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Direct regulation of *egl-1* and of programmed cell death by the Hox protein MAB-5 and by CEH-20, a *C. elegans* homolog of Pbx1

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Hox genes are crucial determinants of cell fates and of body morphology of animals; mutations affecting these genes result in abnormal patterns of programmed cell death. How Hox genes regulate programmed cell death is an important and poorly understood aspect of normal development. In the nematode *C. elegans,* the Hox gene *mab-5* is required for the programmed cell deaths of two lineally related cells generated in the P11 and P12 lineages. We show here that in the P11 lineage, a complex between MAB-5 and the Pbx homolog CEH-20 directly regulates transcription of the BH3 domain gene *egl-1* to initiate programmed cell death; in the P12 lineage, *mab-5* and *ceh-20* apparently act indirectly to initiate programmed cell death. Direct regulation of programmed cell death may be an evolutionarily ancient and conserved function of Hox genes.

KEY WORDS: *C. elegans*, Hox genes, Programmed cell death

INTRODUCTION

Hox proteins specify cell fates along the anteroposterior body axis in metazoans. In many organisms, including *C. elegans*, *Drosophila* and mammals, mutations that affect Hox gene function result in abnormal patterns of programmed cell death (Bello et al., 2003; Clark et al., 1993; Economides et al., 2003; Gavalas et al., 2003; Kenyon, 1986; Lohmann et al., 2002; Miguel-Aliaga and Thor, 2004; Salser et al., 1993; Stadler et al., 2001). Programmed cell death is a widespread and, in most organisms, essential aspect of normal development; mutations that influence whether cells survive or undergo programmed cell death contribute to human disease. Discovering how Hox genes regulate programmed cell death is therefore important for understanding the roles of Hox genes in development and disease.

As homeodomain-containing proteins, Hox proteins are likely to act by regulating transcription. The crucial task is therefore to identify those targets regulated by Hox proteins that determine programmed cell death or survival of individual cells or groups of cells. At present, it is not clear whether, for example, the abnormalities in programmed cell death observed in Hox mutants represent transformations in cell fate that then indirectly manifest as abnormal patterns of programmed cell death, or whether Hox genes directly determine programmed cell death or survival by regulating transcription of cell death genes. Recent data in *Drosophila* indicate that the Hox protein Deformed directly regulates transcription of the proapoptotic gene *reaper* to induce programmed cell death and shape a morphological boundary (Lohmann et al., 2002). Whether this is a general function of Hox genes during development needs to be explored.

The genetic pathway for the execution of programmed cell death in *C. elegans* is well established and highly conserved across animal species (Metzstein et al., 1998). In addition, the essentially invariant pattern of development makes it possible to study cell fates at the level of individual cells (Sternberg and Horvitz, 1984). *C. elegans* therefore presents an excellent model in which to study the mechanisms that regulate cell fates and programmed cell death.

The *C. elegans* genome contains six Hox genes organized as three gene pairs in a contiguous region of chromosome III. As in other metazoans, the Hox genes of *C. elegans* are generally organized along the chromosome in the order in which they are expressed in the animal, with one exception (WormBase website, http://www.wormbase.org, release WS138, 11 February 2005) (Brunschwig et al., 1999; Clark et al., 1993; Wang et al., 1993). The six Hox genes include single genes orthologous to *labial* (*ceh-13*), *sex combs reduced* (*lin-39*) and *antennapedia* (*mab-5*), and three posterior Hox genes (*egl-5*, *php-3*, *nob-1*) (Aboobaker and Blaxter, 2003; Van Auken et al., 2002; Wang et al., 1993). Three Hox genes, *lin-39*, *mab-5* and *egl-5*, for which mutants bearing null alleles are viable, have been extensively characterized (Chisholm, 1991; Clark et al., 1993; Kenyon, 1986; Wang et al., 1993). Mutations affecting these genes result in abnormal patterns of programmed cell death. Specifically, *lin-39* is essential for the survival of six neurons, the VC neurons, generated in the midregion of the ventral nerve cord (Clark et al., 1993), and *mab-5* is essential for the programmed cell death of two cells, P11.aaap and P12.aaap, generated in the posterior ventral nerve cord (Kenyon, 1986); (Pn.aaap, the posterior daughter of the anterior daughter of the anterior daughter of the anterior daughter of any P blast cell). In *lin-39* mutants, the six VC neurons in the midbody of the ventral nerve cord express fates characteristic of their more anterior and posterior lineal homologs [lineal homologs are cells arising at corresponding positions of related lineages, and they typically adopt related fates (Sulston and Horvitz, 1977)], which undergo programmed cell death, suggesting *lin-39* provides spatial information to the developing animal (Clark et al., 1993) and consistent with the functions of Hox genes in other animals (McGinnis and Krumlauf, 1992). A similar role has been suggested for *mab-5* in the posterior ventral nerve cord, where the P(11,12).aaap cells of *mab-5* mutants survive, as do their anterior lineal homologs (Kenyon, 1986).

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How mutations in *lin-39* and *mab-5* determine cell death or survival is not yet known. Characterization of *mab-5* mutants suggests that *mab-5* function is necessary but not sufficient to specify programmed cell death of the P(11,12).aaap cells (Salser et al., 1993). For example, strong loss of function or null mutations in *mab-5* result in survival only of P(11,12).aaap in the ventral nerve cord; programmed cell death of other cells in the ventral nerve cord occurs normally. In mutants that ectopically express *mab-5*, the anteriorly located lineal homologs of the P(11,12).aaap cells do not undergo programmed cell death (Salser et al., 1993). These experiments suggest the existence of factors that prevent programmed cell death of the P(11,12).aaap anterior lineal homologs or that act with *mab-5* in P(11,12).aaap to ensure their death. Given the similarity in cell division patterns and fates of cells generated by the P11 and P12 lineages (Sulston and Horvitz, 1977), it was surprising to find that MAB-5 protein is detected in the P11 blast cell and the five cells ultimately generated by P11 including P11.aaap, but not in the P12 descendants after the first division (Salser et al., 1993). *mab-5* might therefore determine cell fates through different mechanisms in the P11 and P12 lineages, including the P(11,12).aaap programmed cell deaths.

Hox proteins can bind DNA cooperatively with protein co-factors, including the PBC family of homeodomain proteins (Chan et al., 1994). The PBC family, which includes *Drosophila* Extradenticle and the mammalian Pbx proteins (Burglin, 1997), binds to a specific subset of Hox proteins via an interaction between a conserved hexapeptide motif found in the Hox protein (Chang et al., 1995) and a pocket in the co-factor (Piper et al., 1999). The *C. elegans* genome encodes two proteins similar to Pbx, CEH-20 and CEH-40 (WormBase web site, http://www.wormbase.org, release WS138, 11 February 2005), and the Hox proteins LIN-39 and MAB-5 both contain the hexapeptide motif required for interaction with PBC proteins. *ceh-20* cooperates with *lin-39* and *mab-5* during mesoderm differentiation, where a LIN-39/CEH-20 heterodimer directly regulates the *C. elegans* homolog of *twist* (Liu and Fire, 2000), and *ceh-20* and *ceh-40* act partially redundantly during embryonic development (Van Auken et al., 2002). *ceh-20* mutants have multiple defects in vulval development and neuronal migration that in part are similar to those of *lin-39* mutants, but also have distinct defects that suggest *lin-39*-independent functions (Yang et al., 2005).

