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Faculty of Science
Department of Molecular Biology and Biochemistry

Master Thesis

The regulation of transcription by the nuclear receptor NHR-25 in *Caenorhabditis elegans*

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Anotace: In this study, I utilized the defined binding (target) sequence for transcription factor NHR-25 and GFP as a marker to visualize where *nhr-25* is active during the development *in vivo*. After obtaining worm strains carrying these constructs, the expression pattern was analyzed and the specificity of the expression was tested by means of RNAi.

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1. Introduction

1.1 *Caenorhabditis elegans*

A small worm, *Caenorhabditis elegans,* is a member of family *Rhabditidae*, a large and diverse group of nematodes. In contrast with other related pathogenic or parasitic species it is non-parasitic, soil living nematode and it is advantageous for rearing in laboratory conditions.

C. elegans is one of the widely used model organisms in Developmental biology and in Genetics. This small, 1 mm long transparent worm has traits important as an experimental animal: it is easy to rear feeding on *Escherichia coli* on agar or agarose plates, can be stored as a frozen stock, has short life cycle, and it has small genome in six chromosomes and two sexes. The worms are usually kept at 20°C.

Hermaphrodites are self-fertilizing and can have about 300 of offspring during their reproductive period. Hermaphrodites can not fertilize other hermaphrodites but males can mate with hermaphrodites. The males are quite rare in nature but occur spontaneously or by heatshock in the hermaphrodite population by X-chromosome non-disjunction. This can happen with frequency about 1 male per 500 animals.

The development takes 3.5 days and consists of embryonic stage, four larval stages from L1 to L4 (Fig. 1.1) and the adult. Each post-embryonic stage is progressed through molting. In unpreferable conditions like starvation and high density, worms go through the stage of so called dauer-larva in their development; L2 does not develop into regular L3, but into dauer, which is resistant to starvation and outer stress. The development resumes after improving the conditions to L4 and then continues to be the adult.

The worm's genome is 97 Mb with the total number of coding genes 19,000. *C. elegans* has 6 haploid chromosomes, five of them the autosomes (I, II, III, IV, V) and one sex chromosome, X. The sequence of the whole genome became available in 1997 and it was the first model organism to be fully sequenced.

The first discovery with *C. elegans* in the main role as a model organism was the genetic regulation of apoptosis analyzed by Sydney Brenner, John E. Sulston and

H. Robert Horvitz. This process is conserved in other organisms and it can be activated by many different stimulations such as extracellular growth factors, steroid hormones or viral infection. The morphological changes of cells that die in apoptotic way in *C. elegans*, are similar to the changes in mammalian cells.

The worm has the constant number of body cells. At hatching, the larva of L1 stage has 558 cells in hermaphrodite and 560 in male. In postembryonic development, cells proliferate but 131 cells die with apoptosis to produce the 959 somatic cells in the adult hermaphrodite and 1031 cells in the adult male. The neurons and epidermal cells die mostly, less the muscle cells. The apoptosis occurs also in the embryonic stage as well as in the adult (the germ cells).

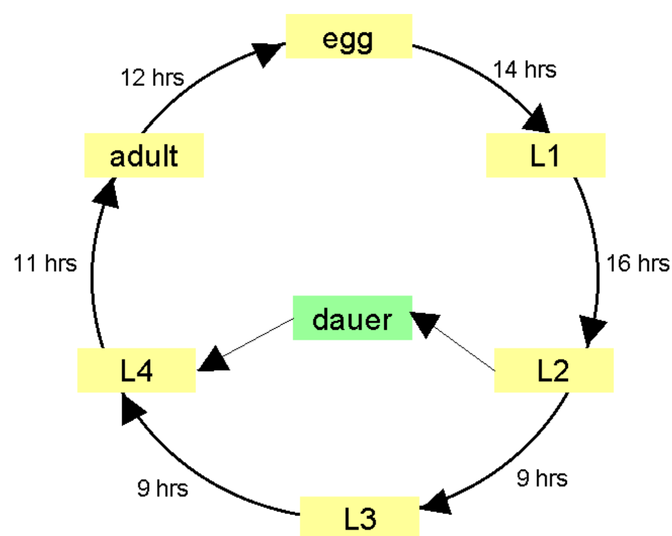

Fig. 1.1 The life cycle of *Caenorhabditis elegans* under laboratory conditions (20°C).

1.2 Transcription factors and enhancer study

Transcription factors are proteins necessary for the transcription. The general transcription factors help to position the RNA polymerase correctly at the promoter, then to separate the strands of DNA and at last to release RNA polymerase from the promoter. These general TFs works on any promoter used by RNA polymerase II. The eukaryotic promoter for RNAP II is characteristic with the presence of TATA box, which binds the proteins like TBP (TATA binding protein) and TAF (TBP-associated factors). Other transcription factors bind on the DNA at enhancer region on specific sequences, which are called binding sites. These are defined for each transcription

factor and also the number of them could be important (more binding sites can enhance the effect of transcription factor).

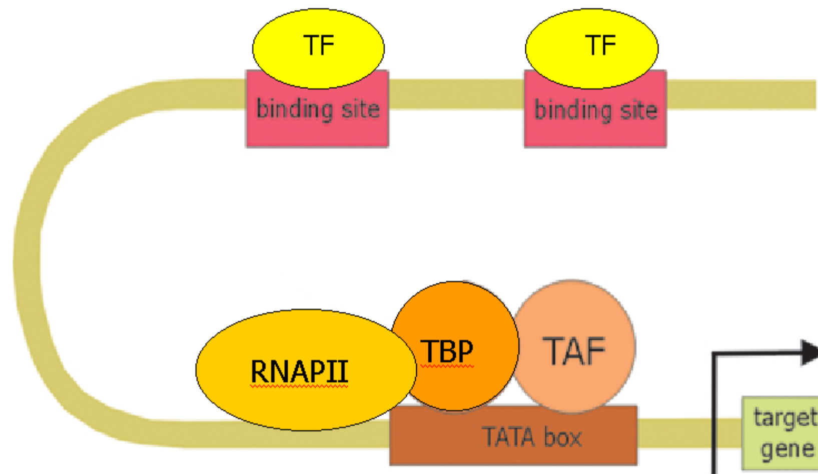

Fig. 1.2 A simple scheme of the transcription machinery on a target promoter and enhancer

Transcription of a gene can be regulated by numbers of proteins in its promotes region which may contain binding sites for various transcription factor. The promoter region which has known binding sites can be tested by adding and/or removing or mutating the element in the natural promoter or creating an artificial promoter containing the binding sites of interest. When a transcription factor acts as an enhancer of its target gene transcription, the activity can be monitored as higher expression of the target protein or a reporter protein (such as luciferase or GFP). With the reporter assay, the vector should have a minimal promoter as it is necessary to activate basal level of transcription. The enhancer sequence(s) should be followed by the minimal promoter and the reporter gene in the vector and the protein expression can be monitored biochemically or visually. The specificity of the binding site can be analyzed by (i) creating a mutation in the binding site and (ii) monitoring the target protein expression in mutant and/or RNAi background of the transcription factor binding to this element.

Advantage of utilizing *C. elegans* is that we can perform this study *in vivo* as we can transform *C. elegans* with GFP expression vector containing the binding site(s) and monitor the activity throughout the development and the localization (cell/tissue type). Two types of minimal-promoter-containing GFP vectors are widely

used in the field (Brodigan et al., 2003; Harfe and Fire, 1998). One utilizes a minimal promoter from *myo-2* and the other from *pes-10*.

1.3 Nuclear receptors in *C.elegans*

Nuclear receptors are an important family of transcription factors, which are usually hormone-gated. They play crucial role in metazoan transcription, when they regulate gene expression in response to specific, lipophilic ligands (steroids, retinoids, bile and fatty acids (Mangelsdorf et al., 1995)). According to their role in the organism, their dysfunction is responsible for many human diseases, including diabetes, obesity, cancer and cardiovascular diseases (Lehrke and Lazar, 2005; Smith and Muscat, 2006).

They have conserved molecular architecture, when the N-terminus contains a DNA binding domain (DBD), which is made of two Cys4 zinc fingers. The more variable C-terminal ligand binding domain (LBD) binds ligands but this terminal part also docks coactivators and corepressors (Mangelsdorf at al., 1995).

Nuclear receptors, which have no ligand or for which no ligand(s) were found yet, are called orphans (Mangelsdorf et al., 1995).

The number of nuclear receptors present in *C. elegans* is unusually high (284 NRs, human 48 NRs, fly 21 NRs). The nuclear receptor superfamily has undergone a dramatic expansion and diversification during the evolution and consequences of this expansion are not known. 279 of these receptors arose from ancestral HNF4 (Robinson-Rechavi et al., 2005). Only 15 of *C. elegans* nuclear receptors are conserved with other organisms (Sluder et al., 1999). They play important role in processes such as dauer formation, neuronal development, sex determination, epidermal development and toxin resistance.

In this huge number of worm nuclear receptors almost all of them are orphans with one exeption. In 2006, Motola et al. reported for the first time that DAF-12 has ligands: 3-keto-cholestenoic acid metabolites of DAF-9, a member of cytochrome P450. These ligands bind and transactivate DAF-12 at nanomolar concentrations and rescue the hormone deficiency of *daf-9* mutants.

1.3.1 NHR-25

NHR-25 is one from the fifteen conserved NRs in C. elegans. It is a homolog of *Drosophila* FTZ-F1 and human SF-1/LHR-1. FTZ-F1 is involved in embryonic segmentation and larval metamorphosis (Broadus et al., 1999; Guichet et al., 1997; Lavorgna et al., 1993; Ueda et al., 1990; Yu e al., 1997). Mammalian SF-1 controls differentiation of the gonad, adrenal gland, pituitary and hypothalamus, when it is also responsible for the steroidogenesis (Parker at al., 2002). It is also shown that members of this family work as a monomer binding protein.

NHR-25 has been shown to be required in the embryogenesis, molting and gonadal differentiation in *C. elegans* (Asahina et al., 2000; Gissendanner and Sluder, 2000;). Also it has an important role in epidermal differentiation (Chen et al., 2004; Silhánková et al., 2005). Embryonic loss of function ends with failure of ventral enclosure and defects in epidermal elongation, when the embryo gets arrested before the two-fold stage. The worms with postembryonic loss of function suffer from molting defects, they have abnormal morphology of seam cell, and vulval differentiation, and defects in gonad. (Gissendanner and Sluder, 2000, Chen et al., 2004; Silhánková et al., 2005, Asahina et al., 2006).

