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mab-3 is a direct *tra-1* target gene regulating diverse aspects of *C. elegans* male sexual development and behavior

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SUMMARY

Sex determination is controlled by global regulatory genes, such as *tra-1* in *Caenorhabditis elegans*, *Sex lethal* in *Drosophila*, or *Sry* in mammals. How these genes coordinate sexual differentiation throughout the body is a key unanswered question. *tra-1* encodes a zinc finger transcription factor, TRA-1A, that regulates, directly or indirectly, all genes required for sexual development. *mab-3* (male abnormal 3), acts downstream of *tra-1* and is known to be required for sexual differentiation of at least two tissues. *mab-3* directly regulates yolk protein transcription in the intestine and specifies male sense organ differentiation in the nervous system. It encodes a transcription factor related to the products of the *Drosophila* sexual regulator *doublesex* (*dsx*), which also regulates yolk protein transcription and male sense-organ differentiation. The similarities between *mab-3* and *dsx* led us to suggest that some aspects of sex determination may be evolutionarily conserved. Here we find that *mab-3* is also required for expression of male-specific genes in sensory neurons of the head and tail and for male interaction with hermaphrodites. These roles in male development and behavior suggest further functional similarity to *dsx*. In male sensory ray differentiation we find that MAB-3 acts synergistically with LIN-32, a neurogenic bHLH transcription factor. Expression of LIN-32 is spatially restricted by the combined action of the *Hox* gene *mab-5* and the *hairy* homolog *lin-22*, while MAB-3 is expressed throughout the lateral hypodermis. Finally, we find that *mab-3* transcription is directly regulated in the intestine by TRA-1A, providing a molecular link between the global regulatory pathway and terminal sexual differentiation.

Key words: *mab-3*, *tra-1*, *dsx*, DM domain, Sex determination, *C. elegans*

INTRODUCTION

Sexual dimorphism is controlled by cascades of regulatory proteins that respond to a primary cue, either chromosomal or environmental, and coordinate the sexual differentiation of diverse tissues. The terminal global regulator in the *C. elegans* sex-determination cascade is encoded by the *tra-1* gene (Hodgkin and Brenner, 1977; Hodgkin, 1987). *tra-1* activity directs female somatic development, and also plays a major role in germline sex determination (Hodgkin, 1987; Schedl et al., 1989). The crucial role of *tra-1* is illustrated by the effects of gain-of-function and loss-of-function *tra-1* mutations, which can cause full sex reversal, irrespective of the activities of upstream regulators (Hodgkin 1980, 1987). Thus *tra-1* can regulate, directly or indirectly, all downstream genes necessary for somatic sexual development. Identifying the genes *tra-1* regulates and determining how they mediate sexual differentiation and behavior are crucial next steps in understanding how *tra-1* controls sexual dimorphism.

tra-1 encodes a DNA-binding protein, TRA-1A, which contains zinc fingers closely related to those of *ci* and *odd paired* of *Drosophila* and the *Gli* genes of vertebrates (Zarkower and Hodgkin, 1992). Thus it is likely that TRA-1A controls sexual fate by activating transcription of female-specific genes and/or by repressing transcription of male-specific genes required for sexual differentiation. TRA-1A also has been proposed to regulate gene expression post-transcriptionally (Graves et al., 1999). *tra-1* controls a wide array of sexually dimorphic features including cell lineages, cell deaths, migrations, morphogenesis and behavior (reviewed in Hodgkin 1988). Several genes have been identified that are required for sexual development, and any of these might be direct targets of TRA-1A regulation. So far, however, only one somatic gene, *egl-1*, has been reported to be directly regulated by TRA-1A (Conradt and Horvitz, 1999). TRA-1A represses *egl-1* transcription in a pair of cells, the HSN neurons, preventing their death in hermaphrodites (Conradt and Horvitz, 1998, 1999). In the vast majority of somatic cells, however, the regulatory targets of TRA-1A and their functions are unknown.

One gene that appears to act downstream of *tra-1*, based on genetic epistasis analysis, is *mab-3* (Shen and Hodgkin, 1988), which has at least two functions in male development. *mab-3* represses vitellogenin (yolk protein) gene transcription in the male intestine, thereby acting as a direct regulator of sexual

differentiation (Yi and Zarkower, 1999). In the nervous system, *mab-3* promotes differentiation of a class of male sense organs of the peripheral nervous system called V rays. In *mab-3* mutant males, the six bilateral pairs of V ray neuroblasts differentiate primarily as hypodermal cells rather than undergoing V ray differentiation to produce sensory neurons and support cells (Shen and Hodgkin, 1988).

mab-3 encodes a protein with two copies of a nonclassical 'zinc finger' DNA-binding motif called a DM domain (Raymond et al., 1998). The DM domain was first identified in the *doublesex* (*dsx*) gene, a downstream sexual regulator in *Drosophila* (Erdman and Burtis, 1993). As expected from its unusual sequence, the DM domain is structurally distinct from other zinc fingers, and it binds in the DNA minor groove (Zhu et al., 2000). In addition to the DM domain, *mab-3* and *dsx* share several other characteristics, suggesting that they may be derived from an ancestral sex-determining gene. Both genes act downstream of the global regulators in their respective sex-determination cascades, controlling a subset of sexually dimorphic features (Baker and Ridge, 1980; Shen and Hodgkin, 1988). Both genes are direct transcriptional regulators of yolk protein genes (Coschigano and Wensink, 1993; Yi and Zarkower, 1999) and are required for differentiation of sex-specific sense organs (Baker and Ridge, 1980; Shen and Hodgkin, 1988). The male-specific isoform of DSX can substitute for MAB-3 in the *C. elegans* male peripheral nervous system, indicating that the two proteins are functionally very similar (Raymond et al., 1998). *dsx* appears to be more widely required than *mab-3*, regulating all external sexually dimorphic features (Baker and Ridge, 1980) and playing a role in the central nervous system that is essential for mating behavior (Villella and Hall, 1996). Based on their extensive similarities, *mab-3* and *dsx* may represent the first example of evolutionary conservation between distantly related sex-determination pathways (Raymond et al., 1998). Recent work suggests that vertebrates use similar genes to control sexual development. Expression of the DM domain gene *Dmrt1* in vertebrates with XX/XY, ZZ/ZW and environmental sex-determining mechanisms is consistent with a role in male sexual development (Raymond et al., 1998, 1999; Smith et al., 1999; De Grandi et al., 2000; Kettlewell et al., 2000; Moniot et al., 2000).

Here, we have investigated the role of *mab-3* in regulating male sexual development by analyzing its expression, its regulation by the sex determination pathway, and its role in nervous system differentiation and function. While the function of *mab-3* in sexual differentiation has been clearly established in the male intestine, where it acts as a transcriptional repressor of yolk protein genes (Yi and Zarkower, 1999), the other functions and regulation of *mab-3* are poorly understood. We find that *mab-3* is a direct target of TRA-1A transcriptional regulation. MAB-3 acts synergistically with the bHLH transcription factor LIN-32 to promote sensory ray neuroblast differentiation. We find that spatial control of sensory ray formation is accomplished by restricting the region of the lateral hypodermis that expresses both MAB-3 and LIN-32. We find that *mab-3* has additional functions, and that, like *doublesex*, it is required for male-specific neuronal gene expression and male sexual behavior. These results demonstrate that *mab-3* has diverse functions in male sexual development.

MATERIALS AND METHODS

C elegans strains and culture

Culture and genetic manipulation of *C. elegans* were performed by standard methods as described previously (Sulston and Hodgkin, 1988). *mab-3* mutants were of genotype *mab-3;him-5(e1490)*.

Reporter and heatshock experiments

Plasmids were coinjected with pRF4 at a concentration of 50-100 ng/μl. pRF4 contains the dominant *rol-6(su1006)* marker (Mello et al., 1991). Except where noted, at least three independent transgenic lines were analyzed in each experiment. To compare expression of *srd-1*::*gfp* and *lov-1*::*gfp* reporters in wild type and *mab-3(null)* males, heritable extrachromosomal arrays of the reporter and pRF4 were established in strains of genotype *mab-3(null);him-5(e1490)*. Transgenic hermaphrodites from these strains were mated with *him-5(e1490)* males, and *mab-3*/+; *him-5* Rol hermaphrodites from these crosses were self-fertilized to generate a mix of one quarter Mab (*mab-3/mab-3*) and three quarters wild-type (*mab-3*/+ and +/+) transgenic males that were examined for reporter gene expression. For *srd-1*::*gfp*, we made 10 lines in *mab-3(e1240);him-5(e1490)* and five lines in *mab-3(mu2);him-5(e1490)*. None expressed *srd-1* in the ray 9 neuron. We crossed two of the *e1240* lines with *him-5* males and tested F_2 self-progeny; in both cases, all of the non-Mab males expressed the reporter and none of the Mab siblings had detectable expression. For *lov-1*::*gfp*, we generated six transgenic lines in *mab-3(e1240;him-5(e1490)*, all of which expressed the reporter in the tail but not in the CEM cells. Two lines were mated with *him-5* males, and in both cases all non-Mab transgenic males expressed the reporter in the CEM cells and no Mab siblings had detectable expression.