In *C. elegans*, the BH3 domain-encoding gene *egl-1* is required for programmed cell death of somatic cells, and expression of *egl-1* is sufficient to induce programmed cell death (Conradt and Horvitz, 1998). The EGL-1 protein physically interacts with the Bcl2 homolog CED-9 to initiate programmed cell death (Conradt and Horvitz, 1998). Two pathways are known that directly regulate expression of *egl-1* and the programmed cell death of specific cells. In the HSN neurons, the TRA-1 transcription factor binds *egl-1* regulatory sequences to prevent programmed cell death (Conradt and Horvitz, 1999). *egl-1* mutations that prevent TRA-1 binding result in expression of *egl-1* in the HSNs, their programmed cell death and an egg-laying defect. In a specific subset of pharyngeal neurons, the *ces-2* and *ces-1* genes act in a negative regulatory cascade to promote programmed cell death (Ellis and Horvitz, 1991). The Snail homolog CES-1 directly represses *egl-1* in a process opposed by the bHLH proteins HLH-2 and HLH-3 (Thellmann et al., 2003). These data demonstrate that the fate of programmed cell death of specific cells can be determined by regulating transcription of *egl-1*.

We report here that, as part of its function in specifying fates in the P11 and P12 cell lineages, a complex containing the Hox co-factor and Pbx homolog CEH-20 and the Hox protein MAB-5 directly regulates *egl-1* expression to induce programmed cell death of P11.aaap.

MATERIALS AND METHODS

Genetic methods and strains

Worms were maintained as described by Brenner (Brenner, 1974) at 20°C. The following mutations were used: LGI, *ced-1(e1735)*; LGII, *muIs16* (Cowing and Kenyon, 1996); LGIII, *ceh-20(ay9)*, *ceh-20(ay42)* and *unc-36(e251)*, *unc-119(ed3)*; LGV, *egl-1(n1084n3082)*; LGX, *ceh-40(gk159)*. The strain NC216 containing the $P_{acr\text{-}5}$*gfp wdEx75* array and the NC190 strain containing the $P_{del\text{-}1}$*gfp wdIs6* integrated array were generously provided by David Miller (Vanderbilt University, Nashville, TN). *ceh-20* alleles were a gift from M. Stern (Yale University, New Haven, CT). The *ceh-40(gk159)* allele was isolated and provided by the *C. elegans* Knockout Consortium (http://celeganskoconsortium.omrf.org). The *ceh-40(gk159)* allele deletes 968 bp of genomic sequence including all of predicted exons 2, 3, 4 and 5. If transcribed and translated, the deletion would be predicted to generate a truncated protein that includes the initial 31 amino acids of CEH-40 followed by missense amino acids and premature termination. Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). Lineage analysis was performed as described by Sulston and Horvitz (Sulston and Horvitz, 1977). Biolistic bombardment was performed as described (Praitis et al., 2001).

Plasmid constructs

The wild-type 7.6 kb *egl-1* genomic region is contained in plasmid pBC08 (Conradt and Horvitz, 1998). Site-specific mutagenesis was performed using the method of Quikchange (Stratagene) and all DNA segments exposed to PCR or mutagenesis were fully sequenced. For the *egl-1* transgene containing mutations in sites 1 and 2 and a deletion in the F23B12.1 predicted phosphatase, a 370 nucleotide *Pfl*F1 fragment was removed from the F23B12.1-coding sequences and the plasmid religated. To construct the pMP0017 $P_{ceh\text{-}20}$*ceh-20:cfp* translational fusion plasmid, a 3.6 kb *Sac*I *Pfl*MI fragment of the *ceh-20* genomic region, including ~1 kb of 5′ sequence and 1 kb of 3′ sequence was amplified by long PCR and cloned into pBSKSII$^+$. Site-specific mutagenesis was used to change the stop codon to an *Asc*I restriction site, into which the *cfp*-coding region from pPD134.96 (kindly provided by Andy Fire, Stanford University, Palo Alto, CA) was cloned. This plasmid was injected at a concentration of 50 ng/μl into *ceh-20(ay42),* where it rescued the egg-laying defect, and together with a *lin-15*-rescuing plasmid into *ced-3(n717); lin-15(n765)* mutants. To construct the $P_{egl\text{-}1}$*histone:gfp* reporter, overlap PCR was used to replace the open reading frame of *egl-1* by an *Age*I restriction site. A fusion of the *R08C7.3* histone 2A gene to *gfp* was inserted into the *Age*I site. This plasmid was microinjected into *ced-3(n717); lin-15(n765)* mutants at a concentration of 10 ng/μl and integrated after gamma irradiation. The *mxIs14* integrated array was mapped to LG X.

Fusion proteins and electrophoretic mobility shift assays

Full length *ceh-20* and *mab-5* cDNAs amplified from wild-type *C. elegans* total RNA were cloned as *Msc*I *Xho*I fragments generated by PCR into a derivative of pSP73 that contains the *Xenopus* β-globin 5′ untranslated region (Swift et al., 1998). Oligonucleotides encoding the myc or FLAG epitopes were then ligated into *Msc*I-digested *mab-5* or *ceh-20* expression plasmids. Those regions of constructs generated by PCR or changed after ligation of the oligonucleotides were completely sequenced. Proteins were generated by coupled transcription and translation in vitro using SP6 polymerase and TnT wheat-germ extract (Promega) and efficiency of protein generation was estimated by synthesis of an aliquot including ^{35}S-methionine followed by autoradiography (for native proteins) or after western blot (for epitope-tagged proteins). For EMSAs, proteins were preincubated for 15 minutes at 37°C in a 10 μl reaction including 20 mM HEPES (pH 7.9), 15% glycerol and 0.2 mM EDTA. Probe (50,000 cpm) in 10 μl with 100 mM KCl, 5 mM DTT, 5 mM $MgCl_2$, 50 ng polydI-dC, 50 ng

E. coli genomic DNA and 1% NP40 was also preincubated for 15 minutes at 30°C. Probe and proteins were combined and incubated on ice for 30 minutes, then separated on an 8% polyacrylamide gel in 0.5×TBE buffer run at 25 mA at 4°C. Probe sequences are available on request.

Sequence comparisons

Family Relations and SeqComp software (Brown et al., 2002) was used to compare sequences in the *egl-1* genes of *C. elegans* and *C. briggsae*.

RESULTS

mab-5 determines aspects of identity in addition to promoting programmed cell death

The P11.aaap and P12.aaap cells are lineal equivalents of the VB motoneurons (Sulston and Horvitz, 1977), which express the predicted acetylcholine receptor subunit *acr-5* and the sodium channel *del-1* (Esmaeili et al., 2002; Winnier et al., 1999). We used *gfp* reporter constructs to examine expression of these markers in *ced-3* mutants, in which the death of the P(11,12).aaap cells was prevented by a block in programmed cell death, and compared the results with those where death of the cells was prevented by mutation of *mab-5*. Thirty out of 30 animals of genotype $P_{del\text{-}1}gfp$; *ced-3(n717)* expressed *gfp* in P11.aaap, while only one out of 30 animals of genotype $P_{del\text{-}1}gfp$; *mab-5(n1384)* did so. Similarly, 24 of 30 animals of genotype $P_{acr\text{-}5}gfp$; *ced-3(n717)* expressed *gfp* in P11.aaap, while only two out of 30 animals of genotype $P_{del\text{-}1}gfp$; *mab-5(n1384)* did so. These data suggest that *mab-5* may determine aspects of P11.aaap identity in addition to inducing programmed cell death of the cell, or that it directly promotes expression of the *acr-5* and *del-1* transgenes.

