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Functional specificity of the nematode Hox gene *mab-5*

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SUMMARY

Hox genes encode evolutionarily conserved transcription factors involved in morphological specification along the anteroposterior body axis of animals. The two most striking features of Hox genes are colinearity and the strong sequence conservation. Among all animals studied so far, the nematode *Caenorhabditis elegans* contains one of the most divergent Hox clusters. The core cluster contains only four members, which in part deviate from the colinearity rule. In addition, orthologous and paralogous nematode Hox sequences diverged substantially. Given these nematode-specific features, we asked how these Hox proteins evolved and how they provide functional specificity. We investigated the role of MAB-5 during ray formation and established an in vivo assay using *Cel-mab-5* regulatory elements to express orthologous, paralogous and chimeric cDNAs in a *Cel-mab-5* mutant background. We show that the MAB-5 ortholog from *Pristionchus pacificus*, but not the *C. elegans* paralogous Hox proteins can rescue *Cel-mab-5*. Experiments with chimeric, truncated and mutagenized Hox proteins suggest the specificity to be conferred by the N-terminal arm and helix I, but not helix II of the homeodomain.

Key words: Hox genes, Evolution, Ray formation, *Caenorhabditis elegans*, *Pristionchus pacificus*

INTRODUCTION

The evolution of animal morphology is in large parts based on evolutionary changes in the regulatory genetic networks that control developmental processes. Basically, molecular alterations in regulatory genes can cause changes in either the expression pattern of downstream target genes or changes of the structure and/or function of the proteins themselves. A large body of evidence in insects, vertebrates and nematodes supports the importance of regulatory changes as caused by mutations in promoter and enhancer elements of the regulatory genes or their targets (Force et al., 1999; Ludwig et al., 2000; Tautz, 2000). By contrast, functional changes of regulatory proteins, such as those encoded by the Hox genes, are only rarely seen in developmental systems (Chauvet et al., 2000; Grandien and Sommer, 2001; Grenier and Carroll, 2000). For example, two recent studies have reported that the evolution of a transcriptional repression domain in an insect Hox protein is involved in the diversification of thoracic and anterior abdominal segments (Galant and Carroll, 2002; Ronshaugen et al., 2002).

The observation that functional changes of Hox proteins are only rarely associated with the evolution of the protein sequences is surprising because the sequence conservation of most transcription factors, in particular Hox proteins, is restricted to the DNA binding region. Most other parts of the proteins are highly divergent and often, only a few islands of conserved amino acids exist. One obvious question therefore is how Hox proteins provide functional specificity. So far, this question has mainly been addressed by studies using insects and vertebrates (Graba et al., 1997) and only rarely in nematodes (Hunter and Kenyon, 1995; Maloof and Kenyon, 1998).

In the nematode *Caenorhabditis elegans*, Hox genes play an important role during the formation of multiple developmental processes (Kenyon et al., 1997). However, nematode Hox clusters and Hox genes show several characteristics that differ from their counterparts in most other animal phyla. First, the Hox cluster of *C. elegans* contains only four core members: *ceh-13*, *lin-39*, *mab-5* and *egl-5*, the *labial*, *Deformed*, *Antennapedia* and *Abdominal-B* orthologs, respectively (Fig. 1A). Second, these genes are scattered over an approximately 300 kb interval with many unrelated genes being interspersed (The Caenorhabditis elegans Sequencing Consortium, 1998). Third, two additional *Abd-B*-like Hox genes, *php-3* and *nob-1*, are located more than 1 Mb away on the same chromosome (Fig. 1A) (Van Auken et al., 2000). Fourth, the two anterior genes *ceh-13* and *lin-39* deviate from the colinearity rule in that *lin-39* is more distal than the *labial*-like gene *ceh-13* (Fig. 1A). To our knowledge, this represents the only proven case in the animal kingdom, in which the colinearity rule is broken. Furthermore, some *C. elegans* Hox genes, such as *ceh-13*, have taken over essential functions during embryogenesis, whereas other core members provide positional information during pattern formation, but are not essential for development. Finally, nematode Hox proteins show only limited sequence conservation. In addition to sequence divergence in the N-terminal and C-terminal regions, the N-terminal arm and

