A cell-specific enhancer that specifies *lin-3* expression in the *C. elegans* anchor cell for vulval development

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Summary

During *C. elegans* vulval development, the anchor cell (AC) in the somatic gonad expresses *lin-3*, activating the EGF receptor signaling pathway in vulval precursor cells (VPCs) and thereby inducing and patterning VPCs. Previous studies with *lin-3* mutants and transgene expression have revealed that the level of LIN-3 in the AC must be precisely regulated for proper vulval development. To understand how *lin-3* expression is achieved in the AC, we identified a 59 bp *lin-3* enhancer sufficient to activate *lin-3* transcription solely in the AC. The enhancer contains two E-box elements, and one FTZ-F1 nuclear hormone receptor (NHR) binding site that is mutated in a vulvaless mutant, *lin-3(e1417)*. Mutagenesis studies show that both E-boxes and the NHR binding site are necessary to express *lin-3* in the AC. In vitro DNA-binding studies and in vivo functional assays indicate that distinct trans-acting factors, including the E-protein/Daughterless homolog HLH-2 and unidentified nuclear hormone receptor(s), are necessary for *lin-3* transcription in the AC and thus are involved in vulval development.

Key words: EGF, LIN-3, HLH-2, E-protein/Daughterless, Nuclear hormone receptor, Anchor cell, Vulval induction

Introduction

C. elegans hermaphrodite vulval development requires a signal from the anchor cell (AC) in the gonad, which acts on three of the six epidermal blast cells termed vulval precursor cells (VPCs) (Kimble, 1981; Kimble and Hirsh, 1979; Sternberg and Horvitz, 1986). In wild-type hermaphrodites, the VPC nearest to the AC, P6.p, adopts the 1° cell fate, while the adjacent P5.p and P7.p adopt the 2° cell fate. The other three distal VPCs, P3.p, P4.p. and P8.p, adopt the non-vulval 3° fate. All VPCs divide once about 4 hours after the L2 molt stage (Sulston and Horvitz, 1977; Wang and Sternberg, 1999). The two daughters of the VPCs that assume the 3° fate then fuse with the hyp7 epidermal syncytium. Daughters of the VPCs that assume the 1° and 2° fates divide again about 2.5 hours later and then a third time during the L3 molt to give rise to eight and seven progeny cells, respectively. The AC signal LIN-3, a member of the epidermal growth factor (EGF) family, is sufficient to induce the VPCs to divide and is necessary to establish an invariant pattern of vulval development (Hill and Sternberg, 1992; Katz et al., 1995). The response to LIN-3 is mediated by the EGF receptor (LET-23)/Ras (LET-60)/mitogen activated protein kinase (MAPK) signal transduction pathway in the VPCs (reviewed by Sternberg and Han, 1998). Overexpression of *lin-3* in the AC causes excess vulval fates to be adopted by the VPCs, which leads to the formation of extra vulval tissues (Hill and Sternberg, 1992; Liu et al., 1999). However, reduced LIN-3 activity in the AC makes the VPCs fail to adopt vulval cell fates (Sulston and Horvitz, 1981; Wang and Sternberg, 1999). Thus, the level of LIN-3 in the AC must be precisely regulated for the proper vulval development. In addition, LIN-3 activity in the AC must be temporally regulated, as LIN-3 induces the patterned proliferation of VPCs only in the early L3 stage in wild-type *C. elegans*.

The LIN-3 protein is synthesized as a transmembrane precursor like other EGF family growth factors, and an unidentified protease(s) has been proposed to cleave the precursor to release the extracellular EGF domain that binds to its receptor, LET-23, in the VPCs (Hill and Sternberg, 1992). Molecular lesions in the *lin-3*-coding region have been identified in seven out of the eight known *lin-3* mutant alleles (Liu et al., 1999). The mutation in the eighth allele, *e1417*, is not in the coding region of *lin-3*. As *e1417* mutants are defective only in the vulval development and LIN-3 from the AC is necessary to induce vulvae, this suggests that the mutation may reside in a regulatory region that is necessary to specify *lin-3* expression in the AC (Ferguson and Horvitz, 1985; Hill and Sternberg, 1992; Horvitz and Sulston, 1980; Liu et al., 1999; Sulston and Horvitz, 1981).

Although *lin-3* was discovered because of its role in vulval development, *lin-3* is also required for growth and viability, hermaphrodite fertility, male spicule development, and cell fate specification of the P12 neuroblast and the uterine uv1 cells (Chamberlin and Sternberg, 1994; Chang et al., 1999; Clandinin et al., 1998; Ferguson and Horvitz, 1985; Jiang and Sternberg, 1998). Studies including laser ablation experiments identified several cells as sources for the induction of the EGF signaling pathway, such as the AC for vulval induction (Kimble, 1981), vulF cells of the primary vulva for uv1 cells specification (Chang et al., 1999), and male F and U cells for spicule development (Chamberlin and Sternberg, 1994).

In this study, we identified the molecular lesion in *lin-3(e1417)* and the regulatory region (59 bp) of *lin-3* that drives AC-specific expression. This enhancer region contains two E-box elements and one FTZ-F1 nuclear hormone receptor (NHR) binding site, both of which are necessary for *lin-3* expression in the AC. The HLH-2 protein, a basic helix-loop-helix (bHLH) protein and *C. elegans* homolog of mammalian E-protein and *Drosophila* Daughterless, binds to both E-box elements. The NHR-25 protein, which is a *C. elegans* homolog of *Drosophila* FTZ-F1 NHR, binds to the wild-type form of the NHR-binding site, but not to the *e1417* form of the site. Blocking *nhr-25* expression using RNAi causes defects in vulval development but does not affect *lin-3* expression in the AC, suggesting that NHR-25 in other cells is important for vulval development and that NHRs other than NHR-25 are necessary for *lin-3* expression in the AC. Blocking *hlh-2* expression using RNAi causes defects in vulval development and also affects *lin-3* expression in the AC, suggesting that *hlh-2* is required for the expression of *lin-3* in the AC.

Materials and methods

General methods and strains

C. elegans strains were handled, maintained and crossed following standard protocols (Brenner, 1974). Experiments were conducted at 20°C unless otherwise indicated. Cell anatomy was observed with Nomarski optics and GFP expression was observed using a Chroma High Q GFP LP filter set (450 nm excitation/505 nm emission) in a Zeiss Axioplan microscope. Photographs were taken with a digital camera and Improvision Openlab software.

Transgenic lines were generated using a standard microinjection protocol (Mello et al., 1991). Each *gfp* construct (100 ng/μl) and a rescue plasmid (50 ng/μl) (pBX, pMH 86 or pDP#MM016B) were co-injected into *pha-1; him-5*, *dpy-20* or *unc-119* animals. After injection, transgenic animals were obtained by growing at 20°C (*pha-1*) or by rescuing a Dpy or Unc phenotype (*dpy-20* or *unc-119*).

