Molecular characterization of the *her-1* gene suggests a direct role in cell signaling during *Caenorhabditis elegans* sex determination

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We have characterized two transcripts from the male-determining *her-1* locus in *Caenorhabditis elegans*. The larger transcript, which appears more important for male development, is predicted to encode a novel 175-amino-acid, cysteine-rich polypeptide with an apparent amino-terminal signal sequence and potential cleavage and glycosylation sites. Expression of a full-length cDNA construct for the larger transcript driven by a body-wall-myosin promoter causes extensive masculinization of all sexually dimorphic tissues in XX (normally hermaphrodite) animals. This activity is dependent on the presence of the *her-1* signal sequence or a substitute synthetic signal sequence in the encoded polypeptide. These results suggest that a secreted product of the *her-1* gene dictates male development.

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The primary signal for sex determination in *Caenorhabditis elegans* is the ratio of X chromosomes to autosomes (X/A ratio) (Nigon 1951; Madl and Herman 1979). Genotypes of XO (X/A = 0.5) and XX (X/A = 1.0) normally dictate male and hermaphrodite development, respectively. The two sexes show extensive dimorphism in most tissues: For example, males have a unilobed testis connected to the cloaca and a complex tail specialized for copulation, whereas hermaphrodites, which are self-fertile, have a bilobed ovotestis connected to the vulva and a simpler tail (for review, see Hodgkin 1988). Genetic analysis has shown that the primary signal is interpreted through a regulatory cascade (Fig. 1) including seven autosomal genes that control somatic as well as germ-line sex determination (for review, see Hodgkin 1987; Hodgkin 1990) and three X-linked genes that regulate both these genes and the genes that mediate X chromosome dosage compensation (for review, see Villeneuve and Meyer 1990). Although molecular information on some of the corresponding gene products has recently become available (see above reviews and Discussion), the nature of the cascade and its relationship to other known developmental regulatory pathways is not yet clear.

The *her-1* gene, which is necessary for normal male development in XO animals (Hodgkin 1980), appears to be the point at which the X-linked regulatory genes con-

trol sex determination. The activity of *her-1*, in turn, controls activity of the terminal regulator *tra-1* as shown in Figure 1, via *tra-2*, *tra-3*, and the *fem* genes, so that in an XO animal *tra-1* function is repressed and male development is permitted. The *her-1* gene is defined by 25 loss-of-function (*lf*) mutations; most of these result in complete transformation of XO animals into self-fertile hermaphrodites and have no effect on XX animals (Hodgkin 1980; Trent et al. 1988). Weak *lf* alleles can result in variably transformed intersexual XO animals. Two dominant gain-of-function (*gf*) mutations at the *her-1* locus (*n695* and *y101*) result in the opposite phenotype: XX animals are variably transformed (masculinized) into pseudomales (Trent et al. 1983, 1988, 1991). This dominant phenotype shows that *her-1* expression in XX animals is sufficient to activate much of the male developmental program.

Trent et al. (1991) cloned the *her-1* gene and identified two colinear *her-1* transcripts present in XO and virtually absent in XX animals, a relatively rare 1.2-kb mRNA and a relatively abundant 0.8-kb mRNA. Both transcripts were shown to be present in XX animals carrying either of the *her-1* (*gf*) alleles or a partial *lf* mutation in either *sdC-1* or *sdC-2*, indicating that these genes act to control *her-1* transcript levels.

Mosaic analysis reported by Hunter and Wood (1992) demonstrated that *her-1* or a gene under its control can

Figure 1. The proposed regulatory pathway governing somatic sexual phenotype in *C. elegans*, based on genetic analysis of sex determining genes. Genes are named for their loss-of-function phenotypes, as follows: *xol* (*XO-lethal*) (Miller et al. 1988), *sdc* (*sex and dosage compensation*) (Villeneuve and Meyer 1987; Nusbaum and Meyer 1989), *her* (*hermaphroditization*) (Hodgkin 1980), *tra*, (*transformer*)

(Hodgkin and Brenner 1977), and *fem* (*feminization*) (Kimble et al. 1984). Barred arrows indicate negative regulation (of either expression or function). Some of these genes also function in sex determination of the germ line, although additional genes are involved and some of the epistatic relationships differ (for review, see Hodgkin 1990). (Adapted from Villeneuve and Meyer 1990.)

function nonautonomously. A *her-1(+)* genotype for a given cell was shown to be neither necessary nor in some cases sufficient for adoption of the male fate in a mosaic XO animal, indicating that *her-1(+)* cells must be able to masculinize *her-1(-)* cells and that *her-1(-)* cells, under some circumstances, can feminize *her-1(+)* cells. These results showed that cell interactions play a role in *C. elegans* sex determination and implicated *her-1* or a gene product under its control in intercellular signaling.

Here we report further characterization of the *her-1* transcripts and their functions, indicating that this gene encodes a small protein that could act as a secreted ligand to determine male development in all tissues.

