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Identification of *cis*-regulatory elements from the *C. elegans* T-box gene *mab-9* reveals a novel role for *mab-9* in hypodermal function

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ABSTRACT

We have identified Conserved Non-coding Elements (CNEs) in the regulatory region of *Caenorhabditis elegans* and *Caenorhabditis briggsae mab-9*, a T-box gene known to be important for cell fate specification in the developing *C. elegans* hindgut. Two adjacent CNEs (a region 78 bp in length) are both necessary and sufficient to drive reporter gene expression in posterior hypodermal cells. The failure of a genomic *mab-9*::*gfp* construct lacking this region to express in posterior hypodermis correlates with the inability of this construct to completely rescue the *mab-9* mutant phenotype. Transgenic males carrying this construct in a *mab-9* mutant background exhibit tail abnormalities including morphogenetic defects, altered tail autofluorescence and abnormal lectin-binding properties. Hermaphrodites display reduced susceptibility to the *C. elegans* pathogen *Microbacterium nematophilum*. This comparative genomics approach has therefore revealed a previously unknown role for *mab-9* in hypodermal function and we suggest that MAB-9 is required for the secretion and/or modification of posterior cuticle.

Introduction

The *mab-9* mutation was originally identified in a screen for worms defective in mating (Hodgkin, 1983). In the developing hindgut of wild-type (WT) *Caenorhabditis elegans*, MAB-9 acts to specify the identity of the male-specific blast cells B and F; in *mab-9* mutants, B and F assume the fates of their two anterior neighbours, Y and U, resulting in grossly abnormal male tails lacking many of the normal internal structures (Chisholm and Hodgkin, 1989). In hermaphrodites this hindgut defect leads to severe constipation because B and F are required for the structural integrity of the rectum. In addition to hindgut and tail abnormalities, *mab-9* worms are defective for backward movement (Chisholm and Hodgkin, 1989; Woollard and Hodgkin, 2000). This is now known to be caused by an axon guidance defect in which *mab-9* mutants fail to correctly form circumferential commissures from the ventral nerve cord (VNC) (Huang et al., 2002; Pocock et al., unpublished). *mab-9* encodes a member of the Tbx20 sub-family of T-box transcriptional regulators (Woollard and Hodgkin, 2000). T-box genes are widely distributed in nature with various developmental roles (reviewed in Papaioannou, 2001) and mutations in a number of T-box genes have been linked to human developmental disorders, including DiGeorge syndrome, Holt–Oram syndrome and Ulnar–Mammary syndrome (reviewed in Packham and Brook, 2003).

We have previously shown that a *mab-9*::*gfp* construct including approximately 5.5 kb of upstream sequence was able to completely rescue the *mab-9* phenotype, indicating that this region includes all regulatory sequences required for correct MAB-9 expression (Woollard and Hodgkin, 2000). Worms rescued with this transgene exhibit GFP expression in nuclei of the rectal epithelial cells B and F (and B- and F-derived cells in males), a subset of ventral nerve cord nuclei and a single head neuron (Woollard and Hodgkin, 2000; Pocock et al., unpublished), as well as 3 or 4 nuclei of the posterior hypodermis.

Since MAB-9 is expressed in a variety of different tissues and performs a number of disparate functions in *C. elegans*, we were interested in dissecting the regulatory region in order to define precise regulatory elements. Study of gene regulation in *C. elegans* is now greatly facilitated by the availability of the genome sequence of the closely related species *Caenorhabditis briggsae*. Although the degree of conservation between the two genomes is generally high for coding sequences, there is generally less conservation outside of coding regions (Stein et al., 2003). Blocks of Conserved Non-coding Elements (CNEs) are thought to be likely domains of gene regulation, by acting as binding sites for trans-acting factors (Wang et al., 1999; Kirouac and Sternberg, 2003).

Here we show that there are six CNEs shared between *C. elegans mab-9* and its nearest *C. briggsae* homologue, *CBG24273*. Firstly, we demonstrate that *CBG24273* is a probable *mab-9* orthologue by comparing expression patterns and rescuing ability. Secondly, deletion of CNEs from the *C. elegans mab-9* rescuing construct reveals two adjacent blocks of homologous sequence comprising a total of 78 bp that are absolutely required for expression in the posterior hypodermis. These sequences are also sufficient to drive hypodermal expression of a GFP reporter when inserted into an expression vector containing minimal promoter elements from the embryonically-

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expressed gene *pes-10* (Seydoux and Fire, 1994). *mab-9* mutant animals containing a construct lacking this 78 bp region are not completely rescued and exhibit several male-specific defects including abnormal cuticle properties. *mab-9* hermaphrodites containing the same construct exhibit reduced susceptibility to infection by the pathogenic bacterium *Microbacterium nematophilum* because bacteria are less able to colonise the rectum of worms lacking posterior hypodermal expression of MAB-9. Thus, our studies reveal a novel role for *mab-9* in hypodermal function in both males and hermaphrodites. This aspect of *mab-9* function would not be revealed in loss-of-function mutants due to the severe morphological defects in such alleles masking more subtle defects in hypodermal morphogenesis and cuticle function.

