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Expression and function of conserved nuclear receptor genes in *Caenorhabditis elegans*☆

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Abstract

The *Caenorhabditis elegans* genome encodes 284 nuclear receptor (NR) genes. Among these 284 NR genes are 15 genes conserved among the Metazoa. Here, we analyze the expression and function of eight heretofore uncharacterized conserved *C. elegans* NR genes. Reporter gene analysis demonstrates that these genes have distinct expression patterns and that a majority of the *C. elegans* cell types express a conserved NR gene. RNA interference with NR gene function resulted in visible phenotypes for three of the genes, revealing functions in various processes during postembryonic development. Five of the conserved NR genes are orthologs of NR genes that function during molting and metamorphosis in insects. Functional studies confirm a role for most of these 'ecdysone cascade' NR orthologs during the continuous growth and dauer molts. Transcript levels for these genes fluctuate in a reiterated pattern during the molting cycles, reminiscent of the expression hierarchy observed in the insect ecdysone response. Together, these analyses provide a foundation for further dissecting the role of NRs in nematode development as well as for evaluating conservation of NR functions among the Metazoa.

Keywords: Nuclear receptor; *Caenorhabditis elegans*; *nhr*; Molting; Dauer

Introduction

Nuclear receptors (NRs) are a diverse class of transcription factors, members of which mediate hormonal signaling processes in vertebrates and insects (Manglesdorf et al., 1995). The hormone-responsive NRs are ligand-regulated, and the mechanisms by which these NRs respond to endocrine hormones and activate or repress the transcription of target genes have been well characterized (Beato et al., 1995; Gronemeyer and Laudet, 1995; McKenna et al., 1999; Rastinejad, 1998). The remaining NRs are 'orphans' that lack known cognate ligands. The spectrum of known NR functions now extends beyond direct transduction of endocrine signals to include responses to a variety of signaling molecules (including xenobiotics), participation in multiple signal transduction pathways, and regulation of diverse physiological and developmental processes (Giguere, 1999).

All NRs share a common structure with specific, conserved domains that function in DNA binding, ligand binding, and transcriptional activation (Gronemeyer and Laudet, 1995). Most of the NRs identified in vertebrates and insects belong to six major subfamilies that are evolutionarily conserved throughout the Metazoa (Laudet, 1997; Nuclear Receptors Committee, 1999), although the extent to which conserved NRs regulate common processes across the Metazoa remains to be determined. In nematodes, the NR superfamily has undergone a dramatic expansion and diversification not observed in other phyla (Sluder et al., 1999). The completed *Caenorhabditis elegans* genome sequence contains 284 confirmed or predicted NR genes, over 5-fold more than the number found in the human or *Drosophila melanogaster* genomes (Maglich et al., 2001). The functional and evolutionary consequences of this expansion and diversification are presently unknown. Within the expanded NR superfamily encoded in the *C. elegans* genome are 15 members of the broadly conserved metazoan NR subfamilies

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(Maglich et al., 2001; Sluder and Maina, 2001; Sluder et al., 1999). Only the NR3 subfamily, which contains the vertebrate steroid receptors, is not represented in *C. elegans*. The utility of *C. elegans* as a model system provides an opportunity to study basic conserved and diverged aspects of NR functions, both biological and biochemical, within the context of a developing multicellular organism.

Despite the extensive knowledge of *C. elegans* biology, little is known about the biological functions of NRs in nematodes. Of the 284 *C. elegans* genes, only five have emerged in forward genetic screens for mutations that disrupt specific developmental processes: *daf-12* (dauer formation), *fax-1* (neuronal development), *sex-1* (sex determination), *unc-55* (neuronal development), and *odr-7* (neuronal development) (Antebi et al., 2000; Carmi et al., 1998; Much et al., 2000; Sengupta et al., 1994; Zhou and Walthall, 1998). Interestingly, four of these genes (*daf-12*, *fax-1*, *unc-55*, and *sex-1*) belong to the evolutionarily conserved subfamilies mentioned above. Reverse genetic studies have described functions for three other conserved NR genes in *C. elegans*: *nhr-23* (molting), *nhr-25* (epidermal development or organogenesis), and *nhr-8* (toxin resistance) (Gissendanner and Sluder, 2000; Kostrouchova et al., 1998; Lindblom et al., 2001). For the other eight conserved NR genes, expression patterns and biological functions have not previously been described.

Five of the *C. elegans* conserved NR genes—*nhr-6*, *nhr-23*, *nhr-25*, *nhr-41*, and *nhr-85*—are orthologs of NR genes that function during molting and metamorphosis in insects (*DHR38*, *DHR3*, *Ftz-F1*, *DHR78*, and *E75/E78*, respectively; see Table 1) (Thummel, 1995). The insect orthologs of *nhr-23*, *nhr-25*, and *nhr-85* are components of the cascade of genes activated in response to the 20-hydroxyecdysone endocrine signal that regulates insect molting and metamorphosis (Richards, 1997). A role for *nhr-23* in the *C. elegans* molting process has been established, and initial characterization has suggested a molting role for *nhr-25* as well (Gissendanner and Sluder, 2000; Kostrouchova et al., 2001). It is presently unknown whether these *C. elegans* 'ecdysone cascade' NRs control nematode molting via a regulatory cascade similar to that of their insect orthologs.

Here we present an initial functional analysis of the eight heretofore uncharacterized *C. elegans* conserved NR genes. We verify that these are indeed expressed genes and, utilizing *GFP* reporter gene analysis, we describe the spatial expression of all eight genes. Using double-stranded RNA-mediated interference with gene function (RNAi), we identify putative functions for *nhr-6*, *nhr-67*, and *nhr-85* under normal growth conditions. We also perform a comprehensive expression study of the *C. elegans* 'ecdysone cascade' NRs during postembryonic development, demonstrating that three of these genes are expressed in temporal patterns reminiscent of the expression hierarchies of NR genes during insect metamorphosis. In addition, we establish a direct role for *nhr-25* during molting and provide evidence that *nhr-23*, *nhr-25*, *nhr-41*, and *nhr-85* are important for the specific larval transition that occurs during dauer formation.

Table 1
Groupings of conserved *C. elegans* NRs[a]

C. elegans NRs	*Drosophila* NRs	DBD identity[b] (%)	Human NRs	DBD identity[b] (%)
NHR-85 (NR1)	E75 (NR1D3)	78	Reverbs (NR1D)[c]	72
SEX-1 (NR1G1)	E78 (NR1E1)	79		74
NHR-23 (NR1F4)	DHR3 (NR1F4)	91	RORs (NR1F)[c]	76
DAF-12	HR96	69	PXR, CAR,	58, 50, 53
NHR-8	(NR1J1)	59	VDR (NR1I)	52, 53, 51
NHR-48 (NR1J)		66		56, 50, 49
NHR-64	HNF4	72	HNF4s	74
NHR-69 (NR2A)	(NR2A4)	71	(NR2A)[c]	68
NHR-41 (NR2D1)	HR78 (NR2D1)	76	TR2, TR4 (NR2C)	75, 78
NHR-67 (NR2E2)	TLL (NR2E2)	79	TLX (NR2E1)	70
FAX-1 (NR2E5)	DmFAX-1 (NR2E5)	90	PNR (NR2E3)	86
UNC-55 (NR2F7)	Seven-up (NR2F3)	76	COUP-TFs (NR2F)[c]	78
NHR-6 (NR4A4)	HR38 (NR4A4)	74	NGFI-Bs (NR4A)[c]	76
NHR-25 (NR5A3)	FTZ-F1 (NR5A3)	81	SF1, LRH1 (NR5A)	76, 78
NHR-91 (NR6A2)	HR4 (NR6A)	68	GCNF (NR6A1)	60

[a] Nuclear Receptors Committee (1999). The NR groupings listed here are supported by the high degree of DBD similarity (Sluder et al., 1999; Sluder and Maina, 2001). *C. elegans* NR orthology assignments are not always supported by the strict criteria of the Nuclear Receptors Committee due to the rapid evolutionary rate of *C. elegans* NR LBDs (no *C. elegans* LBD exhibits >30% amino acid sequence identity to the apparent insect and mammalian orthologs). Genes included in this study are indicated in bold.
[b] Percentage of amino acid identity for the sequences spanning from the first Cys residue through the C-terminal Gly-Met dipeptide of the DBD motif.

Materials and methods

cDNA cloning and characterization

cDNA sequences for *nhr-6*, *nhr-64*, *nhr-67*, *nhr-69*, *nhr-85*, and *nhr-91* have been described elsewhere (Kostrouch et al., 1995; Sluder et al., 1999 for *nhr-6*; M. Robinson-Rechavi, C. V. Maina, C. R. Gissendanner, V. Laudet, and A. E. Sluder, submitted for publication, for the others). In the current study, we identified a previously unknown alternately spliced form of *nhr-91*, *nhr-91β*, detected by reverse transcription (RT)-PCR using a nested set of PCR primers directed to the *nhr-91* 3′ UTR and a primer corresponding to the SL1 *trans*-spliced

leader sequence (Krause and Hirsh, 1987). Oligonucleotide primer sequences and specific PCR conditions are detailed in the online Supplementary Materials and Methods. Briefly, first strand cDNA was prepared from 2 μg of mixed stage total RNA primed with oligo-dT using the ProtoScript™ First Strand cDNA Synthesis Kit (New England Biolabs). This reaction (1 μl) was used in the first (primary) round of PCR amplification. A secondary round of PCR amplification utilized 1/500 of the primary reaction as template.

nhr-41 was initially identified as a predicted gene in the *C. elegans* genome (Y77E11A.5) (Sluder and Maina, 2001) but has not been recovered from existing cDNA libraries. A putative *nhr-41* cDNA was assembled by generating overlapping RT-PCR-amplified cDNA fragments and 3′ RACE (Gissendanner, 2001). To isolate full-length cDNAs for *nhr-41*, RT-PCR was performed as described above using SL1 and a nested set of gene specific primers. A nested set of 3′ UTR primers failed to yield *nhr-41* RT-PCR products, likely due to the small size and A/T richness of the *nhr-41* 3′ UTR. Instead, nearly full-length cDNAs were isolated using a nested primer set in the coding region of the last exon, as defined by 3′ RACE analysis.

New sequences generated during this work have been deposited in GenBank with accession numbers AY349014–AY349018.

GFP reporter gene analysis

Reporter constructs fusing green fluorescent protein (GFP) coding sequences to *nhr-41α* and *β*, *nhr-48*, *nhr-64*, *nhr-67*, *nhr-69*, *nhr-85*, and *nhr-91* (Fig. 1) were generated using a two-step PCR amplification protocol (Cassata et al., 1998). Templates for the amplification of genomic DNA were either 15 ng of N2 genomic DNA (*nhr-41α* and *β*, *nhr-48*, *nhr-67*, *nhr-69*, *nhr-85*, *nhr-91*) or 500 pg of linearized cosmid (*nhr-64*, cosmid C45E1). Primers were designed to incorporate approximately 3–6 kb of sequence upstream of the predicted first ATG of each gene. The Expand™ Long Template PCR System (Roche) was utilized for the PCRs according to the manufacturer's protocol. Oligonucleotide primer sequences and specific PCR conditions are detailed in the online Supplementary Materials and Methods. To avoid producing dominant-negative fusion proteins, the constructs were designed to omit or truncate the coding sequences for the NR DNA-binding domain (DBD).

