The Caenorhabditis elegans Orphan Nuclear Hormone Receptor Gene nhr-2 Functions in Early Embryonic Development

Ann E. Sluder,* Tim Lindblom,* and Gary Ruvkun†

*Department of Cellular Biology, University of Georgia, Athens, Georgia 30602; and †Department of Molecular Biology, Massachusetts General Hospital and Department of Genetics, Harvard Medical School, Boston, Massachusetts 02114

We have identified a Caenorhabditis elegans gene, nhr-2, that is a member of the nuclear hormone receptor superfamily of transcription factors and defines a new subclass of the superfamily. nhr-2 messenger RNA is expressed in the maternal germline and during the first half of embryogenesis. Zygotic expression of nhr-2 begins by the 16-cell stage, making it one of the earliest genes known to be transcribed in the embryo. Immunolocalization detects NHR-2 protein in embryonic nuclei as early as the 2-cell stage. The protein is present in every nucleus until the 16- to 20-cell stage. Subsequently, expression continues in many, but not all, cell lineages, becoming progressively restricted to the anterior and dorsal regions of the embryo and disappearing during the initial stages of morphogenesis. Disruption of nhr-2 function with antisense RNA results in embryonic and early larval arrest, indicating that the gene has an essential function in embryonic development. nhr-2 does not correspond to known mutations mapped to the same genetic interval, and will provide an entry point for further study of a heretofore uncharacterized zygotic gene regulatory pathway.

INTRODUCTION

Two sources of genetic information direct the early development of multicellular organisms. Maternal gene products program the initial events of embryogenesis. These events lead to expression of the zygotic genome, which ultimately assumes control of the developmental process. Establishing differential gene expression among cells of different fates is an important facet of embryogenesis. The maternal controls that pattern the initial expression of embryonic genes employ a combination of asymmetrically distributed determinants and cell interactions (Gurdon, 1992; St. Johnston et al., 1992; Wood et al., 1994). Our understanding of the elaboration of maternally defined patterns into different embryonic gene regulatory events is still incomplete. Embryogenesis in the nematode Caenorhabditis elegans has been described at the level of individual, essentially invariant cell lineages (Sulston et al., 1983), permitting observation of differential gene activity in specific defined cells within a developing multicellular organism. Zygotic transcription can be detected as early as the 4- to 8-cell stage (Edgar et al., 1994; Seydoux et al., 1994), but little is known about the genes expressed, their regulation, or their roles in the execution of processes initiated by maternal gene products (Bowerman, 1995; McGhee, 1995).

Many developmental control genes encode transcriptional regulators, including both maternal and zygotic components of the gene regulatory circuitry (reviewed in Thissen et al., 1992; Davidson, 1994; Lawrence et al., 1994). The nuclear hormone receptor (NHR) superfamily of transcription factors is one class of regulatory proteins represented among known developmental control genes. The defining members of the NHR superfamily were first identified biochemically as receptors for steroid and thyroid hormones (reviewed in Evans, 1988; Mangelsdorf et al., 1995). The ability of these receptors to regulate transcription of target genes is modulated by binding of the hormonal ligands. Additional members of the NHR superfamily have been identified based on sequence similarities to known NRHs (for reviews see Segraves, 1991; Laudet et al., 1992; Mangelsdorf et al., 1995). Some of these have been shown to be receptors for known hormones (Arriza et al., 1987; Giguere et al., 1987; Petkovich et al., 1987; Koelle et al., 1991;
Becker-Andre et al., 1994). Many others, however, are “orphan” receptors for which the ligands, if any, are still unknown. At least some orphan receptors are likely to bind as yet unknown ligands, and the potential for diffusible ligands to establish spatial or temporal domains of NHR protein activity during development provides a possible mechanism for coordinating the fates of diverse cells.

Little is known about the biological roles of most orphan receptors. Genetic analyses and expression studies have indicated that a number of orphan receptors function during embryogenesis. For example, the Drosophila orphan receptor genes knirps and tailless are required during early embryogenesis for establishing abdominal segmentation and specifying terminal structures, respectively. The genes are expressed in the blastoderm-stage embryo in patterns consistent with these genetically defined functions. Expression is also observed during late embryogenesis in domains distinct from those in the early embryo, suggesting additional roles for these genes (Nabauer et al., 1988; Rothe et al., 1989; Pignoni et al., 1990). Similarly, the Drosophila Ftz-F1 orphan receptor has been implicated in embryonic pattern formation (Lavorgna et al., 1991) and in postembryonic development (Ayer et al., 1992; Lavorgna et al., 1993). The mouse homolog of ftz-f1 also functions during both embryonic and postnatal development (Ikeda et al., 1993; Luo et al., 1994). The expression patterns of vertebrate homologs of tailless suggest roles in embryonic development of the central nervous system (Yu et al., 1994; Monaghan et al., 1995). Another orphan receptor, HNF-4, appears to contribute to embryonic endoderm specification in Drosophila (Zhong et al., 1993), Xenopus (Weber et al., 1996), and mouse (Chen et al., 1994).

To examine the roles of other NHR genes in development, we have begun an analysis of the role of orphan receptors in the regulation of zygotic gene expression in the nematode C. elegans. Here we describe the gene nhr-2, which defines a new orphan receptor subclass. nhr-2 expression is detected ACCG* end to provide an entry point to the study of the regulatory pathway(s) in which it functions.

**MATERIALS AND METHODS**

**Nematode Strains**

Nematode strains used included N 2 var. Bristol (wild type) (Brenner, 1974), RW7000 var. Bergerac (Moerman et al., 1984), glp-1(e2414ts) (Priess et al., 1987), fem-2(b2425ts) (Kimble et al., 1984), rol-6(n1270e187) (Park et al., 1986), fer-1(h1c1ts) (Ward et al., 1978), zyg-2(b10) (Wood et al., 1980), emb-10(k12) (Cassada et al., 1981b), emb-14(g43), emb-17(g20), emb-19(g22), and emb-20(g27) (Cassada et al., 1981a). The Caenorhabditis Genetics Center provided some nematode strains. Ann Rose (University of British Columbia) provided the Bristol/Bergerac hybrid strain KR408, dpy-5(h14) unc-29(h2) I, in which the dpy-5 to unc-29 interval is derived from var. Bergerac (Starr et al., 1989). The following strains were also obtained from Ann Rose: Chromosomal duplications (Mckim et al., 1990); KR1293, dpy-5(e61) dpy-14(e188ts) I; dhp12(I;f); KR1280, dpy-5(e61) dpy-14(e188ts) I; dhp313(I;f); KR1284, dpy-5(e61) dpy-14(e188ts) I; dhp15(I;f); KR1282, dpy-5(e61) dpy-14(e188ts) I; dhp16(I;f); KR1772, dpy-5(e61) dpy-14(e188ts) I; hd37(f1); Lethal mutations (McDowall, 1990); KR362, dpy-5(e61) let-388(h88) unc-13(e450); sDp2(I;f); KR430, dpy-5(e61) let-377(h110) unc-13(e450); sDp2(I;f); KR434, dpy-5(e61) let-367(h119) unc-13(e450); sDp2(I;f); KR444, dpy-5(e61) let-378(h124) unc-13(e450); sDp2(I;f); KR446, dpy-5(e61) let-376(h130) unc-13(e450); sDp2(I;f); KR454, dpy-5(e61) let-379(h127) unc-13(e450); sDp2(I;f); KR530, dpy-5(e61) let-393(h225) unc-13(e450); sDp2(I;f); KR612, dpy-5(e61) let-395(h271) unc-13(e450); sDp2(I;f); KR633, dpy-5(e61) let-603(h289) unc-13(e450); sDp2(I;f); KR1339, dpy-5(e61) let-532(h715) unc-13(e450); sDp2(I;f); dpy-5(e61) let-531(h752) unc-13(e450); sDp2(I;f); KR1441, dpy-5(e61) let-530(h798) unc-13(e450); sDp2(I;f).

