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Copulation in *C. elegans* males requires a nuclear hormone receptor

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ABSTRACT

In *Caenorhabditis elegans*, *uncoordinated (unc)-55* encodes a nuclear hormone receptor that is necessary for coordinated movement and male mating. An *unc-55* reporter gene revealed a sexually dimorphic pattern: early in post-embryonic motor neurons in both sexes; and later in a subset of male-specific cells that included an interneuron and eight muscle cells. A behavioral analysis coupled with RNA interference (RNAi) revealed that males require UNC-55 to execute copulatory motor programs. Two mRNA isoforms (*unc-55a* and *unc-55b*) were detected throughout post-embryonic development in males, whereas only one, *unc-55a*, was detected in hermaphrodites. In *unc-55* mutant males isoform *a* rescued the locomotion and mating defect, whereas isoform *b* rescued the mating defect only. Isoform *b* represents the first report of male-specific splicing in *C. elegans*. In addition, isoform *b* extended the number of days that transgenic *unc-55* mutant males mated when compared to males rescued with isoform *a*, suggesting an anabolic role for the nuclear hormone receptor. The male-specific expression and splicing is part of a regulatory hierarchy that includes two key genes, *male abnormal (mab)-5* and *mab-9*, required for the generation and differentiation of male-specific cells. We suggest that UNC-55 acts as an interface between genes involved in male tail pattern formation and those responsible for function.

Introduction

As is true for a number of animal species, cytogenetic, structural and behavioral features distinguish the two sexes of the nematode *Caenorhabditis elegans* from one another. Reproductive behaviors for many species are essential to their evolutionary success; in addition examples exist for vertebrates and invertebrates where variations of male behaviors are used to establish dominance among members of the same sex (Issa and Edwards, 2006). Insight into the relationship between gene networks and the cellular networks that produce these reproductive behaviors has been gained from studies of *Drosophila melanogaster* and *C. elegans*. The organization and activation of gonadal and nongonadal tissues involved in producing different reproductive behaviors for the two sexes of virtually every species studied involves an ancient family of transcription factors called nuclear hormone receptors (e.g. testosterone receptors in vertebrates and the dissatisfaction receptor in *D. melanogaster*) that play multiple roles in coordinating the complex cascade of events (Hoshijima et al., 1991; McEwen, 1992a,b). However until recently nuclear hormone receptors had not been implicated in aspects of reproduction in *C. elegans* (Gissendanner et al., 2008). Here we report that *unc-55*, an orphan nuclear receptor gene of the *coup-tf* family (Zhou and Walthall, 1998), is required for copulation in *C. elegans*. Wildtype males show sexually dimorphic expression and splicing of the *unc-55* mRNA that depends upon transcription factors instrumental in generating male-specific lineages. The role of UNC-55 in copulation does not depend upon its role in locomotion, where it acts as a transcriptional suppressor to modify a genetic program necessary for creating the synaptic pattern that distinguishes two classes of motor neurons from one another (Shan et al., 2005).

C. elegans males possess a pair of spicules, prong-like structures that are inserted into the vulva of the hermaphrodite and anchor the male to the hermaphrodite during copulation. The spicules are analogous in some ways to the mammalian penis, the avian phallus, and the anal fins found in some fish species that display internal fertilization. Associated with each spicule is a pair of protractor muscles (one ventral and one dorsal) that contract to extend the spicule, and a pair of retractor muscles (one ventral and one dorsal) that contract to retract the spicule. The four protractor muscles are innervated by cholinergic motor neurons. This cellular network generates three distinct spicule motor programs: *prodding*, which allows the precise location of the vulva through small rapid protractor contractions; *insertion*, which extends the spicules due to the complete contraction of the protractor muscles and anchors the male to the hermaphrodite; and *withdrawal*, which is the final step and is mediated by the spicule retractor muscles (Garcia et al., 2001). The eight spicule muscles express the reporter gene *Punc-55*::*gfp* and *unc-55* mutant males are unable to copulate, a behavior that requires the spicule muscles; this suggests an anabolic role for the UNC-55

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nuclear receptor in male muscles involved in mating. In addition, UNC-55 is expressed in a male-specific interneuron.

The analysis of structurally abnormal mutant males in *C. elegans* has identified a number of important genes that orchestrate the lineages and differentiation of the somatic cells that distinguish males from hermaphrodites (e.g., lineage defective *(lin)-32* and male abnormal *(mab-5)* (reviewed in Emmons and Lipton, 2003). Mutations in additional genes have been identified that disrupt the execution of the male mating behavior. These genes encode components associated with the production of neurotransmitters, cell signaling cascades and ion channels (Gower et al., 2005; Gruninger et al., 2006). Mutations in these genes affect specific functional steps in mating. We argue that UNC-55 acting in an androgenic and anabolic role performs an essential link between the generation of male-specific lineages and downstream genes involved in activating the motor programs necessary for copulation.

Materials and methods

Strains and growth of C. elegans

Animals were grown on nematode growth medium (NGM)-agar plates as described by Brenner (1974). Worm strains used in this study include: LGI: *unc-55 (e402, e1170, jd3, jd4, jd8, jd13, u411)*; LGII: *unc-52 (e444), rrf-3 (pk1426), mab-9 (e2410)*; LGIII: *egl-5 (n945), mab-5 (e1239), mab-3 (e1240), mab-23 (e2518)*; LGV: *him-5 (e1467)*; LGX: *lin-15 (n765ts), jdIs2 (Punc-55::gfp)*; *him-5 (e1467)*. All males, including control "wildtype" males, were generated from *him-5 (e1467)* unless otherwise noted.

Male mating behavior

A series of behavioral tests were designed to compare the mating of wildtype and *unc-55* mutant males. L4 males were selected and used in mating experiments the following day. In the first assay three adult (24–48 h post L4 stage) *unc-52 (e444)* hermaphrodites were placed in the center of a 1 cm lawn of *E. coli*, and a male was transferred to the edge of the lawn. *unc-52* hermaphrodites were used because they are paralyzed making it possible to videotape couples with a stationary camera and stage (Liu and Sternberg, 1995). The plate was then videotaped for 6 h. The times that males and hermaphrodites spent together and apart were measured from the recordings. In the second assay, backward movement leading to the location of the vulva and spicule insertion was observed under the dissecting microscope (Zeiss, Stemi SV11 with 160× magnification). The ability of wildtype and mutant males to locate the vulva was measured using criteria developed by (Yu et al., 2003; Barr and Sternberg, 1999; Garcia et al., 2001) and again used *unc-52* hermaphrodites. Location of the vulva was scored as successful when the male paused for more than 1 s with its copulatory bursa apposed to the hermaphrodite's vulva. The efficiency of vulva location was calculated as 1 (successful vulva location at the first encounter with the copulatory bursa), 1/2 (successful vulva location at the second encounter), or 1/3 (successful vulva location at the third encounter). All tested males located the vulva within three encounters. In the third assay time was recorded from initial male/hermaphrodite contact to the first copulatory bursa/vulva encounter, if the male did not lose contact with the hermaphrodite body before the first vulva encounter, otherwise time was started again at the next male/hermaphrodite contact. The Wilcoxon (Mann–Whitney) test was used to determine statistical significance between data from wildtype and mutant males in each bioassay. The fourth assay compared mating in aging males: Individual males were placed with eight adult (24–48 h post L4 molt) *unc-52 (e444)* hermaphrodites on a bacterial lawn (2 cm in diameter). Four to six days following the mating, plates were checked, mating was considered successful if coordinated male and hermaphrodite progeny were present. Males were transferred onto new plates with fresh sets of hermaphrodites until the aged males no longer mated successfully.