For heatshock experiments, laid eggs were collected for 8-10 hours from hermaphrodites carrying extrachromosomal arrays of the relevant heatshock transgenes. Eggs were allowed to hatch at 20°C for approx. 12-16 hours and then raised at 20°C with 45 minutes heatshock treatments at 33°C every 12-14 hours until adulthood. The heterogeneous staging of animals and repeated heatshocks are intended to ensure that a significant proportion of animals receive treatment during the appropriate stages of development. Heatshock constructs contained the relevant cDNAs fused to the hsp16-41 promoter (Stringham et al., 1992) in pPD49.78 (gift from A. Fire). Plasmids were pDZ124 for MAB-3, EM#226 for LIN-32 (Zhao and Emmons, 1995) and pKM1034 for HLH-2 (Harfe et al., 1998).

Gel mobility shift assays

For gel mobility shift experiments, probes were generated by PCR amplification with primers MAB PROF1 and WY52 at low dATP concentration (20 μM) and in the presence of 10 μCi [^{32}P] dATP. For the wild-type probe the template was –1497pro::*gfp* (pDZ147) and for the 6 bp mutant probe (Mutant 1) the template was pDZ147M1. Labeled PCR products were purified on a non-denaturing 6% polyacrylamide gel. TRA-1A protein was generated by in vitro transcription and translation of a full-length *tra-1* cDNA (pDZ118) using a T7-based coupled reticulocyte lysate (TNT Coupled Reticulocyte Lysate System, Promega). After in vitro translation of protein, $ZnSO_4$ was added to 50 μM. Gel mobility shift assays were performed as described (Pollock and Treisman, 1990), with incubation for 20 minutes at room temperature before electrophoresis on 4% acrylamide gels/0.5×TBE. Competitor DNAs were generated by PCR using primers MAB PROF1 and WY52 and relevant templates, pDZ147M2, pDZ147M3 or pDZ147M4 (mutations are detailed in Fig. 4). PCR products were eluted and purified from non-denaturing polyacrylamide gels. Competition experiments were performed with 10 fmol of ^{32}P-labeled PCR fragments as probe, and 0, 5, 50 or 500 ng of unlabeled PCR fragments as competitor. Mutant –1497pro::*gfp* plasmids were made by site-directed mutagenesis with the following primers (sequences follow): DZ1000 for Mutant 1; WY49 for Mutant 2; WY50 for Mutant 3; and WY51 for Mutant 4.

Oligonucleotides

MAB PROF1: 5′ CGCAAGCTTCGCAGAGATCACACGATTC-GCGGA 3′
WY52: 5′ CCCCTCCTGTGAAACGGGGCGGGTCCC 3′
DZ1000: 5′ CTCTAATTATCGTCGTGCTGCAGCTTCTCCAA-TCGC 3′
WY49: 5′ CTCTAATTATCGTCGTGTGACATCTTCTATCCAATC-GC 3′
WY50: 5′ CTCTAATTATCGTCGTGTGAGGCATTCTATCCAA-TCGC 3′
WY51: 5′ CTCTAATTATCGTCGTGTTGGGTCTTCTATCCAATC-GC 3′

Reporter plasmids

The rescuing *mab-3*::*gfp* reporter gene –1497pro-*mab-3*::*gfp* (pDZ162) contains *mab-3* genomic sequences from 1497 bp 5′ of the site of splice leader SL1 addition (1508 bp 5′ to the MAB-3 start codon), fused in frame to the *gfp* coding region. These sequences were inserted as a *Kpn*I/*Asc*I PCR fragment into pPD117.01 (gift from A. Fire). In gene –1497pro-*mab-3*::*gfp*, the *gfp* stop codon is followed by a 1755 bp *Eco*RI/*Nhe*I PCR fragment containing *mab-3* genomic sequences including the 3′ UTR and poly(A) addition signals, extending about 400 bp beyond the site of poly(A) addition (Raymond et al., 1998). Promoter-only reporters (e.g., –1497pro::*gfp*) included the indicated amounts of *mab-3* genomic sequences upstream of the site of SL1 addition and the first four codons of *mab-3*, fused in frame to *gfp* in the vector pPD95.67 (gift from A. Fire).

The *lin-32*::*gfp* reporter plasmid, pLIN32GFP, contains *lin-32* genomic sequences from 3.2 kb upstream of the initiation codon to the last codon, fused in frame to gfp in pPD95.67. Other reporter constructs used are *srd-1*::*GFP* (Troemel et al., 1995), *plov-1*::*gfp1* (Barr and Sternberg, 1999) and *ppkd-2*::*gfp1* (Barr and Sternberg, 1999).

RESULTS

Male-specific expression of *mab-3* reporters in the intestine and nervous system

mab-3 mutations cause defects in V ray formation and male intestinal differentiation, but, like *dsx*, *mab-3* might play other sex-specific roles not yet identified. For example, *mab-3* expression in the nervous system might regulate mating behavior. To identify such roles and to investigate how *mab-3* expression is regulated, we first assayed the expression of reporter genes containing *mab-3* promoter and coding sequences fused to a *gfp* cassette. We initially included 1497 bp of genomic DNA 5′ of the *mab-3* AUG, as this amount is sufficient for phenotypic rescue of *mab-3* mutants (Raymond et al., 1998). The reporter gene –1497pro-*mab-3*::*gfp* contains the *mab-3* promoter from –1497 and the entire *mab-3* genomic coding region, and is fused in frame to a *gfp* cassette (Fig. 1A). We also constructed a series of reporter genes containing only *mab-3* promoter sequences and the first four codons of *mab-3*, fused to a *gfp* cassette. These are denoted –1497pro::*gfp*, –1266pro::*gfp*, and so on (Fig. 1B).

The –1497pro-*mab-3*::*gfp* reporter rescues both of the known *mab-3* phenotypes: defective V ray formation in the male tail (Fig. 1C, top); and ectopic expression of yolk in the male intestine (Fig. 1C, bottom). Thus, its expression is likely to approximate that of the endogenous gene. Rescue of yolk expression was not complete, with yolk eventually accumulating in mutant adult males expressing MAB-3::GFP, and thus the fusion protein may be less active than native MAB-3. Early in development, –1497pro-*mab-3*::*gfp* was expressed in head neurons of late embryos and L1 larvae of both sexes (not shown). Later in larval development, starting in L3, it was expressed in the lateral hypodermis (seam) in both sexes (Fig. 2A-D), and male-specifically in the nervous system, both in the head and tail, including the sensory ray neuroblasts R1-R9 in the tail (Fig. 2C; data not shown). Initially there was some expression in the intestine of both sexes, but expression becomes male-specific by the adult stage, when vitellogenin transcription begins in hermaphrodites.

Tissue-specific regulatory elements in the *mab-3* promoter

A reporter containing just the upstream *mab-3* sequences fused to *gfp* (Fig.1B. –1497pro::*gfp*) showed similar but distinct expression. Like –1497pro-*mab-3*::*gfp*, the –1497pro::*gfp* reporter was expressed in the larval intestine, with stronger expression in males, and became completely male-specific by the adult stage (Fig. 2E; data not shown). Expression of –1497pro::*gfp* was much stronger in the intestine than that of –1497pro-*mab-3*::*gfp*, possibly because the MAB-3::GFP fusion protein made by –1497pro-*mab-3*::*gfp* was less stable than GFP alone (Fig. 2E,F). –1497pro::*gfp* was also expressed in neurons in adult males, including, most prominently, the sensory neuron ADF in the head (Fig. 2E), V ray neurons (Fig. 2F) and one hook neuron (Fig. 2G). Unlike –1497pro-*mab-3*::*gfp*, –1497pro::*gfp* expression was not detectable in the lateral hypodermis or the early sensory ray lineages, suggesting that additional regulatory elements may lie within the *mab-3* coding region. Since the promoter-only reporter was expressed sex-specifically in the intestine and in neurons of the head and tail, we conclude that the regulation of *mab-3* expression is primarily transcriptional.