Strains bearing the Bristol-derived duplications hDp12, hDp13, hDp16, and hDp37 over the Bristol/Bergerac hybrid Chromosome I from KR408 were constructed: GR1001, dpy-5(h14) unc-29(h2) I; dhp12(I;f); GR1002, dpy-5(h14) unc-29(h2) I; dhp313(f1); GR1003, dpy-5(h14) unc-29(h2) I; dhp151(f1); GR1004, dpy-5(h14) unc-29(h2) I; dhp16(I;f); and GR1005, dpy-5(h14) unc-29(h2) I; hd37(f1).

**Oligonucleotides**

**HRF-1:** TGYGGXAYGTGAARXTTTYTYAAY (1024-fold degenerate)

**HRF-2:** TGYGGXCTGTYAARXTTTYTYAAY (2048-fold degenerate)

**HRF-3:** RAARAAAXCYTTRAXCXYTTCRCA (4096-fold degenerate)

**PEP2-10:** CGCGGGATCCCCGAGCCAGCADCGCCAGTGGCC

**PEP2-11:** CGCGGGATCCCCGGTTGGGAGCCGTGGTCCGAGAC

**PEP2-15:** TTTGGGTCTATTAGTTGATTTG

**PEP2-16:** GGGTGGCGCATCGGTGAACTTAGG

**PEP2-17:** CATCTACAAAGAGATTCGCTCG

**PEP2-18:** CATGTGCTTACAGATGGGAGCC

**RLR-1:** GGGATTCGGAGCCCGCCACTGATT

**RLR-2:** TAACTATGGCGGGCCCTCAAGATGCC

**RT:** ATCGATGTCGAGCATCGGATCAACAGCGTGAACGCTTCAGTC

**R:** ATCGATGTCGAGCATCGGATC

**GATCCAAAGCTTGTTGAAGCGTGGC

Degeneracies are indicated as follows: Y for C, T; R for A, G; S for C, G; and X for A, C, G, T. HRF-1 and HRF-2 correspond to coding strand sequences; HRF-3 corresponds to antisense sequences (see below). Oligonucleotides PEP2-10–PEP2-18 are derived from nhr-2 sequences (see Fig. 1C). PEP2-10 and PEP2-11 contain the additional sequence CGCGGGATCC at the 5’ end to provide BamHI sites for cloning of the amplified product.

**Identification of Candidate NHR Genes**

We synthesized three degenerate oligonucleotide probes corresponding to a highly conserved block of eight to nine amino acids
within the DNA binding domain of NHRs ("P box," Fig. 1A). The HRF/HR-2 probes will detect genes of the glucocorticoid receptor subclass of the superfamily, while HRF-3 was designed to detect the estrogen, thyroid hormone, and retinoid receptor classes. In computer searches of GenBank these degenerate nucleotide sequences detect only NHR superfamily members. On Southern blots of known genes the HRF oligonucleotides hybridized to NHR genes of the appropriate class but not to genes containing TFI11A-type "zinc finger" DNA binding domains (e.g., Xenopus TFI11A, Ginsberg et al., 1984; Drosophila Krüppel, Rosenberg et al., 1996, data not shown).

The HRF oligonucleotides were used to probe ordered filter grids of cosmid genomic libraries as described (Bürglin et al., 1989). Two separate screens were conducted, one with a pool of 50% HRF-1/50% HRF-2 and one with HRF-3. After hybridization the filters were washed sequentially at 53, 58, and 63°C, and exposed to film following each wash (Bürglin et al., 1989). The 53, 58, and 63°C washes theoretically allowed 3, 2, and 1 mismatches respectively for HRF-1/HRF-2 and 2, 1, and 0 mismatches for HRF-3 (Wood et al., 1985; Jacobs et al., 1988). Fifty-nine hybridizing cosmids were identified, representing 47 loci on the physical genomic map (Coulson et al., 1988). Twenty-six of these loci hybridized to HRF-1/HRF-2 (7 at 53°C, 9 at 58°C, 10 at 63°C), 14 to HRF-3 (at 53°C, 6 at 58°C, 3 at 63°C), and 7 to both probes (6 at 53°C, 1 at 58°C). Southern blots bearing each of these cosmids digested with EcoRI were probed with the HRF oligonucleotides to verify the hybridization to individual cosmids and to identify restriction fragments for subcloning and sequencing. To date 5 of the loci detected with HRF-3 (1 at 53°C, 2 at 58°C, 2 at 63°C) have been found to encode NHR superfamily members. DNA sequence analysis of hybridizing restriction fragments has shown that 2 of the HRF-3 loci (1 at 53°C, 1 at 58°C) and 7 of the HRF-1/HRF-2 loci (1 at 58°C, 6 at 63°C) were false positives in which the probes hybridized to regions of partial homology in what appear to be introns (AS and S. Gottlieb, unpublished). The sequences of the remaining 33 hybridizing clones have not yet been determined.

The cosmid AD7 discussed in this report contains a 1.1-kb EcoRI fragment which hybridized strongly to HRF-3 following the 63°C wash. The other confirmed NHR genes will be described elsewhere.

**PCR Analysis of nhr-2 cDNA**

First-strand cDNA was synthesized from 0- to 12-hr embryo poly(A)⁺ RNA with M-MLV reverse transcriptase (Bethesda Research Laboratories). This cDNA was used as a template for PCR amplification of nhr-2 sequences using primers PEP2-10 and PEP2-11. The 1.2-kb product was subcloned and sequenced; two error-free clones were identified from 12 independent transfectants.

For 5' rapid amplification of cDNA ends (RACE) first-strand cDNA (prepared from mixed stage N2 total RNA) to which the oligo RLR-1 had been ligated using RNA ligase (Dumas et al., 1988), a gift from M. Finney, was amplified with primers RLR-2 and PEP2-15. The products of this reaction were diluted 100-fold and used as the template in a second round of amplification using primers RLR-2 and PEP2-16. The resulting ~145-bp product was gel-purified and subcloned. Ten cloned inserts were sequenced to determine the position of RLR-1 sequences and from that to infer the location of the 5' end of the cDNA. The 10 clones were identical in sequence.

A 3' RACE was performed by an adaptation of the protocol described by Frohman and Martin (1989). First-strand cDNA was synthesized from N2 0- to 12-hr embryo total RNA using primer RT. This cDNA was used as template for PCR amplification with primers R₃ and PEP2-17. A second round of amplification was performed using primers Rₛ and PEP2-18. The ~480-bp product was gel-purified and subcloned. Ten cloned inserts were sequenced in order to determine the position of the 3' end of the cDNA. Two different classes of clones were obtained (6 of one, 4 of the other); the site of poly(A) addition in the two classes differed by 5 nucleotides.