The Hox co-factor *ceh-20* regulates programmed cell deaths

Hox proteins can act with co-factors to alter their binding affinity and specificity (Mann and Affolter, 1998). *ceh-20* and *ceh-40* each encode homologs of the extradenticle/Pbx TALE class of homeodomain proteins (http://www.wormbase.org, release WS138, 11 February 2005). *ceh-20* acts with *lin-39* and *mab-5* to pattern mesoderm (Liu and Fire, 2000), and redundantly with *ceh-40* during embryonic development (Van Auken et al., 2002). *ceh-20* mutants also have defects in neuronal migration that are in part similar to those in *lin-39* mutants (Yang et al., 2005). In the ventral nerve cord, *ceh-20* mutants have defects in programmed cell death consistent with loss of *lin-39* function in the midbody and of *mab-5* function in the posterior (Table 1). In mutants carrying the strong loss-of-function allele *ceh-20(ay42)* (M. Stern, personal communication; null alleles are lethal), the defects include programmed cell death of six cells in the midregion of the ventral nerve cord and survival of two cells in the posterior ventral nerve cord (Fig. 1). We followed cell lineages in *ceh-20(ay42)* mutants and directly observed programmed cell death of the VC neurons in the midbody, as in *lin-39* mutants, and survival of the P(11,12).aaap cells, as in *mab-5* mutants (Fig. 1). *ceh-40(gk159)* mutants have a normal pattern of cell deaths in the ventral nerve cord, and the defects in cell death in mutants carrying a weak allele of *ceh-20, ay9* are not enhanced by

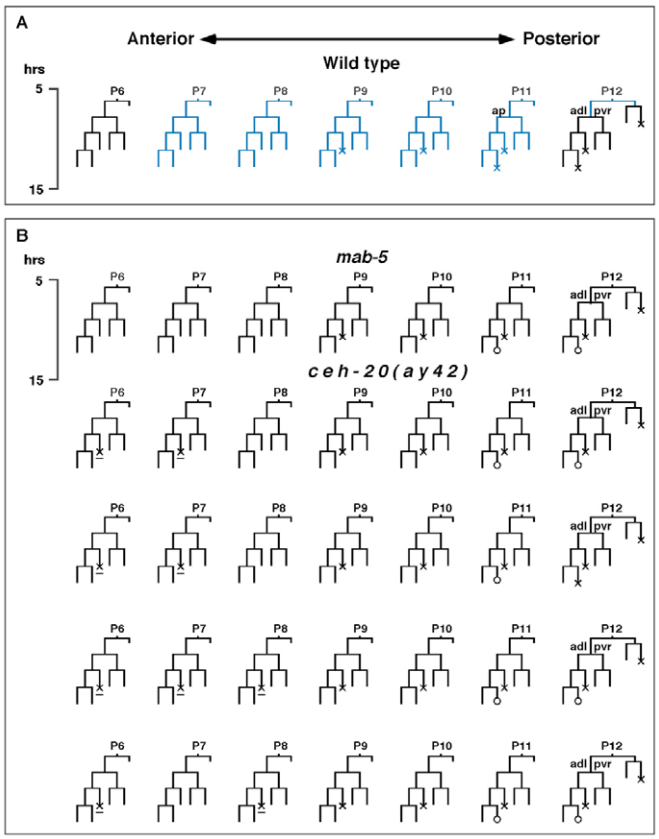


Fig. 1. *ceh-20* mutants have defects in programmed cell death. X indicates cells that die in wild-type animals; X̲ indicates programmed cell deaths of cells that survive in wild type animals; an open circle indicates cells that survive in *ceh-20* mutants but undergo programmed cell death in wild-type animals. (**A**) In the P12 cell lineage of wild-type animals, the P12.a division axis is anterior dorsal left (adl) and posterior ventral right (pvr). (**B**) The P12 lineages of *ceh-20* mutants did not show this evidence of transformation to the P11 fate. *mab-5* lineage is from Kenyon (Kenyon, 1986). Cells in which MAB-5 protein is detected are indicated in blue, with data taken from Salser et al. (Salser et al., 1993).

Table 1. *ceh-20* acts together with Hox genes to determine cell death and survival

Genotype	Number of corpses in ventral nerve cord*		
	Anterior (W, P1, P2)	Midbody (P3-P8)	Posterior (P9-P12)
ced-1	2.8±0.1	0	5.5±0.1
ced-1; egl-1(n1084n3082)	0.1±0.0	0	0
ced-1; mab-5(e1239)	2.4±0.1	0	3.5±0.2
ced-1; ceh-20(ay42)	2.3±0.5	5.2±0.8 (range 4-6)	2.9±0.9
ced-1; ceh-20(ay9)	2.5±0.5	2.9±1.2 (range 1-6)	4.4±1.2
ced-1; ceh-40(gk159)	2.4±0.6	0	5.8±0.8
ced-1; ceh-20(ay9); ceh-40(gk159)	2.7±0.4	2.3±0.5 (range 1-5)	4.7±1.0

*The number of cell corpses in the indicated regions of the ventral nerve cord was determined by observation using Nomarski optics. In wild-type animals, there are three, zero and seven cell deaths in the anterior, midbody and posterior ventral nerve cord, respectively (Sulston and Horvitz, 1977). Mutation of *ced-1* does not completely block engulfment (Ellis et al., 1991; Zhou et al., 2001) and engulfment contributes to cell killing (Reddien et al., 2001), accounting for the small differences between numbers of cell deaths and numbers of cell corpses in otherwise wild-type *ced-1* mutants. The *ced-1(e1735)* allele was used in all strains. For all genotypes, *n*=30.

the *ceh-40(gk159)* deletion, suggesting that *ceh-40* is dispensable for determining the pattern of programmed cell death in the ventral nerve cord (Table 1). In contrast to the highly penetrant defects in *lin-39* and *mab-5* function, we did not observe any transformation of the P12 lineage to the P11 fate, as is observed in *egl-5* mutants (Chisholm, 1991), suggesting that *ceh-20* may not act as the co-factor for *egl-5* in this lineage.

egl-1 expression correlates with the pattern of programmed cell death in the posterior ventral nerve cord

In some *C. elegans* cells, programmed cell death is regulated by controlling transcription of *egl-1*. To begin to determine whether Hox proteins might promote survival or programmed cell death of cells by directly regulating transcription of *egl-1*, we constructed a reporter transgene in which the *egl-1* open reading frame was precisely replaced by a *histone:gfp* fusion (see Materials and methods) and examined expression of this reporter in wild-type and Hox mutant backgrounds. In wild-type transgenic hermaphrodites, expression of the reporter in the posterior ventral nerve cord matches the wild-type pattern of programmed cell deaths, with expression of *gfp* in one nucleus each generated by the P9 and P10 lineages, two nuclei generated by the P11 lineage, and three nuclei generated by the P12 lineage (Fig. 2; data not shown). In *mab-5* and *ceh-20* mutants (see below) the pattern of fluorescent nuclei is changed to match the pattern of programmed cell death in the mutants (Fig. 2; data not shown); specifically, the reporter is not expressed in P(11,12).aaap. These results are consistent with transcriptional regulation of *egl-1* to induce programmed cell death of these cells. The P(11,12).aaap cells do not undergo programmed cell death in *egl-1* mutants (Table 1) consistent with the essential role of *egl-1* in the programmed cell death of somatic cells (Conradt and Horvitz, 1998).