The wild-type strain used in this study is *C. elegans* var. Bristol strain N2. The following mutant strains of N2 were used: *dpy-20(e1282), unc-119(ed4), pha-1(e2123ts); him-5(e1490), dpy-20(e1282) syIs49[zmp-1::gfp; dpy-20(+)]* and *lin-3(e1417)*. Information about these alleles can be found through WormBase (http://www.wormbase.org).

Sequence analysis of *lin-3* genomic region

To identify the *lin-3(e1417)* mutation, 11.4 kb of *lin-3* genomic region including 6 kb of upstream sequence was amplified from N2 and *lin-3(e1417)* animals using PCR. The PCR products were directly sequenced using several internal DNA sequencing primers and the dideoxy chain termination method with a ABI PRISM cycle sequencing kit (Applied Biosystems, Foster City, CA), and the DNA sequencing chromatograms of N2 and *e1417* were compared. When ambiguous sequence differences were observed, sequencing was repeated with another sequencing primer or using the other strand as a template.

Construction of *lin-3::gfp* enhancer assay reporters

lin-3::*gfp* constructs containing different lengths of *lin-3* were prepared by fusing *gfp* after the transmembrane domain of an inactive form of *lin-3*, in which nucleotides encoding two cysteine residues in the EGF domain were changed to those encoding serine residues (Hill and Sternberg, 1992). Two of the constructs contain either 10 kb or 3 kb of 5′ upstream sequences from the first *lin-3* exon. The other two contain either 4 kb or 0.2 kb of 5′ upstream sequences from the putative second promoter in the fourth intron of *lin-3*. The 59 bp of the ACEL (Anchor cell-specific enhancer of *lin-3*) DNA fragments were PCR-amplified from N2 and *lin-3(e1417)* genomic DNA, and the PCR products were cloned into a Δ*pes-10::gfp* enhancer assay vector (Fire et al., 1998). PCR amplification was also used to generate deletion and site-directed mutations in the ACEL. All of the mutations were confirmed by DNA sequencing.

Preparation of recombinant proteins

The entire open reading frames of *hlh-2* and *luciferase* were N-terminally tagged with a FLAG epitope by insertion into a pCMV-Tag2 vector (Stratagene, La Jolla, CA). The FLAG-cDNAs were then inserted into a pFastBacHT vector (Life Technologies, Gaithersburg, MD) in which a $(His)_6$ tag is located at the N terminus to facilitate rapid purification of recombinant proteins. Recombinant proteins were expressed in Sf9 insect cells and purified using nickel agarose according to the manufacturer's instructions (Life Technologies). Purified proteins were stored at –80°C in aliquots.

The NHR-25 proteins were synthesized by transcribing α- and β-*nhr-25* cDNAs in pCMV-Tag2 vectors from a T3 promoter with T3 RNA polymerase and then translating the mRNA in rabbit reticulocyte lysates in the presence of ^{35}S-methionine or cold methionine according to the manufacturer's instructions (Promega Madison, WI).

The proteins were analyzed by SDS-PAGE and immunoblotting as described previously (Hwang et al., 1999). Briefly, proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA), and probed with mouse anti-FLAG immunoglobulin G (IgG) antibodies (1:1000 dilution) and then with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (1:1,000 dilution; Vector Laboratories, Burlingame, Calif.). Antibody binding was detected by enhanced chemiluminescence (ECL reagents; Amersham, Little Chalfont, Buckinghamshire, UK).

Electrophoretic mobility shift assay

The DNA fragment that contains one E-box near the 5′ end, the other E-box in the middle, and a FTZ-F1 binding site between the two E-boxes, was used as the wild-type ACEL probe. The three binding sites were systematically mutated in other probes. Each probe was labeled with ^{32}P-α-dCTP and Klenow DNA polymerase as described (Hwang et al., 1999). Two different buffers were used to form protein-DNA complexes: buffer A (12 mM HEPES, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 4 mM Tris-HCl, 0.6 mM EDTA, 1 mM DTT, 12% (v/v) glycerol) for HLH-2; and buffer B (10 mM HEPES, pH 7.7, 100 mM NaCl, 2 mM DTT, 12% glycerol) for NHR-25.

The binding reaction was initiated by adding proteins in 10 μl of a reaction mixture that contains 1 ng of ^{32}P-labeled probe, 1 μg of BSA and 1 μg of poly (dI-dC)-(dI-dC) to mask the effects of non-specific DNA-binding proteins. After incubating on ice for 30 minutes, the mixture was resolved by non-denaturing polyacrylamide gel electrophoresis in 0.3×TBE at 4°C, and the gel was dried and exposed to a phosphoimager. Anti-FLAG (M2; IBI-Kodak, New Haven, Conn.) antibodies were used in a mobility supershift assay of protein-DNA complex as described previously (Hwang et al., 1999). Briefly, proteins were pre-incubated on ice for 10 minutes with the FLAG antibodies, incubated on ice for 30 minutes with DNA probe, and then resolved by nondenaturing gel electrophoresis.

RNAi experiments

RNAi was performed by soaking synchronized animals in *hlh-2* or *nhr-25* dsRNA solutions (Tabara et al., 1998). RNA was synthesized in vitro using a Ambion MEGAscript kit (Ambion, Austin, TX). Equal amounts of sense and anti-sense strand RNA were denatured at 80°C for 5 minutes, mixed and slowly cooled to room temperature to generate dsRNA.

Animals were grown on 10 cm special NGM plates (Brenner, 1974; but with peptone at 2% (w/v) and cholesterol at 20 mg/l). When most animals were young gravid adults they were collected and treated with hypochlorite. Eggs were then allowed to hatch in M9 and transferred to special NGM plates after 17 to 22 hours (Lewis and Fleming, 1995).

