (32%, *n*=61 larvae) lacked the adherens junction marker. This progressive disappearance of the adherens junction marker from the lateral row results from cell fusion; we were able to observe ongoing dissolution of the adherens junctions between individual lateral cells and the adjacent epidermal syncytium as they were caught in the act of fusion (see Fig. 4H, arrowhead). These ongoing fusions were observed in some cases as late as in newly hatched animals.

The relatively late onset of fusion (i.e. after the time that the normal epidermal syncytial cells fuse; Podbilewicz and White, 1994) suggests that many seam cells that ultimately fuse with surrounding syncytia are initially specified correctly in *elt-5(RNAi)* embryos. This notion was supported by observing expression of SCM, a seam cell-specific marker (Terns et al., 1997). In wild-type embryos, SCM is expressed in all seam cells from the twofold stage through adulthood (Fig. 2G). We found that SCM expression is visible in all seam cells of *elt-5(RNAi)* embryos at the threefold stage (*n*=52), although arrested embryos and larvae show somewhat reduced expression (not shown). These observations revealed that seam cells are not misspecified as syncytial cells at the time of their birth in *elt-5(RNAi)*

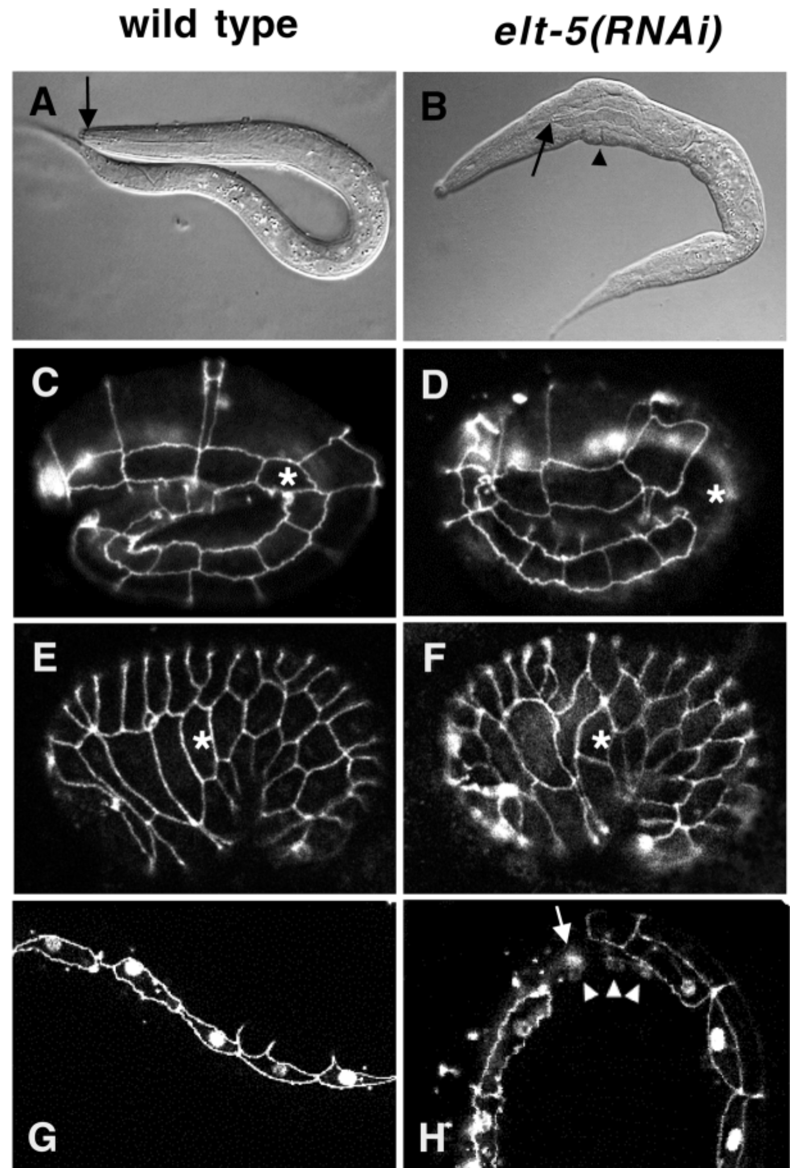

Fig. 2. Phenotypes of *elt-5(RNAi)* embryos and larvae. (A,C,E,G) Wild-type; (B,D,F,H) *elt-5(RNAi)* animals. (A) Nomarski image of wild-type L1 larva. Arrow points to the buccal capsule, which is attached to the anterior end of the worm. (B) *elt-5(RNAi)* L1 larva showing lumps (arrowhead) and a detached buccal capsule (arrow) at the anterior end of the pharynx, which has contracted toward the posterior. (C-F) Embryos stained with mAb MH27 to visualize adherens junctions of epidermal cells. The asterisks mark the V1 seam cell. (C) Lateral view of a wild-type embryo at the ~2.5-fold stage. The row of 10 lateral seam cells, all completely surrounded by adherens junctions, is prominently visible. (D) Lateral view of an *elt-5(RNAi)* embryo at the ~2.5-fold stage. One of the seam cells, V1 (asterisk), does not show adherens junctions, indicating that it has fused with the neighboring hyp7 syncytium on the dorsal and ventral sides. (E) Lateral view of a wild-type embryo slightly past the comma stage. All ten seam cells are visible, although part of the most posterior seam cell, T, is out of focus. (F) Lateral view of an *elt-5(RNAi)* embryo slightly past the comma stage. One of the seam cells, V1 (asterisk), is ventrally misplaced, and its neighbors, H2 and V2, inappropriately contact each other. (G,H) L1-stage larvae expressing the seam cell marker SCM (nuclear signal) and JAM-1::GFP, a maker for adherens junctions. (G) Wild-type larva showing SCM expression in seam cells only. (H) *elt-5(RNAi)* larva showing SCM expression in nuclei of syncytial cells (arrowheads) near a fused seam cell (arrow).

embryos. Consistent with the view that cells that have been specified as seam cells subsequently fuse with the syncytium, we often observed several nuclei in the epidermal syncytium, surrounding the region in which a seam cell had fused, 'ectopically' expressing SCM, albeit at low levels (Fig. 2H, arrowheads). Presumably fusion of SCM-expressing seam cells into neighboring syncytia allows release of some GFP molecules, which are taken up by nearby syncytial nuclei.

Morphological observations indicated that seam development in *elt-5(RNAi)* animals is abnormal even in the seam cells that do not fuse. Seam cells normally produce the alae, bilateral ridges of specialized cuticle superjacent to the seam cells of L1 larvae, dauer larvae, and adults. Wild-type alae are clearly evident at the L1 stage as two parallel ridges running along the body on each side (Fig. 3A,E). We found that the alae in *elt-5(RNAi)* larvae were invariably missing or malformed: 86% of *elt-5(RNAi)* larvae showed no visible alae, and 14% had partial and/or defective alae (*n*=56). For example, Fig. 3B,F show an *elt-5(RNAi)* larva that lacks alae over most of its length and that contains a stretch of ala with several irregularly shaped branches. By correlating the position of missing alae with the JAM-1::GFP pattern, it was evident that seam cells failed to produce alae irrespective of whether or not they had fused (Fig. 3D).