To identify regulatory sequences in the *mab-3* promoter, we examined the expression of six reporters with deletions of sequences 5′ to the *mab-3* coding region (Fig. 1B). Deleting *mab-3* promoter sequences from –1266 to –1497 greatly reduced expression in the intestine without affecting expression in neurons of the head and tail (Fig. 1B; compare Fig. 2E with 2H, and Fig. 2F with 2I). Deletion beyond –566 eliminates neural expression (Fig. 1B). These data indicate that there are at least two regulatory elements in the *mab-3* promoter: one required for intestinal expression and one required for neuronal expression.

mab-3 transcription in the intestine is directly regulated by TRA-1A

The mutant phenotype of *mab-3* and genetic epistasis in the intestine (Shen and Hodgkin, 1988) suggest that *mab-3* acts downstream of *tra-1*. Consistent with this, –1497pro::*gfp* was expressed in the nervous system and tail of adult XO animals, in which *tra-1* is inactive, but not in adult XX animals, in which *tra-1* is active (Fig. 3A). In XX *tra-1(null)* mutants, –1497pro::*gfp* was expressed exactly as in wild-type XO males, suggesting that TRA-1A represses, directly or indirectly, the transcription of *mab-3* in XX animals.

To determine whether the transcriptional regulation of *mab-3* by TRA-1A is direct, we first searched for potential binding sites in the promoter of *mab-3*. These searches identified a single consensus TRA-1A binding site (Zarkower and Hodgkin, 1993) located at –1470, within the region required

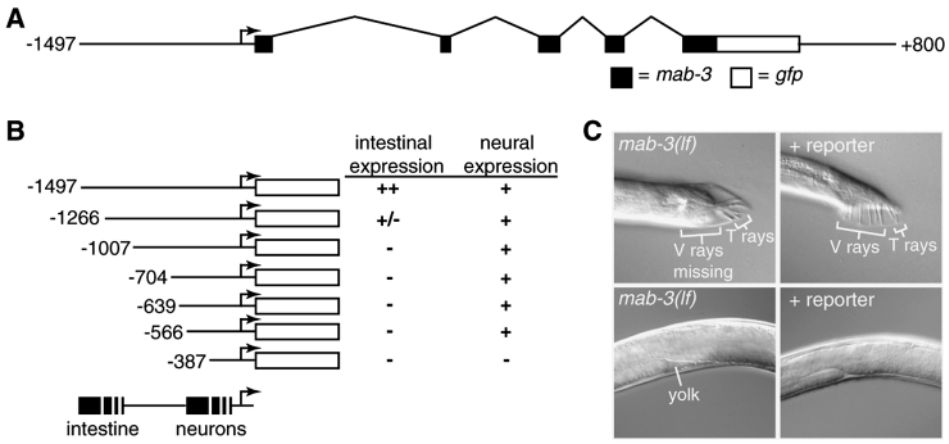

Fig. 1. *mab-3*::*gfp* reporter genes. (A) –1497pro-*mab-3*::*gfp*, which contains *mab-3* genomic sequences from 1497 bp 5′ of the translational start, the entire coding region and a *gfp* cassette, followed by 800 bp of *mab-3* genomic sequences including the 3′ UTR and 3′ flanking sequences. Numbering is relative to the translational start because *mab-3* mRNA is trans-spliced and thus the transcriptional start is unknown. (B) Promoter-only *mab-3*::*gfp* reporters (–1266pro::*gfp*, etc.), with numbering as above. *mab-3* sequences extend from the indicated 5′ nucleotide to the fourth codon of *mab-3*. Expression level of each reporter in the intestine and nervous system is indicated. Below, the broken horizontal line indicates the approximate boundaries of intestine-specific and neuron-specific regulatory elements defined by expression of these reporters. (C) Rescue of *mab-3* tail and intestinal phenotypes by –1497pro-*mab-3*::*gfp* reporter. Top left: DIC (differential interference contrast) image of tail of *mab-3(e1240);him-5(e1490)* adult male, showing lack of V rays and presence of T rays. In this ventral view both sides of the tail are visible. Top right: lateral view of tail of adult male of same genotype carrying an extrachromosomal DNA array containing –1497pro-*mab-3*::*gfp*. All six V rays are present and have correct morphology. Only one side of tail is visible. Bottom left: lateral view of midsection of *mab-3(e1240);him-5(e1490)* young adult male, showing refractile pool of yolk in the coelomic cavity adjacent to the distal gonad arm. Bottom right: lateral view of the same region of young adult male of same genotype and age containing the –1497pro-*mab-3*::*gfp* array, showing lack of yolk accumulation in the coelomic cavity. These animals lack accumulate small droplets of yolk (not shown), indicating incomplete rescue, but consistently produce less yolk than siblings lacking –1497pro-*mab-3*::*gfp*. Incomplete rescue may be due to mosaic expression of –1497pro-*mab-3*::*gfp* within the intestine.

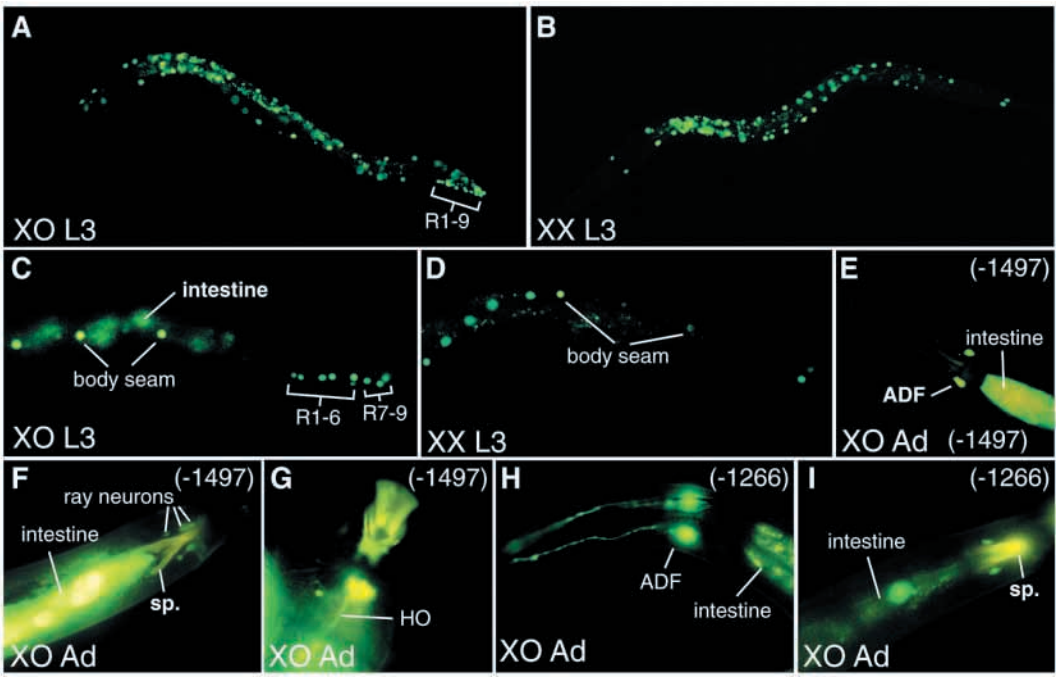

Fig. 2. Expression of *mab-3*::*gfp* reporter genes. (A) L3 XO male expressing –1497pro-*mab-3*::*gfp*. (B) L3 XX hermaphrodite expressing –1497pro-*mab-3*::*gfp*. Intestinal background is due to autofluorescent gut granules. (C) Higher magnification view of L3 male in A, showing expression in body seam, intestine (out of plane of focus) and ray neuroblasts. (D) Higher magnification view of posterior of L3 hermaphrodite in B, showing expression only in hypodermal cells. (E) –1497pro::*gfp* in adult XO male, showing expression in the ADF amphid neuron of the head and in the intestine. (F) Tail of XO adult male showing expression of –1497pro::*gfp* in intestine and sensory ray neurons. sp., copulatory spicules, which are autofluorescent. (G) Tail of adult XO male showing expression of –1497pro::*gfp* in one of the hook neurons (HO). Plume shape at tip of tail is autofluorescence of copulatory bursa. (H) Head of XO adult male showing strong expression of –1266pro::*gfp* in ADF and weaker expression in the intestine. (I) Tail of same animal as in H, showing weak expression of –1266pro::*gfp* in intestine.