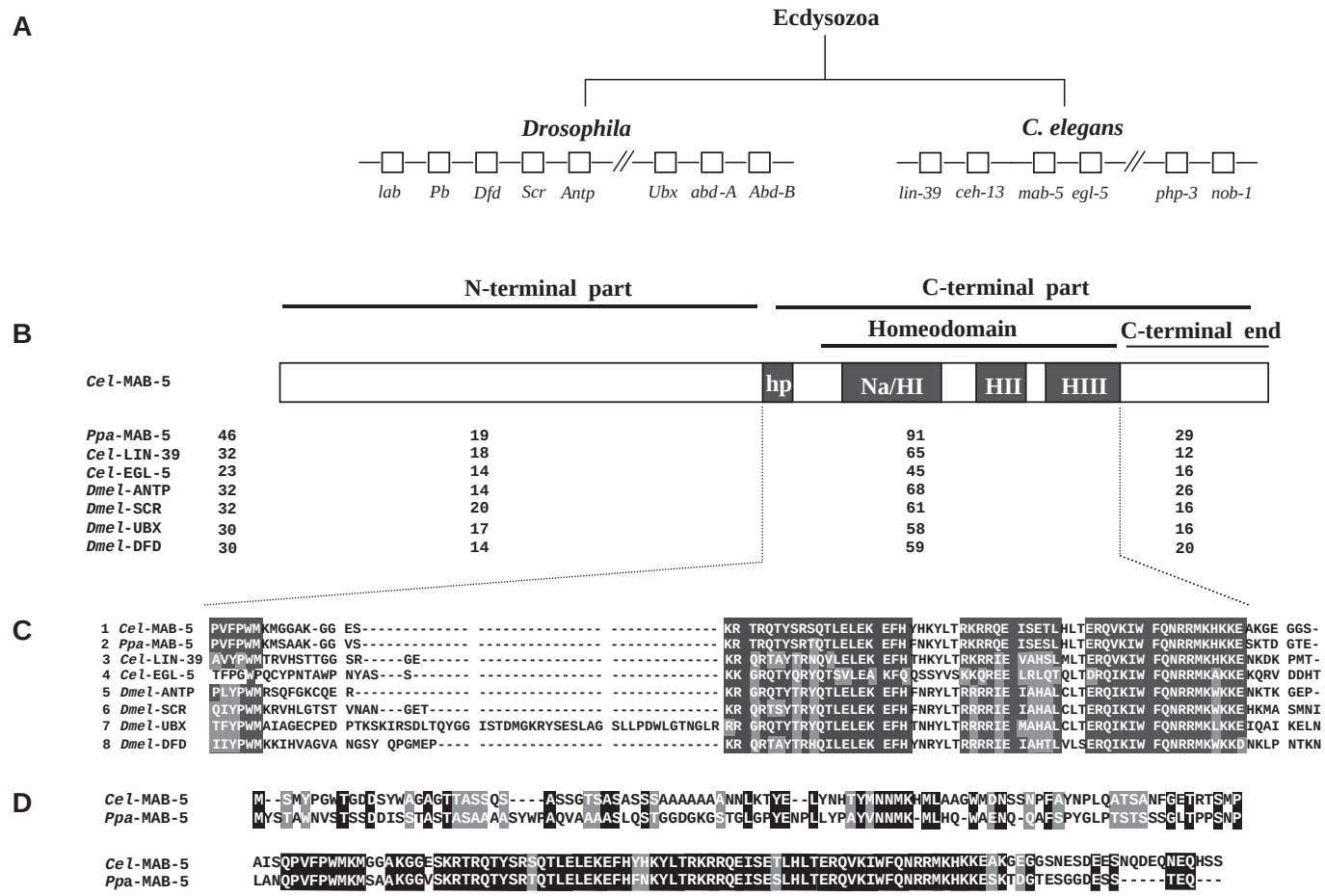

Fig. 1. Comparison of Hox cluster organization and sequence between *C. elegans* and *Drosophila*. (A) *Drosophila* contains eight Hox genes, organized into two subclusters. In *C. elegans*, the four core members are in one cluster, interspersed by many other unrelated genes. Two additional Hox genes, *php-3* and *nob-1*, are located elsewhere in the genome. *ceh-13*, the labial ortholog of *C. elegans*, is not in a distal position in the cluster as it is in other organisms. (B) Hox gene organization and sequence identity (for the different parts of the proteins) of orthologous and paralogous genes at the amino acid level. Hox genes can be subdivided into the N-terminal and the C-terminal parts. Both, hexapeptide (hp) and homeodomain are localized in the C-terminal part of the protein. Within the homeodomain itself, the N-terminal arm (Na) and helices I, II and III (HI, HII, HIII) can be distinguished. The C-terminal end refers to the protein-coding part between the end of the homeodomain and the end of the protein. The numbers to the left indicate percentage of overall sequence similarity; the three following numbers refer to the sequence similarity in the N-terminal part (second number), the hexapeptide and homeodomain region (third number) and the C-terminal end (last number), respectively. (C) Amino acid sequence comparison of the Hox proteins shown in B in the homeodomain region. Black, sequence identity; gray, sequence similarity. (D) Amino acid sequence comparison of *Cel-mab-5* and *Ppa-mab-5* over the complete protein.

helices I and II of the homeodomain, also show sequence differences to an extent that is not seen in most other animal phyla (Fig. 1B,C). Thus, nematode Hox genes show several special features unknown in Hox genes of other organisms. These features are most probably secondary modifications of Hox gene organization, function and sequence, many of which might be the result of the adaptation to the small body size and the simpler bauplan. Given these differences in nematode Hox genes, the question arises of how specificity is provided to particular Hox functions. In insects, original studies had suggested that most of the functional specificity resides within the homeodomain (Furukubo-Tokunaga et al., 1993; Zeng et al., 1993). However, more recent studies have clearly indicated that regions outside of the homeodomain are also of importance (Chauvet et al., 2000).

One simple developmental process in nematodes, in which the evolutionary diversification of Hox proteins can be studied in detail, is the formation of the ray sensilla in males. Rays are sensory structures that are generated specifically in the male by the lateral hypodermis. Generally, the lateral hypodermis is generated by six bilateral pairs of V cells, called V1-V6, which are located along the anteroposterior body axis (Fig. 2) (Sulston and Horvitz, 1977). The anterior cells V1-V4 undergo a stem cell division pattern and form seam cells that produce epidermal ridges in the adult cuticle, called alae. V5 and V6 have initially a division pattern similar to that of V1-V4. However, instead of generating alae, V5 and V6, together with the post-anal blast cell T, generate nine pairs of copulatory sensillae, rays R1-R9 in the male. V5 generates the anterior ray R1 and V6 generates the rays R2-R6, whereas the posterior rays R7-R9 are formed from the T cell (Fig. 2B). The homeotic gene *mab-5* provides positional information during ray

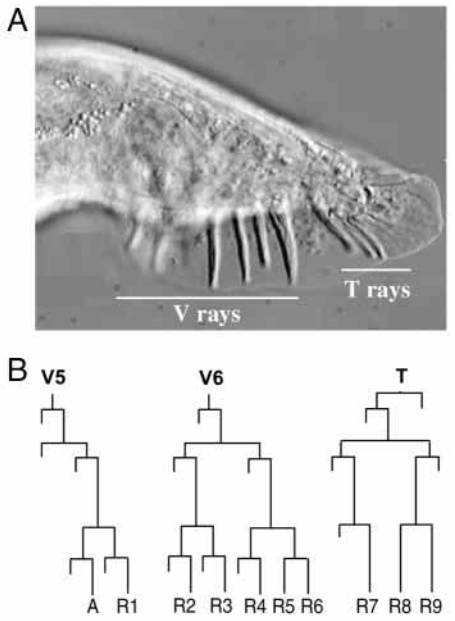

Fig. 2. Morphology and cell lineage of *C. elegans* rays. (A) Ray morphology in an adult male. Lateral view. (B) The nine pair of rays (R1-R9) are formed by the blast cells V5, V6 and T. Anterior is towards the left and dorsal is upwards.

formation. In the absence of MAB-5 protein, V5 and V6 generate alae instead of rays and only the posterior T rays R7-R9 are formed (Costa et al., 1988; Kenyon, 1986). The *mab-5* gene is switched on and off several times in the V5 and V6 lineage, indicating that the gene is regulated in a complex manner during ray specification (Salser and Kenyon, 1996). Thus, MAB-5 not only has a global patterning role for V-rays, but also specifies several V-sublineages giving rise to individual rays. One downstream target of MAB-5 is the *Abdominal-B* ortholog *egl-5*, which is required in certain sublineages of V6 (Chisholm, 1991; Ferreira et al., 1999). Together, the role of MAB-5 during *C. elegans* ray formation provides an easy test system for the functional specificity of Hox proteins: MAB-5 is a central regulatory control gene for V-rays. In addition, the ray system is simple and easy to manipulate by transgenesis, thereby providing the basis for a detailed study of the evolution of protein function.

We address how orthologous, paralogous and chimeric nematode Hox proteins provide functional specificity by testing their rescuing activity during ray formation in *Cel-mab-5* mutants. We show, that orthologous nematode MAB-5 proteins, but not the paralogous Hox proteins from *C. elegans*, can functionally replace *Cel*-MAB-5. Studies with chimeric and truncated Hox proteins suggest that the specificity is conferred by the homeodomain. In vitro mutagenesis experiments further indicate that the N-terminal arm and helix I of MAB-5 are sufficient to provide ray identity. Similar mutagenesis experiments with the neighboring Hox protein LIN-39 suggest that protein domains other than the N-terminal arm and helix I provide specificity of this Hox protein during vulva formation. Together, our data indicate that different domains of nematode Hox proteins provide functional specificity in individual developmental decisions.

MATERIALS AND METHODS

C. elegans strains and culture

Strains were maintained under standard conditions as described (Brenner, 1974). Wild-type *C. elegans* corresponds to the Bristol strain N2. The strain CB3531 with the genotype *mab-5 (e1239)*; *him-5(e1490)* was used for rescue experiments.