Hox genes act upstream of or parallel to the cell death gene *egl-1*

To address where in the genetic pathway for programmed cell death the Hox genes might act, we determined whether loss-of-function mutations in *egl-1* could prevent programmed cell deaths in *lin-39* mutants. The $P_{lin\text{-}11}gfp$ reporter construct is expressed in the six VC motoneurons of wild-type animals (Cameron et al., 2002). *lin-39(n1760);* $P_{lin\text{-}11}gfp$ mutants do not express the reporter in the midbody (Fig. 3), consistent with programmed cell death of the VC neurons in these mutants (Clark et al., 1993). In *lin-39(n1760); egl-1(n1084n3082);* $P_{lin\text{-}11}gfp$ mutants the VC neurons survive and express the $P_{lin\text{-}11}gfp$ reporter, suggesting that *lin-39* acts upstream of or parallel to *egl-1*. We performed similar experiments with *ceh-20* mutants (Fig. 3). Mutants carrying the weak *ay9* allele of *ceh-20* express the reporter in few cells in the midbody, consistent with programmed cell death of many of the VC neurons in these mutants. The VC neurons survive and express the $P_{lin\text{-}11}gfp$ reporter in *ceh-20(ay9); egl-1(n1084n3082);* $P_{lin\text{-}11}gfp$ mutants, but expression of the reporter was weak in some VC neurons, suggesting *ceh-20* may determine other aspects of VC identity in addition to being required for their survival. This suggestion is supported by the phenotype of mutants carrying the strong *ay42* allele of *ceh-20.* In *ceh-20(ay42); egl-1(n1084n3082);* $P_{lin\text{-}11}gfp$ mutants the VC neurons survive but do not express $P_{lin\text{-}11}gfp$ (Fig. 3). Expression of $P_{lin\text{-}11}gfp$ in *lin-39* null alleles but not in *ceh-20* mutants also suggests that *ceh-20* has *lin-39*-independent functions. These data are consistent with the model that *lin-39* and *ceh-20* act through *egl-1* to prevent programmed cell death of the VC neurons.

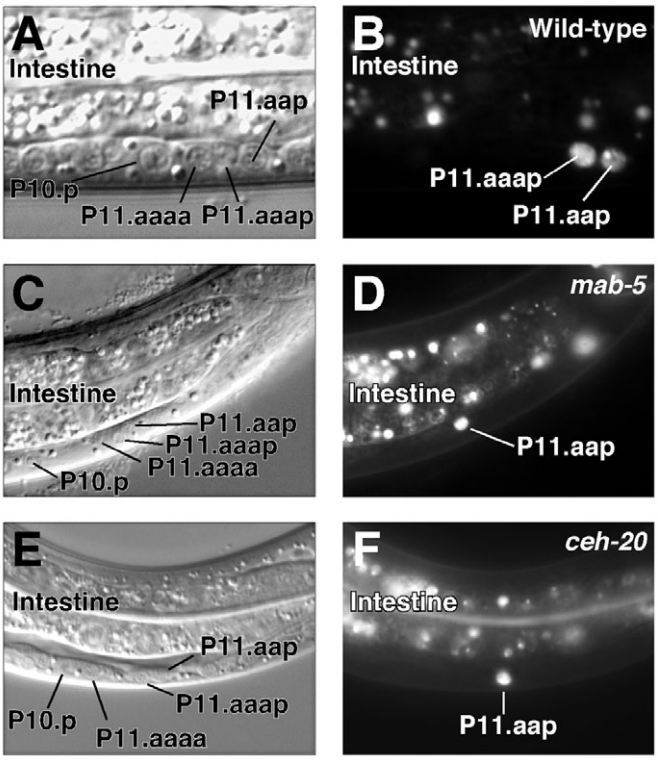


Fig. 2. Transcription of *egl-1* correlates with programmed cell death. Nomarski optics (A,C,E) and epifluorescence (B,D,F) of the posterior ventral nerve cord of L3 stage larvae carrying an integrated $P_{egl\text{-}1}$*histone:gfp* reporter construct localized to nuclei. The descendants of P11.aaa are reproducibly located immediately posterior to the hypodermal cell P10.p. (**A**,**B**) In *ced-3;* $P_{egl\text{-}1}$*histone:gfp* transgenic animals (30 out of 30 animals), *egl-1* is expressed in the two cells that undergo programmed cell death in the P11 lineage (P11.aap and P11.aaap). (**C**,**D**) In *mab-5(n1384); ced-3;* $P_{egl\text{-}1}$*histone:gfp* transgenic mutants, the *egl-1* transcriptional reporter is not expressed (27 of 30 mutants) in P11.aaap, which survives in *mab-5* mutants. (**E**,**F**) In *ceh-20(ay42); ced-3;* $P_{egl\text{-}1}$*histone:gfp* mutants, the *egl-1* reporter is not expressed (58 out of 60 mutants) in P11.aaap, which survives in *mab-5* and *ceh-20* mutants. The P12 lineage descendants arise in the preanal ganglion, which contains other neuronal cells; consequently, we were unable to identify unambiguously P12.aaap. However, the $P_{egl\text{-}1}$*histone:gfp* reporter was expressed in two rather than three cells in the preanal ganglion of *mab-5* and *ceh-20* mutants.

A CEH-20/MAB-5 complex directly regulates *egl-1* expression in P11.aaap

A complex between CEH-20 and a Hox protein could regulate programmed cell death through direct regulation of *egl-1* transcription or indirectly through other cell fate determinants. We developed a rescue assay to define the *egl-1* genomic sequences required to restore a normal pattern of programmed cell death in the ventral nerve cord of *egl-1* mutants, and used this assay to examine candidate elements regulated by a CEH-20/Hox complex. A 7.6 kb genomic fragment of wild-type *egl-1* genomic DNA fully rescued programmed cell death in the ventral nerve cord of *egl-1(n1084n3082)* mutants (Fig. 4; data not shown). We compared *egl-1* genomic sequences between *C. elegans* and the related nematode *C. briggsae* to identify evolutionarily conserved sequences identical to the TGATNNAT consensus sequence

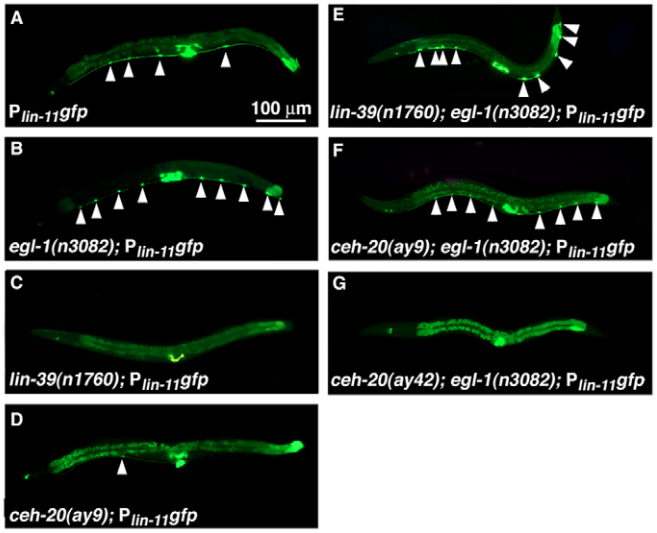


Fig. 3. *lin-39* and *ceh-20* are likely to act through *egl-1* in the VC neurons. For all genotypes, the expression pattern of the $P_{lin\text{-}11}gfp$ reporter was scored in 50 animals. (**A**,**B**) The $P_{lin\text{-}11}gfp$ reporter is expressed in the six VC neurons of wild-type animals [average of 4.4 cells; two VC neurons (VC4 and VC5) are often obscured by vulval and sex muscle expression, and are not scored] and in the VC-like neurons that survive in an *egl-1* mutant (average of 8.7 cells from P2-P12 descendants) (Cameron et al., 2002). (**C**,**E**) VC neurons undergo programmed cell death in *lin-39* mutants (average of 0 cells). VC and VC-like neurons survive and express $P_{lin\text{-}11}gfp$ in *lin-39; egl-1;* $P_{lin\text{-}11}gfp$ mutants (average of 9.0 cells). In *lin-39* mutants, the P cell descendants migrate abnormally far forwards (Clark et al., 1993). (**D**,**F**) Many VC neurons die in *ceh-20(ay9)* mutants. In *ceh-20(ay9); egl-1;* $P_{lin\text{-}11}gfp$ mutants, the VC and VC-like neurons survive and express $P_{lin\text{-}11}gfp$ but the fluorescence is of variable brightness, especially in the anterior nerve cord (average of 7.4 cells). (**G**) In *ceh-20(ay42); egl-1;* $P_{lin\text{-}11}gfp$ mutants, the VC and VC-like neurons survive but do not express $P_{lin\text{-}11}gfp$ (average of 0 cells). Direct observation of cell lineages in the *ceh-20(ay42); egl-1;* $P_{lin\text{-}11}gfp$ mutant confirmed that the VC neurons did not undergo programmed cell death. Arrowheads indicate nuclei of VC and VC-like neurons.