**Isolation of RNA from Staged Cultures**

Wild-type animals were grown at 20°C in 500-ml liquid cultures (Sulston et al., 1988). Embryos isolated by sodium hypochlorite treatment were frozen (−80°C) as 0- to 12-hr embryos or were allowed to hatch overnight without food to establish synchronized cultures. One such culture was harvested without feeding to obtain L1 animals. The remaining cultures were fed and their growth monitored. Prior to harvesting the stage of the animals was verified by examining cuticle alae and the extent of gonad development under Nomarski optics. L2's were collected after ~17 hr of growth, L3's after ~30 hr, L4's after ~43 hr, and adults after ~72 hr. To isolate staged embryos, young synchronized adults containing 0-2 embryos were taken as time "0" for embryo staging. Then, 2.5 hr later (most animals contained 2-6 embryos) the embryos were isolated by sodium hypochlorite treatment and one-half were frozen as 0- to 3-hr embryos. The remainder were incubated in S basal at 20°C for 3 hr, then were frozen as 3- to 6-hr embryos.

Plate cultures of glp-1(e2141ts) and fem-2(b245ts) were started with embryos isolated from cultures grown at 15°C. These were raised at 25°C until ~90% of the animals were adults (~48 hr). The glp-1 mutant hermaphrodites had gonads with adult morphology but no germ cell or embryo production. The fem-2 mutant hermaphrodites contained oocytes but no embryos.

Total RNA was purified by an adaptation of a protocol described in Ausubel et al. (1991). Selection of poly(A)⁺ RNA was as described (Sambrook et al., 1989).

**Preparation of Anti-NHR-2 Antibodies and Immunostaining**

A glutathione S-transferase (GST) NHR-2 fusion was constructed by cloning the nhr-2 cDNA into pGEX-3X (Smith et al., 1988). A maltose binding protein (MBP) NHR-2 fusion was similarly constructed using a derivative of pMAL-RI (New England Biolabs). SDS-polycrylamide gels slices containing the fusion proteins were used to produce rabbit antisera (Harlow et al., 1988). GST/NHR-2 was used for the initial immunization and the first boost; the fusion proteins were used alternately for subsequent boosts.

To produce affinity columns for antibody purification, the fusion proteins were purified by electrophoresis from SDS-polycrylamide gels (Harlow et al., 1988) and coupled to CN Br-activated Sepharose 4B (Pharmacia). Prior to affinity chromatography (Robbins et al., 1984) serum proteases were removed using DEAE-Affiged blue (BioRad) and the combined antibody fractions were passed through a column of total Escherichia coli protein coupled to CN Br-activated Sepharose 4B. The affinity-purified antibodies react specifically with the fusion proteins on Western blots (not shown).

Fixation of animals for immunostaining was as described (Finney et al., 1990) except that the oxidation and reduction steps were omitted for embryos isolated by alkaline hypochlorite treatment and heptane was omitted from the fixation. Indirect immunofluorescence utilized FITC- and rhodamine-conjugated secondary antibodies (Cappel). Identification of embryonic cells was aided by double-staining with the mouse monoclonal antibody OIC1D4, which recognizes P granules (Strome et al., 1983). Embryos immuno-
FIG. 1. nhr-2 gene structure and sequence. (A) Alignment of the predicted NHR-2 DNA-binding domain with a subset of superfamily members representing various subclasses of the family. Amino acid sequences represented by the oligos HRF-1/HRF-2 and HRF-3 (HRF-3a shows alternate amino acids allowed by the degeneracy) are also included. The alignment was generated and edited using the GCG Pileup and Lineup programs (Devereux et al., 1984) and the Boxshade program (developed by Kay Hofman). The P box and D box regions are as defined for classical hormone receptors (Tsai et al., 1994). The T box and A box regions marked are as defined in Rastinejad et al. (1995). We note that an alternative alignment of the second zinc finger module of NHR-2 is possible, placing the seven additional amino acids within the D box region. The alignment shown preserves the D box spacing observed in other superfamily members. Black boxes indicate amino acids identical to those found in NHR-2; gray boxes indicate conservative changes. Species abbreviations used: Ce, C. elegans; Dm, Drosophila melanogaster; Hum, human; Mus, mouse; Rat. GenBank references for the sequences are given in the legend to

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.
D. (B) nhr-2 gene structure (Zn finger motif or C domain as defined in Tsai et al., 1994), determined as described in the text. Exon sequences are indicated by bars, solid bars indicate predicted coding regions, and open bars indicate predicted untranslated regions. The arrow indicates the start and direction of transcription. The nhr-2/lacZ fusion gene described below is also diagrammed. (C) Genomic sequence of nhr-2 (GenBank Accession No. U37424). Exons are in uppercase letters, introns and nontranscribed regions in lowercase letters. The amino acid sequence of the predicted translation product is shown in single-letter code below the DNA sequence. The underlined amino acid sequences represent the core DNA binding domain motif. Features indicated by underlining and labeling of the appropriate DNA sequences are: (1) PCR primer sites used in the amplification of cDNA; those designated “r” were synthesized as the reverse complement of the underlined sequence. Note that the primer PEP2-16 site is interrupted by an intron in the genomic sequence. (2) EcoRI (RI) and HindIII (H3) restriction sites. The HindIII site at which nhr-2 sequences were fused to lacZ is noted. (3) Homology to the HRF-3 degenerate oligonucleotide probe. (4) The consensus poly(A) addition signal preceding the sites of poly(A) addition determined by RACE. The two poly(A) addition sites detected by RACE are indicated by A’s above the last nucleotide preceding the poly(A). (D) Comparison of the predicted NHR-2 core DNA binding domain (underlined in C) to other members of the NHR superfamily. A comparative tree of DNA-binding domain amino acid sequences representing a cross section of the superfamily was generated using the GCG Pileup program. Species abbreviations are as in A. Sequences included are (GenBank accession numbers given in parentheses): AR, androgen

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.
stained with OIC1D4 were fixed with methanol and acetone (Strome et al., 1983); the samples were kept at 4°C at all times.

Construction and Analysis of Transgenic C. elegans Strains

A 5' flanking 6-kb HindIII fragment (Fig. 1B) was subcloned into pPD21.28Δ5 (pPD21.28 of Fire et al., 1990, modified by filling in the SalI site; T. Burgin, B. Reinhart, and GR, unpublished data) to give pAS3. pAS3 (25 μg/ml) was co-injected with the dominant rol-6(su1006) clone pRF4 (175 μg/ml) into mgEx1, mgEx2; mgEx9; mgEx10; and mgEx11. Embryos were fixed and stained with X-gal to determine the pattern of NHR-2/β-galactosidase fusion protein expression. Self-progeny embryos of transgenic hermaphrodites were collected, fixed, and stained in parallel for comparison.

Tests for Complementation of Mutations by the nhr-2 Gene

Transgenic arrays bearing AD7 plus the rol-6(su1006) marker were generated in rol-6(m1270e187). The array mgEx9 was used in the experiments described here.