Plasmid constructs

All DNA constructs were made using standard techniques (Sambrook and Russell, 2001) and verified by restriction mapping or sequencing. In addition, all exons amplified by PCR were verified by sequencing. The pAG1 *Cel-mab-5* construct was generated by PCR amplification from *C. elegans* genomic DNA and cloning of the product into pBIIKS (Stratagene). This construct contains 8.9 kb upstream of the ATG, the 7.6 kb gene and 1.5 kb downstream of the stop codon. A 3.5 kb fragment, which contains the start codon, was isolated from pAG1 by digestion with *Nco*I and *Sac*I and was modified to create a unique *Xho*I site in front of the start codon using the Quikchange technique (Stratagene) according to the manufacturers instructions. This fragment was reintroduced into the basic construct to generate pAG2. A frame-shift mutation in exon 3 was generated by *Nco*I-mediated digestion of pAG2, followed by fill-in and religation, leading to the final pAG3 vector. The *Cel-mab-5*, *Ppa-mab-5*, *Cel-lin-39* and *Cel-egl-5* cDNAs were amplified by PCR, are supposed to represent full length cDNAs and have been verified by sequencing. These cDNAs were amplified by using 5′ primers introducing *Xho*I sites in front of the start ATG and 3′ primers with *Xho*I sites downstream of the stop codons. These modified cDNAs were cloned into pAG3 to generate pAG4, pAG5, pAG6 and pAG7, respectively. We have used a *Cel-mab-5* cDNA of 603 bp in size. This cDNA is shorter than originally reported and was recently corrected in the database (Grandien and Sommer). To create pAG9, the GFP-coding sequence was amplified using PCR from the plasmid pPD95.70 (a gift from A. Fire) and cloned in-frame with the hexapeptide of *Cel-mab-5*. The GFP-mediated expression of pAG9 is in large parts similar to the previously reported *Cel-mab-5* expression pattern (Cowing and Kenyon, 1992). pAG10 contains a truncated cDNA, which lacks the C-terminal end of *Cel-mab-5*, the region 3′ of the homeodomain. Throughout the text we refer to the protein coding part that is located 3′ of the homeodomain as the ‘C-terminal end’. Plasmids pAG11 and pAG12 are chimeric constructs carrying the N-terminal part of *Cel-lin-39* fused with the C-terminal part (hexapeptide, homeodomain and C-terminal end) of *Cel-mab-5* (pAG11) and vice versa (pAG12). Finally, the N-terminal arm helix I and helix II of *Cel-lin-39*, were modified to the corresponding sequences of *Cel-mab-5* by PCR using the Quikchange technique to generate pAG13 and pAG14, respectively.

In the case of *Cel-lin-39*, we used the construct pKG11 (Grandien and Sommer, 2001) to insert *Cel-mab-5* (pLK1) and the modified cDNAs (pLK2 and pLK3). pLK2 and pLK3 were generated in the similar way as pAG13 and pAG14 by modifying the N-terminal arm and helix I (pLK2) and helix II (pLK3) of *Cel-mab-5* into the corresponding sequences of *Cel-lin-39* by *in-vitro* mutagenesis.

Germline transformation

Germline transformation and generation of transgenic *C. elegans* strains was performed as described (Mello and Fire, 1995; Mello et al., 1991). Rescue constructs were injected into *mab-5(e1239)*; *him-5 (e1490)* with the co-injection marker pTG96 (Gu et al., 1998), encoding *sur-5*::GFP (a gift from M. Han). The co-injection marker was kept at a concentration of 50 ng/μl, whereas rescuing constructs were injected 10 ng/μl. The total DNA concentration was kept constant by addition of pBIIKS. It should be noted that transgenic nematode animals contain multiple copies of the transgene.

Ray rescue assay

Transgenic adult males were picked and the tail region was analyzed using Nomarski microscopy as described elsewhere (Kenyon, 1986).

Egg laying rescue assay

The strain MT4498, with the genotype *Cel-lin-39 (n1880)*, was used for rescue experiments. Transgenic L2/L3 animals were picked singly to plates at day 1 and incubated at 25°C. Eggs were counted daily until day 5, where normally no or only a few eggs were still laid. Animals laying at least one egg were scored as rescued (non-Egl). Eggs and newly hatched larvae were counted and removed until the end of the experimental period or until the mother died from internal hatching of progeny. *Cel-lin-39(n1880)* mutant animals are completely egg-laying defective and no eggs are laid.

RESULTS

A 19 kb genomic construct rescues the ray phenotype of *Cel-mab-5*

To study the evolution of the functional specificity of MAB-5 and other Hox proteins, a transformation-rescue system had to be established. We used the *Cel-mab-5(e1239)* loss-of-function allele as a test system. In particular, we used the mutant *Cel-mab-5(e1239); Cel-him-5(e1490)* (CB3531) that generates a high frequency of males because of a mutation in *him-5*. In CB3531, no V-rays are formed (Fig. 3, Fig. 4A). Initial work towards a functional comparison of Hox proteins in nematodes has been based on overexpression studies using a heat-shock promoter (Hunter and Kenyon, 1995). However, another study indicated that the detection of functional differences among related proteins would benefit from a more sensitive system using endogenous regulatory elements of the gene under consideration (Grandien and Sommer, 2001). In addition, the work on the evolution of the neighboring Hox gene *lin-39* indicated the requirement of multiple regulatory elements, spanning a total region of 23 kb of genomic sequence (Grandien and Sommer, 2001). Another significant result of the *lin-39* work was that the importance of regulatory regions cannot easily be defined by expression studies using *lacZ* fusion constructs, but rather requires in vivo studies of protein function by transformation rescue (Grandien and Sommer, 2001). Therefore, we tested the requirements of *Cel-mab-5* regulatory elements by analyzing *Cel-mab-5* transgenes for their ability to rescue the ray phenotype of *Cel-mab-5(e1239)*.

We generated a basic construct pAG1 containing all exons and introns, as well as the 3′ UTR and 9 kb of upstream regulatory sequences of *Cel-mab-5*. When this 19 kb construct was introduced as a transgene into CB3531, rescue of the ray defect in males was seen in all transgenic animals of five independent transgenic lines (Fig. 3). On average, 3.6 V-rays were seen per side of the animal (in the following, the given ray numbers are the observed V-rays, per side and transgenic animal) (Fig. 3). Although most rays had wild-type ray-like morphology, some had the morphology of fused rays as often seen in various mutants. These malformations in the rescued ray pattern are most probably the result of an incorrect temporal and/or spatial expression of *Cel*-MAB-5 in the transgenic arrays. Similar results have also been described in the functional analysis of *Cel-mab-5* (Costa et al., 1988; Hunter and Kenyon, 1995; Salser and Kenyon, 1996; Salser et al., 1993).

The observed rescuing activity of pAG1 specifies the included regulatory elements as sufficient for driving *Cel-mab-5* expression in the V lineage and certifies this construct as a backbone construct for further analysis. To generate a *Cel-mab-5* expression vector, pAG1 was modified in several ways. We introduced a unique *Xho*I restriction site immediately upstream of the start ATG (Fig. 3). This construct, pAG2, retains a rescuing activity of 3.4 V-rays (Fig. 3). To diminish endogenous *Cel-mab-5* gene activity of the pAG2 construct, a frameshift mutation was introduced in the hexapeptide (Fig. 3). The resulting construct, pAG3 did not show any rescuing activity of V-rays, i.e. not a single V-ray was seen (Fig. 3). Ray formation was restored by introducing a *Cel-mab-5* cDNA into pAG3 (pAG4) (Fig. 4B). On average, transgenic animals carrying pAG4 formed 3.2 V-rays (Fig. 3). Thus, pAG3 provides an expression vector, in which open reading frames of genes of interest can be expressed in a way similar to *Cel-mab-5*. Such a construct can be used to test orthologous, paralogous or chimeric Hox proteins.