Given the large gene size of *nhr-6* (Sluder et al., 1999), the *nhr-6*::*GFP* reporter was generated by cloning PCR-amplified *GFP* coding sequences (from pPD95.67) into a deleted variant of a *nhr-6* genomic subclone. The *nhr-6* genomic subclone, pLH1, was generated by removing approximately 17 kb *Sac II* fragment from cosmid C48D5, followed by religation. A deleted variant of pLH1, pLH1Δ, was then generated by removing approximately 7 kb of *nhr-6* genomic sequence from pLH1 by digestion with *Nhe I*, followed by religation. pLH1Δ contains 5.2 kb of sequence upstream of exon 1, and the first five exons. The *GFP* PCR fragment was cloned into the pLH1Δ *Nhe I* site, which resides in the fifth intron of *nhr-6*.

With the exception of *nhr-41α*::*GFP* and *nhr-6*::*GFP*, transgenic lines of *C. elegans* were obtained following injection of 20 μg/ml of reporter construct and 100 μg/ml of pRF4, which contains the dominant visible marker gene *rol-6*(*su1006*) (Mello and Fire, 1995). *nhr-41α*::*GFP* was injected at 5 μg/ml and *nhr-6*::*GFP* was injected at 10 μg/ml. At least two independent transgenic lines were evaluated for each reporter construct, except for *nhr-6*::*GFP* (one line). Transgenic animals were examined at all stages to determine the spatial expression patterns. When multiple lines were examined for a given reporter construct, similar expression patterns were observed in the various lines.

dsRNA interference with gene function (RNAi)

dsRNA was generated by annealing sense and antisense RNA strands synthesized using T3 and T7 polymerases (Ambion MegaScript) from templates with opposing T3 and T7 promoters or by synthesizing dsRNA directly using the HiScribe RNAi Kit (New England Biolabs) on templates containing two opposing T7 promoters. With the exception of *nhr-85*, templates consisted of ligand-binding domain (LBD) coding sequences only, omitting the highly conserved DBD motif. The dsRNAs used in the RNAi experiments exhibit no extended sequence identity with nontarget coding sequences (no blocks of sequence identity were greater than 22 bp), and are thus unlikely to interfere with the function of nontarget genes.

For microinjection experiments, approximately 1–2 mg/ml of dsRNA was injected into both syncytial gonads of L4 or young adult hermaphrodites. RNAi effects on growth and development of the progeny of injected hermaphrodites were assessed and quantitated as described by Gissendanner and Sluder (2000). All RNAi experiments were performed at least twice. For *nhr-67*(*RNAi*), early larvae had a tendency to move out of the bacterial lawn and become lost from the plate, resulting in the high "missing" class for *nhr-67* (Table 2).

For RNAi by feeding experiments, pPD129.36/*nhr* constructs were transformed into HT115 *E. coli* (Timmons et al., 2001). Transformed HT115 *E. coli* was induced to synthesize dsRNA as described (Kamath et al., 2000; Timmons et al., 2001). Developmentally synchronized L1 larvae, derived by hatching embryos in the absence of food (Lewis and Fleming, 1995), were inoculated onto NGM plates with induced HT115. Phenotypes were scored after culturing for 72 h at 20°C. The data presented represent the combined numbers from multiple experiments. For the *nhr-85*(*RNAi*) experiments, phenotypes were scored after culturing for 88–96 h at 20°C. Worms were picked to slides in M9 buffer containing 0.1% sodium azide and scored under high magnification. Due to the variable and incomplete penetrance of the *nhr*-85 RNAi phenotype, a plate coding system was used so that the phenotypes were scored blind. For *daf-7*(*e1372*) RNAi experiments, larvae were cultured at

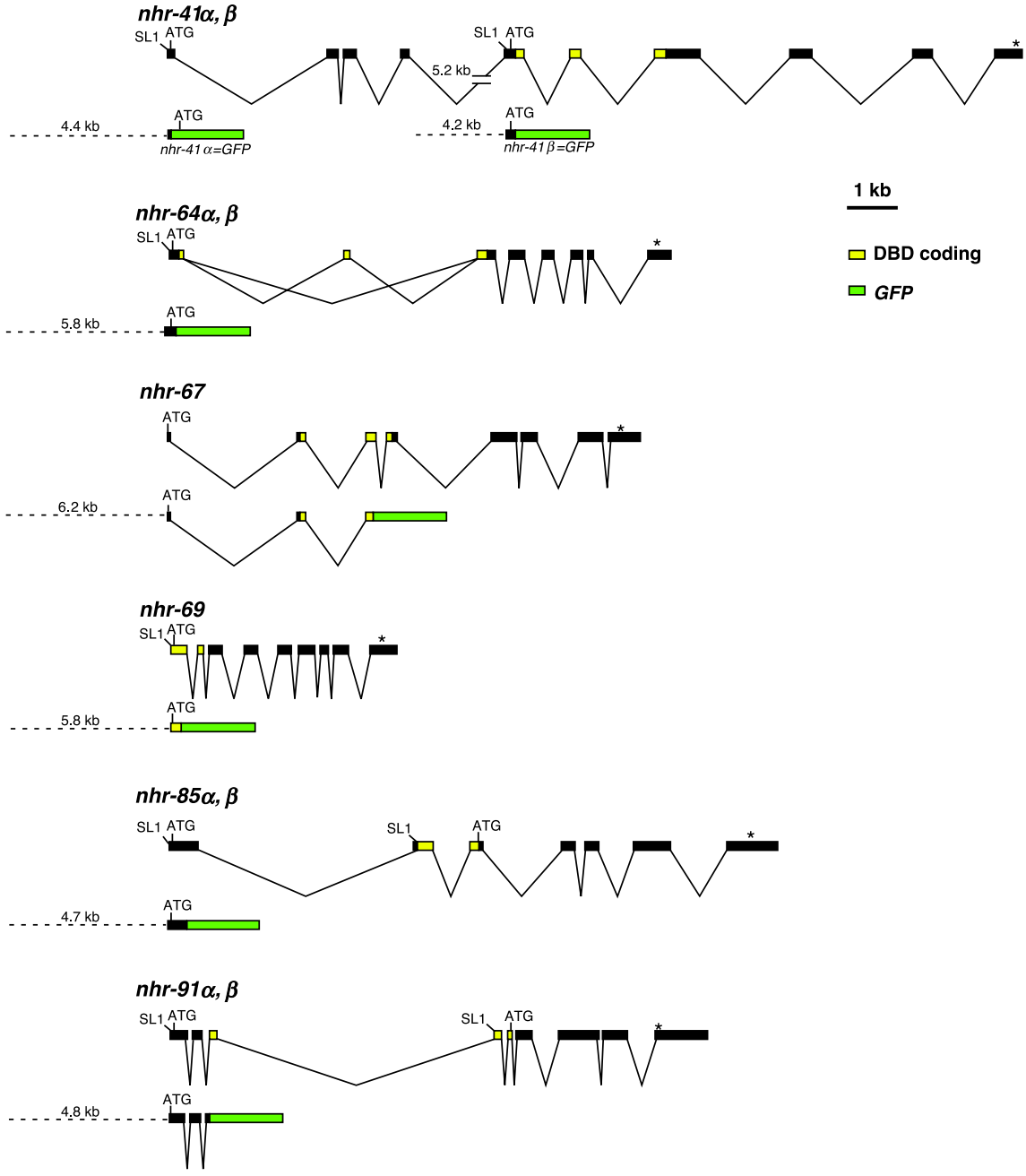


Fig. 1. cDNA structures for *nhr-41*, *nhr-64*, *nhr-67*, *nhr-69*, *nhr-85*, and *nhr-91*. *nhr-41*, *nhr-64*, *nhr-85*, and *nhr-91* have two mRNA isoforms. The alternate isoforms for *nhr-41*, *nhr-85*, and *nhr-91* are produced by the *trans*-splicing of SL1 to different exons, as indicated. *nhr-64β* is produced by the alternate splicing of exon 1 to exon 3. The first ATG downstream of the SL1 splice site is indicated. The DBD coding sequences are indicated in yellow. Structures of the *GFP* reporter gene fusions are shown below each gene. For each reporter gene, the amount of genomic DNA upstream of the first exon is indicated on the dashed lines (not drawn to scale). For *nhr-6* and *nhr-48* (cDNA structures not shown), *GFP* coding sequences in the reporter transgenes were fused at exons 6 and 4, respectively. *nhr-6*::*GFP* has 5.2 kb of genomic DNA upstream of the first exon and *nhr-48*::*GFP* has 3.3 kb of upstream genomic DNA.

25°C for 96 h before scoring. To test for SDS resistance, a portion of the *nhr*(*RNAi*); *daf-7*(*e1372*) animals was incubated in 1% SDS for 30 min at room temperature, washed once with water, plated, and assessed for survival. Negative controls for these RNAi experiments utilized HT115 transformed with pPD128.110, which contains *GFP* coding sequence under a double T7 promoter, or the empty double T7 vector pPD129.36.

RNAi by soaking was performed as described (Maeda et al., 2001). Developmentally synchronous L1 larvae were soaked in 5 μl dsRNA (2–4 mg/ml in soaking buffer, Maeda et al., 2001) for 24 h at 20°C. *GFP* dsRNA, derived from pPD128.110, was used as a negative RNAi control. Larvae were then cultured with OP50 *E. coli* on NGM plates for 72 h at 20°C and observed for phenotypes.