For nonconditional lethal mutations mapped to the same interval as nhr-2, transgenic males bearing mgEx9 were crossed to hermaphrodites carrying the lethal mutation of interest on a genetically marked Chromosome I balanced by a chromosomal duplication (dpy-5(e61); let-367(h124), let-379(h127), let-388(h88), let-393(h225), let-395(h271), let-396(h798), let-397(h752), let-398(h715), and let-399(h289). Self-progeny embryos of transgenic hermaphrodites were collected, fixed, and stained in parallel for comparison.

FIG. 1—Continued

The observed expression pattern was the same for mgEx1 and mgEx2; mgEx2 exhibits a higher degree of meiotic transmission and was used for most experiments.

To produce transgenic embryos inheriting the nhr-2/β-galactosidase fusion gene paternally, males carrying mgEx2 were mated to nontransgenic hermaphrodites, fer-1(hc1ts) hermaphrodites, raised at 25°C and thus incapable of self-fertilization (Ward et al., 1978), were used to minimize the background of (nontransgenic) self-progeny. 25–30 Rol mgEx2 males were placed with 50–60 fer-1 25°C hermaphrodites on each of 6 mating plates at 25°C. The animals were moved to fresh plates and the embryos which had been laid were washed off with M9 buffer at 6- to 12-hr intervals for a total of 36–48 hr. Embryos were fixed and stained with X-gal to determine the pattern of NHR-2/β-galactosidase fusion protein expression. Self-progeny embryos of transgenic hermaphrodites were collected, fixed, and stained in parallel for comparison.

Transgenic arrays bearing AD7 plus the rol-6(su1006) marker were generated in rol-6(m1270e187). The array mgEx9 was used in the experiments described here.

For nonconditional lethal mutations mapped to the same interval as nhr-2, transgenic males bearing mgEx9 were crossed to hermaphrodites carrying the lethal mutation of interest on a genetically marked Chromosome I balanced by a chromosomal duplication (dpy-5(e61); let-x unc-13(s350) I; sDp2(f); see above). F2 cross-progeny were scored for rescue of the lethal Dpy Unc class by the array. Mutations tested included let-367(h119), let-376(h130), let-378(h124), let-379(h127), let-388(h88), let-393(h225), let-395(h271), let-396(h798), let-397(h752), let-398(h715), and let-399(h289). No rescue was observed. While fertile Dpy Unc progeny were recovered from the crosses with let-351(h752) and let-352z(h715), Dpy Uncs were also recovered when these strains were crossed to N2 males. let-377(h110), also mapped to this genetic interval, was not tested because we were unable to observe the lethal class in two different stocks of the strain KR430.

Hermaphrodites homozygous for the maternal effect mutations roller (rol-6(n1270e187)) or seven-up (emb-20(g27)) were crossed to let-377(h110), modiﬁed by ﬁlling in the SalI site; T. Burgin, B. Reinhart, and GR, unpublished data) to give pAS3. pAS3 (25 μg/ml) was co-injected with the dominant rol-6(su1006) clone pRF4 (175 μg/ml) into mgEx1, mgEx2; mgEx9; mgEx10; and mgEx11. Embryos were fixed and stained with X-gal to determine the pattern of NHR-2/β-galactosidase fusion protein expression. Self-progeny embryos of transgenic hermaphrodites were collected, fixed, and stained in parallel for comparison.

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.
Antisense RNA Injections

For in vitro production of RNA, the nhr-2 cDNA was cloned in both sense and antisense directions behind a T7 RNA polymerase promoter in a derivative of the pET3c vector (Studier et al., 1990) from which the copy-control region (between the BglII and PvuII restriction sites) had been deleted. Plasmid pK8 for production of antisense mes-6 RNA was a gift from I. Korf and S. Strome (Indiana University). Plasmid DNAs were linearized with appropriate restriction enzymes to generate templates for runoff production of RNA. RNA (uncapped) was produced using the T7 Megascript in vitro transcription kit from Ambion, Inc. (Austin, TX). Transcription reactions were injected directly without further purification.

Wild-type (N2) hermaphrodites were injected in both distal gonad arms and placed on individual plates to allow egg-laying. Total postinjection broods were counted. Parents were transferred to new plates and eggs and larvae were counted every 12 hr. Embryonic arrest (failure to hatch) was assessed after one day. To assess larval arrest, progeny that grew past the L1 stage were counted after 2–3 days. Arrested L1 larvae were observed on the plates, but were difficult to count accurately due to their transparency and minimal movement.

Material effect sterility (Mes phenotype) resulting from RNA injections was assessed by placing individual progeny of injected animals on separate plates (at least 10 progeny were cloned from each injected parent). These progeny were examined for egg production once they became adults. Mes hermaphrodites could be recognized in the progeny of parents injected with antisense mes-6 RNA, and the selection of animals cloned was biased toward these to verify the Mes phenotype. Of 346 cloned F1 progeny from 7 hermaphrodites injected with antisense mes-6 RNA, 199 were sterile (for individual those of the mutant strains range, 10.3–79.4%). No obviously Mes animals were observed in the progeny of parents injected with antisense nhr-2 RNA; of 83 F1 progeny cloned from 5 injected parents none were sterile.

RESULTS

nhr-2, a C. elegans Member of the Nuclear Hormone Receptor Superfamily, Defines a New Class of Orphan Receptor

Three degenerate oligonucleotide probes corresponding to a highly conserved region of the NHR DNA-binding domain were used to screen C. elegans cosmid genomic libraries for NHR genes (see Materials and Methods). Cosmid AD7 hybridized strongly with the probe HRF-3, and contains a region of exact homology to HRF-3 and a DNA binding domain motif characteristic of the NHR superfamily (Fig.1A). This gene encoding a C. elegans member of the NHR superfamily has been designated nhr-2.

Exons were predicted from the nhr-2 genomic sequence using frequency tables for C. elegans splice sites (Fields, 1990). The structure of the nhr-2 transcript was determined by polymerase chain reaction (PCR) amplification of first-strand cDNA with primers from within predicted exons. The amplification product from the most widely separated primers yielding a product was subcloned and sequenced to verify the predicted splicing pattern. The 5' and 3' ends of the transcript were positioned using RACE (see Materials and Methods). The 3' end of the transcript determined by RACE is immediately preceded by a consensus poly(A) addition signal. No sequences corresponding to the transspliced leader RNA As SL1 (Krause et al., 1987) or SL2 (Huang et al., 1989) were detected at the 5' end of the nhr-2 transcript either in the 5' RACE product or by direct PCR amplification of first-strand cDNA using SL- and nhr-2-specific primers. The predicted transcript size (1614 bp) is consistent with the size of the single nhr-2 mRNA detected on a Northern blot (Fig.2).