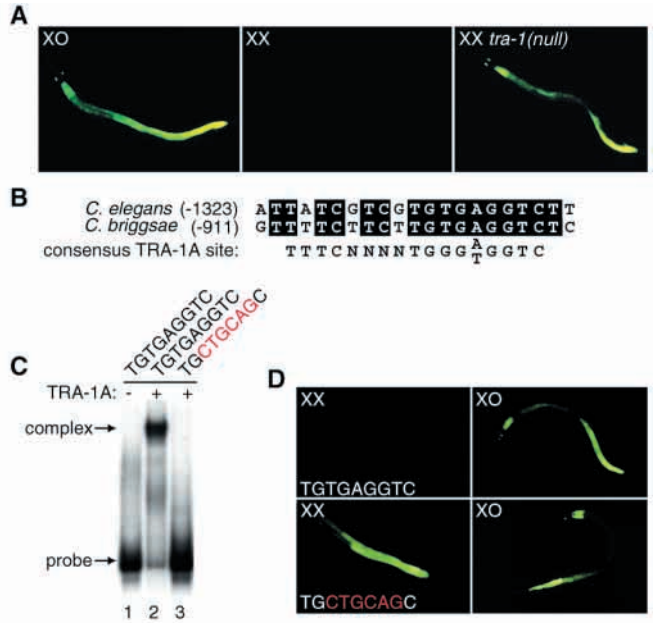

Fig. 3. TRA-1A directly regulates *mab-3* reporter transcription in the XX intestine. (A) Expression of –1497pro::*gfp* in adult XO male (left), XX hermaphrodite (middle) and *tra-1(null)* XX pseudomale (right). (B) Conserved TRA-1A consensus binding site in 5′ sequences of *C. elegans and C. briggsae mab-3* genes. Underneath, the preferred TRA-1A in vitro DNA-binding sequence is indicated. (C) TRA-1A binds the *mab-3* promoter in vitro. Gel mobility shift assays using a labeled fragment of the mab-3 promoter (–1449 bp to –1213 bp). Lane 1: unprogrammed lysate does not shift the wild-type fragment. Lane 2: lysate programmed with TRA-1A cDNA clone efficiently binds the wild-type fragment, forming a complex of reduced electrophoretic mobility. Lane 3: lysate programmed with TRA-1A does not bind a *mab-3* promoter fragment with six nucleotides altered in the TRA-1A consensus site. (D) The TRA-1A site is required in vivo for repression of a *mab-3*::*gfp* reporter. Top panels: –1497pro::*gfp* reporter is expressed in XO adult male (right) but not in XX adult hermaphrodite (left). Bottom panels: –1497pro::*gfp* reporter with mutant TRA-1A consensus site is expressed normally in XO adult male (right) and ectopically in intestine of XX adult hermaphrodite (left).

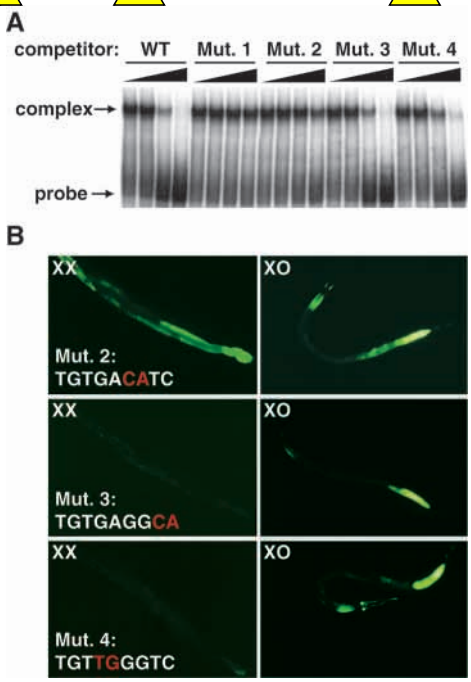

Fig. 4. Strict correlation between TRA-1A binding to *mab-3* promoter in vitro and *mab-3* reporter regulation in vivo. (A) Gel mobility shift analysis of 2 bp mutations in TRA-1A-binding site. A [^{32}P]-labeled –1449 bp to –1213 bp *mab-3* promoter fragment was used as probe in gel mobility shift assays in reactions containing reticulocyte lysates programmed with TRA-1A cDNA and increasing amounts of the indicated unlabeled competitor DNAs. WT indicates the wild-type competitor, containing the sequence TGTGAGGTC. Mutant 1 is the 6 bp mutant described in Fig. 3. Mutants 2-4 contain TGTGACATC, TGTGAGGCA, and TGTTGGGTC respectively, where the altered residues are underlined (red in the figure). For each group of gel lanes, the concentrations of competitor from left to right are 0, 5, 50 and 500 ng. (B) Effect of 2 bp TRA-1A site mutations on *mab-3*::*gfp* reporter expression. –1497pro::*gfp* reporters with each of the three 2 bp TRA-1A-binding site mutations were tested for expression in adult XX and XO animals. Top pair: Mutant 2, which severely affects TRA-1A binding in vitro is expressed in the intestine of XX animals (left) and is expressed normally in XO animals (right). Middle pair: Mutant 3, which has no effect on TRA-1A binding in vitro, is not expressed in XX animals (left), but is expressed normally in XO animals (right). Bottom pair: Mutant 4, which has a slight effect on TRA-1A binding in vitro, is expressed at very low levels in the intestine of XX animals (left) and normally in XO animals.

for intestinal *mab-3* reporter expression. There are also several TRA-1A consensus binding sites located 5′ to –1497 (Clarke and Berg, 1998), but we have not investigated these further. The –1300 site is highly conserved between *C. elegans* and *C. briggsae* (Fig. 3B), suggesting that it may be functionally significant. Although the site in the *C. briggsae mab-3* promoter is about 400 bp closer to the *mab-3* coding region (Fig. 3B), conservation of flanking sequences between the two species (not shown), suggests that these elements are homologous. In a gel mobility shift assay with *mab-3* promoter sequences from –1449 to –1213, TRA-1A bound to a wild-type fragment (Fig. 3C lane 2) but not to one in which the TRA-1A site was mutated at 6 positions (Fig. 3C, lane 3).

To test whether this TRA-1A binding site is required for *mab-3* regulation in vivo, we assayed expression of a –1497pro::*gfp* reporter containing the same 6 bp TRA-1A site mutation described above. In contrast to the wild-type –1497pro::*gfp* reporter, which is expressed only in males (Fig. 3D, top panels), the mutant reporter was expressed in the intestine in both sexes (Fig. 3D, bottom panels). These data suggest that TRA-1A directly represses the transcription of *mab-3* in the intestine. Regulation by *tra-1* in the nervous system does not require this TRA-1A binding site, as the mutant reporter was expressed male specifically in neurons (data not shown). We have not identified other TRA-1A-binding sites within the –1497 promoter region, and most of the neurons that express *mab-3* are present only in males. Thus, regulation of *mab-3* expression in the nervous system by TRA-1A is likely to be indirect.

Deregulation of *mab-3* reporter gene expression by mutation of the TRA-1A binding site strongly suggests that *mab-3* transcription in the intestine is directly regulated by TRA-1A.

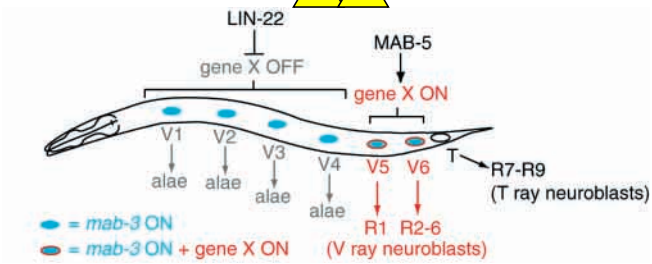

Fig. 5. Model for V ray regulation. Co-expression of *mab-3* (blue) and a second gene, 'gene X' (red) is required for V ray differentiation. MAB-5 promotes V ray formation in the V5 and V6 cells of the posterior lateral hypodermis by promoting the expression of 'gene X', a gene whose expression is restricted to the V5 and V6 cell lineages, promoting the differentiation of the V ray neuroblasts R1-R6. LIN-22 prevents the anterior lateral hypodermis (V1-V4 lineages) from forming sensory rays by preventing the expression of gene X, resulting in differentiation of body seam with cuticular ridges called alae. This model explains how MAB-5 and LIN-22 can restrict V ray formation to the posterior without restricting *mab-3* expression to the posterior. T rays are not regulated by *mab-5* and *lin-22* and thus are not addressed by this model.