Table 2
Microinjection RNAi analysis of conserved NR genes

Experiment	#P0	#F1		Percentage of total brood arresting as			Adult F1 phenotypes
				Embryos	Larvae	Missing	
Mock[a]	6	732	Mean:	0.2 ± 0.4	0	0.8 ± 1.0	none
			Range:	0–0.8		0–2	
nhr-6 (1)	4	598	Mean:	0.7 ± 0.7	0.6 ± 1.1	2.0 ± 1.6	Ovulation defective (20/30 adults examined)
			Range:	0–1.8	0–2.8	0.7–4.2	
nhr-6 (2)	6	739	Mean:	1.8 ± 2.6	ND	ND	Ovulation defective (23/57 adults examined)
			Range:	0–5.5			
nhr-41	7	349	Mean:	6.5 ± 7.3	0.2 ± 0.5	6.8 ± 7.1	none
			Range:	1.4–21.4	0–1.4	0–14.5	
nhr-48	8	927	Mean:	3.1 ± 3.5	0.3 ± 0.5	2.6 ± 1.6	none
			Range:	0.5–3.6	0–1.4	0–5.4	
nhr-64	4	666	Mean:	2.6 ± 0.8	0.8 ± 0.8	2.6 ± 2.7	none
			Range:	1.5–3.2	0–2.0	0–5.2	
nhr-67 (1)	5	508	Mean:	2.6 ± 1.9	19.6 ± 8.0[b]	13.0 ± 6.8	Sickly larvae developed into Sma adults; most adults Pvul/Egl
			Range:	0–5.3	10.5–30.6	5.0–22.5	
nhr-67 (2)	4	432	Mean:	3.9 ± 4.3	34.1 ± 5.4[b]	9.4 ± 4.6	Sickly larvae developed into Sma adults; most adults Pvul/Egl (185/229 examined)
			Range:	2.0–10.6	27.9–43.4		
nhr-69	4	449	Mean:	0.6 ± 0.6	0.25 ± 0.5	0.25 ± 0.5	none
			Range:	0–1.2	0–1.0	0–1.0	
nhr-85 N2	3	236	Mean:	3.6 ± 5.6	0	2.1 ± 1.8	none
			Range:	0–10		0–3.3	
rrf-3(pk1426)[c]	3	178		ND[d]	ND[d]	ND[d]	26/178 Egl
nhr-91	7	887	Mean:	2.0 ± 2.8	0.2 ± 0.4	2.4 ± 1.2	none
			Range:	0–8.1	0–1.1	1.1–4.8	
nhr-64; nhr-69[e]	4	569	Mean:	0.7 ± 0.9	0	1.0 ± 1.6	none
			Range:	0–2.0		0–3.3	

Injections and assessment of lethality were performed as described in Materials and methods. All are injections into N2 except where noted. #P0 = number of hermaphrodites injected. #F1 = total number of progeny scored. The "missing" progeny class is as described by Gissendanner and Sluder, 2000. ND = not determined. Replicate injections were performed for all genes and results were similar among experiments. Replicate data are shown here for *nhr-6* and *nhr-67* because RNAi phenotypes were observed for these genes.

[a] Representative mock experiment is shown; multiple mock injected or uninjected control experiments were performed and results were similar to the data shown here.

[b] Larvae were sickly and slow growing but developed to the adult stage.

[c] Egl phenotypes were not observed in mock-injected *rrf-(pk1426)* animals (2 injected P0s; 160 total F1 progeny scored).

[d] Embryo and larval stages were observed for phenotypes but not quantitated. No phenotypes were observed.

[e] Injection mix contained equal volumes of *nhr-64* and *nhr-69* dsRNA.

N2 dauer RNAi was performed by hatching larvae in the presence of dauer-inducing pheromone and HT115 bearing an RNAi construct. In our experiments, >90% dauer formation was achieved when embryos were hatched in liquid containing dauer pheromone (prepared and titrated as described by Golden and Riddle, 1984). Embryos were hatched in 50 μl S-complete medium (Sulston and Hodgkin, 1988) containing 100 μg/ml carbenicillin, 1 mM IPTG, pheromone, and dsRNA-expressing HT115.

Semiquantitative RT-PCR analysis

Reverse transcription (RT)-PCR expression analysis was performed essentially as described (Jeon et al., 1999; Johnstone and Barry, 1996; Kostrouchova et al., 2001). Synchronized, developmentally arrested larvae were inoculated onto 9 cm NGM plates completely covered with *E. coli* OP50. Larvae (10,000) were inoculated on three plates for each of the time points up to the 27 h time point; 8000 larvae were inoculated onto two plates for each of the 30–39 h time points; 6000 larvae were inoculated onto two plates for each of the 42–51 h time points; and 4000 larvae were inoculated onto two plates for the 54 h time point. Animals (20–30) from the cultures were periodically observed under high magnification with DIC optics to ensure that growth was synchronous and to determine the times that molting occurred. Gonad growth and vulval development (for later larvae) were used to evaluate synchrony of the population. The larval molts were

noted by observing larvae in the molting lethargus (suppressed pharyngeal pumping and movement) and by identification of animals that had molted to the next stage. Animals were harvested from plates with water and total RNA was extracted using the TriPure Isolation Reagent (Roche), according to manufacturer's instructions. For each time point, 1 μg of total RNA from each time point extraction was used in a RT reaction performed using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs) with the provided Oligo dT (VN) primer. The RT reactions were diluted 1/10 with water and 1 μl of the diluted RT was used in the PCR reaction. Oligonucleotide primer sequences and specific PCR conditions are detailed in the online Supplementary Materials and Methods.

Linear-range PCR conditions were determined empirically by altering cycle numbers and input cDNA concentrations. For the final analyses, all PCRs were halted in the linear amplification range using the following cycle numbers: *lin-42* and *ama-1*, 28 cycles; *nhr-23*, *nhr-25α*, and *nhr-41α*, 31 cycles; *nhr-67*, 32 cycles; *nhr-6*, *nhr25β*, and *nhr-41β*, 33 cycles; *nhr-85α*, 34 cycles. PCR analysis was attempted for *nhr-85β* but the cDNA was very difficult to amplify and reproducible PCR conditions could not be established. All PCRs were performed in triplicate.

PCR products were detected by electrophoresis in 1% agarose gels stained with ethidium bromide. Gels were digitally exposed (below pixel saturation) and visualized using a Bio-Rad Gel Doc imager. Bands were quantitated using the Bio-Rad Quantity One software package. For each reaction set, the highest band value was set to one and the remaining band values were expressed as a fraction thereof. The average value at a given time point was divided by the average *ama-1* value for that time point to generate the relative values shown in Fig. 5. Standard deviations were determined using a large sample approximation (Jeon et al., 1999).

Results

The 15 *C. elegans* members of conserved NR subfamilies are summarized in Table 1. For most of these, comparative sequence analyses establish apparent orthologous relationships with insect and vertebrate NRs, as reflected in the assignment to conserved NR subgroups (indicated by letter designations with the major subfamilies NR1-6; Nuclear Receptors Committee, 1999), with some notable exceptions. Although NHR-85 and SEX-1 are more closely related to the vertebrate Rev-erb paralogs and insect E75/E78 paralogs than to other subgroups, the degrees of similarity do not meet the defined criteria for formal placement in these groups. However, the NHR-85 DNA-binding domain (DBD) is more similar to that of E75 than to the E78 DBD, and is more similar to E75 than is SEX-1 (Sluder and Maina, 2001), so for the purposes of this work we will consider NHR-85 to be the likely E75 ortholog. The NR2A subgroup (HNF4) was the source of the massive NR expansion in *C. elegans*, precluding definitive identification of the ancestral HNF4 ortholog by sequence analysis alone (Robinson-Rechavi et al., submitted for publication). However, *nhr-64* and *nhr-69* are the most similar to the vertebrate and insect *HNF4* genes (Robinson-Rechavi et al, submitted for publication; Sluder and Maina, 2001), and these two genes were therefore included in this study.

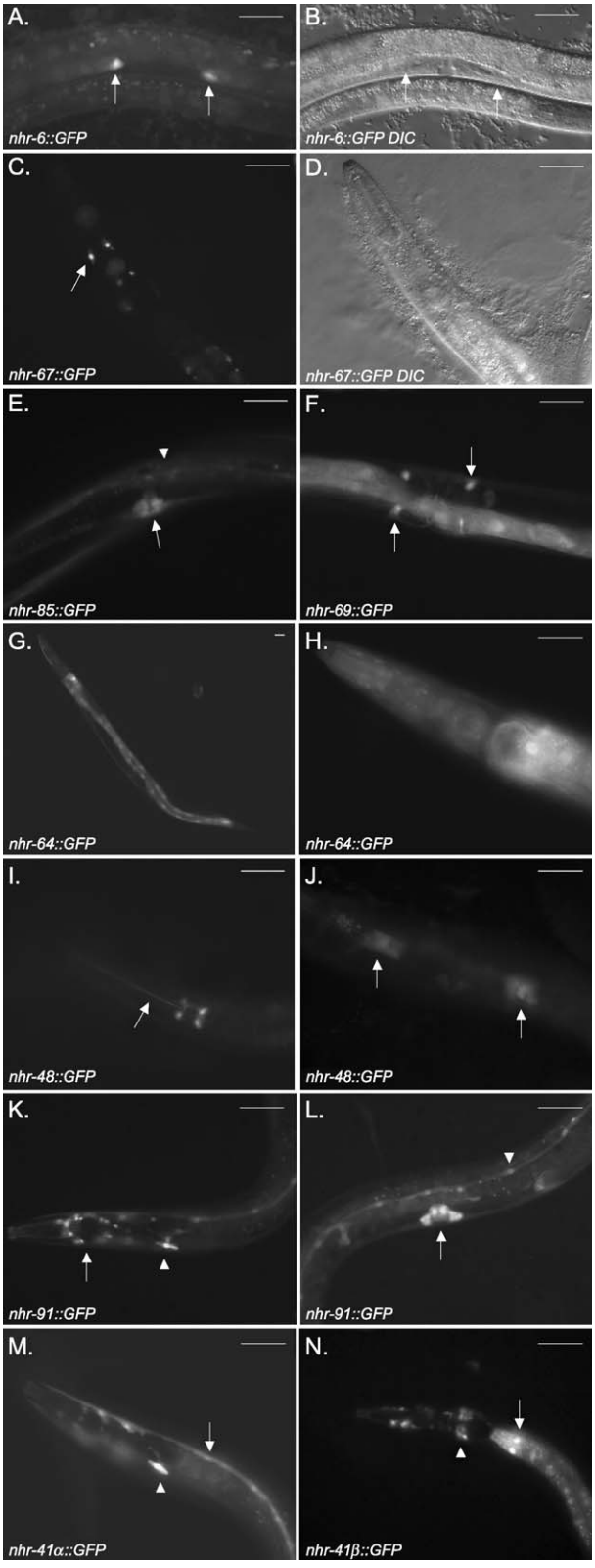
Gene structures of nhr-41, nhr-64, nhr-67, nhr-69, nhr-85, and nhr-91

Of the eight NRs characterized in this study (bold highlighted in Table 1), initial mRNA expression data and cDNA sequences have been previously reported for *nhr-6* and *nhr-48* (Antebi et al., 2000; Kostrouch et al., 1995; Sluder et al., 1999). For the six remaining genes, representation in cDNA libraries and initial RT-PCR and Northern blot analyses confirmed that all are expressed genes (Gissendanner, 2001; Sluder et al., 1999). The analysis of full-length cDNA sequences verified the gene structures for *nhr-41*, *nhr-64*, *nhr-67*, *nhr-69*, *nhr-85*, and *nhr-91* (Fig. 1). The stereotypical NR protein contains an N-terminal transactivation domain (A/B domain), a central DNA binding domain (DBD), and a ligand-binding domain (LBD) positioned C-terminally to the LBD. All of the genes characterized here encode NRs exhibiting this canonical structure. The mRNAs for all of the genes except *nhr-67* are SL1 *trans*-spliced and four genes—*nhr-41*, *nhr-64*, *nhr-85*, and *nhr-91*—encode more than one mRNA isoform (Fig. 1). Multiple mRNA isoforms have also been described for *nhr-48* (Antebi et al., 2000). For *nhr-41*, *nhr-85*, and *nhr-91*, the alternate mRNA isoforms arose by the *trans*-splicing of SL1 to different exons (Fig. 1). The *trans*-splicing of SL1 to exon 5 of *nhr-41* yields a mRNA (*nhr-41β*) that encodes a NR with a truncated A/B domain. The *trans*-splicing of SL1 to exon 2 of *nhr-85* yields a mRNA (*nhr-85β*) that encodes a NR containing only the ligand-binding domain. A similarly truncated NR lacking a DBD is encoded by *nhr-91β*, which arises from the *trans*-splicing of SL1 to exon 4 of *nhr-91*. The alternate mRNA isoform of *nhr-64*, *nhr-64β*, results from the alternate splicing of exon 1 to exon 3. Interestingly, this *nhr-64β* mRNA would also encode a NR lacking an intact DNA-binding domain.

