Development 125, 1617-1626 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV5193

CHR3: a *Caenorhabditis elegans* orphan nuclear hormone receptor required for proper epidermal development and molting

Marta Kostrouchova[1], Michael Krause[2], Zdenek Kostrouch[1] and Joseph Edward Rall[1]

[1]Diabetes Branch, NIDDK, NIH, Bethesda, MD 20892, USA
[2]Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD 20892, USA
*Author for correspondence (e-mail: mwkrause@helix.nih.gov)

Accepted 17 February; published on WWW 1 April 1998

SUMMARY

CHR3 is a *Caenorhabditis elegans* orphan nuclear hormone receptor highly homologous to *Drosophila* DHR3, an ecdysone-inducible gene product involved in metamorphosis. Related vertebrate factors include RORalpha/RZRalpha, RZRbeta and RevErb. Gel-shift studies show that CHR3 can bind the DR5-type hormone response sequence. CHR3 is a nuclear protein present in all blastomeres during early embryogenesis. During morphogenesis, both CHR3 protein and zygotically active reporter genes are detectable in epidermal cells and their precursors. Inhibition of the gene encoding CHR3 results in several larval defects associated with abnormal epidermal cell function, including molting and body size regulation, suggesting that CHR3 is an essential epidermal factor required for proper postembryonic development.

Key words: Nuclear hormone receptor, *Caenorhabditis elegans*, Molting, Hormone response element, Epidermis, CHR3

INTRODUCTION

Steroid/thyroid hormone nuclear receptors are a large family of transcription factors induced by ligands including steroid, thyroid, retinoic acid and ecdysone (Tsai and O'Malley, 1994; Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995). The general structure of these receptors consists of amino-terminal A/B domains of various sizes, a zinc-finger motif responsible for DNA binding (DBD) and dimerization, a hinge region and a carboxy terminal ligand-binding (LBD)/activation domain (Laudet et al., 1992; Freedman, 1992). Many receptors have been isolated for which no physiological ligands have been identified but which show strong sequence similarity to the DBD of nuclear hormone receptors, so called orphan nuclear receptors (O'Malley and Conneely, 1992; Laudet and Adelmant, 1995; Mangelsdorf and Evans, 1995a; Enmark and Gustafsson, 1996). More than 60 genes have been identified to date in *C. elegans* that encode orphan nuclear receptors (Waterston et al., 1992; Waterston and Sulston, 1995; Sengupta et al., 1994; Sluder et al., 1997), including three that we had previously identified (Kostrouch et al., 1995). Here we study one of the three, CHR3.

CHR3 is closely related to the *Drosophila* orphan nuclear receptor DHR3 that plays a major role in the larval-prepupal transition (Koelle et al., 1992; Horner et al., 1995; White et al., 1997; Lam et al., 1997). *Drosophila* metamorphosis is regulated by pulses of the hormone ecdysone (Riddiford, 1993; Thummel, 1996). Ecdysone functions via heterodimers of nuclear hormone receptors (Ecdysone Receptor (EcR) and Ultraspiracle (USP)) to activate directly the transcription of a set of early genes (*E74A, E75A, BR-C*) and DHR3 (Koelle et al., 1991, 1992; Yao et al., 1992, 1993; Horner et al., 1995). DHR3 does not bind ecdysone nor has a ligand been identified for DHR3. DHR3 is a transcriptional repressor of some early genes and a transcriptional activator for another orphan receptor gene encoding FTZ-F1 (Lam et al., 1997; White et al., 1997). Together, these two transcriptional activities of DHR3 reset the hormone signaling pathway for a new response to a subsequent pulse of ecdysone. Several mutations have recently been identified in the *DHR3* gene and demonstrate DHR3 is also an essential embryonic factor (Carney et al., 1997).

In vertebrates, CHR3-related factors include RORalpha/RZRalpha, RZRbeta and RevErb (Giguere et al., 1994; Becker-André et al., 1993; Carlberg et al., 1994). In mice, RORalpha is known to act as a transcriptional activator for at least two genes. It is also important for brain development as demonstrated by *staggerer* mutant mice, which carry deletions of the RORalpha locus (Hamilton et al., 1996). RevErb has been shown to function as a transcriptional repressor and plays a role in adipocyte and muscle differentiation (Chawla and Lazar, 1993; Downes et al., 1995).

We show here that, in *C. elegans*, CHR3 is a ubiquitous nuclear factor in early embryogenesis that becomes largely epidermal specific in late embryogenesis and early larval development. Inhibition of CHR3 results in larval phenotypes attributable to epidermal cell defects, demonstrating that CHR3 is an essential factor for proper postembryonic development, most likely acting in the epidermis.

MATERIALS AND METHODS

Antibody preparation

A His-tagged CHR3 fusion protein was generated by cloning the carboxyl-terminal region (residues 385 to 587) into the vector pRSET B (Invitrogen). This segment of CHR3 does not include the highly conserved DNA-binding domain. Fusion protein was expressed in BL21(LysS) cells after induction for 2 hours. Cell pellets were lysed in denaturing conditions and the CHR3 fusion protein purified by affinity chromatography on Ni-Sepharose columns. Purified CHR3 fusion protein was used to immunize two rabbits and subsequent bleeds (at 4, 6 and 8 weeks postimmunization) were tested by western blot and staining of fixed *C. elegans* embryos for reactivity. Serum from one rabbit (#2865) was positive in both assays whereas the other rabbit serum showed only background staining of embryos. Rabbit #2865 serum was used directly or after partial purification by ammonium sulphate precipitation (50% cut).

Immunocytochemistry

Fixation of embryos for staining with CHR3 antibody used a modification of a procedure by Tabara and colleagues (1996); those modifications were previously detailed (Krause et al., 1997). CHR3 serum was diluted 1:400, applied to specimens overnight at 4°C and detected with either a rhodamine- or FITC-conjugated donkey anti-rabbit IgG secondary antibody (Jackson Immunological). MH27 monoclonal antiserum was a gift of R. Francis and R. Waterston. A commercially available monoclonal antibody against β-galactosidase (Promega) was used to detect *lacZ* reporter gene expression. MH27 and anti-β-gal antibodies were diluted 1:1000 or 1:400 prior to use on embryos fixed in methanol acetone. LIN-26 antibodies, a gift of M. Labouesse, were used diluted 1:1000.

Attempts using the CHR3 antibody to localize CHR3 in larvae and adults, by a variety of fixation methods (Finney and Ruvkun, 1990; Krause et al., 1994; Miller and Shakes, 1996; Krause et al., 1997) have been unsuccessful in giving a consistent and reproducible signal. The variability of staining that we observe prevents us from confidently reporting the distribution of CHR3 postembryonically. A reporter gene construct in which CHR3 has been tagged with GFP (see below) suggests that CHR3 continues to be present in epidermal cells postembryonically.

Cloning

Many of the reporter gene constructs are derivatives of a single parental construct, #3306. #3306 was generated by PCR amplification of a 3.5 kb fragment from the 5′ end of the *nhr-23* gene, digestion with the enzymes *Hin*dIII and *Pst*I, and cloning in frame to the corresponding restriction sites in the *lacZ* reporter vector pPD22.04 (Fire et al., 1990). This fragment begins 1.6 kb upstream of exon I and extends 198 bp into exon II before fusing with *lacZ* sequences. #4281 is a 1 kb *Hin*dIII-*Bgl*II deletion of #3306, leaving 667 bp sequence 5′ to exon I. #4282 is a *Hin*dIII-*Xho*I deletion of #3306, leaving 1,241 bp of sequences 5′ to exon II.

Constructs #4298 and #4271 were generated by subcloning genomic restriction fragments as indicated in Fig. 5 into the GFP reporter vector pPD95.67. Construct #4265 was generated by subcloning a PCR-amplified genomic fragment into the *lacZ* reporter vector pPD95.03.

A GFP-tagged *nhr-23* gene (#4276) was made by PCR amplification of a GFP cassette from the vector pPD79.44 using *Bam*HI restriction enzyme site containing primers. This *Bam*HI fragment was inserted into a unique *Bam*HI site within exon VII of the 11 kb *Stu*I-*Eco*RV genomic clone of *nhr-23* in the vector pVZ-1.