Seam cells fail to differentiate properly and inappropriately express a non-seam marker in *elt-5(RNAi)* embryos

We assessed the range of seam cell characteristics that require *elt-5/6* by analyzing several markers of seam-specific fate. As noted earlier, SCM, a marker of seam fate, is expressed in the seam cells of *elt-5(RNAi)* embryos, implying that seam-specific differentiation is initiated in these mutants. Eight genes that encode nuclear hormone receptors (NRs) are also apparently expressed exclusively in seam cells (Miyabayashi et al., 1999). We found that expression of reporters for some, but not all of these NR genes was diminished or abolished in *elt-5(RNAi)* embryos (Table 1). Expression of three NR reporters, *nhr-75*::GFP, *nhr-81*::GFP, and NHR-82::GFP, was undetectable in all *elt-5(RNAi)* embryos examined (Fig. 4A,B). In contrast, expression of two NR reporters, *nhr-73*::GFP and *nhr-74*::GFP, was only slightly

Table 1. Effects of *elt-5* dsRNA on expression of seam-specific NR genes

	% Embryos expressing GFP		
	– *elt-5* dsRNA (*n*)*	+ *elt-5* dsRNA (*n*)	Ratio (+/– dsRNA)‡
nhr-75::GFP	61 (57)	0 (77)	0
nhr-81::GFP	36 (85)	0 (86)	0
NHR-82::GFP	91 (34)	0 (32)	0
nhr-72::GFP	47 (75)	11 (157)	0.23
nhr-77::GFP	65 (105)	27 (111)	0.42
nhr-89::GFP	63 (106)	31 (148)	0.49
nhr-73::GFP	60 (139)	52 (105)	0.87
nhr-74::GFP	54 (102)	51 (111)	0.94

*Owing to mosaicism of the reporters, not all embryos without *elt-5* dsRNA express GFP. Embryos were scored as positive even if only one cell showed a detectable level of GFP.

‡The ratio was computed by dividing the percentage of dsRNA-treated embryos expressing GFP by the percentage of untreated embryos expressing GFP. This ratio is therefore a measure of the effect of *elt-5* RNA on the reporter expression. (0 corresponds to complete suppression and 1 to no suppression.)

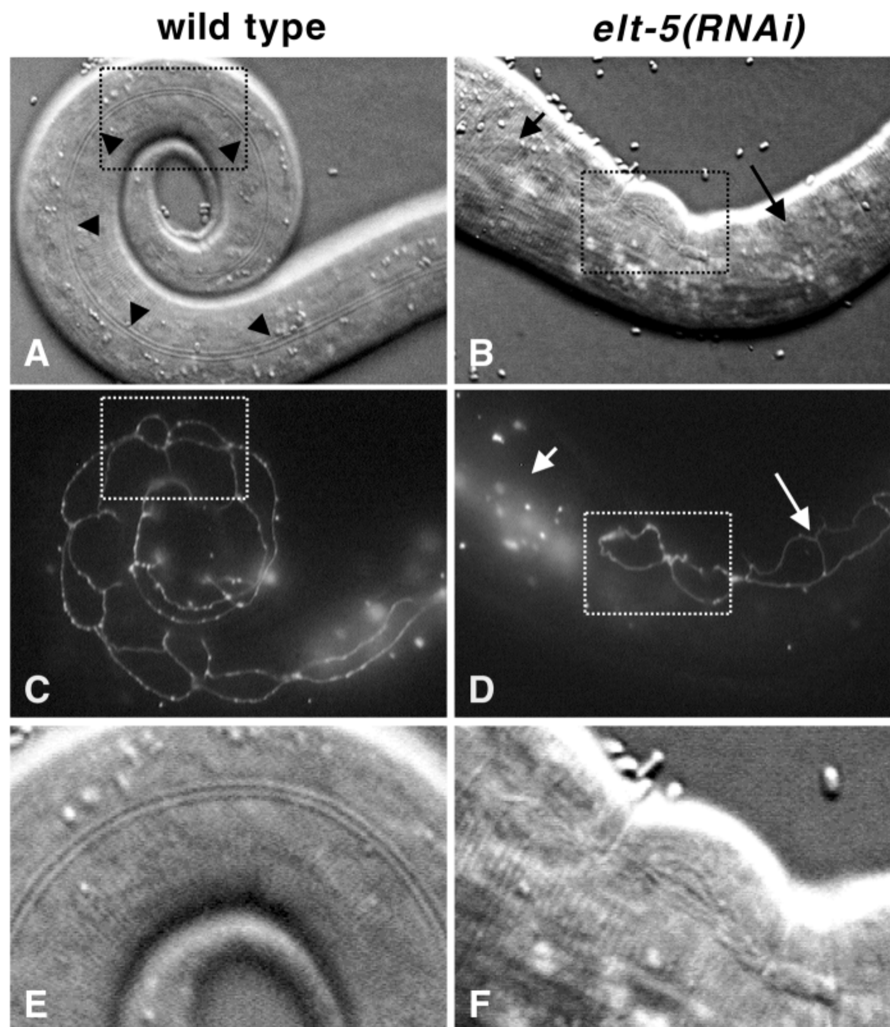

Fig. 3. Alae defects in *elt-5(RNAi)* larvae. (A,C,E) Wild-type; (B,D,F) *elt-5(RNAi)* larvae. (A,B) Nomarski images of L1 larvae. (A) Wild-type larva, showing normal alae, visible as two ridges along the length of the body (arrowheads). (B) *elt-5(RNAi)* larva in which alae are absent over most seam cells (short and long arrow) and malformed over two others (box). (C,D) Fluorescence images of the adherens junctions (revealed with JAM-1::GFP) of the larvae shown in A and B, respectively. (C) All seam cells show adherens junctions in the wild-type larva. (D) Some of the seam cells (short arrow) have fused to the epidermal syncytium in the *elt-5(RNAi)* larva. Some of the unfused seam cells (long arrow) do not have alae. (E,F) Details from A and B, respectively. Areas of detail are marked by the boxes in A,B.

affected, both in terms of the fraction of expressing animals and the level of GFP signal (Fig. 4C,D). The remaining three NR reporters, *nhr-72*::GFP, *nhr-77*::GFP, and *nhr-89*::GFP, gave intermediate results; these were expressed much less frequently in *elt-5(RNAi)* embryos than in wild-type embryos, and expression was barely detectable in only a few cells for those embryos showing any expression (Fig. 4E,F).