Biological functions of the conserved C. elegans NR genes

Reporter gene analysis and double-stranded RNA (dsRNA)-mediated interference with gene function (RNAi) were utilized to assess the biological functions of the conserved NR genes. Spatial expression patterns were determined using transgenic animals bearing *GFP* reporter constructs (Fig. 1). Each gene displayed a distinct expression pattern ranging from expression in one or two cell types to expression in nearly all cells of the animals, although it should be noted that some reporter constructs might not

fully reflect endogeneous gene expression due to the absence of intronic enhancers. All major somatic tissue types, with the exception of body wall muscle, express one or more of these conserved NR genes (Fig. 2).



Multiple RNAi approaches were utilized to probe the genetic functions of the conserved NR genes. dsRNA corresponding to each gene was injected into young adult N2 hermaphrodites and hermaphrodites of the RNAi sensitized strain *rrf-3*(*pk1426*) (Simmer et al., 2002). F1 progeny from these hermaphrodites were then assessed for visible phenotypes (Table 2). In addition to dsRNA delivery by injection, RNAi was performed by feeding hatched L1 larvae with dsRNA-expressing bacteria (Kamath et al., 2000; Timmons et al., 2001) or by soaking larvae in a dsRNA solution (Maeda et al., 2001). N2 and *rrf-3*(*pk1426*) larvae exposed to dsRNA by these methods were then monitored to adulthood for detection of additional or more subtle postembryonic phenotypes. For *nhr-41*, *nhr-48*, *nhr-64*, *nhr-69*, and *nhr-91*, no visible phenotypes were observed under conditions of abundant food supporting continuous larval development. Visible phenotypes were observed for *nhr-6*, *nhr-67*, and *nhr-85* (discussed below; Fig. 3, Table 2). The use of *rrf-3*(*pk1426*) in the RNAi experiments also allowed an assessment of male morphology because *rrf-3*(*pk1426*) hermaphrodites have an elevated number of males among their progeny (Simmer et al., 2002). Morphology of the *rrf-3*(*pk1426*) male gonad and tail structures were phenotypically wild-type for all genes, although mating efficiency was not specifically addressed (data not shown).

Observations for each gene are described in more detail below and are summarized in Table 3.

nhr-6

Expression of the *nhr-6*::*GFP* reporter was limited; robust expression was only observed in the posterior and anterior spermatheca during the L3 and L4 stages (Figs. 2A, B). Weak expression in two chemosensory neurons was variably observed at all stages (data not shown). F1 progeny of P0 hermaphrodites injected with double-stranded *nhr-6* RNA developed normally up to the adult stage (Table 2). However, these adult F1 progeny produced abnormally

Fig. 2. *nhr*::*GFP* reporter gene expression patterns. Epifluorescence micrographs of *GFP* expression for the eight conserved NR genes. All animals are late larvae except where indicated. Scale bar = 10 μm. In all micrographs, anterior is to the left. (A) *nhr-6*::*GFP* expression in the L4 spermatheca (arrows). (B) DIC micrograph of A. (C) *nhr-67*::*GFP* expression in anterior neurons (arrow). The large fluorescent circles are additional expressing neuronal nuclei in a different plane of focus. (D) DIC micrograph of C. (E) *nhr-85*::*GFP* expression in the vulva (arrow) and hypodermis (arrowhead). (F) *nhr-69*::*GFP* expression in uterine nuclei (arrows). Expression in the gut is also evident. (G and H) *nhr-64*::*GFP* expression in the gut and anterior neurons (of an adult animal), respectively. (I) *nhr-48*::*GFP* expression in the pharyngeal gland. Arrow indicates pharyngeal gland process. (J) *nhr-48*::*GFP* expression in the spermatheca (arrows). (K) *nhr-91*::*GFP* expression in anterior neurons (arrow) and excretory cell (arrowhead). (L) *nhr-91*::*GFP* expression in the vulva (arrow) and seam (arrowhead). Expression in the spermatheca, anterior and posterior to the vulva, is also evident. (M) *nhr-41α*::*GFP* expression in the seam (arrow) and excretory cell (arrowhead). (N) *nhr-41β*::*GFP* expression in the gut (arrow) and chemosensory neurons (arrowhead). Expression in head hypodermal nuclei is seen anterior to the chemosensory neurons.

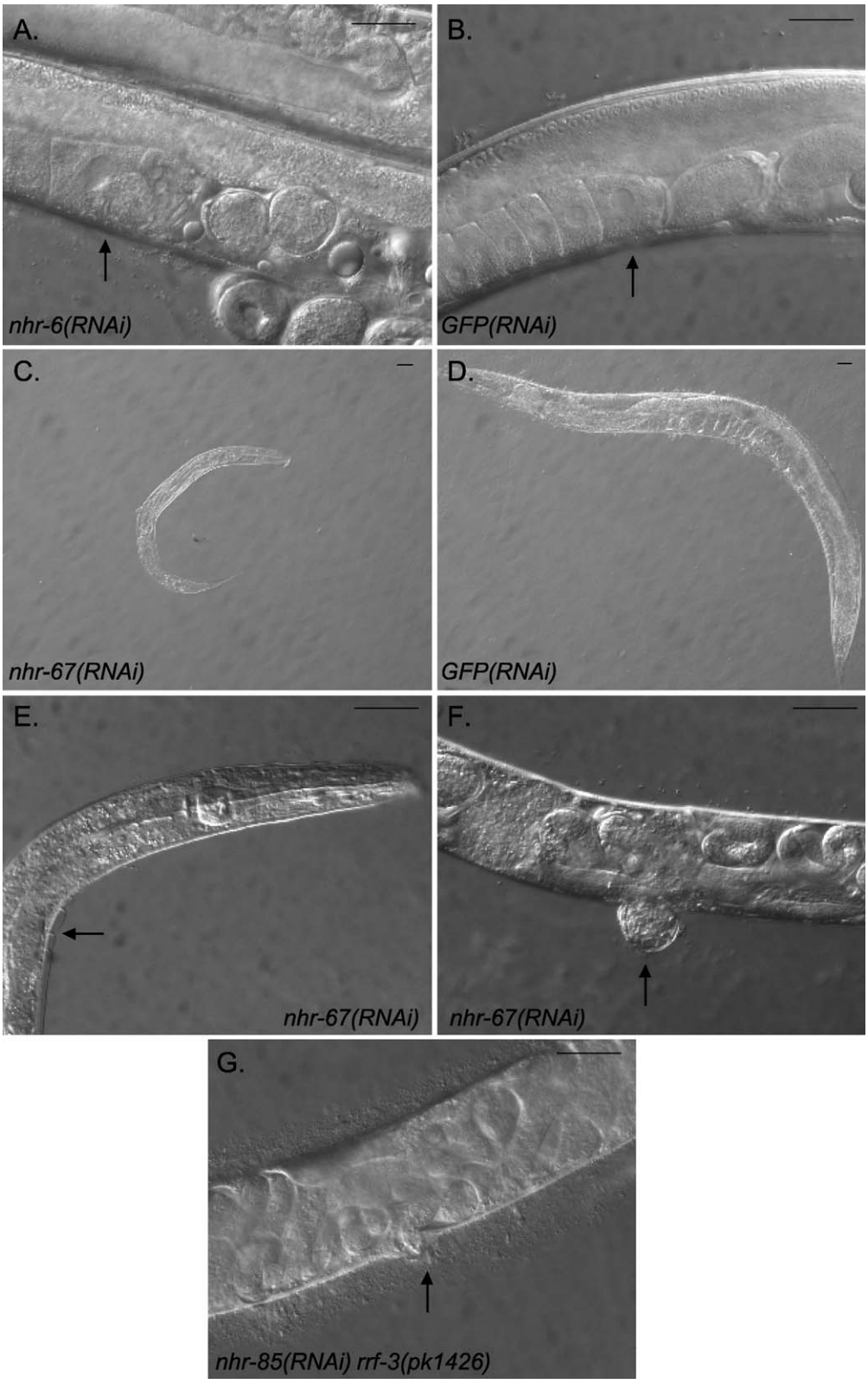


Fig. 3. *nhr-6*, *nhr-67*, and *nhr-85* RNAi phenotypes. DIC micrographs. Scale bar = 10 μm. (A) Ovulation defect in *nhr-6*(*RNAi*) adults. Arrow indicates the most proximal oocyte. Note the fragmentation of the oocyte and abnormally round embryos inside the uterus and outside of the animal. (B) Gonad arm of negative control (*GFP*) RNAi animals. Arrow points to the proximal oocyte. (C) *nhr-67*(*RNAi*) Sma phenotype. Compare to negative control adult in D. (E) Molting defect in *nhr-67*(*RNAi*) Sma adult (arrow). (F) Protruding vulva (arrow) in *nhr-67*(*RNAi*) adult. Note the hatched larvae in the uterus. (G) *nhr-85*(*RNAi*); *rrf-3*(*pk1426*) Egl adult. Arrow indicates abnormally developed vulva.

Table 3
Summary of conserved NR gene expression and function (this study)

nhr	Spatial expression (*nhr*::*GFP* transgene)	Function (RNAi)
nhr-6	Spermatheca, two chemosensory neurons	Ovulation
nhr-41	Seam cells, excretory cell, rectal epithelia (*nhr-41α*), chemosensory neurons, gut, head/tail hypodermis (*nhr-41β)*	Dauer formation
nhr-48	Pharyngeal gland, spermatheca, hypodermis	None detected
nhr-64	Anterior and posterior neurons, ventral nerve cord, pharynx, gut, hypodermis	None detected
nhr-67	Six or seven anterior neurons	Molting, vulva development
nhr-69	Gut, hypodermis, uterus	None detected
nhr-85	Hypodermis, excretory and rectal epithelia, vulva	Egg-laying, dauer formation
nhr-91	Seam cells, spermatheca, vulva, excretory cell, anterior neurons	None detected

small, round eggs (Fig. 3A; 29/29 F1 progeny transferred to individual plates laid abnormal embryos). DIC observation of the germline of 87 *nhr-6* (*RNAi*) adult hermaphrodites revealed that 43/87 had defective oocytes present in the most proximal position relative to the spermatheca (Fig. 3A; compare to control hermaphrodites in Fig. 3B). More distal oocytes appeared normal, although slightly misshapen gonad arms were observed in two adults. The proximal oocytes were often fragmented, although it was not clear whether this fragmentation occurred before or during ovulation. Fertilization of the fragmented oocytes could be the source of the abnormally shaped eggs. This oocyte fragmentation is reminiscent of the ovulation defects resulting from mutations in genes required for ovulation, such as *ceh-18,* which functions during sheath cell development (Rose et al., 1997). Because the *nhr-6*::*GFP* is expressed in the spermatheca, the ovulation defect may be due to defective spermathecal development or function.

nhr-67

Like *nhr-6*::*GFP*, *nhr-67*::*GFP* also exhibited a restricted expression pattern. *nhr-67*::*GFP* expression was only observed in six or seven head neurons (Figs. 2C, D). Nevertheless, *nhr-67* exhibited a robust RNAi phenotype of perturbed larval and vulval development. Significant numbers of abnormal, slow growing larvae were observed among the F1 progeny of *nhr-67* dsRNA-injected hermaphrodites (Table 2). These larvae did not display morphological defects, but were sickly in appearance, uncoordinated in movement, and slow growing, though they did eventually reach the adult stage (see below). 185/229 of adult *nhr-67(RNAi)* animals examined displayed a protruding vulva (Pvul phenotype), indicating defects in vulval development (Fig. 3F). This vulval phenotype varied in severity; the most severely affected animals were incapable of laying eggs and died as the result of embryos hatching within the uterus (matricide). Examination of developing vulvae in *nhr-67(RNAi)* larvae did not reveal any gross abnormalities, suggesting that the Pvul phenotype is due to later events in vulval morphogenesis (data not shown).