bound by Exd/Hox complexes. In this site, the 5′ half mediates binding by the PBC co-factor and the 3′ half mediates Hox binding (Chan and Mann, 1996; Mann and Affolter, 1998). Four such matches were identified (Fig. 4). Transgenic animals carrying an *egl-1* genomic construct in which all four sites had been mutated from TGATNNAT to TCCATGGT had defects in programmed cell death in the ventral nerve cord (Fig. 4). Specifically, one cell in the P11 lineage survived in virtually all transgenic animals, and one cell in the P12 lineage often survived. Mutation of only the candidate Hox co-factor/Hox site at position +5995 relative to the *egl-1* ATG was completely sufficient for this phenotype (Fig. 4; see Fig. S1 in the supplementary material). Mutation of this site in the $P_{egl\text{-}1}$*histone:gfp* reporter resulted in a failure to express *gfp* in P11.aaap, indicating that this site regulates transcription of *egl-1* in this cell (Fig. 4). This site is also conserved in the *egl-1* gene of *Caenorhabditis remanei* (data not shown). Transgenic animals with mutations of two nucleotides in the Hox half site or three nucleotides in the Hox co-factor half site were indistinguishable from the TCCATGGT mutant, suggesting binding by both a Hox and its co-factor is required for regulation (Fig. 4).

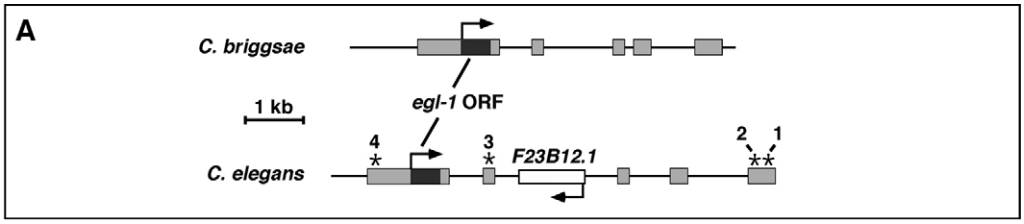
Electrophoretic mobility shift assays with epitope-tagged MAB-5 and CEH-20 proteins generated by coupled transcription and translation in vitro demonstrated specific binding by a CEH-20/MAB-5 protein complex to an oligonucleotide containing the site at +5995 of *egl-1*, and little binding by MAB-5 or CEH-20 alone (Fig. 5). Addition of antibody recognizing the epitope-tagged CEH-20 or MAB-5 altered mobility of the binding complex, consistent with binding of the oligonucleotide in vitro by a CEH-20/MAB-5 complex. Oligonucleotides with the TCCATGGT mutation or specific mutations of the individual half sites competed poorly for binding by the CEH-20/MAB-5 complex, consistent with these mutations disrupting function of the site in vivo.

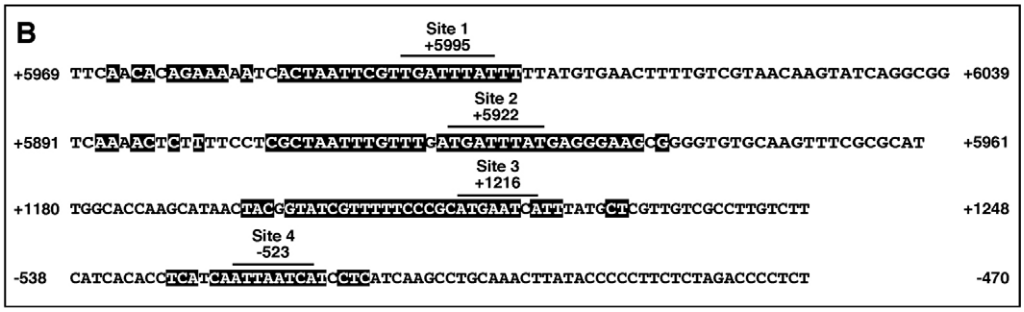
ceh-20 is expressed in P(11,12).aaap and many other cells

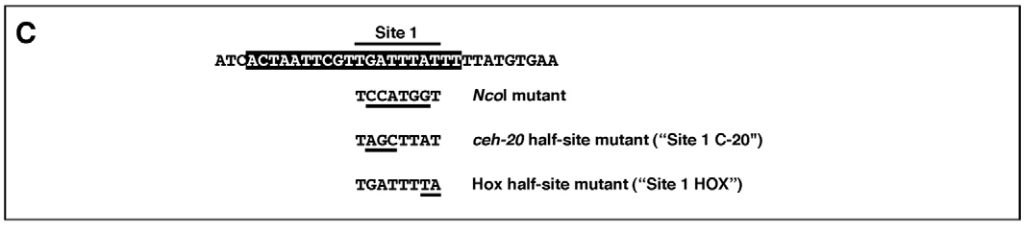
To examine in which cells the *ceh-20* gene is expressed, and specifically to test whether *ceh-20* is expressed in P11.aaap where CEH-20 could interact with MAB-5 to regulate *egl-1*, we generated a rescuing *cfp* reporter construct. We generated transgenic animals that expressed a full-length CEH-20 protein with a fusion of CFP to the C terminus of the protein. Introduction of this construct into *ceh-20(ay42)* mutants rescued the egg-laying defect (data not shown). We introduced this construct into *ced-3* mutants, in which P11.aaap and the other cells that undergo programmed cell death in the ventral nerve cord survive, and examined expression of the reporter construct. Consistent with a recent report of the expression pattern of a similar construct (Yang et al., 2005), we identified broad expression in many nuclei, including most ventral nerve cord neurons. We specifically identified expression in P11.aaap, which undergoes programmed cell death in a *ceh-20*-dependent fashion (Fig. 6). To address the possibility that *ceh-20* is required for expression of *mab-5*, we examined expression of a $P_{mab\text{-}5}gfp$ reporter (Cowing and Kenyon, 1996) in *ceh-20(ay42)* mutants and identified no significant difference (Fig. 6), suggesting that *ceh-20* is not required for expression of *mab-5* in the P11 lineage. However, as *ceh-20(ay42)* is a strong loss of function but not null allele, this does not rule out regulation of Hox genes by *ceh-20*.