Results

Structures of *her-1* transcripts

To confirm that the region identified by Trent et al. (1991) included all the elements required for *her-1* activity, we demonstrated by germ-line transformation that an 8-kb genomic DNA fragment (Fig. 2A; pMPP14-22) can restore *her-1* function to a mutant strain that lacks it. This strain, *him-8(e1489)*; *her-1(y101hv1)*, carries a small deletion eliminating most of the *her-1* gene (Trent et al. 1991) and produces about 37% XO animals as a result of the *him-8* mutation, which causes X-chromosome nondisjunction (Hodgkin et al. 1979). These animals develop as hermaphrodites because of the *her-1* mutation; transformation with the rescuing fragment gave animals that had male morphology and exhibited male mating behavior. Sequencing of ~7 kb of the transforming fragment (Fig. 3) identified four putative exons on the coding strand (determined by Trent et al. 1991), two in the region hybridizing to both transcripts and two in the region hybridizing only to the rarer 1.2-kb transcript. Screening of a cDNA library with appropriate genomic fragments yielded clones including sequences from the two putative 3' exons (3 and 4) but none with sequences from the two putative 5' exons (1 and 2), present only in the rarer transcript. We obtained cDNAs spanning exons 1 and 2 by means of an anchored PCR technique (Frohmann et al. 1988; Loh et al. 1989) using a sequence from exon 3 as the downstream primer (see Materials and methods). To establish that exon 4 is *cis*-

spliced to the first 3 exons to form the large transcript, we used PCR primers specific for exon 1 and exon 4 to amplify cDNA and sequenced the resulting product, confirming the structure shown in Figure 2 for the larger *her-1* cDNA.

Sequencing of cDNAs (see Fig. 3, legend) demonstrated further that the 0.8-kb transcript carries the SL1 trans-splice leader present on the 5' ends of about 10% of *C. elegans* mRNAs (Krause and Hirsh 1987; Bektesh et al. 1988). For the 1.2-kb transcript, the 5' end of the cDNA sequence was the same as the genomic sequence, indicating no splice leader.

To confirm the exon/intron structures shown in Figures 2 and 3 and to define more precisely the 5' ends of each transcript, we carried out mapping experiments using RNase protection (Melton et al. 1984), assaying probes representing appropriate regions of both DNA strands for protection by sequences in embryonic RNA. Representative assays are shown in Figure 4. Consistent with the earlier Northern blot experiments (Trent et al. 1991), total RNA from *him-8* embryos gave strong protection of exon 3 (Fig. 4A) and exon 4 probes. Protection of exon 1 and 2 probes with total RNA was detectable only after long exposures; we used the poly(A)-containing fraction of RNA to obtain stronger signals (Fig. 4B). These experiments gave no evidence for other transcripts and confirmed the predicted splice sites. They revealed no additional exons in any of the predicted introns including intron 2, which at 3.3 kb is much larger than the average *C. elegans* intron (Emmons 1988). However, they showed that both the 5' end of exon 1 (see Fig. 4B, legend) and the 3' end of exon 4 can vary in length, suggesting that promoter elements controlling the large transcript utilize multiple capping sites, and that the transcriptional termination machinery can choose between several poly(A) addition sites.

There are two *her-1* promoters

We have designated as P1 the presumed promoter for the larger transcript (Fig. 2). Although it appears to utilize multiple capping sites, all are upstream of the predicted ATG initiation codon at position 1652 of the genomic sequence (Fig. 3). We attempted to demonstrate P1 promoter activity directly by constructing and injecting the

Figure 2. Physical map of the *her-1* locus and constructs for germ-line transformation experiments. (A) Top line shows a genomic restriction map (Trent et al. 1991). The 7-kb region sequenced extends rightward from the unique *SacI* site to a point just beyond the rightmost *EcoRI* site on the diagram. Boxes below the restriction map show the locations of the four *her-1* exons; white boxes indicate untranslated regions of mRNA and black boxes indicate coding stretches. (ATG) Presumed points of translation initiation. ("P1" and "P2") Approximate locations of the two XO-specific *her-1* promoters described in the text; arrows show the direction of transcription and indicate that P2 is more active than P1. The smallest genomic subclone tested in germ-line transformation rescue (pMPP14-22) is shown below the structure of the *her-1* cDNAs. Promoter test plasmids consist of the indicated *her-1* sequences fused to either a *lacZ* coding sequence (pMPL14, pWLG1) or a construct derived from the larger *her-1* cDNA (see Materials and methods). Abbreviations for restriction enzyme recognition sites are: H3, *HindIII*; Sac, *SacI*; Pst, *PstI*; Bam, *BamHI*; RI, *EcoRI*. Individual exons of the *her-1* gene are numbered; introns (not numbered in the figure) are counted from left to right starting with intron 1 between exons 1 and 2. The small deficiency in *her-1(y101hv1)* strains, used in several experiments referred to in the text, removes sequences from a point to the left of the *PstI* site in the P1 region to a point approximately midway between the H3 and *BamHI* sites at the start of exon 4 (Trent et al. 1991). (B) Plasmids for ectopic expression experiments were constructed as described under Results and Materials and methods. pMPW12-1 (IV) and pMPX174 (VI) contain *her-1* cDNA sequence corresponding to the larger transcript; pMPZ159 (V) and pMPZ162 (VII) contain cDNA sequence corresponding to the smaller transcript.

P1-*lacZ* fusion construct I (Fig. 2A, pMPL14) into *him-8(e1489)* animals, but no β -galactosidase reporter activity was detected by histochemical staining with the chromogenic substrate X-gal (data not shown). Because *her-1* mRNAs driven by P1 are normally present only at low steady-state levels (Trent et al. 1991), our inability to detect expression from P1 may simply reflect its very low activity, although we have not ruled out the possibility that promoter or enhancer elements could be missing from the construct.