The *nhr-67* RNAi phenotypes were also observed when performing postembryonic RNAi by dsRNA feeding. 100% of RNAi animals exhibited either the larval development phenotype or the Pvul phenotype with the associated matricide, indicating that the larval development phenotype in *nhr-67*(*RNAi*) animals is due to a postembryonic function of *nhr-67*. As expected, *nhr-67* RNAi in the *rrf-3*(*pk1426*) background had more severe effects, with 44% of animals forming abnormal larvae ($n = 102$) compared to 11% for N2 ($n = 94$). The abnormal *nhr-67*(*RNAi*) larvae develop into adults that are significantly smaller (Sma phenotype) than control RNAi adults (Fig. 3C). All Sma adults displayed molting defects with portions of incompletely shed larval cuticle remaining attached to the animal (Fig. 3E). These molting defects were first observed at the L3/L4 molt. The cuticle lining the pharynx was frequently not shed, and failure to shed the cuticular lining of the pharynx could inhibit feeding and lead to the formation of small, sickly adults.

How the neural expression of *nhr-67* correlates to the cuticle-shedding defect is not entirely clear, unless *nhr-67* affects neural cues for ecdysis. Similarly, there was no vulval *GFP* expression in the *nhr-67*::*GFP* reporter line. While it is a formal possibility that the vulval phenotype is not directly due to *nhr-67* function in the developing vulva, there is no known requirement for a neural function in vulval development. Alternatively, this reporter construct may not recapitulate the complete *nhr-67* expression pattern, although the reporter construct incorporated all sequences between the 5′ end of the *nhr-67* open reading frame and the next predicted upstream gene.

nhr-85

The *nhr-85*::*GFP* reporter is expressed in the developing vulva, the hypodermis (Fig. 2E), and specialized epithelia of the rectum and excretory duct (data not shown). While *nhr-85* RNAi by injection did not produce phenotypes in the N2 background, 15% of F1 adult progeny of injected *rrf-3*(*pk1426*) hermaphrodites were egg-laying defective and became bloated with eggs (Egl phenotype, Table 2; Fig. 3G). In the more severe cases, matricide occurred due to internal hatching of retained eggs (bagging phenotype). The Egl and bagging phenotypes were confirmed by postembryonic *nhr-85* RNAi in the *rrf-3*(*pk1426*) background. In two separate feeding experiments, the Egl or bagging phenotype was observed in 34% ($n = 114$) and 42% ($n = 79$) of *nhr-85*(*RNAi*); *rrf-3*(*pk1426*) animals, compared to 11% ($n = 158$) and 9% ($n = 79$), respectively, in the control animals. Similar results in *rrf-3*(*pk1426*) animals were also obtained from *nhr-85* RNAi by soaking, with 41% ($n = 39$) of animals displaying an Egl phenotype compared to 4% ($n = 51$) for control animals. Although some older *rrf-3*(*pk1426*) adults exhibited moderate bloat-

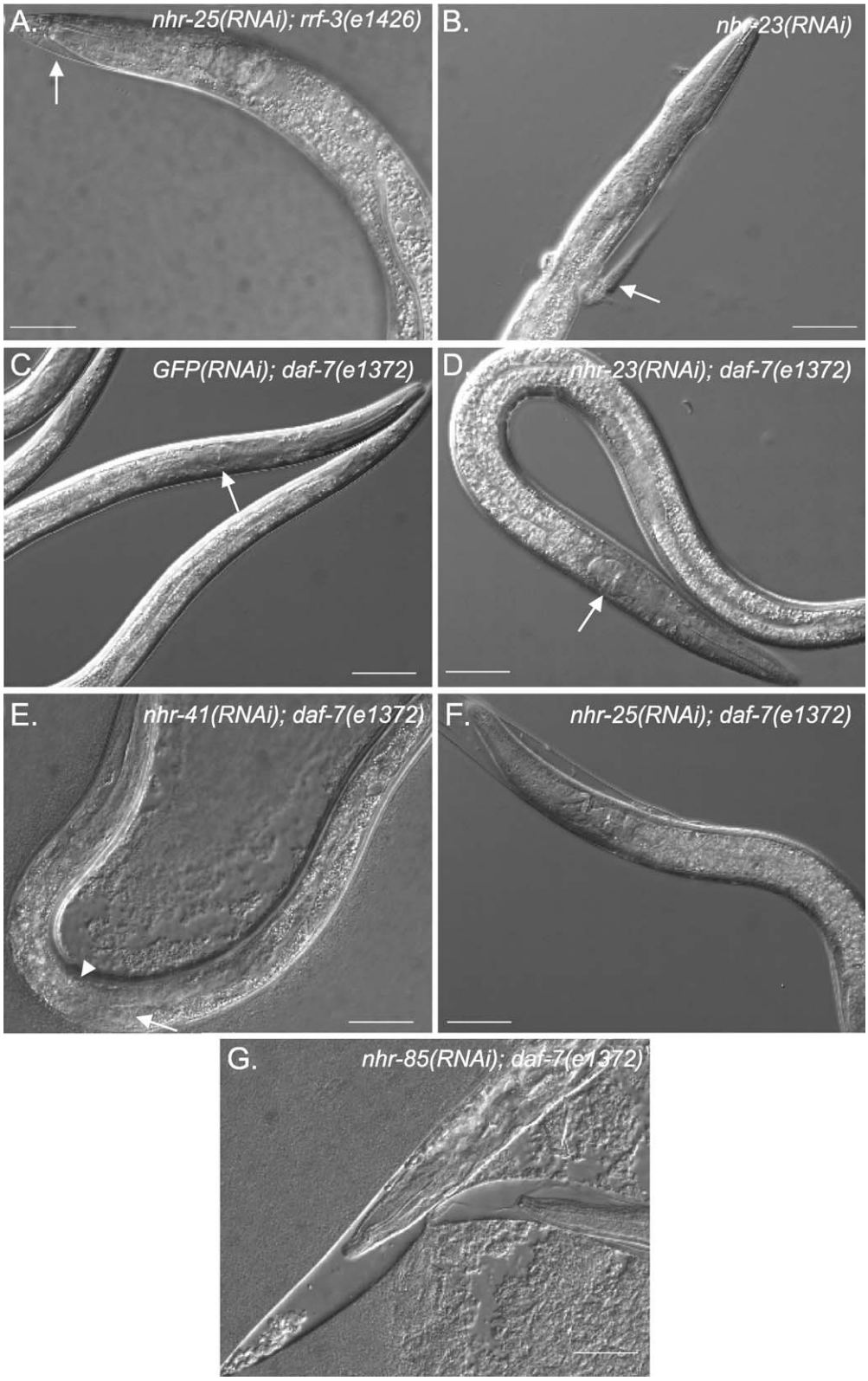


Fig. 4. Molting and dauer formation RNAi phenotypes. DIC micrographs. Scale bar = 10 μm. (A) Young adult *nhr-25*(*RNAi*); *rrf-3*(*pk1426*) animal encased in L4 cuticle (arrow). (B) *nhr-23*(*RNAi*) molting defect. Arrow indicates previous stage cuticle constriction. (C) Normal dauer produced by *GFP*(*RNAi*); *daf-7*(*e1372*) at 25°C. Arrow indicates the thin pharyngeal structure of dauer animals. (D) Abnormal *nhr-23*(*RNAi*); *daf-7*(*e1372*) dauer. Body size defect (compare to C) and abnormally large pharyngeal structure (arrow) are shown in this panel. (E) Abnormal *nhr-41*(*RNAi); daf-7*(*e1372*) dauer. Body size defect and gonadal growth defects are shown in this panel. Arrowhead indicates the point of dorsal turn of gonad, indicative of L4 gonad growth. Arrow indicates distal tip of the L4 gonad. (F) Abnormal *nhr-25*(*RNAi*); *daf-7*(*e1372*) dauer encased in cuticle. (G) Abnormal *nhr-85*(*RNAi*); *daf-7*(*e1372*) dauer exhibiting degeneration of internal tissues.

ing with retained eggs, resulting in a low percentage of control animals scored as Egl, the more severe bagging Egl phenotype was only observed in the *nhr-85*(*RNAi*) animals (with the exception of a single bagging control animal). Protruding and other forms of abnormal vulvae were sometimes observed in Egl *nhr-85*(*RNAi*) adults. Thus, *nhr-85* appears to be required for the development or function of the egg-laying machinery.

nhr-64 and nhr-69

The reporter gene for *nhr-69* is strongly expressed in the gut (Fig. 2F) and hypodermis at all stages. In late L4 and adult, the *nhr-69*::*GFP* is expressed in uterine cells (Fig. 2F) and some *nhr-69*::*GFP* transgenic animals also expressed GFP in the rectal epithelia and the posterior pharynx (data not shown). *nhr-64*::*GFP*, the *nhr* reporter gene with the broadest expression, is expressed in the gut, hypodermis, pharynx, and neurons of the head, tail, and ventral nerve cord (Figs. 2G, H; tail neurons and ventral nerve cord expression not shown). As noted above, no obvious phenotypes were observed for *nhr-64* and *nhr-69* in the various RNAi experiments. A *nhr-64*/*nhr-69* double RNAi experiment also did not reveal any possible redundant functions for these two genes.

nhr-48 and nhr-91

nhr-48::*GFP* expression was observed in the pharyngeal gland (Fig. 2I) in larvae and adults and also in the spermatheca (Fig. 2J) beginning in L4. Some transgenic lines displayed weak hypodermal expression. *nhr-91*::*GFP* displayed expression in the developing vulva, seam cells, spermatheca, excretory cells, and anterior neurons (Figs. 2K, L). No obvious RNAi phenotypes were observed for either *nhr-48* or *nhr-91*.

nhr-41

The gene structure of *nhr-41* is unique in that a large 5.2-kb intron (intron 4) lies upstream of the second, alternate SL1 splice site (Fig. 1). Thus, two *GFP* reporter constructs were generated, containing distinct putative promoters for *nhr-41α* and *nhr-41β*, the latter promoter corresponding to genomic DNA in the large fourth intron. Our analysis suggests different expression patterns for the two *nhr-41* mRNA isoforms, although it is a formal possibility that enhancers in the fourth intron could be acting on both presumed promoters (α and β). The *nhr-41α* reporter gene is expressed in the hypodermal seam, excretory cell (Fig. 2M), and rectal epithelia (data not shown). Varied and inconsistent expression was also observed in head neurons (data not shown). In contrast, the *nhr-41β* reporter gene is expressed consistently in the gut, chemosensory neurons, and head and tail hypodermal cells (Fig. 2N). Thus, the *nhr-41* reporter gene analysis may be revealing distinct functions for the two isoforms. RNAi analysis in continuously developing animals did not reveal possible functions for *nhr-41*. However, *nhr-41* is an ortholog of an insect NR involved in regulation of molting, and its potential functions during larval molting are discussed below.

