Development 127, 5179-5190 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV6473

Overlapping roles of two Hox genes and the *exd* ortholog *ceh-20* in diversification of the *C. elegans* postembryonic mesoderm

Jun Liu and Andrew Fire*

Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, MD 21210, USA
*Author for correspondence (e-mail: fire@ciwemb.edu)

Accepted 20 September; published on WWW 2 November 2000

SUMMARY

Members of the Hox family of homeoproteins and their cofactors play a central role in pattern formation of all germ layers. During postembryonic development of *C. elegans*, non-gonadal mesoderm arises from a single mesoblast cell M. Starting in the first larval stage, M divides to produce 14 striated muscles, 16 non-striated muscles, and two non-muscle cells (coelomocytes). We investigated the role of the *C. elegans* Hox cluster and of the *exd* ortholog *ceh-20* in patterning of the postembryonic mesoderm. By examining the M lineage and its differentiation products in different Hox mutant combinations, we found an essential but overlapping role for two of the Hox cluster genes, *lin-39* and *mab-5*, in diversification of the postembryonic mesoderm. This role of the two Hox gene products required the CEH-20 cofactor. One target of these two Hox genes is the *C. elegans twist* ortholog *hlh-8*. Using both in vitro and in vivo assays, we demonstrated that *twist* is a direct target of Hox activation. We present evidence from mutant phenotypes that *twist* is not the only target for Hox genes in the M lineage: in particular we show that *lin-39 mab-5* double mutants exhibit a more severe M lineage defect than the *hlh-8* null mutant.

Key words: Hox, *mab-5, lin-39, ceh-20, Caenorhabditis elegans*, Mesoderm, *twist*

INTRODUCTION

Anterior-posterior patterns in animals arise from a combined consequence of cellular identities acquired in each of the three germ layers. Members of the Hox family of homeodomain proteins play a central role in this process (see Lewis, 1978; Lawrence and Morata, 1994; Krumlauf, 1994; Biggin and McGinnis, 1997 for review). Each of the three germ layers has a unique pattern of Hox expression, with the eventual pattern of tissues reflecting both autonomous Hox specification in each germ layer and interactions between germ layers (for example, see Bienz, 1994 for review). Two key paradoxes that have arisen in studies of Hox function concern (1) the relatively broad DNA binding specificity exhibited by Hox proteins in vitro, and (2) the ability of Hox proteins with very similar DNA binding properties in vitro to direct distinctive developmental patterns in vivo.

Recent studies have shown that the specificity of Hox factors is augmented in vivo by interaction with a distinctive group of homeodomain cofactors. These cofactors belong to the TALE (three amino acid loop extension) class of homeodomain proteins (Bürglin, 1997; Bürglin, 1998). One group of cofactors is represented by the *Drosophila* EXD and vertebrate PBX proteins (see Mann, 1995; Wilson and Desplan, 1995; Mann and Chan, 1996; Mann and Affolter, 1998 for review). Heterodimerization with EXD/PBX can greatly increase the specificity of DNA binding by Hox factors. In some cases the nature of the interaction with target genes is also changed, with the PBX/Hox dimers exhibiting a genetic activation function not seen with Hox alone (Pinsonneault et al., 1997; Li et al., 1999). Stuctural investigation of this regulatory system has recently begun with the analysis of Hox/EXD/DNA complex by X-Ray crystallography (Piper et al., 1999; Passner et al., 1999).

An additional level of regulation of Hox activity involves a second group of TALE class homeodomain proteins; these factors (HTH in *Drosophila*, MEIS and PREP1 in vertebrates) have been shown to regulate nuclear/cytoplasmic localization of EXD/PBX (Rieckhof et al., 1997; Kurant et al., 1998; Pai et al., 1998; Abu-Shaar et al., 1999; Berthelsen et al., 1999). It has also been shown that HTH/MEIS/PREP1 can form trimeric DNA binding protein complexes with Hox factors and EXD/PBX proteins (Berthelsen et al., 1998; Ryoo et al., 1999; Ferretti et al., 2000).

Although identification of the two families of Hox cofactors helps in explaining the specificity of Hox function in vivo, how Hox/cofactor combinations activate different regulatory networks to specify distinct cellular identity is not well understood. The availability of the entire lineage history of all cells in *C. elegans* (Sulston et al., 1983; Sulston and Horvitz, 1977) and its powerful genetics provide an approach to this question at a single-cell level.

The major *C. elegans* Hox complex contains four genes: *ceh-13*, *lin-39*, *mab-5* and *egl-5*; these are orthologs of the *Drosophila labial*, *Deformed/Sex combs reduced*, *Antennapedia/Ubx/Abd-A* and *Abd-B* genes respectively

(Costa et al., 1988; Schaller et al., 1990; Clark et al., 1993; Wang et al., 1993; Brunschwig et al., 1999). Two additional *Abd-B* homologs have recently been identified at a distinct chromosomal locus (Ruvkun and Hobert, 1998). Each Hox gene has a distinct expression pattern along the anterior-posterior axis of the animal. For the ectoderm, functions of the Hox factors have been extensively studied (see Bürglin and Ruvkun, 1993; Salser and Kenyon, 1994; Kenyon et al., 1997 for review). *C. elegans* also contains a single *exd/pbx* ortholog, *ceh-20* (Bürglin, 1992). As with *Drosophila exd* mutants (Peifer and Wieschaus, 1990; Rauskolb et al., 1995), *ceh-20* mutants share similar ectodermal phenotypes with multiple-loss-of-function *Hox* mutants (E. Chen, M. Robinson and M. Stern, personal communication). The situation in the mesoderm is less clear: although several of the Hox factors are known to be present in developing mesoderm (Wang et al., 1993; Salser, 1995; Ferreira et al., 1999), the roles for Hox and CEH-20 in mesodermal specification remain to be elucidated.

The mesoderm of *C. elegans* produces a variety of cell types including striated muscle, several types of non-striated muscle, and a limited set of non-muscle cells of diverse function. Cells from each of these classes arise both embryonically and postembryonically. Myogenesis in the embryo produces 81 striated bodywall muscles, 37 non-striated pharyngeal muscles, and four gut-associated enteric muscles (Suston et al., 1983). At hatching, a single mesoblast (the M cell) is poised to produce all of the additional non-gonadal mesodermal cells that will be produced during larval development (Sulston and Horvitz, 1977; Fig. 1). In hermaphrodites, M divides in a characteristic and reproducible pattern to produce 14 bodywall muscles, 2 sex myoblasts (SMs) and 2 coelomocytes. The 14 bodywall muscles eventually join the 81 embryonically born bodywall muscles and are used for locomotion. The 2 SMs are born in the posterior of L1 larvae, but migrate towards the anterior and reside at the vulval region. At mid L3 stage the SMs divide and give rise to 8 vulval muscles and 8 uterine muscles, which flank the gonad and are involved in egg laying. The coelomocytes are non-muscle mesodermal cells that behave as macrophages with a scavenger-like function (Chitwood and Chitwood, 1974; Fire et al., 1998a). The M lineage provides a valuable microcosm for genetic and experimental manipulations of mesodermal patterning since it is not essential for viability or for the overall body plan of the worm (Sulston and Horvitz, 1977).

Three of the Hox genes are known to be expressed in the M lineage. Although *mab-5* expression occurs throughout the M lineage, *mab-5* mutants show only a limited set of M lineage defects (Kenyon, 1986; Salser, 1995; Harfe et al., 1998b). *lin-39* is expressed in sex myoblasts and their descendants (Wang et al., 1993; Liu and Fire, unpublished), while *egl-5* expression has been observed in a subset of posterior muscles, and in the M mesoblast of males. No functional analysis of *lin-39* or *egl-5* in mesodermal specification has been reported. In this paper, we demonstrate an essential but redundant role for the Hox genes *mab-5* and *lin-39* in the diversification of the M lineage. We show that this role involves interactions of these factors with the *C. elegans* EXD ortholog CEH-20 and describe one direct target for Hox/CEH-20 complexes, the *C. elegans* ortholog of *twist*.

MATERIALS AND METHODS

C. elegans strains

Strains were maintained and manipulated under standard conditions as described by Brenner (1974). Analyses were performed at 25°C, unless otherwise noted. The following strains were used in this work: *mab-5(e1239)* III (Kenyon, 1986), *lin-39(n1760)* III (Clark et al., 1993), *egl-5(n945)* III (Wang et al., 1993), *mab-5(e1239) egl-5(n945)* III, *lin-39(n1760) mab-5(e1239)/qC1; him-5 (e1490)* III (Wang et al., 1993), *lin-39(n1760) mab-5(e1239) egl-5(n945)/sma-3(e491) mab-5(e1239) egl-5(n945)* III (gift from C. Kenyon), *ceh-20(n2513)/sma-3(e491) unc-32(e189)* III (gift from K. Kornfeld).

Strains carrying integrated cell-type-specific reporter transgenes were used to facilitate identification of specific cell fates within the M lineage:

myo-3::gfp–PD4251(*ccIs4251*)I, active in all bodywall muscles and vulval muscles (Fire et al., 1998b).

hlh-8::gfp–PD4666(*ayIs6*)X and PD4667(*ayIs7*)IV, active in all undifferentiated cells in the M lineage (Harfe et al., 1998b).

egl-15::gfp–NH2447(*ayIs2*)IV, active in adult vm1 muscles (gift from C. Branda and M. Stern).

Nde-box::gfp–PD4655(*ccIs4655*)II, active in eight vulval and eight uterine muscles (Harfe et al., 1998b).

myo-3::secreted gfp (*secreted gfp*)–GS1919(*arIs37*)I and GS2077(*arIs39*)X, used to visualize coelomocytes (gift from J. Fares and I. Greenwald).

Two integrated transgenic lines used in the heat-shock experiments were CF303(*muIs9*) X for *hs::mab-5* (Salser et al., 1993) and CF439(*muIs23*) for *hs::lin-39* (linkage group unknown; Hunter and Kenyon, 1995).

Lineage analysis was performed as described by Sulston and Horvitz (1977).