The amino acid sequence of the predicted NHR-2 DNA binding domain is 57% identical to the human rev-erbα orphan receptor (Miyajima et al., 1988) within the “zinc finger” core DNA binding domain (Fig. 1A). The predicted NHR-2 core DNA binding domain is most similar to that of the retinoid and thyroid hormone receptors and a number of orphan receptors, but is not a clear homolog of any other known NHR DNA binding domain (Fig. 1D). Two additional structural elements C-terminal to the core DNA binding domain, the “T box” and “A box”, also contribute to DNA binding by some NHRs (Rastinejad et al., 1995 and references therein). NHR-2 similarity to other NHRs extends to the T box but not the A box. In contrast, a C. elegans relative...
of the mammalian orphan receptor NGFI-B exhibits 72% amino acid identity to the rat protein within the core DNA binding domain as well as extensive identity in both the T and A boxes (Wilson et al., 1992; Kostrouch et al., 1995). Compared to other known NHRs the predicted NHR-2 protein has seven extra amino acids in the second “zinc finger” module (Fig. 1A). This and a lack of significant similarity between NHR-2 and other NHRs outside regions involved in DNA binding suggest that NHR-2 represents a new subclass of orphan receptor.

**nhr-2 RNA Is Present in Embryos and in the Maternal Germline**

Developmental Northern blot analysis detects a 1.6-kb nhr-2 mRNA in embryos and at low levels in young adult hermaphrodites, but not in larval stages L1 through L4 (Fig. 2). To determine whether the RNA detected in adults was due to maternal transcription or from developing embryos still within the uterus, RNA was prepared from adults of mutants exhibiting defects in germline development. glp-1(e2141ts) hermaphrodites raised at restrictive temperature contain a somatic gonad but no germline (Priess et al., 1987). In fem-2(b245ts) hermaphrodites raised at restrictive temperature the germline produces oocytes but no sperm (Kimble et al., 1984); therefore these animals cannot self-fertilize and contain no embryos in the gonad. nhr-2 message is detected in fem-2 mutant adults but not in glp-1 mutant adults (Fig. 2), indicating that the gene is transcribed in the maternal germline. Analyses of NHR-2 protein expression and of the expression of a nhr-2/lacZ fusion gene in transgenic animals suggest that the gene is also transcribed zygotically (see below).

**NHR-2 Protein Is Expressed during the First Half of Embryogenesis**

To examine the NHR-2 expression pattern, rabbit antisera were raised against NHR-2 fusion proteins produced in E. coli. Immunocytochemistry with affinity-purified antibodies detects NHR-2 in the nuclei of embryos as early as the two cell stage (Fig. 3). The protein is present in every nucleus until the 16- to 20-cell stage and then is no longer detected in the germine precursor P4 and its sister D; at this time the levels of NHR-2 in the other cells increase. No immunostaining is observed in cells that are undergoing or have just completed mitosis, even when both mother and daughter cells exhibit strong NHR-2 expression. This suggests that NHR-2 levels are cell cycle regulated and that protein expression in the daughter cells represents resynthesis from maternal mRNAs and/or by zygotic expression. By the 28-cell stage expression in the E and MS descendants is variable and generally weak (especially in the E cells) when present. Expression in the descendants of ABp and ABpr at this stage is also somewhat variable but can be quite strong. The other 10 AB cells and the 4 C cells exhibit reproducible strong expression; P4 and D continue to exhibit no expression.

Past the 28-cell stage the staining pattern is complex and changes rapidly (Figs. 3F–3K), and definitive cell identifications of all expressing cells at all stages have not been made. At the ~51-cell stage P4 and the descendants of E do not express NHR-2; expression continues in the C cells, many AB cells, and in some MS cells (particularly those in anterior and dorsal positions). As embryogenesis progresses NHR-2 expression becomes restricted primarily to the anterior and dorsal regions of the embryo. Nuclei staining at the ~250-cell stage include (but are not limited to) the Cp descendants that contribute to the hyp7 syncytium and many but not all AB descendants; germ line precursors and intestinal nuclei do not stain. NHR-2 is last detected in one or a few nuclei in the vicinity of the excretory cell before expression ceases during the initial stages of morphogenesis (very early “comma” stage).

No expression of NHR-2 is detected by immunostaining in any developmental stage other than embryos, whereas control antibodies demonstrate that these later stage animals were fixed and permeabilized properly (data not shown). No immunostaining is observed following preabsorption of the affinity-purified antibodies with either GST/NHR-2 or MBP/NHR-2 fusion protein; preabsorption with total E. coli protein containing GST or MBP does not affect the immunostaining (not shown).

**The nhr-2 Promoter Is Active in the Early Embryo**

A nhr-2/lacZ fusion gene was constructed and transformed into worms. The construct contains approximately

---

**FIG. 3.** Immunofluorescent detection of NHR-2 expression. Embryos were immunostained with affinity-purified anti-NHR-2 antibodies. For each panel the left half shows the pattern of staining with anti-NHR-2 antibodies; the right half shows nuclei visualized by DAPI staining of the same embryo. Anterior is to the left in each image. A–K show wild-type embryos. (A) Two cell embryo. NHR-2 is detected in both nuclei. (B, C) 14-cell embryo, dorsal view. E and MS blastomeres have completed division, the C blastomere is in early prophase, and P3 has not yet begun to divide. NHR-2 is detected in every nucleus. Upper (B) and lower (C) planes of focus. (D, E) 24-cell embryo, dorsal view. NHR-2 is not detected in P4 (arrow). D (black arrowhead), and Ea and Ep (white arrowheads). Upper (D) and lower (E) planes of focus. (F, G, H) ~50-cell embryo. Many posterior, internal cells (primarily P4 and E lineage) do not stain, while most if not all AB and C descendants and many MS descendants do. Upper (F), central (G), and lower (H) planes of focus. (I, J) ~200-250-cell embryo, right dorsal view. Hyp7 precursors on dorsal surface stain strongly; many other cells stain more weakly. Upper (I) and lower (J) planes of focus. (K) ~500-cell embryo (early “comma”), ventral view. One cell in the presumptive head region stains. (L) Embryos from msEx9-bearing hermaphrodites. Compare the two ~24-cell embryos, one exhibiting wild-type levels of staining (white arrowhead) and one apparent transgenic embryo exhibiting elevated levels of staining (black arrowhead).
5 kb of upstream sequence as well as nhr-2 coding sequence through the middle of the zinc finger motif (Figs. 1B and 1C). Fusion protein expression is first detected, by either anti-β-galactosidase immunostaining or histochemical staining, at the 16- to 20-cell stage in all cells except P4 and D. Expression becomes progressively restricted in a pattern mirroring that observed for NHR-2, and is ultimately observed in 2-4 cells in the head region before disappearing entirely by early comma stage (Fig. 4). As with native NHR-2, no expression of the fusion protein is detected in cells during or just after mitosis. Unlike the NHR-2 expression detected by immunostaining, fusion protein expression is not observed prior to the 16-cell stage and is never observed in the germ line lineage.

The initial expression of the fusion protein and the early increase in levels of NHR-2 protein exhibit the same spatial and temporal patterns, occurring approximately one cell cycle after zygotic transcription is first detected (Edgar et al., 1994). This suggested that the portion of the NHR-2 expression pattern reproduced by the fusion gene is due to zygotic expression. To establish this, transgenic males carrying the fusion gene were mated to nontransgenic hermaphrodites. Any fusion protein expression in the cross-progeny will arise from zygotic transcription of the gene (barring an unusual paternal contribution). The pattern of fusion protein expression observed in the cross-progeny is indistinguishable from that in the self-progeny of transgenic hermaphrodites (Fig. 5), indicating that zygotic expression of the fusion gene is sufficient to generate the observed pattern. These results in combination with the developmental Northern blot analysis suggest that nhr-2 is transcribed both in the maternal germline and in the developing embryo. NHR-2 protein expression prior to the 16-cell stage likely arises from translation of maternal mRNA, while zygotic transcription is sufficient to produce the later expression.