However, we also considered the possibility that a different factor binds to a site overlapping the TRA-1A site, and that this factor, rather than TRA-1A, regulates *mab-3* transcription. To test this possibility, we made three additional mutant reporters, each with two base pairs altered in the TRA-1A site (Fig. 4). One mutant reporter is predicted to have severely reduced TRA-1A-binding affinity based on in vitro binding studies (Zarkower and Hodgkin, 1993), while the other two are predicted to retain TRA-1A binding. To compare the affinity of the mutant reporters for TRA-1A in vitro, we performed gel mobility shift assays using a labeled wild-type probe and either wild-type or mutant unlabeled competitor. The TRA-1A/DNA complex was efficiently competed by unlabeled wild-type probe and not by the 6 bp mutant (Mutant 1, Fig. 4A). As predicted, Mutant 2, which lacks a critical pair of G residues (Zarkower and Hodgkin, 1993), also did not compete efficiently for TRA-1 binding, while Mutants 3 and 4 competed nearly as well as wild type (Fig. 4A). In vivo, the effects of the 2 bp mutations on *mab-3*::*gfp* regulation paralleled their effects on DNA binding in vitro. Mutant 2, which binds TRA-1A poorly, was expressed in both sexes, while Mutants 3 and 4, which bind TRA-1A well, retained normal male-specific expression. Since the regulation of *mab-3*::*gfp* in vivo correlates precisely with the ability of TRA-1A to bind in vitro, we conclude that TRA-1A directly represses *mab-3* transcription in the hermaphrodite intestine. In the male intestine, where TRA-1A is inactive, MAB-3 directly represses yolk protein gene transcription (Yi and Zarkower, 1999). Thus, in this tissue *mab-3* serves as a direct molecular link between the terminal regulator of the global sex determination pathway, *tra-1*, and the sex-specific structural genes, the vitellogenins.

Combinatorial regulation of V ray formation by *mab-3* and *lin-32*

From the results described above and previous work (Yi and Zarkower, 1999), the role of *mab-3* in sexual differentiation of the intestine is now fairly clear, but its role in sensory ray development is less clear. *mab-3* activity is required for differentiation of the 6 pairs of V ray neuroblasts, R1-R6, acting downstream of genes that determine which region of the lateral hypodermis (the body seam) forms ray neuroblasts. These genes include the *Hox* gene *mab-5* (Kenyon, 1986) and the basic helix-loop-helix (bHLH) transcription factor gene *lin-22*, a homolog of the *Drosophila hairy* gene (Wrischnik and Kenyon, 1997). As shown in Fig. 5, MAB-5 promotes the formation and differentiation of the V ray neuroblasts R1-R6 in the posterior lateral hypodermis, which is derived from the V5 and V6 cells (Kenyon, 1986). LIN-22 has the opposite role, preventing sensory ray formation in the anterior lateral hypodermis, which is derived from the V1-V4 cells (Wrischnik and Kenyon, 1997). Thus, the combined activities of MAB-5 and LIN-22 result in sensory ray formation only in the posterior lateral hypodermis, in the V5 and V6 lineages. Since *mab-3* is required for differentiation of the ray neuroblasts, a simple model would be that these upstream regulators control ray formation by determining where *mab-3* is expressed.

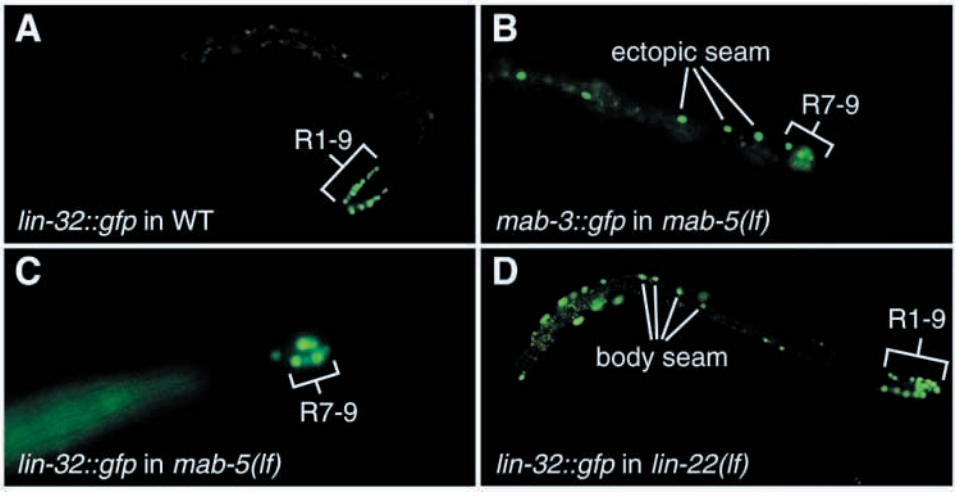

Fig. 6. Expression of *mab-3*::*gfp* and *lin-32*::*gfp* reporters is differentially affected by mutations in spatial regulators of ray formation. (A) *lin-32*::*gfp* reporter in adult *him-5* XO males is expressed in the V and T ray neuroblast cells (Rn) but not in lateral hypodermis. (B) *mab-3*::*gfp* reporter –1497pro-*mab-3*::*gfp* in *mab-5(e1239);him-5(e1490)* L3 male is expressed in T ray neuroblasts (R7-9) and in the ectopic seam cells derived from V5 and V6. (C) *lin-32*::*gfp* in *mab-5(e1239);him-5(e1490)* L3 male is expressed only in the T ray neuroblasts (R7-9), which have recently divided in this animal. (D) *lin-32*::*gfp* reporter in *lin-22(mu2);him-5(e1490)* L3 XO male is expressed both in ray neuroblasts (R1-9) and in lateral hypodermis (body seam). For experiments using the *lin-32*::*gfp* reporter, slightly older animals are shown. This is because the reporter is expressed briefly and weakly in the R1-9 neuroblasts cells before they divide.

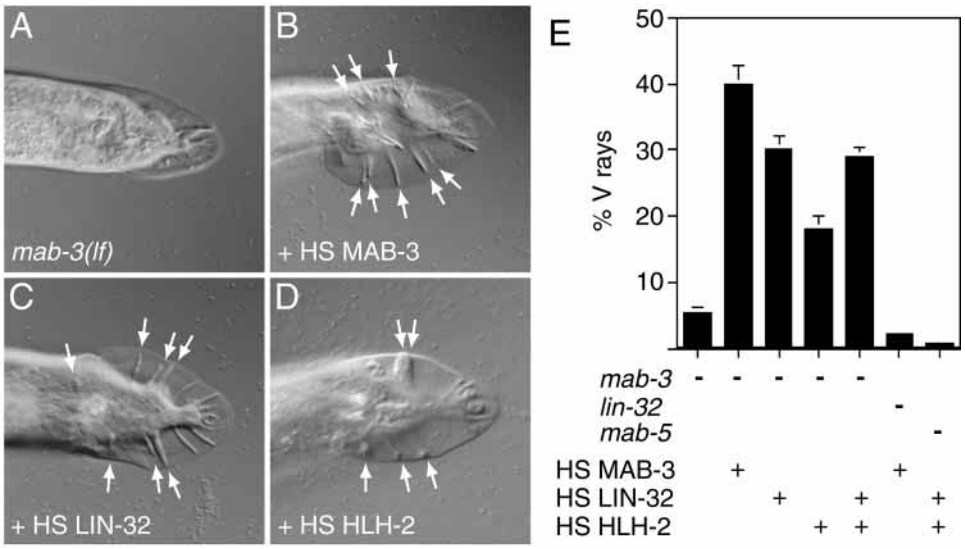

Fig. 7. LIN-32 or HLH-2 overexpression restores V ray formation *in mab-3(null)* males. (A) Ventral DIC image of *mab-3(e1240)* adult male tail, showing lack of V rays and presence of T rays. (B) Ventrolateral view of tail of *mab-3(e1240)* adult male expressing HS-MAB-3, showing partial rescue. Arrows indicate eight V rays. (C) Ventral view of tail of *mab-3(e1240)* adult male expressing HS LIN-32, with seven V rays indicated by arrows. (D) Ventral view of tail of adult *mab-3(e1240)* male expressing HS HLH-2, with five V rays indicated by arrows. Top two rays are fused. (E) Graph of V ray frequency expressed in percentage of normal number (six per side). Genotype and treatment are indicated: -, loss-of-function mutation in the indicated gene; +, heatshock expression of the indicated cDNA. Error bars indicate standard error of the mean. Mutant alleles used were *mab-3(e1240)*, *lin-32(u282)* and *mab-5(e1239)*. As shown in B,C, overexpression of MAB-3, LIN-32 or HLH-2 can partially restore V ray formation to *mab-3* mutant males. Co-expression of LIN-32 and HLH-2 suppresses *mab-3* about as well as LIN-32 alone, when the same total concentration of DNA (50 ng/μl) is injected. Overexpression of MAB-3 does not suppress *lin-32*, and overexpression of LIN-32 and HLH-2 together does not suppress *mab-5*.