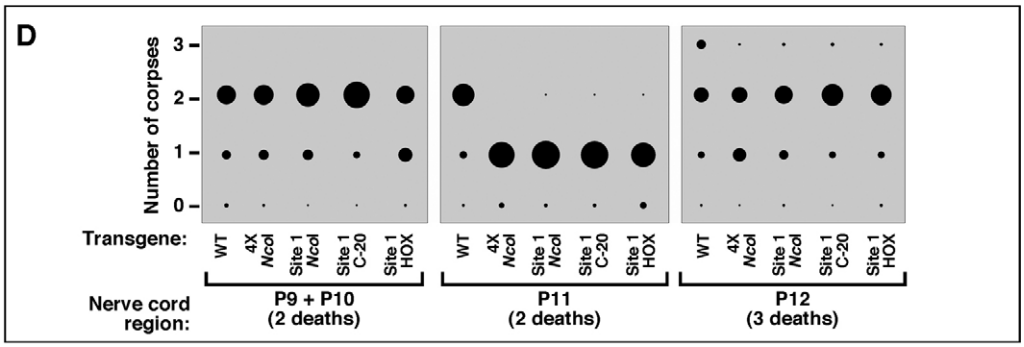
egl-1 is regulated differently in P11.aaap and P12.aaap

To determine precisely which cells are affected by mutation of the site at +5995, we followed P11 and P12 cell lineages in transgenic animals carrying the TCCATGGT mutation at position +5995 of an integrated *egl-1* transgene (Fig. 7). In six out of seven animals, mutation of the site resulted in survival of P11.aaap, which survives in *ceh-20* and *mab-5* mutants. By contrast, in seven of seven animals P12.aaap, which survives in *ceh-20* and *mab-5* mutants, underwent programmed cell death. In three out of seven animals, we observed survival of P12.pp, a cell that in wild-type animals undergoes programmed cell death (Sulston and Horvitz, 1977). MAB-5 protein is present in P11.aaap when the cell is generated (Salser et al., 1993), consistent with binding by a CEH-20/MAB-5 complex to the site at +5995 and direct activation of *egl-1* transcription in this cell. By contrast, in P12.aaap, MAB-5 is likely to act indirectly, as MAB-5 protein is undetectable after the first division in this lineage (Salser et al., 1993), 4 hours prior to the programmed cell death of P12.aaap. Survival of some P12.pp cells in animals with a mutation in the site at +5995 suggests that this site may mediate activation of *egl-1* transcription to induce death of this cell. As P12.pp cell death occurs normally in *mab-5* and *ceh-20* mutants, perhaps a more posterior Hox gene and Hox co-factor [such as EGL-5 and the MEIS homolog UNC-62 (Van Auken et al., 2002)] act upon this site.









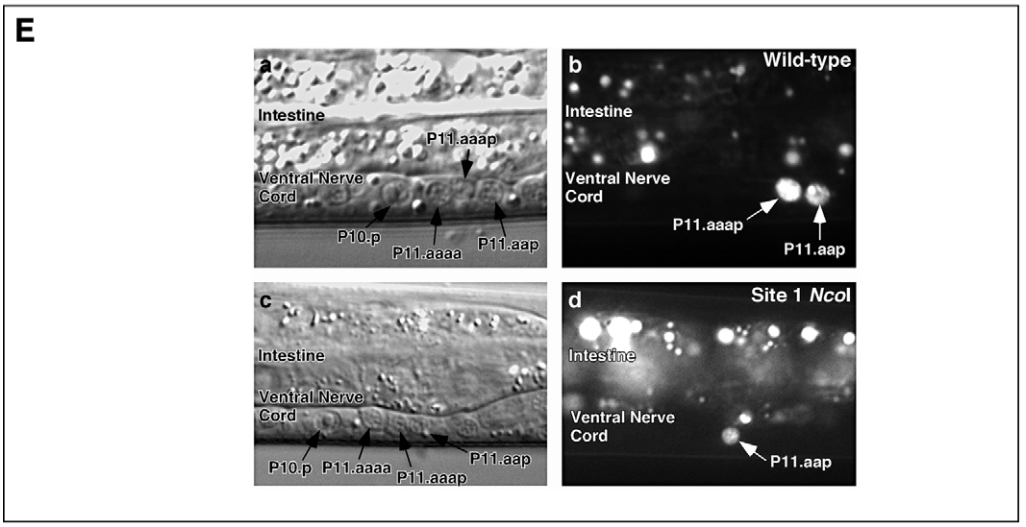


Fig. 4. An evolutionarily conserved site in *egl-1* is required for programmed cell death of specific cells in the P11 and P12 lineages. (**A**) Light-gray boxes in the *egl-1* genes of *C. briggsae* and *C. elegans* indicate regions with evolutionarily conserved sequences. The dark-gray boxes indicate the *egl-1* open reading frame. Numbered asterisks indicate the locations of four evolutionarily conserved matches to the TGATNNAT Hox/Hox co-factor consensus. F23B12.1 encodes a predicted phosphatase that is not present in the *C. briggsae* (or *C. remanei*) *egl-1* region. (**B**) *C. elegans* genomic DNA sequence is shown flanked by nucleotide positions relative to the *egl-1* ATG. The positions of candidate binding sites are indicated. Nucleotides conserved in *C. briggsae* are indicated by black boxes. (**C**) Site 1 at position +5995 from *C. elegans* is shown. Mutated nucleotides are underlined. (**D**) The percentage of transgenic animals with the indicated number of corpses among the descendants of specific P cells. The diameters of the spots are proportional to the percentage of animals with the indicated number of corpses. Transgenic animals were constructed by biolistic transformation (Praitis et al., 2001) of *ced-1(e1735); unc-119(ed3); egl-1(n1084n3082)* mutants with the 7.6 kb genomic DNA of *C. elegans egl-1* (see Materials and methods). Wild-type (WT) indicates introduction of wild-type genomic DNA. In general, 15 animals were scored for each independently derived transgenic line and the data were pooled (11 independent transgenic lines for the wild-type construct; 13 transgenic lines for the Site 1 *Nco*I construct; and six transgenic lines for the others). The F23B12.1 phosphatase was not required for the effects on programmed cell deaths of transgenic animals, and mutations affecting sites 2, 3 and 4 did not alter the pattern of programmed cell deaths in the ventral nerve cord (see Fig. S1 in the supplementary material). Deletion of the 470 nucleotide evolutionarily conserved region, including Site 1 (sequences 3′ of an *Xho*I site), resulted in a phenotype like that of mutations in Site 1. (**E**) DIC (a,c) and epifluorescence (b,d) images of some of the P11.a descendants of transgenic *egl-1(n1084n3082)* mutants carrying either a (a,b) wild-type $P_{egl\text{-}1}$*histone:gfp* reporter or a (c,d) mutant reporter in which Site 1 was changed to an *Nco*I site. Thirty out of 30 transgenic animals with a wild-type reporter expressed *gfp* in P11.aaap, and 29 of 30 expressed *gfp* in P11.aap. By contrast, of 90 descendants of three independent transgenic lines with a Site1 *Nco*I mutant reporter, only 11 expressed *gfp* in P11.aaap, while 83 out of 90 expressed *gfp* in P11.aap. P11.aap undergoes programmed cell death in wild-type animals and in *mab-5* and *ceh-20* mutants.