Heat-shock expression utilized the vector pPD49.78 containing the promoter of hsp16-48 (Stringham et al., 1992). *nhr-23* cDNA coding sequences were amplified by PCR using *Kpn*I restriction site-containing primers and the resulting product cloned in either the sense or antisense orientation in the heat-shock promoter vector.

All transgenic lines were extrachromosomal and were generated by microinjection of reporter plasmid DNA (10-100 ng/μl) along with pRF4 DNA at 50 ng/μl (Mello and Fire, 1995).

RNA-mediated interference (RNAi)

RNA used for the inhibition of *nhr-23* was synthesized from linearized templates using either T3 or T7 RNA polymerase. Reaction products were extracted with phenol and chloroform, precipitated, resuspended at a concentration near 1.0 mg/ml and used without further purification. Annealed sense and antisense RNA was prepared by mixing complementary T3 and T7 RNA polymerase reaction after completion, and heating to 45°C for 5 minutes prior to extraction and precipitation. Two templates were used to synthesize *nhr-23* RNA. One corresponded to the near full-length cDNA clone that includes the DBD. The second template was a 601 bp *Bam*HI-*Hin*dIII fragment, encoding amino acid residues 373-573, which lacked the conserved DNA-binding domain.

Gel shifts

CHR3 protein for gel shifts used a His-tagged fusion protein from a near full-length cDNA clone into the expression vector pRET (Invitrogen) (see antibody production details above). After induction, CHR3 fusion protein was purified by nickel chromatography under native conditions and eluted with increasing amounts of imidazole (50 mM and 200 mM). After dialysis, CHR3 fusion protein was incubated in binding buffer (10 mM Tris (8.0), 40 mM KCl, 1 mM dithiothreitol, 6% glycerol) and 0.5 μg/μl poly(dI-dC) (Pharmacia). Oligos were end labelled with ^{32}P by T4 kinase, annealed with a 10-fold molar excess of an oligo of complimentary sequence. After incubation at room temperature for 15 minutes, 0.1 ng of probe was added to the reaction, incubated at room temperature an additional 10 minutes, followed by electrophoresis at room temperature on 5% polyacrylamide gel in 0.5× TBE running buffer. Oligos used for gel-shift assays were as follows:

DR5 – TCGACGACC**AGGTCA**AAAAA**AGGTCA**CGTTCTA
DR5(1st Mut) – TCGACGACC**AttTaA**AAAAA**AGGTCA**CGTTCTA
DR5(2nd Mut) – TCGACGACC**AGGTCA**AAAAA**AttTaA**CGTTCTA
DR5(Both Mut) – TCGACGACC**AttTaA**AAAAA**AttTaA**CGTTCTA
Mutant A Site – GATATTT**GGGAGT**CTCACA

RESULTS

Genomic organization

The gene encoding CHR3 was first identified by a PCR-based strategy focused on nuclear hormone receptors (Kostrouch et al., 1995). Using a probe derived from the cDNA, we have cloned the gene encoding CHR3; it has been designated *nhr-23* (***n**uclear **h**ormone **r**eceptor*-23) in keeping with *C. elegans* nomenclature. The *nhr-23* gene was mapped by Alan Coulson and colleagues to cosmid C01H6 in the central part of Chromosome I between *unc-14* and *myo-1* (Fig. 1A); the cosmid C01H6 has since been sequenced by the genome consortium (Waterston and Sulston, 1995). A search of the Kohara EST database identified a single clone (#yk4D4.5) from the *nhr-23* locus.

A comparison of the genomic, cDNA and EST sequences revealed the following gene structure for *nhr-23* (Fig. 1B). The gene is composed of 9 exons, spanning about 4 kb, and can encode at least two distinct isoforms of CHR3 that differ at their amino-terminal end. This gene structure is slightly different than that predicted with the sequence analysis program Genefinder (Hillier and Green, personal communication). Because exons 5 and 6 are in the same

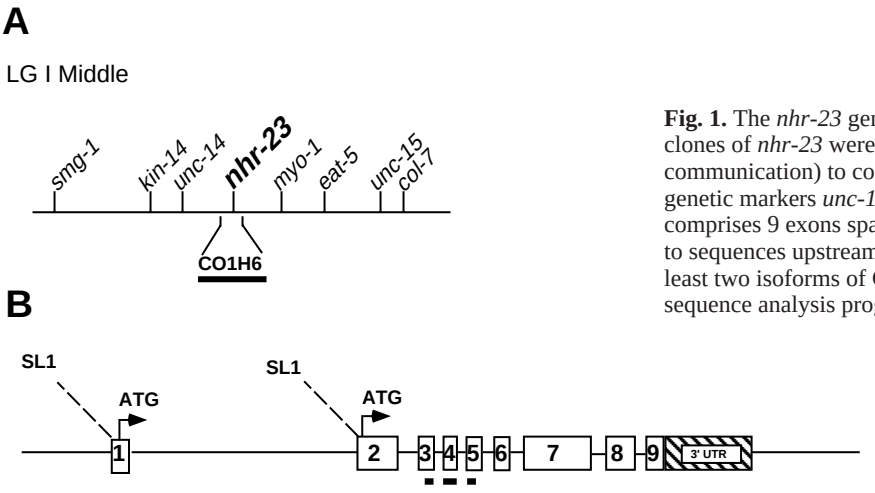

Fig. 1. The *nhr-23* gene. (A) Genomic map position of *nhr-23*. Genomic clones of *nhr-23* were mapped by Coulson and colleagues (personal communication) to cosmid C01H6 on chromosome I located between the genetic markers *unc-14* and *myo-1*. (B) *nhr-23* gene structure. The gene comprises 9 exons spanning ~4 kb. There are two SL-1 *trans*-splice sites to sequences upstream of exon I and exon II as indicated suggesting at least two isoforms of CHR3 can be encoded by this gene. The DNA sequence analysis program Genefinder predicts a continuous exon spanning the regions shown here as exon 5, intron 5 and exon 6; no cDNA clone corresponding to this potential coding variant have been identified. The DNA-binding domain of CHR3 is indicated below the gene by a heavy black line spanning exons 3, 4 and 5.

reading frame and the intervening intron has no in-frame stop codons, Genefinder predicts a single large exon rather than the two exons demonstrated by a cDNA clone.

Based on cDNA sequences and *trans*-splicing patterns, there appear to be at least two promoter start sites; one upstream of exon I and the second within intron I. The transcript initiating upstream of exon I is predominantly *trans*-spliced to SL1 (Krause and Hirsh, 1987) at a site 35 bp upstream of the putative initiation methionine codon. A second transcriptional start site within intron I was demonstrated by a previously described cDNA clone (Kostrouch et al., 1995). This clone demonstrated that SL1 *trans*-splicing could also occur at the 3′ splice acceptor sequence of intron I; translation of this transcript would presumably be initiated at the first methionine codon located 38 bp downstream of the splice site. The utilization of a 3′ splice acceptor site for both *cis*- and *trans*-splicing has been observed previously (Azzaria et al., 1996), although there are no documented cases of SL-1 *trans*-splicing competing directly with conventional *cis*-splicing (Blumenthal and Steward, 1997). In the case of *nhr-23* transcripts, functional promoter studies (see below) indicate the presence of a second promoter start site within intron I that would generate a second transcript that could serve as a substrate for SL1 *trans*-splicing. SL1 splicing to both acceptor sites is easily detectable by RT-PCR amplification and the two products are present in nearly equal amounts from a mixed stage RNA preparation (data not shown). The SL1 *trans*-spliced products have been cloned and sequenced to confirm the splice sites and predicted PCR products.

CHR3 binds to hormone response elements

Nuclear hormone receptors have been shown to bind a consensus DNA sequence, known as a hormone response element (HRE), as homodimers and heterodimers depending on the particular receptor pair (see Forman et al., 1994; Mangelsdorf et al., 1995; Mangelsdorf and Evans, 1995; Thummel, 1995); specificity is frequently determined by the number of nucleotides between the HREs. Several orphan nuclear hormone receptors have also been shown to bind the consensus HRE and, in many cases, these receptors bind a half site sequence as a monomer (Giguère et al., 1994; Schräder et al., 1996; Greiner et al., 1996). Many of the vertebrate receptors of the TR-RAR type and including RORalpha and *Drosophila* DHR3, close relatives of CHR3, prefer the half site sequence PuGGTCA preceded by a 6 bp AT-rich region (Giguère et al., 1994, 1995; Kato et al., 1995; Horner et al., 1995; Lam et al., 1997).