However, this is unlikely, since our reporter gene analysis indicates that *mab-3* is expressed in the lateral hypodermis not only where rays form (V5 and V6 lineages), but also where they do not (V1-V4 lineages). Rather, it is likely that *mab-3* acts together with another gene whose expression is restricted to the posterior body seam under the control of MAB-5 and LIN-22 (‘gene X’ in Fig. 5). We therefore investigated the relationship between *mab-3* and other genes involved in sensory ray formation.

A good candidate for a gene acting with *mab-3* in ray formation is *lin-32*, which encodes a bHLH transcription factor (Zhao and Emmons, 1995). Like *mab-3*, *lin-32* is required for differentiation of the V ray neuroblasts. It also is required in the T ray neuroblasts and is involved in neurogenesis elsewhere in both sexes (Zhao and Emmons, 1995; Zhao, 1996; Emmons, 1999). To investigate the relationship of *mab-3* and *lin-32*, we first compared the expression of *gfp* reporters for both genes in wild-type animals and in mutants with altered V ray formation. A reporter with 3.2 kb of *lin-32* promoter and the two coding exons fused to *gfp* partially rescued V ray formation in a *lin-32(lf)* mutant (not shown). This reporter, denoted *lin-32*::*gfp*, was expressed in all 18 ray neuroblast (Rn) cells in L3 males (Fig. 6A) beginning slightly later than *mab-3*::*gfp*, but unlike *mab-3*::*gfp*, it is not expressed in the lateral hypodermis (compare with Fig. 2A,C). Thus, in wild-type males *lin-32* expression only overlapped that of *mab-3* in the cells that form rays, as predicted if *lin-32* is ‘gene X’.

Next we tested whether *mab-5* is required for expression of *mab-3* or *lin-32* in this region. In *mab-5(lf)* mutants, the posterior hypodermal cells V5 and V6 do not form ray neuroblasts, instead they form an ectopic body seam, and T rays form normally. In *mab-5(lf)* L3 males, the –1497pro-*mab-3*::*gfp* reporter is still expressed in the ectopic body seam and in the remaining T-derived ray neuroblast cells, R7-9. (Fig. 6B). This indicates that while *mab-5* is required for the formation of V ray neuroblasts in the V5 and V6 cell lineages of the tail, it is not required for expression of *mab-3* in those lineages. However, *mab-5(lf)* mutations do eliminate *lin-32*::*gfp* expression in the descendants of V5 and V6, without affecting expression in R7-9 (Fig. 6C). Thus, *lin-32*, but not *mab-3*, requires *mab-5* activity for expression in the lineages that form V rays. This suggests that *mab-5* directs V ray differentiation at least in part by positively regulating *lin-32* expression.

Next we investigated whether *lin-22* prevents anterior V ray formation by limiting the domain of *lin-32* expression to the posterior. As predicted by the model, in *lin-22(lf)* L3 males, *lin-32*::*gfp* was expressed in the anterior lateral hypodermis as well as in the 18 ray neuroblast cells (Fig. 6D). This suggests that the ectopic rays in *lin-22* mutants may result from ectopic *lin-32* expression. Similar results with *lin-32* have been obtained by Zhao (1996). From the comparison of *mab-3* and *lin-32* expression it appears that *lin-22* and *mab-5* specify the domain in which V rays form at least in part by defining the cells of the lateral hypodermis in which *lin-32* is expressed with *mab-3*. The combined expression of *mab-3* and *lin-32* then acts to promote V ray differentiation.

MAB-3 as a potentiator of LIN-32 activity

mab-3 and *lin-32* are both crucial for V ray formation, but two lines of evidence suggest that their functions are distinct, with *lin-32* an essential determinant of sensory ray formation and *mab-3* playing more of a supporting role. First, *mab-3(null)* mutations severely reduce the number of V rays formed, but do not eliminate ray formation completely (Shen and Hodgkin, 1988). In contrast, even non-null *lin-32(lf)* mutations can have more severe effects on ray formation (Zhao and Emmons, 1995). Second, as shown above, *lin-32* expression, regulated positively by *mab-5* and negatively by *lin-22*, is strictly correlated with ray formation, while *mab-3* is expressed

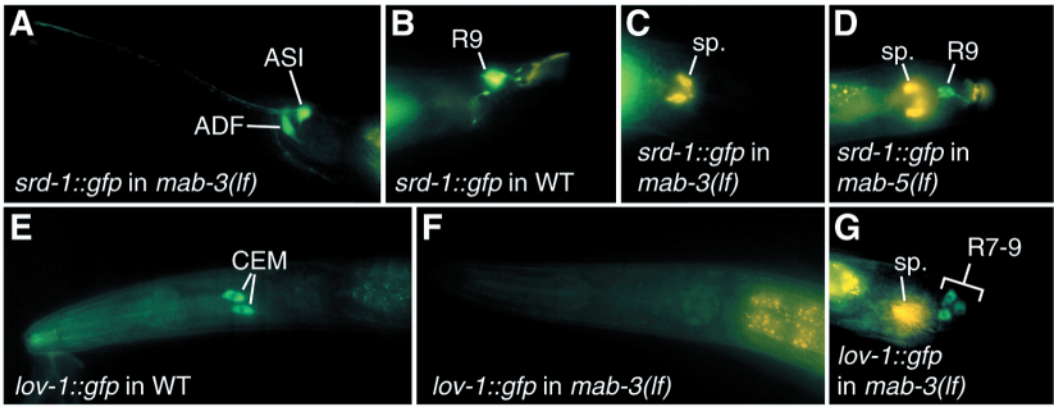

Fig. 8. *mab-3* is required for normal expression of *srd-1* and *lov-1* reporters in male sensory neurons. (A) Head of *mab-3(e1240)* adult male with normal *srd-1*::*gfp* expression in the amphid neurons ADF and ASI. (B) Tail *of mab-3*/+ or +/+ adult male showing expression of *srd-1*::*gfp* reporter in two ray 9 neurons (R9). This reporter is also expressed in the intestine (left of panel). (C) Tail of *mab-3/mab-3* littermate of animals in A and B, showing absence of *srd-1*::*gfp* expression. Spicule autofluorescence is indicated (sp.). (D) Tail of *mab-5;him-5* adult male showing expression of *srd-1*::*gfp* in R9 neurons. (E) Head of *mab-3*/+ or +/+ adult male showing expression of *lov-1*::*gfp* reporter in one of the bilateral pairs of CEM cells (other pair is outside focal plane). (F) Head of *mab-3/mab-3* littermate of animal in D, showing lack *of lov-1*::*gfp* expression in CEM cells. (G) Tail of *mab-3/mab-3* littermate of animals in D and E, showing expression of *lov-1*::*gfp* in T ray neurons (R7-9).

throughout the lateral hypodermis of both sexes. Together these results suggest that *lin-32* plays an instructive role in ray formation, while *mab-3* plays more of a permissive role, possibly acting to enhance the activity of *lin-32*.

To test the idea that *mab-3* enhances *lin-32* activity we asked whether the requirement for *mab-3* in V ray formation can be overcome by elevated expression of LIN-32. We expressed cDNAs encoding MAB-3 and LIN-32 from a heatshock promoter and assayed rescue of the *mab-3* null allele *e1240*. As expected, HS-MAB-3 restored V ray formation to *mab-3(e1240)* males, increasing the V ray number from 5% to 40% of wild type (Fig. 7A,B,E). HS-LIN-32 also suppressed *mab-3(e1240)*, to 30% of the wild type V ray number (Fig. 7C and E). MAB-3 overexpression, however, did not suppress *lin-32(lf)*, suggesting that the two genes do not have identical functions in the V ray lineage (Fig. 7E). We also tested HLH-2, a *C. elegans* E/daughterless homologue (Krause et al., 1997). HLH-2 is expressed in the V ray lineage and thus might serve as a dimerization partner for LIN-32 (J. M. R., data not shown; D. Portman and S. Emmons, personal communication). HS-HLH-2 can suppress *mab-3(e1240)*, but less efficiently than HS-LIN-32 (15% of wild type), and produces rays with abnormal morphology (Fig. 7D,E). Collectively these data suggest a model in which LIN-32 is a crucial determinant of V ray formation and MAB-3 acts to enhance LIN-32 activity in specifying V ray neuroblast cell fate. Normally MAB-3 is required for V ray differentiation, but if sufficient LIN-32 activity is present, MAB-3 can become dispensable. *lin-32* is probably not the only crucial target of *mab-5* in the V ray lineages, however, as expression of LIN-32 and HLH-2 together did not restore V rays to *mab-5* mutants (Fig. 7E).