The cDNA sequences indicated that the 5' end of exon 3 can either be *cis*-spliced to exon 2, forming the 1.2-kb mRNA, or *trans*-spliced to SL1, forming the 0.8-kb mRNA. Examples of alternative *cis*- and *trans*-splicing for wild-type *C. elegans* mRNAs are rare, although mu-

tant constructs engineered in vitro can be forced into this pattern (R. Conrad and T. Blumenthal, pers. comm.). Therefore, it seemed likely that the 0.8-kb transcript might be under the control of a second promoter upstream of exon 3. RNase protection assays of the 5' end of exon 3 (data not shown) revealed a weakly protected band about 35 nucleotides longer than the exon. Observation of this band, expected for a primary transcript that is subsequently *trans*-spliced, supports the existence of a second promoter, which we have designated P2, just upstream of the acceptor splice junction of exon 3. Similar detection of a primary transcript has been reported previously for other *trans*-spliced mRNAs (Graham et al. 1989).

To obtain more direct evidence for P2 activity, we

Figure 4. Results of representative RNase protection experiments. (A) Autoradiograph of a denaturing gel showing protection of *her-1* exon 3 probe fragments (square bracket) and control *act-1* probe (arrowhead) by various total RNA preparations. Antisense RNA probes for *her-1* (spanning exon 3, positions 4821–5603 in Fig. 3) and *act-1* (Krause et al. 1989) were hybridized to total *C. elegans* RNA and digested with RNase as described in Materials and methods (in this experiment $\sim 2 \times 10^5$ cpm of each probe was hybridized to 120–160 μ g of RNA). In most experiments, protected fragments were seen as sets of bands varying in length by 2–15 bases, generally attributed to breathing at the ends of probe–mRNA duplexes causing variable protection of the ends. For example, the protected exon 3 fragments in lane 5 range from 150–165 bases in length. (Lane 1) *her-1* probe, undigested; (lane 2) yeast tRNA carrier hybridized to the *her-1* and *act-1* probes; (lane 3) *mnt12(IV;X)* RNA hybridized to the *her-1* and *act-1* probes; (lane 4) N2 RNA hybridized to the *her-1* and *act-1* probes; (lane 5) *him-8 (e1489)* RNA hybridized to the *her-1* and *act-1* probes; (lane 6) size markers derived from an end-labeled *Sau3A* digest of pBR322; (lane 7) *her-1(y101gf)* RNA hybridized to the *act-1* probe only; (lane 8) *act-1* probe, undigested. The sizes of the molecular weight markers in base pairs are indicated to the right of the gel. (B) Autoradiograph from a similar experiment showing protection of a *her-1* exon 1 fragment (arrow) by various RNA preparations. Antisense RNA probes for *her-1* (spanning part of exon 1 and all of exon 2, positions 1658–1969 in Fig. 3) and *act-1* were used as in A, with the same size markers. Lanes are as in A, except that the *C. elegans* RNAs in lanes 3, 4, 5, and 7 were fractions selected to contain poly(A). A 113-nucleotide protected exon 2 fragment also seen in lane 5 is not included in the reproduced portion of the autoradiograph. The *her-1* probe used in this experiment does not include the transcriptional start site and the 5'-UTR of the large transcript. In similar experiments (not shown) using probes that extend further 5' we observed four sets of male-specific protected exon 1 bands indicating multiple capping sites; the largest was the size expected from the transcriptional start site shown in Fig. 3.

assay, but they do contain low levels of the 0.8-kb transcript, which cannot be attributed to spontaneously arising XO males in the population.

Results obtained with the P2-*lacZ* reporter construct II described above were consistent with these observations. Crossing the integrated construct into an N2 background without the *him-8* mutation yielded an XX strain that showed little or no embryonic staining with X-gal (Table 1). Crossing this strain with males to produce XO offspring again produced staining embryos, indicating that the construct was still active.

The larger her-1 transcript is predicted to encode a polypeptide with a secretion signal sequence

The 1.2-kb transcript contains an open reading frame (ORF) of 525 bp, beginning at an AUG codon 128 nucleotides from the 5'-most transcriptional start site (see Figs. 3 and 4B, legends) and predicted to encode a 175-residue polypeptide with an M_r of ~ 20 kD (Fig. 3). This ORF is spliced in-frame across all three exon–exon splice sites, and includes the ORF found in the smaller transcript (see below). The predicted polypeptide includes at

its amino-terminal end an apparent signal sequence (von Heijne 1986) for secretion into the endoplasmic reticulum (i.e., a basic residue next to the initiator methionine, followed by a stretch of 14 largely apolar residues, terminating in another charged residue, glutamate). Otherwise the polypeptide shows no significant sequence similarities to any proteins in the current data bases (see Materials and methods). It does include, however, two potential sites (residues 98 and 163) for post-translational amino-linked glycosylation (Kornfeld and Kornfeld 1985), as well as four pairs of basic residues that could be sites for post-translational proteolytic processing (Douglass et al. 1984). Because there are no additional membrane-spanning domains, the polypeptide or a processed form of it could be secreted at the cell surface.