Ppa-mab-5 mutants have a ray phenotype similar to *Cel-mab-5*

Previous studies on vulva formation in the nematode

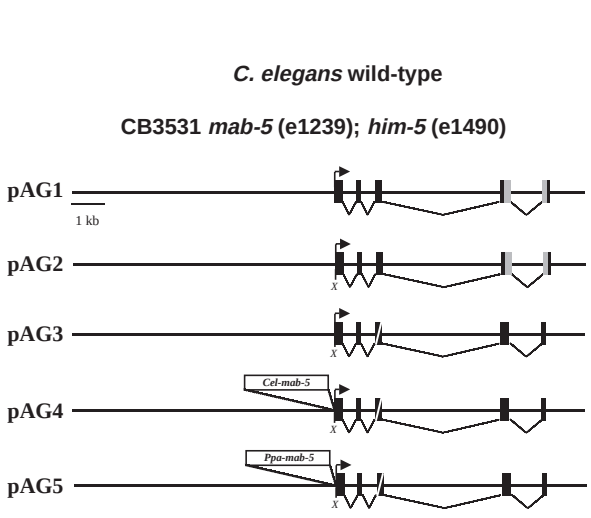

Fig. 3. Summary of ray rescue experiments: part I. All transgenic animals were analyzed in the strain CB3531 carrying the null allele *Cel-mab-5(e1290)*, which forms no V-rays. At least three transgenic lines were tested for each construct. Black rectangles, coding exons; stippled rectangles, introduced frame-shift mutation; *X*, the unique restriction site in pAG2 and all further constructs. Inserted cDNAs in pAG4 and pAG5 are indicated. *n*, number of animals analyzed.

Pristionchus pacificus indicated an important role for the Hox genes *Ppa-lin-39* and *Ppa-mab-5* (Eizinger and Sommer, 1997; Jungblut et al., 2001; Jungblut and Sommer, 1998; Jungblut and Sommer, 2000; Sommer et al., 1998). However, these studies did not investigate the role of *Ppa-mab-5* during ray formation. *P. pacificus* contains nine rays with a spatial pattern that differs from the one seen in *C. elegans* (Fig. 5A) (Sommer, 1996).

To study the role of *mab-5* during ray formation in *P. pacificus*, we used the *Ppa-mab-5(tu31)* allele that represents a strong reduction-of-function mutation (Jungblut and Sommer, 1998). R1-R6 were absent in *Ppa-mab-5* mutant animals, whereas R7-R9 were formed normally (Fig. 5B). These results suggest that *Ppa*-MAB-5 provides positional information for ray specification in a way similar to *Cel*-MAB-5.

Ppa-mab-5 expression restores ray formation in *Cel-mab-5* mutants

Next, we asked whether the *Ppa*-MAB-5 protein, when expressed under the control of the *Cel-mab-5* regulatory elements, could rescue the *Cel-mab-5* mutant phenotype. The similarity between the *Cel-mab-5* and *Ppa-mab-5* mutant ray phenotypes would argue in favor of rescue. However, a sequence comparison between both proteins indicates substantial sequence differences inside and outside of the homeodomain (Fig. 1D).

When a *Ppa-mab-5* cDNA was driven by *Cel-mab-5* regulatory elements, the resulting construct rescued the ray phenotype nearly as well as a *Cel-mab-5* cDNA. On average, 2.8 V-rays were observed (Fig. 3, Fig. 4C). Specifically, all transgenic animals tested had more than two V-rays and no animals were observed without V-rays. These results indicate that the orthologous MAB-5 protein from *P. pacificus* can functionally replace *Cel*-MAB-5 when expressed appropriately.

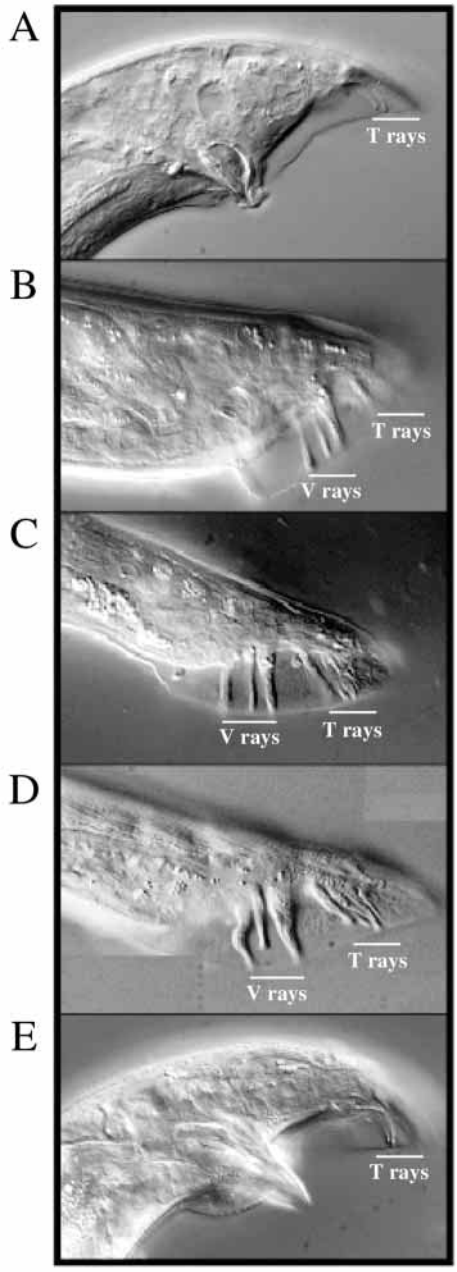

Fig. 4. Ray morphology in *Cel-mab-5(e1290)* animals rescued with various constructs. (A) CB3531, no V-rays are formed. (B) pAG4, a *Cel-mab-5* cDNA rescues ray formation. (C) pAG5, a *Ppa-mab-5* cDNA also rescues ray formation. (D) pAG13, an in vitro mutagenized protein containing the N-terminal arm and helix I of *Cel*-MAB-5 in an otherwise *Cel*-LIN-39 protein rescues ray formation. (E) pAG14, an in vitro mutagenized protein containing helix II of *Cel*-MAB-5 in an otherwise *Cel*-LIN-39 protein shows poor rescue of ray formation. Anterior is towards the left and dorsal is upwards.

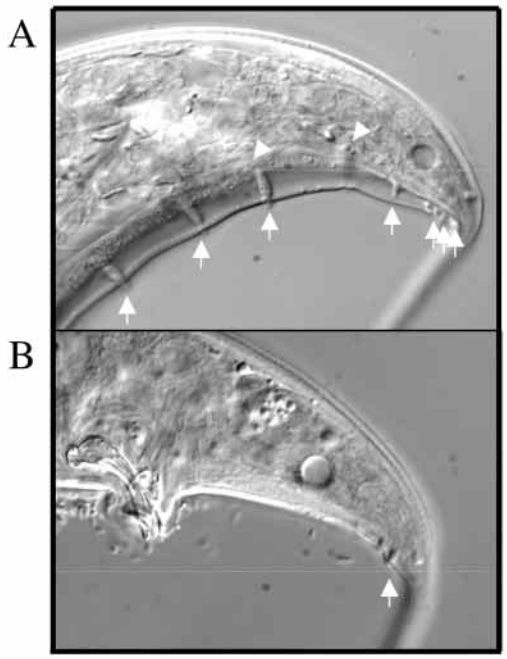

Fig. 5. Ray morphology in *P. pacificus* wild-type (A) and *Ppa-mab-5(tu34)* mutant animals (B). The spacing and position of wild-type rays (arrows) is different from the one in *C. elegans*. Two rays open laterally (arrowheads). R7-R9 are clustered at the tip of the animal. R1-R6 are absent in *Ppa-mab-5*. R7-R9 are present (arrow in B) but not all rays are visible in this plane of focus. Anterior is towards the left and dorsal is upwards.