Orthologs of insect 'ecdysone cascade' NRs function during the C. elegans molting process

Five *C. elegans nhr* genes are orthologs of NR genes that function during the ecdysone response cascade that regulates molting and metamorphosis in insects: *nhr-6* is the *C. elegans* ortholog of *DHR38*, *nhr-23* of *DHR3*, *nhr-25* of *Ftz-F1*, *nhr-41* of *DHR78*, and *nhr-85* is the likely *C. elegans* ortholog of *E75* (Table 1). Initial analyses of *nhr-25* and *nhr-23* demonstrated that both genes are expressed in the hypodermis and may function to regulate molting processes (Asahina et al., 2000; Gissendanner and Sluder, 2000; Kostrouchova et al., 1998).

For *nhr-23* and *nhr-25*, F1 larvae from parents injected with dsRNA corresponding to these genes displayed difficulties in shedding cuticle during the molting process (Asahina et al., 2000; Gissendanner and Sluder, 2000; Kostrouchova et al., 1998). However, these initial observations did not eliminate the possibility that the observed larval defects resulted from defective development of the hypodermis during embryogenesis. *nhr-23* was subsequently shown to have a specific role in the molting process during postembryonic development (Kostrouchova et al., 2001).

To determine if *nhr-25* also has a specific molting function, the postembryonic function of *nhr-25* was inhibited

Table 4
nhr RNAi effects on dauer formation

dsRNA	*daf-7*(*e1372*) at 25°C		N2 + pheromone at 20°C	
	Percent dauers	Percent SDS resistance[a]	Percent dauers	Percent SDS resistance[a]
GFP[b]	100% (*n* = 233)	99% (*n* = 109 dauers tested)	94% (*n* = 68)	100% (*n* = 33 dauers tested)
nhr-6	98% (*n* = 125)	96% (*n* = 50)	97% (*n* = 73)	100% (*n* = 39)
nhr-23[b]	100% (*n* = 346)[c]	0% (*n* = 121)	>90%[c,d]	0% (*n* = 43)
nhr-25[b]	100% (*n* = 312)[c]	0% (*n* = 199)	>90%[c,d]	0% (*n* = 17)
nhr-41[b]	100% (*n* = 175)[c]	0% (*n* = 128)	95% (*n* = 82)[c]	0% (*n* = 60)
nhr-64	100% (*n* = 77)	100% (*n* = 77)	ND	ND
nhr-67	100% (*n* = 79)	96% (*n* = 79)	ND	ND
nhr-69	100% (*n* = 72)	100% (*n* = 72)	ND	ND
nhr-85[b]	97% (*n* = 197)	47% (*n* = 108)	72% (*n* = 78)	100% (*n* = 68)
nhr-91	100% (*n* = 83)	98% (*n* = 83)	ND	ND

[a] Only dauers or dauer-like larvae were assessed for SDS resistance.
[b] For *daf-7*(*e1372*), two replicate experiments were performed with these genes. The numbers from both replicates are combined in the table.
[c] Dauer-like larvae.
[d] Exact proportions not determined. >90% were dauer-like larvae.

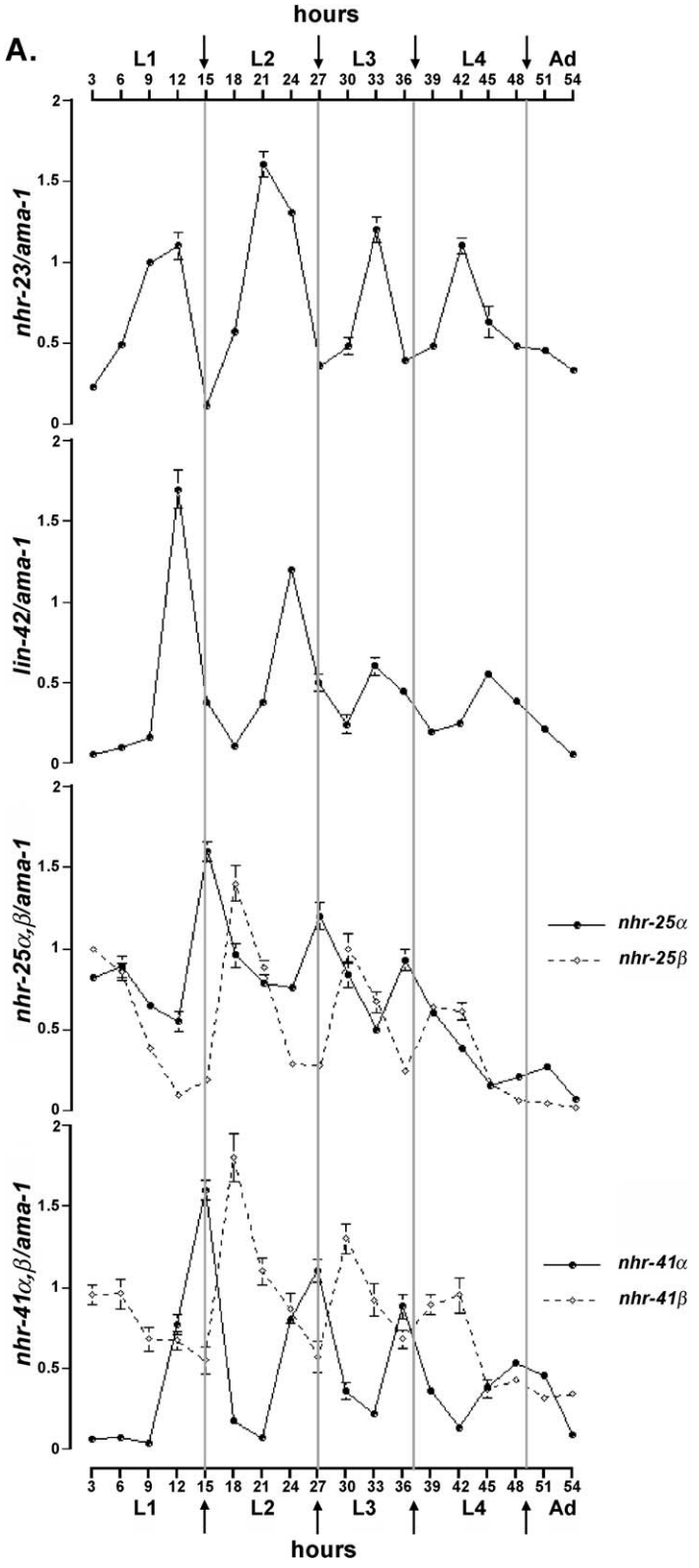


Fig. 5. Temporal RT-PCR expression analysis of conserved NRs. (A) mRNA abundance, relative to *ama-1*, of *nhr*s that exhibit a reiterated expression pattern during larval development. (B) mRNA abundance, relative to *ama-1*, of *nhr*s that exhibit a more irregular pattern of expression. *y*-axis, ratio of *nhr/ama-1* signals; *x*-axis, developmental time points (hours). Arrows and gray lines indicate the times at which molting occurred.

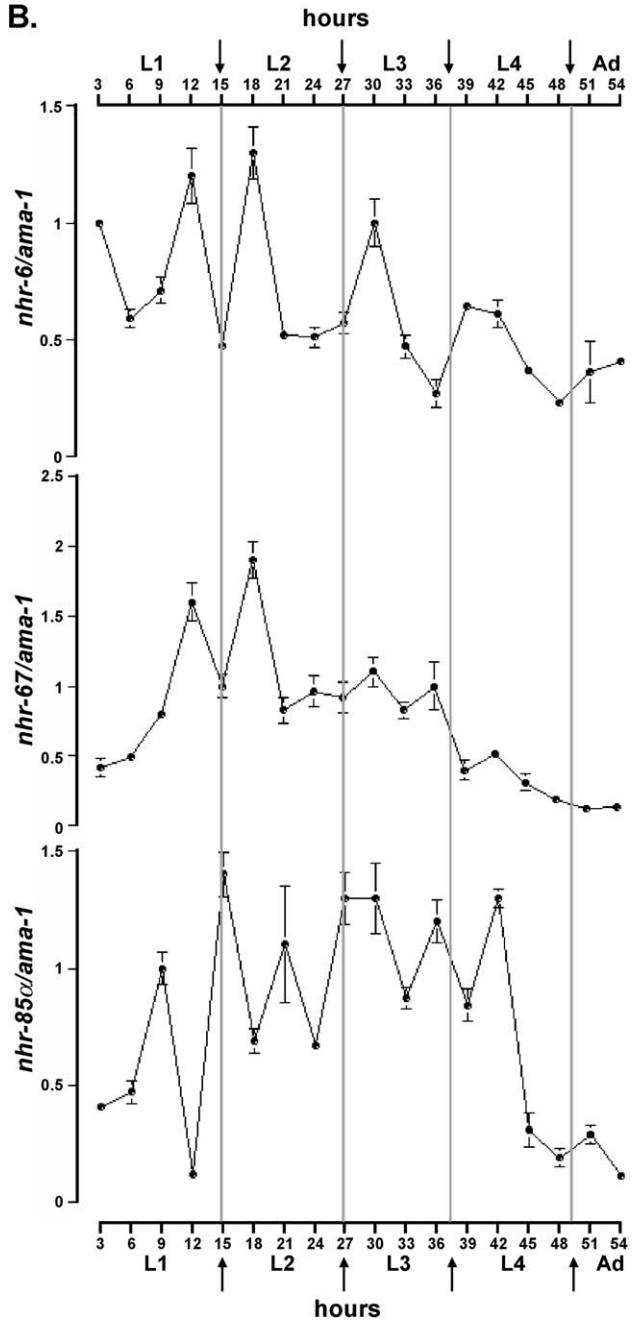


Fig. 5 (*continued*).

by larval RNAi by feeding (as described above). For comparison, RNAi of *nhr-23* was performed in parallel. The *nhr-25*(*RNAi*) animals developed to the adult stage of development ($n = 109$). As has been previously observed (Asahina et al., 2000; Gissendanner and Sluder, 2000), these *nhr-25*(*RNAi*) adults displayed gonad and vulval abnormalities, although most were fertile (data not shown). Further inspection of these animals revealed that the L4 cuticle was not completely molted. Generally, this was observed as extraneous cuticle stuck to the vulva, rectum, and mouth. When the RNAi experiments were performed in the *rrf-3*(*pk1426*) background, a more severe phenotype was observed. In all animals ($n = 113$), movement ceased and the animals were completely encased within both the L4 and adult cuticles (Fig. 4A). While the adult cuticle had been synthesized, the L4 cuticle was not shed. These animals developed an adult gonad but the majority were sterile (14/14 scored by DIC optics), similar to the most severely affected adults observed in RNAi by injection experiments (Gissendanner and Sluder, 2000). In contrast, at this point of development (approximately 72 h postfeeding), *rrf-3*(*pk1426*) larvae fed bacteria expressing *dsGFP* RNA had become normal, gravid adults ($n = 151$). These data indicate that *nhr-25* is required for at least the L4/adult molt. Earlier molting defects are observed in F1 larvae derived from P0 hermaphrodites injected with *nhr-25* dsRNA, suggesting that *nhr-25* also has a role in the earlier molts (Gissendanner and Sluder, 2000).