The 0.8-kb transcript includes a 220-bp ORF that begins at the point of *trans*-splicing (Fig. 3) and corresponds to exons 3 and 4 of the larger transcript. At position 77 of the smaller transcript (5372 in the genomic sequence) is an AUG codon. Initiation of translation at this site would result in a polypeptide of about 10 kD, corresponding to the carboxy-terminal 64 amino acids of the larger polypeptide. Curiously, the first AUG in this tran-

Table 1. Expression of an integrated P2-lacZ fusion construct in XX and XO embryos

Genotype of animals carrying construct	Staining/total embryos
<i>him-8(e1489)</i> (37% XO males) ^a	350/1003
<i>him-8(+)</i> (<0.2% XO males) ^b	2/1071
<i>him-8(e1489);her-1(y101hv1)</i> (37% XO hermaphrodites) ^c	271/1043

^aA *him-8(e1489)* strain carrying chromosomally integrated copies of the P2-lacZ fusion construct III (Fig. 2A, pWLG1) were obtained by germ-line transformation and irradiation of a stably transformed line as described in Materials and methods.

^bTo obtain a strain carrying the construct without the *him-8* mutation, hermaphrodites of the above strain were crossed to N2 males and the desired line was propagated from an F2 hermaphrodite that exhibited the Rol phenotype and produced no male offspring. Retention of the active P2-lacZ fusion construct was verified by mating with N2 males and observing a high proportion of embryos that stained with X-gal.

^cThis strain was obtained by (1) crossing N2 males with hermaphrodites of the strain described in footnote a, (2) crossing F₁ males to *him-8(e1489);her-1(y101hv1)* hermaphrodites, (3) picking Rol F₁ hermaphrodites from the second cross that produced F₂ male progeny, (4) and picking their Rol hermaphrodite siblings to individual plates and retaining a Rol clone that no longer produced males, indicating homozygosity of the *her-1* mutation. Because ~37% of *him-8(e1489);her-1(y101hv1)* hermaphrodites are XO, such strains produce inviable nullo-X embryos, which could account for the lower than expected fraction of total embryos stained. When apparently healthy embryos of ~100 cells (identified by DAPI fluorescence) were counted, 39/100 were found to stain with X-gal.

script is further upstream (5347 in the genomic sequence), immediately followed by a UGA terminator. Because most eukaryotic mRNAs seem to be scanned from their 5' ends for initiation codons (Kozak 1983), a scanning ribosome would have to reinitiate at the second AUG if this transcript is translated conventionally (Peabody and Berg 1986).

Expression of the larger *her-1* transcript driven by a body-wall muscle myosin promoter masculinizes all dimorphic tissues

To further characterize masculinizing effects of the two predicted *her-1* products, we asked whether expression of either of the two *her-1* transcripts in body-wall muscle could affect sex determination of other tissues. Each of the two cDNAs was subcloned into the expression plasmid pPD30.38 (P. Okkema, S. White-Harrison, V. Plunger, A. Aryana, and A. Fire, in prep.), containing an enhancer, promoter, and poly(A) addition signal from the *unc-54* gene, which encodes the major body-wall muscle myosin protein (Epstein et al. 1974). The *unc-54* protein and its mRNA are expressed in body muscle cells, most of which are not sexually dimorphic (Sulston et al. 1983). When worms are transformed with a *lacZ* fusion construct driven by this *unc-54* enhancer-promoter combi-

nation, β -galactosidase activity is detected only in these same muscle cells (Fire et al. 1990; P. Okkema, S. White-Harrison, V. Plunger, A. Aryana, and A. Fire, in prep.).

We coinjected construct IV (Fig. 2B, pMPW12-1), carrying the larger *her-1* cDNA, with marker *rol-6* DNA as above into N2 hermaphrodites, and screened their progeny for Rol transformants. Of the Rol animals from this experiment, 59% showed masculinization (Table 2), ranging from hermaphrodites with slightly truncated tails or intersexual gonads to animals of male size and shape, with male gonads containing only sperm and distinctly masculinized tails (sensory rays, acellular fan, crumpled spicules). One such animal is shown in Figure 6D. The strongly transformed animals did not produce vitellogenin, indicating that the intestine was also masculinized. When we injected the same construct (IV) into hermaphrodites of the *her-1* deletion strain *him-8(e1489);her-1(y101hv1)*, transformed animals produced apparently complete male progeny (Fig. 6C).

These results indicate that the larger *her-1* transcript encodes biologically active *her-1* product. Because the *unc-54* promoter-enhancer is reported to be tissue-specific (Fire et al. 1990), they are also consistent with the

Figure 5. Expression of an integrated *her-1* P2-lacZ fusion construct in embryos of a *him-8(e1489)* hermaphrodite. A line carrying the integrated construct was obtained as described in Materials and methods after germ-line transformation of *him8(e1489)* with pWLG1 (see Fig. 2). About 35% of embryos (presumed XOs) from this line show expression (Table 1). Animals were fixed, stained with X-gal and DAPI (see Materials and methods) and viewed under Nomarski (upper panel) or epifluorescence (lower panel) optics. DAPI staining in the lower panel indicates that the two embryos shown are at approximately the same stage (100–200 nuclei), although epifluorescence in the presumed XO embryo on the left is obscured by the heavy X-gal staining.

Table 2. Dominant sexual transformations in transgenic N2 hermaphrodites

Plasmids	Number of animals with transformed tissues					
	masculinized tail ^a (AB-derived)	masculinized vulva ^b (AB-derived)	masculinized intestine ^c (E-derived)	masculinized gonad (MS-derived)	masculinized sex muscles (MS-derived)	masculinized germ line ^d (P ₄ -derived)
<i>unc-54-her-1</i> (pMPW12-1)	19/44	18/44	25/44	25/44	4/27	13/44
<i>HSP16-her-1</i> (pMPX174)	22/22	9/21	11/22	15/21	3/4	14/20

^aWeakly transformed animals were missing the tail spike, whereas strongly transformed animals had sensory rays, acellular fan, mating hook, and spicule material.