1.3.2 NHR-23

NHR-23 (CHR-3) is an another conserved nuclear receptor and is a homolog of *Drosophila* DHR3, which mediates pre-pupal to pupal transition (Lam et al., 1999). It has its homolog in vertebrates, too, RORα,β,γ, which are involved in many processes like Purkinje cell generation, bone maintenance, circadian rhytms and thymopoesis (Jetten et al., 2001). In *C. elegans* it has an important role in epidermal differentiation, molting, collagen synthesis and mail tail development (Kostrouchova et al., 1998; Kostrouchova et al., 2001). Loss of *nhr-23* function has consequences such as severe molting defects, disrupted collagen synthesis and abnormal tail development (Kostrouchova et al., 2001).

1.4 RNA interference

The great discovery of RNA interference (RNAi) by double stranded RNA (dsRNA) was achieved by C. C. Mello and A. Fire (Fire at al., 1998) and has been a big breakthrough in biology. The principle seems to be very old and widely used by many organisms to face the threat of inserting transposones and retroviruses into their genome as well as to protect chromatin and histones to be modified. RNAi has also the function in regulation of translation.

This posttranscriptional mechanism, when dsRNA is introduced into the organism, small interfering RNAs are generated by Dicer, leads to the degradation of specific homologous mRNAs by RISC complex and consequently to the down-regulation of the expression of the targeted gene (Figure 1.3) so the gene is silenced (so called "gene knock down"). RNAi mechanism was first investigated in *C. elegans*, then it was also found in *Drosophila* (Kennerdell and Carthew, 1998), mouse (Wianny and Zernicka-Goetz, 2000), plants (Waterhouse et al., 1998) and many others. The RNAi has been employed in investigation of gene function and now it emerges for usage in medicine.

The RNAi in *C. elegans* can be accomplished by four different ways: by injection when the dsRNA is injected into the gonad arm of the worm or the body cavity, by soaking when the worms are submerged in the solution of dsRNA; by feeding when they are fed on bacteria expressing dsRNA and by transformation of a vector containing palindrome sequence of a targeted gene. The unique feature of the RNAi in *C. elegans* is the systemic effect of RNAi. The dsRNA can be uptaken into any tissue of the body (except neurons) and the animal will be affected. This systemic RNAi effect is due to the transmembrane protein SID-1, which is responsible for the income of dsRNA inside of the cell.

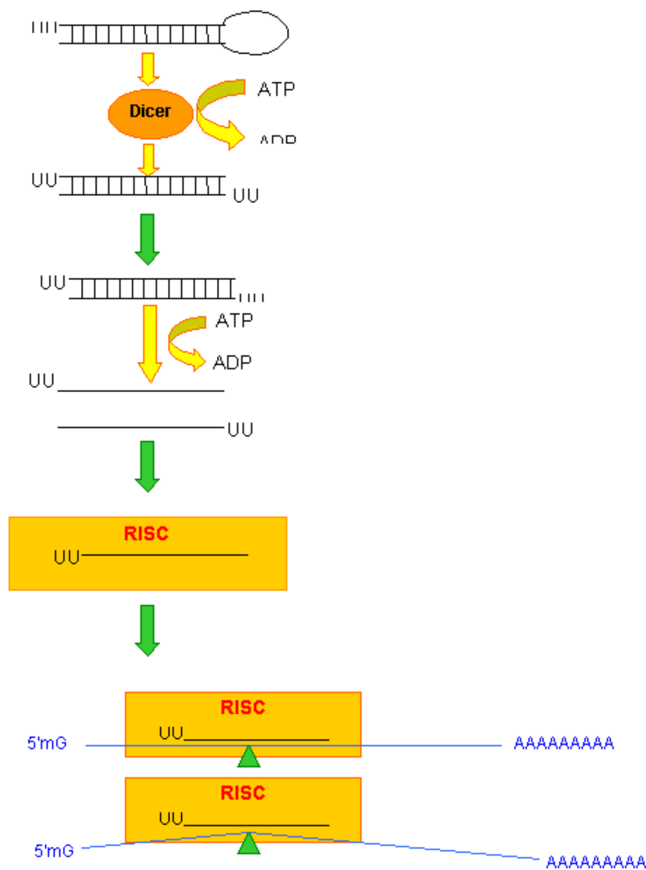

Fig. 1.3 The mechanism of RNA interference.

1.5 Development of *C. elegans* intestine

The intestine is one of three major epithelial organs in *C. elegans*. It is a simple tube, made from 20 cells only, which all come from one ancestor cell – from embryonic blastomere E, which is one of two daughter cells of EMS (mesendodermal precursor). Some maternal regulatory pathways work on the cell fate decision leading to E blastomere (Schnabel and Priess, 1997; Bowerman, 1998; Labouesse and Mango, 1999).

This blastomere is specified to form the intestinal cells in eight-cell stage of embryogenesis (Sulston et al., 1983), when it undergoes 5 rounds of division until the hatching. At the hatching time, the L1 intestine has 20 cells, situated bilaterally symmetrically in pairs, organized in eight intestinal rings (II-IX). The four most anterior intestinal cells form together the first intestinal ring (I). Each of them has a single diploid nucleus (Hedgecock and White, 1985) with large nucleoli. At the beginning of the L1 lethargus, all the nuclei except 6 most anterior of them replicate

their DNA and divide, forming finally 30-34 diploid nuclei. Later during the development the nuclei undergo endoreduplication, which repeats before each of the three remaining molts.

No role in development of the gut has been reported for NHR-25 and NHR-23 so far.

2. Materials and Methods

2.1 Culture of *C. elegans*

The worms were cultured on nematode growth medium (NGM) plates fed on *E. coli* strain OP50 at 20°C as described (Brenner, 1974).

2.2 DNA clones and DNA amplification for cloning

DNA clones (pOLDO-luciferase) containing two copies of either NHR-25 binding sites and ROR binding sites (gift of M. Van Gilst, Fred Hutchinson Cancer Research Center, Seattle, WA) were used to subclone into two GFP vectors, pPD107.97 and pPD107.94 (Fig. 2.1, gift of A. Fire, Stanford University, USA), developed for *C. elegans* transformation. This GFP vectors contain minimal promoters from *myo-2* gene (pPD107.97) and from *pes-10* gene (pPD107.94). They should have no activity by themselves alone. DNAs were amplified either by transforming bacteria by heatshock or recovering from a frozen stock, then plasmid DNAs were isolated and purified with QIAprep Spin Miniprep Kit (QIAGEN).

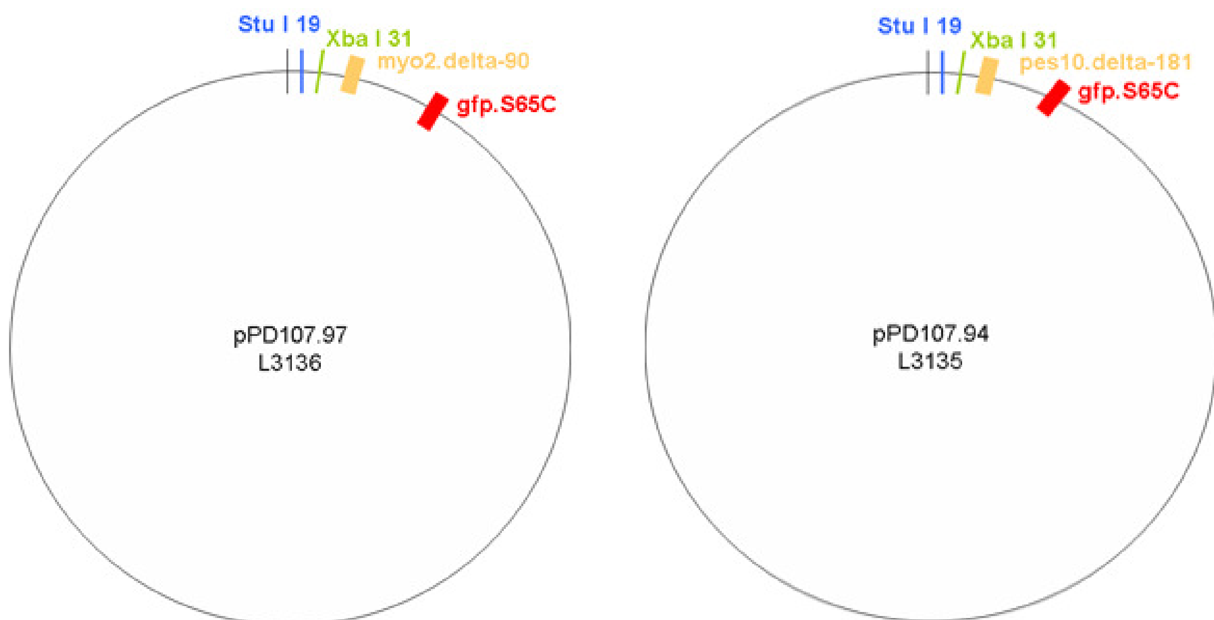

Fig. 2.1 The maps of GFP vectors used for cloning.

2.3 Cloning

Directional cloning using Xba I (3' overhang) and Stu I (blunt) was performed. The double digests of the vector containing two copies of NHR-25 binding site and ROR binding sites create the fragment of 275 bp with the binding sites. The GFP vectors were digested with the same set of enzymes and linealized. Single cut with each of the enzymes alone was also performed to see if each enzyme works. The reactions were done in total of 20 µl volume with 2 µl of 10x M buffer, 2 µl of BSA (Takara), 0.5 µl of Xba I and Stu I (both Takara), and 500 ng of DNA and incubated at 37 °C for 2 hours or overnight and then, the digestion was terminated by heat (at 70°C water bath for 15 min).

1Kb Plus DNA Ladder (Invitrogen) for distinguishing the linear DNA (cut fragments) and Supercoiled DNA Ladder (Invitrogen) for distinguishing the circular DNA (uncut fragments) were used as size markers. 0.8 µl of 10x DNA Loading Buffer was added in each sample and run the electrophoresis in TAE buffer. 4% low-temperature-melting agarose was used to obtain fragments of 275 bp inserts (both for NHR-25 and for ROR) and 0.8 % was used to obtain 8,260 bp (vector pPD107.97) and 8,480 bp (vector pPD107.94) linealized DNAs. Gels were melted and ligation was performed in the gel using T4 ligase (Takara) at 15 °C overnight. The ligation reaction was melted and TCM was added and then transformed into *E. coli* DH5α cells by heatshock. Colonies were checked by colony PCR for the insert and correct colonies were picked to grow in 5 ml LB medium and plasmids were isolated using QIAprep Spin Miniprep Kit (QIAGEN). The correct clones were further confirmed by sequencing. Clones are designated as 94TGA, 97TGA, 94TCT and 97TCT (see detail in the Results).