Gel-shift experiments with bacterially expressed, purified His-tagged CHR3 fusion protein showed optimal binding activity to a PuGGTCA direct repeat with a 5 bp spacer (DR5) (Fig. 2). Much weaker binding to direct repeats with other spacing, as well as to monomeric sites, could also be demonstrated (data not shown). DR5 binding by CHR3 required two intact direct repeats; mutating either repeat alone or both together eliminated binding under our gel-shift conditions. The binding activity of CHR3 to DR5 could be competed by self, but not by unrelated oligos or oligos containing mutated HREs.

CHR3 distribution during embryogenesis

Polyclonal rabbit antibodies were raised against a bacterially produced, His-tagged CHR3 fusion protein (residues 270-553)

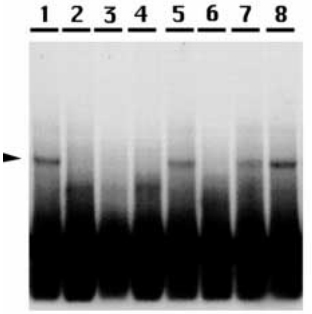

Fig. 2. CHR3 binds a hormone response element (HRE) in vitro. His-tagged CHR3 fusion protein was incubated with intact or mutated oligonucleotides containing a direct repeat HRE separated by 5 bps (DR5). Lane 1+8, DR5; lane 2, first HRE mutated; lane 3, second HRE mutated; lane 4, both HRE elements mutated; lanes 5-7, CHR3 binding to DR5 with a 100-fold molar excess of unlabeled DR5 (lane 6) or a 20-fold molar excess of an unrelated competitor oligo (lane 7) added prior to probe addition. Arrow marks position of CHR3-specific shifted species.

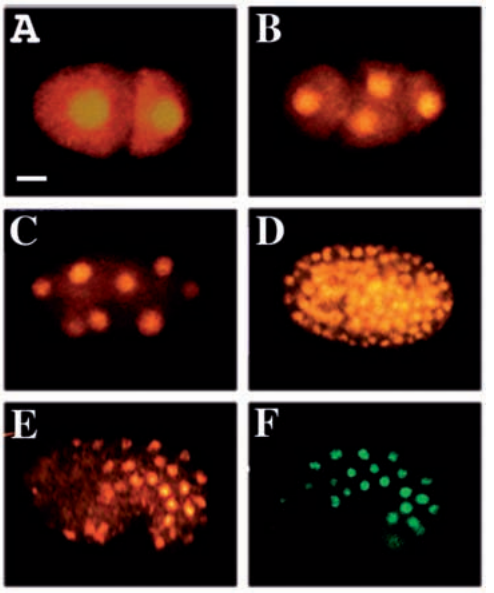

Fig. 3. Distribution of CHR3 during embryogenesis. Embryos were stained with anti-CHR3 in A-E. Staining with CHR3 antiserum is nuclear with variable levels of cytoplasmic background staining. Intensity of staining is uniform among blastomeres until the comma stage of embryogenesis at which time epidermal cells and their precursors exhibit more pronounced staining. (A) 2-cell embryo; (B) 4-cell embryo; (C) ~16-cell embryo; (D) ~200-cell stage embryo; (E) lateral view of 1.5-fold embryo with the head to the left and dorsal side at top. Note the three rows of intensely staining nuclei corresponding to epidermal nuclei. (F) A 1.5-fold embryo oriented as in E stained with an antibody recognizing LIN-26 to highlight the positions of epidermal nuclei. Bar, ~10 μm.

lacking the conserved DNA-binding domain. The specificity of the antiserum for CHR3 was demonstrated by multiple criteria. First, the antiserum specifically recognizes the CHR3 fusion protein by western blot analysis whereas preimmune serum does not. Second, the signal from CHR3 antibody in fixed embryos is eliminated by preincubation of the antiserum with purified CHR3 fusion protein but not by an unrelated His-tagged transcription factor fusion protein. Third, overexpression of CHR3 from a heat-shock promoter in transgenic strains is easily detected with the antiserum in the tissues in which that heat-shock promoter is known to be most active. Fourth, the distribution of CHR3 in late stage embryos determined by antibody staining mimics the tissue distribution of *nhr-23* reporter gene expression in transgenic strains.

CHR3 is a nuclear protein, consistent with its role as a transcriptional regulator. CHR3 is present in all blastomeres of the embryo from at least the 2-cell stage until approximately the 200-cell stage (Fig. 3A-D). The presence of CHR3 in 2-cell embryos suggests a maternal contribution, a notion confirmed by in situ hybridization (see below). At around the 200-cell stage, the ubiquitous distribution of CHR3 begins to fade in most blastomeres and a more restricted pattern begins to emerge in which the most prominent staining nuclei are epidermal cells and their precursors (Fig. 3E,F). The epidermal staining pattern of CHR3 antibody is maintained throughout embryogenesis, while the staining of all other nuclei fades to background levels.

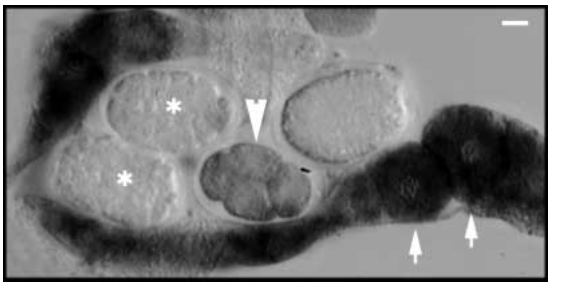

Fig. 4. Maternal expression of *nhr-23* detected by in situ hybridization. A *nhr-23* antisense DNA probe was hybridized to an extruded adult gonad courtesy of B. Harfe and A. Fire (Seydoux and Fire, 1995; Harfe and Fire, 1998). Strong hybridization is seen throughout the gonad and in forming oocytes (small arrows). Weaker expression is detected in embryos until the 4-cell stage (arrowhead) after which it is undetectable in early embryos (e.g. embryos with asterisk). A weak hybridization signal is detected again in late stage embryos after the bean stage of embryogenesis (not shown, see text). Bar, ~10 μm.

In *C. elegans*, epidermal cells originate from two founder blastomeres, AB and C. The early epidermal derivatives of these founder blastomeres are located in the dorsal region near the posterior of the embryo (for complete description of cell fate see Sulston et al., 1983). At approximately the 350-cell stage (270 minutes postfertilization), these cells begin to migrate ventrally to enclose the developing embryo (Sulston et al., 1983; Williams et al., 1997). Dorsal epidermal cells in the midbody, designated Hyp7, interdigitate and fuse to form a single large syncytium. Smaller multinucleate epidermal cells are also present in the head (Hyp 1-6) whereas mostly mononucleate epidermal cells (Hyp 8-11) are present in the tail (Sulston et al., 1983). Lateral and ventral epidermal cells of the embryo, designated V (seam cells) and P respectively, are stem cell-like. In addition to functioning as epidermis, the V and P cells divide postembryonically to generate additional epidermal and non-epidermal cell types (Sulston et al., 1983).

