REF-1, a protein with two bHLH domains, alters the pattern of cell fusion in 
*C. elegans* by regulating Hox protein activity

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**SUMMARY**

Hox genes control the choice of cell fates along the anteroposterior (AP) body axis of many organisms. In *C. elegans*, two Hox genes, *lin-39* and *mab-5*, control the cell fusion decision of the 12 ventrally located Pn.p cells. Specific Pn.p cells fuse with an epidermal syncytium, hyp7, in a sexually dimorphic pattern. In hermaphrodites, Pn.p cells in the mid-body region remain unfused whereas in males, Pn.p cells adopt an alternating pattern of syncytial and unfused fates. The complexity of these fusion patterns arises because the activities of these two Hox proteins are regulated in a sex-specific manner. MAB-5 activity is inhibited in hermaphroditic Pn.p cells and thus MAB-5 normally only affects the male Pn.p fusion pattern. Here we identify a gene, *ref-1*, that regulates the hermaphroditic Pn.p cell fusion pattern largely by regulating MAB-5 activity in these cells. Mutation of *ref-1* also affects the fate of other epidermal cells in distinct AP body regions. *ref-1* encodes a protein with two basic helix-loop-helix domains distantly related to those of the *hairy/Enhancer of split* family. *ref-1*, and another *hairy* homolog, *lin-22*, regulate similar cell fate decisions in different body regions along the *C. elegans* AP body axis.

Key words: *C. elegans*, *ref-1*, Hox genes, Cell fusion, *hairy*, E(spl)

**INTRODUCTION**

How individual cells interact to coordinate the formation of complex tissues is a fundamental biological question. Much of the *C. elegans* epidermal layer, the hypodermis, is composed of several multinucleate cells (syncytia) that are formed by the fusion of mononucleate cells throughout embryonic and postembryonic development (Podbielwicz and White, 1994; Shemer and Podbielwicz, 2000; Sulston and Horvitz, 1977). One such syncytium, hyp7, extends over most of the length of the worm and contains 133 nuclei, close to 15% of all somatic nuclei in the worm. How is the fusion of all these cells coordinately regulated to allow formation of hyp7? Genes that affect fusion of epidermal cells during *C. elegans* development include those that provide temporal information (heterochronic genes; Ambros and Horvitz, 1984; Bettinger et al., 1997), determine polarity of certain cell divisions (such as *egl-20*, *lin-44* and *cam-1*; Forrester et al., 1999; Herman and Horvitz, 1994; Whangbo et al., 2000) and control anteroposterior (AP) cell fate (genes of the *C. elegans* Hox cluster). In this study, we focus on genes that regulate cell fusion in a position-specific manner along the AP body axis.

To understand how the hyp7 syncytium is generated, we have been studying the regulation of the fusion decision of one group of cells called the Pn.p cells that line the ventral surface of the worm during the first larval stage (L1) (Fig. 1A). Pn.p cell fusion is regulated by two genes of the *C. elegans* Hox gene cluster. The Hox cluster consists of six genes: *ceh-13*, *lin-39* and *mab-5*, homologs of *Drosophila* labial, Sex combs reduced and Antennapedia, respectively, and *egl-5*, *php-3* and *nob-1*, three *Abdominal-B* homologs (Brunschwig et al., 1999; Chisholm, 1991; Clark et al., 1993; van Auken et al., 2000; Wang et al., 1993). In *C. elegans*, as in other organisms, the Hox genes regulate the choice of cell fates along the AP body axis (Krumlauf, 1994; Lawrence and Morata, 1994; McGinnis and Krumlauf, 1992). However, the simple Hox gene expression pattern in *C. elegans* is insufficient to explain the complex Pn.p cell fusion pattern. This is due to the sex-specific, post-translational regulation of two Hox genes, *lin-39* and *mab-5* (Salser et al., 1993). In hermaphrodites, MAB-5 is inactive and only LIN-39 influences Pn.p cell fusion fate. In males, both LIN-39 and MAB-5 are active, but the two proteins interact in an unusual way to control cell fusion. It is quite likely that in most species, Hox proteins interact with each other and with other factors to generate more complexity than their expression patterns alone would allow (Duncan, 1996). Understanding how these interactions modulate Hox protein activity is therefore necessary to understand fully how an animal body plan is laid out.