From orthologs to paralogs: *Cel-lin-39* and *Cel-egl-5* cannot restore ray formation in *Cel-mab-5* mutants

Orthologous nematode Hox proteins when compared with the paralogous Hox proteins within *C. elegans*, can often only be aligned properly by comparing the homeodomain region (Fig. 1B,C). The non-DNA binding regions of *Cel-mab-5*, *Ppa-mab-5*, *Cel-lin-39* and *Ppa-lin-39*, respectively, show only limited similarities. In particular, the degree of amino acid identity between *Cel*-MAB-5 and *Ppa*-MAB-5 is only 19% and 29% in the N-terminal and C-terminal region, respectively. At the same time, *Cel*-MAB-5 and *Cel*-LIN-39 have in the corresponding region an amino acid identity of 18% and 12%, respectively. In addition to the non-DNA binding regions, the homeodomains of paralogous Hox proteins in *C. elegans* also differ much more from one another than their counterparts in insect Hox proteins. For example, the homeodomain sequences of *Dfd*, *Antp* and *Ubx* in *Drosophila* differ at only five amino acid positions (92% identity), nearly all of which are located in the N-terminal arm and helix I (Fig. 1C). By contrast, the homeodomains of LIN-39, MAB-5 and EGL-5 in *C. elegans* differ at several amino acids in the N-terminal arm and helix I, but also in helix II (Fig. 1C). As a result, the sequence identity is only 65% between *Cel*-MAB-5 and *Cel*-LIN-39, and 45% between *Cel*-MAB-5 and *Cel*-EGL-5 (Fig. 1B). The most important difference between the paralogous proteins in *Drosophila* and *C. elegans* is that sequence differences in *Drosophila* occur only in the N-terminal arm and helix I, whereas in *C. elegans* also helix II differs substantially. As helix II has been suggested to be crucial for protein-protein interactions (Mann and Affolter, 1998; Mann and Chan, 1996), these sequence differences might be important to provide functional specificity.

We sought to determine if the paralogous Hox proteins from *C. elegans* could take over the function of *Cel-mab-5* during ray formation when expressed in a similar way to the *Cel*-MAB-5 protein. First, we tested a construct containing the *Cel-lin-39* cDNA (pAG6) (Fig. 6). We have shown previously that this *Cel-lin-39* cDNA is sufficient to rescue a *Cel-lin-39* mutant (Grandien and Sommer, 2001). When expressed under the regulatory elements from the *Cel-mab-5* gene, the *Cel-lin-39* construct pAG6 showed poor rescue of ray structures with an average of 0.4 V-rays (Fig. 6). Specifically, 68% of transgenic animals showed no rescue (0 V-rays), a result never seen in transgenic animals of constructs containing orthologous MAB-5 proteins. Next, we tested a construct containing a cDNA of *Cel-egl-5* (pAG7). This construct also showed poor rescue of ray structures. On average, 0.5 V-rays were formed (Fig. 6). Together, these results indicate that the paralogous Hox

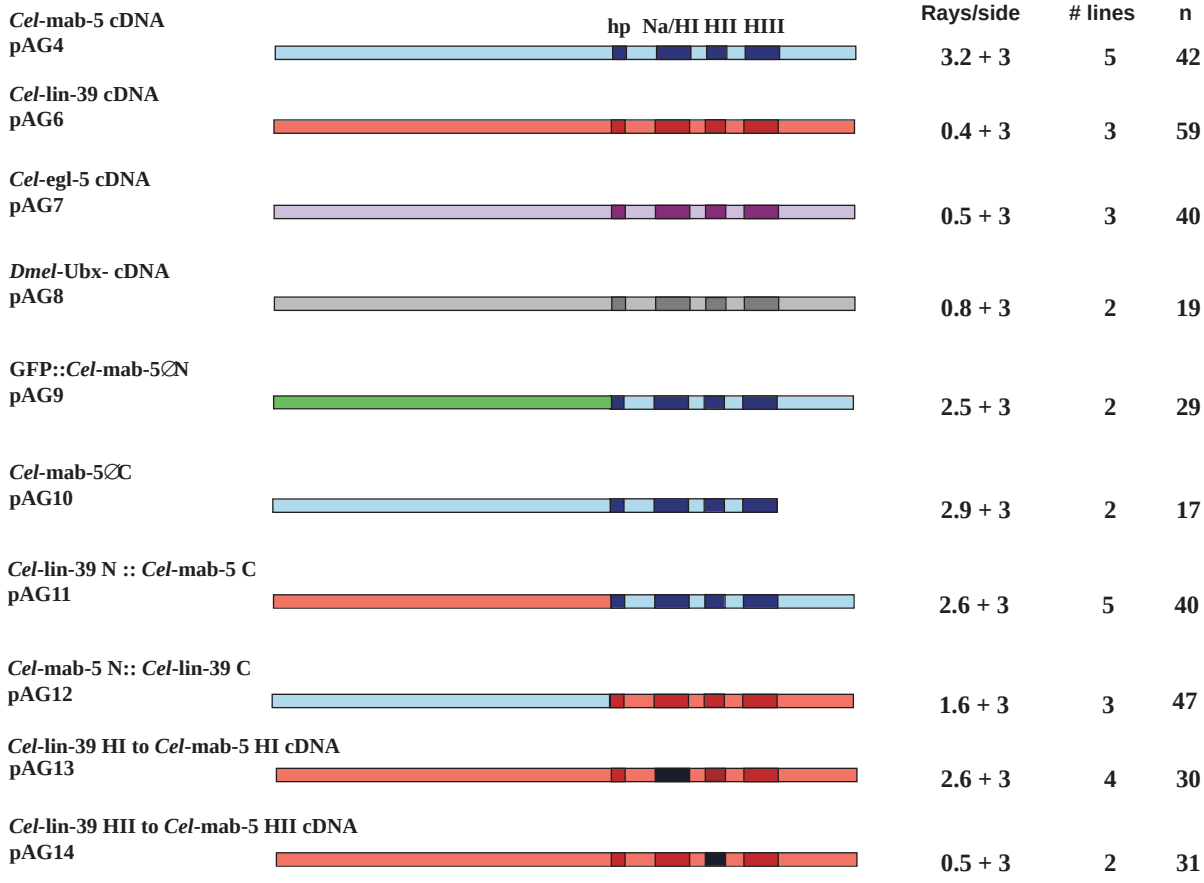

Fig. 6. Summary of ray rescue experiments: part II. All transgenic animals were analyzed in the strain CB3531 carrying the null allele *Cel-mab-5(e1290)*, which forms no V rays. At least three transgenic lines were tested for each construct. Only the cDNAs that have been inserted into the unique restriction site of pAG3 are shown. *mab-5* (blue), *lin-39* (red), *egl-5* (pink) and Ubx (gray), as well as the other Hox genes are shown with an individual color code, using specific darker colors for the hexapeptide and the helices of the homeodomain. The region of GFP fused to *mab-5* is shown in green. See text for details.

proteins *Cel*-LIN-39 and *Cel*-EGL-5 cannot substitute for *Cel*-MAB-5 and suggest the existence of important functional differences between these paralogous Hox proteins. Finally, we tested a construct containing a cDNA of the *Drosophila* gene *Ubx* under the control of *Cel-mab-5* regulatory elements (pAG8) (Fig. 6). The construct pAG8 also showed poor rescue of ray structures with on average 0.8 V-rays (Fig. 6).