The foregoing observations indicate that *elt-5/6* is essential for many, but not all, aspects of seam cell differentiation. To investigate the possibility that they may also participate in specifying seam identity, we examined the expression of the *elt-3*::GFP reporter, which accurately reflects expression of endogenous ELT-3 (Gilleard et al., 1999). At the 1.5-fold stage, *elt-3*::GFP is expressed in all major epidermal cells except seam cells (Fig. 4G). In contrast, we found that seam cells often express *elt-3*::GFP ectopically in *elt-5(RNAi)* embryos (Fig. 4H). As most seam cells in *elt-5(RNAi)* embryos are still unfused at this stage, the expression of *elt-3*::GFP cannot be simply the consequence of seam fusion, but apparently reflects a partial transformation in fate of seam cells into non-seam cells. In nearly all (19/20) 1.5-fold stage embryos examined in detail, at least one unfused seam cell, and an overall average of 27% of the unfused seam cells, expressed *elt-3*::GFP. Of particular note, the V3 seam cell was the most likely to express *elt-3*::GFP (80% of V3s examined). However, V3 was not the most likely to fuse. One explanation for this behavior is that, of all the seam cells, V3 is the most closely related to P cells: its sister and all its cousins are P cells (Sulston et al., 1983). It is therefore possible that in the absence of *elt-5/6*, V3 often adopts the fate of its sister and cousins (i.e. the P cell fate) and therefore both expresses *elt-3* and remains unfused.

These findings indicate that *elt-5/6* is required to maintain the identity of seam cells. Its role in repressing cell fusion may reflect conversion to a syncytial epidermal fate in the absence of seam-specifying information.

elt-5 and *-6* are expressed in seam cells

To assess the expression patterns of *elt-5* and *-6*, we created several transcriptional and translational reporter constructs (Fig. 5). An *elt-5* translational fusion construct (pKK52), in which the *elt-5* promoter drives expression of a GFP::ELT-5 fusion protein, shows a complex and dynamic expression pattern that can be divided into three major components. First, pKK52 expression begins at the 28-cell stage in all four granddaughters and 16 great-great granddaughters of the MS and AB founder cells, respectively (Fig. 6A); this expression continues in many, possibly all, of their descendants until around the time of hatching. Second,

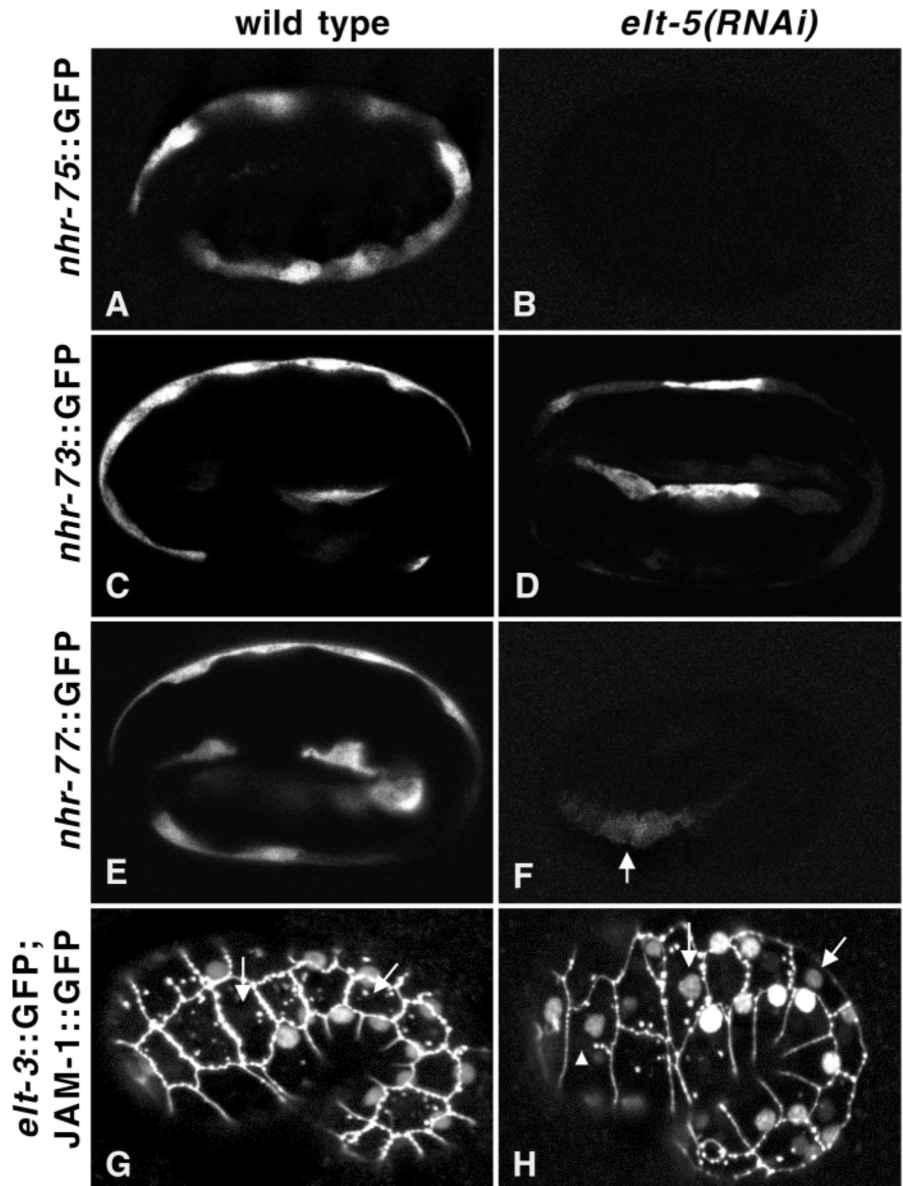

Fig. 4. Embryonic expression of seam and non-seam epidermal markers. Wild-type expression patterns are shown in the left panels, expression in *elt-5(RNAi)* embryos is shown on the right. (A-F) Approximately threefold stage embryos carrying NR reporters. (A,B) *nhr-75*::GFP is expressed in seam cells in wild-type (A) but not in *elt-5(RNAi)* (B) embryos. (C,D) *nhr-73*::GFP is expressed at high levels in both wild-type (C) and *elt-5(RNAi)* (D) embryos. (E,F) *nhr-77* is expressed at high levels in seam cells in wild-type (E) and weakly and sporadically in *elt-5(RNAi)* (F) embryos. In the *elt-5(RNAi)* embryo, a single seam cell shows a barely detectable level of GFP expression (arrow). (G,H) *elt-3*::GFP expression in wild-type and *elt-5(RNAi)* embryos at the ~1.5-fold stage. JAM-1::GFP expression in adherens junctions was included to help in identifying seam cells. The arrows indicates two seam cells, H2 and V3, in G,H. (G) *elt-3*::GFP is expressed in all non-seam major epidermal nuclei, and is excluded from all seam nuclei in a wild-type embryo. (H) *elt-5(RNAi)* embryo showing ectopic expression of *elt-3*::GFP in seam cells. Eight seam nuclei show expression, two of which are indicated by the arrows. A gap in the adherens junction of H0 (arrowhead) reveals that the cell is undergoing fusion.

expression becomes more pronounced in seam cells about 1 hour after their birth. This seam expression remains strong throughout embryonic and larval development (Fig. 6B), but becomes slightly reduced in adults. Third, robust expression is also seen in several cells in the head region, at least some of which are cells in the nervous system (neurons and/or support cells), beginning at approximately the comma stage (Fig. 6B) and continuing through adulthood. For simplicity, we will refer to this component of the expression pattern as nervous system expression, although we have not determined the precise identity of these cells.