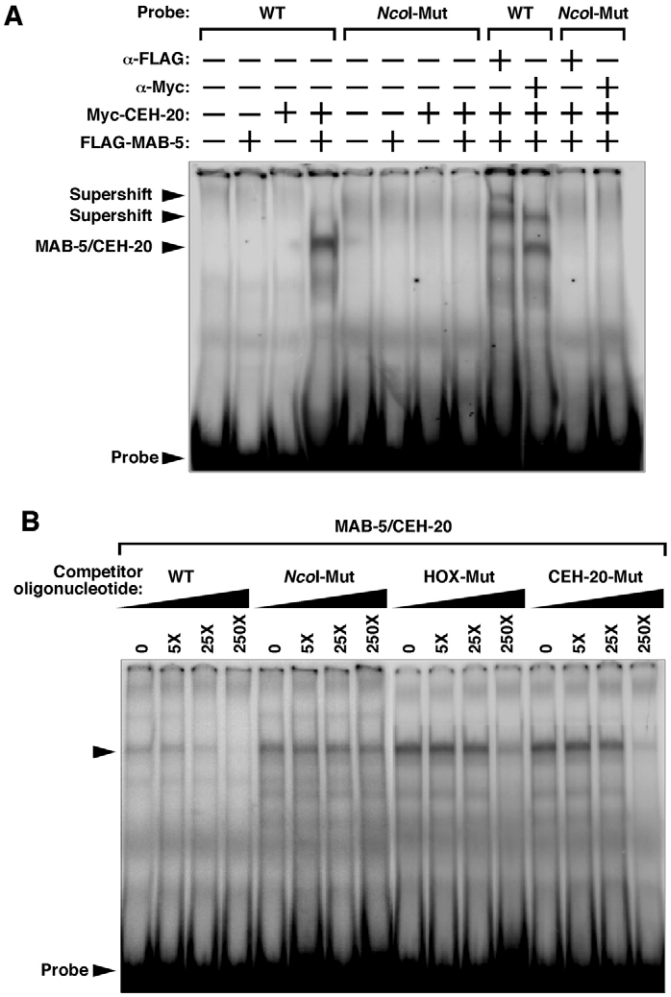


Fig. 5. A CEH-20/MAB-5 complex binds the Hox/Hox co-factor site at +5995 in *egl-1*. (**A**) Electrophoretic mobility shift assays were performed with epitope-tagged CEH-20 and MAB-5 proteins using wild-type and mutant Site 1 ^{32}P-labeled oligonucleotides. The mutant oligonucleotide probe contains the *Nco*I mutation described in Fig. 4C. (**B**) CEH-20/MAB-5 proteins were used in an in vitro binding reaction with a ^{32}P-radiolabeled oligonucleotide containing Site 1. Cold competitor oligonucleotide was included in the binding reactions at the indicated molar ratios. The *Nco*I-Mut, HOX-Mut and CEH-20-Mut mutant probes correspond to those in Fig. 4C.

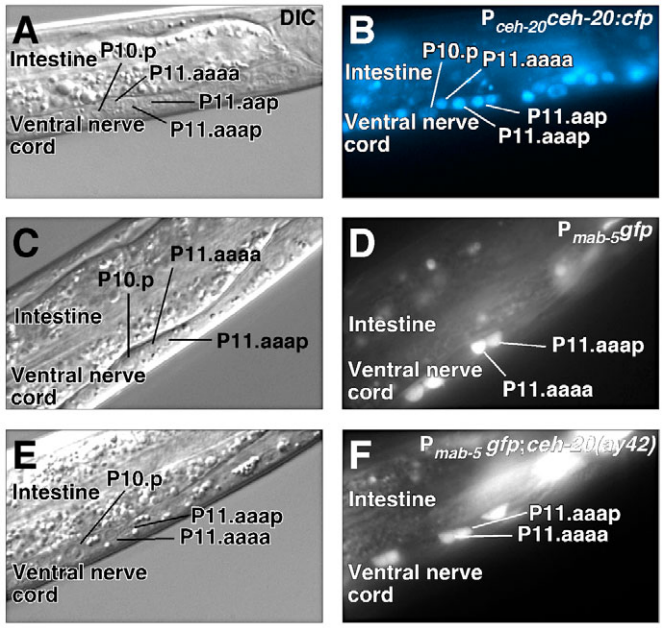


Fig. 6. CEH-20 is expressed in P11.aaap and may not be required for expression of a P$_{mab-5}$*gfp* reporter. DIC (A,C,E) and epifluorescence (B,D,F) images. (**A**,**B**) The posterior ventral nerve cord of a late L2 stage animal carrying a rescuing P$_{ceh-20}$*ceh-20:cfp* fusion gene. (**C**,**D**) Of the 30 otherwise wild-type *ced-3* mutants carrying the P$_{mab-5}$*gfp* reporter, 28 and 26 animals expressed *gfp* in P11.aaaa and P11.aaap, respectively. (**E**,**F**) Of the 60 *ceh-20(ay42)* mutants carrying the P$_{mab-5}$*gfp* reporter, 51 and 50 mutants expressed *gfp* in P11.aaaa and P11.aaap, respectively.

DISCUSSION

The Hox gene *mab-5* is essential for programmed cell death of two lineally related cells in the P11 and P12 cell lineages. Based on the following six observations, we propose that a CEH-20/MAB-5 complex directly activates *egl-1* transcription in P11.aaap to initiate programmed cell death. First, *egl-1* is specifically transcribed in P11.aaap and is essential for the death of this cell. Second, mutations affecting *ceh-20* or *mab-5* result in a failure to transcribe *egl-1* and survival of P(11,12).aaap. *ceh-20* most probably acts through *egl-1* to control programmed cell death of the VC neurons, and it seems likely that *ceh-20* also acts through *egl-1* in P(11,12).aaap. Third, mutations affecting an evolutionarily conserved regulatory sequence in *egl-1* result specifically in a failure to transcribe *egl-1* in P11.aaap and in survival of this cell. Fourth, this sequence includes a consensus Hox/Pbx site that binds a CEH-20/MAB-5 complex in vitro. Fifth, mutations that disrupt *egl-1* function in vivo disrupt binding by CEH-20/MAB-5 in vitro. Sixth, MAB-5 and CEH-20 proteins are both present in P11.aaap.

The programmed cell death of P12.aaap seems to be regulated in a distinct fashion, despite the fact that, like P11.aaap, this cell is also dependent upon *mab-5* and *ceh-20* for its death. Mutations that nearly completely prevent programmed cell death of P11.aaap do not affect death of P12.aaap. Although this could be due to our not having identified regulatory sites in *egl-1* through which CEH-20/MAB-5 might act in P12.aaap, this finding is consistent with the selective expression of MAB-5 in P11.aaap but not P12.aaap (Salser et al., 1993) and with indirect regulation of *egl-1* by CEH-20/MAB-5 in P12.aaap.

Despite the similar patterns of cell division and cell fates in P11, P12 and their descendants, different mechanisms determine these lineages. In first larval stage animals, the P11 and P12 blast cells migrate into the ventral nerve cord from lateral positions, and laser ablation studies indicate that both cells are initially each capable of adopting the P12 fate (Sulston and White, 1980). Multiple intercellular signaling pathways, including EGF/EGFR-like (LIN-3/LET-23) and Wnt/Wnt receptor (LIN-44/LIN-17) pathways specify the P12 fate, in part by acting through the Hox protein EGL-5 (Jiang and Sternberg, 1998). *egl-5* is a homolog of Abd-B (Aboobaker and Blaxter, 2003; Chisholm, 1991; Wang et al., 1993). In *egl-5*-null mutants P12 is transformed to the P11 fate (Chisholm, 1991), and *egl-5* represses expression of *mab-5* in the P12 lineage after the first division (Salser et al., 1993). Less is known about how