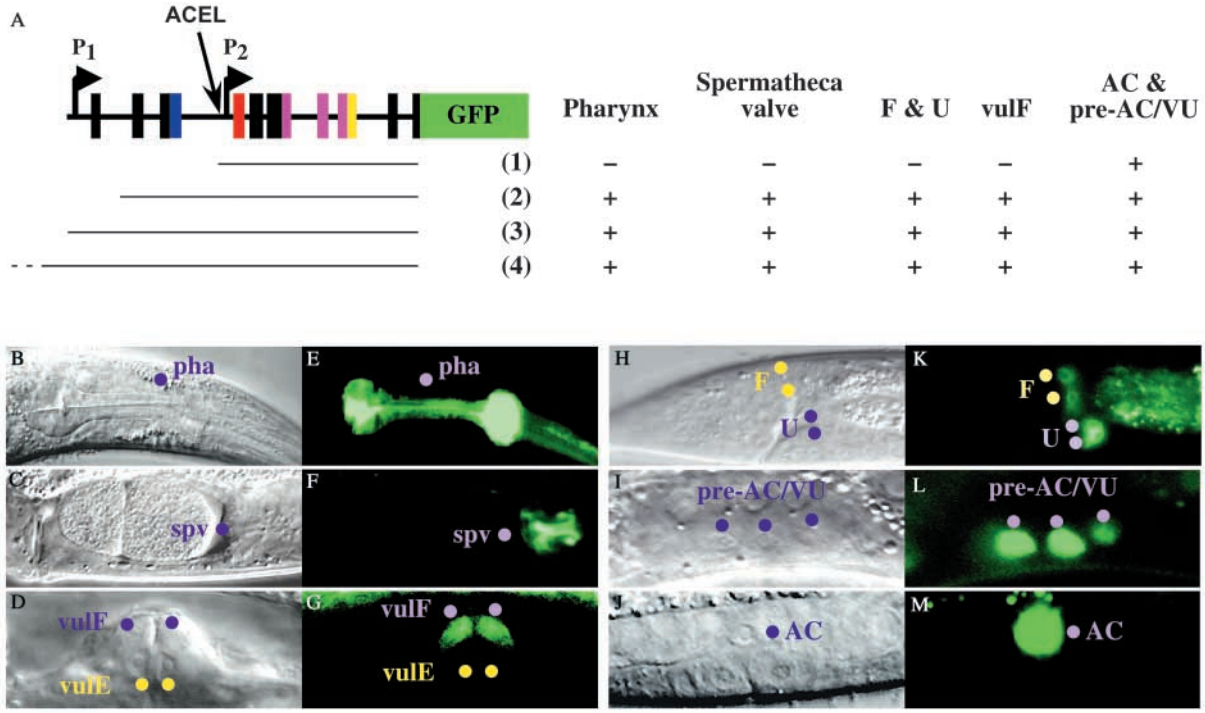

Fig. 1. Spatial and temporal expression pattern of *lin-3*. (A) Different extents of 5′ noncoding and coding regions of *lin-3* were cloned into a *gfp* reporter construct as described in the Materials and methods. Construct 1 begins near the 3′ end of the fourth intron, construct 2 begins from the 3′ half of the second *lin-3* intron, and constructs 3 and 4 contain the first intron and the first promoter. The two arrows indicate *lin-3* promoters; blue and red boxes represent two alternative signal peptides right after translational initiation codons; purple boxes are exons encoding an EGF domain; and yellow box represents a domain that exists only in one form of *lin-3* and is created by alternative splicing. The arrow from ACEL (anchor cell-specific enhancer of *lin-3*) marks the position of an enhancer element that drives the expression of *lin-3* in the AC. Cells expressing *lin-3::gfp* were summarized as –, no expression; and +, expression. (B-M) The expression of *lin-3::gfp* in pharynx (pha), spermatheca valve (spv), a subset of the primary vulval cells (vulF), male tail (F and U cells), and in the anchor cell (AC) and the pre-anchor (AC)/ventral uterine precursor cells (VU). (B-D,H-J) Nomarski images; (E-G,K-M) corresponding images of *lin-3::gfp* expression.

Synchronized L1 animals were harvested from the plates at different stages. The larvae were then either soaked immediately in *hlh-2* or *nhr-25* dsRNA solutions, or grown on regular plates for 10-16 hours and then soaked in the dsRNA solutions. After the soaking, animals were transferred to regular plates and phenotypes were scored at several developmental stages.

Results

Expression pattern of *lin-3::gfp*

As the first step towards understanding the molecular mechanisms underling the regulation of *lin-3* gene expression, we expressed LIN-3::GFP fusion proteins in which different extents of the 5′ noncoding and coding regions of *lin-3* were fused to *gfp* (Fig. 1A). The longest construct, containing 10 kb of 5′ upstream region from the first *lin-3* exon, expresses *lin-3::gfp* in pharynx; spermathecal-uterine junction core cells and later in the spermatheca valve; pre-anchor (AC)/ventral uterine precursor (VU) cells and later in the anchor cell in the somatic gonad; vulF cells of the 1° vulval lineage cells; and F, U and some of the B progeny cells in the male tail (Fig. 1B-M). This expression pattern was not affected by the different genetic backgrounds (*dpy-20*, *pha-1* and *unc-119*) rescued by the corresponding co-injected rescue plasmids, implying that the *gfp* expression pattern is established by the *lin-3* regulatory region. LIN-3 expression in different cells was temporally distinct as well. Expression in the pharynx was observed throughout post-embryonic stages. Spermathecal-uterine junction core cells, which later form the spermatheca valve, started expressing *lin-3::gfp* at the late L3 larval stage. As previously described using a *lin-3::lacZ* reporter (Chang et al., 1999), *lin-3::gfp* was transiently expressed in the vulF, but not in the vulE, cells of the 1° vulval lineage during the early and mid L4 stages. In the male tail, *lin-3::gfp* is expressed in the F and U cells from the L2 stage and in some B cell descendants at later stages. As described with the *lin-3::lacZ* reporter (Hill and Sternberg, 1992), during the early L3 stage when VPCs are induced by *lin-3* from the AC, *lin-3::gfp* is expressed only in the AC in somatic gonad.. Interestingly, *lin-3::gfp* was expressed in the pre-AC/VU cells at the mid to late L2 stages (Fig. 1) but the expression disappeared in the VU cells after AC/VU cell fate determination, leaving the AC as the sole source for LIN-3.

The region from the 3′ half of the second intron to near the 3′ end of the fourth intron is necessary to express *lin-3* in all of the above cells (Fig. 1A, compare constructs 1 and 2). The putative second *lin-3* promoter, which was identified by the isolation of a *lin-3* transcript encoding a polypeptide with an alternative N terminus, is located at the 3′ end of the fourth intron (Liu et al., 1999). Deletion of the upstream region of the second promoter eliminated *lin-3::gfp* expression in the cells described above except in the AC and the pre-AC/VU cells (Fig. 1A, construct

1). Thus, the region that controls *lin-3* expression in the AC can be separated from the region that controls the expression in other cells, and an enhancer that allows *lin-3* to be expressed in the AC may be located near the second promoter.