At the end of the first larval stage, some of the 12 Pn.p cells fuse with the hyp7 syncytium in a sex-specific pattern. In hermaphrodites, anterior (P1.p and P2.p) and posterior P(9-11).p cells fuse with the hyp7 syncytium while the six central cells P(3-8).p remain unfused (Fig. 1B; Sulston and Horvitz, 1977). These six unfused cells, the vulval precursor cells, remain competent to develop further, and some of these cells
generate the hermaphrodite vulva later in development (Sulston and Horvitz, 1977; Sulston and White, 1980). The Pn.p cell fusion pattern is different in males, with P1.p, P2.p, P7.p, and P8.p fusing with hyp7 and P(3-6).p and P(9-11).p remaining unfused (Fig. 1C; Maloof and Kenyon, 1998; Pn.p cell fusion. lin-39 is expressed in P(3-8).p in both hermaphrodites and males (Fig. 1; Maloof and Kenyon, 1998; Wang et al., 1993). In hermaphrodites, lin-39 prevents fusion of those Pn.p cells in which it is expressed and therefore P(3-8).p remain unfused (Fig. 1B; Clark et al., 1993; Wang et al., 1993). Thus, in a lin-39 mutant, all hermaphrodite Pn.p cells fuse with the hyp7 syncytium and are unable to generate a vulva. The regulation of Pn.p cell fusion in males is more complex because both lin-39 and mab-5 can affect the fusion decision. mab-5 is expressed in P(7-11).p in both sexes, but only functions in males (Salser et al., 1993; Wang et al., 1993). Acting alone, either Hox gene is able to prevent fusion of those cells within which it is expressed: P(3-6).p for lin-39 and P(9-11).p for mab-5 (Fig. 1C). However, when cells express both Hox genes (P7.p and P8.p), those cells fuse with hyp7, much like cells that contain neither Hox gene (P1.p and P2.p) (Fig. 1C). The ability of these two Hox genes to negate each other’s effects in males occurs post-translationally (Salser et al., 1993). That is, LIN-39 and MAB-5 proteins can somehow inhibit each other’s activity when both proteins are present in the same cell. Moreover, the relative levels of the two proteins do not matter because the two proteins are still capable of inhibiting each other when one of the Hox genes is strongly overexpressed (Salser et al., 1993). This result argues against a model in which the two Hox proteins simply sequester each other and, as a consequence, titrate each other’s activity. Instead, something else appears to be limiting in this cell fate decision. One possibility is that both proteins bind to regulatory sites in the same target gene, which in turn encodes a protein that directly affects cell fusion. In this model, the binding of either protein alone influences the activity of the fusion gene, whereas the binding of both Hox proteins together does not.

In summary, Hox protein activity is regulated in two key ways to control the Pn.p cell fusion decision. First, MAB-5 is present in the same cells in both sexes but only functions in male Pn.p cells. Thus, something keeps MAB-5 inactive in the hermaphrodite Pn.p cells. Second, both Hox proteins can interact to inhibit each other when present in the same Pn.p cell in males.

To identify genes that affect Pn.p cell fusion by regulating Hox protein activity, we isolated mutations that alter the Pn.p cell fusion pattern. One such mutation, ref-1(mu220) (REGulator of Fusion-1) prevents fusion of posterior Pn.p cells in hermaphrodites, largely, but not completely, by affecting the sex-specific activity of MAB-5. ref-1 mutants also exhibit a defect in the specification of the fate of a hypodermal cell located on the lateral surface of the worm in this same posterior body region as well as other defects in the anterior part of the worm. We have cloned ref-1 and determined that it encodes a transcription factor with two basic helix-loop-helix (bHLH) domains, both of which are distantly related to the hairy Enhancer of split [E(spl)] subfamily of such proteins.

**MATERIALS AND METHODS**

**Worm strains and construction of ref-1; Hox double mutants**

Standard worm culture and genetic techniques were used (Brenner, 1974; Wood, 1988). All experiments were carried out at 20°C. Strains used in this study [alleles referenced in Hodgkin, 1997, or as described below] are: N2 wild-type var. Bristol, CF121: unc-4(e120) blt-1(e769) II, SP635; mndF66/mnc1 dpy-10(e128) unc-52(e444) II, SP619; mndF57/mnc1 dpy-10(e128) unc-52(e444) II, SP719; mndF83/mnc1 dpy-10(e128) unc-52(e444) II, SP542; mndF52/mnc1 dpy-10(e128) unc-52(e444) II, SP629; mndF59/mnc1 dpy-10(e128) unc-52(e444) II, SP645; mndF63/mnc1 dpy-10(e128) unc-52(e444) II (Sigurdson et al., 1984), CF262: lin-39(n1760)/qC1 dpy-19(e1259) glp-1(q339) III, him-5(e1490) V, CF911: mab-5(e2088) III, TU202: egl-5(mu203) II, CF398: lin-39(n1760) mab-5(e1239)/qC1 dpy-19(e1259) glp-1(q339) III, CF311: mab-5(e1239) egl-5(n945) III, him-5(e1490) V (Chisholm, 1991), MT7238: lin-39(n1760) mab-5(e1239) egl-5(n945)/smg-3(e491) mab-5(e1239) egl-5(n945) III (from A. Chisom), CB3256: mab-5(e1751) III, MT668: lin-12(n137)/unc-32(e189) III, him-5(e1467) V, CF260: lin-2(mu2) IV, lin-1(e490) V, him-5(e1490) V, and the STS mapping strain RW7000 (Williams et al., 1992). The him-5 mutation causes worms to generate male progeny at high frequency (Hodgkin et al., 1979).

To construct strains containing both ref-1(mu220) and the various Hox single, double, and triple mutations, ref-1(mu220) was crossed to the various Hox mutant strains, and animals homozygous for the mu220 allele were first recovered in the F2 descendants (either by using the misshapen head phenotype or the ectopic pseudovulval phenotype in a lin-12(n137+)/+ background). The animals were then stained with the MH27 monoclonal antibody (see below) to confirm that they were homozygous for mu220. Animals homozygous for the Hox mutations were subsequently identified among the progeny of these mu220 homozygotes (using the Egl phenotype for lin-39 and egl-5 and the QL descendant migration defect for mab-5). All Hox mutant alleles used are predicted to be null alleles by genetic, DNA sequence and/or immunofluorescence criteria (Chisholm, 1991; Clark et al., 1993; Ferreira et al., 1999; Salser and Kenyon, 1996; Wang et al., 1993). Animals heterozygous for the Hox mutations were subsequently identified among the progeny of these mu220 homozygotes (using the Egl phenotype for lin-39 and egl-5 and the QL descendant migration defect for mab-5) because the two proteins are still capable of inhibiting each other when one of the Hox genes is strongly overexpressed (Salser et al., 1993). That is, LIN-39 and MAB-5 proteins can somehow inhibit each other’s activity when both proteins are present in the same cell. Moreover, the relative levels of the two proteins do not matter because the two proteins are still capable of inhibiting each other when one of the Hox genes is strongly overexpressed (Salser et al., 1993). This result argues against a model in which the two Hox proteins simply sequester each other and, as a consequence, titrate each other’s activity. Instead, something else appears to be limiting in this cell fate decision. One possibility is that both proteins bind to regulatory sites in the same target gene, which in turn encodes a protein that directly affects cell fusion. In this model, the binding of either protein alone influences the activity of the fusion gene, whereas the binding of both Hox proteins together does not.