Feeding RNA interference (RNAi)

Bacteria strain [HT115 (DE3)] with *unc-55* (F55D12.4) RNAi vector was obtained from HGMP Resource Center, UK (created by Dr. Julie Ahringer). The control bacterial strain used was HT115 with an empty L4440 vector. Feeding RNAi experiments were performed following a protocol reported in Kamath et al. (2001). The genotype of control worms and worms subjected to RNAi is *jdEx4(Pflp-13::gfp);rrf-3 (pk1426)* (Simmer et al., 2002). Males from this background were maintained by male/hermaphrodite mating. A small portion of *rrf-3* males (less than 10%) show abnormal tail morphologies and were not chosen for control or RNAi experiments. A positive control used to test the efficacy of *unc-55* RNAi took advantage of the ability of UNC-55 to suppress the expression of the neuropeptide transgene, *Pflp-13::gfp* in the 13 VD motor neurons (Shan et al., 2005). Ectopic expression of *Pflp-13::gfp* expression in the motor neurons in worms subjected to RNAi indicated the success of the RNAi in neurons (Fig. 3B). Male mating tests were set up using *unc-52* hermaphrodites, and with the corresponding bacteria (males from RNAi bacteria were on mating plates with RNAi bacteria; males from control bacteria or OP50 were on mating plates with control bacteria or OP50).

Identification of the unc-55 isoforms

Total RNA was extracted using the TRIzol LS Reagent (Life Technology) using the accompanying protocol. cDNA was then prepared using Superscript II system (Life Technologies) and the protocol attached. cDNA obtained from reverse transcription was then used as a template to amplify full-length *unc-55* cDNA clones. PCR products were cloned in the pGEM-T vector (Promega). At least eight different clones from each of the samples were sequenced to confirm the full-length isoforms. PCR products were subjected to DdeI, which, cuts specifically the *unc-55a* isoform from *C. elegans* and *C. briggsae* or PleI, which cuts specifically the *unc-55a* isoform from *C. remanei*) to display isoforms in agarose gel.

Generation of transgenic worms

A 2.4 kb *unc-55* promoter and the 1.1 kb unc-55a/b cDNA were PCR amplified. All *gfp* vectors were constructed using pPD117.01 plasmid (obtained from Dr. Andrew Fire). These two fragments were digested respectively with SphI/SacII and SacII/AgeI, and then cloned into pPD117.01 between the SphI and AgeI sites via a common SacII site. Primers for PCR amplification of 2.4 kb *unc-55* promoter are *Punc-55-5* (ATCGGCATGCGACATGGC AGGGAGAGCAAG) and *Punc-55-3* (ATCGCCGCGGATCCTGCATCTGAAATTTT AGAC). RT-PCR primers to amplify *unc-55* cDNA were *unc-55-a/b*-5′ (ATCGCCGCGGATGCAGGAT GGCTCATCAGGTG) and *unc-55-a/b*-3′ (ATCGACCGGTGAATTTCTG-GAAGGTA GTTGGG). All PCR products and final *gfp* plasmids were sequenced for confirmation. Transgenic constructs were individually injected into the gonad of *unc-55;him-5* hermaphrodites following the protocol described by Mello et al. (1991). For each construct, all lines gave similar GFP expression patterns; results shown are representative. Names of these lines are: *jdEx7 (Punc-55::unc-55a::gfp)*, *jdEx8 (Punc-55::unc-55b::gfp)*, *jdEx9 (Punc-55::unc-55a::gfp+Punc-55::unc-55b::gfp)*.

Phalloidin-rhodamine staining

Phalloidin-rhodamine staining was performed following a protocol modified from Goh and Bogaert (1991). Details of this protocol were published in Plunkett et al. (1996).

Laser ablation

Two precursor cells in the somatic gonad, Z1 and Z4, were killed by laser ablation in the early L1 stage using a (Laser Science VSL 337) dye laser and Coumarin 440 dye. Following laser surgery the worms were recovered and allowed to develop into young adults. The success ablation was indicated by the absence of any gonad structure.

Confocal microscopy

Preparations were viewed on a Zeiss (Thornwood, NY) LSM (laser scanning confocal microscope) 510. To observe rhodamine-tagged phalloidin and *Punc55::gfp* in the same preparation, we scanned for GFP and rhodamine fluorescence sequentially to avoid the possibility of overlapping excitation. 3-d renderings