The homeodomain of MAB-5 provides functional specificity

Given the different rescuing activities of orthologous and paralogous nematode Hox proteins, we next asked which parts of the proteins are providing ray specificity. Is the N-terminal part of the MAB-5 protein dispensable for function? Can chimeric Hox proteins function properly during ray formation? Are there several regions within the homeodomain that are of importance? To address such questions, we generated two sets of constructs. In one construct, we truncated the MAB-5 protein and fused the C-terminal part containing the homeodomain to GFP (pAG9). pAG9 does not contain the N-terminal part of a Hox protein and can therefore be used to determine whether or not this part of the protein is dispensable for function. In a second set of constructs, we generated chimeric proteins containing the N-terminal part of one Hox protein and the C-terminal part including the homeodomain of another.

We found that the GFP-MAB-5 ΔN construct pAG9 rescued the ray phenotype of *Cel-mab-5*. On average, we observed 2.5 V-rays in transgenic pAG9 animals (Fig. 6). This rescuing activity is very similar to that of the *Ppa-mab-5* construct pAG5, indicating that the C-terminal part of MAB-5 is sufficient for ray function. However, this result does not rule out the possibility that regions other than the homeodomain also contribute to protein function. Recent studies in insect have indicated an important role for a transcriptional repression domain located in the C-terminal end of the *Ubx* protein involved in the diversification of thoracic and anterior abdominal segments (Galant and Carroll, 2002; Ronshaugen et al., 2002).

To study if the C-terminal end of MAB-5 has any role in ray formation, we generated a construct with a truncation of the C-terminal end (pAG10). However, pAG10 showed rescue of ray structures, with (on average) 2.9 V-rays (Fig. 6). This result is a further indication of the importance of the homeodomain and suggests that the very C-terminal end of the MAB-5 protein is dispensable for ray formation.

To study the importance of the N-terminal and the C-terminal half of MAB-5 in a different way, we generated two chimeric Hox proteins. The construct pAG11 contains the N-terminal domain of *Cel*-LIN-39 and the C-terminal region, including the homeodomain of *Cel*-MAB-5. We found that pAG11 has a rescuing activity very close to that of the pAG4 and pAG5 constructs. Specifically, 2.6 V-rays were generated and there were no animals without V-rays (such as in the *Cel-lin-39* construct) (Fig. 6). This result confirms that the C-terminal part of *Cel*-MAB-5 is sufficient for ray formation.

The opposite construct pAG12 contains the N-terminal part of *Cel*-MAB-5 and the C-terminal part of *Cel*-LIN-39. Testing the construct pAG12, we observed an intermediate rescuing activity of 1.6 V-rays, which was not seen in any other construct (Fig. 6). Transgenic animals differed strongly in their rescuing ability showing no rescue, i.e. no V-rays to good rescue (three V-rays) (Fig. 6). Specifically, in ~33% of transgenic animals no rescue was observed. These results suggest that the N-terminal region of *Cel*-MAB-5 can also contribute to ray specificity.

The N-terminal arm and helix I of the homeodomain provide most of the specificity of *Cel*-MAB-5

The comparison of the rescuing activities of the orthologous, paralogous and chimeric Hox proteins described above, indicates that the C-terminal part of *Cel*-MAB-5 is sufficient for ray formation. In particular the comparison between pAG6 and pAG7 with that of pAG9 and pAG10 suggests the specificity to be conferred by the homeodomain. As indicated above, *C. elegans* paralogous Hox proteins differ not only in the N-terminal arm and the neighboring region of helix I as in *Drosophila*, but also in helix II (Fig. 1). To determine if both regions of the homeodomain provide specificity to MAB-5 function, we tested both regions by in vitro mutagenesis. Specifically, we mutated the N-terminal arm and helix I of *Cel*-LIN-39 to the sequence of *Cel*-MAB-5. The resulting construct pAG13 contains this small sequence motif characteristic for *Cel*-MAB-5 in an otherwise *Cel*-LIN-39 protein (Fig. 6). The second construct was generated using the same strategy, this time modifying helix II of *Cel*-LIN-39 towards *Cel*-MAB-5 (pAG14).

pAG13 and pAG14 differed strongly in their rescue activity. Whereas pAG13 with the N-terminal arm and helix I of *Cel*-MAB-5 showed a strong rescue with 2.6 V-rays, the rescue of pAG14 is poor (Fig. 6). Most pAG14 transgenic animals show no rescue, a result that is reminiscent of the *Cel-lin-39* construct itself. By contrast, all transgenic animals of the pAG13 construct showed a good rescue, similar to the *Cel-mab-5* constructs pAG1 and pAG4. Together, these results suggest that although the N-terminal arm and helices I and II show similar sequence differences between paralogous Hox proteins, the functional specificity for ray formation resides mostly within the N-terminal arm and helix I.

MAB-5 protein cannot substitute for LIN-39 during vulva formation

The experiments described above clearly indicate that paralogs of *C. elegans mab-5* cannot restore ray formation in *Cel-mab-5* mutants. One obvious question therefore is, whether this result holds true for other nematode Hox functions as well. To answer this question, we performed similar experiments using vulva formation as a test system. The Hox gene *Cel-lin-39* plays a crucial role during vulva cell fate specification and has been studied in detail in *C. elegans* and *P. pacificus* (Eizinger and Sommer, 1997; Maloof and Kenyon, 1998; Salser and Kenyon, 1996; Sommer et al., 1998). As *Cel-lin-39* and *Ppa-lin-39* differ in their functional specificity during vulva formation in both species, we have previously used a similar assay to replace *Cel-lin-39* in the vulva and to identify those parts of the gene, regulatory versus protein-coding regions, that provide species-specific functions (Grandien and Sommer, 2001).

To study if paralogous Hox genes of *C. elegans* can substitute for *Cel-lin-39* during vulva formation, we have used the assay system previously established (Grandien and Sommer, 2001). We generated a construct (pLK1) in which the

Cel-mab-5 cDNA is introduced into the *Cel-lin-39* backbone construct pKG11 (Grandien and Sommer, 2001) that contains all regulatory elements of the *Cel-lin-39* gene (Fig. 7A,B). Consistent with our data on ray formation, pLK1 does not rescue vulva formation. All transgenic animals tested are egg-laying defective with no eggs being laid (Fig. 7B). By contrast, if *Cel-lin-39* itself is expressed under the control of *Cel-lin-39* (pKG12) vulva formation and the egg-laying defect is restored in 63% of transgenic animals (Fig. 7B).

The second major conclusion of our work described above is that the functional specificity for ray formation resides mostly within the N-terminal arm and helix I of *Cel*-MAB-5. Is the specificity conferred by the N-terminal arm and helix I a general property of Hox proteins in *C. elegans* or do different domains of Hox proteins provide specificity in individual developmental decisions? To determine if the N-terminal arm/helix I and/or helix II of the homeodomain provide specificity to LIN-39 function during vulva development, we tested both regions by in vitro mutagenesis. In the constructs pLK2 and pLK3, we mutated the N-terminal arm/ helix I and helix II of *Cel*-MAB-5 to the sequence of *Cel*-LIN-39, respectively. pLK2 shows poor rescue of egg-laying, whereas pLK3 shows no rescue at all (Fig. 7B). Specifically, 6% of transgenic animals carrying the construct pLK2 were egg-laying positive with, on average, 23 eggs being laid. This is a poor rescue when compared with pKG12, in which 63% of transgenic animals are egg-laying positive with on average 84 eggs being laid (Fig. 7B). In addition, the rescuing activity of pLK2 in the vulva is severely lower than the rescuing activity of pAG13 in the rays (Fig. 6). Together, these data indicate that the functional specificity of the Hox proteins *Cel-lin-39* and *Cel-mab-5* during vulva and ray formation is provided by different domains of the Hox proteins.