In summary, Hox protein activity is regulated in two key ways to control the Pn.p cell fusion decision. First, MAB-5 is present in the same cells in both sexes but only functions in male Pn.p cells. Thus, something keeps MAB-5 inactive in the hermaphrodite Pn.p cells. Second, both Hox proteins can interact to inhibit each other when present in the same Pn.p cell in males.

**Screen for Pn.p cell fusion mutants**

To identify mutations affecting Pn.p cell fusion, lin-12(n137?); him-5(e1467) young adults were mutagenized with 50 mM EMS for 4 hours. F2 progeny were picked to individual plates and F3 and F4 progeny were screened for extra pseudovulvae. One of the mutants isolated was ref-1(mu220), which often generated extra pseudovulvae in the posterior body region. The ref-1; lin-12 animals failed to mate as either males or hermaphrodites, probably because of mating defects present in lin-12 animals (Greenwald et al., 1983). In order to outcross the ref-1 mutant, these animals were further treated with a second dose (1.25 mM) of EMS. Progeny of mutagenized worms occasionally laid eggs. Animals on plates containing eggs were transferred repeatedly to fresh plates for several generations, at which point worms containing a putative lin-12(n137) suppressor had overrun the population. These animals mated successfully. The lin-12 suppressor identified was tightly linked to lin-12, as the Lin-12 phenotype was not observed during outcrossing. All characterization of ref-1 described in this paper, with the exception of the lineages described in Fig. 4, was carried out in a ref-1(mu220) strain that had been outcrossed three times and that was genotypically wild-type for lin-12.

**Characterization of ref-1(mu220) phenotypes**

Animals were stained with the monoclonal antibody MH27 to determine whether Pn.p cells had fused with hyp7 or remained
unfused (Francis and Waterston, 1991; Kenyon, 1986). Staged populations of animals were stained in early L2 after the lateral V cells had divided 2-3 times (about 16 hours for wild-type worms at 20°C; somewhat longer for worms carrying several mutations). P12.p behaves differently than the anterior Pn.p cells and thus was not scored for cell fusion. To determine if the H1 seam cell was fused, newly hatched animals were stained with MH27. Staining with the LIN-39 antiserum was carried out as described by Maloof and Kenyon (Maloof and Kenyon, 1998). Analysis of the V5 and V6 cell lineages, which was performed to observe ectopic V6 postdeirid formation, was carried out from late L1 just prior to the second V cell division to the middle of L2 after postdeirid production appeared to be complete. The ectopic postdeirid phenotype was only about 10% penetrant in a strain that was outcrossed three times; however it was somewhat more penetrant (30%) in a strain outcrossed only once, suggesting that there may be a modifier of this phenotype in the mutagenized background. Hermaphrodite alae and male ray production were scored in young adults. The Fisher Exact Test (determined using Statview Software, SAS) was used to calculate P values for all experiments.

Mapping and cloning ref-1

Both the Pn.p cell fusion and ectopic V6 postdeirid ref-1 phenotypes were found to be recessive; the Pn.p cell fusion defect was assayed in mapping experiments. ref-1 was first mapped using STS mapping (Williams et al., 1992) between two polymorphisms, stp36 and map1, and was tightly linked to a third polymorphism, stp98, on the center of LG II. Chromosomal deficiencies were then used to refine the map position. The Pn.p cell fusion defect was generated when ref-1(mu220) was placed in trans to the deficiencies mnDf66, mnDf57, mnDf83 and mnDf29 but not mnDf59 and mnDF63. Three factor mapping was then used to place ref-1 between unc-4 and bli-1. Transformation rescue of the Pn.p cell fusion phenotype was obtained with pools of cosmids located in this region. Ultimately, rescuing activity of the Pn.p cell fusion phenotype was positioned on a single cosmid, T01E8. This cosmid also completely rescued the ectopic postdeirid and misshapen head phenotypes. A candidate open reading frame for ref-1 identified by the C. elegans sequencing project was T01E8.2, which encodes a putative transcription factor. A 10.9 kb AvrII-Ng004V subclone (pSA139) encompassing this gene plus 7.0 kb upstream and 2.5 kb downstream sequence was injected into ref-1(mu220) mutants. Injection of the T01E8 cosmid at concentration >10 ng/μl or of this subclone at concentration >3 ng/μl induced strong embryonic lethality, suggesting that misexpression and/or overexpression of this gene was toxic. 3/3 lines generated with this 10.9 kb subclone injected at 3 ng/μl gave approximately 50% rescue of the Pn.p fusion phenotype (n>300 all lines, P<0.0001). Control injection of an otherwise identical DNA construct lacking the coding sequence (NcoI deletion-pSA155) injected at 50 ng/μl completely failed to rescue 9/9 lines. One of the lines rescued with the 10.9 kb subclone was tested for rescue of other ref-1(mu220) phenotypes. The misshapen head phenotype was rescued in 89% of the animals (3/322 with array, 54/465 without array, P<0.0001) and the V6 postdeirid phenotype was rescued in 48% of worm sides (11/232 with array, 39/442 without array, P=0.063). The formation of the wild-type V5 postdeirid was also prevented at low frequency (7/232 worm sides) in the rescued lines, suggesting that a small amount of misexpression or overexpression of wild-type ref-1 was capable of inhibiting postdeirid production in V5 as well as V6.