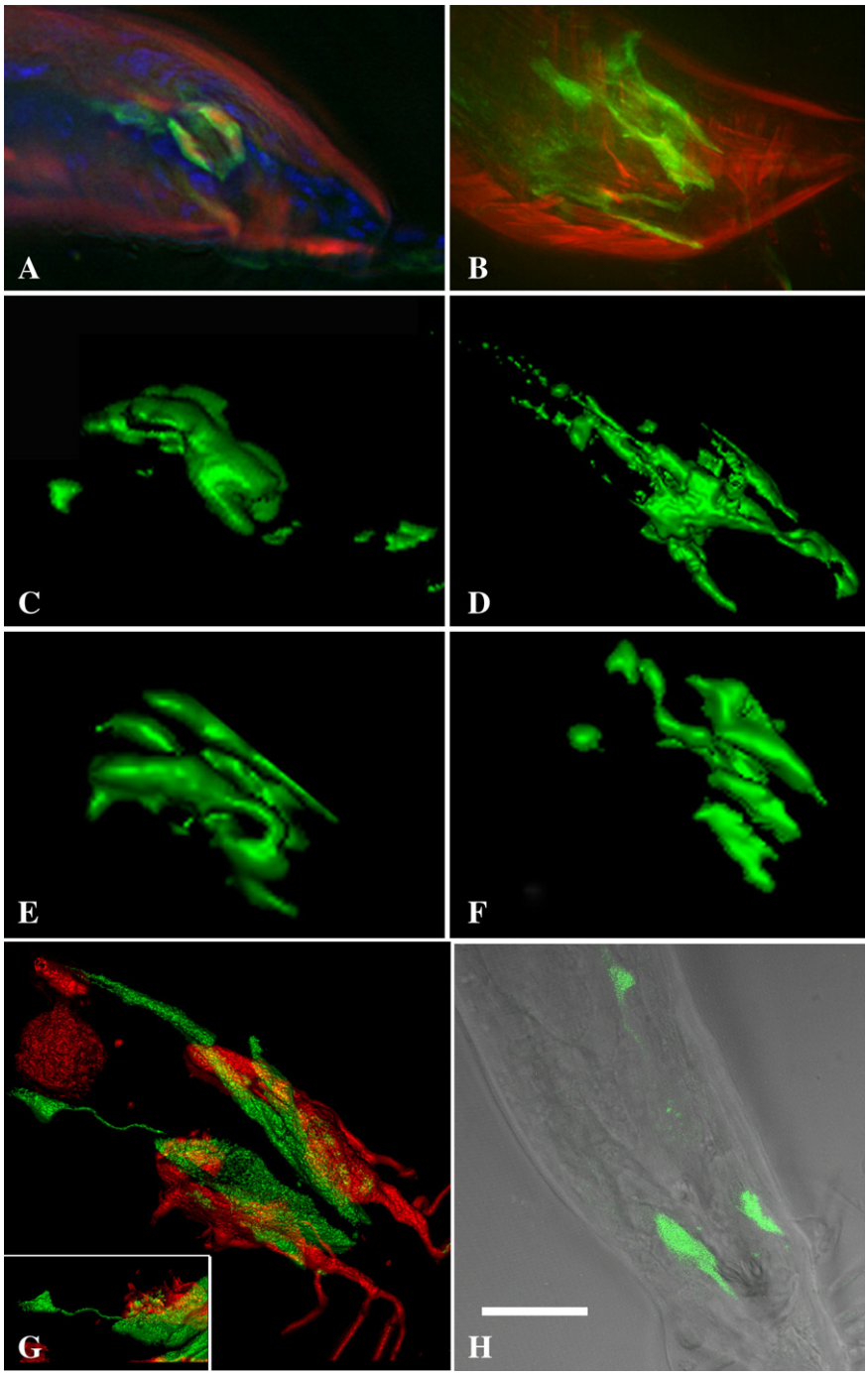

Fig. 1. Male-specific pattern of gene expression and cell identification of *Punc-55::gfp*. (A and B) Co-localization of *Punc-55::gfp* in a subset of *unc-55(+)* male-specific muscles and a neuron tentatively identified as CP9. In the projections in panels A and B *Punc-55::gfp* is green and rhodamine conjugated phalloidin binding to the muscle thin filaments is red. In panel A, cell bodies are stained with the nuclear stain Hoechst. (A) Shows a male with the spicules retracted; (B) shows an adult male with spicules extended. (C–F) Three-dimensional renderings of the spicule retractor and protractor muscles visualized with *Punc-55::gfp*. (C and D) *unc-55(+)* spicules retracted and extended, respectively; (E and F) *unc-55 (e402)* spicules retracted and partially extended, respectively. (G) 3-d rendering from the dorsal perspective, *Punc-55::gfp* (green) and *Ppkd-2::rfp* (red). The inset shows a ventral perspective of the cell body of a neuron, tentatively identified as CP9, projecting posteriorly to the middle of the preanal ganglion where it branches and terminates in a region where the post-synaptic terminals of the ray sensory neurons project. (H) Nomarski image showing the ventral posterior region of the male tail shown in panel G. The anterior (upper) green cell body is the neuron featured in panel G, the more posterior paired fluorescence are the ventral spicule muscles. The cal bar is 20 μm, anterior is to the left, panels A–F is viewed from the left, panel G shows a dorsal perspective, inset a ventral perspective and panel H is ventral.

shown in Figs. 1C–G were made using the Zeiss LSM 510 3-d software.

Results

More than 100 genes have been identified in *C. elegans* through mutants that are unable to move normally. These have been designated uncoordinated (*unc*). The males of many *unc* mutants also have difficulties in mating. Jonathan Hodgkin (1983) devised a system for evaluating the mating efficiency (ME) of mutant males, and four categories were designated, ME 0 to 3, in increasing order of mating success (in term of percentage of progeny sired from mating). Approximately half of the *uncs* including *unc-55 (e402)* are unable to sire progeny (ME=0). We tested six additional *unc-55* strains and found that they too were unable to sire progeny due to unsuccessful copulation. *unc-55* mutants show ventral uncoordinated backward movement due to defects in the cross-inhibitory network (Walthall and Plunkett, 1995; Shan et al., 2005). Is the inability of *unc-55* mutant males to copulate due to the locomotion defect, or to defects independent of locomotion? We examined the expression pattern of the reporter *Punc-55::gfp* in males to begin to distinguish between these two possibilities.

Sexually dimorphic expression of Punc-55::gfp in adult males

We observed *unc-55* expression in the spicule protractor and retractor muscles and in the male-specific neuron CP9. *Punc-55::gfp* adult males were fixed and stained with phalloidin-tagged rhodamine and we observed partial colocalization between the muscle stain and the *Punc-55::gfp* (Figs. 1A and B). Shown in each panel is a projection of the paired protractor and retractor muscles inserting onto the proximal bulb of one of the two spicules (Sulston, Albertson and Thomson, 1980). In Fig. 1A cell bodies were stained with Hoechst (blue) and the spicule was retracted, colocalization was observed around the insertion site of the two contracted retractor muscles inserting onto the proximal portion of the spicule. In Fig. 1B the imaged spicule was extended and the colocalization was apparent in the contracted protractor muscles. Typically the *Punc-55::gfp* stained the entire cell, whereas the phalloidin was only visible in contracted muscles. Comparison of GFP expression patterns in *unc-55(+)* and *unc-55 (e402, e1170* and *jd8)* males revealed no distinguishable structural alterations between mutant and wildtype males. Figs. 1C–F show 3-d renderings of *unc-55(+)* animals with spicules retracted (C) and extended (D); *unc-55 (e402)* mutants with spicules retracted (E) and extended (F).

The cell body of a single neuron located in the anterior region of the preanal ganglion expressed transiently *Punc-55::gfp* (cell body is present in Figs. 1C, F and G). This neuron has been tentatively identified as CP9 based upon the position of the cell body, the posterior projecting process that branches and terminates in a rosette fashion in the preanal ganglion, which is in close apposition with the ray neuron terminals viewed in the inset of Figs. 1G and in H (Sulston et al., 1980; Gower et al., 2005). The ray sensory projections and the juxtaposed projections were viewed with *Ppkd-2::rfp* (Barr and Sternberg, 1999).

Developmental regulation of unc-55 expression

mab-3, *mab-5*, *mab-9* and *mab-23* all encode transcription factors that orchestrate patterns of differentiation in the male tail (reviewed in Emmons and Lipton, 2003). *Punc-55::gfp* was crossed into loss-of-function mutant backgrounds for each of the genes *mab-3 (e1240)*, *mab-5 (e1239)*, *mab-9 (e2410)* and *mab-23 (e2518)* and males expressing *Punc-55::gfp* reporter examined. Despite morphological abnormalities due to alterations in male tail structure (data not shown) expression of the reporter was observed in all except *mab-5*. Thus MAB-5 expression is required for the expression of *unc-55* in the male tail. *Punc-55::gfp* expression was observed in the ventral nerve cord motor neurons of all of the *mab* mutants in L2 animals (data not shown). Mutations in *mab-5* alter sex myoblast migration patterns and the differentiation of posterior P lineages, which give rise to many cells in the prenanal ganglion (Kenyon 1986; Salser et al., 1993; Salser and Kenyon, 1996). Thus the requirement of *mab-5* for *Punc-55::gfp* expression in male tail may be a permissive condition. *Punc-55::gfp* is still expressed in male tail region in *mab-3*, *mab-9*, and *mab-23* mutants. The GFP positive cells are mis-located, with morphological defects. Presently, we do not believe there is any transformation to other male-specific cell types within these cells. It is possible that they are immature muscle cells located at wrong positions.