DISCUSSION

We have analyzed the functional specificity of nematode Hox proteins by studying the role of MAB-5 during ray formation. Hox proteins have been intensively studied in insects and vertebrates, but little is known about how Hox proteins provide specificity to their many specific roles during nematode development. Nematodes provide an interesting example, as studies in *C. elegans* have indicated that several aspects of Hox genes, including their organization in the cluster, their function and sequence, differ strongly from Hox genes in other phyla. It remains unknown, however, whether the derived features of *C. elegans* Hox genes are representative for all nematodes. More detailed studies on Hox genes in other nematodes, including animal and plant parasitic species, are necessary to reveal if other nematode species differ from *C. elegans* in cluster organization and Hox gene sequence.

We have addressed the question of the functional specificity of nematode Hox genes by investigating ray formation in males. To overcome the problems often associated with overexpression studies of constitutive promoters, we have used a strategy recently developed for the analysis of the function of the neighboring Hox gene *lin-39* (Grandien and Sommer, 2001). cDNAs and chimeric Hox genes were expressed in a 19 kb vector backbone that contains sufficient cis-regulatory elements for rescue of a *Cel-mab-5* mutant by the *Cel-mab-5* gene itself. *Cel-mab-5* is transcriptionally regulated in the V5/V6 lineage and is required multiple times in various sublineages (Salser and Kenyon, 1996). As a result, none of the tested cDNAs, not even *Cel-mab-5* itself, was able to restore ray formation completely, reflecting the complex requirements of *Cel-mab-5*. Although some of the rays of transgenic animals were malformed, the average number of

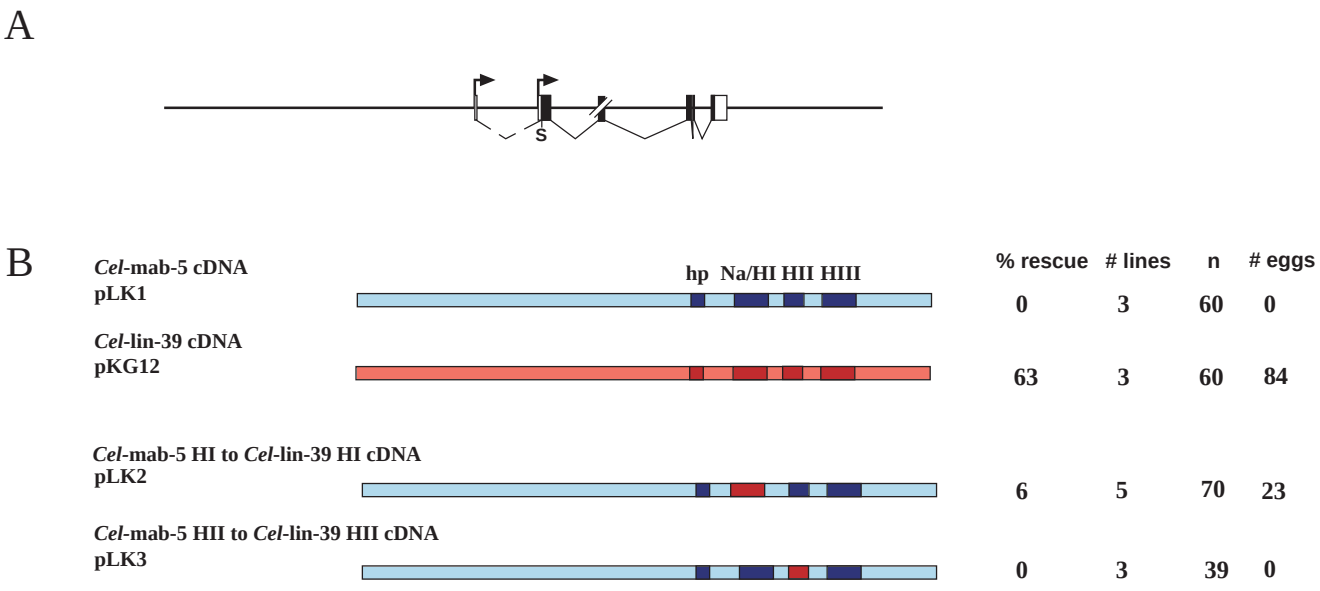

Fig. 7. Summary of vulva rescue experiments. (A) Organization of the basic *Cel-lin-39* construct pKG11 (Garndien and Sommer, 2001). The construct contains more than 10 kb upstream region, all exons and introns and the 3′-UTR of *Cel-lin-39*. As in the case of *Cel-mab-5*, the endogenous gene has been silenced by a frame-shift mutation and cDNAs can be inserted into a unique *Sma*I (S) site. (B) The four constructs with *Cel-lin-39*, *Cel-mab-5* and chimeric cDNAs inserted into pKG11. See text for details. All transgenic animals were analyzed in the strain MT4498 carrying the null allele *Cel-lin-39(n1880)*, which is completely egg-laying defective. At least three transgenic lines were tested for each construct.

rays generated as well as the range of ray numbers seen in transgenic animals provided an easy measurement of the rescuing activity of individual transgenes.

In a previous study, Hunter and Kenyon (Hunter and Kenyon, 1995) analyzed the ability of *Drosophila* Hox proteins to specify cell fates in *C. elegans* by overexpression under a heat shock promoter. Although this study mainly focused on neuronal cells, rays have also been investigated. However, the limited control of gene expression from constitutive promoters did not provide the sensitivity to distinguish between good and poor rescuing activity in the comparison of *mab-5*, *Antp* and *lin-39* (Hunter and Kenyon, 1995). The average rescue of V-rays was lower than in constructs using endogenous regulatory elements as reported in this study, and no significant difference was observed between orthologous and paralogous proteins. The lack of sensitivity most probably results from the limited control of gene expression in a spatiotemporal way.

Orthologous versus paralogous proteins: sequence and function evolve differently

The comparison of orthologous and paralogous Hox proteins clearly shows that only MAB-5 orthologs, but not MAB-5 paralogs can functionally substitute for *Cel*-MAB-5 when expressed similar to the endogenous gene. Considering the sequence similarity of orthologous and paralogous Hox proteins in nematodes (Fig. 1), these results suggests that it is not the overall sequence similarity that determines functional specificity. Rather, discrete small domains of the protein are of fundamental importance.

Our work strongly suggests that most of the functional specificity for ray formation is conferred by the N-terminal arm and helix I of the homeodomain. This result is surprising as the sequence comparison of the homeodomains of nematode Hox genes shows substantial sequence divergence not only in the N-terminal arm and helix I, but also in helix II. Thus, only a subset of those parts of the homeodomain that differ in sequence between nematode paralogous Hox proteins provide functional specificity, whereas other evolving regions are not required for ray specification at all.