An *elt-6* transcriptional reporter (pKK41) is expressed in the same groups of cells as the *elt-5* translational reporter (pKK52), but the relative expression levels are different. Whereas the *elt-5* reporter is strongly expressed in both seam cells and the nervous system during the comma through pretzel stages (Fig. 6B), the *elt-6* reporter is strongly expressed only in the nervous system (Fig. 6E). Only weak expression of the *elt-6* reporter is apparent in seam cells and in the AB and MS descendants during embryogenesis, but the seam expression becomes stronger during larval development (not shown). Strong expression of

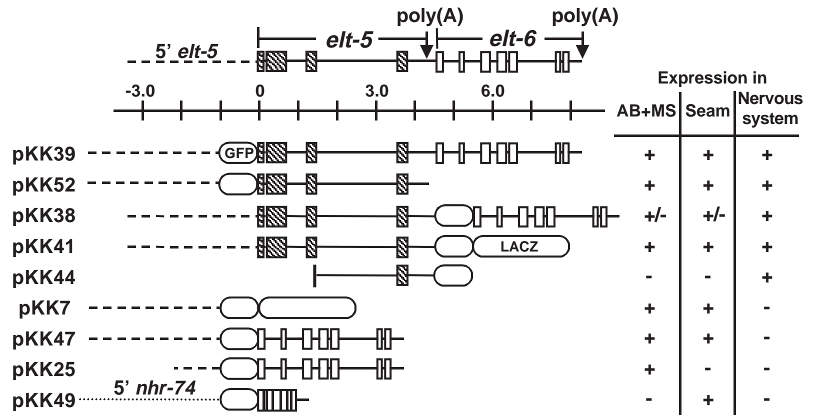

Fig. 5. Summary of *elt-5* and *-6* reporter construct expression. The exon-intron structures of the *elt-5* and *-6* genes and approximate distances in kb are shown at the top. Short and long oval shapes represent GFP and *lacZ*-coding regions, respectively. +/- indicates weak expression. See Materials and Methods for a more detailed description of the constructs. See text for a description of the AB+MS lineage, seam and nervous system expression.

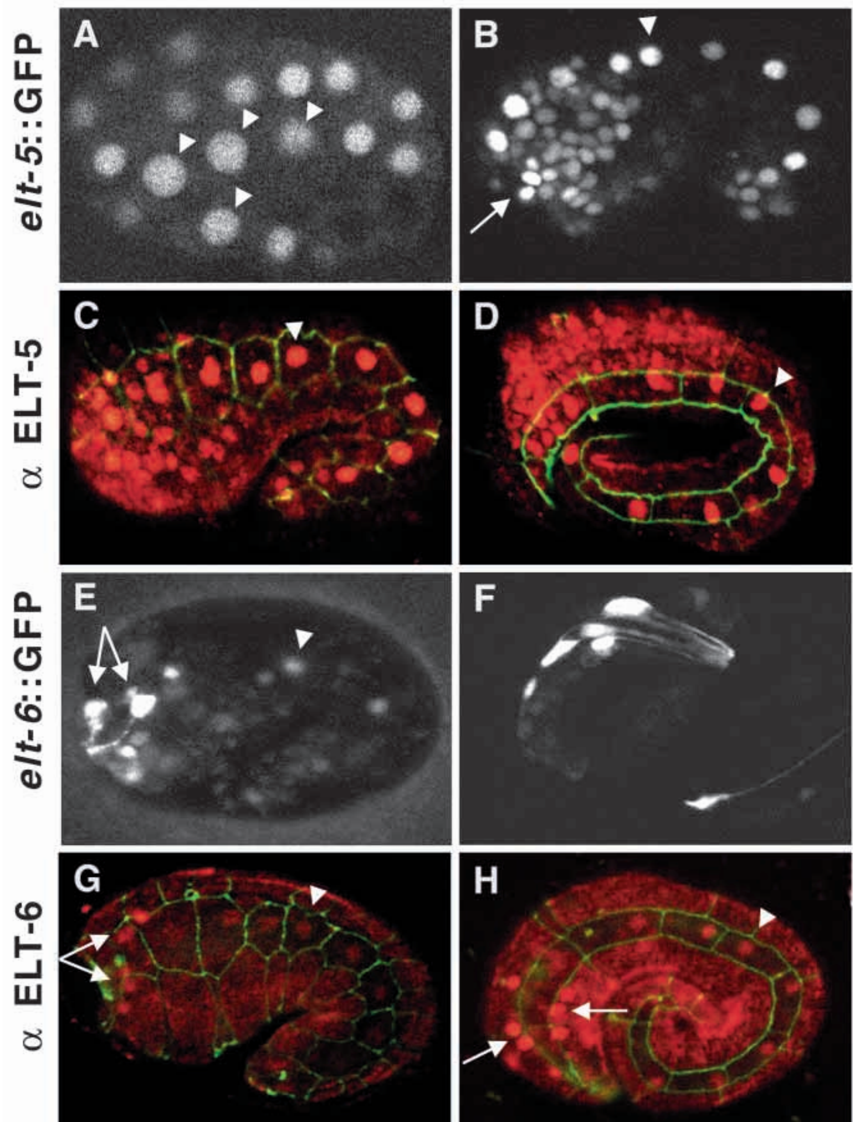

Fig. 6. Expression patterns of *elt-5* and *-6* reporters and endogenous ELT-5 and -6 proteins. (A,B) Expression of the *elt-5* translational fusion, pKK52. (A) Ventral view of a 28-cell stage embryo. GFP is seen in the nuclei of all four MS granddaughters (arrowheads) and all 16 AB great great granddaughters. Only 14 AB descendants are visible in this focal plane. (B) Lateral view of a comma-stage embryo. Strong GFP expression is present in seam cells and several cells in the head, tentatively identified as neurons and/or neuronal support cells. Somewhat weaker expression is observed in many other cells, mostly in the head and tail regions; these are likely to be descendants of the AB and MS founder cells. (C,D) Embryos at the ~1.5-fold (C) and ~threefold (D) stages stained with anti-ELT-5 (red) and MH27 (green). High levels of ELT-5 are detected in all seam cells and in many other cells in the head and tail regions at these stages. (E,F) Expression patterns of the *elt-6* transcriptional fusion reporter, pKK41. (E) Embryo at the ~1.5-fold stage shows strong GFP expression in several cells in the head region, tentatively identified as neurons and/or support cells, and much weaker expression in seam cells. (F) Head region of an L1-stage larva showing long processes in GFP-expressing cells. These are likely to be neurons and/or support cells. GFP is also present in seam cells at this stage, but they are not visible in this focal plane. (G,H) Embryos stained with anti-ELT-6 (red) and MH27 (green) at the ~1.5-fold (G) and ~2.5-fold (H) stages. The staining pattern is similar to the expression pattern of the reporter construct shown in E. The arrowheads point to a seam cell (V2) in B-E,G,H, and the arrows point to cells in the nervous system.