In the absence of the male gonad a decrease in the duration of spicule protractor muscle contraction during copulation has been reported (Garcia et al., 2001). This result suggests the occurrence of signaling between gonadal cells and associated somatic cells. To determine whether the male gonad is necessary for the expression of *Punc-55::gfp* in somatic tissues we ablated gonad precursors (Z1 and Z4) in newly hatched males. The pattern of *Punc-55::gfp* expression and the morphology of the cells was indistinguishable from that of unablated animals (data not shown). Thus, the absence of the male gonad in *C. elegans* had no effect on the developmental expression of *Punc-55::gfp* in male-specific cells.

Mating behavior of wildtype and unc-55 mutant males

We examined the mating behavior of *unc-55* mutant males and found they were defective in two substeps of the copulatory sequence, spicule prodding and spicule insertion. Other substeps appeared to be normal. To compare the levels of interest of males in mating, we observed wildtype/*him*-5 males, *unc-55* and *(e1170* and *e402)* mutant males. We measured the time that a single male spent together and apart from a single hermaphrodite. On average, mutant males were observed with the hermaphrodites for 150±109 min as compared to 53±41 min for the wildtype (Table 1; Fig. 2A). The longest continuous contact time for mutant males was 243 min, whereas the longest continuous contact time for wildtype males was 79 min (Table 1). Mutant and wildtype males stayed separated from hermaphrodites for similar amounts of time between mating attempts (Table 1). As a result, mutant males spent a larger proportion of time (87.84±11.21%) with the hermaphrodite as compared to 56.49±29.65% for wildtype males (Fig. 2A).

Once the mutant male contacted the hermaphrodite, it searched for the vulva by backing with the copulatory bursa closely apposed to the hermaphrodite's body, as has been reported for wildtype males (Emmons and Sternberg, 1997; Simon and Sternberg, 2002; Lints and Emmons, 2002). Backing in *unc-55* males was comparatively slow and

Table 1
Comparison of mating between wildtype and *unc-55* males

	Wildtype males	*unc-55 (e1170)* males	*P* value
Average time a male spends with hermaphrodite during each contact	53.81±41.61 min *n*=13 males, 26 trials	150.3±109.4 min *n*=12 males, 26 trials	<.01
Average duration of longest contact for each male	79.69±63.72 min *n*=13	243.2±81.59 min *n*=10	<.01
Average time between male mating trials	45.26±38.19 min *n*=13 males, 23 trials	32.65±30.19 min *n*=12 males, 20 trials	0.27
Average duration of longest break time for each male during each trial	52.33±41.61 min *n*=12	38.55±35.47 min *n*=12	0.29

Parameters of male mating behavior were compared between wildtype and *unc-55 (e1170)* males. Each individual virgin male was allowed access to three adult paralyzed hermaphrodites in a circle of bacteria with a radius of 0.5 cm. Video-taping was started with the initial male/hermaphrodite contact and continued for 3–7 h. min, minutes; s, seconds. Data are presented with mean±S.D. *P* values were generated with Wilcoxon test.

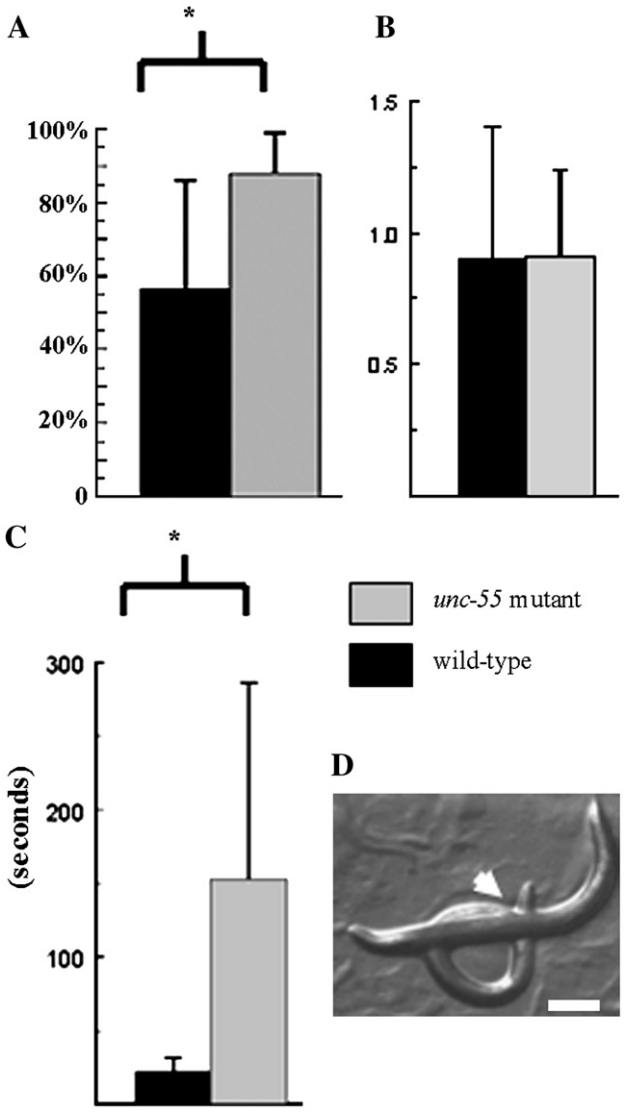

Fig. 2. Comparison of wildtype and *unc-55* male mating behavior. (A) Comparison of the proportion of time males and hermaphrodites spent together and apart (*P value <0.01). (B) Number of passes required for males to locate the vulva. (C) The average amount of time required by males to locate the vulva after contacting the hermaphrodite (*P value <0.01). (D) Micrograph of an *unc-55 (e1170)* male having located the hermaphrodite vulva (white arrow) (scale bar, 100 μm).

periodically they readjusted their body position, as a result they took longer to locate the vulva (Fig. 2C). Typically wildtype males located the vulva on the first encounter (Yu et al., 2003). The same was true for *unc-55* males (Figs. 2B–D). Once *unc-55* males located the vulva they kept the copulatory bursa in direct apposition for periods of time ranging from several seconds to more than 10 min. The final location of the vulva utilizes two steps (Liu and Sternberg, 1995). First, tactile cues detected by the post-cloacal and hook sensilla are used to identify the immediate region around the vulva. Second, the spicules initiate rapid periodic movements, prodding, which lead to the insertion of the spicules through the opening of the vulva (Garcia et al., 2001). Neither prodding nor insertion of the spicules was observed while the copulatory bursa was apposed to the hermaphrodite vulva in *unc-55* mutant males. Very slow prodding was observed in three of eleven *unc-55* males. The failure of *unc-55* mutant males to execute copulation with the spicules provides the most likely explanation for their inability to sire progeny.