By the 1.5-fold stage of embryogenesis, the Hyp7, V and P nuclei are easily identified as three lateral rows on each side of the embryo (Fig. 3F). By antibody detection, these bilaterally symmetric three rows are clearly CHR3 positive as are nuclei in the head and tail regions corresponding to Hyp nuclei. This epidermal staining pattern was further confirmed by double staining with a monoclonal antibody (MH27) that recognizes desmosomes (Francis and Waterston, 1985). Desmosomes are a particularly striking feature of the epidermal cells beginning after embryonic cell division has been completed and morphogenesis has begun.

nhr-23 expression

The expression of *nhr-23* was assayed by in situ hybridization. *nhr-23* RNA is easily detectable in the germline and oocytes confirming that *nhr-23* is a maternal gene product (Fig. 4). *nhr-23* RNA is also detected in 2- and 4-cell embryos but then becomes undetectable in subsequent stages of early embryogenesis. By the comma stage of embryogenesis, a faint in situ signal for *nhr-23* appears on the surface of embryos, consistent with expression in the epidermis. However, because the epidermis covers the entire embryo at this stage, it is

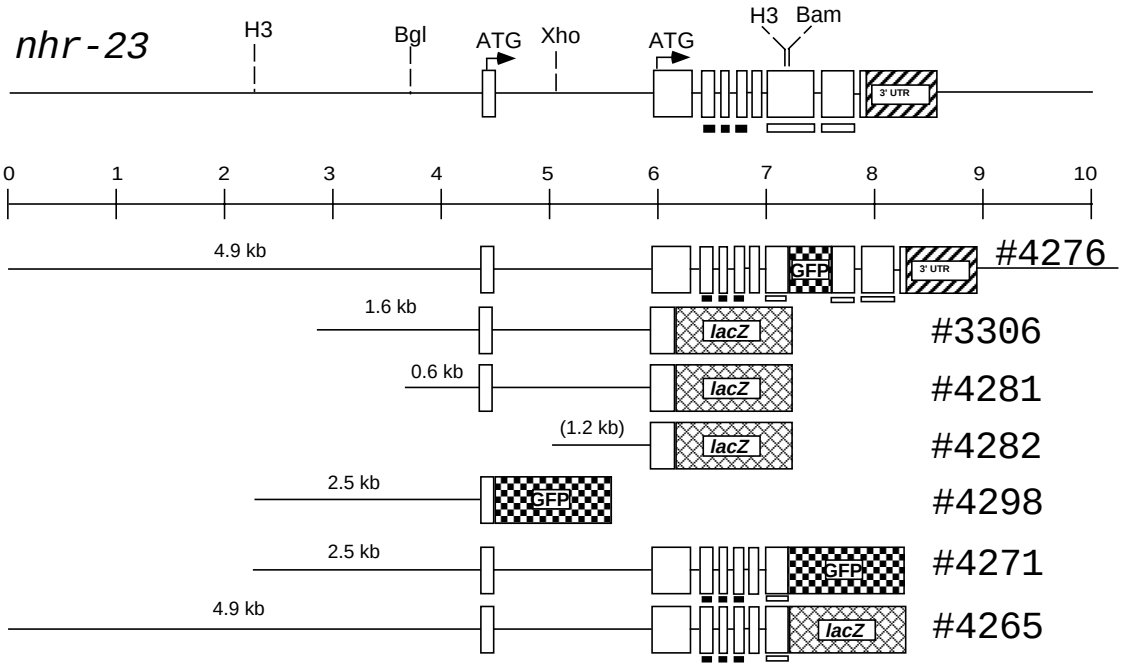

Fig. 5. *nhr-23* reporter gene constructs. The exon/intron structure of the *nhr-23* gene encoding CHR3 is shown at top with approximate distances in kb shown immediately below the gene. The coding regions corresponding to the DNA-binding domain and ligand-binding domain are indicated below the gene with a black line and open box, respectively. Restriction enzyme sites used in generating several of the constructs are indicated. Reporter genes in which *nhr-23* sequences have been fused to either *lacZ* or green fluorescent protein (GFP) are shown and identified by the number to the right. Each construct was used to generate a minimum of two independent transgenic lines using the maker plasmid pRF-4 (Mello and Fire, 1995). Expression patterns are described in the text.

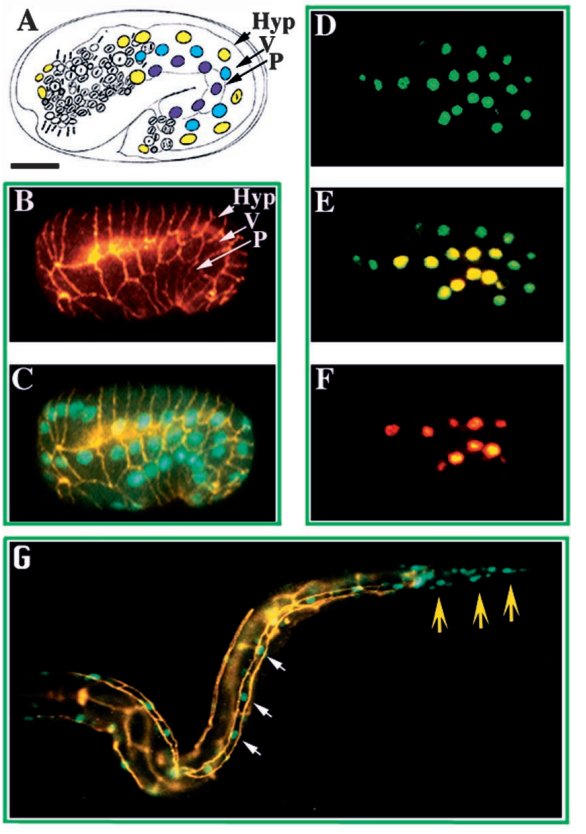

Fig. 6. Expression of *nhr-23* reporter genes in epidermal cells during late embryogenesis and larval stages. (A) Schematic diagram of nuclear positions in a 1.5-fold embryo (adapted from Sulston, 1983) in which epidermal nuclei have been colored. Some of the epidermal nuclei form a distinctive set of three rows on either side of the embryo at this stage; the rows are composed of nuclei from Hyp7 (top row, yellow), V cells (middle row, blue) and P cells (bottom row, purple). Additional epidermal cells are located in the head and tail of the animal. (B,C) A lateral view of a comma-stage embryo harboring the transgene construct #4276 (see Fig. 4). The embryo was fixed and stained with the antibody MH27 (B) to visualize the borders of epidermal cells. GFP fluorescence (C) of the *nhr-23* reporter marks the nuclei of all epidermal cells. (D-F) Comparison LIN-26 antibody staining of epidermal nuclei (D) to expression of a *nhr-23::lacZ* reporter gene (construct #3306) in a transgenic embryo (F) detected using an anti-β-galactosidase antibody. E is a merged image of LIN-26 and β-galactosidase-positive nuclei showing overlap of the two patterns in V and P nuclei (yellow). Not all epidermal nuclei are marked by the *nhr-23* reporter gene due to mosaicism of expression. (G) A transgenic larva harboring a GFP-tagged *nhr-23* gene (construct #4276) stained with MH27. Expression of the reporter gene in seam cells (epidermal) is indicated by small arrows; expression in tail epidermal cell is indicated by large arrows. Bar, ~10 μm.

difficult to distinguish a faint in situ hybridization signal from background hybridization.

An additional method to assay *nhr-23* expression, and to dissect promoter elements, utilizes reporter genes in which segments of the *nhr-23* gene have been fused with either β-galactosidase or green fluorescent protein (GFP) (Fig. 5). These reporter gene constructs were used to generate non-integrated transgenic strains with at least two independent lines analyzed for each construct. Expression of the reporter gene was mosaic in all lines, with less mosaicism observed in lines made with constructs containing larger amounts of *nhr-23* flanking sequences.

Reporter gene constructs that begin at least 1.6 kb upstream of exon I and include all of intron I were consistently expressed in epidermal cells and their precursors (Fig. 6). Expression in epidermal cells and precursors was clearly evident by the comma stage of embryogenesis and continued through larval stages. Construct #3306 showed the earliest expression, beginning at about the 50-cell stage in one or two unidentified nuclei. Based on the lineage of epidermal cells, it is possible that these early nuclei are Cpa and Caa; however, we have been unable to confirm this independently.

Epidermal expression of *nhr-23* reporter genes can be achieved with as little as 600 bp of sequence upstream of exon I provided that intron I sequences are also included (construct #4281). However, this construct shows additional expression in nuclei of the pharynx and distal 4-6 nuclei of the intestine, two common sites of ectopic expression (Krause et al., 1994). Expression was completely eliminated by removing intron I (see #4298) and only ectopic (pharynx and intestine) expression was observed when mostly intron I sequences (construct #4282) were used to drive expression. These results demonstrate that sequences both upstream and within intron I are together necessary for epidermal expression. The ectopic expression observed with construct #4282 suggests the presence of promoter activity within intron I and/or the first part of exon II. Recall that *trans*-spliced leader sequences found on cDNA clones suggested that at least two transcriptional starts sites function for *nhr-23*, one upstream of exon I and a second within intron I (described above).

