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The study of expression and function of selected nuclear receptors in *Caenorhabditis elegans*

PhD thesis summary

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Prague 2010

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PhD thesis sent: 1.11.2010

Defense of PhD thesis:

Place of defense: 1st Faculty of Medicine, Charles University
Purkyňův ústav
Albertov 4, Prague 2,

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This PhD thesis was elaborated within the PhD program for studies in Biomedicine at the Institute of Inherited Metabolic Disorders, 1st Faculty of Medicine, Charles University, Prague.

This work was supported by the grant projects 304/08/0970 and 304/07/0529 awarded by the Czech Science Foundation and by Grant 0021620806 from the Ministry of Education and Youth of the Czech Republic.

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Podpis:

Identifikační záznam:

VOHÁNKA, Jaroslav. *Studium exprese a funkce vybraných jaderných receptorů v modelovém organismu Caenorhabditis elegans. [The study of expression and function of selected nuclear receptors in Caenorhabditis elegans]*. Praha, 2010, 96s., 4s příl. Disertační práce (Ph.D.) Univerzita Karlova v Praze, 1. lékařská fakulta, Ústav dědičných metabolických poruch. Vedoucí závěrečné práce MUDr. Marta Kostrouchová, CSc.

Abstrakt*:*

V genomu *Caenorhabditis elegans* je více než 280 jaderných hormonálních receptorů (NHRs) v porovnání se 48 jadernými receptory u člověka a 18 jadernými receptory u octomilky (*Drosophila*). Většina jaderných hormonálních receptorů u *C. elegans* patří do skupiny suplementárních (podpůrných) jaderných receptorů (supnrs), které vznikly následnou duplikací jednoho původního genu. Evoluční tlak, který vedl ke zmnožení jaderných hormonálních receptorů u nematod, stejně tak i funkce většiny supnrs nejsou známé. V této práci byla studována exprese sedmi genů organisovaných v klastru na chromosomu V: *nhr-206, nhr-208, nhr-207, nhr-209, nhr-154, nhr-153* a *nhr-136*.

Metodou reverzní transkripce-kvantitativní PCR a použitím transgenních linií nesoucích fůzní geny (obsahující pravděpodobné promotory) s GFP bylo zjištěno, že všech sedm genů tohoto klastru je exprimováno a pět z těchto genů má částečně se překrývající expresní profil. Exprese byla lokalizována v jícnu, ve střevě, v určitých neuronech, v análním svěrači a v samčích specifických buňkách.

Čtyři geny v tomto klastru jsou zachovány u *C. elegans* a u *C. briggsae*, zatímco tři geny jsou přítomné pouze u *C. elegans*, což ukazuje na poměrně nedávnou genovou expanzi.

V naší práci jsme zjistili, že jak část zachovaných tak všechny nezachované geny v tomto klastru odpovídají transkripčně na hladovění a tato odpověď je tkáňově specifická. Naše výsledky ukazují, že rozrůznění časové, prostorové a metabolické genové exprese je ve skupině suplementárních jaderných receptorů spojeno s evolučním tlakem.

Abstract:

The genome of *Caenorhabditis elegans* encodes more than 280 nuclear hormone receptors (NHRs) in contrast to the 48 NHRs in humans and 18 NHRs in *Drosophila*. The majority of the *C. elegans* NHRs are categorized as supplementary nuclear receptors (supnrs) that evolved by successive duplications of a single ancestral gene. The evolutionary pressures that lead to the expansion of NHRs in nematodes, as well as the function of the majority of supnrs, are not known. Here, we have studied the expression of seven genes organized in a cluster on chromosome V: *nhr-206, nhr-208, nhr-207, nhr-209, nhr-154, nhr-153 and nhr-136*.

Reverse transcription-quantitative PCR and analyses using transgenic lines carrying GFP fusion genes with their putative promoters revealed that all seven genes of this cluster are expressed and five have partially overlapping expression patterns including in the pharynx, intestine, certain neurons, the anal sphincter muscle, and male specific cells.