During locomotion *unc-55* mutant males move backward differently than *unc-55* hermaphrodites. Hermaphrodites coil ventrally within 1.5 s upon initiating backward movement (Walthall and Plunkett, 1995), whereas *unc-55* males initially maintain a rigid posture for a few seconds before assuming an asymmetric ventral posture. We used paralyzed hermaphrodites in all the mating assays, and *unc-55* males maintained contact with these hermaphrodites for long periods rarely attempting to move forward. We suspect that the slow backward movement enhanced the likelihood of vulval location once the copulatory bursa encountered the vulva and obviates the need to move forward.

unc-55 expression is required continuously in male tail cells for male mating

RNAi provides an alternative means to assess the importance of UNC-55 in male mating. RNAi is a reverse genetic technique that blocks the function of a selected gene (Fire et al., 1998). We coupled RNAi and the developmental stage-specific differences between the expression of *unc-55* in the motor neurons and the male tail to separate the roles of *unc-55* in locomotion from male mating. Three groups consisting of either young adult or L4 stage *rrf*-3 males that were *unc-55(+)* were selected. Group 1 was a control group; males were selected from a plate that had been fed bacteria containing the empty vector for two generations. Group 2 was an experimental group of males from a plate that had been fed double-stranded *unc-55* RNA expressing bacteria for two generations. In Group 3, young adult males were fed with RNAi bacteria for 48 h. Each group of males was then placed in the presence of *unc-52* hermaphrodites. 91% of the *rrf-3* males in the Group 1 mated successfully, whereas the percentage of males that mated successfully for the two experimental groups was 30% for Group 2 and 50% for Group 3, the difference between the control group and the experimental groups was statistically significant (Fig. 3A).

In a second set of RNAi experiments, L3 males (Group 4) were selected from a group of transgenic animals expressing *Pflp-13::gfp* in which the mothers and progeny had been fed RNAi bacteria. Successful suppression of *unc-55* in the VD motor neurons resulted in their expression of *Pflp-13::gfp* ectopically (Shan et al., 2005). We selected males that expressed GFP in the VD and DD motor neurons and showed a ventral coiling phenotype typical of *unc-55* mutants

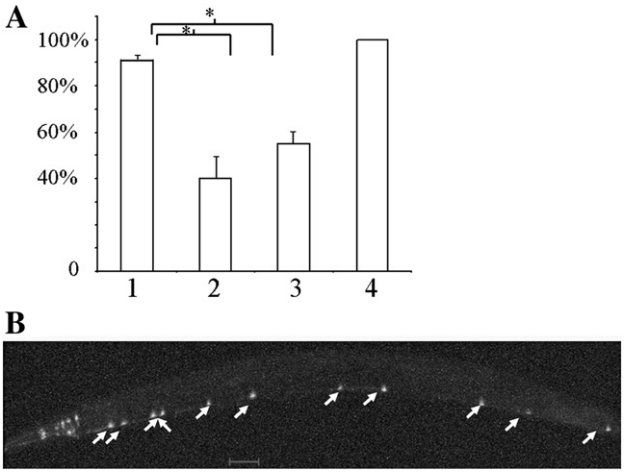

Fig. 3. RNA interference and male mating. (A) Mating ability of *rrf-3* males (n=22) with control RNAi (1); *rrf-3* males (n=32) from mothers that had been fed *unc-55* RNAi bacteria (2); *rrf-3* males (n=33) fed *unc-55* RNAi for two days after their last molt (3); *rrf-3* males (n=8) showing ectopic *Pflp-13::gfp* in the L3 stage were then transferred onto control food for later mating tests (4). (*P value less than 0.05 compared to control, X^2-test.) (B) Micrograph of *C. elegans* with the *Pflp-13::gfp* transgene following *unc-55* RNAi showing the VNC neuron cell bodies expressing ectopic GFP indicating a release from the suppression mediated by UNC-55 and successful RNAi in neurons. Scale bar: 20 μm.

(Fig. 3B). These males were then fed OP50 and allowed to grow into adulthood. All eight of the ventral coiler males sired progeny.

Using developmental stage-specific RNAi, we created uncoordinated males that could mate (Group 4), coordinated males that had their mating efficiency compromised (Group 3) and uncoordinated males with reduced mating capability (Group 2). Thus, UNC-55 is required in male tail cells and the locomotion defect is not responsible for the inability of males to copulate.

Two unc-55 isoforms in males

In *Drosophila*, a number of genes involved in sex determination, male pattern formation and mating have male and female-specific transcripts (reviewed in Lalli et al., 2003; Baker et al., 2001). However, sexually dimorphic alternative splicing in *C. elegans* has not been reported to our knowledge. Using RT-PCR to amplify full-length *unc-55* cDNAs from a mixed population of *him-5* males and hermaphrodites two products were produced, a larger *unc-55* isoform *(unc-55a)*, and a shorter one *(unc-55b)* (Fig. 4A). Hermaphrodites express only the *unc-55a* isoform, which was described previously (Zhou and Walthall, 1998). The *unc-55b* isoform is male-specific. RT-PCR experiments were performed on two populations containing males, one group of *him-5* L2 animals and a second group of adult males. Both isoforms were present in both populations (Fig. 4A). These results show that both *unc-55a* and *unc-55b* are expressed in males, whereas only *unc-55a* is expressed in hermaphrodites. Both isoforms were observed in populations of *him-5* L2 animals (a stage when *Punc-55::gfp* expression is observed only in motor neurons) suggesting that although *unc-55b* is male-specific, it may not be exclusive to male-specific lineages, although this view is challenged by our following results.

We reasoned that if the male-specific splicing of the *unc-55* mRNa is coordinately regulated with genes encoding transcription factors that regulate male-specific developmental events. RT-PCR was used to detect the two isoforms in the following mutant backgrounds: *mab-3*, *mab-5*, *mab-9*, *mab-23* and *egl-5* (Emmons and Lipton, 2003; Woollard and Hodgkin, 2000). *mab*-3, *mab-5*, *egl-5* and *mab-23* were reported to affect male-specific muscle lineages (Lints and Emmons, 2002; Chisholm, 1991; Ferreira et al., 1999). RT-PCR of *mab-3*, *mab-23* and *egl-5* populations containing *him-5* showed that both isoforms were