^bSpecific cells were not lined; vulval induction was scored by the presence of a normal vulva or a mid-ventral hernia.

^cSex of the intestine was assessed either by the accumulation of pseudocoelomic yolk protein visible by Nomarski microscopy or by the presence of developing embryos in utero (assuming that yolk protein is necessary for embryogenesis; Hunter and Wood 1990), or both.

^dOverproduction of sperm.

suggestion that *her-1* product or some other molecule under its control is secreted and able to act on other tissues. However, because only low levels of *her-1* product may be required for activity, based on the apparent weakness of the P1 promoter, we cannot rule out the possibility of masculinization attributable to low-level ectopic expression of the construct in nonmuscle cells.

Expression of the smaller her-1 transcript may be unimportant for male development

Construct V (Fig. 2B, pMPZ159), carrying the smaller *her-1* cDNA under control of the *unc-54* promoter-enhancer appeared to have no masculinizing effect in ex-

pression experiments similar to those above. In this and the following experiments, presence of the construct in the transformed animals was verified by PCR as described in Materials and methods. We would not expect the resulting polypeptide to be secreted, and because body wall muscle is not sexually dimorphic, we would not be able to score cell-autonomous masculinization in these cells. The *unc-54* promoter is active in the dimorphic sex muscles that arise during larval development (Hodgkin 1988), but expression of *her-1* in these cells could be too late to affect their sexual fates. As a more general test for function of the smaller transcript, we made the constructs VI and VII (Fig. 2B, pMPX174 and pMPZ162) containing the *her-1* cDNAs fused to a *C. elegans* HSP16 heat shock promoter (Stringham et al. 1992; D. Dixon, S. Harrison, and A. Fire, in prep.). Subjecting transformed lines made with these constructs to a heat-shock during embryogenesis (see Materials and methods) confirmed the above results: We observed strong masculinization with the larger cDNA construct pMPX174 (Table 2) but no masculinization with the smaller cDNA construct.

Expression of the smaller transcript may not be necessary for male development. As described above, we observed complete masculinization of XO animals from the *her-1* deficiency strain *him-8(e1489);her-1(y101hv1)* after transformation with construct II (Fig. 2A, pWLF1), which lacks the P2 promoter region and the splice acceptor sequence adjacent to exon 3 in the genomic sequence. This result suggests that at least when this *her-1* construct is present on an extrachromosomal array, expression from the P1 promoter is sufficient to direct normal male development.

Masculinization by the larger her-1 polypeptide requires presence of a signal sequence

To ask whether the putative *her-1* signal sequence is required for masculinizing activity, we modified the masculinizing cDNA construct I (pMPW12-1), which en-

Figure 6. Nomarski photographs showing masculinization of animals expressing the 1.2-kb *her-1* transcript under the control of a body-wall-muscle-specific promoter. (A and B) Normal male and hermaphrodite (N2) tails, respectively, for comparison (lateral views). (C) F1 offspring (presumably XO) of a *him-8(e1489)her-1(y101hv1)* animal transformed with pMPW12-1 (Fig. 2, construct IV), showing apparently complete masculinization of the tail (lateral view). (D) XX F1 offspring of an N2 animal transformed with pMPW12-1 and *rol-6* marker, showing partial masculinization of the tail (ventral view).

codes the complete *her-1* large polypeptide (Fig. 7, top), to a truncated derivative (pMPE21-2), which encodes the same polypeptide without the signal sequence. In transformation experiments similar to those described above, this construct showed no detectable masculinizing activity of either XX or XO *her-1*(-) animals. Therefore, the amino-terminal sequence of the normal translation product is important for masculinizing activity, either as a signal sequence or fulfilling some other necessary function. To distinguish between these alternatives, we used a plasmid constructed to substitute a heterologous secretion signal plus several more amino acids for the normal amino terminus of the *her-1* product. The plasmid (pPD52.81; A. Fire, unpubl.) was designed to encode a synthetic sequence with features similar to known eukaryotic signal sequences and to place the resulting cDNA under the control of the *unc-54* promoter as above. An appropriate fragment of *her-1* cDNA was cloned into this plasmid to give an in-frame fusion to the *her-1* polypeptide sequence (Fig. 7, pMPD24-1), starting at residue 16. The amino-terminal sequence of the resulting polypeptide bears little resemblance to the *her-1* signal sequence, which is completely missing. In germline transformation experiments this plasmid strongly masculinized both XX and XO animals of the *her-1* deficiency strain *him-8(e1489);her-1(y101hv1)*. These results suggest that the normal amino terminus of the *her-1* product is required as a signal sequence for masculinizing activity.

Figure 7. Evidence that *her-1* function requires a signal sequence. Shown are amino-terminal polypeptide sequences encoded by the full-length larger *her-1* cDNA construct IV (Fig. 2) and constructs modified to test the requirement for a signal sequence in *her-1*-mediated masculinization. The upper diagram shows the probable cleavage site (von Heijne 1986) for removal of the normal presumed signal sequence, which would leave an amino-terminal Thr residue (!). The amino terminus of the second polypeptide, with no signal sequence, would be expected to remain as shown with 6 additional residues preceding the normal amino-terminal Thr. The third polypeptide has a substituted synthetic signal sequence and an additional amino-terminal sequence that would be expected to remain after cleavage. The exclamation point (!) in the second and third diagrams indicates the leftmost extent of normal *her-1* sequence in the predicted polypeptides. (See text for additional description of the plasmids.)