To confirm that ref-1 was T01E8.2, double stranded RNA was generated from a full length cDNA clone (see below) and injected into the gonad of wild-type hermaphrodites. Such RNA interference (RNAi) (Fire et al., 1998) induced grossly misshapen heads (15% of animals, n=98) and ectopic V6 postdeirids (4% of animals, n=116) in progeny of injected parents.

To identify the molecular lesion in ref-1(mu220), we isolated and sequenced cDNA clones generated by RT-PCR (Frohman, 1993) from wild-type and ref-1(mu220) DNA. The oligo(dT) primer was used to obtain the 3’ end and the spliced leader sequence SL1 primer to obtain the 5’ cDNA end. Only a single isoform was isolated from several clones; it exactly matched the Genefinder predicted intron/exon structure. A single point mutation was identified in cDNA products derived from ref-1(mu220) DNA (see Results).
had more than six pseudovulvae in a lin-12(n137) background (see Materials and Methods). One such mutant, ref-1(mu220), frequently had between one and three ectopic pseudovulvae in the posterior body region (compare Fig. 2A with B). To determine whether these ectopic pseudovulvae corresponded to extra unfused Pn.p cells, ref-1(mu220) worms were stained with MH27, a monoclonal antibody that labels a component of adherens junctions and thus outlines unfused cells (Francis and Waterston, 1991; Kenyon, 1986). P9,p and P10,p sometimes (and P11,p rarely) remained unfused in ref-1(mu220) animals (Fig. 2C,D and Table 1). Anterior Pn,p cells (P1,p and P2,p) and other lateral hypodermal cell fusions in the worm were largely unaffected, suggesting that this mutation affects the pattern of Pn,p cell fusion and not cell fusion more generally. ref-1 also did not affect the male Pn,p cell fusion pattern (data not shown).

The ref-1(mu220) mutation was recessive, suggesting that the mutation likely reduces or eliminates gene activity (also see below). Moreover, when mu220 was placed in trans to a chromosomal deficiency, the mu220 phenotype was not enhanced, suggesting that mu220 is a strong loss-of-function (lf) allele. Thus we infer that the wild-type function of ref-1 is to allow the posterior Pn,p cells to fuse correctly in hermaphrodites. In wild-type worms, some Pn,p cells that remain unfused in L2 do fuse with hyp7 later in development (for example, P(3-6),p in males and P3,p in 50% of hermaphrodites). Likewise, in a lin-12(+) background, the 'unfused' posterior Pn,p cells in ref-1(mu220) remained unfused through L2 but did subsequently fuse with hyp7 by the time the vulval precursor cells divide in L3, as determined by MH27 staining. Thus, ref-1 specifically affects Pn,p cell fusion early but not later during larval development.

**MAB-5 is inappropriately active in the Pn,p cells of ref-1(mu220) hermaphrodites**

Because the pattern of Pn,p cell fusion is regulated by Hox genes, we tested if alterations in Hox gene expression or Hox protein activity were responsible for the Pn,p cell fusion phenotype in ref-1. We first tested whether the Pn,p cell fusion defect in the ref-1 mutant was due to misexpression of lin-39 in the posterior of the worm. We stained ref-1 mutant animals with an antiserum to LIN-39 (Maloof and Kenyon, 1998) and

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<th>Table 1. Posterior Pn,p cells remain unfused in ref-1(mu220) hermaphrodites</th>
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*C. elegans* early L2 larvae were stained with the MH27 antibody to score Pn,p cell fusion. Indicated are the percentage of Pn,p cells that remain unfused in the listed mutants. The number of animals scored is listed on the right.
found little or no detectable difference in *lin-39* expression between wild-type and *ref-1(mu220)* animals (Table 2). To confirm that misregulation of *lin-39* was not responsible for the *ref-1* Pn.p cell fusion defect, we constructed the *ref-1*; *lin-39* double mutant. Normally, all Pn.p cells fuse with hyp7 in a *lin-39* mutant (Table 1). If inappropriate expression of *lin-39* were responsible for the extra unfused cells in the *ref-1* mutant, then removal of *lin-39* should suppress the *ref-1* Pn.p cell fusion defect. However, in the *ref-1*; *lin-39* double mutant, not only did P(9-11).p often remain unfused but P7.p and P8.p did as well (Table 1), revealing a *ref-1* mutant phenotype that was masked when the wild-type *lin-39* gene copy was present. Thus, P(7-11).p could all remain unfused inappropriately in *ref-1(mu220)* mutants in a *lin-39*-independent fashion (although *lin-39* can play a role under other special conditions: see below). Interestingly, this region corresponds to the domain of *mab-5* expression (Salser et al., 1993; Wang et al., 1993), raising the possibility that MAB-5, which is normally inactive in hermaphrodite Pn.p cells, might be inappropriately preventing Pn.p cell fusion in the *ref-1(mu220)* hermaphrodites.