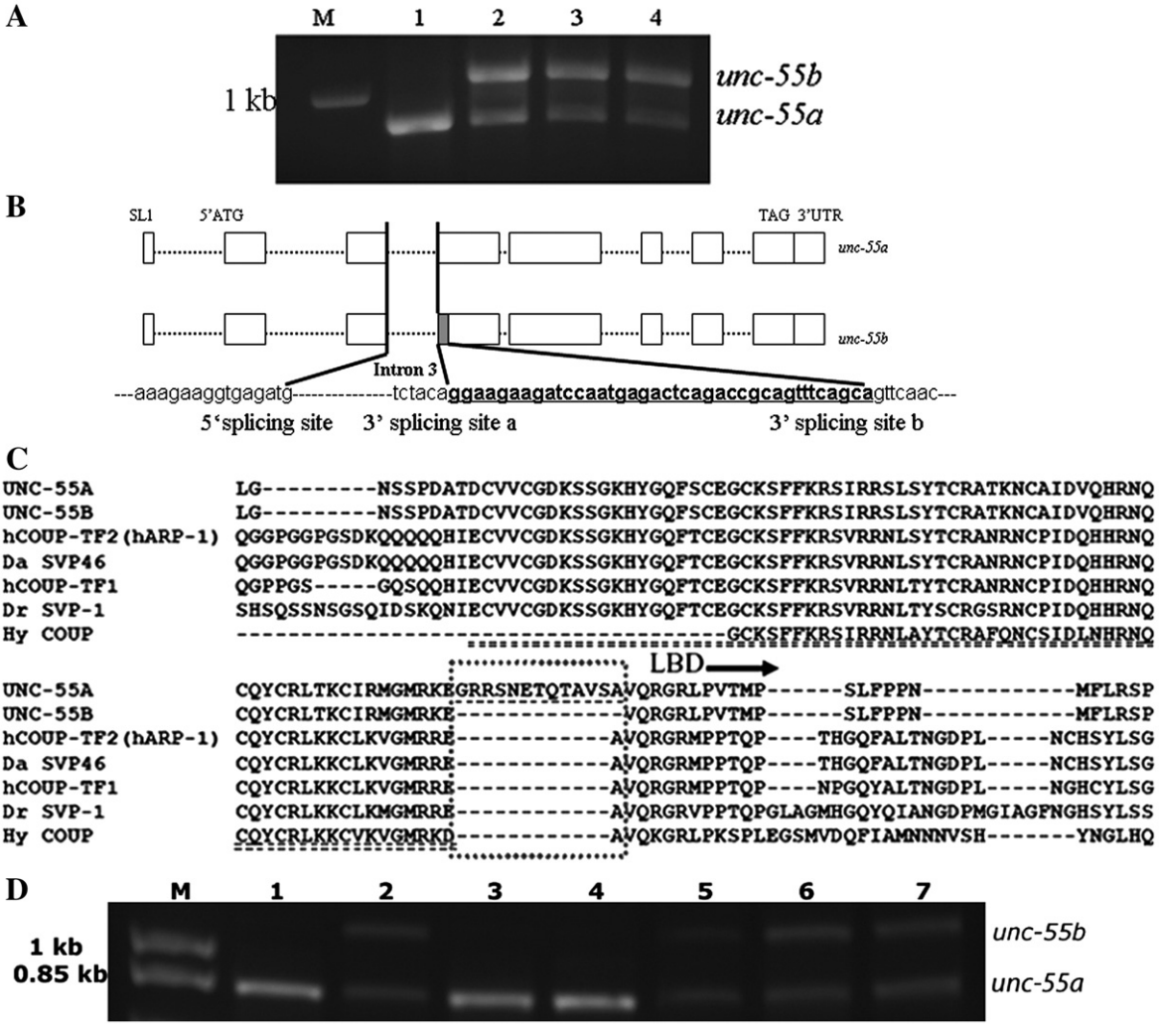

Fig. 4. *unc-55* isoforms in *C. elegans* male. (A) RT-PCR products showing isoforms of *unc-55* transcripts in mixed stage N2 hermaphrodites (lane 1), *him-5* mixed stage population of both males and hermaphrodites (lane 2), *him-5* L2 population (lane 3), and N2 adult males (lane 4). The 1.1 kb bands represent *unc-55b*, and the 0.8 kb bands represent *unc-55a* restriction fragment following digestion with DdeI, which cuts *unc-55a*, but not *unc-55b*. (SL1, splice leader sequence 1). (B) The arrangement of exons and introns that compose pre-mRNA and produces the two isoforms *unc-55* isoforms. (C) Partial alignment of UNC-55A and UNC-55B with other COUP-TF proteins. The unique hinge sequence found in UNC-55A but not in panel B or any other COUP-TFs is marked (box with dotted lines). The highly conserved DNA binding domain (DBD) is underlined twice. The start point of the LBD is marked. Dr, *Drosophila*; Da, *Danio*; h, human; Hy, *Hydra*. The alignment analysis was performed using the CLUSTAL program. (D) Both isoforms were present in *mab-3*, *mab-23*, *egl-5*, and *him-5* populations with males (lanes 2, 5, 6, and 7 respectively), whereas only one isoform *(unc-55a)* was present in N2 hermaphrodites, *mab-5*, and *mab-9* populations with males (lanes 1, 3, and 4 respectively).

present, whereas *mab-5* and *mab-9* mutant populations expressed only the *unc-55a* isoform (Fig. 4D). The *Punc-55::gfp* expression pattern and the RT-PCR result for *mab-5* mutants suggest that *unc-55b* is male tail specific. The observation of *unc-55b* in the L2 male population may be due to the presence of *unc-55b* mRNA in male-specific cells of L2 animals. *Punc-55::gfp* expression in the male tail is observed early in L3 animals (data not shown). *mab-9* is also expressed in the L2 male tail (Woollard and Hodgkin, 2000). The absence of isoform (*b*) in *mab-9* mutant males, who express *Punc-55::gfp* in the male tail, suggests that although *mab-9* does not regulate *unc-55* expression in the male tail, male-specific splicing is regulated in conjunction with male-specific regulatory networks.

The difference between the *unc-55a* and *b* isoforms is the utilization of an alternate 3′ splice acceptor site located in the fourth exon of the *unc-55* pre-RNA (Fig. 4B). 39 extra nucleotides present in exon 4 of isoform *a* include six potential splice acceptor sites (AG); therefore the spliceosome in males must not have access to the first five acceptor sites (Fig. 4B). In hermaphrodites, the spliceosome uses the first acceptor site encountered. Exon 4 contributes the hinge domain that separates the DNA binding domain from the ligand-binding domain (Fig. 4B). The hinge functions in homo-dimerization and cofactor recruitment (Horlein et al., 1995; Chen and Evans, 1995; Bailey et al., 1998). Based on the nucleotide sequence, *unc-55a* encodes a protein (UNC-55A) of 370 amino acids (aa), and *unc-55b* is 39 bp shorter and encodes a protein (UNC-55B) of 357 aa. The 13 aa (GRRSNETQTAVSA) are included in the hinge region (Fig. 4C).

Features and functions of the two unc-55 isoforms

Because *unc-55b* is a male-specific isoform, and *unc-55a* is present in both males and hermaphrodites, we hypothesized that *unc-55a* would be necessary for locomotion in both males and hermaphrodites, whereas *unc-55b* would be necessary for male mating. This hypothesis was tested by using an *unc-55* upstream regulatory region to express either the *unc-55a* or *unc-55b* isoform in an *unc-55 (e1170)*; *him-5* mutant background. Both transgenic isoforms were fused with the *gfp* reporter at the C-terminus. Three independent transgenic lines were obtained for each translational isoform and the two translational reporters were co-injected to create a third set of lines. The expression and rescue patterns that we report were internally consistent. Consistent with the first prediction of the hypothesis, the *unc-55a* isoform rescued locomotion, whereas *unc-55b* did not (Table 2); however the prediction for male mating was not correct, as both isoforms restored mating. *unc-55a* rescued mating more efficiently in young adults (72%) than *unc-55b* (29.5%) (Table 2). Thus the essential role of UNC-55 in male mating can be performed by either isoform; whereas only isoform *a* rescued the defect in locomotion. Consistent with this, isoform *a* suppressed *Pflp-13::gfp* expression in the VD motor neurons, whereas isoform *b* did not (data not shown). Males rescued with isoform *b* were uncoordinated, and the decreased efficiency in siring progeny that was observed in three different transgenic strains may be the result.

Table 2
Rescue of male mating and locomotion by the two *unc-55* isoforms

Males	*him-5 (e1467)*	*unc-55 (e1170) him-5*	Isoform A	Isoform B	A+B
Percentage of males mated successfully	100% $n=100$	0% $n=100$	72.2%* $n=36$	29.5%* $n=78$	75%* $n=36$
Locomotion	Wildtype	Ventral coiler	Wildtype	Ventral coiler	Wildtype

The two isoforms were injected individually *Punc-55a::gfp* (isoform A) or *Punc-55:: unc-55b::gfp* (isoform B) and together *Punc-55::unc-55a::gfp + Punc-55::unc-55b::gfp* (isoforms A+B) into *unc-55 (e1170;him-5)* hermaphrodites. Male progeny showing *gfp* expression in male tail cells from the three strains were placed with *unc-52* hermaphrodites. Varying degrees of successful mating were observed for each of the transgenic strains. Three isoform A lines rescued male mating from 69.4 to 72.2%; three isoform B lines rescued male mating from 26.9 to 29.5%. We used the data from the lines shown best rescue for comparison. $P<0.05$ (*) when compared to mutant *unc-55* males.