A point mutation in *lin-3* abolishes its expression in the AC

As the *lin-3(e1417)* mutation causes only a defect in vulval development and the molecular lesion of the *lin-3(e1417)* allele was not identified in the coding region of *lin-3* (Liu et al., 1999), we searched for the *e1417* mutation in the non-coding region of *lin-3*, expecting that the *e1417* mutation might lead to the identification of an AC-specific enhancer element for *lin-3*. We compared genomic DNA sequences of *lin-3* from wild-type and *e1417* mutant animals, and identified a G to A change located just 5′ to the second promoter (labeled ‘G to A’ in Fig. 2A). The *lin-3::gfp* construct that expresses GFP only in the AC (Fig. 1A, construct 1) also includes the region of the ‘G to A’ change. The 5′ end of the *lin-3* sequence in the *lin-3::gfp* construct 1 was 54 bp upstream from this lesion. Both findings suggested that an enhancer for the AC-specific expression of *lin-3* is located just 5′ to the second promoter.

To further dissect this putative AC-specific *lin-3* enhancer region, we compared the DNA sequence of the region from *C. elegans* with that from *C. briggsae*, expecting that important *cis*-acting elements in the enhancer might be conserved in these nematodes, which have similar patterns of vulval formation (Delattre and Felix, 2001). As shown in Fig. 2B, DNA sequence identity is much higher within exons than within this intron. However, a region in the intron is also highly conserved. The conserved region extends 59 bp in *C. elegans* and 54 bp in *C. briggsae* (cacctg…cacaggtgtt’ in Fig. 2B), and contains putative binding sites for basic helix-loop-helix (bHLH) transcription factors (‘caggtg’), POU homeodomain proteins (‘tattnnatgc’), and FTZ-F1 nuclear hormone receptor (NHR) proteins (‘tcaaggtca’). The *e1417* mutation is located in the predicted FTZ-F1 binding site.

To test the potential for this conserved region to function as an AC-specific enhancer, wild-type and *e1417* forms of the putative enhancer region (59 bp in *C. elegans*) were fused with a Δ*pes-10::gfp* enhancer assay vector (Fig. 2A). The Δ*pes-10::gfp* vector itself expresses *gfp* in cells near the tail, thus providing a way to identify transgenic animals containing extra-chromosomal arrays. When the wild-type form of the putative enhancer was fused with the Δ*pes-10::gfp*, 83% of the animals expressed *gfp* in the AC. By contrast, *gfp* expression in the AC was not observed in any of the animals that contain extrachromosomal arrays of either Δ*pes-10::gfp* itself (15 animals) or the *e1417* form of the putative enhancer fused with Δ*pes-10::gfp* (33 animals) (Fig. 2A). Therefore, this 59 bp region, ACEL (AC-specific enhancer of *lin-3*), is an AC-specific enhancer, and the *e1417* change of G to A in this region inactivates the enhancer activity.

E-box and FTZ-F1 nuclear hormone receptor binding sites in the ACEL are necessary for *lin-3* expression in the AC

Deletion and site-directed mutagenesis studies suggest that three *cis*-acting elements in the ACEL are necessary to express *lin-3::gfp* in the AC (Fig. 3). Mutation of the FTZ-F1 binding site (*e1417* mutation) as well as deletions on either side of the

A

ACEL	% of worms expressing GFP in the AC
–	0 (15)
wild type	83 (24)
‘G to A’	0 (33)