Plasmid constructs

mab-5 promoter constructs

7.5 kb of the *mab-5* promoter sequence (−7485 to −1) was amplified through long range PCR (Boehringer Mannheim) using genomic DNA as template. Primers JKL-192 and JKL-209 were used for the amplification, which resulted in the addition of unique *Ngo*MI and *Not*I sites at the ends of the PCR fragment. This fragment was cloned into pBS/KS$^+$ and used as the *mab-5* promoter in the following plasmids:

pJKL443.1: *mab-5 promoter::mab-5 cDNA::unc-54 3′UTR*
pJKL419.5: *mab-5 promoter::lin-39 cDNA::unc-54 3′UTR*
pJKL436.3: *mab-5 promoter::egl-5 cDNA::unc-54 3′UTR*
pJKL439.5: *mab-5 promoter::ceh-13 cDNA::unc-54 3′UTR*
pJKL418.4: *mab-5 promoter::hlh-8 cDNA::unc-54 3′UTR*
pJKL420.2: *mab-5 promoter::hlh-8 cDNA::smg suppressible 3′UTR*
pJKL481.9: *mab-5 promoter::gfp-lacZ::unc-54 3′UTR*

The unc-54 3′UTR is functional in all somatic tissues (Fire et al., 1990); the segment used for these constructs was derived from pPD96.85; the smg suppressible 3′UTR was derived by an extended coding region of *let-858* out of frame (Kelly et al., 1997). The GFP-β-gal fusion in pJKL481 contains a nuclear localization signal (derived from pPD107.94), thus resulting in nuclear localized GFP fluorescence. The cDNAs used were derived from the following plasmids:

mab-5: p198 (Costa et al., 1988), *lin-39*: p15.121A (Wang et al., 1993), *egl-5*: p160.111 (Wang et al., 1993), *ceh-13*: yk466g11 (gift from Yuji Kohara), *hlh-8*: pBH48.20 (Harfe et al., 1998b).

Heat-shock constructs

The following constructs were used for ectopic production of the corresponding coding regions upon heat-shock treatment:

hs::mab-5: pHS*mab-5* (Salser et al., 1993), *hs::lin-39:* pHS*lin-39* (Hunter and Kenyon, 1995), *hs::hlh-1:* pPD50.63 (Harfe et al.,

1998b), *hs::hlh-2:* pKM1035 (Krause et al., 1997), *hs::hlh-8:* pBH48.8 (Harfe et al., 1998b).

hlh-8 promoter constructs

Two plasmids, pBH56.55 and pBH52.05 were used as *hlh-8::gfp* reporters. Each contains 517 bp of the *hlh-8* upstream sequence. In pBH52.05, GFP was fused in frame to the first 9 amino acids of HLH-8, whereas in pBH56.55, GFP was fused in frame to only the ATG of HLH-8. Transgenic animals containing either of these two plasmids allowed visualization of the M lineage by GFP expression. Slight differences were seen between the constructs (pBH52.05 gave a lower level of GFP fluorescence than pBH56.55, and occasionally, pBH56.55 gave ectopic GFP expression in bodywall muscle cells). To generate mutant versions of the *hlh-8* promoter, the following mutations were introduced into these two plasmids. Names of plasmid pairs containing each set of mutations are listed, with a pBH52.05 derivative first and a pBH56.55 derivative second.

site 1 Hox half site mutant: pJKL454.1, pJKL459.1

site 1 CEH-20 half site mutant: pJKL458.2, pJKL463.1

site 1 double Hox and CEH-20 half sites mutant: pJKL480.2, pJKL479.2

site 2 mutant: pJKL455.1, pJKL460.1

site 3 mutant: pJKL456.1, pJKL461.1

site 4 mutant: pJKL457.1, pJKL462.1

sites 2, 3 and 4 triple mutants: pJKL478.2, pJKL477.3

Heat-shock experiments

Two different heat-shock protocols were used.

Heat-shock protocol A

To examine the effect of ectopic expression of *mab-5* or *lin-39* on *hlh-8* promoter activity, the following strains were constructed:

ayIs7 (hlh-8::gfp) IV; *muIs9 (hs::mab-5)* X;

ayIs6 (hlh-8::gfp) X; *ccEx[hs::lin-39 + rol-6(d)]*;

muIs23 (hs::lin-39); *ccEx[hlh-8::gfp + rol-6(d)]*;

ayIs7 (hlh-8::gfp) IV or *ayIs6 (hlh-8::gfp)* X animals were used as controls.

In each set of experiments, mixed staged animals were heat-shocked at 37°C for 30 minutes to 1 hour and allowed to recover for 2 to 6 hours. The expression pattern of GFP was then examined and compared to that of control animals that have undergone the same heat-shock treatment. Ectopic expression of *hlh-8::gfp* in embryonic muscle precursor cells was observed.

Heat-shock protocol B

To examine whether forced expression of *lin-39* was able to rescue the M lineage defects in *mab-5(0)* or *lin-39(0) mab-5(0)* mutants, mutant animals carrying different GFP markers (*hlh-8::gfp, egl-15::gfp* or *secreted gfp*) were used to generate transgenic lines carrying the *hs::lin-39* transgene. The transgenic animals were then heat-shocked for multiple rounds from mid-embryo stage to adulthood, with each round being 32°C for 20 minutes followed by 20°C, 3 hours 40 minutes. Animals not carrying the transgene array but subjected to the same treatment were used as negative controls. Transgenic animals carrying the *hs::mab-5* transgene were used as positive controls. Similar heat-shock conditions were used to assay whether ectopic expression of *hlh-1*, *hlh-2* and *hlh-8*, either singularly, or in combination, could rescue the M lineage defects in *mab-5(0)* or *lin-39(0) mab-5(0)* mutants.

RNAi

In a number of cases (Fig. 2), loss-of-function analysis of phenotypes using traditional mutants was confirmed using RNA-mediated interference (Fire et al., 1998b). cDNA clones used to generate dsRNAs were: p15.121A (Wang et al., 1993): *lin-39*, pJKL422.1: *ceh-20*. pJKL422.1 was derived from EST clone yk219d1 (a gift from Yuji Kohara) by deletion of extraneous non-*ceh-20* sequences.

Fusion proteins and gel-shift assays

lin-39 and *ceh-20* ORFs were cloned into expression vectors pRSETC and pRSETA respectively. 6xHis-tagged fusion proteins were generated using the *E. coli* BL21(DE3)pLysS cells (Invitrogen) and purified on a Ni affinity-column under standard denaturing conditions (Invitrogen). The resulting proteins were of the expected molecular mass and were approximately 95% pure, as assayed by SDS-PAGE followed by Coomassie staining. These proteins were renatured and used in gel-shift assays using conditions described by Chang et al. (1995). The *mab-5* ORF appeared toxic to both *E. coli* and yeast.

RESULTS

An essential role for Hox genes *mab-5* and *lin-39* in diversification of the postembryonic mesoderm

We first investigated the roles of Hox genes in patterning of the M lineage by examining the pattern of differentiated cells produced by the lineage in strains carrying different combinations of mutations in these genes.

Using a set of cell fate-specific reporter constructs (Fig. 1, Materials and Methods), we found that each single mutant retained the ability to carry out extensive M lineage diversification. The M lineage in null mutant *egl-5(n945)* or *lin-39(1760)* animals was normal, whereas limited M lineage defects were observed in null *mab-5(e1239)* mutants as reported previously: the M-derived coelomocytes and one or two bodywall muscles transformed to the sex myoblast fate (Harfe et al., 1998b). These results suggested that none of the three individual genes was essential for extensive M lineage diversification.

In contrast to the single mutants, a *lin-39(n1760) mab-5(e1239) egl-5(n945)* triple mutant showed a pronounced and severe defect in the M lineage. These animals produced no postembryonic coelomocytes, none of the body wall muscles normally derived from the M lineage and they lacked differentiated vulval and uterine muscles (Fig. 2). Instead of the 32 cells normally produced by the M lineage, we found either no identifiable product of the M lineage (20% of animals) or 1-4 large elongated cells in the posterior, which appeared myogenic (expressing *myo-3* and *egl-15* reporter constructs) but lacked the morphology of any normal muscle class.

The *lin-39* and *mab-5* genes, but not *egl-5*, appear to be the key factors in specifying the M lineage. Comparison of the *lin-39(n1760) mab-5(e1239)* double mutant with the *lin-39(n1760) mab-5(e1239) egl-5(n945)* triple mutant showed a similar range of phenotypes. All observed phenotypes were comparable in the two strains, although there was a somewhat higher fraction of animals in the double mutant showing the 1-4 residual myogenic products of the M lineage (Fig. 2). In similar assays for M lineage diversification, we saw no difference between *mab-5(e1239) egl-5(n945)* double mutants and *mab-5(e1239)* single mutants (Fig. 2). These results indicate that *egl-5* does not play a critical role in patterning the hermaphordite M lineage.