Table 2. Effects of *elt-5* or *-6* dsRNA on *elt-5* and *-6* reporter expression

				% Embryos expressing GFP		
	Reporter for	*elt-5*-coding region	*elt-6*-coding region	– dsRNA (*n*)	+ *elt-5* dsRNA (*n*)	+ *elt-6* dsRNA (*n*)
pKK39	*elt-5*	+	+	76 (45)	0 (30)	61 (31)
pKK47	*elt-6*	–	+	56 (80)	69 (54)	0 (108)
pKK41	*elt-6*	+	–			
Nervous system				64 (28)	58 (43)	n.d.
Seam/AB/MS				64 (28)	7 (43)	n.d.

Owing to mosaicism of the reporters, not all embryos untreated with dsRNA express GFP. Embryos were scored as in Table 1. Almost all embryos either expressed GFP at moderate to strong levels in many cells, or did not express any GFP at all.

the *elt-6* reporter in the nervous system continues throughout larval development (Fig. 6F).

To confirm the expression patterns obtained with the reporter constructs, we raised antibodies against peptides specific for ELT-5 and -6. For each protein, two peptides were selected from regions of little or no similarity between the two proteins. Anti-ELT-5 staining is readily detected in the nuclei of seam cells during mid- to late-embryogenesis (Fig. 6C,D). At these stages, many unidentified cells in the head region also stained, consistent with the pattern seen for the GFP reporters (Fig. 6B). This staining is eliminated in *elt-5(RNAi)* embryos (not shown). We have been unable to obtain consistent and reliable staining of early embryos, larvae and adults, and therefore have not confirmed the reporter expression pattern at these stages. Anti-ELT-6 staining is most readily seen in several cells in the head and is faint in seam cells (Fig. 6G,H), consistent with the GFP reporter data. *elt-6* dsRNA eliminates all nuclear staining (not shown).

Monocistronic transcription of *elt-6* messages and modularity of tissue-specific enhancers

As described above, despite the apparent operon-like organization of the *elt-5* and *-6* genes, we could not find evidence that *elt-6* cDNA is *trans*-spliced to SL2. Previous studies have shown that some downstream genes in apparent operons are transcribed monocistronically under the control of their own promoters (Gilleard et al., 1997). To test whether *elt-6* can be transcribed monocistronically, we constructed an *elt-6* reporter (pKK44), which includes the unusually large (>2 kb) last intron and the last exon of *elt-5* upstream of the *elt-6* ATG (see Fig. 5). pKK44 is expressed strongly in some cells in the nervous system but is expressed in neither seam cells nor in early AB and MS descendants (not shown). Because pKK44 lacks the 5′ end and the first three exons of *elt-5*, this result indicates that *elt-6* messages can be transcribed alone.

The result with pKK44 also suggests that enhancer sequences for seam, AB and MS expression are separable from those for expression in the nervous system. Indeed, we found that an *elt-5* transcriptional reporter (pKK7) that includes only the 3.4 kb sequences upstream of the *elt-5* ATG (see Fig. 5) is expressed in the early AB and MS lineages and in seam cells, but is not expressed in the nervous system. This pattern is complementary to the expression pattern of pKK44, demonstrating that separable enhancer regions regulate *elt-5* and *-6* expression in different groups of cells. Whereas enhancers for seam, AB and MS are contained in the 3.4 kb region upstream of *elt-5*, those for the nervous system reside in the 3.1 kb region upstream of *elt-6* (perhaps within the last, large intron of *elt-5*).

Efficacy of RNAi and evidence for tissue-specific monocistronic versus dicistronic transcription

To determine the effectiveness and specificity of the *elt-5* and *-6* dsRNAs, we injected them into strains carrying various *elt-5* and *-6* GFP reporter genes (Table 2). Expression of an *elt-5* translational fusion (pKK39) was reduced to undetectable levels in *elt-5(RNAi)* embryos, suggesting that RNAi results in a strong loss-of-function or null phenotype. Furthermore, although *elt-6* dsRNA did not cause an observable phenotype, we confirmed that *elt-6* dsRNA was also effective; expression of an *elt-6* translational fusion (pKK47) was eliminated by *elt-6* dsRNA. In contrast, consistent with only moderate similarity between the two genes (~60% identity overall), *elt-5* dsRNA did not significantly affect expression of the *elt-6* translational fusion, pKK47, which lacks the *elt-5* coding region, and *elt-6* dsRNA likewise did not alter expression of an *elt-5* fusion (pKK39).

Previous studies have suggested that in some cases RNAi targeted against one gene in an operon can inhibit expression of another in the same operon (Bosher et al., 1999). We therefore explored the possibility that *elt-5* dsRNA interferes with expression of both *elt-5* and *-6* and found that, indeed, *elt-5* dsRNA blocks expression of the *elt-6* fusion pKK41 (which contains the *elt-5*-coding region) in seam cells and in early AB and MS descendants, but not in the nervous system. (This probably does not relate to the relative insensitivity of the nervous system cells to RNAi (Tavernarakis et al., 2000), as we were able to eliminate expression of *elt-6* in these cells using *elt-6* dsRNA (data not shown).) Because pKK41 does not include any portion of *elt-6*, this is not due to cross-hybridization: instead, this result implies that *elt-5* dsRNA eliminates expression of *elt-6* in some (although not in all) cell types. However, *elt-6* dsRNA did not affect expression of an *elt-5* fusion (pKK39) that includes both *elt-5*- and *elt-6*-coding regions, suggesting that a fraction of the *elt-5* transcripts are monocistronic. In summary, our results suggest that in seam cells and in early AB and MS descendants *elt-5* is either transcribed alone or co-transcribed with *elt-6*, while in the nervous system *elt-5* and *-6* are each transcribed monocistronically (although there may be some dicistronic transcripts in the nervous system as well). Collectively, our findings suggest that the apportionment of monocistronic versus dicistronic transcription of this pair of genes is regulated in a tissue-specific manner.