Four genes in this cluster are conserved between *C. elegans* and *C. briggsae* whereas three genes are present only in *C. elegans*, the apparent result of a relatively recent expansion. Interestingly, we find that a subset of the conserved and non-conserved genes in this cluster respond transcriptionally to fasting in tissue-specific patterns. Our results reveal the diversification of the temporal, spatial, and metabolic gene expression patterns coupled with evolutionary drift within supnr family members.

Klíčová slova: *Caenorhabditis elegans; Caenorhabditis briggsae;* jaderný hormonální receptor; genová exprese; hladovění

Keywords: *Caenorhabditis elegans; Caenorhabditis briggsae;* nuclear hormone receptor; gene expression; fasting

ACKNOWLEDGEMENTS

I would like to thank my supervisor MUDr. Marta Kostrouchová, CSc. for her scientific approach and understanding throughout my study.

I am grateful to Prof. MUDr. Milan Elleder, DrSc., the Institute of Inherited Metabolic Disorders for giving me the opportunity to work on this thesis.

I would like to thank MUDr. Zdeněk Kostrouch, CSc. and Dr. Michael Krause for their support and advice all along my work on the topic.

I thank all my colleagues at the Institute, namely to Ing. Michal Pohludka, Ph.D., František Behenský, Mgr.Kateřina Šimečková,Ph.D., Mgr. Eva Brožová, Ph.D., Lenka Brtvová, Hana Prouzová and Eliška Machalová for their help and friendship.

Vectors L4440, pPD95.67 were a kind gift from Dr. Andrew Fire (Stanford University School of Medicine, Stanford CA, USA).

Content:

1. The aim of the study

The aim of the study was to analyze a cluster of seven genes localized on chromosome V in *Caenorhabditis elegans,* which were predicted as nuclear hormone receptors. All seven receptors belong to the Class I of *C.elegans* nuclear hormone receptors based on their P-box sequences (Gilst 2002). NHR-206, NHR-208, NHR-207 have P box sequence CNGCKA and form a small subgroup 12, and four receptors NHR-209, NHR-154, NHR-153 and NHR-136, which have P box sequence CNGCKT form the subgroup 8, (Van Gilst, 2002).

The goal of this study was

a) to determine whether all these receptors are functional NHRs

b) to characterize and localize their expression

c) to find their function

2. Abbreviations

AF-1 activation function-1

AF-2 activation function-2

C.briggsae Caenorhabditis briggsae

C.elegans Caenorhabditis elegans

cDNA complementary DNA

CGC *Caenorhabditis* Genetics Center

CTE carboxy terminal extension

DAPI diamidinophenyl indole

DBD DNA binding domain

DNA deoxyribonucleic acid

dsRNA double stranded RNA

EcR ecdysone receptor

E. coli Escherichia coli

e.g. exempli gratia

Fig. figure

gDNA genomic DNA

GFP green fluorescent protein

HNF4 hepatocyte nuclear factor 4

HRE hormone response element

IPTG isopropyl-β-D-thiogalactopyranoside

kb kilobase

LBD ligand binding domain

Mb Megabase

mRNA messenger RNA

NCoR nuclear receptor corepressor

NGF nerve growth factor

NGM nematode grow medium

NHR nuclear hormone receptor

NLS nuclear localization signal

NR nuclear receptor

PBS phosphate buffered saline

PCR polymerase chain reaction

PPAR peroxisome proliferator-activated receptor

qPCR quantitative PCR

RACE rapid amplification of cDNA ends

RAR retinoic acid receptor

RXR retinoid X receptor

RNA ribonucleic acid

ROR RAR-related orphan receptor

rRNA ribosomal RNA

RT-PCR reverse transcription polymerase chain reaction

RT-qPCR real-time quantitative polymerase chain reaction

SD standard deviation

SL1 splice leader 1

SL2 splice leader 2

SMRT silencing mediator of retinoid and thyroid receptors

SRC-1 steroid receptor coactivator-1

ssRNA single stranded RNA

supnr supplementary nuclear receptor

TR thyroid receptor

tRNA transfer RNA

TTBS Tris Tween Buffered Saline

VDR vitamin D receptor

3. Introduction

3.1. Suitable model organism - *Caenorhabditis elegans*

Using a model organism helps us to understand biological functions and phenomena because of the conservation of several metabolic and developmental pathways over the course of evolution. Studying model organisms can be informative, but care must be taken when generalizing from one organism to another. Nowadays model organisms are widely used to explore gene regulatory cascades and causes and treatments for human diseases.