Postembryonic RNAi for *nhr-23* generated the same suite of phenotypes as described previously (Kostrouchova et al., 2001). N2 animals subjected to *nhr-23* RNAi arrested as L4 animals with a variable dumpy (Dpy) phenotype and constrictions of L3 cuticle ($n = 108$; 10/10 scored by DIC optics) (Fig. 4B). When *nhr-23* RNAi was performed in the *rrf-3*(*pk1426*) background, molting defects were observed one stage earlier at the L2/L3 molt (data not shown).

Molting defects were never observed in *nhr-6*(*RNAi*), *nhr-41*(*RNAi*), or *nhr-85*(*RNAi*) larvae (although cuticle defects were occasionally observed with *nhr-85* RNAi), leaving unclear what role, if any, these genes may have in the *C. elegans* molting cycle. The initial RNAi experiments for *nhr-6, nhr-23*, *nhr-25*, *nhr-41*, and *nhr-85* were performed during the continuous growth life cycle, during which *C. elegans* undergoes four molts before reaching the adult stage. Under adverse environmental conditions, *C. elegans* executes an alternate life cycle, entering a diapausing state marked by the formation of a specialized third-stage larva, the dauer (Riddle and Albert, 1997). Dauer larvae differ markedly in body morphology and cuticle structure from the L3 larvae formed during the continuous growth molts.

To test if *nhr-6*, *nhr-23*, *nhr-25*, *nhr-41*, and *nhr-85* function during the specialized dauer molt, RNAi for these genes was performed in *daf-7*(*e1372*) larvae. *daf-7* encodes a TGFβ homolog that transduces environmental signals and promotes non-dauer development (Ren et al., 1996). The *e1372* mutant is temperature sensitive, and at 25°C *daf-7*(*e1372*) larvae constitutively enter the dauer stage. *daf-7*(*e1372*) L1 larvae fed bacteria expressing dsRNA for *nhr-23*, *nhr-25*, *nhr-41*, and *nhr-85* fail to form normal dauers (Figs. 4D, E, F, G and Table 4). Dauers are resistant to treatment with 1% SDS (Riddle and Albert, 1997), and *daf-7*(*e1372*) larvae fed bacteria expressing *dsGFP* RNA formed 100% phenotypically wild-type, SDS-resistant dauers at 25°C. In contrast, *nhr-23*(*RNAi*); *daf-7*(*e1372*), *nhr-25*(*RNAi*); *daf-7*(*e1372*), *nhr-41*(*RNAi*); *daf-7*(*e1372*), and *nhr-85*(*RNAi*); *daf-7*(*e1372*) dauers were SDS-sensitive (Table 4). Except for *nhr-85*(*RNAi*); *daf-7*(*e1372*), the morphology defects and SDS sensitivity were 100% penetrant. Approximately one-half of the *nhr-85*(*RNAi*); *daf-7*(*e1372*)

dauers were SDS resistant and also appeared morphologically wild-type. RNAi in the *daf-7*(*e1372*) background for *nhr-6*, *nhr-64*, *nhr-67*, *nhr-69*, and *nhr-91* resulted in morphologically normal, SDS-resistant dauers, indicating that the dauer defects observed for *nhr-23*, *nhr-25*, *nhr-41*, and *nhr-85* are specific effects and are not due to a general incompatibility between dauer formation and the RNAi process.

The defective RNAi dauer morphologies for these genes were distinct. *nhr-23*(*RNAi*); *daf-7*(*e1372*) dauers exhibited the most severe phenotypes and were defective in most features of dauer morphogenesis, including radial shrinkage of the hypodermis, pharyngeal remodeling, and dauer alae synthesis (n = 30 scored; Figs. 4C, D). In addition, some animals exhibited L4 gonad growth and vulva development. However, these animals eventually died and did not reach the adult stage, although they never exhibited the cuticular defects observed in *nhr-23* RNAi experiments in the N2 background. *nhr-41*(*RNAi*); *daf-7*(*e1372*) dauers were similar to *nhr-23* but exhibited slightly more normal morphology (n = 28 scored) with weak or nonexistent alae (data not shown) and increased gonad growth (Fig. 4E). Some *nhr-41*(*RNAi*); *daf-7*(*e1372*) dauers were also stuck in cuticle. *nhr-25*(*RNAi*); *daf-7*(*e1372*) dauers had a more normal dauer morphology (some radial shrinkage, pharyngeal remodeling, dauer gonad) and synthesized dauer alae (n = 30 scored). However, these animals were frequently stuck in cuticle and displayed tissue degeneration (Fig. 4F). The abnormal *nhr-85*(*RNAi*); *daf-7*(*e1372*) dauers were nearly identical to *nhr-25*(*RNAi*) dauers in that they were stuck within a cuticle and often exhibited tissue degeneration (generally more severe than for *nhr-25*(*RNAi*); n = 10 scored) (Fig. 4G).

To verify that these dauer formation RNAi phenotypes can occur in wild-type animals, RNAi was performed on N2 larvae grown in the presence of dauer-inducing pheromone (Golden and Riddle, 1982). The SDS sensitivity and the dauer morphology defects described above were also observed in *nhr-23*, *nhr-25*, and *nhr-41* RNAi dauer larvae induced by pheromone exposure (Table 4 and data not shown). *nhr-85* RNAi dauer larvae induced by pheromone were 100% SDS resistant, in contrast to the incompletely penetrant SDS sensitivity observed in *nhr-85*(*RNAi*); *daf-7*(*e1372*) dauers. This result suggests that the *daf-7*(*e1372*) background is sensitized for *nhr-85* RNAi, possibly due to either enhanced RNAi efficiency in *daf-7*(*e1372*) animals or a dauer developmental anomaly of *daf-7*(*e1372*) mutants that prevents normal dauer development in larvae exposed to *nhr-85* dsRNA. The failure of *nhr-23*, *nhr-25*, and *nhr-41* RNAi larvae to form normal dauers in both N2 and *daf-7*(*e1372*) backgrounds suggests that these genes function during the dauer molt or the dauer formation process.

Temporal larval expression of the 'ecdysone cascade' NRs

In insects, the ecdysone cascade NRs display a specific temporal expression and functional hierarchy during the metamorphic molts (Richards, 1997). To assess whether the *C. elegans* orthologs of these ecdysone cascade NRs also display a specific temporal expression hierarchy during larval development, temporal expression profiles were analyzed for the seven "ecdysone cascade" *nhr* mRNAs: *nhr-6*, *nhr-23*; *nhr-25α*, and *β*; *nhr-41α* and *β*; and *nhr-85α*. *lin-42*, a *C. elegans period* homologue that has been shown to cycle relative to the molts (Jeon et al., 1999), was included as a positive control and for comparison. Because *nhr-67*(*RNAi*) animals also display a molting defect, the mRNA temporal expression profile was examined for *nhr-67* as well.

Synchronous cultures of *C. elegans* at 20°C were harvested every 3 h until the adult stage was reached, and total RNA was extracted. Specific mRNA species were detected by RT-PCR. To determine the relative abundance for these mRNAs during larval development, the levels of RT-PCR product were standardized to that of a control mRNA, *ama-1*, which encodes the large subunit of RNA polymerase II (Bird and Riddle, 1989). This semiquantitative RT-PCR approach is intended to determine expression fluctuations for a given mRNA across developmental time. Only a comparison of the timing of expression fluctuations, not of absolute expression levels, can be made among mRNA species. This approach has previously been used to establish temporal expression profiles for the cuticle collagen genes (Johnstone and Barry, 1996), *lin-42* (Jeon et al., 1999), and *nhr-23* (Kostrouchova et al., 2001). The temporal expression profile of *lin-42* observed in this study is nearly identical to the *lin-42* profile previously reported (Jeon et al., 1999). Although the previously described expression profile for *nhr-23* was based on worm cultures raised at 25°C (Kostrouchova et al., 2001), so that a direct comparison of absolute developmental times cannot be made with our data, the expression profiles are qualitatively similar.

Distinct temporal patterns emerged from the RT-PCR analysis (Fig. 5). The mRNAs for *lin-42*, *nhr-23*, and *nhr-41α* exhibit a low-high (peak)-low profile during the L1 intermolt that is precisely reiterated at later stages (Fig. 5A). The mRNAs for *nhr-25α*, *nhr-25β*, and *nhr-41β* have a high-low-high (peak) expression profile during the L1 stage (and into early L2) that is also reiterated (Fig. 5A). *nhr-25α* and *nhr-41β* also show a downward trend in baseline expression as development proceeds. For all six mRNAs, there are peaks of expression that occur at precise intervals relative to the molt at all larval stages: *nhr-25β* and *nhr-41β* peak at the beginning of the intermolt; *nhr-23* peaks at mid-intermolt, slightly preceding the intermolt peak in *lin-42* expression; and *nhr-25α* and *nhr-41α* peak at the time the larval molts occur. Two mRNAs, *nhr-6* and *nhr-85α*, show a more irregular expression pattern with respect to the molt (Fig. 5B); however, it should be noted that *nhr-6* mRNA has consistent relatively lower expression values at the times the molt takes place. *nhr-67* also does not display a reiterated, oscillating expression pattern, although two early peaks are observed at the end of the L1 stage and at the beginning of L2 (Fig. 5B).

Discussion

We have characterized the expression and function of eight genes that encode orthologs of vertebrate and insect NRs. These conserved genes have distinct spatial expression patterns. Some of these genes—*nhr-41*, *nhr-48*, *nhr-64*, *nhr-69*, and *nhr-91*—are expressed in several different cell types and likely participate in multiple transcriptional pathways regulating nematode development and cell function. RNAi analysis revealed requirements for *nhr-6*, *nhr-67*, and *nhr-85* in nematode reproduction. In addition, *nhr-67* and *nhr-85* have distinct functions during larval development, and *nhr-41* is specifically required for development of the alternate dauer larval stage (discussed below).