Discussion

her-1 encodes a small protein that may require secretion for its activity

We have shown that the two previously described transcripts (Trent et al. 1991) of the masculinizing gene *her-1* are produced from separate promoters, designated P1 and P2, both active in XO animals and essentially inactive in XX animals. The larger transcript, from P1, is predicted to encode a polypeptide with an apparent amino-terminal signal sequence and potential glycosylation and cleavage sites, suggesting that it could be post-translationally processed and secreted. We have shown that expression of the full-length *her-1* cDNA from the large transcript under the control of a myosin heavy-chain promoter reported to be tissue specific causes masculinization of all dimorphic tissues in XX animals. We have shown further that this activity is dependent on the presence of the *her-1* signal sequence or a substitute synthetic signal sequence in the encoded polypeptide. These results suggest that the larger product of the *her-1* gene may act in a secreted form to dictate male development.

The smaller transcript, predicted to encode a polypeptide corresponding to the 64 carboxy-terminal amino acids of the larger polypeptide, does not appear to have masculinizing activity. Moreover, expression of the larger cDNA from constructs that lack P2 in the absence of an endogenous *her-1* gene results in development of fertile XO males, suggesting that transcription from P1 is sufficient for complete male development. Although these results do not rule out involvement of the smaller transcript in normal sex determination, they suggest that its functions are not essential for male development.

Is transmembrane signaling involved in *C. elegans* sex determination?

The molecular results reported here provide a potential explanation for the conclusion from genetic mosaic analysis by Hunter and Wood (1992), described in the introductory section, that the *her-1* gene could act nonautonomously to determine male sexual development. On the basis of previous genetic evidence, the most likely target of *her-1* action is the *tra-2* gene or its products (see Fig. 1). Okkema and Kimble (1991) have cloned *tra-2* and shown that, in adults, the steady-state levels of the major transcript are 15-fold lower in males than in hermaphrodites, although it is expressed in both sexes. More recently, Kuwabara et al. (1992) have shown that the major somatic product of *tra-2* predicted from sequencing is a large protein with an apparent signal sequence and several other hydrophobic domains capable of spanning the plasma membrane. As previously pointed out (Hunter and Wood 1992; Kuwabara et al. 1992; Kuwabara and Kimble 1992), these results suggest that the *tra-2* product could be the cell-surface receptor for a *her-1*-encoded ligand.

The earlier genetic evidence (for review, see Hodgkin 1987) indicated that in the absence of *her-1* activity (the

normal situation in XX animals), the *tra-2* gene product acts with the help of the *tra-3* product to inhibit the activity or expression of one or more of the masculinizing *fem* gene products, thereby allowing *tra-1* action to direct hermaphrodite development (see Fig. 1). Two of the three *fem* gene products, *fem-1* (Spence et al. 1990) and *fem-3* (Ahringer et al. 1992) have been cloned and sequenced; both are predicted to encode hydrophilic presumably cytoplasmic proteins that show no sequence similarity to known proteins except for six repeats of a *cdc-10/SWI6*/ankyrin motif in the *fem-1* product. The *tra-1* gene acts cell autonomously (Hunter and Wood 1990) and is predicted to encode two proteins with zinc-finger domains similar to those of the human *GLI* and *Drosophila cubitus interruptus* Dominant genes (Zarkower and Hodgkin 1992).

Secreted *her-1* protein could bind to the extracellular domain of a *tra-2*-encoded receptor, inhibiting the activity of its cytoplasmic domain and thereby allowing the *fem* gene products to inhibit *tra-1* to enable male development. Alternative mechanisms can also be imagined, however; for example, *her-1* product could interact with some other cell-surface receptor, which would relay the signal to the *tra-2* protein. The gene for such a receptor could have escaped identification in the previous extensive genetic screens for sex transformer mutants if it were redundant in function with another gene product or if it had other functions essential for viability. Direct inhibition of *tra-2* function by binding of *her-1* product is merely the simplest model pending further investigation.

Neither the *her-1* nor the *tra-2* (Kuwabara et al. 1992) predicted translation products shows significant sequence similarity to any known proteins, with the possible exception of slight similarity between the *tra-2* product and the product of the *Drosophila* segment-polarity gene *patched* (*ptc*). The *her-1* product, however, does not show significant similarity to the product of the *Drosophila* *hedgehog* (*hh*) gene (Lee et al. 1992), postulated (Ingham et al. 1991) to encode a ligand for the putative *ptc*-encoded receptor (Hooper and Scott 1989; Nakano et al. 1989).

The interaction proposed in the simple model above would be somewhat unusual in that the *her-1* product inhibits *tra-2* function. Although ligand–receptor interactions generally activate receptor functions, a few cases of naturally occurring protein ligands that inhibit their cognate receptors have been reported or postulated. Two examples are *pp63*, the inhibitor of the insulin receptor tyrosine kinase (Auberger et al. 1989) and the interleukin-1 receptor antagonist (Hannum et al. 1990). Another could be Mullerian Inhibiting Substance (MIS), which may act in mammalian male gonad development by binding to and inhibiting an EGF-tyrosine kinase receptor (Cigarroa et al. 1989).