To further characterize M lineage products in the *lin-39(n1760) mab-5(e1239)* double mutant, we followed the cells continuously from hatching to early L2 stage by direct observation using Nomarski optics. In the majority of newly hatched mutant L1 larvae, M appeared at the correct position (although a fraction appeared ventralized). Since the final

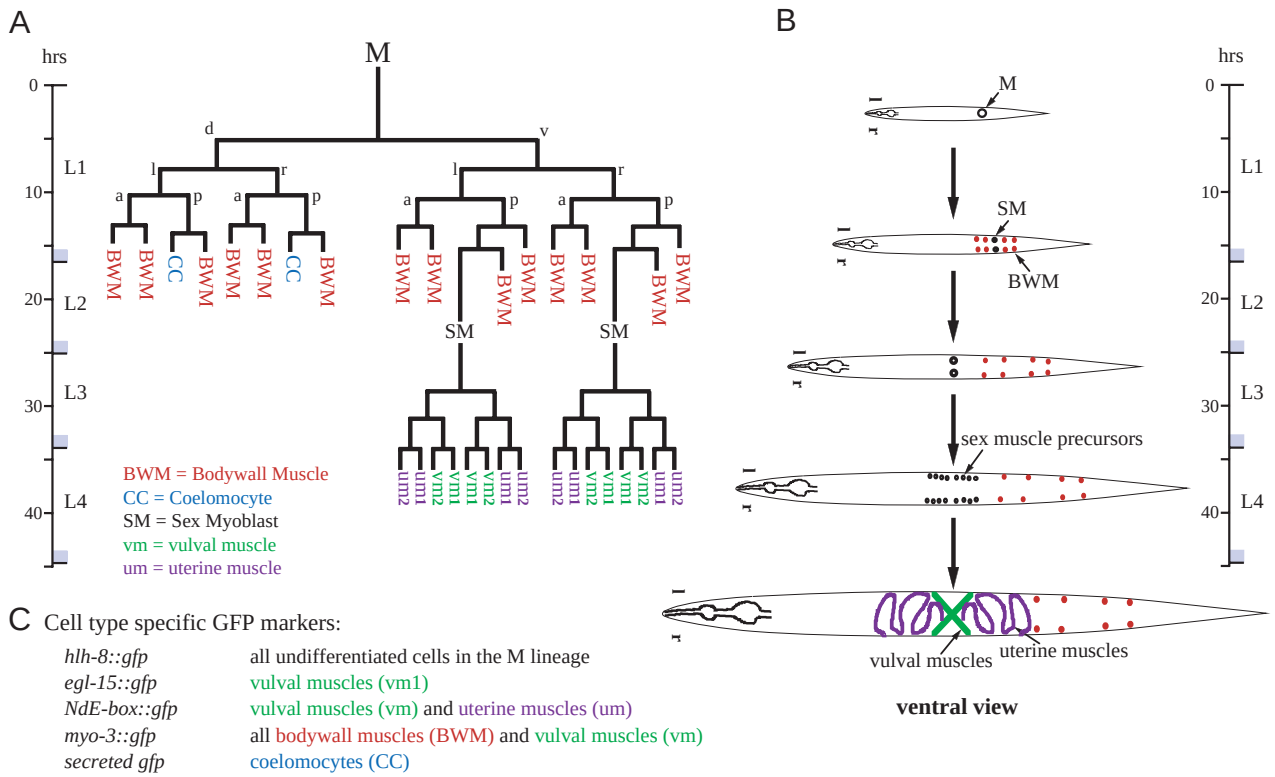


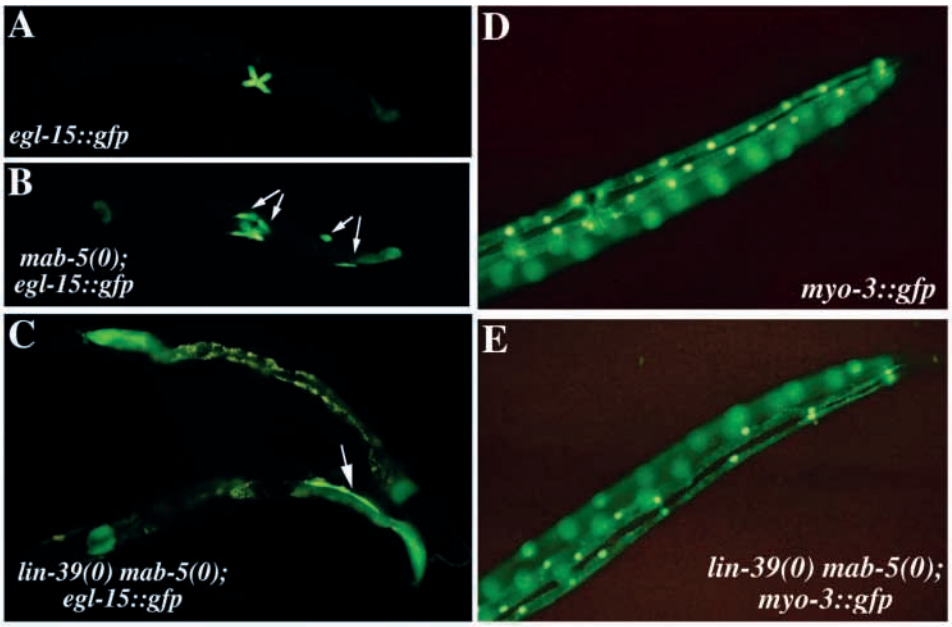
Fig. 1. The *C. elegans* hermaphrodite postembryonic M lineage. Times indicated are hours post-hatching at 25°C (from Sulston and Horvitz, 1977). (A) The M lineage with all the differentiated cell types. (B) A schematic ventral view of the M lineage through larval development. l and r represent left and right respectively. (C) GFP reporter constructs used to follow specific cell types in the M lineage (see Materials and Methods for detail).

position of M is a result of a posterior-directed cell migration during embryogenesis (Sulston et al., 1983), this indicates that the mutant M cells expressed aspects of their normal fates needed for migration during embryogenesis. In four of seven lineaged mutant animals, M did not divide at all until early L2 stage; in the remaining three animals, M divided once. In wild type animals, M divides to produce 18 cells during this time. The M lineage defect in the double mutant is not due to a postembryonic arrest, since cells in the somatic gonad divided with an apparently normal time course. There were also cell shape defects in the mutant animals: from the early L1 stage, M had begun to adopt an elongated shape (while in wild type the cell is spheroidal at this point). In the four lineaged animals where M failed to divide, the cell continued to elongate through the L2 stage. In the three lineaged animals where M divided (dorsal-ventral in two and anterior-posterior in one), the two resultant cells also became elongated.

The 1-4 cells produced by the M lineage in *lin-39(n1760) mab-5(e1239)* mutant animals are not readily classified as equivalent to any cell type in the normal animal. Although the elongated shapes initially adopted by these cells are somewhat characteristic of sex myoblasts, the cells fail to migrate and fail to carry out the division program of sex myoblasts. The subsequent differentiation of these cells appeared to occur precociously: in the L2 stage the cells express a set of reporters (*egl-15::gfp*, *myo-3::gfp* and *NdE-box::gfp)* which are characteristic of differentiated sex muscles. These reporters are normally expressed in differentiating sex muscles from the L4 stage. Taken together, our observations of the M lineage in *lin-39(n1760) mab-5(e1239)* mutant animals suggest that several cell cycles that are normally a part of the lineage are replaced by precocious differentiation, producing a series of cellular stages with each sharing a limited set of properties with a specific M-lineage-derived cell type (first sex myoblasts and then sex muscles).

Functional equivalence of Hox factors MAB-5 and LIN-39 in the M lineage

The synergism of *lin-39* and *mab-5* mutations suggested a partial redundancy between the two gene products in postembryonic mesoderm development. To ask whether this redundancy resulted from a functional equivalence between the two factors, we performed a series of experiments in which forced expression of one Hox family member was carried out in a genetic background lacking endogenous *lin-39* and *mab-5* activity. Rescue was assayed by direct analysis of differentiated descendants of the M lineage using specific integrated reporter constructs (Fig. 1; see Materials and Methods for details of reporter constructs used). We found that expression of either MAB-5 or LIN-39 from a heat shock promoter using a periodic heat shock regimen to maintain levels of MAB-5 or LIN-39 was sufficient for full or partial rescue of the M lineage defects (Fig. 3). Expression of either coding region from a 7 kb segment of the *mab-5* promoter also gave rescue (although rescue was somewhat less effective in this case; Fig. 3). The differences in rescue activity may reflect timing differences in the function of this promoter segment and the requirement for Hox activity in the M lineage. Experiments



F

Genetic background	Phenotype: M-derived Coelomocytes	M-derived Bodywall muscles	Vulval muscles (vm1)	Vulval and uterine muscles
WT	2	14	4	16
lin-39(0)	2	14	4	16
lin-39(RNAi)	2	14	4	16
mab-5(0)	0-1	12-13	4 + extra	16+ extra
egl-5(0)	2	14	4	16
mab-5(0) egl-5(0)	0-1	12-13	4 + extra	16+ extra
lin-39(0) mab-5(0)	0	0	0 (18%) 1-2* (82%)	0 (0%) 1-4* (100%)
lin-39(RNAi) mab-5(0)	0	0	0 (20%) 1-2* (80%)	0 (0%) 1-4* (100%)
lin-39(0) mab-5(0) egl-5(0)	0	0	0 (51%) 1-2* (49%)	0 (20%) 1-4* (80%)
lin-39(RNAi) mab-5(0) egl-5(0)	0	0	0 (48%) 1-2* (52%)	0 (22%) 1-4* (78%)
*ceh-20(n2513)***	0 (93%) 1 (7%)	0 (85%) >1 (10%)	0 (64%) 1-2* (24%) >3 (12%)	0 (26%) 1-4* (63%) >5 (11%)
ceh-20(RNAi)	0	0	0	0

Fig. 2. M lineage phenotypes of Hox and *ceh-20* mutants. For all panels in this and subsequent figures, *lin-39(0), mab-5(0)* and *egl-5(0)* represent *lin-39(n1760), mab-5(e1239)* and *egl-5(n945)* respectively, except where noted. All animals, except for the top one in C, are oriented with anterior to the left. (A-C) Vulval muscle phenotypes of (A) wild-type, (B) *mab-5(0)* and (C) *lin-39(0) mab-5(0)* animals visualized using *egl-15::gfp*, which labels vm1 vulval muscles. Note the extra vulval muscles in *mab-5(0)* animals (arrows in B), and the lack of vulval muscles (top animal in C) or the presence of one highly elongated *egl-15::gfp*-positive cell (arrow, bottom animal in C) in *lin-39(0) mab-5(0)* animals. (D,E) Bodywall muscle phenotypes of (D) wild-type and (E) *lin-39(0) mab-5(0)* animals visualized by *myo-3::gfp*, which labels all bodywall and vulval muscles. Note the decreased number and increased spacing of muscle nuclei in the double mutants. (F) Summary of M lineage phenotypes. The number of M-derived cells were assayed using cell type-specific GFP markers as described in Materials and Methods. For each genotype, >70 animals were examined for M-derived coelomocytes (*secreted gfp*). 7-15 animals were counted for M-derived bodywall muscles (*myo-3::gfp*), >90 animals were assayed for the number of vm1 vulval muscles (*egl-15::gfp*), and >80 animals were examined for the number of vulval and uterine muscles (*NdE-box::gfp*). The percentage represents the number of animals in the population examined that exhibited the phenotype indicated. In cases where the percentage is not noted, 100% of the animals exhibited that phenotype. *****Although there were no sex muscles in the presumptive vulval region, there were 1-4 highly elongated or irregularly shaped, 'sex-muscle-like', cells in the posterior of some *lin-39(0) mab-5(0), lin-39(0) mab-5(0) egl-5(0)* or *ceh-20(n2513)* mutants. **The M lineage phenotypes of *ceh-20(n2513)* animals were approximately 90% penetrant, approximately 10% of the animals exhibited partial formation of sex muscles (1-3 vm1s) in the presumptive vulval region. '*ceh-20 (RNAi)*', progeny from hermaphrodite animals injected with double stranded *ceh-20* RNA. After RNAi, all progeny were viable and showed 100% penetrance with respect to all M lineage defects. Moreover, all progeny lacked the 'sex-muscle-like' elongated cells which were present in *lin-39(0) mab-5(0), lin-39(0) mab-5(0) egl-5(0)* or *ceh-20(n2513)* animals.