There are many model organisms. One of the first model systems for molecular biology was the bacterium *Escherichia coli*, a common constituent of the human digestive system. Several of the bacterial viruses (bacteriophages) that infect *E. coli* also have been very useful for the study of gene structure and gene regulation (e.g. phages Lambda and T4). From eukaryotes, several yeasts, particularly *Saccharomyces cerevisiae* (baker's yeast), have been widely used in genetics and cell biology, largely because of the simplicity with which yeast can be grown. The cell cycle in a simple yeast is very similar to the cell cycle in human cells and is regulated by homologous proteins. The fruit fly *Drosophila melanogaster* is studied, because it is easy to grow, has various visible congenital traits and has a polytene (giant) chromosome in its salivary glands that can be examined under a light microscope. The nematode *Caenorhabditis elegans* is studied because it is a simple organism, it has short generation time, it has very defined patterns of development involving fixed numbers of cells, it has a transparent body, which could be rapidly assayed for abnormalities.

3.1.1. Main features

Caenorhabditis elegans is a small nematode living in soil across the world. The *C. elegans* in nature feeds on bacteria. In laboratory conditions the main food is a non pathogenic strain of *E.coli*. *C.elegans* has a short life cycle and has a lot of progeny. Due to these properties *C.elegans* became a useful model organism for genetic and developmental studies (Brenner, 1974).

C. elegans has a constant number of somatic cells. Adult hermaphrodites have 959 somatic nuclei, adult males 1031. *C. elegans* was the first organism where full cell lineage was described and the first multicelular organism in which the whole genome was sequenced. Worms have small size and could be easily cultivated under laboratory conditions. They can be grown in Petri dishes on nematode growth medium (NGM) seeded with *Escherichia coli*.

3.1.2. Life cycle

C.elegans has two sexes: a self-fertilizing hermaphrodite (XX) and a male (XO). Males produce only sperm and can fertilize hermaphrodites. Hermaphrodites produce both sperm and oocytes and are capable of self-fertilization, but cannot fertilize each other. Males are found at low frequency (approximately 0,1%). They arise spontaneously by non-disjunction of sex chromosomes.

An adult hermaphrodite lays about 300 embryos. They hatch in twelve hours into L1 larvae. *C. elegans* develops during four larval stages (punctuated by molt) into an adult animal. The development is completed during three - four days (Fig. 1). The adult animals are approximately 1 mm long and live for an additional 14 days. Due to unfavorable environmental conditions a specific larval stage like dauer larvae can occur. Dauer larvae (instead of L3 larvae) do not feed and survive several months. When food becomes available, dauer larvae molt to be normal L4 larvae (Hope, 1999).