**Correlation of Physical and Genetic Maps in the nhr-2 Region**

The position of cosmid AD7 on the physical genomic map places nhr-2 on Chromosome I between the genes dpy-5 and bli-4 (Fig. 6A). The genetic interval containing nhr-2 was further defined by mapping flanking restriction map polymorphisms (RFLPs) relative to the endpoints of free chromosomal duplications. A Southern blot was prepared bearing genomic DNA from the strains N2 (Bristol), RW7000 (Bergerac), KR408, GR1001, GR1002, GR1003, GR1004, and GR1005. In strain KR408 the dpy-5 to unc-29 interval of Chromosome I is derived from Bergerac (Starr et al., 1994).
The strains GR1001-GR1005 carry Bristol-derived Chromosome I duplications and the KR408 Chromosome I. The duplications have genetically defined right-hand endpoints in the region containing nhr-2. hDp16 extends the farthest, followed, in order of decreasing extent, by hDp12, hDp37, hDp13, and hDp15 (McKim et al., 1990). The blot was probed to determine the extents of these duplications relative to Bristol/Bergerac RFLPs of known physical map positions.

The polymorphism stP124, one of the “sequence-tagged sites” described by Williams et al. (1992), lies approximately 60 kb to the right of nhr-2. The Bristol allele of this locus was present with hDp12 and hDp16, but not with hDp15, hDp13, or hDp37 (Fig. 6B). The right boundary of the genetic interval containing nhr-2 is thus defined by the right endpoint of hDp12, the shortest duplication that covers stP124.

The mgP29 polymorphism is approximately 80 kb to the left of nhr-2; the restriction map of the Bergerac allele of mgP29 is consistent with it being an insertion of the transposon Tc1 (data not shown). The Bristol allele of mgP29 was observed with hDp12 and hDp16 but not with hDp15 and hDp13 (Fig. 6B). The results for hDp37 were inconclusive due to a low level of meiotic transmission of this duplication compounded by a background of the Bristol allele (even in the Bergerac strain) from frequent apparent somatic excision of the mgP29 Tc1. These results place the left boundary of the nhr-2 genetic interval at the right endpoint of hDp13, the longest duplication that does not cover mgP29.

**nhr-2 Does Not Correspond to Known Genetic Loci in the Same Interval**

The lethal complementation groups let-367, let-376, let-378, let-379, let-388, let-393, let-530, let-531, let-532, let-533, and let-534 are located in the same interval as nhr-2. However, these loci are not syntetic with nhr-2, suggesting that nhr-2 represents an additional genetic locus.

**FIG. 6.** Correlation of physical and genetic maps in the nhr-2 region. (A) Positions of clones representing nhr-2 (cosmid AD7), mgP29 (cosmid C08C11), and stP124 (phage RW#L124) on the current physical map of the C. elegans genome (Coulson et al., 1988, 1991) are shown in the upper diagram. The genetic map aligned below indicates the positions of the lethal complementation groups (McDowall, 1990) and RFLPs (this work) relative to the extent of chromosomal duplications. (B) A Southern blot bearing Hpa-di-gested genomic DNA from the strains N2 (Bristol; lane 1), RW7000 (Bergerac; lane 2), KR408 (lane 3), GR1003 (hDp15; lane 4), GR1002 (hDp13; lane 5), GR1005 (hDp37; lane 6), GR1001 (hDp12; lane 7), and GR1004 (hDp16; lane 8) was hybridized with 32P-labeled DNA probes and exposed to X-ray film. Bands representing Bristol and Bergerac alleles of the polymorphisms are indicated by Br and BO, respectively. The upper panel shows detection of the stP124 polymorphism by hybridization with oligonucleotide 093 (Williams et al., 1992). In the lower panel the mgP29 polymorphism is detected by hybridization with cosmids C08C11. The Bristol allele band of mgP29 was quantitated using a Molecular Dynamics 300A densitometer; for each lane values were normalized to the nonpolymorphic band just below to account for any differences in amounts of DNA on the blot. Relative to KR408, the levels of Bristol allele detected in the various strains were as follows: N2, 480%; RW7000, 115%; KR408, 100%; GR1003, 121%; GR1002, 148%; GR1005, 168%; GR1001, 275%; GR1004, 241%.

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.
532, and let-603 have been mapped to the genetic interval containing nhr-2 (Fig. 6A, McDowall, 1990); no existing chromosomal deficiencies remove the nhr-2 region. To determine if nhr-2 corresponds to any of the genes mapped to the same interval we tested for complementation of the lethal mutations by mgEx9, a transgenic extrachromosomal array bearing cosmids AD7 (see Materials and Methods). No complementation was observed for any of these mutations, even though mgEx9 transgenic embryos exhibit a two- to three-fold elevation in NHR-2 protein levels as judged by immunostaining, indicating that the array does support expression of the protein (Fig. 3L). These results have been partially confirmed by the subsequent rescue of let-367, let-376, let-378, let-393, let-395, let-530, let-532, and let-603 with cosmids mapped closely to one side or the other of AD7 and by the failure of a second array spanning nhr-2 to complement any of the lethal mutations against which it has been tested to date (J. McDowall and A. Rose, personal communication).

The maternal effect lethal complementation groups zyg-2, emb-10, emb-14, emb-17, emb-19, and emb-20 have been broadly mapped on the right-hand half of Chromosome I (Wood et al., 1980; Cassada et al., 1981a,b). nhr-2 does not appear to correspond to any of these genes because mgEx9 also failed to complement temperature-sensitive mutations in these genes (see Materials and Methods). However, nhr-2 may not be expressed maternally from mgEx9 and zygotic expression may not be sufficient for rescue of the mutant phenotype (see Discussion), so it is still possible that nhr-2 is represented by one of these maternal effect mutations.

**Disruption of nhr-2 Function Results in Embryonic and Early Larval Developmental Arrest**

Injection of antisense RNA into the syncytial region of the hermaphrodite gonad has been used to disrupt the function of maternal effect genes in the early C. elegans embryo (Guo et al., 1995; Lin et al., 1995). Many of the postinjection progeny of an injected hermaphrodite exhibit a phenotype consistent with disruption of the targeted gene. Though the mechanism by which the injected RNA interferes with gene activity is still unclear, the injections have resulted in reliable phenocopies of known mutant phenotypes.

The maternal and early embryonic expression of nhr-2 makes its function a likely target for disruption by RNA injection. Injection of antisense nhr-2 RNA into the syncytial gonads of wild-type hermaphrodites causes embryonic arrest in the progeny of injected animals (Table 1). In addition, many of the progeny that hatch then arrest as L1 larvae. As has been observed by others (Guo et al., 1995), injected sense RNA has the same effect as does injected antisense RNA. The embryonic and larval arrest phenotypes also result from injection of a shorter antisense RNA that does not contain sequences for the DNA binding domain motif, the only region of nhr-2 exhibiting detectable homology to other known C. elegans sequences. Control injections of in vitro transcription reactions programmed with cloning vector alone do not cause arrest (Table 1). Specificity of the effects of injected RNA was further demonstrated with antisense mes-6 RNA, which does not cause embryonic or larval arrest (Table 1). mes-6 function is required maternally for germline development, and mes-6 mutants exhibit a grandchildless phenotype (Capowski et al., 1991). Disruption of mes-6 function by antisense RNA injection causes progeny of injected hermaphrodites to be sterile (I. Korf and S. Strome, personal communication). We observed a significant number of sterile progeny following antisense mes-6 injections, but not following antisense nhr-2 injections (see Materials and Methods).