For several of the reporter constructs (#4265, #4271, #4276), it was difficult to establish transgenic lines and, once established, the strains had a high frequency of embryonic and early larval lethality making the strains difficult to maintain. Most, if not all, of the lethal animals from these strains had relatively high levels of reporter gene expression and were associated with severe defects in morphogenesis and elongation. Such an effect was never observed in constructs lacking the DBD of CHR3, but was always observed in lines carrying constructs with intact DBD and lacking, or having an interrupted, region corresponding to the LBD. Once established (when possible), these stains had fairly weak reporter gene activity. It seems likely that the difficulties associated with these particular reporter constructs resulted from a dominant negative effect of the encoded CHR3/reporter fusion since many receptors with intact DBD in combination with mutations in the LBD function result in a dominant negative phenotype (Herskowitz, 1987).

Inhibition of CHR3 function

Gene function in *C. elegans* can be studied by both classical genetic methods (Brenner, 1974) as well as several reverse genetic approaches (Zwaal et al., 1993; Guo and Kemphues, 1995). To date, no mutations in *nhr-23* have been identified nor do any small deficiencies of the region exist to aid in our understanding of CHR3 function. We have, therefore employed a molecular technique, known as RNAi (Rocheleau et al., 1997), to interfere with *nhr-23* gene function using sequence-specific RNAs.

RNAi is based on earlier observations that antisense RNA transcribed in vitro from a cloned copy of a gene of interest can induce a phenotype similar to a null mutation in the progeny of adult hermaphrodite animals into which it has been microinjected (Guo and Kemphues, 1995, 1996; Lin et al., 1995, Krause et al., 1997). Surprisingly, injection of sense RNA, often used as a control in vertebrate studies, has the same effect as that induced by the antisense strand suggesting that the mechanism of inhibition in *C. elegans* is different from that in vertebrates. Fire and colleagues have recently shown that RNAi of zygotic genes can be greatly enhanced by injecting a double stranded duplex of annealed sense and antisense RNA for the target gene (Fire et al., 1998).

We find that injection of either *nhr-23* sense or antisense RNA alone results in larval defects in approximately 30% (n=1088) of the progeny of injected animals; this frequency is boosted to greater than 80% (n=599) when sense and antisense RNA are annealed prior to injection. The phenotypes of affected larvae include small size, difficulties in shedding old cuticles after molts and larval arrest at the L3 or L4 stage (Fig. 7). The defect in shedding the cuticle at molts is particularly striking and animals can be found in which up to three previous cuticle segments are still attached near the tail. These unshed cuticles are often bunched together at discrete sites along the body length and result in

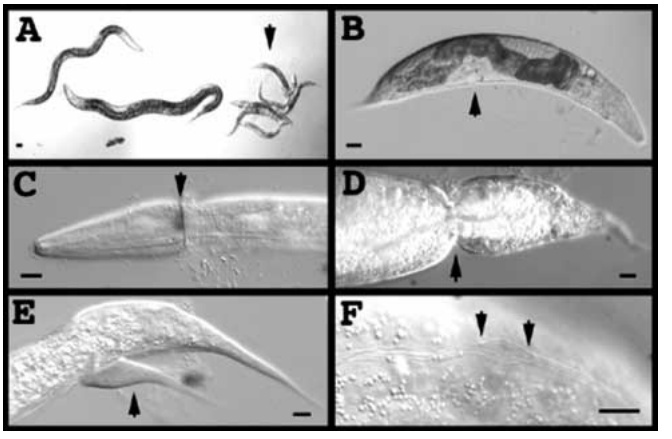

Fig. 7. Postembryonic defects associated with RNA-mediated interference (RNAi) of *nhr-23*. (A) The slow growth of affected animals (cluster under arrow) is compared with similarly aged, unaffected siblings to their left. (B) A late stage L4 larva showing the typical Dumpy (short and fat) phenotype. The arrow marks the forming vulva. Molting defects resulting from inhibition of *nhr-23* are shown in C-F. (C) Constriction and bunching of old cuticle in the head region. (D) Severe constriction (arrow) of unshed cuticle(s) in an adult animal. (E) A tail cuticle from a previous larval stage (arrow) remains attached to this animal at a lateral position near the tail. (F) Branched alae (arrows) in a seam cell of a rare animal that survived to adulthood. Bar, ~20 μm.

a constriction, or 'collar', that becomes more severe as the body size increases (Fig. 7C,D).

We have followed individual affected animals (n=5) through the first larval molt to understand better the defects in cuticle shedding. Affected animals enter lethargus and initiate separation of the old cuticle from the new in the head region, undergo head contractions and twists like wild-type animals and expel the buccal cavity lining. Separation between old and new cuticle in the tail region is also seen in affected animals. However, unlike wild-type, *nhr-23(RNAi)* affected animals fail to puncture the old cuticle in the head region so that, when they exit lethargus and begin pharyngeal pumping, they are unable to eat because their mouth is still encased in the old cuticle. The inability to shed the old cuticle is also problematic in the tail region since it interferes with defecation resulting in some animals accumulating material in the space between the old and new cuticle in the tail. The inability to eat may be the primary cause of larval arrest and death of affected animals. Some animals are able eventually to break through the old cuticle at the head and tail allowing these animals to feed and grow. However, there is an inability to shed the cuticle from the remainder of the body of the animal and this is likely the cause of the old cuticle 'collars' in the head and attached tail cuticles that we frequently observe.

Less than 1% of affected larvae (n=735) resulting from *nhr-23(RNAi)* survive to adults and in all cases they have a strong Dumpy (short and fat) phenotype. Some of these adults look otherwise normal, move well and are fertile whereas some have multiple defects and are sterile. Included in the adult defects that we observe are problems in gonad morphogenesis and seam cell (lateral epidermis) development. The seam cells in these Dumpy adult animals are sometimes misplaced such that they are stacked along the dorsal/ventral axis rather than forming a continuous row of adjacent cells. In addition, the alae of adult cuticles are occasionally branched (Fig. 7F). The number of seam cells of affected animals appears normal based on direct observation and staining of these Dumpy adults with LIN-26 and MH27 antibodies (data not shown).

At low frequency (up to 7%; n=831), we also observed embryonic defects and lethality following *nhr-23(RNAi)*. Eggshells of affected embryos were often spherical and embryos usually died prior to morphogenesis. Occasionally embryos would die later in embryogenesis displaying elongation and morphogenetic defects. Dead embryos may be seen following any microinjection into the adult germline and the frequency that we observe for this non-specific, 'background' lethality is 1-2%. Because the frequency of embryonic lethality is higher than background following *nhr-23(RNAi)*, it may represent a low penetrant phenotype resulting from specific inhibition of the gene.

Specificity of RNAi relies on gene sequence homology. Since more than 60 orphan nuclear receptors have been identified in *C. elegans*, there is a potential for cross-inhibition among family members because they are all related by sequence within the highly conserved DNA-binding domain. To test this, we injected *nhr-23* RNA spanning codons for amino acid residues 373-573 that lacked the DNA-binding domain (residues 162-227). This RNA was capable of inducing the same phenotypes as full-length *nhr-23* RNA, including a small percentage (up to 5%; n=792) of lethal embryos, many with spherical egg shells identical to those observed following injection of near full-length *nhr-23* RNA. A search of the *C. elegans* genomic database with this 200 amino acid CHR3 peptide failed to identify any closely related sequences other than CHR3 itself, so non-specific cross reactivity seems unlikely.

Ectopic/overproduction of CHR3

Since inhibition of *nhr-23* function with RNAi demonstrated a role for CHR3 in molting, we were interested to see if ectopic and/or overproduction of CHR3 would result in abnormal phenotypes. We constructed transgenic lines carrying a plasmid in which the CHR3-encoding cDNA was fused to a heat-shock promoter. The heat-shock promoter is expressed in all tissues (Stringham et al., 1992), but shows particularly strong expression in intestinal cells and neurons. Induction of CHR3 in these transgenic strains was confirmed by CHR3 antibody staining of embryos; a 2 hour shock at 31°C resulted in large amounts of CHR3, which was nuclear localized.

Heat-shock-induced overexpression of CHR3 at various periods of development resulted in a low level of defects but significantly greater than in control animals. Embryos were most sensitive to heat-shock-induced CHR3, resulting in dead embryos characterized by numerous vacuoles indicative of general necrosis. There was no obvious specificity of this toxicity to a particular tissue type. Overexpression of CHR3 during early larval development usually had no effect; in some experiments, however, the treatment resulted in lethality associated with a blister phenotype (17%, n=354) in which the cuticle separated from the epidermis.