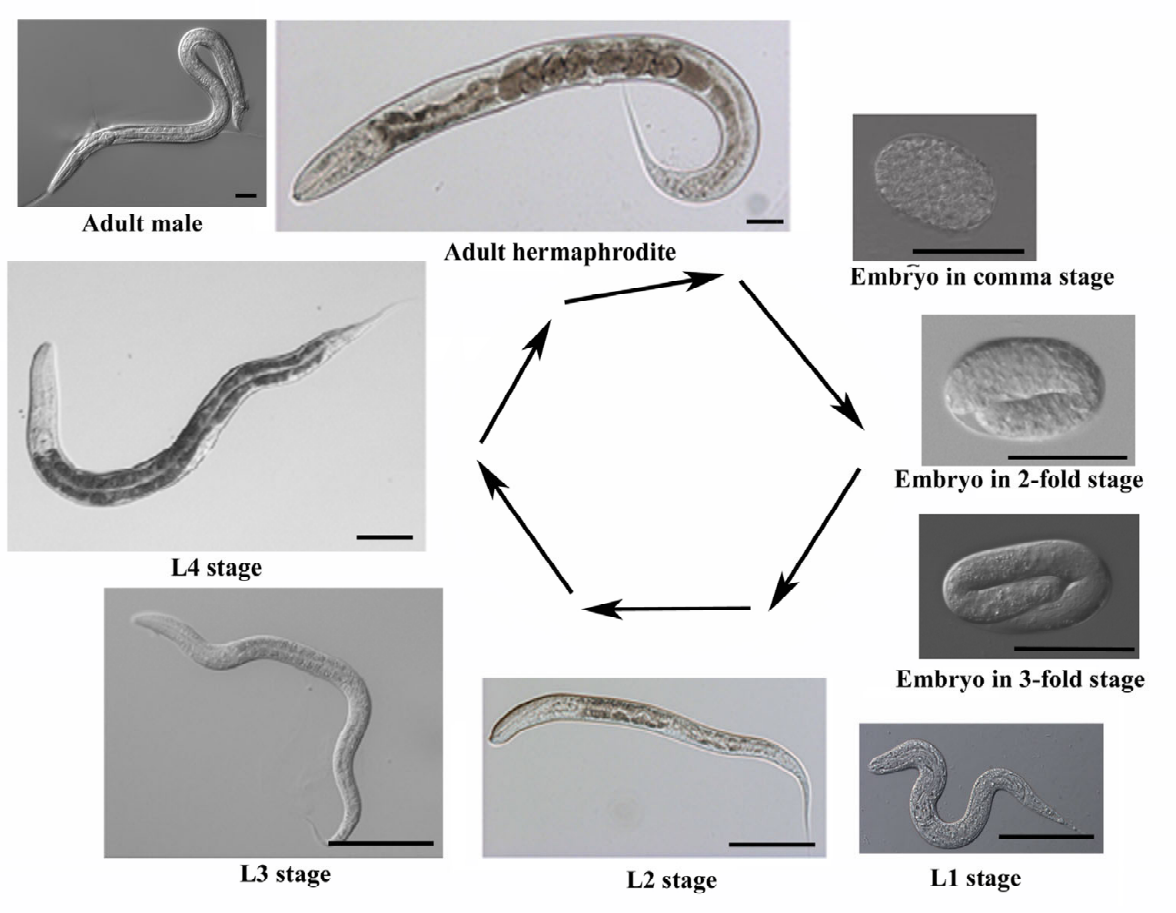

Fig. 1: *C.elegans* developmental stages.

The individual *C.elegans* stages: embryos (comma, 2-fold and 3-fold), larval stages (L1 larva, L2 larva, L3 larva, L4 larva) and adult hermaphrodite and adult male. Scale bar: 40 µm

3.1.3. Development

The development of *C.elegans* has two distinct periods. The first period starts with mitotic cell divisions generating cells that form the first larval stage (558 cells in the hermaphrodite) and the second period consist of morphological changes. (Sulston et al., 1983).

3.1.3.1. First part of embryogenesis

First embryonic divisions generate six cells in various sizes. These cells are called founder cells: AB, E, MS, C, D, and P_4 (Fig.2). Each "stem" cell gives rise to a predetermined number of cells by a series of synchronous and asymmetrical divisions. The state of

determination is clonally inherited (Deppe et al., 1977). E gives rise to intestinal cells, D to body-wall muscle cells and P_4 to germ lines. Other founder cells produce several cell types.