ELT-6 can rescue the lethality of *elt-5(RNAi)* animals, revealing an apparently continuous post-embryonic requirement for *elt-5*

Given the strong similarity of the ELT-5 and -6 proteins,

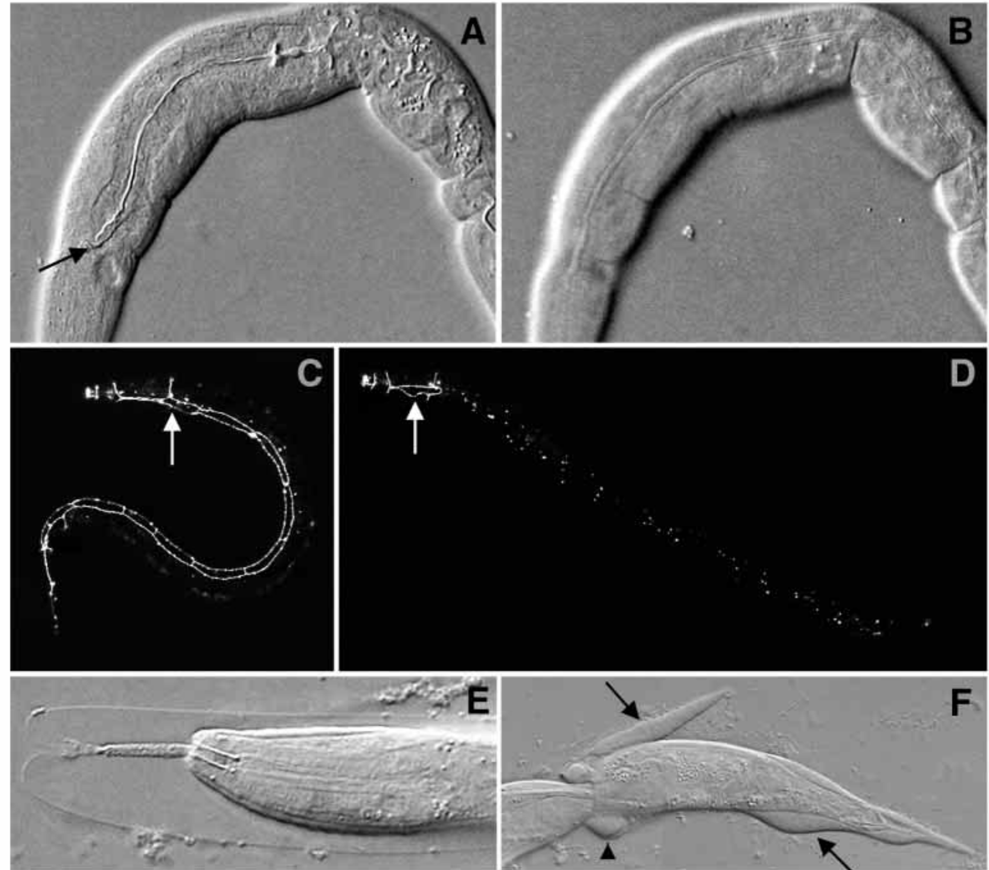

Fig. 7. Rescue by ELT-6 restores normal alae and uncovers post-embryonic defects in *elt-5(RNAi)* animals. (A,B) L1-stage *elt-5(RNAi)* larva in which the *nhr-74*::GFP::ELT-6 fusion (pKK49) was expressed in seam cells. (A) Interior view showing that the buccal capsule (arrow) is not attached. (B) Surface view showing that alae are restored in larvae expressing GFP::ELT-6 in seam cells (compare with Fig. 3B,F). (C,D) Fluorescence image of epidermal adherens junctions (JAM-1::GFP) in L2-stage larvae. (C) Wild-type larva showing that adherens junctions surround all seam cells. (D) *elt-5(RNAi)* larva carrying a construct (pKK25) that drives ELT-6::GFP expression in AB and MS descendants. All but one of the seam cells (arrow) lack visible adherens junctions and have therefore fused with the surrounding epidermal syncytium. Arrows point to the H0 seam cell in C,D. (E,F) Molting defects in *elt-5(RNAi)* larvae expressing GFP::ELT-6 in AB and MS descendants but not in seam cells (pKK25). (E) Larva arrested at the L2-L3 molt showing its inability to break through the old cuticle, resulting in a plugged mouth. (F) Larva arrested at the L3-L4 molt with cuticle in the tail region still attached. The space between the old and new cuticle has collected waste material (arrows) and the old cuticle has formed a constriction (arrowhead).

we explored the possibility that they are functionally interchangeable. To test whether *elt-6* can rescue the absence of *elt-5*, we created a GFP::ELT-6 fusion, driven by the 3.4 kb upstream region of *elt-5* (pKK47, Fig. 5), which allowed us to drive expression of ELT-6 in seam cells and AB and MS descendants even in the presence of *elt-5* dsRNA. Most *elt-5(RNAi)* animals expressing pKK47 (52 of 57 animals carrying the construct, as assessed by GFP expression) appeared wild type at hatching, and many (48/57) grew up to be viable adults. These rescued animals appeared to develop normally with the exception that many of them lacked a vulva (not shown), as will be described in more depth in a separate publication. These results demonstrate that *elt-5* and *-6* are functionally interchangeable through most of development and that expression of ELT-6 in seam cells and in AB and MS descendants is sufficient to rescue *elt-5(RNAi)* animals to viability.

We next asked whether the *elt-5(RNAi)* phenotypes we observed were attributable to the activity of the gene in seam cells, in AB and MS descendants, or both. Because all seam cells are derived from the AB lineage, it is not possible to completely separate the two components. We therefore created two rescuing constructs, pKK25 and pKK49, with partially overlapping, but largely complementary expression patterns (Fig. 5). In construct pKK25, expression of a GFP::ELT-6 translational fusion is driven by the 1.2 kb upstream promoter of *elt-5*. This construct is expressed in AB and MS descendants from early embryogenesis until about the time of hatching and is expressed in seam cells during embryogenesis only as part of the broad AB expression pattern. By the late L1 stage, however, no GFP is detectable. We created another construct, pKK49, in which the GFP::ELT-6 fusion protein is driven by the *nhr-74* promoter. This promoter, which is expressed only in seam cells from about the comma stage through adulthood (Miyabayashi et al., 1999), was chosen because it is not significantly affected by *elt-5* dsRNA (Table 1). The sum of the expression patterns of the two constructs closely resembles that of pKK47, the GFP::ELT-6 fusion construct containing the 3.4 kb *elt-5* promoter. Indeed, we found that most (31/44) *elt-5(RNAi)* embryos carrying both pKK25 and pKK49 were rescued and grew up to become fertile adults.

While each of these constructs alone was not sufficient for viability, they allowed us to assess the temporal and tissue requirements for *elt-5/6*. All (n=54) *elt-5(RNAi)* animals expressing GFP::ELT-6 only in seam cells (from construct pKK49) arrested as embryos or larvae by the early L1 stage, the stage at which *elt-5(RNAi)* animals normally arrest. The arrested animals showed the Pun phenotype (Fig. 7A), which is presumably the cause of lethality. However, the alae defects normally seen in *elt-5(RNAi)* larvae were rescued (31/33 larvae showed normal alae; Fig. 7B), implying a cell-autonomous function of *elt-5/6* in alae formation. In addition, the Lpy phenotype was partially rescued: the animals were only slightly lumpy, indicating that *elt-5/6* activity in seam cells contributes to proper morphogenesis.