To control for heat-shock effects, similar temperature shifts were given to either wild-type N2 animals or to a transgenic strain harboring the heat-shock promoter fused to an unrelated transcription factor (*hlh-2*). There was some lethality associated with heat-shock treatment of these control strains during embryogenesis and some L1 animals had a Nob (no back end) phenotype. Both of these effects had been previously associated with heat-shock treatment of wild-type embryos (D. Dichoso and D. Shakes, personal communication). However, the fraction of lethal embryos was lower for N2 and *hs:hlh-2* strains and neither strain gave rise to blistered larvae following heat shock.

DISCUSSION

The genomic organization and transcription of *nhr-23* are of some interest. Seven of the eight introns are approximately 50 bp in length which is normal for *C. elegans* (Blumenthal and Steward, 1997). Intron I, however, at some 1.8 kb is unusually long; probably explained by the fact that it also appears to contain a promoter sequence for one of at least two transcripts from the gene. Clearly the intron I promoter is not alone sufficient to explain the temporal or spatial specificity of *nhr-23* expression. This specificity results primarily from a second promoter/enhancer region that is mostly located within the 0.6 kb preceding the first transcriptional start site; additional sequences further upstream appear to be required for absolute tissue specificity. Both transcripts are SL-1 *trans* spliced and are present in roughly equal amounts.

CHR3 is capable of binding a hormone response element (HRE) of a direct repeat of PuGGTCA separated by 5 bp

(DR5). Binding of CHR3 to DR5 requires that both repeats of the HRE are intact. This is a somewhat surprising result for two reasons. First, *Drosophila* DHR3 is capable of binding a DR3 HRE but not a DR5 HRE and second, DHR3 is able to bind a single HRE element as a monomer (Horner et al., 1995). CHR3 requires both HRE direct repeats for binding suggesting a requirement for dimerization or cooperativity for CHR3 binding, at least in the context of the DR5 oligo we have used as a probe. The difference between the binding specificity of the CHR3 and DHR3 may reflect intrinsic properties of the two proteins or may simply reside in the differences in gel-shift conditions and probe sequences used in these respective studies. Further DNA-binding studies will be necessary to address the nature of the differences between CHR3 and DHR3 target site specificity.

The *nhr-23* gene is maternally expressed and CHR3 protein is present in the nuclei of all blastomeres during the cellular proliferation phase, or first half, of embryogenesis. The results presented here do not provide compelling evidence for a function of CHR3 during early embryogenesis. RNA-mediated interference (RNAi) of *nhr-23* results in a low level (7%) of embryonic lethality with affected embryos frequently having spherical eggshells suggesting a maternal defect. This low level of affected embryos is inconsistent with an essential maternal function for CHR3 because maternal genes are extremely sensitive to RNAi (Guo and Kemphues, 1995; Lin et al., 1995; Rocheleau et al., 1997). However, it is possible that maternal *nhr-23* roles in oogenesis are redundant with one or more other genes so that this function is only partially revealed by inhibition of *nhr-23*.

CHR3 is required for proper molting

Upon hatching, *C. elegans* larvae are covered by a cuticle secreted by the epidermis. The cuticle is composed primarily of collagens expressed from a repertoire of an estimated 150 genes (Cox et al., 1984; Johnstone, 1994; Kramer, 1994). During each larval molt, a new cuticle is synthesized and the old cuticle shed. The cuticle of each stage is composed of a unique set of collagen genes and three stages (L1, dauer, adult) can be distinguished in the light microscope by the pattern of alae (small ridges) produced by lateral epidermal cells (seam cells). It is unknown exactly how the old cuticle is detached biochemically from the epidermis during molting and whether this detachment occurs prior to, or concurrent with, the production of new cuticle.

Shedding of the old cuticle appears to begin at the mouth of the animal where secretory vesicles from pharyngeal gland cells, combined with spasmodic contractions of the pharynx, weaken and then break the old cuticle (Singh and Sulston, 1978). The animal then sheds the old cuticle from head to tail as it moves. Several mutations have been identified genetically that alter the stage specificity of cuticles (eg. heterochronic mutations; see (Ambros and Horvitz, 1984; Ambros, 1997; Liu et al., 1995); however, no mutations have been identified that affect the physical process of molting.

Normal molting is disrupted by *nhr-23(RNAi)*, most likely due to a reduction or loss of function of *nhr-23* in the epidermal cells. Both CHR3 protein and *nhr-23* reporter genes are specifically detected in epidermal cells during late embryogenesis and *nhr-23* reporter genes continue to be expressed in epidermal cells throughout larval development.

What is the role of CHR3 in the epidermis? CHR3 is not required for cells to adopt an epidermal cell fate; mutant embryos following *nhr-23* inhibition are initially covered by an epidermis and cuticle and the epidermal cell number appears normal at hatching based on LIN-26 antibody staining. Two of many possible functions for CHR3 are (1) to regulate the onset of molting, by perhaps activating a collagenolytic pathway or (2) to regulate collagen gene expression (positively or negatively) at one or more stages of development. Defects in either one of these functions might result in the phenotypes that we observe.

The inability of *nhr-23(RNAi)* animals to completely shed their old cuticles suggests that complete lysis of the matrix that attaches cuticle to the epidermal cells is a primary defect of loss of CHR3 function (see Fig. 7). The blistering of some larval cuticles in response to ectopic and/or the overproduction of CHR3 is consistent with a role of CHR3 in cuticle detachment. Further support for this model is that the close homolog of CHR3 in *Drosophila* has a role in ecdysis. However, with DHR3, the involvement appear to be indirect and the result of inhibition of some genes and the activation of other genes. Hence the role of CHR3 in *C. elegans* molting could also be indirect. This model is difficult to assess in *C. elegans* currently, as little is known about the genes and gene products responsible for breaking the attachment of the cuticle in this animal. However, the phenotype of *nhr-23(RNAi)* animals should serve as an entry point into the study of the molecular and genetic mechanisms of molting.

There are, of course, numerous other possible mechanisms of CHR3 action. For example, the Dumpy phenotype resulting from the inhibition of *nhr-23* suggests that improper ecdysis may be linked to a disruption in collagen synthesis. There are 30 Dumpy mutations that have been previously defined genetically (although none map to the *nhr-23 locus*). Several of these Dumpy genes have been cloned and shown to encode mutant collagen genes (eg. *dpy 10*, *dpy-13*) (Levy et al., 1993; von Mende et al., 1988). If collagen genes were a direct target of CHR3 action, their misexpression after *nhr-23* inhibition could alter the normally well-orchestrated assembly of cuticle and result in a Dumpy phenotype. Only a few of the estimated 150 collagen genes of *C. elegans* have been analyzed for sequences required for proper expression. Promoter analysis and comparisons of flanking genomic sequences of several cuticle collagen genes has failed to identify elements required for expression that include a HRE capable of CHR3 binding (Cox et al., 1989; Liu et al., 1995; Gilleard et al, 1997). A role of CHR3 in the regulation of collagen gene expression, if any, will only become clear after sequence elements regulating collagen genes are better defined, specific candidate collagen gene targets are identified and analyzed, and the function of new cuticle in the detachment of the old cuticle from the epidermis is understood in greater detail.

Evolutionary considerations

The relatively simple body plan, small genome size and genetics of *C. elegans* should allow one to identify functions and target genes for orphan receptors that otherwise would be hard to discern in more complex developmental systems. Although different in specific detail, the more generalized role of these receptors during development would presumably be conserved among the invertebrate and vertebrate homologs. In

this context, it is interesting that CHR3 is important for molting in the nematode given the role of its closest known homologs in *Drosophila* (DHR3) and *Manduca* (MHR3). Both DHR3 and MHR3 are ecdysone-inducible factors (Riddiford, 1993; Palli et al., 1992) and DHR3 has been shown to play a critical role in *Drosophila* pupation (Thummel, 1995; Lam et al., 1997; White et al., 1997). DHR3 does not bind ecdysone directly, but rather serves as one of many factors in an ecdysone-induced cascade of transcriptional activation and repression. The close homology of CHR3 to DHR3, coupled with the facts that DHR3 is integral to metamorphosis and CHR3 is involved in molting, suggests that these two phenomena may be more closely related than would be immediately apparent. The absence of detectable ecdysone in *C. elegans* leaves open the question of what factor(s) are serving to trigger CHR3 function. It will be interesting to learn more about the genes acting upstream and downstream of *nhr-23* to see how exactly CHR3 has been integrated into developmental programs in the nematode. The detrimental effects of nematodes in many agricultural crops, coupled with the importance of CHR3 in normal nematode development, suggest that inhibitors of CHR3 function could have considerable economic value.