A

expression construct	# of lines tested	mutant background	integrated reporter construct	rescue result
mab-5 p::mab-5	2	*lin-39(0) mab-5(0)*	*egl-15::gfp*	+
mab-5 p::lin-39	3	*lin-39(0) mab-5(0)*	*egl-15::gfp*	+/-*
mab-5 p::egl-5	2	*lin-39(0) mab-5(0)*	*egl-15::gfp*	-
mab-5 p::ceh-13	2	*lin-39(0) mab-5(0)*	*egl-15::gfp*	-
hs::mab-5	2	*mab-5(0)*	*egl-15::gfp*	+
hs::mab-5	1	*mab-5(0)*	*secreted gfp*	+/-
hs::mab-5	2	*lin-39(0) mab-5(0)*	*egl-15::gfp*	+
hs::lin-39	3	*mab-5(0)*	*egl-15::gfp*	+
hs::lin-39	3	*mab-5(0)*	*secreted gfp*	+/-
hs::lin-39	3	*lin-39(0) mab-5(0)*	*egl-15::gfp*	+
mab-5 p::mab-5	2	*mab-5(0)*	*hlh-8::gfp*	++
mab-5 p::mab-5	3	*lin-39(0) mab-5(0)*	*hlh-8::gfp*	++
mab-5 p::lin-39	2	*mab-5(0)*	*hlh-8::gfp*	++
mab-5 p::lin-39	2	*lin-39(0) mab-5(0)*	*hlh-8::gfp*	++
mab-5 p::egl-5	2	*mab-5(0)*	*hlh-8::gfp*	-
mab-5 p::ceh-13	2	*mab-5(0)*	*hlh-8::gfp*	-
hs::mab-5	1	*mab-5(0)*	*hlh-8::gfp*	++
hs::lin-39	3	*mab-5(0)*	*hlh-8::gfp*	++
mab-5 p::hlh-8	2	*mab-5(0)*	*egl-15::gfp*	-
hs::hlh-8	2	*mab-5(0)*	*egl-15::gfp*	-
hs::hlh-8	2	*lin-39(0)mab-5(0)*	*egl-15::gfp*	-
hs::hlh-2	1	*mab-5(0)*	*egl-15::gfp*	-
hs::hlh-2	1	*lin-39(0) mab-5(0)*	*egl-15::gfp*	-
hs::hlh-8+hs::hlh-2	2	*mab-5(0)*	*egl-15::gfp*	-
hs::hlh-8+hs::hlh-2	2	*lin-39(0) mab-5(0)*	*egl-15::gfp*	-
hs::hlh-8+hs::hlh-2	2	*mab-5(0)*	*secreted gfp*	-
hs::hlh-8+hs::hlh-1	3	*lin-39(0) mab-5(0)*	*egl-15::gfp*	-
hs::hlh-8+hs::hlh-1+hs::hlh-2	2	*lin-39(0) mab-5(0)*	*egl-15::gfp*	-

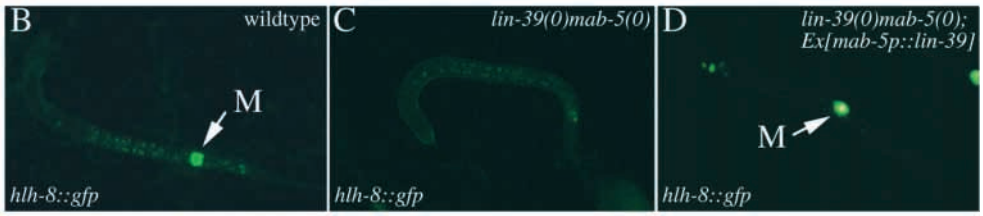


Fig. 3. Rescue of Hox mutant defects by forced expression of individual coding regions. (A) Summary of rescue data from forced expression of Hox genes (*mab-5*, *lin-39*, *egl-5* and *ceh-13*), *hlh-8*, *hlh-2* and *hlh-1*, using either the *mab-5* promoter (*mab-5p*) or the heat-shock promoter (*hs*). In each case, transgenic lines with over 50% transmission efficiency were chosen for analysis. 'Rescue result' describes the presence or absence of a normal reporter expression pattern. ++, >50% complete rescue of early loss of *hlh-8::gfp* expression; +, 10-15% complete rescue and 20-30% partial rescue; +/–, around 5% complete rescue and 5% partial rescue. In each case, >100 animals were examined. Partial rescue of later defects might reflect timing of the heat shock regimen and of *mab-5* promoter activitiy (the 7.5 kb *mab-5* promoter segment used for these experiments was primarily active early in the M lineage). *The fluorescence intensity of the *egl-15::gfp* reporter was also weaker in this case. (B-D) *hlh-8::gfp* expression pattern in L1 larvae. (B) Wild type, (C) *lin-39(0) mab-5(0)*, (D) *lin-39(0) mab-5(0); Ex[mab-5 promoter::lin-39]*. Arrows point to the M mesoblast. Note the rescue of *hlh-8::gfp* expression in M cell in (D). All animals are oriented with anterior to the left. Similar results were observed in *mab-5(0); Ex[mab-5 promoter::lin-39]* animals.

in which a short pulse of heat in late embryogenesis was used to transiently produce LIN-39 or MAB-5 resulted in transient activation of the *hlh-8* reporter in the L1 stage but not to the later production of vulval muscles expressing *egl-15::gfp* (data not shown). The ability of LIN-39 and MAB-5 proteins to function in the M lineage appeared specific: forced expression of two other Hox factors (CEH-13 or EGL-5) under the control of the 7 kb *mab-5* promoter did not result in rescue of any M lineage defects in the *lin-39(n1760) mab-5(e1239)* double mutant (Fig. 3). These results suggest that LIN-39 and MAB-5 proteins share specific structural properties and/or activities that allow either protein (in the absence of the other) to direct diversification in the M lineage.

The *C. elegans* EXD/PBX ortholog CEH-20 acts as a Hox cofactor in M lineage diversification

In both *Drosophila* and vertebrates, Hox proteins function with a homeodomain protein cofactor, EXD/PBX, to regulate target gene expression (see Mann, 1995; Wilson and Desplan, 1995; Mann and Chan, 1996; Mann and Affolter, 1998 for review). We tested the role of the unique *C. elegans exd/pbx* ortholog *ceh-20* (Bürglin, 1992) in patterning the M lineage using a strong loss-of-function mutation (*n2513*; E. Chen, M. Robinson and M. Stern, personal communication) and RNA-mediated interference (RNAi; Fire et al., 1998b).

Both the *n2513* mutation and *ceh-20(RNAi)* produced M lineage defects that were similar to *lin-39(n1760) mab-5(e1239)* double mutants. M lineage defects were approximately 90% penetrant in the *n2513* mutant and 100% penetrant following *ceh-20* RNAi (Fig. 2). Although M was present, all M-derived cells, including the 14 bodywall muscles, the 16 sex muscles and the 2 coelomocytes, were missing in the strongly affected animals.

The similarity in M lineage phenotypes between *ceh-20* and

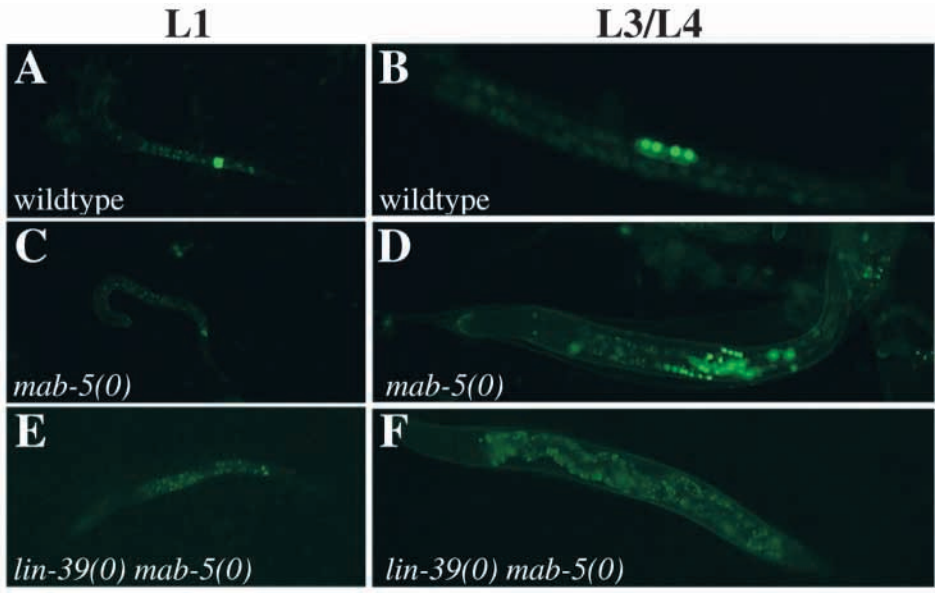


Fig. 4. *hlh-8::gfp* expression pattern in wild type and Hox mutant larvae. (A,B) Wild-type, (C,D) *mab-5(0)* and (E,F) *lin-39(0) mab-5(0)* animals carrying the integrated *hlh-8::gfp* reporter construct in L1 (A,C,E) and L3/L4 (B,D,F) larvae. Note the absence of M lineage reporter expression in the double mutants in both L1 and L3/L4 stages. All animals are oriented with anterior to the left. Similar phenotypes were observed in *lin-39(0) mab-5(0) egl-5(0)* as well as in *ceh-20* mutants (data not shown).