B

```
CB: ttacatct..atagttggaa.cacctg..caatttatgctgccata
    |  | |||   || ||  |  |||||   | |||||||||
CE: t..c.tctccCta.ttcaatgcacctgtgtattttatgctg.....

caggattt..gtgtgaccctga........t.cacaggtgttctcgctta
  |  |||   ||||||||||          ||||||||||    ||||
..gttttttcttgtgaccctgaaaactgtacacacaggtgtt....ctta

gaaatttcccgcaagacatt....gaaaattac..caaagaaagtactgt
  ||| || |   || ||||   |||||  ||     ||||||  | | |
ccaatgtctc...aggcatttttggaaaagtaatattaagaaa..attat

aca.attgccaaggcttctt..atatttcttttaactactttcatg..aa
||| |||        |||||  |||  |    ||  | |||       |
acatatt........ttcttgaata...c...gaa.aaattt......a.

gaATGTCTC.GTTATTCGGTTCCAGAACGACTT.TTGATTGCATTTG
 ||||| || || | ||| |||| ||||||||| ||| | ||||||||
.aATGT.TCGGTAAATCGATTCCTGAACGACTTCTAG.TCGCATTTG
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Fig. 2. The anchor cell (AC)-specific enhancer element of *lin-3*. (A) The wild-type, but not ‘*G to A*’, form of ACEL can activate Δ*pes-10::gfp* transcription in the AC. A base change from guanine (G) to adenine (A) was identified in the *lin-3(e1417)* allele (‘*G to A*’). The wild-type and ‘*G to A*’ forms of the putative ACEL elements (59 bp) were cloned into a Δ*pes-10::gfp* enhancer assay vector. The number in parenthesis represents the number of animals in which *gfp* expression in the AC was examined. (B) Conservation of the ACEL sequence between *C. briggsae* (CB) and *C. elegans* (CE). The intron sequence (3′ part of the fourth intron of *lin-3*) is in lower case letters and the exon sequence (5′ part of the fourth exon of *lin-3*) is in upper case letters (purple underline). The *e1417* mutation, indicated with a dot, is located in the conserved region of the FTZ-F1 NHR binding site, underlined in green. E boxes are underlined in red. Binding sites of POU homeodomain proteins are underlined in blue. The unbroken blue line indicates the site conserved in both species; the broken blue line represents the site that exists only in *C. elegans*.

FTZ-F1 binding site eliminated *gfp* expression in the AC (Fig. 3A). Consistent with the deletion analysis, mutation of the E-box (‘CACCTG’ to ‘CACCAA’) on either side of the ACEL impaired the ability of the ACEL to express *gfp* in the AC (Fig. 3B, constructs 9 and 11). Thus, both of the E-boxes on either side of the FTZ-F1 binding site were necessary to express *lin-3::gfp* in the AC. By contrast, site-directed mutagenesis of the predicted POU binding sites did not affect the AC-specific expression (Fig. 3B, constructs 2-7). Furthermore, RNAi against all three POU homeodomain genes in *C. elegans* (*ceh-6*, *ceh-18* and *unc-86*) did not affect the *lin-3::gfp* expression in the AC or interfere with vulval induction (data not shown). Thus, we exclude the possibility POU proteins from playing a role in the AC-specific *lin-3* expression. Mutations in other non-conserved regions did not affect the *lin-3::gfp* expression in the AC (Fig. 3B).

HLH-2, ACEL and vulval development

Both E-boxes in the ACEL consist of the sequence motif, ‘CACCTG’. This represents the major binding sequence for E-protein/Daughterless, which can form homo- or heterodimers

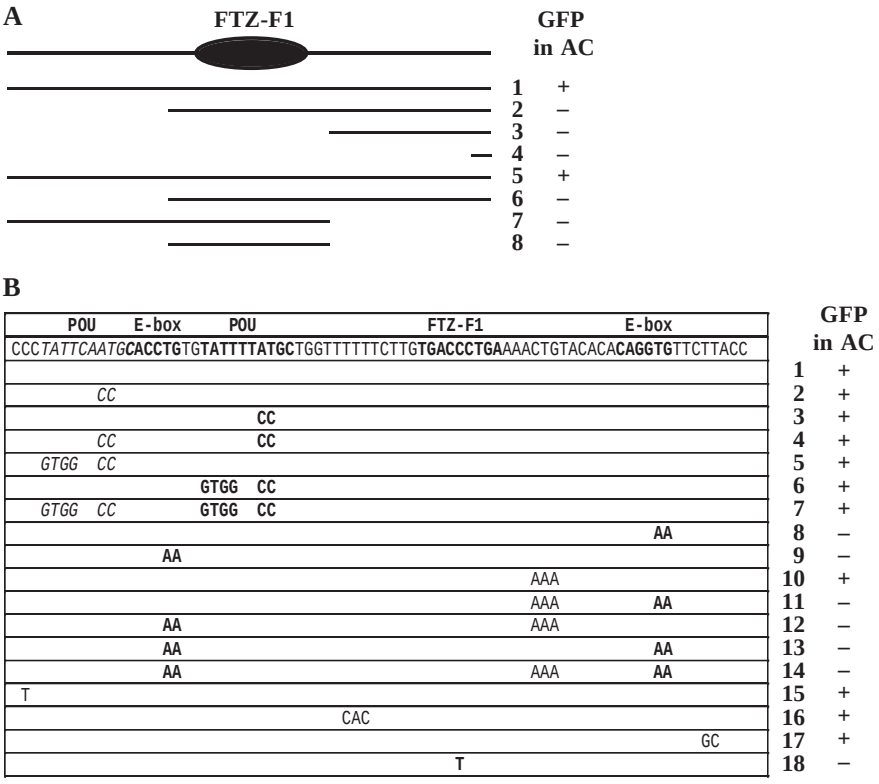

Fig. 3. Deletion and site-directed mutagenesis analyses of ACEL. (A) Deletion analysis of ACEL. Deletions from the 5′ end of the ACEL were made with the endogenous *lin-3* promoter (constructs 1-4). Deletions from the 5′ or the 3′ end of the ACEL were generated by PCR and the DNA fragments were fused with a Δ*pes-10::gfp* enhancer assay vector (constructs 5-8). (B) Site-directed mutagenesis analysis of ACEL. Mutations in the ACEL were generated using PCR and the PCR products were cloned into the Δ*pes-10::gfp* vector. Construct 1 has no mutations and the others (2-18) have changes in the ACEL as indicated. For each construct, about 30 animals that express *gfp* in the tail were examined for *gfp* expression in the AC.

with other bHLH proteins (Massari and Murre, 2000; Ohsako et al., 1994). The *C. elegans* ortholog of E-protein/Daughterless, *hlh-2*, is expressed in the AC (Karp and Greenwald, 2003), which suggested that HLH-2 protein may bind to E-boxes in the ACEL and activate *lin-3* expression in the AC. To test this hypothesis, we purified FLAG tagged-HLH-2 protein, with FLAG tagged-Luciferase protein as a control, using a baculovirus system and performed an electrophoretic mobility shift assay (EMSA) (Fig. 4). FLAG-HLH-2 protein bound to the DNA probes containing at least one intact E-box (Fig. 4C, lanes 2-4) but not to the probe in which both E-boxes were mutated (lane 5). FLAG-Luciferase did not bind to the ACEL DNA probe in the EMSA (lane 1). Thus, HLH-2 binds to both E-boxes in the ACEL. The FLAG-HLH-2 protein/DNA probe complex was supershifted by adding anti-FLAG antibodies, confirming the presence of FLAG-HLH-2 protein in the complex (lanes 6-8). The complex with the first mutated probe (probe B in Fig. 4B) migrated faster than the complex with the second mutated probe (probe C in Fig. 4B), as indicated by a comparison of the migrations of B (FLAG-HLH-2 protein-DNA probe complex) in lanes 3 and 4, and in lane 7 versus 8 (Fig. 4C). As only one wild-type E-box is in the near 5′ end of probe B and in the middle of probe C, the mobility difference probably reflects a hydrodynamic difference of the complexes because of the DNA bending upon binding of HLH-2 to the DNA probe (Crothers et al., 1991; Kahn and Crothers, 1993). Less of the HLH-2 protein-DNA complex was detected with probes containing only one E-box than with the probe containing two E-boxes (compare B and Supershift in lanes 2 and 6 with those in lanes 3, 4, 7 and 8 in Fig. 4C). Therefore, the HLH-2 complex with the probe containing two E-boxes appears to be more stable in an EMSA than the complex with the probes that have only one wild-type E-box. Consistent with this observation, we also observed dissociation of the complex during gel electrophoresis (Fig. 4C).