mab-3 regulates gene expression in male head and tail sensory neurons

Expression of *mab-3* reporters in male neurons outside the sensory ray lineages suggests that *mab-3* might have additional functions in the male nervous system. In the V ray lineages, *mab-3* clearly is important for neuroblast differentiation. Other male sensory neurons that express *mab-3*, while present in *mab-3* mutants, might not function normally. To test this possibility, we examined the expression in *mab-3* mutant males of three genes with known or suspected roles in male mating behavior. The first gene, *srd-1*, encodes a putative chemosensory receptor protein that is expressed male-specifically in the ADF amphid neuron of the head and one of the ray 9 neurons of the tail, and in both sexes in the ASI amphid neuron (Troemel et al., 1995). Since *mab-3* reporters also are expressed male-specifically in ADF and the T ray neuroblasts, the *srd-1* gene is a good candidate to be regulated by *mab-3*. In *mab-3(null)* adult males, *srd-1*::*gfp* was expressed normally in ASI and ADF in the head (Fig. 8A), but no expression was detectable in the ray 9 neuron in the tail (Fig. 8B,C). *srd-1*::*gfp* was expressed in ray 9 in *mab-5* mutant males (Fig. 8D), demonstrating that the loss of expression in this cell in *mab-3* mutant males is not simply a consequence of disrupted V ray formation.

We also tested the expression of *lov-1*, a gene encoding a putative cell-surface protein required for normal male mating behavior (Barr and Sternberg, 1999), and its close homolog *pkd-2*. Both genes are expressed in the CEM neurons of the male head, and in the hook neuron HOB and ray neurons of the tail (Barr and Sternberg, 1999). *pkd-2*::*gfp* expression appeared normal except in rays 1-6, which are missing in *mab-3(null)* (not shown). However, *lov-1*::*gfp* expression was not detectable in the CEM cells of *mab-3* mutant males (Fig. 8E,F). *lov-1*::*gfp* is expressed normally in R7-9 of *mab-3* mutant males (Fig. 8G). Thus *mab-3* is required in both the head and the tail of males for expression of neuronal genes.

mab-3 is required for normal male interaction with hermaphrodites

Both the male-specific neuronal expression of *mab-3* and the defects in *lov-1*::*gfp* and *srd-1*::*gfp* expression in *mab-3* mutants suggest a potential requirement for MAB-3 activity in male-specific sensory neuron function. We therefore tested the ability of *mab-3* mutant males to interact with hermaphrodites.

The lack of V rays in *mab-3* mutant males complicates this task, as V rays are crucial mediators of early steps in mating, and thus *mab-3* mutant males are incapable of copulation for mechanical reasons. As a result, it is not possible to assess the ability of *mab-3* males to perform the different sub-behaviors of copulation. Instead we focused on earlier steps of attraction to hermaphrodites.

Direct observation of *mab-3* mutant males in the presence of hermaphrodites suggests a deficit in attraction to and interaction with hermaphrodites (not shown). To evaluate this deficit more quantitatively, we used the leaving assay of Lipton and Emmons (personal communication) to measure the interaction of males with paralyzed hermaphrodites. In this assay, *him-5(e1490)* (wild-type) males placed in the presence of paralyzed hermaphrodites on a small bacterial lawn remained on the lawn (probability of leaving, P_L=0.006/hr), while those plated with paralyzed males left at a much higher rate (P_L=0.040/hr) (Table 1). This difference in leaving rate between males plated with hermaphrodites and those plated with males suggests that wild type males detect hermaphrodite-specific cues that retain them on the bacterial lawn (Lipton and Emmons, unpublished). *mab-3* mutant males are insensitive to these cues and failed to discriminate between the two sexes, leaving males (P_L=0.169/hr) and hermaphrodites (P_L=0.176/hr) at equivalent high rates. The rate at which *mab-3* mutant males left worms of both sexes is higher than the rate at which wild-type males left males. This might indicate a general sensory defect unrelated to sexual attraction. However, since *mab-3* males respond normally to volatile attractants and repellents, food, and mechanical stimuli (data not shown), a general sensory deficit is unlikely. Another possibility is that *mab-3* males have defects not only in attraction to hermaphrodites, but also in detection of non-sex specific nematode cues.

To exclude the possibility that the failure of *mab-3* males to interact with hermaphrodites is due to the absence of V rays, we determined leaving probabilities for *mab-5* mutants, which also lack V rays and have other tail defects but have normal development of sensory neurons in the head. Although *mab-5* males left hermaphrodites at a significantly higher rate (P_L=0.044/hr) than did wild-type males (P_L=0.006/hr), they did distinguish between males (P_L=0.089/hr) and hermaphrodites (P_L=0.044/hr). Because *mab-5* males are unable to copulate but still are preferentially attracted to hermaphrodites, it is likely that the interaction has contact-independent components. The observation that *mab-3* males did not respond to hermaphrodites under conditions in which *mab-5* males were selectively retained indicates that sensory structures other than the V rays contribute to detection and response to the hermaphrodite cue and that *mab-3* is required for this process.

To determine whether loss of *lov-1* expression might contribute to the defective interaction of *mab-3(null)* males with hermaphrodites, we tested *lov-1(sy582Δ)* males (Barr and Sternberg, 1999) in the leaving assay. *lov-1(sy582Δ)* males left paralyzed males (P_L=0.043/hr) and hermaphrodites (P_L=0.004/hr) at rates indistinguishable from wild type, indicating that *lov-1* is dispensable for hermaphrodite detection in this assay. This result suggests that *lov-1* carries out a MAB-3-regulated function separate from hermaphrodite detection or redundant with other targets of MAB-3 regulation.

DISCUSSION

TRA-1A controls sexual development via *mab-3* and other regulators

Genes that are transcriptionally regulated by TRA-1A serve as a bridge between the global sex-determination cascade and the terminal effectors of sexual differentiation, and defining the molecular basis of these interactions is essential to a detailed understanding of sexual development. Other than *mab-3*, however, *egl-1* is the only somatic target of TRA-1A regulation reported (Conradt and Horvitz, 1999). Regulation of these two genes hints at the varied strategies that may be used by *tra-1* to control sexual development. *egl-1* is a general apoptosis regulator whose transcription is directly repressed by TRA-1A in a single pair of cells, the HSNs, preventing their death in XX animals (Conradt and Horvitz, 1998, 1999). Similarly, TRA-1A regulates vitellogenin expression by direct transcriptional repression of *mab-3* in the XX intestine (Yi and Zarkower, 1999). In other cells, such as the V ray and T ray neuroblasts and ADF, TRA-1A appears to regulate *mab-3* expression indirectly, and in the lateral hypodermis, *mab-3* expression is independent of TRA-1A. Both *mab-3* and *egl-1* are negatively regulated by TRA-1A, demonstrating that the protein is a transcriptional repressor. It is not known whether TRA-1A can both repress and activate transcription, like the related transcription factors CI and GLI (Johnson and Scott, 1998), a point that should eventually be clarified by the identification of additional direct TRA-1A targets.

Our analysis of the role of *mab-3* in regulation of *srd-1* and *lov-1* expression indicates additional complexity in downstream sexual regulation. *srd-1* expression in ray 9 in the tail is dependent on *mab-3*, but its expression in ADF in the

Table 1. *mab-3* mutant males are defective in their interactions with hermaphrodites

Genotype	P_L(M)/hour*	P_L(H)/hour‡	P_L(M)/P_L(H)§
him-5(e1490)	0.040	0.006	6.7
mab-3(e1240); him-5(e1490)	0.169	0.176	0.96
mab-5(e1239); him-5(e1490)	0.089	0.044	2.0
lov-1(sy582Δ); him-5(e1490)	0.043	0.004	10.8

*P_L(M)=P_L(male): probability of individual males of indicated genotype leaving five paralyzed *unc-119;him-5* males over a 9-hour period, expressed as leaving rate per hour. For *lov-1;him-5*, results of two trials of 15 individual males each were averaged. For all other genotypes, results of six trials of seven individual males each were averaged. Leaving is defined as migrating to the boundary of a 100 mm culture plate.

‡P_L(H)=P_L(hermaphrodite): probability of individual males of indicated genotype leaving five paralyzed *unc-119;him-5* hermaphrodites over a 9-hour period, performed in parallel with trials using paralyzed males.