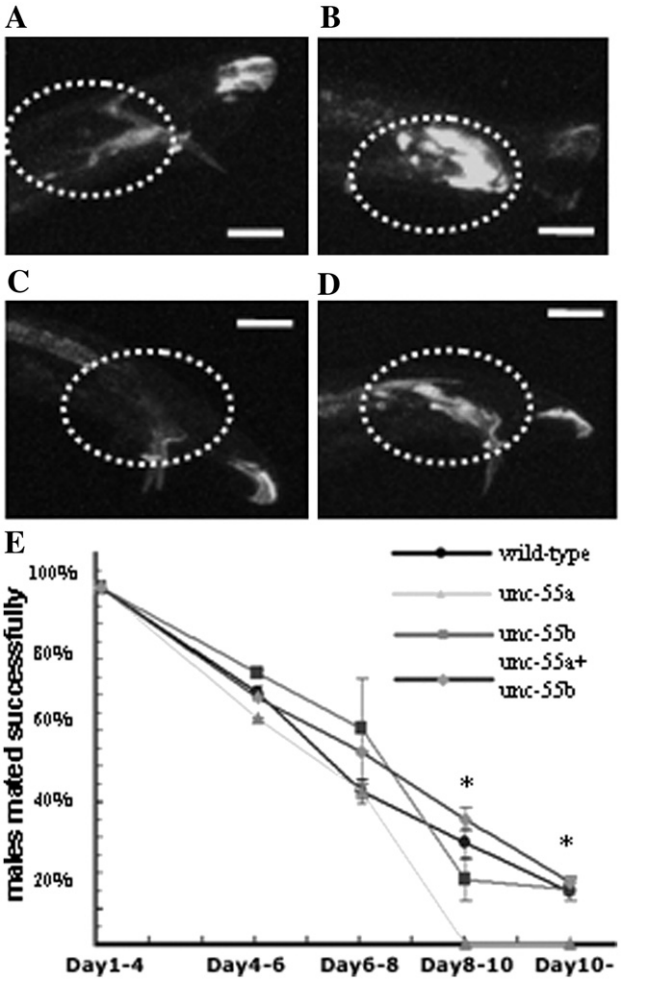

Fig. 5. Time course of expression and mating rescue ability of *unc-55a* and *unc-55b* isoforms in males. (A and B) *Punc-55:*:UNC-55A::GFP (A) and *Punc-55:*:UNC-55B::GFP (B) expression in an adult *unc-55 (e1170)* male (one day after the final molt). (C and D) *Punc-55:*:UNC-55A::GFP (C) and *Punc-55:*:UNC-55B::GFP (D) expression in an adult *unc-55 (e1170)* male 8 days after the final molt. GFP cells in the male tail region are surrounded with a dotted oval. Outside of the ovals, signals are generated from auto-fluorescence in the gut, spicules, and rays. Scale bar represents 50 μm. (E) Comparison of the longevity of mating abilities in *him-5* males (circles) and *e1170* transgenic males rescued with *unc55* isoform A (triangles); isoform B (squares); and isoforms A and B (diamonds) over a ten day period following the last molt. (*P value <0.05).

We found that the translational reporter expressing UNC-55B sustained mating ability as males aged. In male tail cells, the *Punc-55::unc-55b::gfp* expression was more intense and was expressed longer than that of *Punc-55::unc-55a::gfp* in both *unc-55 (e1170)* and wildtype backgrounds (Fig. 5A). Because of the increased duration of *Punc-55::unc-55b::gfp* expression, we compared the mating ability of four groups of males over a ten day period: 1) *him-5* (control), 2) *unc-55 (e1170)* males rescued with *Punc-55::unc-55a::gfp*; 3) *unc-55 (e1170)* rescued with *Punc-55::unc-55b::gfp*; and 4) *unc-55 (e1170)* rescued with both isoforms. Rescued males (24 h after their final molt) were placed with *unc-52 (e444)* hermaphrodites for three days, on day 4 the males were transferred to a new plate with virgin hermaphrodites. The first time point represents the progeny of hermaphrodites mated by males from day 1 to day 4, the second represents progeny from days 4 to 6, the third, days 6 to 8, the fourth, days 8 to 10 and the last time point is from males after day 10. Mating success was determined by the presence of coordinated males and hermaphrodites among the *unc-52* hermaphrodite progeny (Fig. 5E). Following day 6, the mating

success of *unc-55a* transgenic males declined more rapidly than the other three groups, and by day 8 none of the males in this group mated successfully. The mating ability of *unc-55b* and *unc-55a* plus *unc-55b* transgenic males followed a time course that was very similar to wildtype males (Fig. 5). This result was correlated with longer and brighter fluorescence of UNC-55::GFP in both the male tail cells and motor neurons (Fig. 5B). Because the 39 nucleotides associated with the intron 3/exon 4 boundary, or the resulting 13 amino acids added to the hinge region represent the only difference, their presence must be involved with destabilizing isoform A (UNC-55A).

Discussion

We have analyzed the role of a nuclear receptor, UNC-55, in male mating through a comparative study of the mRNAs, *gfp* reporter expression patterns, and behavior of wildtype and mutant males. One surprising finding from RT-PCR experiments was the existence of two isoforms: in addition to the *(unc-55a)* isoform previously described, a male-specific isoform *(unc-55b)* was revealed (Fig. 4; Zhou and Walthall, 1998). *unc-55a* is larger and rescues defects in locomotion and male mating (Table 2); whereas *unc-55b* rescues male mating only. Gender-specific alternative splicing is critical for both primary and secondary sex determination events in *D. melanogaster* (reviewed in Billeter et al., 2006).

The male-specific isoform *b* was not detected in male populations in two mutant backgrounds, *mab-5* and *mab-9*, however both strains expressed isoform *a* in the motor neurons and both strains exhibit coordinated movement. The absence of isoform *b* in these *mab* mutant backgrounds suggests the existence of a male-specific pathway for creating isoform *b*. We have not identified factors responsible for choosing the alternative splice acceptor sites in *C. elegans* males, however the existence of male-specific alternative splicing for *unc-55* could serve as a starting point for further exploration of this phenomenon. Tissue, developmental stage and male-specific alternative splicing have now been reported for a number of genes in *C. elegans* (this study; Snow and Larsen, 2000; Zahler, 2005 for a summary).