Embryos arrested by nhr-2 antisense injection fail to undergo morphogenesis, but frequently display twitching movements indicative of body wall muscle differentiation. Structures characteristic of pharyngeal and gut differentiation are also observed in arrested embryos (Figs. 7B and 7C). Thus some developmental processes proceed in these embryos, indicating that their arrest arises from disruption of specific events and not from general cell death. Antisense nhr-2-induced arrested larvae exhibit sluggish and uncoordinated movement, are often squat in appearance (Fig. 7E), and frequently show regions of the cuticle separated from the underlying tissue (Fig. 7F). These observations suggest that lack of nhr-2 gene activity causes defects in neuroectodermal differentiation.

The results of these RNA injections indicate that nhr-2 has an essential role in the developing embryo. The two classes of arrest phenotypes may be due to variation in the level of antisense nhr-2 RNA-mediated interference with nhr-2 function. The early embryonic arrest phenotype may occur in animals where there has been complete disruption of nhr-2 function, whereas the later larval arrest phenotype may result from partial disruption of nhr-2 function. Several factors, including instability of the injected RNA, uneven partitioning of the RNA within the developing embryo, and partial alleviation of the effects of the RNA by zygotic nhr-2 expression, may contribute to a high fraction of the presumed weak antisense phenotype in these experiments. Similar variations in phenotypic severity have been observed following antisense RNA disruption of other zygotically expressed genes (M. Krause, personal communication). Full analysis of nhr-2 function will require the identification of mutant alleles. The phenotypes revealed by these antisense experiments delimit the mutant phenotypes likely to result from nhr-2 mutations, and the mapping of nhr-2 to the dpy-5 bli-4 region suggests where such genetic analysis should be focused.

**DISCUSSION**

We have identified a C. elegans gene, nhr-2, that encodes a member of the nuclear hormone receptor superfamily. The highest levels of NHR-2 protein expression occur in 16- to 50-cell embryos, when many cell fates are being established (Sulston et al., 1983), and disruption of nhr-2 function results in embryonic and early larval developmental arrest. NHR-2 is thus a strong candidate to participate in the regu-
TABLE 1
Antisense RNA Disruption of nhr-2 Function

<table>
<thead>
<tr>
<th>RNA injected</th>
<th>n</th>
<th>Mean</th>
<th>Range</th>
<th>% Embryo</th>
<th>% Larval</th>
<th>Total %a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisense nhr-2</td>
<td>19</td>
<td>Mean</td>
<td>8.0 ± 8.3</td>
<td>0–29.8</td>
<td>7.8–53.3</td>
<td>10.9–57.8</td>
</tr>
<tr>
<td>Sense nhr-2</td>
<td>7</td>
<td>Mean</td>
<td>6.3 ± 8.2</td>
<td>0.9–24.4</td>
<td>7.6–35.3</td>
<td>21.5–37.2</td>
</tr>
<tr>
<td>Short antisense nhr-2</td>
<td>5</td>
<td>Mean</td>
<td>11.6 ± 6.2</td>
<td>4.8–19.0</td>
<td>19.3–37.3</td>
<td>28.0–49.1</td>
</tr>
<tr>
<td>Negative control</td>
<td>5</td>
<td>Mean</td>
<td>1.3 ± 1.6</td>
<td>0–3.8</td>
<td>0–3.1</td>
<td>0–5.4</td>
</tr>
<tr>
<td>Antisense mes-6</td>
<td>7</td>
<td>Mean</td>
<td>1.1 ± 0.8</td>
<td>0–2.1</td>
<td>0</td>
<td>0–0.1</td>
</tr>
</tbody>
</table>

Note. RNAs were injected into the syncytial gonad (both arms) of young adult hermaphrodites. Total postinjection broods of injected animals were counted as described under Material and Methods. Arrest is expressed as percentage of the total brood. n is the number of injected parents (only animals surviving >48 hr after injection were included). Injected animals laid an average of ~150 eggs each. Short nhr-2 antisense RNA contained sequences corresponding to the C-terminal portion of the NHR-2 protein and omitted sequences corresponding to the conserved DNA binding domain motif. Negative control injections were of in vitro transcription reactions programmed with cloning vector alone.

*For some plates the sum of total larvae and arrested embryos was less than the initial number of eggs laid. This was most pronounced for nhr-2 RNA injections, and we suspect that the discrepancy arises from the degeneration of arrested embryos or larvae. Thus the levels of lethality could actually be somewhat higher than those presented.

Orphan Receptor in C. elegans Embryogenesis

The expression of NHR-2 in cells with diverse developmental fates suggests that it may participate in several regulatory pathways, regulating distinct sets of genes in different cells, perhaps in heterodimeric combinations with other NHR proteins. The gene will provide an entry point for genetic analysis of the developmental regulatory pathways within which it functions.

Based on the sequences of the zinc finger DNA binding domain motif the NHR superfamily can be divided into at least three subfamilies (see Laudet et al., 1992). The predicted NHR-2 DNA binding domain is more closely related to the retinoid and thyroid hormone receptors and a number of orphan receptors than it is to the classical steroid receptors (Figs. 1A and 1D). Like the retinoid and orphan receptors, NHR-2 appears to be primarily localized in the nucleus (in contrast to the steroid receptors, which can form cytoplasmic complexes with heat shock proteins in the absence of ligand, Tsai et al., 1994).

The DNA sites recognized by the hormone receptors, which bind DNA as homo- or heterodimers, consist of direct or inverted repeats of a core hexanucleotide element. Though some orphan receptors are able to bind DNA as monomers (e.g., Wilson et al., 1993), their binding sites also contain a core hexanucleotide element. The NHRs can be classed into subgroups that recognize the same core element, and the amino acid sequence of the P box, the region of the DNA binding domain that contacts the DNA, can be used to predict core preference (reviewed in Freedman et al., 1993). The NHR-2 P box sequence (CEGCKG) is identical to that of the thyroid hormone, retinoid, and vitamin D receptors (see Laudet et al., 1992), suggesting that, like these proteins, NHR-2 will bind to the core element sequence AGGTCA.