lin-39(n1760) mab-5(e1239) suggested several plausible models. One model that was readily tested was that CEH-20 might activate *mab-5* and *lin-39* gene expression in the M lineage. A *mab-5::gfp-lacZ* reporter construct (driven by the 7.5kb *mab-5* promoter used in the rescue assays above) was used to examine *mab-5* activity, while anti-LIN-39 antibody staining was used to assess LIN-39 localization. We found no change in *mab-5* or *lin-39* expression in the M lineage in *ceh-20(n2513)* animals (data not shown). In addition, forced expression of *mab-5* or *lin-39* using the heatshock promoter failed to rescue M lineage defects or *hlh-8*::*gfp* reporter activity in *ceh-20(RNAi)* animals (data not shown). These results suggest that the major contribution of CEH-20 is not as a regulator of *mab-5* and *lin-39* expression.

Intriguingly, we found one difference in phenotype between *ceh-20(RNAi)* and the *lin-39(n1760) mab-5(e1239)* double-null mutant (Fig. 2). The *egl-15::gfp* and *NdE-box::gfp* positive, 'sex muscle-like' cells present in *lin-39(n1760) mab-5(e1239)* double mutants were absent in *ceh-20(RNAi)* animals (n>100). Although we cannot rule out the possibility that treatment with dsRNA targeted against *ceh-20* interferes with additional genes, there are no genes with sufficient homology to *ceh-20* in the nearly complete genome sequence (*C. elegans* sequencing consortium, 1998) that could serve as common RNAi targets. Hence, the greater severity of the *ceh-20(RNAi)* phenotype suggests that there might be additional partners for *ceh-20* in the M lineage. This is reminiscent of the situation in *Drosophila*, where EXD has been shown to act as a cofactor for non-Hox homeodomain proteins such as Engrailed (Peifer and Wieschaus, 1990; van Dijk and Murre, 1994).

A critical Hox/CEH-20 target site in the promoter of the *C. elegans twist* ortholog *hlh-8*

To further understand the role of *mab-5*, *lin-39* and *ceh-20* in mesodermal diversification, we investigated the relationship between these genes and *hlh-8*, a lineage-specific regulatory factor involved in patterning the M lineage. *hlh-8* encodes the *C. elegans twist* ortholog and is active in undifferentiated cells throughout the M lineage (Harfe et al., 1998b). *hlh-8* null mutants have variable defects in the M lineage, including alterations in early cleavage planes within the lineage, incomplete differentiation of sex muscles, variable numbers of M-derived bodywall muscles and lack of expression of two *hlh-8* targets, *egl-15* and *ceh-24* (Corsi et al., 2000). Several previous observations with *mab-5* had suggested that *hlh-8* might act downstream of Hox function in the M lineage: (a) *mab-5* mutants lack *hlh-8* reporter expression in the early M lineage (this expression re-appears later in the lineage) and (b) forced expression of *mab-5* can activate an *hlh-8* reporter in muscle precursors (Harfe et al., 1998b).

We extended the connection between *hlh-8* and Hox function by examining *hlh-8* reporter activity in various Hox mutant combinations. We found the *hlh-8* reporter to be completely off in the M lineage in *lin-39(n1760) mab-5(e1239)* double mutants (Fig. 4). These mutants retained normal *hlh-8* expression in a set of non-muscle cells in the head. Loss of *hlh-8* activity throughout the M lineage was similarly observed in the *ceh-20(n2513)* mutant and following *ceh-20* RNAi (data not shown).

To test the hypothesis that *lin-39* and *mab-5* shared the ability to activate *hlh-8* in the lineage, we forced expression of *lin-39* using the *mab-5* promoter or a heat shock promoter. As with MAB-5 (Harfe et al., 1998b), early embryonic expression of LIN-39 with a heat-shock promoter was sufficient to activate ectopic *hlh-8* reporter expression in embryonic muscle precursors (data not shown, see Materials and Methods). Later forced expression of either *lin-39* or *mab-5*, using the heat-shock promoter or the *mab-5* promoter, could rescue the loss of *hlh-8::gfp* expression in *lin-39(n1760) mab-5(e1239)* mutants (Fig. 3). This rescue appeared to be specific to *mab-5* and *lin-39*: forced expression of *egl-5* or *ceh-13* using the *mab-5* promoter failed to rescue the loss of *hlh-8* expression (Fig. 3). These results indicate a necessary and sufficient role in activating *hlh-8* expression that can be fulfilled by either *lin-39* or *mab-5*.

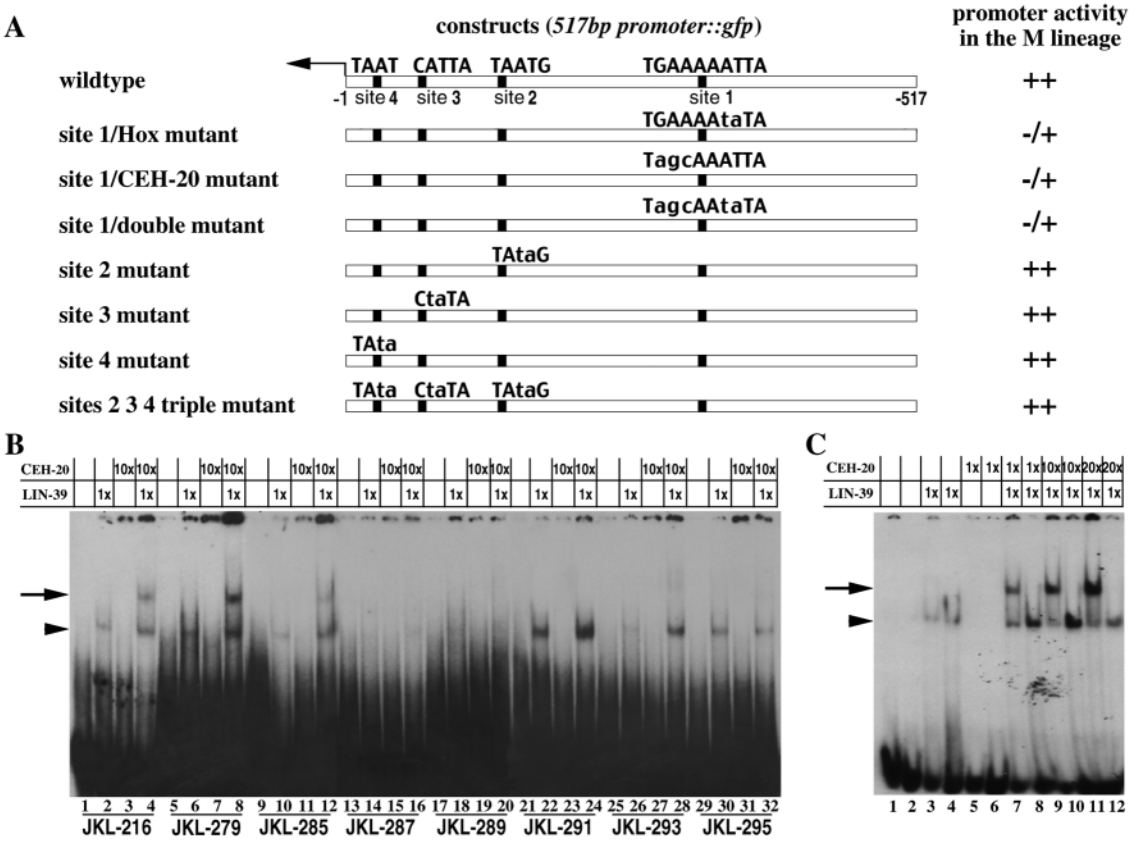
The *hlh-8* promoter contains four candidate Hox binding sites. A 517 bp fragment of the *hlh-8* promoter, which is sufficient for M-lineage specific expression of reporter genes, has four TAAT (or ATTA) sequences resembling core binding sites for Antennapedia type homeodomain proteins (Fig. 5A).

None of these sites matched the reported Hox/EXD consensus site (TGATNNATNN; Mann and Affolter, 1998). However, site 1 (TGAAAAATTA) contains a 3/4 match to the consensus half site (TGAT) for EXD factors (Mann and Affolter, 1998). A set of mutant promoters with clustered alterations in one or more of the Hox sites was created in vitro and tested in the animal using a GFP reporter. As shown in Fig. 5A, mutations in sites 2, 3 and 4 had little or no effect on the activity of the promoter. However, mutations in site 1, including those in the Hox or the CEH-20 half site, or both together, significantly reduced the level of reporter expression in the M lineage. These results suggested that site 1 is critical for *hlh-8* promoter activity in vivo.

A direct interaction between a physiologically critical site in the *hlh-8* promoter and LIN-39/CEH-20

To test if *hlh-8* was a direct target of Hox/CEH-20 dimers, recombinant LIN-39 and CEH-20 proteins were generated and purified from *E. coli*, and in vitro gel mobility shift assays were performed using sequences from the *hlh-8* promoter (as described in Materials and Methods, we were unable to generate full length MAB-5 proteins in *E. coli* or yeast).