The roles of UNC-55 in male mating and locomotion are independent of one another. This conclusion is based upon three lines of evidence. First, *unc-55* mutant males performed the initial steps of mating that require backward movement and were able to locate the vulva (Fig. 2). Second, stage-specific *unc-55* RNAi generated uncoordinated males capable of mating, or coordinated males whose mating ability was reduced significantly (Fig. 3). Third, transgenic *unc-55* mutant males expressing only the male-specific isoform *(unc-55b)* were able to mate even though they were uncoordinated. Thus the presence of UNC-55 in spicule muscles and a neuron, tentatively identified as CP9, correlated with successful male mating. The role of CP9 has not been determined but its structure suggests that it could be involved with integrating afferent information from ray sensory neurons with the activation of appropriate efferent responses (Fig. 1G).

unc-55 mutant males were unable to perform any of the spicule motor programs. Spicule prodding is the final step in locating the vulva and it culminates in spicule insertion, the first step in copulation (Garcia et al., 2001). Recent genetic approaches in *C. elegans* have focused on identifying and characterizing mutations in genes that disrupt the execution of the copulatory motor programs. This approach has revealed a number of critical components. The cholinergic response in the spicule muscles is mediated by members of the inositol triphosphate (IP3) signaling cascade. In response to activation of the ACh receptors the GαQ homologue EGL-30 activates EGL-8, a phospholipase C homologue that generates IP3. Reporter constructs for *egl-8* showed expression in the spicule protractors as well as CP8 or CP9 neurons, and in the same publication the inositol triphosphate receptor gene *(itr-1)* was shown to be expressed in several male-specific muscles including both the spicule protractors and retractors (Gower et al., 2005). This pathway culminates in the regulation of the activities of two types of calcium channels, the ryanodine receptor (UNC-68) that mediates spicule prodding and the α-subunit of the L-type voltage-gated channel (EGL-19) that mediates spicule insertion into the vulva (Garcia et al., 2001). Another study has investigated *unc-103*, which encodes a voltage-gated K^+ channel that is expressed widely in both males and hermaphrodites. Reporter constructs showed expression in the SPC and PCB motor neurons as well as the spicule protractors, the target of these motor neurons (Garcia and Sternberg, 2003). A dominant loss-of-function mutation in *unc-103 (sy557)* was isolated that caused mutant male spicules to be "protraction constitutive" (*prc*). Mutations in *lev-11*, which encodes the only *C. elegans* tropomyosin homologue, suppressed the *prc* phenotype (Gruninger et al., 2006).

The individual genes in these networks are pleiotropic in *C. elegans* and have homologues in all metazoans. Some have multiple promoters that are responsible for tissue-specific expression; others produce multiple isoforms that expand the functional activities of the gene. All are expressed in at least a subset of the male-specific cells that express the *unc-55* reporter and mutant males show defects in their ability to insert their spicules into a hermaphrodite. Four lines of evidence suggest that UNC-55 is necessary to maintain and activate the copulation motor programs rather than participate in the differentiation of the cellular network. First, RNAi of *unc-55* introduced in adult *him-5* males reduced their mating effectiveness (Fig. 3). Second and third, there was an increase in the duration and intensity of GFP fluorescence for the UNC-55B::GFP reporter as compared to the UNC-55A::GFP reporter in both motor neurons and the male tail cells (Fig. 5). This correlated with the ability of *unc-55 (e1170*; *him-5)* males expressing only the UNC-55B::GFP isoform to mate successfully for at least three additional days when compared to *e1170* males expressing only the UNC-55A::GFP isoform. Finally, comparison of the morphology of wildtype and mutant strains of *unc-55 (e1170*, *jd8* and *e402)* revealed no obvious structural alterations in the neuron or the origins, insertions or thin filament organization of the copulatory muscles (Figs. 1C–F). Whether these muscles lose their innervation in *unc-55* mutant background, or lose critical receptors for a neurotransmitter is still an open question.

The continuous requirement of UNC-55 in spicule muscles and the observation that *unc-55b* extends male mating ability as the animals age suggests that UNC-55 acts in an anabolic role to maintain functional male spicule muscles. Age-related muscle loss is an unfortunate phenomenon commonly observed in animals (Lee et al., 2007). The role of *unc-55b* in maintaining function in aging sex muscles could serve as a model for further investigation of this phenomenon.

unc-55 is a member of a gene family that includes COUP-TF in mammals, and *seven-up* in *D. melanogaster* and zebrafish (*Dania reo*) (Mlodzik et al., 1990). The absence of COUP-TF in developing mammalian skeletal muscle progenitors resulted in disrupted myogenesis (Bailey et al., 1998), other related orphan nuclear hormone receptors have been implicated in multiple regulatory roles in muscle including metabolic activity, cytokine production and endurance (Smith and Muscat, 2006). In mice, three genes encode COUP-TF paralogs I, II and III, respectively (reviewed in Tsai and Tsai, 1997; Sluder and Maina, 2001). These three COUP-TFs have different features and functions. *unc-55* is the only representative of the COUP-TF family in the *C. elegans* genome and the roles of the two isoforms partially overlap as described earlier. Typically, the COUP-TFs are potent transcriptional repressors that often form heterodimers with RXR and NGFI-Bα. However there are reports that COUP-TFs can function as transcriptional activators (Malik and Karathanasis, 1995; Pipaon et al., 1999; Fernandez-Rachubinski and Fliegel, 2001). Members of the COUP-TF gene family function as homo- or heterodimers and bind to a common *h*ormone *r*esponse *e*lement (HRE) that is composed of direct or inverted repeats of AGGTCA. UNC-55 repressed the expression of one target gene in a class of motor neurons involved in locomotion (Shan et al., 2005). However the HRE of that target gene, although

conserved, was not repeated (half site) (Shan et al., 2005). *dissatisfaction*, a fly orphan nuclear receptor found to function in male sexual behavior also appears to repress gene expression via binding to a half site (Pitman et al., 2002). No target genes in the male tail regulated by UNC-55 have been identified.

The addition of amino acids to the hinge sequence observed in isoform *a* has not been seen in orthologs outside of the taxon Nematoda, although the exon sequences flanking the insert were conserved (Fig. 4). These results suggest that the modification to the hinge sequence in UNC-55A occurred in the nematode clade and more than likely became important for the differentiation of motor neurons secondarily. The addition of a discrete domain to the hinge region of the protein expanded the role for UNC-55 to include generating the features that distinguish two functionally related motor neuron classes from one another (Shan et al., 2005). Both isoforms were present in *C. briggsae* hermaphrodites and *C. remanei* females (data not shown), thus the mRNA appears to be alternatively spliced in these two species but not in a gender-specific manner. Alternative splice products that make discrete functional modifications for different isoforms have been shown in *Pristionchus pacificus* for *mab-5* paralogs as well (Gutierrez et al., 2003).

Sex steroids and the corresponding nuclear receptors play both "organizational" roles during critical developmental periods and "activational" roles during adulthood (McEwen, 1992a,b; Pilgrim and Hutchison, 1994). In *C. elegans* UNC-55 plays an organizational role in developing motor neurons and an activational role in male copulation. Although UNC-55 is considered an orphan nuclear receptor, the existence of a ligand cannot be ruled out, as investigators recently identified the first ligands for a nuclear hormone receptor in *C. elegans* (Motola et al., 2006).

Whether a ligand for UNC-55 exists or not, this androgenic nuclear hormone receptor appears to play an anabolic role in male muscles that is necessary for copulation. In mammals, masculine sexual behaviors facilitated by additional muscle growth are often governed by nuclear hormone receptors (Morris et al., 2004; McEwen, 1992a,b). It will be interesting to identify downstream target genes regulated directly or indirectly by UNC-55 to determine whether correlations exist with the actions of orthologous nuclear hormone receptors.

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