A priori, it might not have been expected that transmembrane signaling should be required to determine cellular sexual identities in *C. elegans*, a simple animal with few cells, many of whose developmental fates are determined cell autonomously (Sulston et al. 1983). One

possible explanation is that intercellular signalling by the *her-1* product evolved to provide an error correcting mechanism for cells that aberrantly adopt a female fate in an XO animal. Such an error could result from misreading of the X/A primary signal, the nature of which is not yet understood. An ambiguous X/A reading could be compounded by the recent suggestion that active *tra-2* product, when uninhibited by *her-1* action, may indirectly up-regulate its own synthesis (Okkema and Kimble 1991), which would further push an aberrant cell in an XO animal toward a female fate. In *Drosophila*, misreading of X/A is presumably corrected by the *Sxl* gene, which dictates both sex determination and dosage compensation after its activity level is set to the male or female mode by the X/A ratio (Cline 1984). Because inappropriate dosage compensation is a cell-lethal condition, cells that assess X/A aberrantly and set *Sxl* incorrectly die and do not contribute to the imago. In a fixed lineage animal like *C. elegans*, such a cell death mechanism could not be used, but the paracrine effects of a *her-1*-encoded ligand could serve the same purpose. If an occasional cell in an XO animal aberrantly assesses its X/A ratio such that its *her-1* gene is not expressed, secretion of the ligand by neighboring cells would reset the aberrant cell's sex determination pathway to the male mode, thereby ensuring a complete set of the male-specific structures required for mating. This proposal might require that there also be some nonautonomous mechanism for correcting the dosage compensation error that would result from misreading the X/A ratio, also possibly a cell-lethal condition in *C. elegans* (Villeneuve and Meyer 1990).

Alternatively, cell signaling in present-day *C. elegans* sex determination may have no adaptive value. In some species of plant parasitic nematodes, sex is determined not by genotype but rather by conditions in the infested plant (Triantaphyllou 1973), requiring a mechanism for transduction of signals from the environment. A transmembrane signaling step in *C. elegans* could be an evolutionary holdover from an ancestor with environmentally determined sex.

Cell interactions are implicated in a growing number of developmental events in *C. elegans*, including specification of embryonic (Priess and Thomson 1987; Schnabel 1991; Wood 1991) and post-embryonic lineages (e.g. Seydoux and Greenwald 1989; Waring and Kenyon 1990) and induction of vulval development in hermaphrodites (for review, see Horvitz and Sternberg 1991). Our results support a role for cell–cell signaling in *C. elegans* sex determination as well and suggest a mechanism by which it may occur.

Materials and methods

C. elegans strains, culture, and general methods

Techniques for growing, handling, and microscopy of *C. elegans* have been described previously (Brenner 1974; Sulston and Hodgkin 1988; Trent et al. 1988, 1991). All strains were derived from *C. elegans* var. Bristol (N2). The following mutations used in this study were obtained from our collection or from the

Caenorhabditis Genetics Center: Linkage group (LG) II, *rol-6(su1006)*; LGIV, *him-8(e1489)*; LGV, *her-1(y101hv1)*, (*e1520*). The translocation *mnT12* is a fusion of chromosomes IV and X (Sigurdson et al. 1986).

DNA and RNA isolation and characterization

Total RNA was isolated from embryos using guanidinium isothiocyanate (Trent et al. 1991). Poly(A)⁺ RNA was isolated using oligo(dT) cellulose (Sambrook et al. 1989; Trent et al. 1991). Standard techniques were used to isolate restriction fragments for radiolabeling or subcloning (Sambrook et al. 1989). DNA sequencing was performed using chain terminators (Sanger et al. 1977) and modified T7 DNA polymerase (Sequenase 2.0, U.S. Biochemicals). Nested deletions for sequencing large sections of genomic DNA were generated in vivo using a Tn9–GalE system (Ahmed 1984a,b; Gold BioTechnology, St. Louis, MO).

Isolation of *her-1* cDNA clones

cDNA clones corresponding to the 0.8-kb *her-1* transcript were obtained by exhaustively screening a *him-8* (~37% XO) embryonic *λgt11* cDNA library (Schauer and Wood 1990) with cloned genomic DNA fragments including exons 3 and 4 (pCT111), which had been ³²P-labeled by use of random primers (Feinberg and Vogelstein 1983; Benton and Davis 1977).

cDNA clones corresponding to the 1.2-kb transcript were obtained using a modified RACE protocol (Frohmann et al. 1988; Loh et al. 1989). A primer complementary to the 5'-end of exon 3, JH3, was used to prime first-strand cDNA synthesis (see below for list of primer sequences). After tailing with terminal deoxynucleotidyl transferase and dATP, an empirically determined amount of this cDNA was used as template for PCR. The upstream primer was a 9:1 mixture of MPAN-1 and *Xba*I primer-adaptor, and the downstream primer, JH2, was a segment overlapping the primer used for cDNA synthesis. Amplified fragments were rendered blunt-ended with *Escherichia coli* DNA polymerase I (Klenow fragment). The reaction mixture was extracted once with an equal volume of phenol–CHCl₃, precipitated with ethanol, and fractionated on a 1.2-ml spin column of Sephadex G-50. The resulting duplex cDNA was either (1) ligated to an excess of phosphorylated linkers and digested with appropriate restriction enzymes, or (2) digested without adding linkers for subcloning and DNA sequencing.