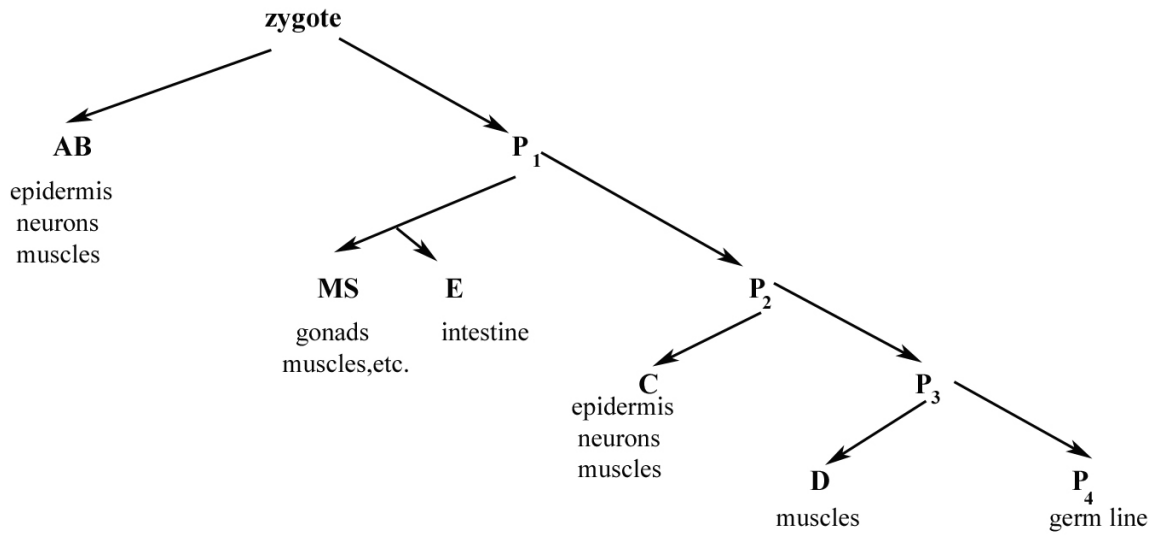

Fig. 2: Initial cell division in *C.elegans*

All founder cells are marked. Their future descendants are noted. The first phase of embryogeneses (time 0 to 150 min) spans the time between zygote formation to the generation of embryonic founder cells.

3.1.3.2. Second part of embryogenesis

This period of embryogenesis consists of morphogenetic changes. The ovoid embryo is squeezed into the elongated shape of L1. Elongation proceeds through several morphological forms: the comma, 1.5-fold, 2-fold, and 3-fold stages. In the 3-fold stage the L1 cuticle is formed. Cells differentiate and the embryo begins to move in the chitin shell during elongation.

3.1.3.3. Post-embryonic development

During post-embryonic development, *C. elegans* develops through four larval stages to adulthood. This development lasts 4 days. In the end of each larval stage the old cuticule is shed and a new one is formed under the old cuticle. Metamorphosis is not involved in *C.elegans* post-embryonic development. All structures are formed while maintaining the same overall structures already generated during embryogenesis (Hope, 1999). From L3 stage the germ cells in the gonad

proliferate and the mature gonads are formed in adult stage. The hermaphrodites have two gonad arms and a spermatheca on each side of the gonads. The males have only one male gonad. The number of somatic cell nuclei increased and reached 959 cells in hermaphrodites and to 1031 in males.

3.1.4. Genetics

3.1.4.1. Basic introduction

Wild-type *C.elegans* hermaphrodites contain five pairs of autosomes and one pair of X chromosomes. Males contain five pairs of autosomes and a single X chromosome. The condensed chromosomes are cytogenetically indistinguishable. The *C.elegans* chromosomes have diffuse kinetochores without a visible constriction (Albertson and Thomson, 1982). Recombination occurs in the sperm of males and in both sperm and oocytes of hermaphrodites (Brenner, 1974).

The genome has a relatively small size - approximately 100 Mb (one-thirtieth the size of mammalian genomes). The *C.elegans* Genome Sequencing Consortium (at the Sanger Institute, Cambridge, UK [http://www.sanger.ac.uk/Projects/ C_elegans/] and at the Genome Center, Washington University of St Louis, MI, USA [http://genome.wustl.edu/]) were established to determine the entire *C.elegans* DNA sequence. The essentially complete sequence was published in Science in December 1998 (Hodgkin J.,1998). The sequence was annotated using ACeDB software [http://www.acedb.org]. The *C.elegans* research community uses several databases to share recent data such as Caenorhabditis elegans WWW Server [http://elegans.swmed.edu/], WormBase [http://www.wormbase.org/], WORMATLAS [http://www.wormatlas.org/] and many others.

Genes in *C. elegans* are short and have an average of five introns. Exons comprise of 27% of the genome. About 42% of the predicted protein products match those of organisms in other phyla.

3.1.4.2. Operons and trans-splicing

C.elegans and its relatives differ from other animals in having operons. Approximately 20% of *C.elegans* genes are organized in operons which contain 2-8 genes (Blumenthal et al., 2002). Polycistronic *C.elegans* operons are processed by internal cleavage. Polyadenylation

follows to form 3´ends of mRNA. At the same time, approximately 100 bp downstream of the newly created 3´end, trans-splicing creates the 5´end of the downstream mRNA (Liu et al., 2003). During the trans-splicing, a short RNA leader is attached to the 5´end of mRNA. Two types of splice leaders are described in *C.elegans* – SL1, which is trans-spliced to the 5´ends of monocistronic genes and to the first genes in operons and SL2, which is trans-spliced to genes downstream in the operons.