To investigate whether MAB-5 might be active in Pn.p cells of *ref-1* hermaphrodites, we used the gain-of-function mutation *mab-5(e1751)* to misexpress *mab-5* in all Pn.p cells. In particular, P1.p and P2.p, cells which do not normally express *lin-39*, *mab-5*, or *egl-5*, do express *mab-5* in a *mab-5(e1751)* background (Salser et al., 1993). In otherwise wild-type animals, *mab-5(e1751)* hermaphrodites still exhibit a wild-type Pn.p cell fusion pattern because MAB-5 is inactive in wild-type hermaphrodites (Table 3). However, in the *ref-1*; *mab-5(e1751)* strain, P1.p and P2.p often remained unfused (Table 3). This indicated that, in *ref-1* mutants, MAB-5 can function inappropriately in hermaphrodite Pn.p cells. To confirm that MAB-5 was affecting cell fusion in the *ref-1* mutant, we examined the Pn.p cell fusion phenotype of *ref-1*; *mab-5(lf)* double mutants (Table 3). In *mab-5(lf)* single mutants, P9.p and P10.p still fused with hyp7, as in wild type (Table 3). P11.p, however, is sometimes transformed into a copy of its posterior neighbor, P12.p (Kenyon, 1986). Thus, P11.p sometimes remained unfused (as determined by MH27 staining) in a *mab-5(lf)* mutant (Table 3). In the *ref-1*; *mab-5(lf)* double mutant, the severity of the *ref-1* P9.p and P10.p cell fusion defect was strongly reduced (by 60%, *P*<0.0001). Thus, inappropriate activation of MAB-5 was at least partially responsible for the Pn.p cell fusion defect in *ref-1* worms. However, P9.p and P10.p still sometimes remained unfused in this double mutant,
indicating that ref-1 also played another role in the regulation of Pn.p cell fusion.

**REF-1 and MAB-5 function redundantly to inhibit lin-39 expression in the posterior body region**

Although the addition of a lin-39 mutation to ref-1 mutant animals did not suppress the ref-1 Pn.p cell fusion defect (see above), the Pn.p cell fusion defect in ref-1; lin-39 mab-5 triple mutant animals was substantially weaker than in ref-1; lin-39 or ref-1; mab-5 double mutant animals ($P<0.0001$) (Table 4). Why does lin-39 appear to play a role in the ref-1 Pn.p cell fusion defect when a mab-5 mutation is present in the strain but not when it is absent? To resolve this question, we examined the lin-39 expression pattern in ref-1; mab-5 double mutant animals using a LIN-39 antisense. In addition to the Pn.p cells, many neurons lie along the ventral surface of the worm. lin-39 is expressed at high levels in P(3-8),p and in all ventrally located neurons that lie in that body region. Only relatively weak lin-39 expression is found in a few neurons posterior to P8,p in the ventral cord in either wild-type, ref-1, or mab-5 mutant animals (Table 2) and lin-39 is never expressed in the posterior Pn.p cells in these three strains. In contrast, more neurons express lin-39 at substantially higher levels in the posterior ventral cord in the ref-1; mab-5 double mutant (Table 2), indicating that the two genes function redundantly to inhibit posterior lin-39 expression. Moreover, at low frequency (approximately 4% of animals), weak levels of lin-39 were detected in P9,p or P10,p in the ref-1; mab-5 double mutant animals. The ref-1; lin-39 mab-5 Pn.p cell fusion data suggest that lin-39 is affecting more than 4% of the animals; a low level of lin-39 undetectable by our antisera may be responsible for this (undetectable levels of lin-39 are sufficient to prevent Pn.p cell fusion in egl-27 mutant animals; Ch’ng and Kenyon, 1999). Thus, in addition to its primary role of preventing MAB-5 from affecting Pn.p cell fusion, ref-1 also functions with mab-5 to prevent lin-39 expression in the posterior body region, thereby also allowing posterior Pn.p cells to fuse.

**egl-5 can also affect Pn.p cell fusion in ref-1 mutant animals**

A few Pn.p cells still remain unfused in the ref-1; lin-39 mab-5 triple mutant. To test if other Hox genes besides lin-39 or mab-5 might influence Pn.p cell fusion, we examined the effect of an egl-5 mutation in a ref-1 background. egl-5 is expressed in P12,p and causes it to adopt a fate distinct from the other Pn.p cells (Chisholm, 1991). However, in an otherwise wild-type background, egl-5 does not affect the other 11 Pn.p cells. The introduction of an egl-5 mutation into ref-1 animals caused a 35% decrease in the P9,p and P10,p cell fusion defect ($P=0.0001$) (Table 4) suggesting that egl-5 is also partially responsible for keeping posterior Pn.p cells unfused in the ref-1 mutant. Consistent with this possibility, the Pn.p cell fusion defect in the ref-1;mab-5 egl-5 triple mutant was substantially weaker than in ref-1; mab-5 or ref-1; egl-5 animals alone (Table 4) ($P<0.0001$). However, a small percentage of posterior Pn.p cells still remained unfused in this triple mutant. egl-5 is not normally expressed in the cells affected by ref-1 (Ferreira et al., 1999), and we found no detectable change in egl-5 expression in ref-1 mutant animals using an egl-5::lacZ fusion. Perhaps a low level of egl-5 that is undetectable by this reporter construct is misexpressed in ref-1 mutant animals. egl-5 is
misexpressed more anteriorly in mab-5 mutant worms (Ferreira et al., 1999). However, any egl-5 that is misexpressed in a mab-5 background does not affect Pn.p and P10.p cell fusion in ref-1(+) worms (Table 4). This suggests that ref-1 might affect EGL-5 activity as well as egl-5 expression.

The Pn.p cell fusion pattern in animals lacking all three of these Hox genes, lin-39, mab-5 and egl-5, was very similar to that of the ref-1; lin-39 mab-5 egl-5 quadruple mutant (P<0.99). Surprisingly, there are still unfused Pn.p cells in the Hox triple mutant. While it is unclear why some Pn.p cells remain unfused in this background (it could be due to an uncharacterized interaction between the three Hox genes or due to the severe sickness of the strain), the Pn.p cell fusion defect was similar whether or not the ref-1 mutation was present, suggesting that the ref-1 Pn.p cell fusion defect might be due entirely to alterations in the activity of these three Hox proteins.