Materials and methods

Strains and worm maintenance

All *C. elegans* strains used were derived from the wild-type (WT) Bristol strain N2. The *C. briggsae* strain AF16 (Fodor et al., 1983) was used where appropriate. Worm manipulations were performed as previously described (Sulston and Hodgkin, 1988). For microscopy, worms were mounted on slides with thin pads of 2% agarose melted in 0.5% 1-phenoxy 2-propanol (Sigma) in M9 buffer. Animals were picked to a small drop of 0.2% 1-phenoxy 2-propanol on the pad and covered with an 18×18 mm coverslip. Worms were visualised using a Zeiss Axiophot microscope fitted with DIC optics. Supplementary Table 1 describes all strains used in this study.

Sequence comparisons

The *C. briggsae* protein identified on Wormbase (http://www.wormbase.org/) by BlastP, as the nearest homologue of *mab-9* is CBP12816, the product of *CBG24273*. Aligned sequences consisted of 5′ sequence (5.5 kb for *mab-9*, 4.3 kb for CBG24273), plus entire protein coding sequence. We also included 1 kb of downstream sequence in the alignment. Alignments were performed by Pairwise Blast (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) using default parameters. Conserved sequences (CNEs) of 25 bp or greater were selected for analysis. Blast alignment of potential regulatory elements was confirmed by means of the Sockeye genomics application (www.bcgsc.bc.ca/sockeye) (Montgomery et al., 2004).

Transgenic animals

Injections were performed in *C. briggsae* or *C. elegans*, using 2–20 ng/μl DNA, as previously described (Mello and Fire, 1995) along with the dominant injection marker *rol-6* (50–100 ng/μl). Transformants were selected on the basis of the dominant *rol-6* phenotype and allowed to self. Stable transmitting lines were examined at the F3 generation or later by epifluorescence and DIC microscopy. Several transgenic lines were examined for each construct and a representative of each is described in this report. Where appropriate, transgenic arrays were integrated using gamma irradiation (Evans, 2006). The integrated *mab-9::gfp* strain *mab-9(e2410); him-8(e1489); eIs34[eEx85(mab-9::GFP+rol-6)]* has been previously described (Woollard and Hodgkin, 2000).

GFP/RFP reporters and rescuing constructs

A *C. briggsae mab-9* promoter-only GFP reporter construct was generated by means of a PCR method (Hobert, 2002). Template was acquired by the single-worm lysis method (Fay and Bender, 2006). PCR was carried out using oligonucleotide primers designed to include the 5′ regulatory sequence of interest, with the reverse primer having a tag complementary to 21 bp of the standard Fire lab GFP vector polylinker sequence (http://www.addgene.org/Fire_Lab). A GFP PCR product was obtained using primers described by Hobert, 2002, using a Fire lab vector with or without nuclear localisation signal, as required. A nested PCR, using products of the above 2 reactions as template, was then performed to generate a single promoter–GFP fusion, which was gel-purified if necessary before injection. Primers to generate tagged *C. brig-mab-9* promoter: Forward 5′-GCAACCGAAAATCTCTTTATGG-3′, reverse 5′-CGACCTGC AGG-CATGCAAGCTGTCCTCTAGAAGAATTTCAGCAG-3′. Nested PCR forward primer: 5′-ATG-CATCGCCCATTAGAAAAGTGACCATACAG-3′. Transgenic lines described in this study are the *C. briggsae* line *AW244* and the *C. elegans* line *AW245*. The *C. elegans mab-9* promoter-only GFP construct pAW150 was made by PCR amplifying the *mab-9* regulatory region using primers containing a 5′*Pst*I site and 3′*Bam*HI site (Forward 5′-GGAACTGCAGCTCCCATCTAAAAAGAGCCG-3′; reverse 5′-GGCGGATCCCAAAATTTAG-TATCTTATGG-3′) and subcloning into Fire Lab vector *pPD96.04*. The corresponding transgenic line described in this study is AW246. The *dpy-7::rfp* transcriptional fusion *pAW289* was made to confirm the hypodermal identity of cells. 249 bp of *dpy-7* promoter sequence (Gilleard et al., 1997) (−373 to −125) was amplified from genomic DNA (obtained by single-worm lysis) using primers oTB7 and oTB8 (containing *Hin*dIII and *Xba*I tags, respectively) and TA cloned into pCRII-TOPO (Invitrogen). The promoter was subcloned into Multiple Cloning Site (MCS) I of pPD49.26 to give the plasmid *pAW288*. mRFP was amplified from pHK210NLS (a kind gift from Hiroshi Kagoshima) using oTB9 and oTB10 (includes SacI tag) and TA cloned into pCRII-TOPO. mRFP(NLS) was subcloned into the MCSII of *pAW288* to generated the plasmid *pAW289*. Primer sequences were as follows: oTB7 5′-AAGCTTTG ACCTCTCGGGAACAATC-3′, oTB8 5′-TCTAGAAGAA-CAGGGTGTGATAAATGAATTG-3′, oTB9 5′-GCCAAAGGACCCAAAGGTAT-3′, oTB10 5′-GAGCTCGGCGCTCAGTTGGAATTCTT-3′. The corresponding transgenic lines described in this study are *AW247* and *AW255*. To make a minimal promoter construct driving GFP from CNE-DE, the region including CNEs D and E was amplified by standard PCR. Forward and reverse primers included tags corresponding to *Hin*dIII and *Xba*I recognition sites, respectively. Forward primer: 5′-AAGCTTTCTC AGATCATAAACCATTATCT GTG-3′. Reverse primer: 5′-TCTAGAATTGACTAATTATCATCAC ACATTCTT-3′. The PCR product was first TA-cloned into the vector pPCR2.1-Topo (Invitrogen) using the manufacturer's standard protocol. The CNE was then subcloned, using *Hin*dIII and *Xba*I, into the Fire lab minimal promoter vector pPD107.94, to give plasmid *pAW339*. The corresponding transgenic line described in this study is *AW248*. A genomic *Cbr-mab-9* rescuing construct was generated by PCR from *AF16 C. briggsae* worms, using primers to include the upstream regulatory sequence of *CBG24273*, full protein-coding sequence and approximately 1 kb of downstream sequence. Forward primer: 5′-GCAACCGAA AATCTCTTTATGTG-3′. Reverse primer: 5′-TCGGATCTCCATTCATCCTC-3′. The corresponding transgenic line described in this study is *AW249*. The genomic rescuing *mab-9::gfp* construct *pAW118* used in this study has been described previously (Woollard and Hodgkin, 2000). The complete genotypes of transgenic strains are shown in Supplementary Table 1.