In contrast, most (36/42) *elt-5(RNAi)* larvae expressing GFP::ELT-6 in AB and MS descendants (from construct pKK25) developed beyond the early L1 stage, but many (32/42) had missing or malformed alae. Those that developed beyond early L1 (*n*=36) arrested growth by the L3-L4 molt. Of particular significance, by the time these larvae arrested, nearly all of their seam cells had fused with the surrounding epidermal syncytia (Fig. 7C,D). Some larvae (5/36) showed no visible seam cell boundaries at all, and the rest contained between one and four seam cells with distinct boundaries (overall the mean number of unfused seam cells in 36 larvae was 1.8). In addition, the arrested larvae were defective in molting: though they apparently initiated the molting process, they were unable to shed their old cuticle completely (Fig. 7E,F). Many of these were still encased in the old cuticle, and because their mouth was blocked they were unable to eat; this is presumably the cause of lethality (Fig. 7E). The old cuticle was often wrapped around the body of these larvae, forming a constriction, and sometimes the space between the old and new cuticle filled up with waste material (Fig. 7F).

Taken together, the results obtained with these two rescuing constructs (pKK49 and pKK25) imply that *elt-5/6* activity in seam cells is essential for proper alae formation, suppression of seam fusion and molting. They further suggest that early L1 lethality is attributable either to *elt-5/6* activity in AB and MS descendants or to expression in very early seam cells, before pKK49 gives robust expression in seam cells ~2 hours after their birth. Finally, they reveal an apparently continuous requirement for *elt-5/6* in maintaining seam identity and/or repression of their fusion throughout post-embryonic development.

DISCUSSION

An apparently redundant pathway specifies seam epidermal cells in the embryo

We have shown that ELT-5 and -6 (or ELT-5 alone) are required in seam cells to promote a number of differentiated seam cell characteristics. These include production of alae, expression of several seam-specific genes, repression of a gene that is expressed in non-seam epidermis and maintenance of seam cells in an unfused state. These factors appear to be part of a regulatory system that links ELT-1, which broadly specifies the general epidermal fate, to the genes imparting the unique characteristics to seam cells.

One of the conspicuous defects in *elt-5(RNAi)* animals is the inappropriate fusion of seam cells with the epidermal syncytia. Cell fusion occurs in many cell types in a tightly regulated fashion in *C. elegans* (Podbilewicz and White, 1994); however, its function is not well understood and only a small number of genes that regulate it have been found (Alper and Kenyon, 2001; Clark et al., 1993; Kenyon, 1986; Wang et al., 1993). Our results demonstrate that *elt-5/6* plays a major role in repressing both embryonic and larval epidermal cell fusion. The later fusion of the seam cells into a single lateral syncytium at the L4 molt (Podbilewicz and White, 1994) must involve a mechanism that bypasses the fusion-repressive function of ELT-5/6. However, this repressive function presumably remains active in L4 larvae and adults, as the seam cells, while fusing with each other, remain separate from the other

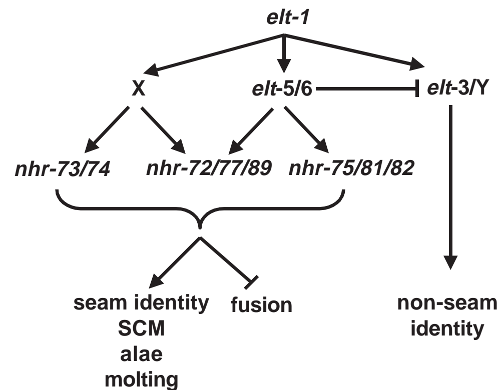

Fig. 8. Model for specification and patterning of the *C. elegans* AB-derived epidermis. *elt-1* is required to specify all major epidermal cells. A set of factors, including *elt-5/6* and an unknown factor (X), directs some epidermal cells to become seam cells by activating partially redundant sets of nuclear hormone receptors and perhaps other transcription factors. *elt-5/6* repress *elt-3* expression in seam cells. We hypothesize that ELT-3, together with another factor (Y), specifies a non-seam fate. No information is available for how syncytial versus P cell fate is apportioned between the group of non-seam epidermal cells.

epidermal syncytia. Thus, the homotypic fusion of seam cells appears to involve a regulatory process distinct from that controlling fusion with the epidermal (hyp) syncytia.

Several findings suggest that the inappropriate fusion of seam cells with the epidermal syncytia is not simply the result of a failure to repress fusion per se. In particular, the failure of seam cells to express several seam-specific markers and the observation that *elt-3*::GFP is inappropriately expressed in unfused seam cells imply a (partial) loss of seam identity. Our finding that some seam-specific markers are largely unaffected, however, suggests that a combination of regulatory inputs from *elt-5/6* and at least one other factor is required to specify and maintain the entire identity of seam cells. Such a factor might simply act redundantly with ELT-5/6, or it may activate a unique set of target genes that, together with the ELT-5/6 targets, contribute to the full suite of genes that define seam cell identity. It is noteworthy that the ELT-1 protein is present in major epidermal cells after their epidermal cell fate is specified, and that ELT-1 expression is stronger in seam cells than in syncytial or P cells (Page et al., 1997). One interesting possibility is that ELT-1 may function in seam cell specification or differentiation as well as in general epidermal cell specification. Alternatively, *elt-5/6* may be essential for all aspects of seam cell specification and the residual seam characteristics observed in *elt-5(RNAi)* animals might be the result of incomplete inactivation of the *elt-5/6* function. Although we have shown that ELT-5/6 expression is undetectable, based on both GFP reporter expression or immunostaining, it is possible that undetectably low levels of ELT-5/6 can provide partial function in *elt-5(RNAi)* animals.

A model for seam epidermal development in the *C. elegans* embryo is presented in Fig. 8. *elt-1* is required to specify all major epidermal cells, which then adopt one of three fates: seam, P or syncytial. Our present results suggest that *elt-5/6*, together with an unknown factor (Factor X in Fig. 8), may direct seam cells to adopt their appropriate identity. We have

found that a transcriptional *elt-5*::GFP reporter is expressed in many cells in *elt-1(–)* mutants (K. K. and J. H. R., unpublished), suggesting that at least the widespread AB and MS expression of the reporter does not require ELT-1; however, it seems likely that *elt-5/6* expression in seam cells per se requires ELT-1. Although we have not tested this possibility directly, their respective mutant phenotypes support the hypothesis that *elt-1* functions upstream of *elt-5/6* in seam cells. (As suggested above, *elt-1* may also function in parallel with *elt-5/6* in seam cells.) Given its expression pattern, *elt-3* may function in syncytial and P cell specification or differentiation. However, deletion of *elt-3* does not produce any noticeable phenotype, suggesting pervasive functional redundancy of this gene (Gilleard and McGhee, 2001). ELT-3 and another factor (Factor Y in Fig. 8) may specify a general non-seam fate, and other factors may then apportion syncytial and P cell identities to the appropriate cells.