Despite these similarities to the retinoid, thyroid hormone, and orphan receptors, two characteristics of the NHR-2 sequence indicate that it represents a new subclass of orphan receptor. First, NHR-2 has seven extra amino acids relative to other NHRs in the second zinc finger module (see Fig. 1A). These extra amino acids could participate in protein–protein interactions in a dimer interface (Luisi et al., 1991; Schwabe et al., 1993; Rastinejad et al., 1995), contributing to positioning of the dimers on the DNA, or in other intra- or intermolecular interactions. Second, outside the DNA binding domain, NHR-2 exhibits no significant similarity to known members of the NHR superfamily. In this regard NHR-2 joins the Drosophila proteins Knirps, Knirps-related, and Egon (Nauber et al., 1988; Oro et al., 1988; Rothe et al., 1989) and the C. elegans ODR-7 protein (Sengupta et al., 1994) as orphan members of the NHR superfamily lacking similarity to known ligand-binding domains. The sequence thus provides no direct clue concerning the existence or identity of a possible NHR-2 ligand, and NHR-2 function may not require a ligand. However, since the ligand-binding domains of superfamily members are less highly conserved than the DNA binding domains (see Laudet et al., 1992), absence of a recognizable ligand-binding domain in a divergent member such as NHR-2 does...
not rule out the existence of a ligand. If NHR-2 transcriptional activity is ligand regulated, the presence of NHR-2 in early blastomeres suggests that graded levels of that ligand could specify distinct blastomere fates. The lethal phenotypes that result from nhr-2 inactivation are consistent with such an early function, but more sophisticated functional analysis of the NHR-2 region corresponding to the NHR superfamily ligand-binding domain is necessary to explore possible ligand regulation of NHR-2 activity.

Immunostaining detects NHR-2 protein in every nucleus prior to the 16-cell stage, beginning as early as the 2-cell stage. This early expression is most likely due to translation of maternal mRNA because it is not reproduced by the nhr-2/lacZ transgene and lacZ transgenes in general are not expressed well in the C. elegans germline (Fire et al., 1990; Hope, 1991). Consistent with maternal contribution of nhr-2 gene activity, we find expression of nhr-2 in the germline (Fig. 2). The translation of maternal nhr-2 transcripts probably occurs after fertilization because no NHR-2 is detected by immunostaining in unfertilized oocytes (T.L. and A.S., data not shown). The maternal contribution of nhr-2 and the antisense nhr-2 phenotypes suggests that mutations in nhr-2 may cause maternal effect lethal or zygotic lethal phenotypes, depending on whether maternal or zygotic nhr-2 gene activity is sufficient.

Zygotic expression of nhr-2, as defined by a nhr-2/lacZ fusion gene, begins by the 16- to 20-cell stage in all cells except the germline precursor P4 and its sister D, making it one of the earliest genes known to be transcribed in the embryo. NHR-2 expression becomes progressively restricted to anterior and dorsal regions of the embryo as embryogenesis proceeds. The restriction of nhr-2 expression proceeds until at the early stages of morphogenesis only one or a few cells in the presumptive head region continue to express the gene. After that stage, nhr-2 is no longer expressed.

Zygotic expression of NHR-2 does not correlate in a straightforward manner with cell lineage or type, and thus must be established via nonlinear mechanisms or by the integration of multiple lineage speculations. One possible mechanism for establishing such an expression pattern would be autoregulation, in which the initial, relatively low levels of maternally derived NHR-2 serve to activate zygotic expression of the gene. Either a localized ligand source or interactions with other regulatory proteins could then generate the asymmetric zygotic expression pattern. Such autoregulation has been observed for thyroid hormone and retinoic acid receptors (reviewed in Tata et al., 1993; Giguere, 1994).

FIG. 7. Arrest phenotypes resulting from antisense nhr-2 RNA injections. (A) DIC microscopy of normal N2 embryos. Developmental stages represented are premorphogenesis (left), threefold stage following completion of elongation (center), and the early stages of morphogenesis (right). (B, C) Arrested embryos from parents injected with antisense nhr-2 RNA. Pharyngeal structures are indicated by arrowheads. The arrow indicates birefringent gut granule. (D) Normal L1 larva (N2). (E, F) Arrested L1 larvae from parents injected with antisense nhr-2 RNA. Arrow in F indicates region of separation of the cuticle from underlying tissue. Bars indicate 2 μm. B, C scale as in A; F scale as in E.
Disruption of nhr-2 function with antisense RNA leads to embryonic and early larval developmental arrest. Arrested embryos exhibit differentiation of muscle, pharynx, and gut, but fail to undergo morphogenesis and elongation. Arrested larvae often have cuticular defects and are short and fat, a phenotype which can result from defects in elongation of the embryo (Priess et al., 1986). Elongation is driven by enclosure of the embryo by the epidermis (Priess et al., 1986), and both the embryonic and larval arrest phenotypes are suggestive of defects in epidermal development. Consistent with this hypothesis, cell lineages that give rise to the epidermis are among those that express NHR-2 after gastrulation.

The nhr-2 lethal phenotypes are induced by injection of nhr-2 antisense RNA into the maternal germ line. Such germ line injections have been shown to inactivate both maternal (Guo et al., 1995) and zygotic (M. Krause, personal communication) gene activities. This antisense analysis predicts that a loss of function nhr-2 mutation would cause either a maternal effect lethal or zygotic lethal phenotype. Several maternal effect lethal and zygotic lethal mutations that map to the genetic interval containing nhr-2 have embryonic or larval lethal mutant phenotypes (McDowall, 1990), but none were complemented by transgenic nhr-2. There are several possible reasons why nhr-2 is not represented in this set of lethal mutations. First, there appears to be a general problem in germline expression of genes on transgene arrays, which is essential for rescue of maternal effect mutations. Thus if mutations in nhr-2 cause maternal effect lethality, we may need to establish this by phenotypic analysis and sequencing nhr-2 mutant alleles rather than by germline rescue. In addition, mutants with strict maternal effect phenotypes would not have been isolated in the near saturation zygotic lethal screen in which many of the candidate lethal alleles in the region were identified. If maternal nhr-2 contribution is sufficient to allow viable development of zygotic homozygous nhr-2 mutants, such mutants would be missing from this collection. Finally, statistical analysis indicates that the large set of lethals from which the tested mutations were drawn represents at most 75% of the essential genes in this region of Chromosome I (Howell et al., 1990; McDowall, 1990). To establish the nhr-2 null phenotype, targeted mutagenesis (Zwaal et al., 1993) of nhr-2 is underway. Further analysis of nhr-2 function should provide insights into the regulation of zygotic gene expression during a pivotal period of embryogenesis.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Alan Coulson for placing previously unmapped cosmids on the physical map, Shott Gottlieb for contributing to cosmid analysis, Joe Gatto for technical assistance, Susan Strome for providing ant-P granule antibodies and the clone for mes-6 antisense RNA production, Charles Gove for assistance in photographic production, and David Greenstein and Michael Bender for critical reading of the manuscript. We thank Ann Rose and members of her laboratory for generously sharing strains and unpublished information. Kim McCall and Corinna Thiel contributed to the construction of some strains and clones as parts of rotation projects. A.S. thanks members of the Ruvkun laboratory and Erica Golemis for many helpful discussions. Thomas Burglin provided much useful advice concerning protein homologies and degenerate screening. A.S. was supported in part by a postdoctoral fellowship from the Damon Runyon–Walter Winchell Cancer Fund. G.R. was supported in part by an American Cancer Society faculty research award. This work was also supported by grants from Hockest, A.G., and the Human Frontiers Science Program to G.R., and by a March of Dimes Basil O’Connor Starter Scholar Award and a grant from the University of Georgia Research Foundation to A.S.

REFERENCES


Received for publication December 19, 1996
Accepted February 20, 1997

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.