2.4 Sequencing

Sequencing of DNA was performed using Big Dye terminator system kit v 1.1 (Perkin Elmer). Reaction mix was prepared according to the instruction of manufacturer with a small modification. Briefly, 4 µl of Big Dye, 4µl of 2.5x sequencing buffer and 3.2 µl of 1 µM M13 reverse primer was premixed and then 1µg of DNA was added. The

total volume of the reaction was set to 20µl. The sequencing reaction was purified with G50 AutoSeq column (Amersham) then dried by the vacuum centrifuge (Labnet Dyna Vap) for 20-30 min. The sequencing was performed on an ABI 310 automatic sequencer. Obtained sequences were analyzed with a program Chromas Lite and further processed using DNASTAR (EditSeq, MegAling, MapDraw).

2.5 Preparation of DNA for the worm transformation

The DNA for injection was prepared with QIAGEN Plasmid Midi Kit according to the instruction of the manufacturer. For injection, total concentration of DNA in 1 µl was 100 ng and the final volume of 20 µl was prepared. For the construct 94TCT, injection mix was prepared with 1600 ng of the construct, 400 ng of pRF4 in 1x injection buffer. For 94TGA and 97TCT injection mixtures, I had to optimize the DNA concentrations and 1100 ng of each construct was mixed with 500 ng of pBlueScript and 400 ng of pRF4 in 1x injection buffer. The pRF4 plasmid carries *rol-6(su1006)* mutation and is routinely used as a dominant visible marker (roller) for transformation of *C. elegans* (Mello and Fire, 1995). Just before injection, the DNA mix was filtered through Ultrafree-MC column (0.1 µm, Millipore).

2.6 Transformation of the worms

The DNA transformation with microinjecting was first discovered in 1982 by Kimble et al. This can be achieved by the germline transformation, when the syncitial gonad is injected with DNA using a needle (Stinchcomb et al., 1985, Mello et al., 1991). The effect of transformation can be analyzed in F1 progeny and later generations as injected DNA can be inherited and amplified as an extrachromosomal array.

The wild type N2 worms from synchronized cultures were used for transformation. They were kept on NGM plates with OP50 in 20°C to reach the stage of young adult. Injection needle (GDC-1, Narishige) was pulled by a needle puller (Narishige) and about 1.5 µl of purified and freshly filtered DNA was placed at the tip of the injection needle.

The worm was put from the original NGM OP50 plate to one new NGM plate without bacteria to clean its body from bacteria and placed on the agarose injection pad with a drop of mineral oil (SIGMA). The worm is fixed on the agarose. The pad was placed under the injection microscope (Olympus) with the objective 60x, the gonadal arm and the tip of the needle were aligned on the same focal plane. The gonad was injected as quickly as possible, because the animal gets dry during the time of staying on the pad in the oil. After taking away the pad from the microscope, the worm was removed from the agarose with a drop of S-basal with gelatin. The injected animal was put on a new NGM OP50 plate and next day separated each one on new NGM OP50 plates.

2.7 Integration

The transformed worms do not have the new DNA construct integrated into their genome as its extrachromosomal nature, so the expression of green fluorescent protein is a mosaic. To integrate it into the chromosomes, the UV irradiation is used. I applied the UV irradiation with intensity 30 mJ/cm^2 on L4 larvae or young adults from synchronized plates of each strains. After that, worms were placed onto new OP50 plates and then all the progeny was collected to screen for the integrants. The F2 generation should be 100% rollers, if integrated.

2.8 Crossing

After obtaining the integrated strain, I backcrossed them with the wild type to cancel possible mutations caused by irradiation. For this, I used single-cross method; I placed on special mating plate (2% agarose plate, 6cm, with 20 µl drop of concentrated bacteria) two males of wild type N2 and one L4 hermaphrodite of the integrated strain.

2.9 RNAi

2.9.1 Preparing the bacteria for RNAi

The transformed bacterial cells from frozen stock were streaked on LB plates containing antibiotics (carbenicillin, tetracyclin) and incubated overnight in 37°C. There were three kinds of cells; the transformed with a plasmid containing *nhr-25* or *nhr-23* in a double T7 vector (pPD129.36, gift of A. Fire) and transformed with the vector only as a control. Next day one colony from each plate was picked and grew in 5 ml LB medium with antibiotics (100 µg/ml CRB, 12.5 µg/ml TET) and incubated overnight at 37°C again. Third day of the procedure, 500 µl of overnight culture was put in 20 ml LB (100 µg/ml CRB, 12.5 µg/ml TET), incubated for 2-4 hours in 37°C until OD_{600} reached 0.4. The dsRNA was induced by adding IPTG (0.4 mM) and then incubated for 4 hours in 37°C. Then the induction was spiked with additional antibiotics (100 µg/ml CRB, 12.5 µg/ml TET) and IPTG (0.8 mM final concentration). Before using those cells for experiments, 1 ml of the liquid culture was centrifuged, the pellet was resuspended again with pipetting and plated on RNAi plates (30 µl per 1 small plate). For RNAi, special plates were used to grow worms; NGM plates with antibiotics (50 µg/ml CRB, 12.5 µg/ml TET) and IPTG (0.4 mM).

2.11 Microscopy

For routine work and checking the worms, stereo-microscope Olympus SZX12 was used. For checking the phenotype of RNAi affected worms and to see detail GFP expression pattern in transgenic worms, the fluorescent microscope Zeiss Axioplan2 equipped with DIC was used. Images were captured by the software Analysis and processed using Adobe Photoshop.

The collected worms in certain stage for observation were anestetised with the mixture of 0.2 % tricaine and 0.02% tetramisole and put on 5% agar pads. Then they were checked in Nomarski view (DIC) and fluorescence in magnification 63x.

2.12 Electron microscopy

After applying the RNAi it was decided to look closer on the worms with transmission electron microscopy. Worms were paralysed with 8% ethanol and fixed in glutaraldehyde (2.5%, 0.2M buffer, 3 overnights). After that, they were picked into triplets and a drop of 2% agar was dropped onto them. then washed three times 15min with the washing solution) then further treated with OsO_4 (4%) was mixed with washing solution in ratio 1:1 (2 hrs) followed by additional washing (three times 15 min). Dehydratation was done with the series of acetone (30%, 50%, 70%, 80%, 90%, 95%, 100%, each for 15 min). Then the worms were embedded into the resin (resin and acetone, 1:2, resin and acetone 1:1, resin and acetone 2:1) and at the end into pure resin (24 hrs). They stayed 48 hrs at 60°C and then they were cut into semithin sections, which were colored with toluidin blue. After checking the semithin sections for position and state of the worms, they were cut to ultrathin sections and contrasted on the nets with uranyl acetate and lead citrate. The nets were before use washed with 50% vinegar acid (5 min), water with detergent (5 min), distilled water (5 min), acetone (5 min) and ultrasound (5 min), then dried on the filter paper.

The sections were examined on TEM JEOL 1010. Images were captured by the software Analysis and processed using Adobe Photoshop.

2.13 Solutions and abbreviations

LB medium – Luria-Bertani medium

IPTG – isoprophylthio-β-D-galactoside

CBR – carbenicillin

TET – tetracyclin

NGM plates – 3 g NaCl, 2.5 g Bacto Peptone, 17 g agarose/agar, 5 μg cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM potassium-phosphate (pH. 6.0) in 1 liter

10x injection buffer – 20% polyethyleglycol, 200 mM K-phosphate, 30 mM K-citrate

TCM buffer – 10 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$

0.2M buffer (PB) – 14.32 g Na_2HPO_4.12 H_2O in 200 ml of H_2O, 1.36 g KH_2PO_4 in 50 ml of H_2O, mix both solutions and set the pH to 7.2, filter

washing solution – 50 ml of 0.2M buffer (PB), mix with 50 ml of H_2O and 4 g of glucose

3. Results

3.1 Sequence of NHR-25 binding sites

My previous work revealed that the NHR-25 binding site in the luciferase vector (pOLDO-luciferase) is TGAAGGTCA (thesis of Merglova 2007). The sequence of ROR/NHR-23 binding site is TCTAGGTCA.

The binding sequence TGAAGGTCA is responsible for transcriptional activation in transfected human cell line (Asahina et al., 2006). While AGGTCA is classic hormone response element, universal to many other nuclear receptors, TGA at its 5'-seems to be NHR-25 specific as mutations in them (TCTAGGTCA) could no longer transactivate in the presence of NHR-25 (Asahina et al., 2006).

3.2 Cloning and transformation of 2x*NHR-25bs::GFP* construct and 2x*RORbs::GFP* construct

The fragments containing the two tandem NHR-25 binding sites and the two ROR binding sites were cloned into GFP-vectors pPD107.97 and pPD107.94 (Table 1, Fig. 3.1) between the restriction sites of Stu I and Xba I, so that each binding sites in two different minimal promoters (4 constructs) can be tested. Clone 97TGA was prepared during previous bachelor study, and other three were made in this study.

The efficiency of cloning was very low and after four rounds of transformation of the bacterial cells we got colonies from all three types (Table 2). All of the obtained colonies of 94TCT (3), 97TCT (1) and 15 chosen colonies of 94TGA were checked with colony PCR and, in case of 94TGA and 94TCT, two colonies were chosen for sequencing. The sequencing revealed that all new constructs have the correct inserts.

Table 1: Overview of constructs.

GFP vector	vector size	minimal promoter	insert size	final size
pPD107.94	8492 bp	pes-10	275 bp	8755 bp
pPD107.97	8272 bp	myo-2	275 bp	8535 bp

Fig. 3.1 A Scheme of cloning of 2xNHR-25 binding sites and 2xROR binding sites into GFP vector pPD107.97.

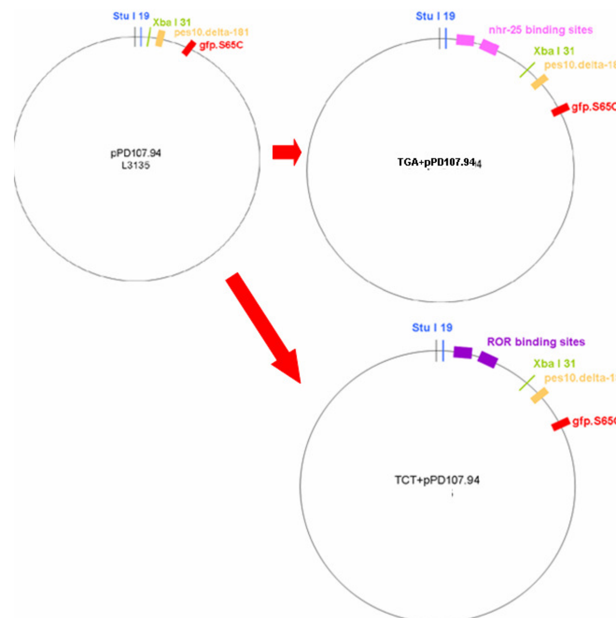

Fig. 3.1 B Scheme of cloning of 2xNHR-25 binding sites and 2xROR binding sites into GFP vector pPD107.94.