As there is no null mutant of *hlh-2*, and RNAi against *hlh-2* causes embryonic lethality (Fraser et al., 2000; Krause et al., 1997), we examined the phenotypes of animals in which *hlh-2* expression was blocked at post-embryonic stages by soaking larval stages of animals in *hlh-2* dsRNA solution. In this and subsequent RNAi experiments, animals expressing *lin-3::gfp* in the pharynx were considered for the analysis. When L1 larvae expressing *lin-3::gfp* were soaked in the dsRNA solution (n>100), all of the animals showed severe defects in gonadal development, did not develop vulvae, were sterile, and did not have any ACs as judged by morphological criteria under Nomarski optics and by lack of the expression of AC markers (Inoue et al., 2002). Thus, the *hlh-2* RNAi with L1 larvae could not conclusively show that the defect in vulval induction was due to the lack of *lin-3* expression in the AC.

To circumvent the effect of *hlh-2* RNAi on early gonadal development, we carried out the RNAi experiments with L2 and L3 animals. Animals treated with *hlh-2* dsRNA at these later stages showed normal gonadal morphology and were fertile. However, mid- to late L2 stage animals soaked in the dsRNA induced a partial vulvae (Fig. 5F) and did not show *lin-3::gfp* expression in the gonad. As *hlh-2* RNAi was initiated at the stages in which *lin-3::gfp* was already expressed in the AC, the incomplete vulval induction most probably reflects a functional reduction of *lin-3* expression in the AC (roughly similar to ablation of the AC during vulval induction). When the *hlh-2* RNAi was performed with early L3 stage animals, 45% (17/38) of the animals induced VPCs normally but showed defects in the vulval-uterine connection (Fig. 5G), 18% (7/38) induced a partial vulvae, and 37% induced a normal vulvae and vulval-uterine connection. About 40% (6/15) of the animals with normal vulval induction showed little or no expression of *lin-3::gfp* in the AC (Fig. 5B), suggesting that the AC-specific *gfp* expression had been eliminated by *hlh-2* RNAi after vulval induction. In control RNAi experiments, only one out of 1 more than 100 animals examined did not

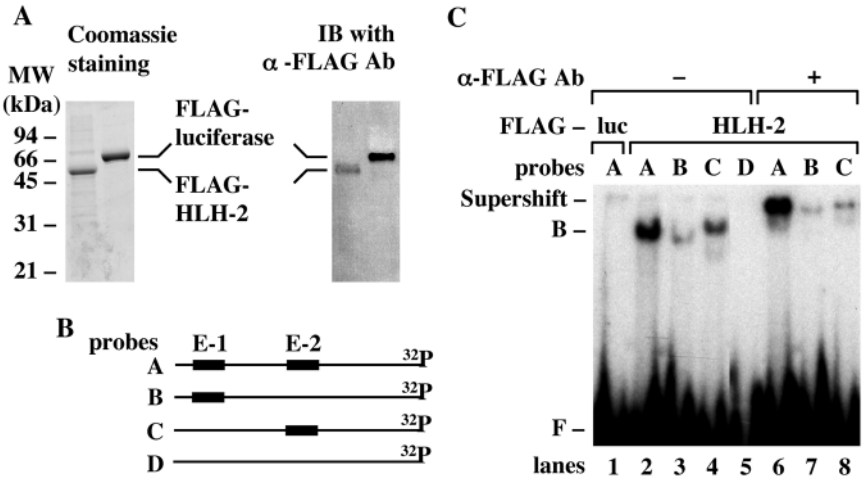

Fig. 4. HLH-2 binds to both E-boxes in the ACEL. (A) Synthesis and purification of FLAG-Luciferase and FLAG-HLH-2 proteins. Both proteins were expressed in insect cells using a baculovirus expression system and then purified using Ni^2-columns. The purified proteins were resolved on an SDS-PAGE gel for Coomassie staining and immunoblotting with anti-FLAG antibodies. (B) Probe A contains both E-boxes, probes B and C contain only one E-box, and probe D does not contain any E-boxes. The probes contain an extra 70 bp at the 3′ end of the ACEL. Thus, the first E-box (E-1) is near the 5′ end of the probe and the second E-box (E-2) is in the middle of the probe. (C) An EMSA that shows the binding of the HLH-2 protein to both E-boxes in the ACEL. The purified proteins were incubated with the wild-type and the mutated DNA probes of the ACEL (Fig. 3B). F indicates the migration of free DNA probe, B is the FLAG-HLH-2 protein/DNA probe complex, and Supershift is created by adding anti-FLAG antibodies (lanes 6 to 8).

express *lin-3::gfp* in the AC, suggesting a low frequency of the mosaic loss of *lin-3::gfp* expression in the AC. Taken together, the *hlh-2* RNAi experiments demonstrate that the continuous expression of *hlh-2* is necessary to maintain the expression of *lin-3* in the AC.

NHR, ACEL and vulval development

FTZ-F1, a member of the NHR superfamily, binds to a nine base pair consensus sequence (Lavorgna et al., 1991). As shown in Fig. 6A, eight base pairs in the predicted FTZ-F1 binding site of the ACEL are identical to the consensus sequence of known FTZ-F1 binding sites. The nonconsensus G to A in the fourth position of the ACEL has also been observed in other NHR-binding sites (Segraves, 1991). The *lin-3(e1417)* mutation is located in this predicted FTZ-F1 binding site (Fig. 2B). The *e1417* mutation changes the conserved G at the fifth position to A, a substitution not observed in other bona fide FTZ-F1 binding sites.

The *nhr-25* gene, the *C. elegans* FTZ-F1 ortholog, is expressed in the AC (Gissendanner and Sluder, 2000). Based on its expression in the AC and its DNA-binding specificity, we predicted that NHR-25 binds to the ACEL. Two different forms of *nhr-25* transcripts have been detected (Asahina et al., 2000; Gissendanner and Sluder, 2000). The α-form contains an intact DNA binding domain and the β-form has a partially deleted DNA-binding domain (Fig. 6B,C). As shown in an EMSA, the α-form bound to the wild-type ACEL DNA probe (lane 4, Fig. 6D), but not to the *e1417* form of the probe (lane 8). The β-form bound to neither probe (lanes 3 and 7). Thus, NHR-25 indeed binds to the AC-specific enhancer of *lin-3*, and the *e1417* mutation impairs this binding.

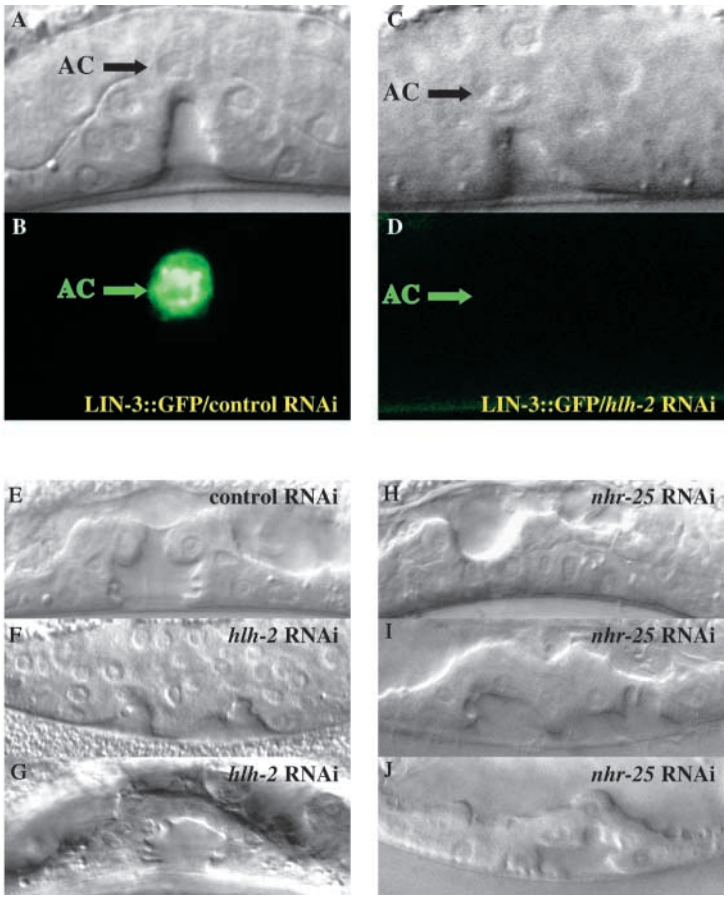