The ref-1 mutant also has other defects in distinct AP body regions

ref-1 mutants also exhibit other defects that, unlike the Pn.p cell fusion defect, occur in both sexes. ref-1(mu220) larvae occasionally have misshapen heads. These head defects vary considerably, ranging from small notches or lumps in the side of the worm head to strongly bent heads (Fig. 3). Animals with severely bent heads move in an uncoordinated fashion. Gross defects such as those depicted in Fig. 3 occur in approximately 10% of ref-1 mutant animals although much more subtle head shape defects are more common.

In a newly hatched worm, ten seam cells are located along both lateral surfaces of the worm (from anterior to posterior: H0, H1, H2, V1-V6, T – Fig. 4, top). To further study the structure of the ref-1 mutant head, we stained newly hatched ref-1 mutant animals with the MH27 antiserum and found that the H1 seam cell occasionally fused inappropriately with either hyp7 or another head syncytial cell (data not shown). This defect may not be responsible for the misshapen head phenotype since some animals in which all seam cells were unfused still had misshapen heads.

Although posterior seam cells did not display any defects in newly hatched ref-1(mu220) mutants, a defect was present later in development in the posteriorly located V6 cell. Normally V5 is the only seam cell that generates a neuronal structure called a postdeirid (Fig. 4). At low frequency in ref-1(mu220) worms, V6 also generates a postdeirid-like structure (Fig. 4) indicating a partial transformation of V6 to V5. This lineage transformation of V6 to a V5-like fate is probably responsible for later defects observed in the posterior body region of ref-1 adults. Seam cells in adult hermaphrodites secrete a cuticular structure known as alae; small gaps were sometimes seen in the alae in the V6 body region in ref-1 mutants, consistent with a loss of part of the wild-type V6 cell lineage. In males, the V5, V6, and T seamscells on each side of the animal generate 9 copulatory structures called rays. ref-1 mutants occasionally lack two V-cell-derived rays. This phenotype is expected in animals in which cells in one branch of the V6 lineage generated a postdeirid instead of adopting their normal fate (generating cells that produce rays).

ref-1 encodes a protein with two bHLH domains that are distantly related to the hairy/E(spl) family

ref-1(mu220) was mapped to a 1.5 map unit region between unc-4 and bli-1 on the center of chromosome II and cloned by transformation rescue (see Materials and Methods). The cosmid T01E8 and subclones of this cosmid containing the predicted open reading frame T01E8.2 rescued the Pn.p cell fusion, misshapen head, and ectopic postdeirid defects of ref-1(mu220) worms. This open reading frame encodes a protein with two putative basic helix-loop-helix (bHLH) domains, suggesting that REF-1 protein functions as a transcription factor. RT-PCR products from wild-type and mu220 animals

![Fig. 6. Mutation of two C. elegans hairy homologs, ref-1 and lin-22, causes similar phenotypes in different body regions. At top is a schematic view of a worm (anterior to left) with seam cells indicated on the lateral surface and Pn.p cells indicated on the ventral surface. ref-1(mu220) phenotypes in black, lin-22 phenotypes in gray. ref-1 mutants have Pn.p cell fusion defects in the posterior region of hermaphrodites while lin-22 mutants have Pn.p cell fusion defects more anteriorly in males. Similarly, ref-1 mutants sometimes generate an ectopic postdeirid derived from V6 in the posterior while lin-22 mutants form ectopic postdeirids more anteriorly. Finally, ref-1 mutants, but not lin-22 mutants, have head defects that include fusion of the H1 seam cell and misshapen heads.](image-url)
were generated and sequenced (see Materials and Methods) to verify the predicted intron/exon structure for this gene and to identify the molecular lesion in the mu220 allele, a G→A transition that alters a conserved arginine in the first basic region (Fig. 5A,B). To verify that this open reading frame was ref-1, RNA interference (Fire et al., 1998) was carried out with double stranded RNA generated from a full length ref-1 cDNA clone. Injection of ref-1 dsRNA into wild-type hermaphrodites caused the formation of misshapen heads as well as ectopic V6 postdeirids in the progeny of injected animals (see Materials and Methods).

Both ref-1 bHLH domains are distant members of the hairy/E(spl) subfamily of such bHLH domains (Fig. 5B; a phylogenetic tree containing the first bHLH domain can be found in the on-line supplementary material from Ruvkun and Hobert, 1998). Both ref-1 bHLH domains lack a conserved proline that is normally found in the basic region of these family members (Fig. 5B; Wainwright and Ish-Horowicz, 1992). ref-1 also lacks an obvious orange domain that provides specificity in some hairy/E(spl) family members (Dawson et al., 1995). ref-1 does contain a FRPWE motif at its COOH terminus (Fig. 5A), which may be a variant of the COOH-terminal WRPW motif found at the end of nearly all hairy family members (Fisher et al., 1996; Paroush et al., 1994; Wainwright and Ish-Horowicz, 1992).

The presence of two bHLH domains in ref-1 is unusual and we have been unable to identify proteins with this structure in other organisms. However, we have identified a small family of such proteins (Fig. 5C and Ruvkun and Hobert, 1998) in C. elegans. All these bHLH domains are distant members of the hairy/E(spl) family that lack the conserved proline. None of these predicted proteins other than REF-1 contains a good WRPW-like motif at their putative COOH terminus. The functions of these other genes have not been determined.