Table 2: Preparation of constructs

construct	colonies	colony PCR	correct size	sequenced	correct seq.
94TGA	100	15	15	2	2
94TCT	3	3	2	2	1
97TCT	1	1	1	1	1

Numbers of obtained and tested colonies for colony PCR, positive clones (correct size), sequenced clones and clones with correct sequence were shown.

The transformed bacteria with the correct sequences were amplified in liquid LB medium and the plasmid DNAs were isolated and purified for transformation to the worms. The N2 worms at young adult stage were injected. The transformation of the worms was successful, although it took long time – especially the 97TCT strain wanted 17 repeats of injecting (Table 3).

Rolling progenies were transferred to new plates and checked their progenies for inheritance of this transgene. After three more generations when the number of rolling worms in the brood increased, they were checked under the fluorescent microscope if the binding sites are active and they show the GFP expression *in vivo*. They were, at least in three of four obtained strains.

Table 3: The number of attempts for transformation

construct	injections	injected worms	rolling F1	line
94TGA	10	94	5	1
94TCT	1	7	1	1
97TCT	17	183	6	1

Numbers of repeats of injection, total injected worms, obtained rolling progeny, and number of successful lines for each construct were shown.

3.3 Integration and backcrossing

Newly generated three transgenic worm strains and the 97TGA strain which was made previously, were integrated into the worm chromosome (see Materials and Methods). Out of four strains, the integration was successful only in two of them. For

unknown reason the integration of strains 94TGA and 97TCT failed. The numbers of worms, which underwent irradiation, the number of attempts and results are shown in Table 4.

Table 4: Results of integration

strain carrying	integration	irrad. worms	integrated line
94TGA	10	125	0
97TGA	3	101	15
94TCT	4	98	1
97TCT	4	59	0

The number of repeats, irradiated L4s and young adults and obtained lines were shown.

The successfully integrated lines (94TCT, 97TGA) were then backcrossed with wild type animals to clean up potentially damaged chromosome by UV irradiation.

3.4 Expression analyses *in vivo*

Obtained strains were checked under fluorescent microscope for their GFP expression. GFP positive cells for all four strains are summarized in Table 5. Weak expression in posterior gut cells (< 4 cells) were considered as background expression of the minimal promoter as it is known for *pes-10* (Brodigan et al., 2003). GFP expression observed was nuclear as both vectors (pPD107.94 and pPD107.97) contain nuclear localization sequence (NLS).

Table 5: Summary of GFP positive cells in each strains.

strain	gut			neurons		n
	anterior(%)	posterior(%)	background(%)	head(%)	tail(%)	
94TGA	4 (5)	3 (4)	11 (13)	2 (2)	2 (2)	82
97TGA*	0 (0)	1 (2)	4 (10)	0 (0)	0 (0)	42
94TCT*	97 (57)	139 (82)	26 (15)	28 (16)	79 (46)	170
97TCT	0 (0)	0 (0)	0 (0)	1 (1)	3 (2)	126

The number of worms showing GFP positive cells in postembryonic stage are shown.
* integrated strains

3.4.1 94TGA

In nonintegrated strain 94TGA carrying NHR-25 binding sites in *pes-10* minimal promoter vector, GFP signal was not so significant and showed mosaic expression pattern due to the nature of extrachromosome array. Some expression was observed in coma stage of embryonic development in epidermal tissue (Fig. 3.2 H, I) and continues to be in postembryonic development. During first larval stage, few most posterior gut cell nuclei and sometimes the most anterior gut cell nuclei, show the production of GFP (Fig. 3.2 B, D). In later stages, the level of fluorescence decreases and in L4s and adults, the expression is barely visible only in last gut cell nucleus.

In other tissues, GFP was rarely detected, though some of the animals have signals in some of the head and tail neurons (Fig. 3.2 D, F). In very few animals of stage L2-L3, expression was seen in hypodermal cells (Fig. 3.2 J), possibly in seam cells where NHR-25 plays very important role.

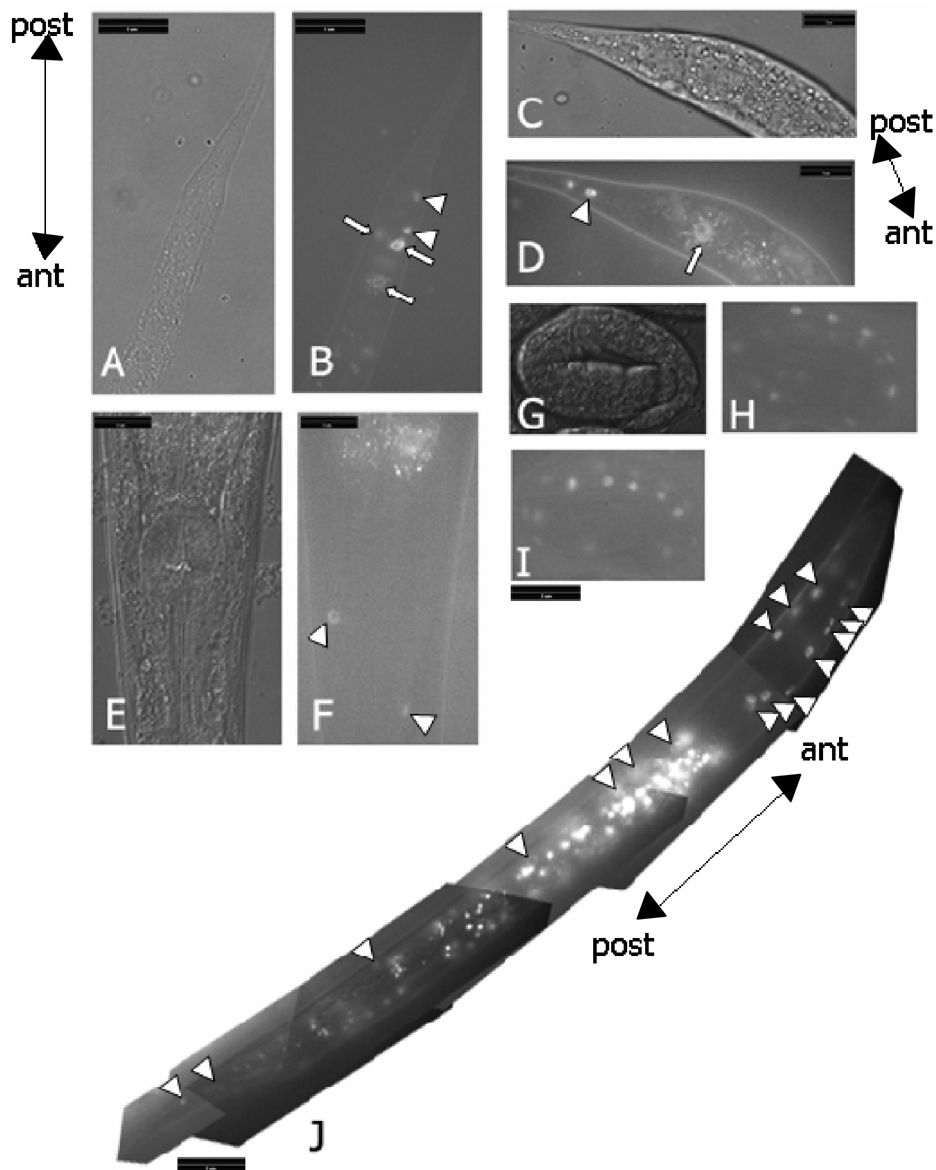

Fig. 3.2 Expression pattern of 94TGA in wild type animals. (A and B) L1 stage, gut expression (arrow), tail neurons (arrowheads), (C and D) L3 stage, gut expression (arrow) and tail neurons expression (arrowhead), (E and F) adult stage, head neurons (arrowheads), (G – I) embryonic expression in hypodermal cells, (J) L2 stage, hypodermal expression (arrowheads), A, C, E and G are DIC images. Ant means anterior of the worms, post is posterior. Scale bars 10 μm.

3.4.2 97TGA

Integrated strain with NHR-25 binding sites in the other minimal promoter (from *myo-2*) vector showed nearly no expression. Only very rarely was seen low expression in the posterior gut in the larvae (Fig. 3.3 D) as background expression of the minimal promoter alone.

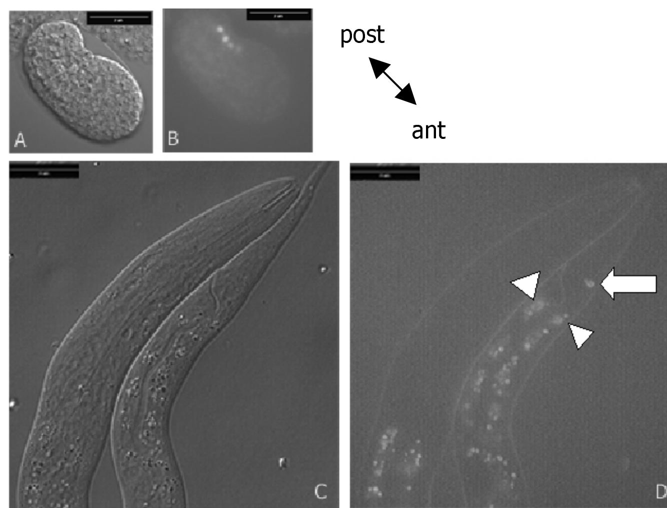

Fig. 3.3 GFP expression of 97TGA in the wild type animals. (A and B) coma stage, (C and D) posterior gut (arrowheads), likely background expression of the minimal promoter and tail neuron (arrow) in L2 larva. A and C are DIC images. Granular signals are autofluorescent in the gut granules. Ant means anterior, post is posterior. Scale bars 10 µm.

3.4.3 94TCT

Expression pattern of 94TCT was the strongest from all four constructs. The expression begins in coma stage, most probably in the gut cells (Fig. 3.5 D). After hatching, the expression was very strong in the gut. The nuclei of first two to four gut cells, which form the first intestinal ring, showed the GFP expression in over 55 percent of the worms (Fig. 3.5 H). This expression decreased during postembryonic development and no green gut nuclei was observed in the adults (Fig. 3.4). 82% of worms show very high level of GFP expression in posterior intestine in range from one to fifteen cells clearly different from background expression (Table 5, Fig. 3.5 F).

The expression also decreased in L4 to adult stages (Fig. 3.4). 94TCT worms also showed expression in the head and tail neurons (Fig. 3.5 F, J) with much higher penetrance compared to 94TGA (Table 5).