Finally, although the nematoda have been considered a primitive phylum, which branched off from the main line of platyhelminths early on (see Willmer, 1990 for an excellent summary of possible phylogenies), the existence of a large number of genes for nuclear receptors suggests that the nematodes have a rather complicated developmental program. The presence of HOX genes in *C. elegans* is in line with this contention (Wang et al., 1993). The small size and apparently simplistic body belie a complex cell integration system.

We thank Adam Antebi for help in analyzing mutant phenotypes, Brian Harfe for in situ hybridization, George Poy for the synthesis of oligos, Andy Fire for vectors, Ross Francis and Robert Waterston for the MH27 antibody, Michele Labouesse for the LIN-26 antibody and Ann Sluder for *nhr* gene info. Thanks also to Robert Littlejohn for technical assistance during the course of this work and to Adam Antebi, Eric Baehrecke and Sheryl Sato for comments on the manuscript.

REFERENCES

Ambros, V. (1997). Heterochronic Genes. In *C. elegans II*, (ed. D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess), pp. 501-518. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Ambros, V. and Horvitz, H. R. (1984). Heterochronic mutants of the nematode *Caenorhabditis elegans*. *Science* **226**, 409-416.

Azzaria, M., Goszczynski, B., Chung, M.A., Kalb, J.M. and McGhee, J.D. (1996). A *fork head*/HNF-3 homolog expressed in the pharynx and intestine of the *Caenorhabditis elegans* embryo. *Dev. Biol.* **178**, 289-303.

Becker-André, M., Andre, E. and DeLamarter, J. F. (1993). Identification of nuclear receptor mRNAs by RT-PCR amplification of conserved zinc-finger motif sequences. *Biochem. Biophys. Res. Commun.* **194**, 1371-1379.

Blumenthal, T. and Steward, K. (1997). RNA Processing and Gene Structure. In *C. elegans II*, (ed. D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess), pp. 117-146. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans. Genetics* **77**, 71-94.

Carlberg, C., Hooft, van, Huijsduijnen, R, Staple, J. K., DeLamarter, J. F. and Becker, A. M. (1994). RZRs, a new family of retinoid-related orphan receptors that function as both monomers and homodimers. *Mol. Endocrinol.* **8**, 757-770.

Carney, G. E., Wade, A. A., Sapra, R., Goldstein, E. S. and Bender, M. (1997). DHR3, an ecdysone-inducible early-late gene encoding a *Drosophila* nuclear receptor, is required for embryogenesis. *Proc. Natl. Acad. Sci. USA* **94**, 12024-12029.

Chawla, A. and Lazar, M. A. (1993). Induction of Rev-ErbA alpha, an orphan receptor encoded on the opposite strand of the alpha-thyroid hormone receptor gene, during adipocyte differentiation. *J. Biol. Chem.* **268**, 16265-16269.

Cox, G. N., Kramer, J. M. and Hirsh, D. (1984). Number and organization of collagen genes in *Caenorhabditis elegans*. *Mol. Cell. Biol.* **4**, 2389-2395.

Cox, G. N., Fields, C., Kramer, J. M., Rosenzweig, B. and Hirsh, D. (1989). Sequence comparison of developmentally regulated collagen genes of *Caenorhabditis elegans*. *Gene* **76**, 331-344.

Downes, M., Carozzi, A. J. and Muscat, G. E. (1995). Constitutive expression of the orphan receptor, Rev-erbA alpha, inhibits muscle differentiation and abrogates the expression of the MyoD gene family. *Mol. Endocrinol.* **9**, 1666-1678.

Enmark, E. and Gustafsson, J. A. (1996). Orphan nuclear receptors--the first eight years. *Mol. Endocrinol.* **10**, 1293-1307.

Finney, M. and Ruvkun, G. (1990). The *unc-86* gene product couples cell lineage and cell identity in *C. elegans*. *Cell* **63**, 895-905.

Fire, A., Harrison, S. W. and Dixon, D. (1990). A modular set of lacZ fusion vectors for studying gene expression in *Caenorhabditis elegans*. *Gene* **93**, 189-198.

Fire, A., Xu, S., Montgomery, M.K., Driver, S.A. and Mello, C.C. (1998). Potent and specific genetic interference by double stranded RNA in *C. elegans*. *Nature* **391**, 806-811.

Forman, B. M., Chen, J., Blumberg, B., Kliewer, S. A., Henshaw, R., Ong, E. S. and Evans, R. M. (1994). Cross-talk among ROR alpha 1 and the Rev-erb family of orphan nuclear receptors. *Mol. Endocrinol.* **8**, 1253-1261.

Francis, G. R. and Waterston, R. H. (1985). Muscle organization in *Caenorhabditis elegans*: localization of proteins implicated in thin filament attachment and I-band organization. *J. Cell Biol.* **101**, 1532-1549.

Freedman, L. P. (1992). Anatomy of the steroid receptor zinc finger region. *Endocr. Rev.* **13**, 129-145.

Giguère, V., Tini, M., Flock, G., Ong, E., Evans, R. M. and Otulakowski, G. (1994). Isoform-specific amino-terminal domains dictate DNA-binding properties of ROR alpha, a novel family of orphan hormone nuclear receptors. *Genes Dev.* **8**, 538-553.

Giguère, V., McBroom, L. D. and Flock, G. (1995). Determinants of target gene specificity for ROR alpha 1: monomeric DNA binding by an orphan nuclear receptor. *Mol. Cell. Biol.* **15**, 2517-2526.

Gilleard, J. S., Barry, J. D. and Johnstone, I. L. (1997). cis- Regulatory requirements for hypodermal cell-specific expression of the *Caenorhabditis elegans* cuticle collagen gene *dpy-7*. *Mol. Cell. Biol.* **17**, 2301-2311.

Greiner, E. F., Kirfel, J., Greschik, H., Dörflinger, U., Becker, P., Mercep, A. and Schüle, R. (1996). Functional analysis of retinoid Z receptor β, a brain-specific nuclear orphan receptor. *Proc. Natl Acad. Sci. USA* **93**, 10105-10110.

Guo, S. and Kemphues, K. J. (1995). par-1, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* **81**, 611-620.

Guo, S. and Kemphues, K. J. (1996). A non-muscle myosin required for embryonic polarity in *Caenorhabditis elegans*. *Nature* **382**, 455-458.

Hamilton, B. A., Frankel, W. N., Kerrebrock, A. W., Hawkins, T. L., FitzHugh, W., Kusumi, K., Russell, L. B., Mueller, K. L., van, B. V., Birren, B. W., Kruglyak, L. and Lander, E. S. (1996). Disruption of the nuclear hormone receptor RORalpha in staggerer mice. *Nature* **379**, 736-739.

Harfe, B. D. and Fire, A. (1998). Muscle and nerve-specific regulation of a novel NK-2 class homeodomain factor in C. elegans. *Development* **125**, 421-429.

Herskowitz, I. (1987). Functional inactivation of genes by dominant negative mutations. *Nature* **329**, 219-222.

Horner, M. A., Chen, T. and Thummel, C. S. (1995). Ecdysteroid regulation and DNA binding properties of *Drosophila* nuclear hormone receptor superfamily members. *Dev. Biol.* **168**, 490-502.

Johnstone, I. L. (1994). The cuticle of the nematode *Caenorhabditis elegans*: a complex collagen structure. *BioEssays* **16**, 171-178.

Kato, S., Sasaki, H., Suzawa, M., Masushige, S., Tora, L., Chambon, P. and Gronemeyer, H. (1995). Widely spaced, directly repeated PuGGTCA elements act as promiscuous enhancers for different classes of nuclear receptors. *Mol. Cell. Biol.* **15**, 5858-5867.

Koelle, M. R., Talbot, W. S., Segraves, W. A., Bender, M. T., Cherbas, P.

and Hogness, D. S. (1991). The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67**, 59-77.