§Ratio of probabilities. Higher number indicates selective attraction to hermaphrodites, and value close to 1.0 indicates no preference. Differences between PL(M) and PL(H) for *him-5*, *mab-5;him-5* and *lov-1;him-5* males are statistically significant, as determined by student's *t* test ($P \leq 0.03$ for 8-hour timepoint).

head is not. However, since *srd-1* expression in ADF is sexually dimorphic (Troemel et al., 1995), it must somehow be regulated by *tra-1*. This regulation might be via a downstream regulator other than *mab-3*, or alternatively TRA-1A might directly repress *srd-1* in ADF. Likewise, expression of *lov-1* in the CEM cells of the male head is dependent on *mab-3*, but expression in tail neurons is not. Expression of the close *lov-1* homolog *pkd-2* in the CEM cells is independent of *mab-3*, despite the fact that *lov-1* and *pkd-2* reporters have virtually identical expression patterns (Barr and Sternberg, 1999). Clearly *tra-1* controls the expression of terminal products of sexual differentiation via a complex network of downstream regulators, and in at least some cases, *tra-1* controls a given downstream target gene in different cells by very different means.

TRA-1A may repress transcription by interfering with enhancer activity

The identification of TRA-1A-binding sites in *mab-3* and *egl-1* suggests a general mechanism for TRA-1A repression of sexually dimorphic transcription. In *mab-3* reporters, a TRA-1A site 1.3 kb upstream is necessary for transcriptional repression by TRA-1A in the intestine, and sequences outside the promoter region are not required for this regulation. A TRA-1A site essential for repression of *egl-1* is instead located 5.6 kb downstream of the *egl-1* transcriptional unit (Conradt and Horvitz, 1999). This demonstrates that TRA-1A can repress transcription through binding sites on either side of the coding region of genes and at (for *C. elegans*) a considerable distance. In both cases, there is significant conservation between *C. elegans* and the related nematode *C. briggsae* of sequences flanking the TRA-1A-binding site. These are sequences that are not required for TRA-1A binding, suggesting that other factors bind nearby. A simple model consistent with the available genetic and molecular data is that TRA-1A binding represses transcription by interference with transcriptional activators that bind to nearby regulatory sequences. Binding of TRA-1A might prevent either the association of these regulators with DNA or their activity once bound. This model also provides a means for TRA-1A, which is presumed to be expressed in most or all cells, to control the expression of a given gene in one cell or group of cells but not elsewhere. If a target gene contains multiple tissue-specific enhancer elements, TRA-1A will only repress transcription directed by those elements adjacent to TRA-1A binding sites. TRA-1A may also employ other repression mechanisms, such as interaction with transcriptional co-repressor complexes, but this has not been tested.

mab-3 as a permissive factor in sensory ray formation

mab-3, in concert with other genes including *lin-32*, is required for V ray differentiation in the male tail. Genetic epistasis analysis and comparison of mutant phenotypes (Shen and Hodgkin, 1988; Zhao and Emmons, 1995; Wrischnik and Kenyon, 1997) indicate that *mab-3* and *lin-32* act later in ray neuroblast differentiation than *mab-5* and *lin-22*, but whether their expression is regulated by these genes and how they interact functionally has been unclear. Using reporter genes, we found that *lin-32* expression was regulated positively by *mab-5* and negatively by *lin-22*, while *mab-3* expression, in contrast, appeared to be independent of these genes. Thus, the crucial determinant of where V rays form appears to be *lin-32* rather than *mab-3*.

Several lines of evidence suggest that *mab-3* acts to enhance the activity of *lin-32* to promote ray formation. First, *mab-3(null)* mutant males, while severely defective in V ray formation, do produce a small number of V rays, and thus *mab-3* is not absolutely essential for ray formation. Second, *lin-22* mutations caused the ectopic expression of *lin-32* in the anterior lateral hypodermis (Fig. 6), but this causes ectopic ray formation only if *mab-3* is also present (Shen and Hodgkin, 1988; Wrischnik and Kenyon, 1997). Third, we found that ectopic expression of LIN-32 could restore V ray formation to *mab-3(null)* mutants. This result must be interpreted with caution as it involves overexpression, but it suggests that *mab-3* is dispensable for V ray formation if sufficient LIN-32 is present. The reciprocal is not the case: MAB-3 overexpression did not suppress ray defects in *lin-32* mutants. This result argues against models in which *mab-3* and *lin-32* perform the same function in ray formation. In such models the total activity of MAB-3+LIN-32, rather than the activity of one protein or the other, is crucial for ray formation. Ectopic HLH-2 expression also restored V ray formation to *mab-3* mutants, but less efficiently, perhaps by increasing the concentration of a complex with LIN-32.

Our results are most consistent with a model in which *mab-3* plays a permissive role in V ray formation in concert with *lin-32*. In wild-type males, *mab-5* directly or indirectly activates *lin-32* expression only in the V5- and V6-derived neuroblasts R1-R6. The combined expression of *mab-3* and *lin-32* in R1-R6 results in their differentiation into V rays. In the anterior body seam (V1-V4 lineages), *mab-3* is expressed but *lin-32* is not, because it is repressed by *lin-22*, and this prevents sensory ray formation. The repression of *lin-32* by *lin-22* could be direct, or it may be mediated by *mab-5*, as *lin-22* mutants ectopically express *mab-5* in the V1-V4 lineages (Wrischnik and Kenyon, 1997). Co-expression of *mab-3* is necessary for full *lin-32* activity, but this requirement can be bypassed by elevating the level of LIN-32 expression. This model predicts that ectopic LIN-32 expression in the anterior body seam should result in ectopic rays, which has been shown to be the case (Zhao and Emmons, 1995). The regulation of *lin-32*, an *achaete-scute* homolog, by *lin-22*, a *hairy* homologue, suggests that the regulatory relationship of these genes may be conserved between flies and worms (Skeath and Carroll, 1991; Orenic et al., 1993; Wrischnik and Kenyon 1997)

mab-3 might potentiate the activity of *lin-32* by any of several mechanisms, which are not mutually exclusive. One possibility is that MAB-3 and LIN-32 physically interact to generate a more active form of LIN-32. A second possibility is that MAB-3 regulates a gene that affects the activity of LIN-32. It could repress an inhibitor of LIN-32 or activate an enhancer of LIN-32 activity. A third possibility is that MAB-3 and LIN-32 may regulate some of the same downstream targets, and that more LIN-32 is required to achieve proper regulation of these genes when MAB-3 is absent. Mechanistic studies and searches for regulatory targets of MAB-3 and LIN-32 should help address these possibilities.

mab-3 is required for male behavior

An intriguing finding of the work reported here is that *mab-3*

reporters were expressed in a number of sensory neurons in the male head and the tail whose formation is not prevented by *mab-3* mutations. All of these cells are good candidates for mediating male mating behavior, and indeed some have been shown to be required for specific aspects of male mating (Ward et al., 1975; Liu and Sternberg, 1995). This raises the possibility that *mab-3* plays additional behavioral roles in the male nervous system.

We tested the possible role of *mab-3* in male mating behavior using two approaches. First, we investigated whether *mab-3* is required for the expression of genes implicated in male mating behavior. Of three genes assayed, *mab-3* was required for normal expression of two: *lov-1* in the head and *srd-1* in the tail. Second, we investigated whether *mab-3* males exhibit defective interaction with hermaphrodites. While wild-type males show a strong preference for hermaphrodites over males, we found that *mab-3* males were not attracted to either sex and rapidly left. This defect cannot result entirely from lack of V rays, as *mab-5* mutants still show a preference for hermaphrodites over males. Taken together, these results strongly suggest that *mab-3* is required in the nervous system for expression of genes that mediate early, and perhaps also later, steps of male mating behavior. In this regard, *mab-3* further resembles *doublesex*, which is required for male courtship behavior in *Drosophila* (Villella and Hall, 1996). Additional assays will be needed to distinguish whether *mab-3* mutant males are defective in taxis to hermaphrodites, sustained interaction with hermaphrodites once located, or both. It also will be important to determine in which cells *mab-3* is required for which aspects of male behavior. The finding that *mab-3*, like *dsx*, is required for male mating behavior further suggests that these two genes may be conserved from an ancient sexual regulator.

It is now clear that *tra-1* coordinates sexual development and behavior via a group of downstream regulatory genes including *egl-1* and *mab-3*. These genes provide an interface between the global sex-determination pathway, with *tra-1* at its terminus, and the expression of the genes responsible for terminal differentiation and function of sexually dimorphic cells throughout the animal. *mab-3* serves as a direct link between *tra-1* and terminal differentiation in the intestine, and as an indirect link in the nervous system, playing key roles in both the formation and the function of male neurons. Even by regulating both *mab-3* and *egl-1* expression, *tra-1* directs the sexually dimorphic development of only a small proportion of cells. An important goal for the future will be to identify the genes that link *tra-1* to sexually dimorphic development elsewhere in the animal.

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