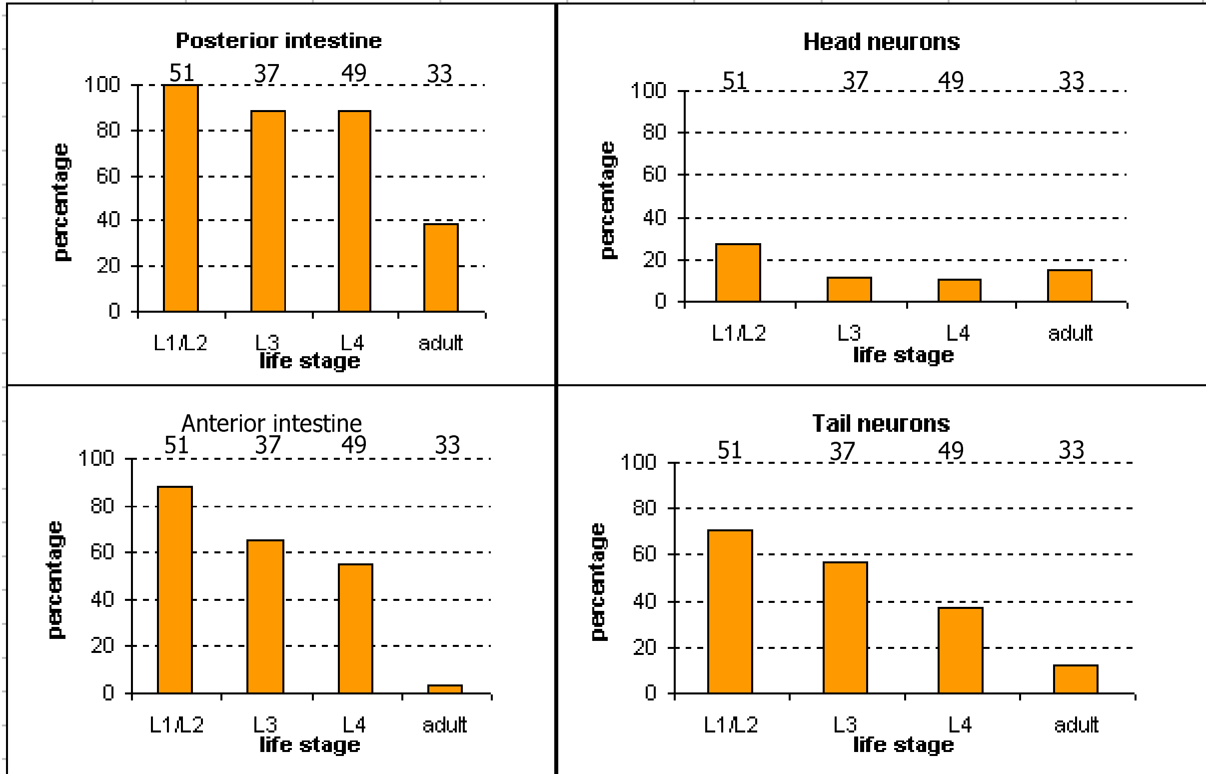

Fig. 3.4 Developmental profiles of 94TCT GFP expression in the gut and neurons. Worms exhibiting background GFP level were not included. The numbers above the bar is N.

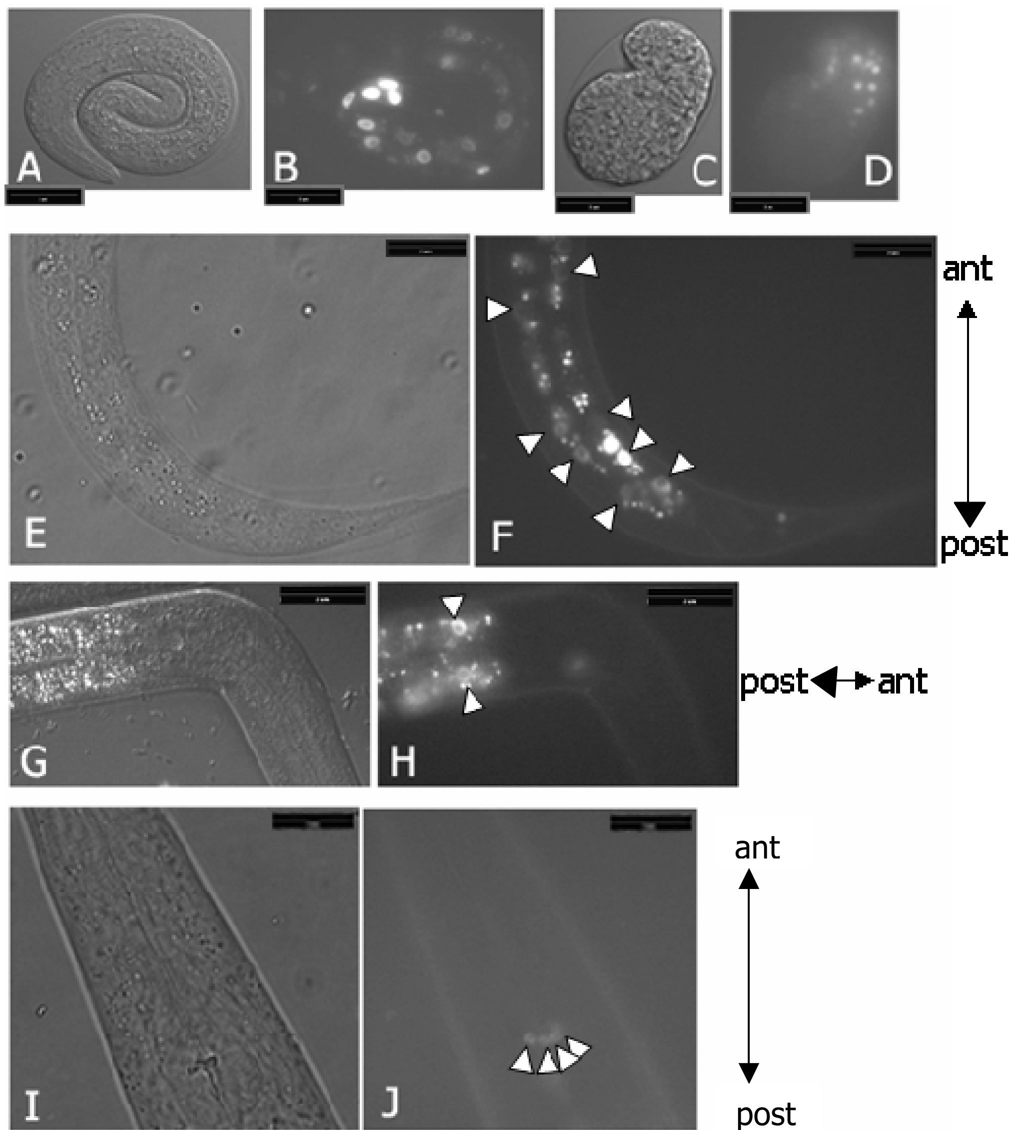

Fig. 3.5 Expression pattern of 94TCT in the wild type animals. (A and B) strong gut expression in L1 stage, (C and D) coma stage, expression is most probably in the gut, (E and F) expression in the gut (arrowheads) and tail neuron (arrow) in L2 stage, (G and H) expression in the anterior gut (arrowheads) in L2 stage, (I and J) head neurons in the adult (arrowheads). A, C, E, G and I are DIC images. Granular signals are autofluorescent in the gut granules. Ant means anterior of the worms, post is posterior. Scale bars 10 μm.

3.4.4 97TCT

The second strain with ROR binding sites is an non-integrated line and has no expression at all in any of the embryonic or postembryonic stages (Table 5). Thus it failed to confirm the expression pattern seen in 94TCT strain.

3.5 RNAi

To see whether the reduced level of NHR-25 and NHR-23 can influence the expression of 94TCT and 97TGA, RNAi against *nhr-25* and *nhr-23* was performed as described in Materials and Methods. The worms were observed in all stages from L1 to adult. In integrated strains 94TCT and 97TGA were subjected to RNAi three times independently and RNAi on non-integrated strains 97TCT and 94TGA was repeated twice.

Worms on *nhr-25* RNAi showed expected morphological defects described before (Asahina et al., 2000; Asahina et al., 2006) such as molting defects in every stage (the old cuticle gets constricted around head, tail and midbody and remains unshed). The vulva defects were evident as well and the adult worms could not lay eggs properly and died as "bag of worms" (Fig. 3.6 B).

nhr-23 RNAi caused more severe defects. Molting was disrupted in all stages, and old cuticle was hindering the proper development (Fig. 3.6 C) as described previously (Kostrouchova et al., 2001). The inside of the body was disturbed with strange shaped gut and the worms were not able to develop normal gonad and lay eggs. They were smaller and dumpy.

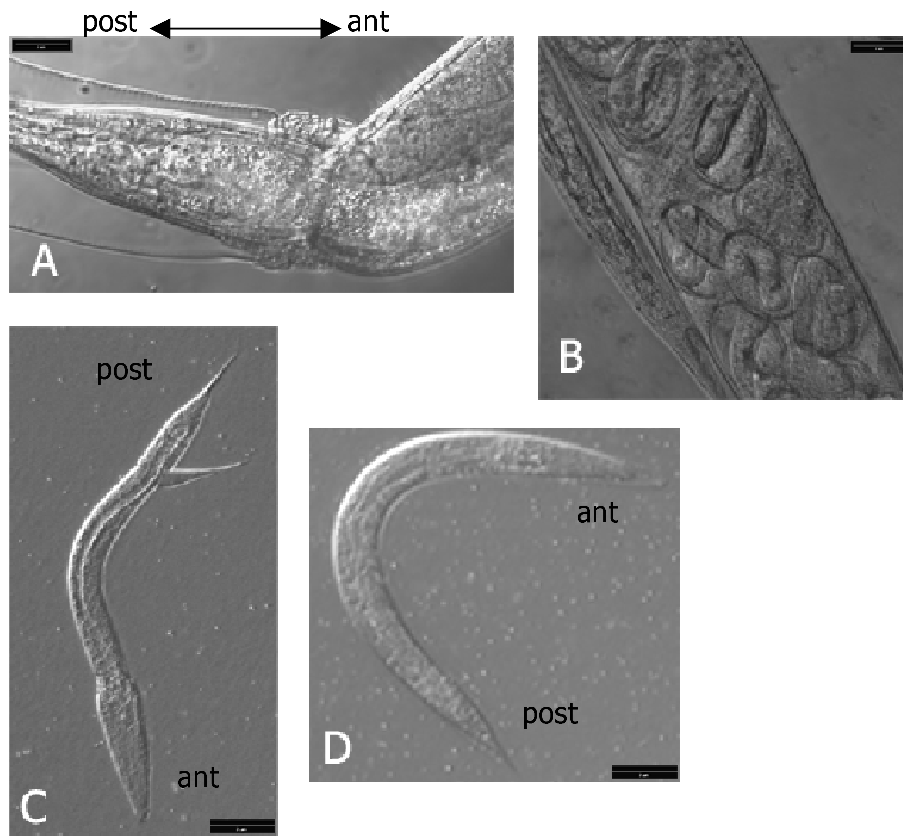

Fig. 3.6 Defects observed in larvae on *nhr-25* and *nhr-23* RNAi. (A) molting defect in *nhr-25(RNAi)* worm of L4 stage, (B) vulval defect in *nhr-25(RNAi)* worm, (C) molting defects (arrows) in *nhr-23(RNAi)* of L3 worm, (D) molting defect (arrow) in *nhr-25(RNAi)* L3 worm. Ant means anterior of the worms, post is posterior. Scale bars A and B 10 μm, C and D 20 μm. All images are DIC images.