DISCUSSION

To understand how the hyp7 syncytium is generated, we have been studying the regulation of the fusion decision of the Pn.p cells. Pn.p cell fusion is controlled by two genes of the C. elegans Hox gene cluster, lin-39 and mab-5 (Clark et al., 1993; Wang et al., 1993). The pattern of Pn.p cell fusion is more complex than the simple expression patterns of these Hox genes would allow because Hox protein activity is regulated in a sexually dimorphic manner (Salser et al., 1993). This is presumably accomplished by the interaction of these two Hox proteins with each other and also with other factors. In this work, we have identified one such factor, REF-1, that is required for proper regulation of posterior Pn.p cell fusion in hermaphrodites.

REF-1 alters Hox protein activity in hermaphrodites

One of the ways that AP diversity is generated in C. elegans Pn.p cells is by altering Hox protein activity in a sex-specific manner. MAB-5 normally only affects Pn.p cell fusion in males. In principle, this regulation could be accomplished by the direct interaction of genes in the sex determination pathway with MAB-5. For example, the zinc finger protein TRA-1, a downstream effector in the sex determination pathway (Hunter and Wood, 1990; Zarkower and Hodgkin, 1992), could bind directly to a cell fusion promoter and in doing so prevent MAB-5 from binding and affecting transcription in hermaphrodites. Alternatively, other unidentified factors could act either alone or with TRA-1 to modulate Hox protein function. This second model appears to be correct because REF-1 is such a factor. REF-1 affects sex-specific Hox protein activity in the Pn.p cells, but the ref-1 mutant has no other obvious sexual transformations. ref-1 may act either downstream or in parallel to the sex determination pathway to affect MAB-5 activity.

ref-1 interacts with mab-5 in two different ways to ensure that posterior Pn.p cells fuse properly. First, ref-1 acts negatively on MAB-5 to ensure that MAB-5 does not affect hermaphrodite Pn.p cell fusion. Second, ref-1 acts positively and redundantly with mab-5 to prevent lin-39 expression in posterior Pn.p cells, which also results in the proper fusion of these cells. In addition to its interactions with mab-5, ref-1 can also influence the ability of EGL-5 to regulate Pn.p cell fusion.

It is interesting to note that the male Pn.p cell fusion pattern is not altered in the ref-1 mutant. Since wild-type REF-1 normally affects MAB-5 activity, this suggests that ref-1 either

### Table 4. lin-39, mab-5 and egl-5 affect Pn.p cell fusion in ref-1(mu220)

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C. elegans early L2 larvae were stained with the MH27 antibody to score Pn.p cell fusion. Indicated are the percentage of Pn.p cells that remain unfused in the listed mutants. The number of worms scored is listed on the right. Some of the data in this Table are also presented in Tables 1 and 3. All Hox mutant alleles used are likely to be null, as described in Materials and Methods.

### Regulation of cell fusion in C. elegans

1801
is not expressed or is inactive in males. The fact that in males, LIN-39 and MAB-5 inhibit each other when expressed in the same cell raises a paradox: why doesn’t this mutual antagonism occur in ref-1 hermaphrodites where MAB-5 is now active? If the two proteins did inhibit one another in ref-1 hermaphrodites, then P7.p and P8.p would fuse occasionally. However, in ref-1 mutants, those cells always remain unfused. One possibility is that still another factor is required for the interaction between LIN-39 and MAB-5 and that factor also acts in a sex-specific manner.

**ref-1 and egl-27 have overlapping functions in the Pn.p cells**

REF-1 is required for complete inhibition of MAB-5 activity in hermaphrodites. However, not all Pn.p cells in the posterior remain unfused in ref-1(mu220) animals, raising the possibility that other factors may also be required for this inhibition (one caveat is that the mu220 allele is a missense mutation that may not be completely null). One such other factor is egl-27, which encodes a component of a chromatin remodeling complex (Ch’ng and Kenyon, 1999; Herman et al., 1998; Solari et al., 1999). egl-27 regulates Pn.p cell fusion in both sexes in several ways, including regulation of MAB-5 activity and regulation of the expression of lin-39, mab-5 and egl-5. In particular, posterior Pn.p cells remain unfused in egl-27 mutant hermaphrodites in part due to inappropriate activation of MAB-5. egl-27 is also required for the ability of LIN-39 and MAB-5 to inhibit each other in males. Thus it is possible that egl-27 and ref-1 act in either the same or parallel pathways to control different aspects of the Pn.p cell fusion decision.

**ref-1 encodes an unusual bHLH protein with some similarity to the Hairy/E(spl) family**

ref-1 encodes a protein with an unusual structure in that it contains two predicted bHLH domains. A family of such predicted proteins is present in the *C. elegans* genome. Interestingly, the first and second bHLH domains within a given protein tend to be less similar to each other than to the corresponding bHLH domain in other family members. This suggests that this family may have arisen from the duplication of a single gene in which the bHLH domain had already been duplicated. These bHLH domains are most similar to those of the hairy/E(spl) family bHLH domains, although they are substantially diverged. The bHLH domains in ref-1 are only about 30% identical to the bHLH domains of other hairy family members; in contrast, the bHLH domain of lin-22, another *C. elegans* hairy homolog, is 60% identical to other family members. ref-1 contains a FRPWE domain at its COOH terminus, a variant of the conserved WRPW domain found at the COOH terminus of hairy family members (Fisher et al., 1996; Paroush et al., 1994). This domain is used by hairy to recruit the corepressor protein Groucho (Fisher et al., 1996; Paroush et al., 1994). The *C. elegans* groucho homolog, unc-37, is also a corepressor in other cell fate decisions (Pflugrad et al., 1997), although unc-37(e262) does not affect hermaphrodite Pn.p cell fusion (data not shown). While this is not a null allele of unc-37 (null alleles are lethal), it raises the possibility that the FRPWE motif in REF-1 may no longer function in recruiting groucho.