Oligonucleotides containing site 1 formed a complex with LIN-39 and CEH-20, generating a band which showed distinctly retarded gel mobility (Fig. 5B). Appearance of the putative ternary complex depended on both CEH-20 and LIN-39 proteins. LIN-39 alone produced an apparent binary



D

Oligonucleotides		Binding to LIN-39	Binding to LIN-39/CEH-20
	CEH-20 Hox		
JKL-216:	GTTTCGATAGTTGAAAAATTACCGCGGAAAAAAGAATTTGAGAG	+	+
JKL-279:	----------ctgct	+	+
JKL-281:	----------tctagc-----	+	+
JKL-283:	----------atcgtc----------	+	+
JKL-285:	----------cgcagt----------	+	+
JKL-287:	----------ac----------	-	-
JKL-289:	----------cg----------	-	-
JKL-291:	----------gg----------	+	(-/+)
JKL-293:	----------gc----------	+	-
JKL-295:	----------gt----------	+	-
JKL-255:	----------agc----------	+	-
JKL-297:	-----tcttac----------	+	+
JKL-299:	caacg----------	+	+
JKL-218:	GTTTCGATAGTTGAAAAtaTACCGCGGAAAAAAGAATTTGAGAC	+	-
	EXD Hox		
JKL-248:	ACCGCGTTGATTAATGACCAGACCGGAT	+	++
JKL-250:	-------ctc----------	+	-

complex with site 1 oligonucleotides, while no complex was produce with CEH-20 alone. Analysis of sequences required for formation of the site 1/CEH-20/LIN-39 complex (Fig. 5B) demonstrated involvement of the putative CEH-20 site (TGAA) (Fig. 5B; JKL-295, JKL-293). The putative Hox site (aaATTA) was also required for formation of the ternary complex, and for the binding of LIN-39 protein to site 1 as a binary complex (Fig. 5B; JKL-287, JKL-289).

Comparison of site 1 with a consensus binding site for HOX/EXD dimers (TGATNNATNN; Mann and Affolter, 1998) showed a single base pair difference in the EXD half site (TGAA instead of TGAT). The difference seems unlikely to reflect divergence in HOX/EXD recognition sequence between nematodes and other systems, since a canonical ANTP/EXD binding site (Knoepfler et al., 1996) formed a ternary complex efficiently with LIN-39 and CEH-20 (Fig. 5C). The greater affinity of LIN-39/CEH-20 to the canonical ANTP/EXD binding site than to site 1 may reflect differences in core sequence or in the four degenerate bases, which could alter the relative spacing or orientation of CEH-20 and Hox sites.

Interestingly, we found a class of mutation in the Site 1 Hox binding site (JKL-218 and possibly JKL-291) that retained Hox binding but lost the ability to form a ternary complex. These mutations may shift the position of the Hox site on the DNA (i.e. creating a new Hox site), or may alter the geometry of the Hox/DNA interaction so as to prevent formation of the ternary complex. In the case of JKL-218, the mutated site had lost the ability to function in vivo as part of the promoter (Fig. 5A). A similar situation was observed with sites 2 and 3 in the *hlh-8* promoter; these two sites bound to LIN-39 in vitro but showed no ternary complex formation with LIN-39/CEH-20 (data not shown). Like the JKL-218 mutant of site 1, the natural sites 2 and 3 showed no evident contribution to in vivo activity of the promoter (Fig. 5A). We can make no conclusion concerning the significance of LIN-39:DNA complexes lacking CEH-20 that are formed in vitro: these complexes might fail completely to form in vivo, they might form only transiently, or they might persist but fail to activate gene expression in the M lineage. At least for the *hlh-8* promoter, our data support a model in which a ternary complex of LIN-39 with CEH-20 on a defined site (site 1) is critical for activation of gene expression in the M lineage.

Fig. 5. The *hlh-8* promoter contains a critical Hox/CEH-20 target site. (A) Wild-type and mutant *hlh-8 promoter::gfp* constructs and their M lineage promoter activity assayed in vivo. In the diagrams of promoter constructs, only the four putative Hox binding sites are highlighted. Core sequences are in upper case; mutations in lower case. Promoter activity was assayed by examining GFP in at least seven independent transgenic lines: ++, bright M lineage GFP in >95% of animals; –/+, faint GFP signals in a low fraction of progeny (3/35 for the CEH-20 half site mutant, 2/100 for the HOX half-site mutant, and 0/100 for the double mutant). Quantitative measurement of fluorescence indicated that the faint signals in the few positive animals from the CEH-20 and Hox half-site mutants were approximately 40-fold lower than signals from the wild-type *hlh-8* promoter. (B) Gel mobility shift assays using purified LIN-39 and CEH-20 proteins and dsDNA oligonucleotides (sequences shown in D). 1× and 10× represent 50 ng and 500 ng of purified proteins used respectively. A more rapidly migrating complex (arrowhead) required only LIN-39 protein and sequences in the Hox half site (lanes 14, 18, 26, 30); a more slowly migrating complex (arrow) required CEH-20 and LIN-39 proteins and sequences in the Hox and CEH-20 half sites (lanes 16, 20; 28, 32). (C) Mobility shifts with oligonucleotides with a canonical ANTP/EXD composite site (JKL-248; lanes 1,3,5,7,9,11) and an EXD half site mutant (JKL-250; lanes 2,4,6,8,10,12). A more rapidly migrating complex (arrowhead) was dependent on LIN-39 protein but independent of CEH-20 and the EXD binding site; a more slowly migrating complex (arrow) was dependent on both LIN-39 and CEH-20, and on the EXD half site. Under the conditions of this assay, the ANTP/EXD composite site was apparently more effective than *hlh-8* site 1 in complexing with CEH-20/LIN-39, as evidenced by the efficiency of the band shift and the requirement for a lower concentration of CEH-20 (compare lane 4 in B and lane 7 in C). (D) A summary of mobility shift assays. + and – represent presence or absence of a mobility shift (–/+, in some experiments we observed a faint band corresponding to an apparent ternary complex between JKL-291 and LIN-39/CEH-20). Oligonucleotide JKL-216 covered site 1 of the *hlh-8* promoter (core sequences underlined). JKL-279 to JKL-299 were derived from JKL-216: mutated residues are shown in lower case with dotted lines indicating un-mutated residues. JKL-248 contained a canonical EXD/HOX site; JKL-250 was a derivative of JKL-248 with the EXD half site mutated. A StyI linker was present on each double stranded oligonucleotide shown in the figure; this linker had no evident effect on binding, since equivalent results were obtained using a wild type site 1 oligonucleotide without the linker (JKL-300: GTTTCGATAGTTGAAAAATTACCGCGGAAAAAA).

Evidence for additional targets of Hox/CEH-20 in the M lineage

To test if *hlh-8* might be the only target for the Hox genes (*lin-39* and *mab-5*) and *ceh-20* in the M lineage, we asked whether forced expression of *hlh-8* was able to rescue M lineage defects in *lin-39(n1760) mab-5(e1239)*. As shown in Fig. 3, expression of *hlh-8* using the *mab-5* or heat-shock promoter was insufficient to rescue the M lineage patterning defects. This result suggested that additional targets exist in the M lineage for the Hox genes.

Two additional *hlh* genes were considered as possible Hox targets in the M lineage. CeE/DA (the *C. elegans* ortholog of Daughterless) is encoded by the *hlh-2* gene and can form heterodimers with the *hlh-8* product CeTwist in vitro and in vivo (Krause et al., 1997; Harfe et al., 1998b). CeMyoD is encoded by the *hlh-1* gene and plays critical roles in patterning of the M lineage (Krause et al., 1990; Harfe et al., 1998a). Forced expression of *hlh-8* together with *hlh-2* and/or *hlh-1* using a heat shock promoter was insufficient to rescue the M lineage defects in *lin-39(n1760) mab-5(e1239)* animals (Fig. 3). Although these experiments do not rule out *hlh-2* and *hlh-1* as potential Hox targets, additional targets must be critical for patterning of the M lineage.

DISCUSSION

Essential roles of Hox genes and *ceh-20* in diversification of the postembryonic mesoderm

We have studied the function of Hox genes and the *C. elegans exd* ortholog *ceh-20* in patterning of the postembryonic mesoderm. Our results showed an essential and redundant role of two Hox genes *mab-5* and *lin-39* in diversification of the postembryonic mesoderm, with *ceh-20* being a cofactor for these two Hox genes.

The M lineage defects of *lin-39 mab-5* double mutants and

ceh-20 mutants are intriguing. These defects did not appear to be a result of homeotic transformation of the fate of M or its descendants. Instead, the mutants exhibit either (1) a loss of all differentiated M-lineage descendants or (2) the precocious production of abnormal mesodermal fates with certain properties of later M lineage products. The precocious appearance of large cells that exhibit SM- and sex muscle-like characteristics suggests that this program might be a default state of M in the absence of Hox function.

The shared role of MAB-5 and LIN-39 in the M lineage appeared to be specific to these two Hox factors. First, forced expression of either *lin-39* or *mab-5*, but not of the neighboring Hox genes *ceh-13* and *egl-5*, was sufficient to activate ectopic expression of M lineage reporters. Second, *egl-5* mutants (which are viable either alone or in combination with *lin-39* and *mab-5* mutants) had no M lineage defects on their own and showed no synergistic effects with *lin-39* and *mab-5*.

Mesodermal roles of Hox and *exd* genes have also been shown in *Drosophila*. In the visceral mesoderm, *Ubx* and *abd-A* are involved in morphogenesis of the midgut (see Bienz, 1994; Frasch and Nguyen, 1999 for review). In this case, a few targets for Hox genes have been described: Ubx in the visceral mesoderm is directly required with an EXD cofactor for activating expression of the signaling molecule *dpp* (Capovilla et al., 1994; Chan et al., 1994). In the somatic mesoderm, *Ubx* and *abd-A* can each promote the formation of specific sets of muscle precursors (Greig and Akam, 1993; Michelson, 1994). None of the *Drosophila* Hox mutants or combinations that have been analyzed show as drastic an effect on postembryonic mesoderm as was seen with the *lin-39 mab-5* double mutant in *C. elegans*. This apparent discrepancy may reflect a fundamental difference between the biological systems; alternatively, a more drastic postembryonic requirement for Hox factors in the *Drosophila* mesoderm might have been missed due to the embryonic lethality of multiple-Hox mutants.