3.5.1 GFP expression driven by binding sites was affected by RNAi

The green fluorescent pattern of the strains was affected with the RNAi. Most interesting data were given from the strain 94TCT. There was no significant difference in early larval stages but the reduction of GFP expressing posterior gut cells in L3 stage was more prominent with *nhr-23(RNAi)* compared to control (vector) and *nhr-25* RNAi. At L4 stage, the difference with control is even bigger though *nhr-25(RNAi)* also shows the reduction (Table 6, Fig. 3.7, Fig. 3.8 D). Although the number of GFP expressing worms was decreasing during the normal development, this reduction occurred much faster in *nhr-23* and *nhr-25* RNAi treated worms and completely abolished in the adults (Fig. 3.7). The neuronal expression was not affected and it is not surprising as neurons are not sensitive to feeding RNAi (Table 6).

Table 6. RNAi effect on GFP expression in worms carrying 94TCT

RNAi	stage	gut			neurons		n
		anterior (%)	posterior (%)	background (%)	head (%)	tail (%)	
vector	L1/L2	45(88)	51(100)	0(0)	14(27)	36(71)	51
	L3	24(65)	4(11)	33(89)	4(11)	21(57)	37
	L4	27(55)	5(10)	44(89)	5(10)	18(37)	49
	adult	1(3)	17(52)	13(39)	5(15)	4(12)	33
	total	97(57)	26(15)	139(82)	28(16)	79(46)	170
nhr-25	L1/L2	39(87)	45(100)	0(0)	7(16)	9(20)	45
	L3	21(64)	28(85)	5(15)	1(3)	12(36)	33
	L4	4(8)	20(42)	24(50)	1(2)	11(23)	48
	adult	0(0)	0(0)	5(38)	1(8)	6(46)	13
	total	64(46)	93(67)	34(24)	10(7)	38(27)	139
nhr-23	L1/L2	51(86)	58(98)	1(2)	11(19)	23(39)	59
	L3	29(59)	35(71)	11(22)	1(2)	22(45)	49
	L4	3(33)	5(56)	3(33)	0(0)	4(44)	9
	adult	0(0)	0(0)	0(0)	1(7)	1(7)	15
	total	83(63)	98(74)	98(74)	13(10)	50(38)	132

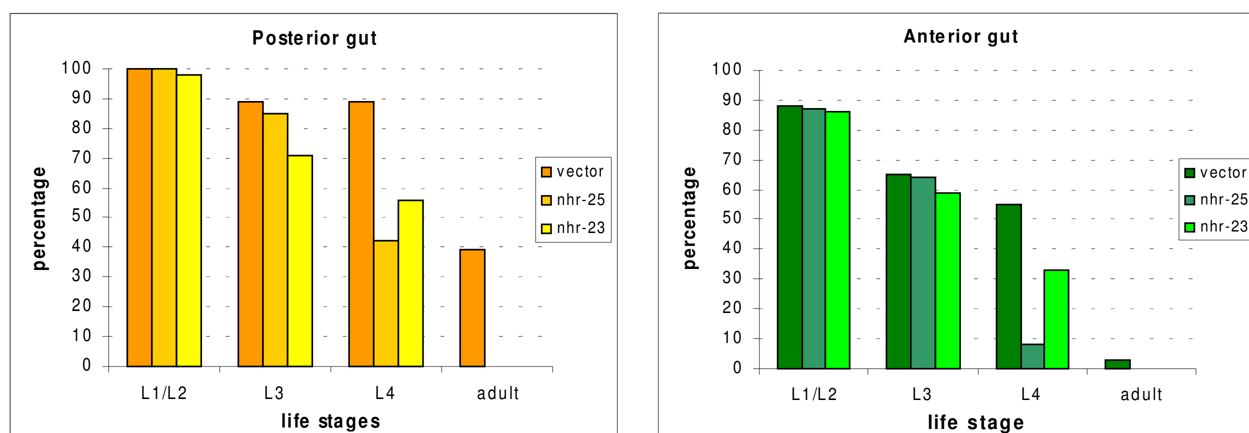

Fig. 3.7 RNAi effect on worms carrying 94TCT during postembryonic development. Percentage of worms expressing GFP in the posterior or anterior gut cells.

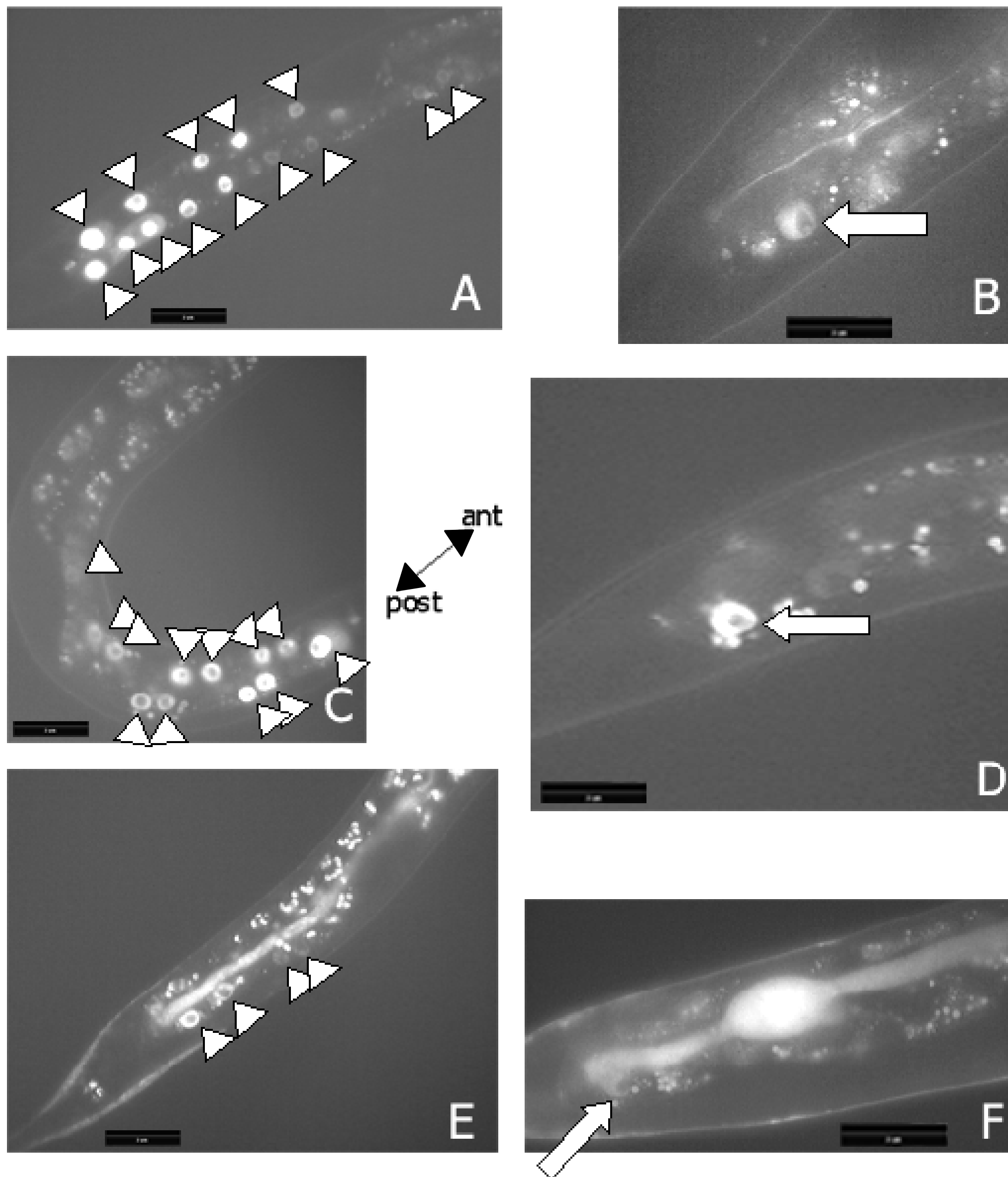

Fig. 3.8 GFP expression on RNAi treated worms carrying 94TCT. (A) Control, L3 larva, strong expression in the nuclei of enterocytes (arrowheads), (B) Control, L4 stage, nucleus of enterocyte (arrow). (C) *nhr-25(RNAi)*, L3 larva, strong expression in the nuclei of enterocytes (arrowheads). (D) *nhr-25(RNAi)* L4 larva, nucleus of the enterocyte (arrow). (E) *nhr-23(RNAi)* L3 larva, nuclei of enterocytes (arrowheads). (F) *nhr-23(RNAi)* L4 worm, nucleus of the enterocyte (arrow). GFP signals in *nhr-23(RNAi)* worms is much lower than in *nhr-25(RNAi)* and control worms. Ant means anterior, post is posterior. Scale bars 10µm.

Due to weaker expressions of GFP in other strains, it was quite problematic to evaluate the level of RNAi effect on the expression. The complete table of all strains tested for RNAi is shown below (Table 7).

Table 7. The summary of RNAi results of all four strains.

strain	RNAi	gut			neurons		n
		anterior(%)	posterior(%)	background(%)	head(%)	tail(%)	
94TGA	vector	4(5)	3(4)	11(13)	2(2)	2(2)	82
	nhr-25	0(0)	9(11)	6(8)	1(1)	2(3)	79
	nhr-23	2(3)	11(14)	2(3)	2(3)	4(5)	77
97TGA	vector	0(0)	1(2)	4(10)	0(0)	0(0)	42
	nhr-25	0(0)	0(0)	4(8)	0(0)	1(2)	51
	nhr-23	0(0)	1(2)	3(6)	0(0)	0(0)	53
94TCT	vector	97(57)	139(82)	26(15)	28(16)	79(46)	170
	nhr-25	64(46)	93(67)	34(24)	10(7)	38(27)	139
	nhr-23	83(63)	98(74)	15(11)	13(10)	50(38)	132
97TCT	vector	0(0)	0(0)	0(0)	1(1)	3(2)	126
	nhr-25	0(0)	0(0)	0(0)	0(0)	0(0)	124
	nhr-23	0(0)	0(0)	0(0)	1(1)	2(1)	142

Strains with 94TGA, 97TGA and 97TCT showed almost no GFP expression and neither *nhr-25* nor *nhr-23* RNAi altered those expression. Therefore NHR-25 and NHR-23 do not seem to work as repressors on these elements.