Fig. 5. The *hlh-2* and *nhr* genes are involved in vulval induction and the expression of *lin-3::gfp* in the AC. (A-D) Defective *lin-3::gfp* expression in the AC was caused by soaking animals in the dsRNA solution against *hlh-2*. Transgenic animals that contain *lin-3::gfp* extrachromosomal arrays were soaked in the control dsRNA solution (RNA synthesized from blank vectors) and in the dsRNA solution against *hlh-2*. (B,D) GFP expression in the AC was examined in the animals that express *gfp* in pharynx at the early L4 stage. (E-G) Defects in vulval induction and vulval-uterine connection in animals treated with dsRNA against *hlh-2*. (E) Wild-type vulvae with control RNAi. (F) Defective vulval induction after *hlh-2* RNAi. (G) Defective vulval-uterine connection after *hlh-2* RNAi. (H-J) Defects in vulval induction and vulval-uterine connection in animals treated with dsRNA against *nhr-25*. (H) Defective vulval induction after *nhr-25* RNAi. (I,J) Defective vulval-uterine connection after *nhr-25* RNAi.

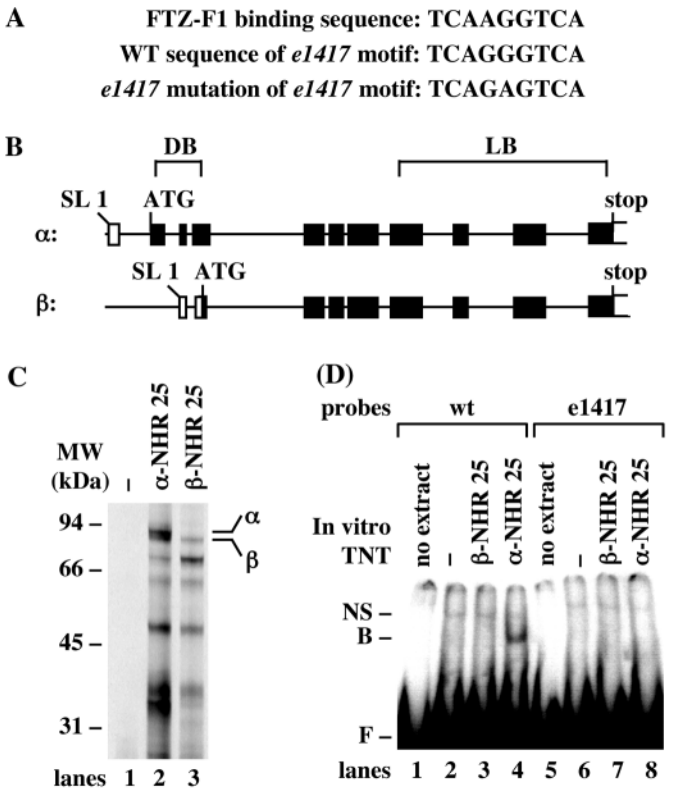

Fig. 6. NHR-25 binds to the wild-type, but not the *e1417*, form of ACEL. (A) A cis-element in the ACEL, which contains the *e1417* mutation site, is similar to the FTZ-F1 binding site consensus. (B) Two forms (α- and β-) of *nhr-25* cDNA. DB represents a DNA binding domain and LB a ligand-binding domain. Both messages are trans-spliced with SL1 RNA. The α-form contains an intact DNA-binding domain and the β-form partially deletes the domain. (C) Synthesis of both forms of the NHR-25 protein. The proteins were synthesized using in vitro transcription and translation (TNT) in rabbit reticulocyte lysates with ^{35}S-methionine. The proteins were visualized by autoradiography after SDS-PAGE. (D) EMSA showing the binding of NHR-25 to the wild-type ACEL DNA probe. The α- and β- NHR-25 proteins, which were synthesized using in vitro TNT with cold methionine, were incubated with the ^{32}P-labeled wild-type (wt) or *e1417* form of ACEL DNA probes. The reaction mixtures were separated on a non-denaturing polyacrylamide gel and the radioactivity signals were detected by phosphoimager. F indicates the migration of free DNA probes, NS indicates the migration of a non-specific protein/DNA probe complex, and B is the NHR-25/DNA probe complex. An equal amount of non-specific protein/DNA probe complex (NS) was observed in all of the binding reactions, regardless of the synthesis of NHR-25, showing that equal amounts of the reticulocyte lysates were used for the binding assay.

To determine whether *nhr-25* is the NHR necessary to express *lin-3* in the AC, we performed a RNAi experiment similar to that performed with *hlh-2* as the null mutant of *nhr-25* is also not viable (Asahina et al., 2000). RNAi with L1 larvae resulted in a severe defect in gonadal development and a complete failure to induce vulvae. RNAi using later stages of animals caused defects in vulval induction and in the vulval-uterine connection (24/32 animals) similar to the RNAi against *hlh-2* (Fig. 5H-J). However, *nhr-25* RNAi did not eliminate *lin-3::gfp* expression in the AC, even in the animals that had defects in vulval induction. We cannot rule out the possibility that *nhr-25* RNAi slightly decreases the *lin-3::gfp* expression. Failure of the *nhr-25* RNAi to eliminate the LIN-3::GFP signal in the AC can be interpreted in two different ways. First, the NHR-25 protein in the AC may be too stable to be completely eliminated by RNAi. Alternatively, as there are about 270 *nhr* genes in the *C. elegans* genome, another NHR protein(s) may be involved in expressing *lin-3* in the AC (Sluder and Maina, 2001). Because *nhr-25* is also expressed in the VPCs (Asahina et al., 2000; Gissendanner and Sluder, 2000) and its null mutant has not been rescued, most probably because NHR-25 plays an important role in germ line development (Asahina et al., 2000), we cannot determine by mosaic analysis whether the defects in vulval induction by *nhr-25* RNAi result from a partial reduction of *lin-3* expression in the AC or from inhibiting a function of NHR-25 in the VPCs.

Discussion

Regulated expression of the EGF ligand, LIN-3, in *C. elegans*

The expression pattern of *lin-3* suggests that its precise spatial and temporal expression is a major way of activating EGF signaling in specific cells in *C. elegans*. The *lin-3* gene is expressed in the cells known as sources of the induction signal for the EGF receptor signaling pathway: the AC for vulval development (Hill and Sternberg, 1992), the male F and U cells for developing male spicules (Chamberlin and Sternberg, 1994), and the vulF cells of the 1° vulva to specify the fate of uv1 cells (Chang et al., 1999). The *lin-3* gene is also expressed in the spermatheca in which the LIN-3/LET-23 EGF receptor signaling pathway regulates ovulation (Bui and Sternberg, 2002; Clandinin et al., 1998). The functional significance of *lin-3* expression in the pharynx has not yet been determined. However, as expression in the pharynx has been observed for several downstream genes of the EGF signaling pathway, including the EGF receptor, *let-23* (Baylis et al., 1999; Clandinin et al., 1998; Dent and Han, 1998), it is likely that *lin-3* is involved in an unidentified process in the pharynx. The germline expression of *lin-3* was detected by RNase protection assay but not by the *lin-3::gfp* transgene, probably because the transgene became silenced in the germ cells (B.J.H. and P.W.S., unpublished). The *lin-3* expression in the germ line and/or in the pharynx may be required for animal viability (Ferguson and Horvitz, 1985).