High throughput genomic data from microarray analysis and SAGE (serial analysis of gene expression) showed that genes in operons in *C.elegans* have positively correlated gene expression (Jones et al. 2001).

3.1.4.3. Protein-coding genes

Protein-coding genes are distributed over the whole genome. The genome encodes approximately 22,000 genes. Most of them are short genes covering around 3 kb. Genes contain usually short exon (median size 123 bases) and intron (median size 47 bases) sequences. Alternative splicing is observed. The number of splicing isoforms per gene is low. More than 90% of genes with alternatively spliced forms have only one or two isoforms (Spieth and Lawson, 2006).

3.1.4.4. Pseudogenes

Pseudogenes are non-functioning copies of genes in genomic DNA. Two types of pseudogenes can be found in the *C.elegans* genome. Processed pseudogenes are created by reverse transcription of mRNA into cDNA and then these cDNAs are inserted into gDNA. Processed pseudogenes appear sporadicly in the *C.elegans* genome while unprocessed pseudogenes are more common and are produced by gene duplication and subsequent disablement. They usually have frameshifts which are followed by STOP codons (Harrison et al., 2001). The number of pseudogenes in *C.elegans* (561 annotated) is still not well estimated.

3.1.4.5. RNA genes

Through the whole genome there are genes which code 5S, 18S, and 26S rRNA genes. A high amount of RNA genes (approximately 150 copies) are localized on chromosome I.

The 5S rRNAs are transcribed separately by RNA polymerase III. (Nelson and Honda, 1985; Sulston and Brenner, 1974). One copy of the 18S/5S/26S rRNA repeat unit is present in the chromosome I sequence assembly and fifteen copies of the 5S rRNA gene are included in the chromosome V sequence. Additionally, the mitochondrial DNA contains one 18S rRNA gene and one 26S rRNA gene.

About 608 nuclear tRNA and 22 mitochondrial tRNA genes are encoded in the *C.elegans* genome. They usually have only one exon (Spieth and Lawson, 2006).

3.1.4.6. The *C.briggsae* genome

The genome of another nematode *Caenorhabditis briggsae* is closely related to the *Caenorhabditis elegans* genome. The areas of sequence encoding proteins are mostly conserved between the two species while most intergenic and intronic sequences are divergent. BLASTP matches and conserved gene order (synteny) have revealed 62% of *C. briggsae* genes to have orthologs in *C. elegans*. About 27% of *C. briggsae* genes have multiple matches in *C. elegans* genome and represent various gene families. Of the remaining 11% of genes, 7% have very weak similarity to *C. elegans* (with a BLASTP *E* value of over 10^{-5}), whereas 4% appear to be unique. *C. elegans* operons are also highly conserved in *C. briggsae*, with the arrangement of genes being preserved in 96% of cases. (Stein et al., 2003).

Caenorhabditis elegans and Caenorhabditis briggsae diverged approximately 80-110 million years ago and are morphologically almost indistinguishable (Stein at al., 2003).

Most gene families have similar sizes between *C. elegans* and *C. briggsae* , as suggested by TRIBE-MCL (a cluster algorithm which provides gene family identification based on sequence similarity results)(Stein et al., 2003). However, there are exceptions that some gene families are larger in *C.briggsae* than in *C.elegans* . For example, there are many more putative chemosensory genes in *C.elegans* than in *C.briggsae* (Chen et al., 2005; Stein et al., 2003). Some families have similar sizes in *C.briggsae* and *C.elegans* , while others, including the srab family (Serpentine receptor) and the srz family (Serpentine receptor class Z) (Chen et al. 2005; Thomas et al. 2005), have twice as many genes in *C.elegans* than in *C.briggsae*. Such gene family size differences resulted largely from massive tandem duplications of individual genes in the *C.elegans* genome. Tandem duplications were also observed in *C.briggsae* but both the ratio and frequency of expansion was not as dramatic in *C.briggsae* as in *C. elegans*. Clustering analysis showed that for gene clusters with five or more *C.elegans* and *C.briggsae* genes, there were 202 clusters (corresponding to gene

families) with gene number differences of at least 2-fold between *C.elegans* and *C.briggsae* (Stein et al., 2003).