Specification of the epidermis and its subdivision into the different epidermal cell types by a sequential cascade of GATA factors is reminiscent of the regulatory events that specify the endoderm (E founder cell) and major mesoderm (MS founder cell) precursors in very early *C. elegans* embryos. In the latter case, a pair of redundant GATA factors, MED-1 and -2, dictate the identity of the EMS cell, and therefore both of its daughters, E and MS (Maduro et al., 2001). Following division of EMS, the fate of the E cell is, in turn, regulated by the END-1 and -3 GATA factors (Zhu et al., 1997; M. Maduro and J. H. R., unpublished). A cascade of three other GATA factors (ELT-2, -4, and -7) is subsequently activated in the E lineage (Fukushige et al., 1998; Fukushige and McGhee, personal communication; K. Strohmaier and J. H. R., unpublished). In contrast, it seems unlikely that additional GATA factors act downstream of ELT-3 and ELT-5/6 in the epidermis: the expression patterns of all 11 GATA factors predicted from the genomic sequence are now known and only ELT-1, -3, -5 and -6 are present in the epidermis.

ELT-5/6 is apparently required continuously for post-embryonic development of seam cells and molting

We observed post-embryonic developmental defects when low doses of *elt-5* dsRNA were used or when the embryonic defects seen at high doses were rescued by forced expression of ELT-6 in selected cells under a partial *elt-5* promoter. Under the latter condition, almost all seam cells fuse with the surrounding syncytium during larval development, suggesting that ELT-5/6 may be continuously and non-redundantly required to specify or maintain the identity of seam cells post-embryonically.

We also found that *elt-5(RNAi)* animals rescued for embryonic or early L1-stage lethality by expression of ELT-6 under a partial *elt-5* promoter had difficulty completing molting and arrested before reaching adulthood. These defects were rescued by additional, seam-specific expression of ELT-6. These results suggest that seam cells play an essential role in molting. Previous studies have identified two genes essential for proper molting. *lrp-1* loss-of-function mutant larvae show molting problems similar to those we observed in *elt-5(RNAi)* larvae (Yochem et al., 1999). LRP-1 is similar to gp330/megalin, a member of the low-density lipoprotein receptor family. LRP-1 is expressed on the apical surface of the main body epidermal syncytium, hyp7, and genetic mosaic analysis has indicated that it is required in hyp7. RNAi of another gene, *nhr-23*, which encodes CHR3 (a nuclear hormone receptor), causes similar problems in molting (Kostrouchova et al., 1998). CHR3 is highly similar to *Drosophila* DHR3, which is induced by ecdysone, a molting hormone and plays an essential role in metamorphosis. CHR3 is expressed in all epidermal cells including seam cells during late embryogenesis and larval stages. It seems likely that the molting process in *C. elegans* larvae is regulated by a network of genes that acts in both seam and syncytial cells. It will be interesting to learn how *elt-5* and -*6* act in such a network and if any of the many known seam-specific nuclear hormone receptors also participate in the regulation of molting.

elt-5 and -*6* may be transcribed both monocistronically and dicistronically in a tissue-dependent manner

elt-5 and -*6* are spaced ~130 bp apart and are organized in an apparent operon; however, our results suggest that they do not form a typical operon. We could not find evidence that *elt-6* messages are *trans*-spliced to SL2 or to other non-SL1 leaders. Most downstream genes in operons are *trans*-spliced to SL2 exclusively or to a mixture of SL1 and SL2 (Spieth et al., 1993; Zorio et al., 1994). A few exceptions in which the downstream genes are *trans*-spliced exclusively to SL1 involve pairs of genes with no intercistronic sequences (Williams et al., 1999). Further evidence that *elt-6* need not be transcribed as part of a polycistronic transcript comes from our identification of an apparent transcription initiation site ~120 bp upstream of the *elt-6 trans*-splice site. Nevertheless, it seems likely that a fraction of *elt-6* messages are transcribed dicistronically. In support of this possibility, we found that *elt-5* dsRNA inhibited expression of reporters for both *elt-5* and -*6* in seam cells, and AB and MS descendants (*elt-6* dsRNA did not affect *elt-5* reporter expression). Recent evidence suggests that for certain dicistronic genes, RNAi targeted against one gene can interfere with both (Bosher et al., 1999). Accumulation of dicistronic *elt-5* and -*6* pre-mRNA may allow *elt-5* dsRNA to inhibit *elt-6* gene activity. (The reason *elt-6* dsRNA does not inhibit *elt-5* gene activity may be because a substantial portion of *elt-5* is transcribed monocistronically.) Intriguingly, such an effect would appear to be tissue specific: while *elt-6* messages may be co-transcribed with *elt-5* messages in seam cells and descendants of AB and MS, allowing *elt-5* dsRNA to interfere with them, this does not appear to be the case in the nervous system. As ELT-6 is most strongly expressed in the nervous system, our inability to detect SL2 *trans*-splicing on *elt-6* cDNAs may simply reflect the relative abundance of monocistronic versus dicistronic *elt-6* transcripts; monocistronic transcripts may be the predominant form in the nervous system.

elt-5 and -*6* are functionally interchangeable and may perform additional functions

We found that the *elt-5(RNAi)* defects can be rescued by expression of *elt-6*, implying that the two gene products can function interchangeably. In addition, we have found that widespread expression of either ELT-5 or -6 can result in an excess of cells expressing a seam-specific marker (SCM; K. K. and J. H. M., unpublished), suggesting that either *elt-5* or -*6* alone may be sufficient to initiate seam differentiation in non-seam cells. It is unclear, however, if the two genes in fact perform redundant functions under normal conditions. The low

level of *elt-6* expression in seam cells and descendants of AB and MS may be insufficient to provide the essential functions in those cells. Although the two genes can apparently function interchangeably, they may be responsible for different activities depending on where they are most abundantly expressed, which might account for the preservation of both genes during evolution.

Although we have focused on the role of *elt-5/6* in seam cell development, several findings indicate that these genes may have additional functions in other cell types. First, they are expressed in several different cell types. Second, we observed a vulvaless phenotype in *elt-5(RNAi)* animals rescued by GFP::ELT-6 (as will be described in a subsequent report). Third, seam-specific expression of GFP::ELT-6 using the *nhr-74* promoter did not rescue all embryonic defects in *elt-5(RNAi)* animals. Although we cannot rule out the possibility that earlier expression of ELT-6 in seam cells than that directed by the *nhr-74* promoter might fully rescue the embryonic *elt-5(RNAi)* defects, a plausible interpretation is that *elt-5/6* performs an unknown essential function in early AB and MS descendants. It will be interesting to investigate whether *elt-5/6* performs distinct functions in different types of cells, and if so, how specificity for particular cell types is achieved.

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Note added in proof

We have recently found that *elt-5* corresponds to the previously identified gene *egl-18* (K. K., C. G. Wood and J. H. R., unpublished).

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