Functional equivalence of *mab-5* and *lin-39* in the M lineage

Our rescue experiments suggested partially overlapping roles for *mab-5* and *lin-39* in the M lineage. The modest M-lineage defects seen in *mab-5* single mutants, compared with the lack of any M-lineage defects in *lin-39* single mutants suggest that under normal circumstances the contribution of *mab-5* may be somewhat more substantial at early time points. One conceivable explanation for the 'either/or' requirement would involve cross-regulation between Hox genes. In particular, we have tested the possibility that *lin-39* expression in the M lineage only occurs in the absence of functional *mab-5*. This is apparently not the case, as *mab-5* mutants show an apparently normal pattern of M lineage staining with antibodies to LIN-39 (date not shown).

Several types of interactions between *lin-39* and *mab-5* activities in determining cell fate have been reported. In a subset of Pn.aap cells that normally express both *lin-39* and *mab-5*, the *lin-39* activity is dominant, preventing *mab-5* from functioning in these cells (Salser et al., 1993; Clark et al., 1993). A distinct interaction is seen in male Pn.p cells, where *lin-39* and *mab-5* are both expressed and act combinatorially to specify a fate that is different from that specified by either alone (Salser et al., 1993; Wang et al., 1993). A third situation (Clandinin et al., 1997; Maloof and Kenyon, 1998) is seen in hermaphrodite vulval precursor cells, for which the loss of Hox (*lin-39*) activity after specification results in a failure to differentiate; in this lineage, *lin-39* and *mab-5* activities have the capability to promote distinct and non-overlapping consequences in terms of cell fate. The functional and simultaneous requirement in the M lineage for either *mab-5* or *lin-39* function represents a further degree of freedom in using these genes to build an organism.

The highly conserved structure of Hox factors is consistent with a view that these genes have evolved by duplication of a single precursor gene (Bürglin, 1994). Under these circumstances, it is not surprising that certain roles for Hox factors would still be maintained as shared (or redundant) between several genes in the cluster (for example, Michelson, 1994; Greig and Akam, 1995; Casares et al., 1996; Favier et al., 1996; Barrow and Capecchi, 1999). While the individual genes might have acquired position-specific roles based on their acquisition of intricate patterns of expression, it is certainly conceivable that the entire family (or a large subset) will have maintained a shared role equivalent to that of the ancestral (and unique) Hox factor. While the role of that factor will remain a mystery, the appearance of Hox factors in the developing embryo just prior to the start of differentiation suggests that the ancestral factor could have played a role in developmental timing, perhaps modulating the start of differentiation in a subset of cells.

The *C. elegans twist* ortholog *hlh-8* is a direct and critical target of Hox genes and *ceh-20* in the postembryonic M lineage

Our studies of the function of *mab-5*, *lin-39* and *ceh-20* in patterning of the postembryonic mesoderm led to the identification of a direct target for these genes, the *C. elegans twist* ortholog *hlh-8*. We identified a critical site in the *hlh-8* promoter that is a binding site for the LIN-39/CEH-20 protein complex. The similarity between core binding sequences for *Drosophila* ANTP and DFD proteins in vitro (Ekker et al., 1994), and the functional equivalence of *mab-5* and *lin-39* in activating *hlh-8* expression in the M lineage, strongly suggest that this site is also a binding site for MAB-5/CEH-20.

Although *hlh-8* is a target for Hox/CEH-20 function in the M lineage, it is not the only such target. Several indirect observations demonstrate the existence of additional targets. One line of evidence comes from the observation that forced expression of *hlh-8* in *lin-39(n1760) mab-5(e1239)* mutants failed to rescue the M lineage defects. An independent line of evidence comes from a comparison of mutant phenotypes: *lin-39(n1760) mab-5(e1239)* mutants showed a more severe patterning defect in the M lineage than null *hlh-8(nr2061)* mutants (Corsi et al., 2000; this work): (1) While *lin-39(n1760) mab-5(e1239)* animals lack both M-derived coelomocytes, the majority of *hlh-8(nr2061)* mutants (76%) contain normal numbers of M-derived coelomocytes. (2) While *lin-39(n1760) mab-5(e1239)* mutants lack all M-derived bodywall muscle, *hlh-8(2061)* mutants produce variable number of these cells. (3) Sex muscles can be produced in *hlh-8(nr2061)* mutants, although they are not fully differentiated.

The identity of other Hox targets in the M lineage is not known. We are currently using a genetic approach to identify additional candidates.

We thank QueeLim Ch'ng, Ann Corsi, Jamie Fleenor, Brian Harfe, Cynthia Kenyon, Yoji Kohara, Kerry Kornfeld, Mike Krause, Julin Maloof, Siqun Xu for *C. elegans* strains, cDNA clones and antibodies; Ann Corsi, David Eisenmann, Cynthia Kenyon, Kerry Kornfeld, Steve Kostas, Mike Krause, Steve Salser, Cynthia Wolberger for helpful discussions and suggestions; Cathy Branda for instruction in lineaging methods; Estella Chen, Matthew Robinson and Michael Stern for communicating unpublished results and for sharing information concerning *ceh-20*(*n2513)*; and Thomas Bürglin, Ann Corsi, David Eisenmann, Manfred Frasch, Jenny Hsieh, Steve Kostas, Mike Krause, Richard Mann, Susan Parrish, Judy Yanowitz, Mariana Wolfner and two anonymous reviewers for critical comments on the manuscript. Some strains used in this study were obtained from the *C. elegans* Genetics Center (CGC), which is supported by a grant from the NIH Center for Research Resources. This work is supported by NIH grants (R01GM37706 to A. Z. F.) and (F32HD08331 to J. L.).

REFERENCES

Abu-Shaar, M., Ryoo, H. D. and Mann, R. S. (1999). Control of the nuclear localization of Extradenticle by competing nuclear import and export signals. *Genes Dev.* **13,** 935-945.

Barrow, J. R. and Capecchi, M. R. (1999). Compensatory defects associated with mutations in *Hoxa1* restore normal palatogenesis to *Hoxa2* mutants. *Development* **126,** 5011-5026.

Berthelsen, J., Zappavigna, V., Ferrettti, E., Mavilio, F. and Blasi, F. (1998). The novel homeoprotein Prep1 modulates Pbx-Hox protein cooperativity. *EMBO J.* **17,** 1434-1445.

Berthelsen, J., Kilstrup-Nielsen, C., Blasi, F., Mavilio, F. and Zappavigna, V. (1999). The subcellular localization of PBX1 and EXD proteins depends on nuclear import and export signals and is modulated by association with PREP1 and HTH. *Genes Dev.* **13,** 946-953.

Bienz, M. (1994). Homeotic genes and positional signaling in the *Drosophila* viscera. *Trend Genet.* **10,** 22-26.

Biggin, M. D. and McGinnis, W. (1997). Regulation of segmentation and segmental identity by *Drosophila* homeoproteins: the role of DNA binding in functional activity and specificity. *Development* **124,** 4425-4433.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77,** 71-94.

Brunschwig, K., Wittmann, C., Schnabel, R., Bürglin, T., Tobler, H. and Muller, F. (1999). Anterior organization of the *Caenorhabditis elegans* embryo by the *labial*-like Hox gene *ceh-13*. *Development* **126,** 1537-1546.

Bürglin, T. R. (1992). New motif in PBX genes. *Nature Genet.* **1,** 319-320.

Bürglin, T. R. and Ruvkun, G. (1993). The *Caenorhabditis elegans* homeobox gene cluster. *Curr. Opin. Genet. Dev.* **3,** 615-620.

Bürglin, T. R. (1994). A comprehensive classification of homeobox genes. In *Guidebook to the Homeobox Genes* (ed. D. Duboule), pp. 25-71. Oxford, England: Oxford University Press.

Bürglin, T. R. (1997). Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. *Nucl. Acid Res.* **25,** 4173-4180.

Bürglin, T. R. (1998). The PBC domain contains a MEINOX domain: Coevolution of Hox and TALE homeobox genes? *Dev. Genes Evol.* **208,** 113-116.

***C. elegans* Sequencing Consortium** (1998). Genome sequence of the nematode *Caenorhabditis elegans*: a platform for investigating biology. *Science* **282,** 2012-2018.

Capovilla, M., Brandt, M. and Botas, J. (1994). Direct regulation of *decapentaplegic* by *Ultrabithorax* and its role in *Drosophila* midgut morphogenesis. *Cell* **76,** 461-475.

Casares, F., Calleja, M. and Sanchez-Herrero, E. (1996). Functional similarity in appendage specification by the *Ultrabithorax* and *abdominal-A Drosophila* HOX genes. *EMBO J.* **15,** 3934-3942.

Chan, S.-K., Jaffe, L., Capovilla, M., Botas, J. and Mann, R. S. (1994). The DNA binding specificity of Ultrabithorax is modulated by cooperative interactions with Extradenticle, another homeoprotein. *Cell* **78,** 603-615.

Chang, C.-P., Shen, W.-F., Rozenfeld, S., Lawrence, H. J., Largman, C. and Cleary, M. L. (1995). Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. *Genes Dev.* **9,** 663-674.

Chitwood, B. and Chitwood, M. (1974). *Introduction to Nematology*. Baltimore, Maryland: University Park Press.

Clandinin, T. R., Katz, W. S. and Sternberg, P. W. (1997). *Caenorhabditis elegans* HOM-C genes regulate the response of vulval precursor cells to inductive signals. *Devel. Biol.* **182,** 150-161.

Clark, S. G., Chisholm, A. D. and Horvitz, H. R. (1993). Control of cell fates in the central body region of *C. elegans* by the homeobox gene *lin-39*. *Cell* **74,** 43-55.

Corsi, A. K., Kostas, S. A., Fire, A. and Krause, M. (2000).*Caenorhabditis elegans* Twist plays an essential role in non-striated muscle development. *Development* **127,** 2041-2051.

Costa, M., Weir, M., Coulson, A., Sulston, J. and Kenyon, C. (1988). Posterior pattern formation in *C. elegans* involves position-specific expression of a gene containing a homeobox. *Cell* **55,** 747-756.