PCR Primers: 5' to 3' are as follows: *Xba*I primer/adaptor: GTCGACTCTAGATTTTTTTTTTTTTT; MPAN1: GGCT-CGAGGTGCTAGTATGATT; JH3: CACATCTTCTCCAG-AATCG; JH2: GAATCGTTTGGTCGTTGCC; MP3: GAAC-TTATCAAAGATGCAGCTG; MP6: TTCAGACTCCTCGGA-AAGTTTCG; MP10: GGACCCAAAGGTATGTTTCG.

Transcript mapping by RNase protection

RNase protection assays were performed using standard procedures (Melton et al. 1984; Sambrook et al. 1989). Genomic fragments spanning an 8-kb region and including all the predicted exons were subcloned into suitable vectors containing phage T7 and T3 promoters. These were used for in vitro transcription with phage T3 or T7 RNA polymerase and ³²P-α-UTP to generate asymmetric RNA probes labeled to high specific activity. To allow comparisons of different assays and estimation of transcript levels, amounts of mRNA in the hybridization reactions were normalized by comparing the signal of the fragments protected by *her-1* probes to those protected by a probe specific for the *C. elegans* actin gene *act-1* (Fig. 4).

Plasmid constructions

pMPL14, *pWLG1*: The *lacZ* fusion constructs, which carry a nuclear localization sequence in addition to the *lacZ* coding sequence, were derived from plasmids described by Fire et al. (1990).

pWLF1: To obtain a full-length cDNA corresponding to the larger *her-1* transcript, two primers, MP3 in exon 1 and MP6 in exon 4, were used to amplify the correct fragment from *him-8* cDNA. After digestion with the appropriate enzymes, the fragment was fused to flanking cDNA segments in a tripartite ligation to generate pMPP13-1. pMPP13-1 was used as a source of full-length 1.2-kb cDNA for construction of pWLF1 and the plasmids listed below.

pMPW, *pMPX*, and *pMPZ* plasmids: The ectopic expression vectors (Fig. 2B) into which *her-1* cDNA fragments were inserted for muscle-specific expression or expression in response to heat shock contain the indicated promoters (see Results) and the 3'-untranslated region from the *unc-54* gene, flanking insertion sites for coding sequences (D. Dixon, S. White-Harrison, and A. Fire, in prep.). The plasmid used for expression of a *her-1* polypeptide with substituted signal sequence (pPD52.81; A. Fire, unpubl.) contained the *unc-54* promoter and enhancer region, the signal sequence cassette, and a polylinker cloning site.

DNA-mediated transformation of *C. elegans* and histochemical staining of transgenic animals

Transgenic worms were generated essentially as described by Mello et al. (1991). Plasmid DNAs at a concentration of 100–200 μg/ml were mixed with Fire's injection buffer (minus the Lucifer Yellow) (Fire 1986) and injected into the distal syncytial gonads of young adult hermaphrodites. The dominant marker *rol-6(su1006)* on the plasmid pRF4 was co-injected with the plasmid to be tested for masculinization in order to identify transformed animals among the F1 progeny of these animals (based on their Rol phenotype). When possible, F2 Rol animals were picked to establish stable lines carrying heritable extra-chromosomal arrays.

To assess whether non-masculinized Rol transgenic animals contained both co-injected plasmids, individual animals that had been examined by Nomarski optics (400×) for masculinization were analyzed by single worm PCR using a primer (MP10) specific for the vector carrying the *her-1*-derived construct and a primer specific for *her-1* (MP6) (Williams et al. 1992). All Rol cotransformants tested also were found to contain the co-injected *her-1*-derived construct.

To obtain lines with chromosomally integrated constructs, young adult hermaphrodites carrying an array were subjected to γ-irradiation (3800 rad), distributed to individual plates and screened for production of 60–75% Rol F2 progeny (integrated constructs often show incomplete penetrance of the dominant Rol phenotype), consistent with Mendelian transmission of the *rol-6* marker (Krause et al. 1990). Linkage of this marker to a chromosomal gene was then established in appropriate crosses to confirm integration.

Populations of animals containing *lacZ* fusions were grown on Lac[−] bacteria, harvested, and frozen in 2% paraformaldehyde, 0.1% glutaraldehyde on dry ice for 5 min. After rapid thawing and rinsing in PBS, the fixed animals were immersed in cold acetone for a further 5 min, air dried, and then stained with X-gal for 4–24 hr at 16–37°C (depending on the experiment) as described by Fire et al. (1990).

Heat shock inductions

Small staged populations of embryos from animals transformed

with a heat shock promoter construct and *rol-6* marker DNA (see text) on NGM plates were heat-shocked in an air incubator for 2 hr at 33°C or 1 hr at 37°C twice a day. Between heat shocks, animals were grown at 20°C. Sexual phenotypes of Rol adults were scored by Nomarski microscopy as described by Doniach (1986).

Data base searches

Nucleic acid and protein sequence data bases at the GenBank on-line e-mail server (Palo Alto, CA) were searched for similarity with *her-1* cDNAs and their predicted translation products. The FASTA algorithm (Pearson and Lipman 1988) was used to search GenBank 72.0, GenPept 72.0, and Swiss-Prot 22.0. The BLAST algorithm (Altschul et al. 1990) server at NCBI was used to search Swiss-Prot 23.0, PIR 34.0, GenPept 73.1, GenBank 73.1, and EMBL 32.0. No significant similarities were detected.

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Note

The sequences reported here, including genomic sequence not shown in Figure 3, have been deposited in the EMBL data library (accession number pending).

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Molecular characterization of the her-1 gene suggests a direct role in cell signaling during *Caenorhabditis elegans* sex determination.

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