The spatially and temporally regulated cell-specific expression of the EGF ligand *lin-3* reflects an activation mechanism for the EGF receptor signaling pathway in *C. elegans* which is distinct from that observed in *Drosophila*. In *Drosophila*, the activation of EGF receptor signaling is regulated by cleavage of the ligands in specific cells rather than by cell-specific ligand expression (Freeman, 1997; Gabay et al., 1997; Ghiglione et al., 2002; Urban et al., 2002). The main activating EGF ligand, Spitz, is expressed in most tissues during fly development (Rutledge et al., 1992), including all developing photoreceptors (Tio et al., 1994). However, its processing is tightly controlled by Rhomboid and Star proteins, expression of which is restricted to specific cells (Freeman et al., 1992; Heberlein et al., 1993).

AC-specific *lin-3* transcription

We have identified a 59 bp enhancer element (ACEL) that

directs *lin-3* expression in the AC, consisting of two E-boxes (bHLH protein binding sites) and one FTZ-F1 nuclear hormone receptor-binding site. We found that the *C. elegans* E-protein homolog, HLH-2, binds to the enhancer element to activate the *lin-3* transcription, and that NHR-25 also binds to the enhancer. E-protein/Daughterless proteins generally recognize target DNA sequences as a heterodimer with other bHLH proteins (Massari and Murre, 2000). However, we prefer the model that a HLH-2 homodimer activates *lin-3* transcription in the AC as purified HLH-2 proteins alone recognize the E-box (Zhang et al., 1999), and *hlh-2* is expressed in the AC but not in the VU cells (Karp and Greenwald, 2003). The *nhr-25* gene is expressed in the AC (Gissendanner and Sluder, 2000), and its protein binds to the wild-type form but not to the *e1417* form of the NHR binding site in the ACEL (Fig. 6). However, *nhr-25* appears not to be the NHR that activates *lin-3* transcription in the AC as RNAi against *nhr-25* did not eliminate *lin-3::gfp* expression in the AC. About 270 *nhr* genes were predicted in *C. elegans* and most of them are not pseudogenes (Sluder and Maina, 2001); this contrasts with 21 *nhr* genes in *Drosophila* and 50 in human (Sluder and Maina, 2001). All of the *C. elegans* NHRs are orphan receptors for which ligands have not been identified, but evidence indicates the presence of unidentified ligands such as steroids, metabolic intermediates and external materials from the environment (Sluder and Maina, 2001). Furthermore, although the amino acid sequences of the ligand-binding domains in *C. elegans* NHRs are evolutionarily less conserved than those of the DNA-binding domains (Clarke and Berg, 1998), structural modeling indicates that many of the *C. elegans* ligand binding domain sequences are compatible with the X-ray crystal structures of the known ligand-binding domains (Francoijs et al., 2000), suggesting they may bind to ligands.

The two transcriptional regulatory activities necessary for *lin-3* expression in the AC (Fig. 7) might reflect distinct regulatory inputs that program the appropriate time, place and level of *lin-3* expression. The presence of a NHR-binding site in the ACEL makes it conceivable that unidentified NHR ligand(s) responding to physiological conditions and environments might control vulval development by activating *lin-3* expression in the AC. As *lin-12*/Notch signaling is involved in the fate determination of the AC (Seydoux and Greenwald, 1989; Wilkinson et al., 1994), it is interesting to know how or whether the *lin-12* signaling is coupled to the AC-specific *lin-3* expression. Several pieces of evidence suggest such a coupling. The expression pattern of *lag-2*, a *lin-12* ligand that is involved in the AC/VU cell fate determination, overlaps with that of *lin-3* in the somatic gonad. The *lag-2* gene is also expressed in the pre-AC/VU cells before the cell fate determination, but only in the AC after the fate determination (Wilkinson et al., 1994). It was shown that this kind of *lag-2* expression pattern is established by the interaction between *lag-2* and *lin-12* during the AC/VU cell fate determination (Seydoux and Greenwald, 1989; Wilkinson et al., 1994). As the AC-specific *lin-3* expression is established at the time of the cell fate determination, it is likely that *lin-12* signaling is also involved in establishing the AC-specific *lin-3* expression. The mechanism that establishes the *lag-2* or *lin-3* expression in the AC is not understood well, but interestingly a well-known Notch downstream pathway, which is involved in the specification of sensory organ precursors in the *Drosophila* peripheral nervous system, involves the binding of bHLH proteins to E-boxes (Heitzler et al., 1996; Kunisch et al., 1994; Parks et al., 1997). Therefore, the findings that E-boxes in the ACEL are necessary for the AC-specific *lin-3* expression and that the expression patterns of *lag-2* and *lin-3* overlap in the somatic gonad suggest that a similar kind of Notch downstream pathway may exist to specify *lag-2* or *lin-3* expression in the *C. elegans* AC.

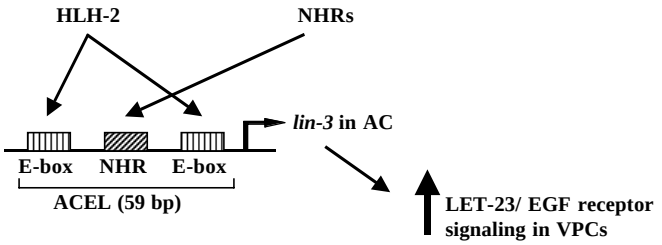

Fig. 7. A model for the AC-specific *lin-3* expression. The AC-specific expression of *lin-3*, which is essential to induce proper fates of VPCs, is established via an enhancer element (ACEL, 59 bp) that contains two E-boxes and one FTZ-F1 NHR binding site. NHRs activate *lin-3* transcription via the FTZ-F1 NHR binding site in the ACEL, which is the site of *lin-3(e1417)* mutation. HLH-2 binds to both E-boxes in the ACEL and activates the transcription of *lin-3* in the AC, which activates the EGF signaling pathway in the VPCs.

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