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

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Acceptance: 23 September 2003

Development 131, 143-151

Published by The Company of Biologists 2004
doi:10.1242/dev.00924

Summary

During C. elegans vulval development, the anchor cell (AC) in the somatic gonad expresses lin-3, activating the EGF receptor signaling pathway in vulvar 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 vulvar 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 Daughtercless, 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 NRHs other than NHR-25 are necessary for lin-3 expression in the AC. Blocking hhh-2 expression using RNAi causes defects in vulval development and also affects lin-3 expression in the AC, suggesting that hhh-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, pMHI 86 or pDP#MM106B) 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) syls49[mp1::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 dyeoxy 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 pApex-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 hhh-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 S9 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 35S-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 32P-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 MgCl2, 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 32P-labeled probe, 1 µg of BSA and 1 µg of poly (dl-dC)-(dl-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 phosphorimager. 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 non-denaturing gel electrophoresis.

RNAi experiments

RNAi was performed by soaking synchronized animals in hhh-2 or nhr-25 dsDNA 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/ml). 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).
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 underlying 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 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.

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.

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...
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 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...caagctgt ‘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 Δpes-10::gfp enhancer assay vector (Fig. 2A). The Δpes-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 Δpes-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 Δpes-10::gfp itself (15 animals) or the e1417 form of the putative enhancer fused with Δpes-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 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 on 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).

FTZ-F1, 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
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 FLAGHLH-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 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 vulva 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
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. 6](image-url)

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) Defective vulval induction after nhr-25 RNAi. (I,J) Defective vulval-uterine connection after nhr-25 RNAi.
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 totally 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 vul1 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; Cladnin 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; Cladnin 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 (Francois 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.

We are grateful to X. Karp and I. Greenwald for communicating unpublished data on *hhli-2*; to Y Kohara for *nhr-25* CDNA; and to A. Fire for *gfp* constructs. We thank J. Lee and H. Y. Yoon in W. Dunphy’s laboratory for helping with phosphomimager analysis and expressing proteins in insect cells. Great appreciation for carefully reading the manuscript is given to C. Bastiani, B. Gupta, T. Inoue, Y. Kee, J. S. Kim, N. Moghal and S. Verneyo. This work was supported by the Howard Hughes Medical Institute with which P.W.S. is an investigator and B.J.H. was an associate.

References