Site-directed mutagenesis

Conserved upstream and intronic CNEs were deleted individually from the *mab-9::gfp* rescuing construct *pAW118* by means of the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). As the full-length rescuing construct is too large for direct mutagenesis (18 kb), mutagenesis was carried out on smaller fragments, with the mutagenised fragments being subcloned back into the *pAW118* plasmid. Successful removal of CNEs was confirmed by PCR and sequencing. Plasmids used in this work are *pAW340* (*mab-9ΔCNE-D::gfp*), *pAW341* (*mab-9ΔCNE-E::gfp*) and *pAW342* (*mab-9ΔCNE-DE::gfp*). Transgenic strains generated are *AW250* (*mab-9(e2410)*; *him-8(e1489)*; *ouEx82[mab-9ΔCNE-D::gfp+rol-6]*), *AW251* (*mab-9(e2410)*; *him-8(e1489)*; *ouEx83 [mab-9ΔCNE-E::gfp+rol-6]*, *AW252* (*mab-9(e2410)*; *him-8* (*e1489)*; *ouEx84[mab-9ΔCNE-DE::gfp+rol-6]*) and *AW253* (*mab-9(e2410)*; *him-8(e1489)*; *ouEx85[mab-9ΔCNE-DE::gfp+rol-6+pdpy-7::rfp]*). Transgenic array *ouEx84[mab-9ΔCNE-DE::gfp+ rol-6]* was integrated to give the strain *AW254* (*mab-9(e2410)*; *him-8(e1489)*; *ouIs1 (ouEx84)*).

Dye-filling assay

Mixed staged worms were washed from seeded NGM plates in sterile water. Following 3 washes in water, followed by brief centrifugation, animals were incubated at 20 °C for 30 min in a solution of 10 μg μl^{-1} DiI (1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) in water. To allow clearing of DiI from the gut, worms were washed briefly in water and transferred to seeded NGM plates for at least 60 min (Shaham, 2006). Animals were mounted for microscopy and examined by epifluorescence.

Lectin staining

Worms were stained with a variety of conjugated lectins, as described previously (Gravato-Nobre et al., 2005). Briefly; adult worms were picked to 1 ml of PBS-T (phosphate-buffered saline containing 0.5% Triton X-100) and washed twice for 5 min. The supernatant was removed and the worms incubated in approximately 30 μl of 50 μg μl^{-1} fluorescein-conjugated lectin for 45 min at 25 °C. Excess lectin was removed by washing 3 times in PBS-T. Worms were mounted for microscopy as described above and examined.

Growth on M. nematophilum

To assess sensitivity of worm strains to infection by *M. nematophilum*, worms were grown on NGM plates seeded with lawns of 10% *M. nematophilum*: 90% OP50 (for brood size assays) or 100% *M. nematophilum* for colonisation assays (Hodgkin et al., 2000). Worms were picked at the L4 stage and allowed to grow at 25 °C. Brood sizes were assessed by counting all viable progeny.

Syto13 staining

Adult hermaphrodite worms grown in the presence of *M. nematophilum* were washed 3 times with TBS and incubated for 1 h at room temperature. Worms were incubated for 1 h in 10 μM Styo13 (Molecular Probes), followed by 3 washes in TBS (Hodgkin et al., 2000).

Results

C. briggsae CBG24273 is a probable orthologue of mab-9

The *C. briggsae* gene predicted by Wormbase to be the closest match to *mab-9* is *CBG24273*. In order to test whether *CBG24273* is