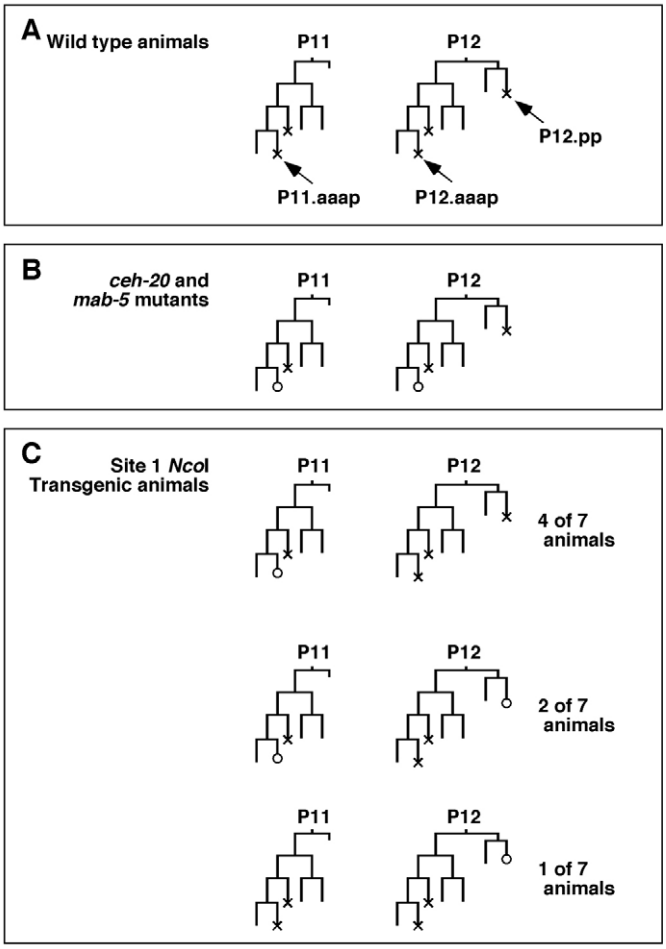


Fig. 7. Site 1 at position +5995 in *egl-1* is required for programmed cell death of P11.aaap and, in some animals, of P12.pp. P11 and P12 cell lineages were observed of seven *egl-1* mutants carrying the *egl-1* Site1 *Nco*I mutant transgene. The pattern of programmed cell deaths of this integrated line was representative of those of 13 other transgenic lines, some of which were also integrated, containing the Site 1 *Nco*I mutation. (**A**) P11 and P12 cell lineages of wild-type animals. (**B**) P11 and P12 cell lineages of *mab-5* and *ceh-20* mutants. (**C**) P11 and P12 cell lineages of transgenic animals. The fraction of animals displaying each lineage is shown.

the P11 fate is specified. Direct activation of *egl-1* transcription by CEH-20/MAB-5 in P11.aaap is one molecular mechanism operative in the P11 lineage that is inactive in the P12 lineage.

In *mab-5* and *ceh-20* mutants, the P(11,12).aaap cells survive, as do their anterior lineal equivalents. Survival of these cells could reflect a spatial transformation towards the fate of a VB motoneuron, which is the fate adopted by P1-P10.aaap (Sulston and Horvitz, 1977). Partial reconstruction of the posterior ventral nerve cord of a *mab-5* mutant indicated that the 'undead' P11.aaap cell extended an axon like that of a VB motoneuron (Kenyon, 1986), consistent with a homeotic transformation. We find that if the programmed cell death of P11.aaap is prevented by a mutation in *ced-3*, it expresses *acr-5* and *del-1*, markers of the VB motoneuron fate (Esmaeili et al., 2002; Winnier et al., 1999). In *mab-5* mutants, however, P11.aaap generally does not express these markers, suggesting that the transformation may be aberrant. Survival of P11.aaap in *mab-5* mutants probably reflects a specific defect in activation of *egl-1* transcription and programmed cell death. In this capacity, *egl-1* acts as a realizator gene (Garcia-Bellido, 1977) regulated by *mab-5* and *ceh-20* to effect morphogenesis, in this case by deleting a single cell from the developing animal. Previous target genes directly regulated by Hox proteins in *C. elegans* include only transcription factors (Koh et al., 2002; Liu and Fire, 2000). Identification of *egl-1* as a realizator gene activated by a Hox co-factor/Hox complex to induce programmed cell death in *C. elegans* supports the suggestion that non-homeotic targets of Hox proteins are common and evolutionarily ancient (Hombria and Lovegrove, 2003).

Mutations in the Hox gene *lin-39* also result in defects in programmed cell death; specifically, the six VC neurons of the midregion of the ventral nerve cord require *lin-39* for survival. We find that *ceh-20* is also required for survival of the VCs. Our data also suggest that *ceh-20* may determine one aspect of VC identity, expression of *lin-11*, through a mechanism that is *lin-39* independent. This suggestion is consistent with *lin-39*-independent functions for *ceh-20* in vulval development and neuronal migration (Yang et al., 2005). The mechanism through which *lin-39* and *ceh-20* regulate VC survival is not clear at present. Analogous to P11.aaap, a LIN-39/CEH-20 complex could directly repress transcription of *egl-1* in the VC neurons to ensure survival of the VC neurons. To confirm this model, regulatory sites in *egl-1* through which a LIN-39/CEH-20 complex acts must be identified. An alternative model is that LIN-39 and CEH-20 regulate *egl-1* indirectly, as MAB-5 apparently does in P12.aaap.

In *Drosophila*, the Hox genes *deformed* and *abd-B* activate transcription of *reaper* to induce programmed cell death and establish segment boundaries (Lohmann et al., 2002). *reaper* and other functionally related genes initiate programmed cell death in *Drosophila* primarily by inhibiting the action of DIAP1, a RING domain-containing protein that ubiquitinates the caspase DRONC and promotes its degradation (Ditzel et al., 2003; Goyal et al., 2000; Wang et al., 1999; Wilson et al., 2002). In mammals, developmental control of apoptosis is mediated in many cases by Bcl2 family members, particularly BH3 domain-encoding genes that are regulated in response to diverse stimuli (Puthalakath and Strasser, 2002). Our demonstration here of direct regulation of the BH3 domain-encoding gene *egl-1* by a Hox co-factor/Hox protein complex to initiate programmed cell death in *C. elegans* suggests the hypothesis that mammalian BH3 domain-encoding genes may be similarly regulated by Hox co-factor/Hox complexes as part of normal development of cells and tissues.

Previously, two pathways have been identified that directly regulate *egl-1* and programmed cell death of specific cells of *C. elegans;* both pathways contain genes whose human homologs are oncogenes (Conradt and Horvitz, 1999; Metzstein et al., 1996; Metzstein and Horvitz, 1999; Thellmann et al., 2003). Mutations affecting a mammalian homolog of *ces-2*, hepatic leukemia factor (HLF), may contribute to oncogenesis by preventing programmed cell death of malignant cells through an evolutionarily conserved mechanism. HLF is altered by the rare t(17;19) translocation in children with acute lymphoblastic leukemia (Inaba et al., 1992). The E2A-HLF fusion protein generated by the translocation inhibits programmed cell death of malignant precursor B lymphoblasts (Inaba et al., 1996). Based upon the genetic pathway established in *C. elegans* in which CES-2 acted through the Snail family member CES-1 to regulate programmed cell death, a human homolog of CES-1, SLUG, was identified and shown to mediate the actions of the fusion protein in preventing cell death of the malignant cells (Inukai et al., 1999).

We report here that the third pathway that directly regulates *egl-1* also contains a human oncogene. A human homolog of *ceh-20* is *Pbx1*, an oncogene initially identified at the t(1;19) breakpoint in children with acute lymphoblastic leukemia (Kamps et al., 1990; Nourse et al., 1990), the most common form of cancer in children. How mutations of *Pbx1* promote leukemogenesis is not yet known. Some *egl-1* homologs act during normal hematopoiesis to prevent the development of hematopoietic malignancy. For example, *Bid*-deficient mice develop a myeloproliferative disease that progresses to leukemia in many mice (Zinkel et al., 2003), and *Bad*-deficient mice develop diffuse large B cell lymphoma (Ranger et al., 2003). Hox genes have many well described functions during normal hematopoiesis and are frequently affected by chromosomal translocations in hematopoietic cancers (Grier et al., 2005). How expression of the BH3 domain-encoding genes is regulated during hematopoietic development is not yet clear, but direct regulation by Hox co-factor/Hox complexes of BH3 gene function to promote or prevent programmed cell death is an intriguing possibility.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/4/641/DC1

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