Ekker, S. C., Jackson, D. G., von Kessler, D. P., Sun, B. I., Young, K. E. and Beachy, P. A. (1994). The degree of variation in DNA sequence recognition among four *Drosophila* homeotic proteins. *EMBO J.* **13,** 3551-3560.

Favier, B., Rijli, F. M., Fromental-Ramain, C., Fraulob, V., Chambon, P. and Dolle, P. (1996). Functional cooperation between the non-paralogous genes *Hoxa-10* and *Hoxd-11* in the developing forelimb and axial skeleton. *Development* **122,** 449-460.

Ferreira, H. B., Zhang, Y., Zhao, C. and Emmons, S. W. (1999). Patterning *of Caenorhabditis elegans* posterior structures by the *Abdominal-B* homolog, *egl-5*. *Devel. Biol.* **207,** 215-228.

Ferretti, E., Marshall, H., Popperl, H., Maconochie, M., Krumlauf, R. and Blasi, F. (2000). Segmental expression of Hoxb2 in r4 requires two separate sites that integrate cooperative interactions between Prep1, Pbx and Hox proteins. *Development* **127,** 155-166.

Fire, A., Harrison, S. W. and Dixon, D. (1990). A modular set of *lacZ* fusion vectors for studying gene expression in *Caenorhabditis elegans*. *Gene* **93,** 189-198.

Fire, A., Ahnn, J., Kelly, W., Harfe, B., Kostas, S., Hsieh, J., Hsu, M. and Xu, S. (1998a). GFP applications in *C. elegans*. In *GFP: Green Fluorescence Protein Strategies and Applications* (ed. M. Chalfie and S. Kain). pp153-168. John Wiley and Sons, New York.

Fire A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998b). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391,** 806-811.

Frasch, M. and Nguyen, H. T. (1999). Genetic control of mesoderm patterning and differentiation during *Drosophila* embryogenesis. *Adv. Devel. Biochem.* **5,** 1-47.

Greig, S. and Akam, M. (1993). Homeotic genes autonomously specify one aspect of pattern in the *Drosophila* mesoderm. *Nature* **362,** 630-632.

Greig, S. and Akam, M. (1995). The role of homeotic genes in the specification of the *Drosophila* gonad. *Curr. Biol.* **5,** 1057-1062.

Harfe, B. D., Branda, C. S., Krause, M., Stern, M. J. and Fire, A. (1998a). MyoD and the specification of muscle and non-muscle fates during postembryonic development of the *C. elegans* mesoderm. *Development* **125,** 2479-2488.

Harfe, B. D., Gomes, A. V., Kenyon, C., Liu, J., Krause, M. and Fire, A. (1998b). Analysis of a *Caenorhabditis elegans* Twist homolog identifies conserved and divergent aspects of mesodermal patterning. *Genes Dev.* **12,** 2623-2635.

Hunter, C. P. and Kenyon, C. (1995). Specification of anteroposterior cell fates in *Caenorhabditis elegans* by *Drosophila Hox* proteins. *Nature* **377,** 229-232.

Kelly, W. G., Xu, S., Montgomery, M. K. and Fire, A. (1997). Distinct requirements for somatic and germline expression of a generally expressed *Caenorhabditis elegans* gene. *Genetics* **146**, 227-238.

Kenyon, C. (1986). A gene involved in the development of the posterior body region of *C. elegans*. *Cell* **46,** 477-487.

Keynon, C. J., Austin, J., Costa, M., Cowing, D. W., Harris, J. M., Honigberg, L., Hunter, C. P., Maloof, J. N., Muller-Immergluck, M. M., Salser, S. J., Waring, D. A., Wang, B. B. and Wrischhik, L. A. (1997). The dance of the *Hox* genes: patterning the anteroposterior body axis of *Caenorhabditis elegans*. *Cold Spring Harb. Symp. Quant. Biol.* **62,** 293-305.

Knoepfler, P. S., Lu, Q. and Kamps, M. P. (1996). Pbx1-Hox heterodimers bind DNA on inseparable half-sites that permit intrinsic DNA binding specificity of the Hox partner at nucleotides 3′ to a TAAT motif. *Nucl. Acids Res.* **24,** 2288-2294.

Krause, M. W., Fire, A., Harrison, S. W., Priess, J. and Weintraub, H. (1990). CeMyoD accumulation defines the bodywall muscle cell fate during *C. elegans* embryogenesis. *Cell* **63,** 907-922.

Krause, M., Park, M., Zhang, J.-M., Yuan, J., Harfe, B., Xu, S.-Q., Greenwald, I., Cole, M., Paterson, B. and Fire, A. (1997). A *C. elegans* E/Daughterless bHLH protein marks neuronal but not striated muscle development. *Development* **124,** 2179-2189.

Krumlauf, R. (1994). *Hox* genes in vertebrate development. *Cell* **78,** 191-201.

Kurant, E., Pai, C.-Y., Sharf, R., Halachmi, N., Sun, Y. and Salzberg, A. (1998). *dorsotonals/homothorax*, the *Drosophila* homologue of *meis1*, interacts with *extradenticle* in patterning of the embryonic PNS. *Development* **125,** 1037-1048.

Lawrence, P. A. and Morata, G. (1994). Homeobox genes: their function in *Drosophila* segmentation and pattern formation. *Cell* **78,** 181-189.

Lewis, E. (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* **276,** 565-570.

Li X., Murre, C. and McGinnis, W. (1999). Activity regulation of a Hox protein and a role for the homeodomain in inhibiting transcriptional activation. *EMBO J.* **18,** 198-211.

Maloof, J. N. and Kenyon, C. (1998). The Hox gene *lin-39* is required during *C. elegans* vulval induction to select the outcome of Ras signaling. *Development* **125,** 181-190.

Mann, R. S. (1995). The specificity of homeotic gene function. *BioEssays* **17,** 855-863.

Mann, R. S. and Chan, S-K. (1996). Extra specificity from *extradenticle*: the partnership between HOX and PBX/EXD homeodomain proteins. *Trends Genet.***12,** 257-262.

Mann, R. S. and Affolter, M. (1998). Hox proteins meet more partners. *Curr. Opin. Genet. Dev.* **8,** 423-429.

Michelson, A. M. (1994). Muscle pattern diversification in *Drosophila* is determined by the autonomous function of homeotic genes in the embryonic mesoderm. *Development* **120,** 755-768.

Pai, C-Y., Kuo, T-S., Jaw, T. J., Kurant, E., Chen, C-T., Bessarab, D. A., Salzberg, A. and Sun, Y. H. (1997). The Homothorax homeoprotein activates the nuclear localization of another homeoprotein, Extradenticle, and suppresses eye development in *Drosophila*. *Genes Dev.* **12,** 435-446.

Passner, J. M., Ryoo, H. D., Shen, L., Mann, R. S. and Aggarwal, A. K. (1999). Structure of a DNA-bound Ultrabithorax-Extradenticle homeodomain complex. *Nature* **397,** 714-719.

Peifer, M. and Wieschaus, E. (1990). Mutations in the *Drosophila* gene *extradenticle* affect the way specific homeo domain proteins regulate segmental identity. *Genes Dev.* **4,** 1209-1223.

Pinsonneault, J., Florence, B., Vaessin, H. and McGinnis, W. (1997). A model for *extradenticle* function as a switch that changes HOX proteins from repressors to activators. *EMBO J.* **16,** 2032-2042.

Piper, D. E., Batchelor, A. H., Chang, C.-P., Cleary, M. L. and Wolberger, C. (1999). Structure of a HoxB1-Pbx1 heterodimer bound to DNA: role of the hexapeptide and a fourth homeodomain helix in complex formation. *Cell* **96,** 587-597.

Rauskolb, C., Smith, K. M., Peifer, M. and Wieschaus, E. (1995). *extradenticle* determines segmental identities throughout *Drosophila* development. *Development* **121,** 3663-3673.

Rieckhof, G., Casares, F., Ryoo, H. D., Abu-Shaar, M. and Mann, R. S. (1997). Nuclear translocation of Extradenticle requires *homothorax*, which encodes an Extradenticle-related homeodomain protein. *Cell* **91,** 171-183.

Ruvkun, G. and Hobert, O. (1998). The taxonomy of developmental control in *Caenorhabditis elegans*. *Science* **282,** 2033-2041.

Ryoo, H. D., Marty, T., Casares, F., Affolter, M. and Mann, R. S. (1999). Regulation of Hox target genes by a DNA bound Homothorax/Hox/Extradenticle complex. *Development* **126,** 5137-5148.

Salser, S. J. (1995). Role of Hox gene expression in patterning cell fates and migrations along the *Caenorhabditis elegans* anteroposterior body axis. Ph.D. thesis, Univ. of California, San Francisco.

Salser, S. J., Loer, C. M. and Kenyon, C. (1993). Multiple HOM-C gene interactions specify cell fates in the nematode central nervous system. *Genes Dev.* **7,** 1714-1724.

Salser, S. J. and Kenyon, C. (1994). Patterning *C. elegans*: homeotic cluster genes, cell fates and cell migrations. *Trends Genet.* **10,** 159-164

Schaller, D., Wittmann, C., Spicher, A., Muller, F. and Tobler, H. (1990). Cloning and analysis of three new homeobox genes from the nematode *Caenorhabditis elegans*. *Nucl. Acids Res.* **18,** 2033-2036.

Sulston, J. E. and Horvitz, H. R. (1977). Post-embryonic lineages of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **56,** 110-156.

Sulston, J. E., Schierenberg, E., White, J. G. and Thomas, J. N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100,** 64-119.

van Dijk, M. A. and Murre, C. (1994). *extradenticle* raises the DNA binding specificity of homeotic selector gene products. *Cell* **78,** 617-624.

Wang, R. B., Muller-Immergluck, M. M., Austin, J., Robinson, N. T., Chisholm, A. and Kenyon, C. (1993). A homeotic gene cluster patterns the anteroposterior body axis of *C. elegans*. *Cell* **74,** 29-42.

Wilson, D. S. and Desplan, C. (1995). Cooperating to be different. *Curr. Biol.* **5,** 32-34.