Why does ref-1 contain two bHLH domains? bHLH proteins typically function as dimers with the HLH domains acting as a dimerization region and the basic region contacting DNA (Littlewood and Evan, 1998). Such dimers could be homodimers or heterodimers between different bHLH proteins. Some proteins lacking the basic domain act as repressors by sequestering partner bHLH proteins in a complex that does not bind DNA (Littlewood and Evan, 1998). hairy family members, in contrast, are often active repressors that recruit Groucho to inhibit transcription (Fisher et al., 1996; Paroush et al., 1994; Van Doren et al., 1994). Since the mu220 allele affects a conserved residue in the first basic region, it is likely that ref-1 functions by binding DNA and that at least the first bHLH domain is required for this interaction. The second bHLH domain, if functional, could interact with the first bHLH domain to regulate it or could interact with other bHLH proteins. The other *C. elegans* genes that encode proteins with two bHLH domains are candidate genes for this interaction.

**The phenotypes of ref-1 are similar to the phenotypes of lin-22 and other hairy/E(spl) genes**

The molecular identity of ref-1 is intriguing in light of the other ref-1 mutant phenotypes. In *Drosophila*, hairy acts as both a primary pair rule gene that specifies the fate of alternate segments and also as a regulator of neuron formation later in development (Ingham et al., 1985; Moscoso del Prado and Garcia-Bellido, 1984). Two *C. elegans* hairy homologs, ref-1 and lin-22, also affect cell fate and neuron formation; moreover, they both do so in distinct AP body regions (Fig. 6). For example, ref-1 mutants have a hermaphrodite Pn.p cell fusion defect in the posterior Pn.p cells (although ref-1 can influence Pn.p cell fate in more anterior Pn.p cells as revealed in a lin-39 or mab-5(e1751) background). In contrast, lin-22 mutants have a male-specific Pn.p cell fusion defect in more anterior Pn.p cells. In lin-22 mutants, P7.p and P8.p remain unfused during the first larval stage (data not shown). In addition, P(3-8).p all continue to remain unfused inappropriately and divide later in development (Fixsen, 1985). Like flies carrying a mutation in hairy, ref-1 and lin-22 mutants also generate ectopic neuroblasts. Specifically, ectopic postdeirids are generated by the posterior V6 lateral seam cell in ref-1 mutants and by the anterior V1-V4 seam cells in lin-22 mutants (Fixsen, 1985; Waring et al., 1992; Wrischnik and Kenyon, 1997). These phenotypes are consistent with a partial (in the case of ref-1) or complete (in the case of lin-22) transformation of the respective V cells into a V5-like cell fate. No other single mutation can cause the transformation of V6 to a V5-like fate, although the double mutant combination of lin-22 and pal-1 (a caudal homeobox transcription factor homolog) does so (Waring and Kenyon, 1991; Waring et al., 1992). We observed no synergy between lin-22 and ref-1. ref-1; lin-22 double mutant worms had phenotypes that were simply the summation of the phenotypes of the individual mutants (data not shown). This observation is consistent with the hypothesis that while ref-1 and lin-22 have similar functions, they act in distinct AP body regions.

ref-1 mutants have defects not found in lin-22 in still another AP body region, the head (Fig. 6). ref-1 mutants exhibit a misshapen head defect and also have defects in the specification of the proper fate of the anterior H1 seam cell. While hairy acts as a pair rule gene affecting the fate of every other segment in *Drosophila* (Ingham et al., 1985), in some sense, these two *C. elegans* hairy homologs are behaving more
like gap genes (Nüsslein-Volhard and Wieschaus, 1980) in that they are required for the specification of cell fates in distinct AP body domains. However, since these mutations affect a limited repertoire of phenotypes, it is also possible that ref-l affects cell fate in a cell-type-specific manner.

Role of ref-1 in Hox protein regulation and the generation of complexity along the AP body axis

The modulation of Hox protein activity allows the generation of a more complex AP body plan than the simple expression patterns of the Hox genes might allow. Several examples of Hox genes interacting with each other and with other factors have been described. One of the most extensively studied cases of Hox protein activity regulation is that of extradenticle and homothorax. extradenticle binds to several Drosophila Hox proteins and alters their affinity for different DNA binding sites (Chan et al., 1994; Peifer and Wieschaus, 1990; Pinsonneault et al., 1997; Rauskolb et al., 1993; Rauskolb and Wieschaus, 1994). homothorax regulates the nuclear localization of this protein complex (Pai et al., 1998; Rieckhoff et al., 1997).

Drosophila teashirt and an isofrom of cap ‘n’ collar can regulate the activity of the Hox genes Sex combs reduced and Deformed, respectively, although it is unclear if this effect is direct (Andrew et al., 1994; de Zuluetla et al., 1994; McGinnis and Ragnhildstveit, 1998; Veraksa et al., 2000). egf-27 and ref-l are both able to modulate MAB-5 activity in C. elegans in a cell-specific manner. Since they are both transcription factors, they could directly bind to a cell fusion promoter to affect the Pn.p cell fusion decision or they could act indirectly, by regulating the expression of other proteins that in turn directly modulate Hox protein activity. Either way, the result is that a relatively simple Hox gene expression code is elaborated into a more complex Pn.p cell fusion pattern.

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