3.1.5. Anatomy

3.1.5.1. Basic anatomy

C. elegans is a simple organism with a tubular shape of body. Its surface is covered with cuticle, which is made mainly from collagens. The cuticle is secreted by epidermis, also called hypodermis. The epidermis surrounds the animal. Under the epidermis are four longitudinal rows of body wall muscle cells. They run subventrally and subdorsally. The intestinal tract consists of mouth, bilobed pharynx, intestine, rectum and anus. 302 *C. elegans* neurons are organized in a circumpharyngeal nerve ring, ventral and dorsal nerve cords, sensory receptors and ganglia. The reproductive system of adult hermaphrodites has two gonads. Each gonad consists of an ovary, oviduct, spermatheca, which leads to the uterus. The adult male reproductive system consists of a single testis, which is connected via vas deferens with the rectum. They form a cloaca. (Sulston and Horvitz 1977)

3.1.5.2. Epithelia

C. elegans has two major forms of extracellular matrix: the cuticle, which forms the exoskeleton and basement membranes. Basement membranes support the mechanical stability of tissues and organs (Kramer, 2005). The body form of the animal is supported by epithelial cells together with secreted cuticle. The epithelial cells are connected by belt desmosomes, which have an adherent-junction like structure (Priess and Hirsh, 1986). The epithelial cells in *C. elegans* are present in the alimentary tract, in epidermis, in interfacial cells and in somatic gonads.

3.1.5.2.1. Epidermis

Epidermis in *C. elegans* is an external epithelial layer of cells. They secret the cuticle. Epidermis is divided into three parts: a) major epidermis, which consists of dorsal hypodermis, ventral hypodermis and seam cells, b) small epidermal syncytia, which covers

the head and the tail and c) interfacial epidermis, which connects internal epithelial organs to the epidermis (Michaux et al., 2001).

3.1.5.2.1.1. Epidermal precursors

For the initial specification of epidermis the GATA family zinc finger transcription factor ELT-1 is important. It is expressed from about the 28-cell stage in cells that will give rise to, among other cells, the major epidermal cells (Page et al., 1997). The major epidermal precursors are located on the early embryo (Chisholm, 2005). The expression of regulatory genes ELT-3, ELT-5, ELT-6 and LIN-26 is important to keep their proper fates. The epidermal cells die in lin-26 mutants and cause embryonic lethality with defects in ventral enclosure and elongation (Labouesse et al., 1994, Labouesse et al., 1996).

3.1.5.2.1.2. Elongation

In the second part of embryogenesis the embryo changes its shape very dramatically. It starts to elongate and change from bean shape to worm-like shape. The elongation has two phases called early and late elongation.

Early elongation starts during embryogenesis when the embryo develops from comma to two-fold stage. In this process the epidermis plays the crutial role and provides the driving power for elongation (Priess and Hirsh, 1986). After venral enclosure actin filaments and microtubules are reposed circumferentially untill the elongation is complete. Their contractions result in contractions of epidermal cells and cell shape changes (Priess and Hirsh, 1986). This mainly happens in seam cells – in lateral epidermis while dorsal and ventral epidemal cells do not actively constrict. They probably react on contraction of seam cells and forces transmitted through adherents junctions (mutations in genes coding proteins of adherents junctions lead to ventral enclosure and elongation defects) (Chisholm, 2005).

Late elongation (beyond the two-fold stage) is driven by different mechanisms. In this process muscle cells are involved. Mutants lacking muscle function fail to elongate after this stage. The failure of elongation could be the consequence of a failed interaction between muscle and hypodermal cells (Williams and Waterston, 1994).

After elongation, epidermal cells begin to secret the cuticle, which keeps epidermal cells in place. Defects in cuticle secretion lead to late-onset defects in elongation (Priess and Hirsh, 1986).