3.5.2 Analyses of *nhr-23(RNAi)* using electron microscopy

DIC observation of *nhr-23(RNAi)* together with the strong expression of 94TCT (containing ROR/NHR-23 binding sites) led me to analyze ultrastructure of inside of *nhr-23(RNAi)* worms. Control (*vector(RNAi)*), *nhr-25(RNAi)* and *nhr-23(RNAi)* adult worms were subjected for electron microscopy and morphology of the gut, the lumen and epidermis was analysed. (Fig. 3.10, Fig. 3.11, Fig. 3.12).

In control worms, the middle of the body was cut and large gonad was observed and the gut lies very near the body wall (Fig. 3.10). The nuclei of the enterocytes seemed to be normal in size and shape and the cytoplasm contained

many granules, yolk and lipid vacuoles. The cell membranes and cell-cell contacts were normal.

The cuticle structure, namely the thickness, composition of layers and alae on the surface was normal.

nhr-25 silenced worms had gut very similar to these control worms, without any defects or abnormalities. Defect was seen in the cuticle, which was evidently thinner than in the controls, and had no alae (Fig. 3.11 C), which corresponds with presence of molting defects and problems with cuticle

In *nhr-23* silenced worms, the lumen of the gut showed similar morphology to the other two, but in the posterior part of the gut, the abnormal number of granules and lipid vacuoles was found (Fig. 3.12 B). *nhr-23* may have some yet unknown function in the gut. Abnormally high number of granules and vacuoles were also found in surrounding tissues like muscles and hypodermal cells.

The cuticle development was also affected, so that the cuticle was thicker in the lateral locality, where alae should be (Fig. 3.12 C). Alae was not found, only some kind of excrescence was localized in this area.

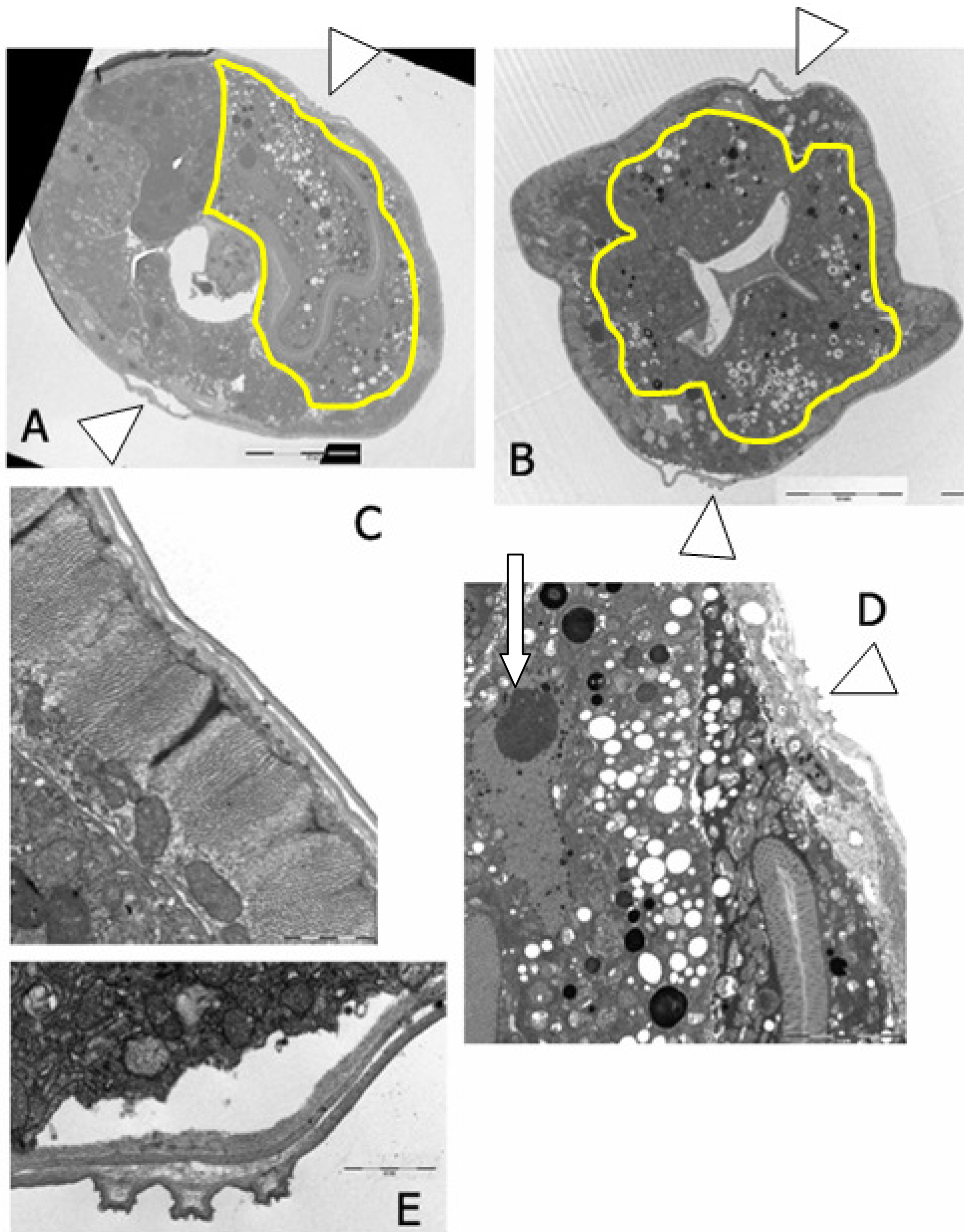

Fig. 3.10 Tramission electron micrography of adult control (*vector(RNAi)*) worms. (A) Section in middle of the body. Scale bar 20 μm. (B) Ultrasection of the anterior gut at the place of the first intestinal ring. Scale bar 10 μm. (C) Cuticle structure is smooth and has normal thickness. Scale bar 1 μm. (D) Ultrasection through middle gut, part of the lumen and the nucleus of the enterocyte (arrow). Scale bar 5 μm. (E) Detail of the alae in the middle of the body. Scale bar 1 μm. Yellow line indicate border of the gut and alae was indicated by arrowheads.

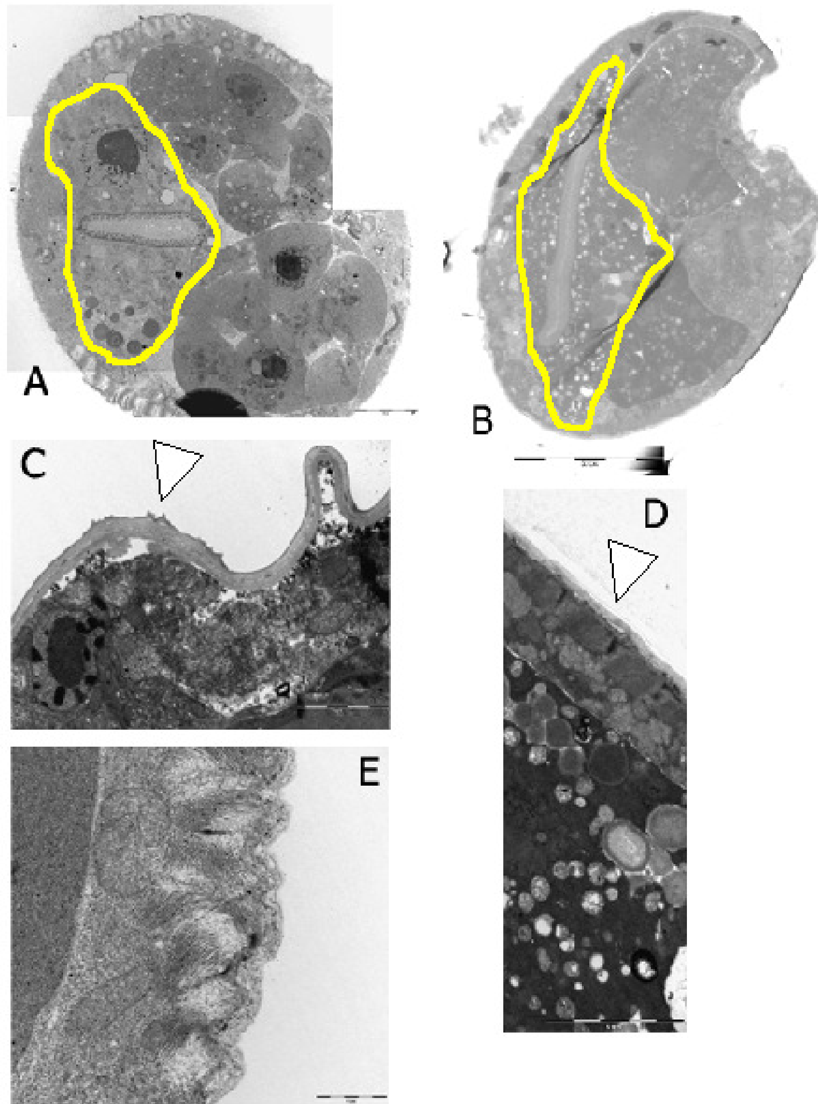

Fig. 3.11 Ultrathinsection of adult worms after *nhr-25* RNAi. (A) Section in middle of the body. Scale bar 5 µm. (B) Different adult worm, section through middle gut. Scale bar 20 µm. (C) Abnormal cuticle arrowhead indicates the place of alae without proper alae. Scale bar 2 µm. (D) Section through middle gut, thin cuticle (arrowhead). Scale bar 5 µm. (E) Detail of the cuticle in the middle of the body, it is not smooth, but wavy. Scale bar 1 µm. Yellow line indicates gut border.