Koelle, M. R., Segraves, W. A. and Hogness, D. S. (1992). DHR3: a *Drosophila* steroid receptor homolog. *Proc. Natl. Acad. Sci. USA* **89**, 6167-6171.

Kostrouch, Z., Kostrouchova, M. and Rall, J. E. (1995). Steroid/thyroid hormone receptor genes in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **92**, 156-159.

Kramer, J. M. (1994). Structures and functions of collagens in *Caenorhabditis elegans*. *FASEB J.* **8**, 329-336.

Krause, M., Harrison, S. W., Xu, S. Q., Chen, L. and Fire, A. (1994). Elements regulating cell- and stage-specific expression of the *C. elegans* MyoD family homolog hlh-1. *Dev. Biol.* **166**, 133-148.

Krause, M. and Hirsh, D. (1987). A trans-spliced leader sequence on actin mRNA in *C. elegans*. *Cell* **49**, 753-761.

Krause, M., Park, M., Zhang, J. M., Yuan, J., Harfe, B., Xu, S. Q., Greenwald, I., Cole, M., Paterson, B. and Fire, A. (1997). A *C. elegans* E/Daughterless bHLH protein marks neuronal but not striated muscle development. *Development* **124**, 2179-2189.

Lam, G. T., Jiang, C. and Thummel, C. S. (1997). Coordination of larval and prepupal gene expression by the DHR3 orphan receptor during *Drosophila* metamorphosis. *Development* **124**, 1757-1769.

Laudet, V. and Adelmant, G. (1995). Nuclear receptors. Lonesome orphans. *Curr. Biol.* **5**, 124-127.

Laudet, V., Hanni, C., Coll, J., Catzeflis, F. and Stehelin, D. (1992). Evolution of the nuclear receptor gene superfamily. *EMBO J.* **11**, 1003-1013.

Levy, A. D., Yang, J. and Kramer, J. M. (1993). Molecular and genetic analyses of the *Caenorhabditis elegans dpy-2* and *dpy-10* collagen genes: a variety of molecular alterations affect organismal morphology. *Mol. Biol. Cell.* **4**, 803-817.

Lin, R., Thompson, S. and Priess, J. R. (1995). *pop-1* encodes an HMG box protein required for the specification of a mesoderm precursor in early *C. elegans* embryos. *Cell* **83**, 599-609.

Liu, Z., Kirch, S. and Ambros, V. (1995). The *Caenorhabditis elegans* heterochronic gene pathway controls stage-specific transcription of collagen genes. *Development* **121**, 2471-2478.

Mangelsdorf, D. J. and Evans, R. M. (1995). The RXR heterodimers and orphan receptors. *Cell* **83**, 841-850.

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schultz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R.M. (1995). The nuclear receptor superfamily: the second decade. *Cell* **83**, 835-839.

Mello, C. and Fire, A. (1995). DNA Transformation. In *Caenorhabditis elegans: Modern Bilogical Analysis of an Organism*, (ed. H. F. Epstein and D. C. Shakes), pp. 452-482. San Diego: Academic Press.

Miller, D. M. and Shakes, D. C. (1995). Immunofluorescence Microscopy. In *Caenorhabditis elegans: Modern Bilogical Analysis of an Organism*, (ed. H. F. Epstein and D. C. Shakes) pp. 365-394. San Diego: Academic Press.

O'Malley, B. W. and Conneely, O. M. (1992). Orphan receptors: in search of a unifying hypothesis for activation. *Mol. Endocrinol.* **6**, 1359-1361.

Palli, S. R., Hiruma, K. and Riddiford, L. M. (1992). An ecdysteroid-inducible *Manduca* gene similar to the *Drosophila* DHR3 gene, a member of the steroid hormone receptor superfamily. *Dev. Biol.* **150**, 306-318.

Riddiford, L. M. (1993). Hormones and *Drosophila* development. In *The Development of Drosphila melanogaster*, (ed. M. Bates and A. Martinez Arias), pp. 899-939. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Rocheleau, C. E., Downs, W. D., Lin, R., Wittman, C., Bei, Y., Cha, Y-H., Ali, M., Priess, J. R. and Mello, C. C. (1997). Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. *Cell* **90**, 707-716.

Schräder, M., Danielsson, C., Wiesenberg, I. and Carlberg, C. (1996). Identification of natural monomeric response elements fo the nuclear receptor RZR/ROR. *J. Biol. Chem.* **271**, 19732-19736.

Sengupta, P., Colbert, H. A. and Bargmann, C. I. (1994). The *C. elegans* gene *odr-7* encodes an olfactory-specific member of the nuclear receptor superfamily. *Cell* **79**, 971-980.

Seydoux, G. and Fire, A. (1995). Whole-mount in situ hybridization for the detection of RNA in *Caenorhabditis elegans* embryos. In *Caenorhabditis elegans: Modern Bilogical Analysis of an Organism*, (ed. H. F. Epstein and D. C. Shakes), pp. 323-338. San Diego: Academic Press.

Singh, R. N. and Sulston, J. E. (1978). Some observations on moulting in *Caenorhabditis elegans*. *Nematologica* **24**, 63-71.

Sluder, A. E., Lindblom, T. and Ruvkun, G. (1997). The *Caenorhabditis elegans* orphan nuclear hormone receptor gene *nhr-2* functions in early embryonic development. *Dev. Biol.* **184**, 303-319.

Stringham, E. G., Dixon, D. K., Jones, D. and Candido, E. P. (1992). Temporal and spatial expression patterns of the small heat shock (*hsp16*) genes in transgenic *Caenorhabditis elegans*. *Mol. Biol. Cell* **3**, 221-233.

Sulston, J. E., Schierenberg, E., White, J. G. and Thomson, J. N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100**, 64-119.

Tabara, H., Motohashi, T. and Kohara, Y. (1996). A multi-well version of in situ hybridization on whole mount embryos of *Caenorhabditis elegans*. *Nucleic Acids Res.* **24**, 2119-2124.

Thummel, C. S. (1995). From embryogenesis to metamorphosis: the regulation and function of *Drosophila* nuclear receptor superfamily members. *Cell* **83**, 871-877.

Thummel, C. S. (1996). Files on steroids--*Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet.* **12**, 306-310.

Tsai, M. J. and O'Malley, B. W. (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* **63**, 451-486.

von Mende, N., Bird, D. M., Albert, P. S. and Riddle, D. L. (1988). *dpy-13*: a nematode collagen gene that affects body shape. *Cell* **55**, 567-76.

Waterston, R., Martin, C., Craxton, M., Hyunh, C., Coulson, A., Hillier, L., Durbin, R., Green, P., Shownkeen, R., Halloran, N., Metzstein, M., Hawkins, T., Wilson, R., Berks, M., Du, Z., Thomas, K, Thierry-Mieg, J. and Sulston, J. (1992). A survey of expressed genes in *Caenorhabditis elegans*. *Nature Genetics* **1**, 114-123.

Waterston, R. and Sulston, J. (1995). The genome of *Caenorhabditis elegans*. *Proc. Nat. Acad. Sci. USA* **92**, 10836-10840.

Wang, B. B., Muller, I. M., Austin, J., Robinson, N. T., Chisholm, A. and Kenyon, C. (1993). A homeotic gene cluster patterns the anteroposterior body axis of *C. elegans*. *Cell* **74**, 29-42.

White, K. P., Hurban, P., Watanabe, T. and Hogness, D. S. (1997). Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. *Science* **276**, 114-117.

Williams, M. E., Malik, A. N. and Hardin, J. (1997). An actin-mediated two-step mechanism is required for ventral enclosure of the *C. elegans* hypodermis. *Development* **124**, 2889-2901.

Willmer, P. (1990). *Invertebrate Relationships; Patterns in animal evolution*. Cambridge: Cambridge University Press.

Yao, T. P., Segraves, W. A., Oro, A. E., McKeown, M. and Evans, R. M. (1992). *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell* **71**, 63-72.

Yao, T. P., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J. D., McKeown, M., Cherbas, P. and Evans, R. M. (1993). Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. *Nature* **366**, 476-479.

Zwaal, R. R., Broeks, A., van, M. J., Groenen, J. T. and Plasterk, R. H. (1993). Target-selected gene inactivation in *Caenorhabditis elegans* by using a frozen transposon insertion mutant bank. *Proc. Natl. Acad. Sci. USA* **90**, 7431-7435.