Functions for the other NR genes may only be revealed in RNAi screens that are targeted for specific processes. For example, several NR genes, including the conserved NR genes *daf-12*, *nhr-25*, and *nhr-8*, were identified in a RNAi screen for genes required for proper fat metabolism (Ashrafi et al., 2003). The design of specific experiments may be informed by the *GFP* reporter gene expression patterns described here, by mRNA expression changes observed in genome-wide expression analyses (Hill et al., 2000; Jiang et al., 2001; Wang and Kim, 2003), or by the functions of orthologous NRs in other species. There are several examples of conservation of function between *C. elegans* NRs and their vertebrate or insect orthologs (Van Gilst et al., 2002). Most striking are the *C. elegans* NRs required for molting (described below) and the function of *nhr-8.* The latter is required for toxin resistance in *C. elegans*, a function very similar to that of its vertebrate ortholog PXR (Lindblom et al., 2001). Based on expression analysis, NHR-67 may exhibit a conserved neural function as related members of the TLL/TLX family regulate neural development and function in both vertebrates and insects (Pignoni et al., 1990; Yu et al., 1994). The *C. elegans HNF-4* homologs *nhr-64* and *nhr-69* are also candidates to exhibit phylogenetically conserved functions: HNF-4 NRs in vertebrates are endodermally expressed and function in multiple metabolic pathways (Giguere, 1999). *nhr-64* and *nhr-69* are both expressed in the *C. elegans* endoderm (gut), suggesting the potential for roles in endodermal metabolic processes.

Complexity in the biochemical activities of the *C. elegans* conserved NRs is suggested by the alternate NR forms that are encoded by alternately spliced mRNAs. The *nhr-41*α mRNA encodes a NR protein that has an additional 82 amino acids in its N-terminal A/B domain, a region known to be involved in transcriptional activation by NRs (Gronemeyer and Laudet, 1995). The alternate β mRNA isoforms of *nhr-64*, *nhr-85*, and *nhr-91* encode truncated NRs lacking DNA-binding domains. This particular NR coding structure is fairly common among the *C. elegans* conserved NRs—mRNA isoforms encoding this type of truncated NR have been previously described for *nhr-8*, *nhr-25*, *nhr-48*, and *daf-12* (Antebi et al., 2000; Gissendanner and Sluder, 2000). NRs consisting of only ligand-binding domains can dimerize with and negatively affect the transcriptional activity of other NRs (Lee et al., 2000; White et al., 1997), and these truncated *C. elegans* NRs may have a similar function.

Regulation of nematode molting by NRs

Molting plays a central role in the life cycle of nematodes, providing for the formation of stage-specific cuticles and cuticle elaborations with specialized functions in feeding, locomotion, and diapause. This is particularly apparent in parasitic species that have specific larval stages that are morphologically and physiologically suited for long-term survival and infection of a host (Bird, 1987). Despite the importance of molting, relatively little is known about the regulation of this process in nematodes.

Nematodes are part of a proposed evolutionary clade, the Ecdysozoa, that contains all molting invertebrates, including insects (Aguinaldo et al., 1997). In insects, events in the molting cycle are regulated by 20-hydroxyecdysone (20E), the active form of ecdysone (Riddiford, 1985). 20E signaling leads to a hierarchical cascade of gene transcription in target cells (Ashburner et al., 1974; Richards, 1997). NRs play a prominent role in this ecdysone response, which has been well characterized genetically and molecularly in *D. melanogaster* (Thummel, 1995, 2001). The receptor for 20E is a heterodimer of two NRs, EcR (ecdysone receptor) and Usp (ultraspiracle) (Koelle et al., 1991; Yao et al., 1993). The expression of four NR genes—*E75*, *E78*, *DHR3*, and *Ftz-F1*—is induced in response to 20E (Richards, 1997). Two additional NR genes, *DHR78* and *DHR38*, also regulate molting events and adult cuticle formation, respectively, but their precise positions within the 20E pathway have yet to be determined (Fisk and Thummel, 1998; Kozlova et al., 1998).

Strikingly, orthologs of *EcR* and *Usp* are absent from the *C. elegans* genome (Sluder and Maina, 2001), raising the question of whether the hormonal signaling events that initiate molting in insects are conserved in *C. elegans.* The absence of *Usp* in *C. elegans* is especially surprising because *Usp* is the ortholog of vertebrate *RXR*, and this NR group (NR1B) is considered one of the more ancient NR groups (Escriva et al., 2000). Interestingly, *EcR* and *Usp* homologs are found in filarial nematodes and there are data to suggest the existence of endogenous ecdysteroids in the parasitic filarid and ascarid nematodes (Cleator et al., 1987; Fleming, 1985; Mendis et al., 1983; Sluder and Maina, 2001, Shea et al., submitted for publication). In addition, specific effects on molting and reproduction have been observed in filarids exposed to exogenous ecdysteroids or to inhibitors of ecdysone synthesis (Barker et al., 1989; Warbrick et al., 1993). Putative orthologs of *DHR38*, *E75*, and *E78* have also been identified in filarids, suggesting that many aspects of the ecdysone signaling cascade could be conserved in filarids (C. Maina and K. Crossgrove, unpublished; Crossgrove et al., 2002). While the ecdysone receptor components are not present in *C. elegans,* the presence of orthologs for the other

NRs in the pathway suggests that the downstream NR functions may be conserved in *C. elegans*, even though the signals that initiate the molting cycle may have diverged.

The data presented here, as well as in previous studies, demonstrate that molting functions of at least some of these 'ecdysone cascade' NRs appear to be conserved in *C. elegans.* The molting defects observed in *nhr-23* and *nhr-25* RNAi animals occur at ecdysis, suggesting that the cuticle shedding process is disrupted in these animals. The overall similarity in the molting phenotypes between *nhr-23*(*RNAi*) and *nhr-25*(*RNAi*) animals suggests that these two NRs regulate similar genes, such as those encoding cuticle collagens (as has been shown for NHR-23, Kostrouchova et al., 2001) or proteases that may be required for cuticle shedding.

Under adverse conditions, *C. elegans* enters an alternate life cycle at the end of the L1 stage, culminating in the formation of a diapausing dauer larva at the L2 to L3 molt (Riddle and Albert, 1997). We have demonstrated that most of the *C. elegans* orthologs of the ''ecdysone cascade'' NRs are also required for dauer development. The observed dauer-defective phenotypes were of two general classes: abnormal dauer morphology and ecdysis defects. *nhr-23* and *nhr-41* RNAi dauers displayed severe dauer morphology defects, suggesting that *nhr-23* and *nhr-41* have critical roles in development of the specialized structure of the dauer larva. *nhr-25* and *nhr-85* RNAi dauer animals exhibited ecdysis defects that, in most cases, were associated with degeneration of internal tissues. However, despite tissue degeneration, the overall morphology of these defective dauer larvae appeared phenotypically wild type. Therefore, the roles of *nhr-25* and *nhr-85* in dauer development may be restricted to the molting process.

The mRNAs of three of the *C. elegans* 'ecdysone cascade' NR genes—*nhr-23*, *nhr-25*, and *nhr-41*—are expressed in an oscillating, reiterated pattern during the larval molting cycles. This oscillation is similar to the reiteration of cuticle collagen gene expression observed during larval development (Johnstone and Barry, 1996) and is consistent with a functional role for these NRs in regulating the molting cycle. The oscillating expression pattern of *nhr-41* suggests that this gene may play a role in molting regulation, despite the absence of an RNAi molting phenotype during continuous growth. The potential molting role for *nhr-41* is intriguing as, unlike *nhr-23* and *nhr-25,* it is expressed in nonepidermal cell types. Most interesting is the neural expression of *nhr-41*, which may indicate a neural regulatory role upstream of the molting processes executed in the hypodermis.

Other NR genes exhibited more complex temporal expression profiles with no obvious relation to the molting cycle. The absence of a reiterated expression pattern for the 'ecdysone cascade' NRs *nhr-6* and *nhr-85*, along with the absence of an RNAi phenotype, could suggest that these genes do not have molting functions during the continuous growth life cycle, though *nhr-85* may have a function during the dauer molt. However, oscillating mRNA expression would not seem to be an absolute requirement for molting function, as NRs have the potential to be regulated posttranslationally by a ligand. Although postembryonic *nhr-67* function is required for larval molting, *nhr-67* mRNA expression levels do not oscillate with each molt. The *Drosophila* homolog of *nhr-67*, *tll*, is required for the development of embryonic termini and the central nervous system (Pignoni et al., 1990). Our data suggest a potential additional function in molting for TLL NRs in the Ecdysozoa.

Several possible expression hierarchies can be proposed for the *C. elegans* 'ecdysone cascade' NRs that exhibit cycling expression profiles. For example, the *nhr-25* and *nhr-41* expression peaks occur at the molt or early in the larval intermolt, and the activity of these two genes could define either the beginning or end of a putative NR molting regulatory pathway. The different mRNA isoforms of *nhr-25* and *nhr-41* have distinct patterns, suggesting that the different protein isoforms have distinct functions. It is tempting to speculate that the LBD-only NR encoded by *nhr-25β* may function as an inhibitor, through dimerization, of another NR that is expressed during molting, similar to the proposed function of the LBD-only NR, E75B, in inhibiting DHR3 during the metamorphic molts in *Drosophila* (White et al., 1997). Genetic analysis will be required for full delineation of any NR hierarchy regulating *C. elegans* molting. Interestingly, the pattern of *nhr-23* and *nhr-25* mRNA expression during the intermolts is similar to the mRNA expression patterns of *DHR3* and *βFtz-F1* during the second larval instar of *D. melanogaster* (Sullivan and Thummel, 2003). *nhr-23* and *DHR3* are both expressed at the middle of the intermolt while *nhr-25* and *βFtz-F1*are expressed at the end of the intermolt. This suggests that a conserved temporal transcriptional hierarchy contributes to the regulation of larval molting in both nematodes and insects.

lin-42, a homolog of the *Drosophila* circadian rhythm gene *period*, is required for the timing of adult cell fate in *C. elegans* (Jeon et al., 1999). *lin-42* mRNA is expressed in a reiterated, fluctuating pattern, with peaks that occur before each molt. In addition, *lin-42* mutants exhibit an ecdysis defect at the L4 to adult molt. The oscillating expression and the ecdysis defect suggest a role for *lin-42* in molting regulation. *lin-42* is one of several genes in the heterochronic pathway that regulates the timing of postembryonic developmental events (Ambros, 2000). The heterochronic and molting pathways both control the timing of distinct events during postembryonic development, but the mechanisms by which these two pathways interact and are coordinated remain unknown. We compared the *lin-42* expression profile with the oscillating NR profiles, finding that *lin-42* expression peaks after that of *nhr-23* but before the *nhr-25* and *nhr-41* expression peaks. The temporal placement of *lin-42* relative to the NR regulatory hierarchy proposed here will provide a context in which to probe heterochronic and molting gene interaction through genetic analysis.

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