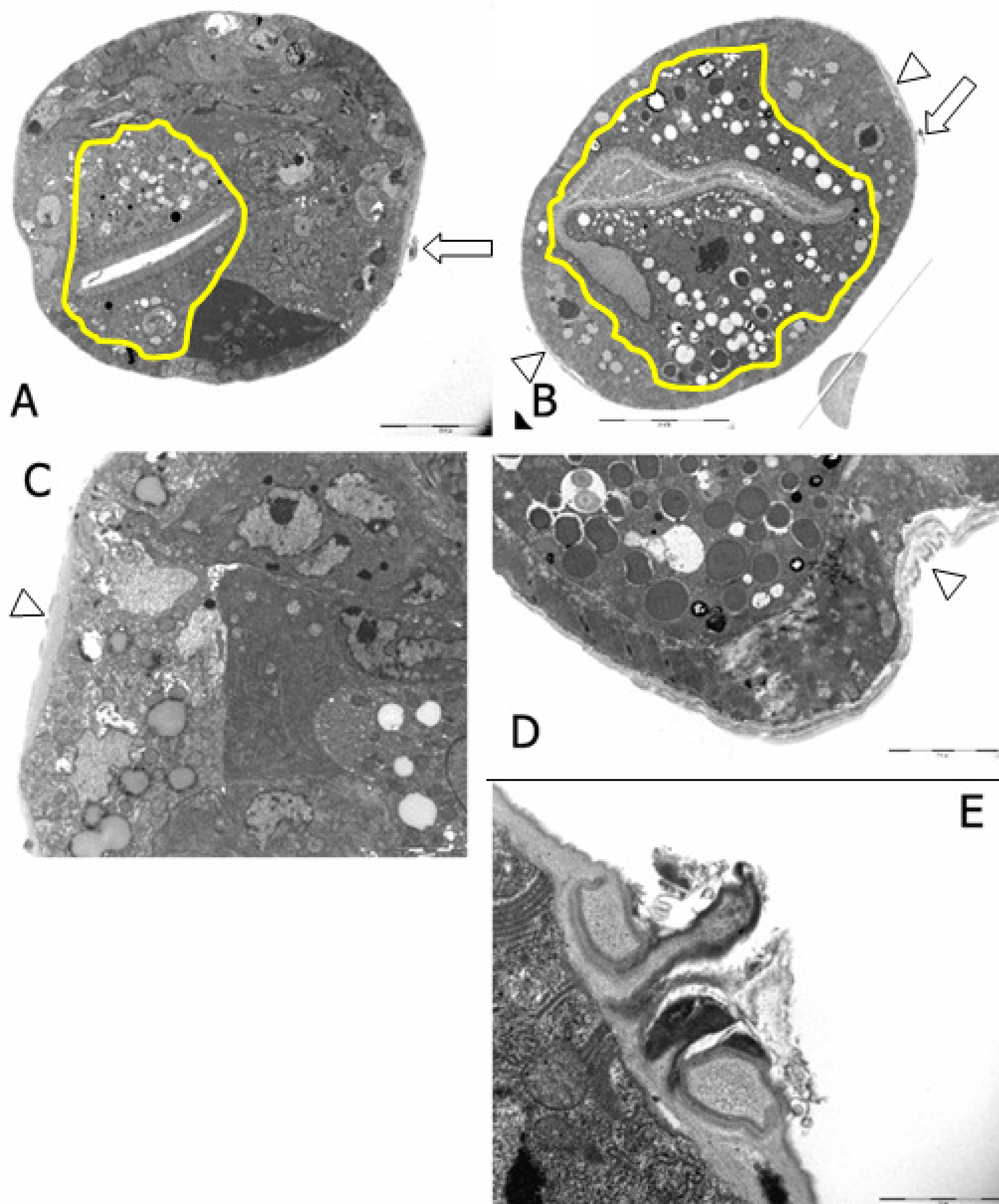

Fig. 3.12 Ultrathinsection of adult worms after *nhr-23* RNAi. (A) Section in the middle of the body of the worm, inside of the lumen is visible. Arrow indicates the cuticle defect. Scale bar 10 µm. (B) Section through posterior gut in different adult worm, arrowheads indicate the place of missing alae and arrow indicate cuticle defect. Many unusual granules were observed in the gut. No proper development of the gonad is seen. Scale bar 10 µm. (C) Cuticle is thick without fully formed alae (arrowhead). Scale bar 2 µm. (D) Defective cuticle, thick and disorganized (arrowhead). Scale bar 5 µm. (E) Detail of the cuticle defect. Scale bar 1 µm. Yellow lines indicate the border of the gut, arrowheads indicate the place of alae.

4. Discussion

The objective of this work was to search further for the function of nuclear receptor NHR-25 in transcriptional regulation in *C. elegans*. This was tested by the analysis of NHR-25 binding sites (enhancer) activity *in vivo* by transforming *C. elegans* and the specificity of the activity was tested by RNA interference.

4.1 Creation of the strains

GFP vectors has been used for gene expression analyses in *C. elegans*. There are two vectors with different minimal promoters currently available and widely used for enhancer studies (Brodigan et al., 2003). One is a minimal promoter from *myo-2* and the other is from *pes-10*. As minimal promoters may have some background activity in certain tissues, both vectors were used to clone a fragment containing either NHR-25 (2xTGAAGGTCA) or ROR/NHR-23 (2xTCTAGGTCA) binding sites. ROR binding site sequence is similar to NHR-25 binding element except it has two bases difference and these bases are known to be responsible for NHR-25 specificity in vitro. Therefore it was thought to be an ideal control to test the binding site specificity *in vivo*.

The transformation was successful, after some problems all four designed strains were obtained. Integration, however, was successful only in one half – I got two strains integrated, one was 97TGA with *NHR-25bs::myo-2::GFP* and 94TCT with *RORbs::pes-10::GFP*. The reason for not getting integrants for 94TGA and 97TCT is not clear. One possibility is the toxicity of the plasmid due to high copy number but it is unlikely as I could obtain integrants for 97TGA and 94TCT. The position of integration on the chromosome is accidental, so it is possible that this plasmid was integrated preferably into some improper place and the animals died.

4.2 Expression of GFP in the worm

GFP expression in the gut was detected in strains carrying 94TGA, 97TGA and 94TCT. In two of them (94TGA, 97TGA) it was most probably only background

caused by the plasmid alone (Harfe and Fire, 1998) because the number of GFP positive intestinal cell nuclei hardly ever exceeded three. However, in 94TCT, the GFP signal was much stronger and the number of GFP positive nuclei reached even fifteen. We considered this expression pattern as a true signal. The problem is that we could not confirm this expression pattern with the other construct, 97TCT as worms carrying this construct practically did not show any expression. While we cannot exclude the possibility completely that 94TCT GFP expression is an artefact of the minimal promoter, there is the significant difference between 94TGA and 94TCT (they are with same minimal promoter and the only difference is the four bases of binding sites) expression, therefore it does not seem to be a simple artefact.

The expression of GFP was observed in neurons in the head and the tail. In the tail area, there are few groups of neurons, organized in front of or behind the anus. In front of it, in location of pre-anal ganglion, was never seen any fluorescent nucleus. Neurons behind anus in region of dorso-rectal ganglion and lumbar ganglion had green nuclei very often. It was difficult to identify the cells as they are small and seems to change the expression profile depends on the developmental stages.

To our surprise, no strong expression in the epidermal tissue was detected with any of the constructs. We had expected to see the expression in the seam cells and hyp cells as both NHR-25 and NHR-23 have important roles in the epidermis (e.g. differentiation of epidermal cells and cuticle synthesis/deposition shown in RNAi study). In only one strain, 94TGA, the GFP expression was found in hypodermal cells. Unfortunately, integration of this strain failed and the we could not confirm the expression.

In vivo (worm), though the element of TGAAGGTCA can react to NHR-25 in human cell lines, it is possible that NHR-25 regulates target genes through slightly different sequences. TCAAGGTCA is also known to bind protein such as Ftz-F1, the *Drosophila* homolog og NHR-25. The affinity for the NHR-25 binding to these elements could be different and when it is weak, it may be difficult to see GFP signal in the worm.

Each tissue may be regulated by slightly different binding sequence ar the combination of the binding sites (generally transcription factors regulate target gene expression through not only one binding sequence). This means more binding sites

for the same transcription factor or for a couple of different transcription factors. We can often find in natural promoters, a series of binding sites and transcription factors share the promoter and act in a cooperative way, sometimes synergistically and sometimes repressively. It is possible that more elements are required to see the authentic enhancer activity in the epidermis.

It is known the more binding sites, the stronger activation of target gene. For this study we used 2x TGAAGGTCA as an enhancer element. It is possible theat when the number of binding sites is increased (e.g. 7x), the signal could be more prominent.

4.3 Specificity of the expression of *RORbs::pes-10::GFP*

As 94TCT (*RORbs::pes-10::GFP*) showed strong expression in the gut, RNAi against *nhr-23* was performed to test the specificity of the binding site. The effect of RNAi of *nhr-23* was visible at L3 stage (reduction of GFP expressing cells) and not with control or *nhr-25(RNAi)*, but at L4 and adult stage, the effect was also seen with *nhr-25(RNAi)*. Thus we could not prove the specificity of the ROR/NHR-23 binding site *in vivo*. However, the reduction was significant compared to control and it is possible that both NHR-25 and NHR-23 has some roles in regulating RORbs-dependent transcription.

The specificity of 94TCT expression by NHR-23 in the worms was not conclusive but the transactivation of TCTAGGTCA element by NHR-23 has not been tested and it would be interesting to test it *in vitro* (human cell line). But it has been tested that this element cannot be activated by NHR-25.

4.3.1 Potential new function of NHR-23 in the gut

Ultrastructure analyses of *nhr-23* RNAi treated worms revealed higher number of granules and other compartments of the posterior region of the gut. These granules were also found in other tissues (e.g. epidermis). It almost looks like that the granules came out to the body cavity and were engulfed by the epidermis. This suggests some possible unknown function of NHR-23 in the gut. This phenotype was

not found with either control or *nhr-25* RNAi treated worms, though other cuticle defects due to *nhr-25* silencing was evident.

The cuticle after *nhr-23* RNAi was thicker than that in controls, forming no alae and it was impossible to distinguish layers of old (L4) and newly synthesized adult cuticle and rather looked as if these layers had merged into one. This observation is yet different from cuticle phenotype seen in *nhr-25(RNAi)* worms (Silhankova et al., 2005) though silencing of both genes cause molting and cuticle abnormality. This may also hint that the differential roles in the epidermis between NHR-25 and NHR-23.

5. Conclusion

The artificial enhancer elements in a GFP vectors were successfully transformed into the worms. Two different enhancer elements (one for NHR-25 and one for ROR/NHR-23) in two different GFP expression vectors (containing minimal promoters from either *myo-2* or *pes-10*) were tested. The strongest GFP expression was observed in *RORbs::pes-10::GFP* transformed worms, the expression occurred in the intestinal cells and neurons in the head and the tail. In other transformed worms, the expression was very weak or absent, it is possible that 2x binding sites may not be sufficient for *in vivo* enhancer study in *C. elegans*.

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