Two different explanations might account for this observation. Helix II might be of importance in the functional specificity of other developmental structures. Indeed, nematode Hox genes are known to be required in multiple developmental decisions. In the case of *mab-5*, previous studies indicated a role in posterior Pn.p-cell specification in males, in P(7,8).p specification in hermaphrodites, in Q cell migration, in the regulation of cell fusion in the ventral epidermis and in the specification of various neuronal cells (Costa et al., 1988; Hunter and Kenyon, 1995; Kenyon, 1986; Kenyon et al., 1997; Salser et al., 1993). Furthermore, similar in vitro mutagenesis experiments of *Cel-lin-39* in the hermaphrodite vulva indicate that regions other than the N-terminal arm and helices I and II are of importance for generating vulva specificity (see below). In addition, recent studies in insects have shown that the functional requirement of the very C-terminus of *Ubx* is only required in a small subset of *Ubx* functions (Grenier and Carroll, 2000).

Another potential explanation for the absence of helix II function during ray formation could be that the observed sequence differences between nematode Hox proteins might be neutral. As long as amino acid substitutions do not interfere with any of the important functions of a protein, they might be tolerated. Therefore, mutations resulting in such amino acid substitutions might be fixed in natural populations.

Although the functional specificity of the chimeric MAB-5/LIN-39 proteins is largely determined by which homeodomain they express, chimeric proteins retain activities characteristic of their non-homeodomain parts. In particular, the chimeric protein containing the N-terminal part of MAB-5 and the homeodomain of LIN-39 had an intermediate rescuing activity of on average 1.6 V-rays, a value not seen with any other construct. This result suggests a role for the N-terminal part in ray specificity. However, the mechanism by which the N-terminal part of MAB-5 confers ray specification remains unknown. At the same time, our data also indicate that when the C-terminal part of MAB-5 is fused to GFP, ray rescue is observed in the absence of the N-terminal part of the protein. Thus, a completely artificial construct is able to rescue if the DNA-binding and protein-protein interaction domains are present.

The structural analysis of Hox proteins has provided valuable insight into how these transcription factors function during development. The homeodomain folds into three α-helices, the third one of which binds to the major groove of the DNA. It is this region of the homeodomain that is most highly conserved with regard to sequence. Helices I and II, however, are not involved in DNA binding and have been suggested to be required for protein-protein interactions (Mann and Affolter, 1998; Mann and Chan, 1996). The N-terminal part of the homeodomain, the N-terminal arm contacts the minor groove of the DNA and has also been indicated to form protein-protein interactions. Thus, the region that confers specificity to MAB-5 function during ray formation is involved in DNA binding and protein-protein interactions. Therefore, one can speculate that the association with other proteins strongly affects the ability of nematode Hox proteins to act upon downstream target genes. Recently, the *extradenticle* homologs *ceh-20* and *ceh-40*, as well as the *Homothorax* homolog *unc-62* have been shown to interact with the posterior-group Hox proteins NOB-1 and PHP-3 (Van Auken et al., 2002). In addition, an overlapping role of *Cel-mab-5* and *Cel-lin-39* with *Cel-ceh-20* has been described for the diversification of the postembryonic mesoderm (Liu and Fire, 2000). Other recent studies in insects, however, indicate that some Hox functions act independently of *extradenticle* (Galant et al., 2002).

Finally, it should be noted that, although the *Ppa-mab-5* cDNA can rescue the formation of rays, this cDNA is not sufficient to produce the *P. pacificus* pattern of rays in a *C. elegans* background. Thus, although MAB-5 is instructive for ray formation, the responding pattern depends on the genetic architecture of the receiving species, in this case *C. elegans*.

Cel-MAB-5 evolution is not representative for other *C. elegans* Hox genes

We have addressed the question of whether the specificity conferred by the N-terminal arm and helix I is a general property of Hox proteins in *C. elegans*. After studying *Cel*-LIN-39 during vulva formation by in vitro mutagenesis, there is no indication that the specificity of this Hox gene is conferred in the same way as in *Cel*-MAB-5. The critical construct pLK2, in which the N-terminal arm and helix I of *Cel*-MAB-5 have been mutated into the corresponding region

of *Cel*-LIN-39, shows poor rescue when compared with *Cel-lin-39* (pKG12) itself and *Cel-mab-5* (pAG13) during ray formation. Thus, our data support the hypothesis that Hox specificity in different developmental decisions is provided by different parts of the proteins. This observation also holds true for insects, in which different parts of the Hox proteins have been shown to be required for individual functions (Chauvet et al., 2000). In the case of *Cel*-LIN-39, additional studies will be required to localize those regions of the protein responsible for providing vulval specificity. Although our previous work showed that the C-terminal end of the protein is dispensable for vulva formation (Grandien and Sommer, 2001), studies as detailed as those described here for *Cel-mab-5* will be necessary to determine the regions that provide specificity to *Cel-lin-39*.

Differential evolution of *Ppa-mab-5* functions

P. pacificus is a second nematode besides *C. elegans*, in which large-scale mutagenesis screens have been carried out to identify genes involved in pattern formation and cell fate specification (Eizinger et al., 1999). *Ppa-mab-5* was one of the first genes to be studied in detail in *P. pacificus* (Jungblut et al., 2001; Jungblut and Sommer, 1998; Jungblut and Sommer, 2000). In contrast to *C. elegans*, *Ppa-mab-5* mutants have a strong vulva phenotype, resulting in the ectopic differentiation of P8.p, a vulval precursor cell that remains epidermal in wild-type animals. We have recently shown that the ectopic differentiation is not dependent on the inductive signal from the somatic gonad but rather relies on signaling from the misspecified mesoblast M (Jungblut et al., 2001). The blastomere M gives rise to all mesodermal structures formed during postembryogenesis, including body wall muscles, sex muscles and coelomocytes. Although *Cel-mab-5* mutants also have M cell lineage defects, the cell lineage alterations in *Ppa-mab-5* are much stronger and affect nearly the complete M lineage. Thus, *Cel-mab-5* and *Ppa-mab-5* mutants differ strongly in both, their vulval and muscle phenotypes.

The ray phenotype of *Ppa-mab-5* as shown in this study, represents the first *mab-5* function that is conserved between *Cel-mab-5* and *Ppa-mab-5*. R1-R6 are missing in *mab-5* mutants in both species and the severity of the phenotype is also similar. Together, the comparison of the *mab-5* phenotypes in vulva, muscle and ray specification indicates that genes can evolve with regard to certain functions, while other functions are retained. Most likely, such patterns of evolutionary diversification of gene function are achieved by changing gene regulation. It remains unknown, however, if some of the novel functions of *Ppa-mab-5* also require a different function of the *Ppa*-MAB-5 protein. A direct analysis of these functions awaits the establishment of transgenic technology in *P. pacificus*.

In summary, our work provides the first detailed analysis of the functional specificity of nematode Hox genes by studying orthologous, paralogous and chimeric proteins for their role in ray formation. We have shown that besides the limited sequence conservation of nematode Hox proteins, the N-terminal arm and helix I of MAB-5 are sufficient to induce ray formation when provided in an otherwise LIN-39 protein. Thus, although the homeodomain is the most highly conserved part of nematode Hox proteins, it is this part of the protein that confers most of the functional specificity. At the same time, similar studies with LIN-39 during vulva formation suggest the importance of regions other than the homeodomain in providing functional specificity. Although still in their infancy, these studies support the view that there is no common mechanism in providing specificity to nematode Hox proteins.

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