3.1.5.2.1.3. Major epidermis

The major epidermis of the embryo consists of the dorsal hyp7 syncytium, the ventral P cells, and the lateral seam cells (Koh and Rothman, 2001). During the postembryonic development, 110 epidermal cells fuse with hyp7 and the dorsal epidermis with 133 nuclei forms the biggest syncytium in *C.elegans* (Podbilewicz and White, 1994).

The ventral hypodermis contains 12 P blast cells during hatching. During postembryonic development, these cells divide and some daughter cells join the dorsal hyp7 syncytium while other daughter cells give rise to either ventral cord motoneurons (form ventral nerve cord) or vulval cells in the hermaphrodite (Sulston and White, 1988). Finally, the hyp7 epidermal syncytium replaces the P cells on the ventral part of the body.

3.1.5.2.1.4. Seam cells

The lateral epidermis is formed by seam cells. Seam cells of L1, dauer, and adult stages produce specific cuticular ridges called alae (linear ridges). Alae are located at the lateral sides of the animal and run along the whole body. Their function is to make the movement of the animal easier.

The lateral epidermis at L1 larval stage has 10 seam cells at each side. They are called $H_0 - H_2$ (located in the head area), $V_1 - V_6$ and T (located in the body and tail region). Almost all of them (except the H_0) are blast cells.

Seam cells divide at each larval stage and one of the daughter cells, which is a stem cell, while the second migrate on the top of their sister and later fuse with hyp7 or become a neuroblast or a ray cell. When seam stem cells reach their seam stem neighbors the division starts again (Podbilewicz and White, 1994). At the end of the L4 stage, seam cells fuse. Fusion occurs between sister seam cells in the anterior and posterior parts of the cells (Podbilewicz and White, 1994) (Fig.3).

In males, different divisions of V_5, V_6 and T seam cells occur in L3 stage. Seam cell daughter cells give rise to the sensory rays of a male tail and seam cells coming from divisions of these three cells do not fuse and do not form alae (Sulston and Horvitz, 1977).

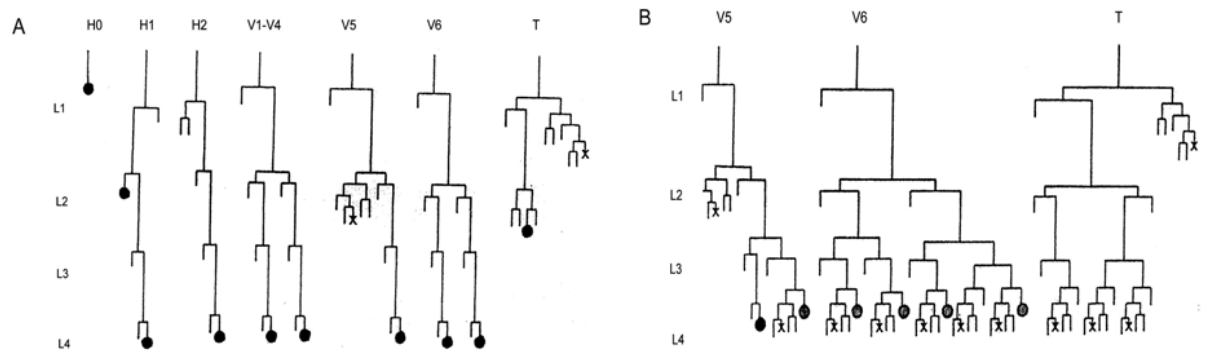

Fig. 3: A seam cells divisions

A: A schema of *C.elegans* hermaphrodite seam cells divisions. The black dots indicate seam cells.
B: A schema of *C.elagans* male. Division of V5, V6, and T seam cells

3.1.5.2.1.5. Epidermal syncytia covering head and tail

The epidermis of the head is formed by six concentric annular syncytia called hyp1 to hyp6 and surrounds the buccal cavity. These syncytia contain from two to six nuclei. The tip of the head has many sensory endings. The sensilla are bound to the cuticle of hyp2 and hyp3. The epidermis of the tail is formed by four mononucleate cells called hyp8, hyp9, hyp11 and the binucleate hyp10 (White, 1988).

3.1.5.2.1.6. Interfacial epidermal cells

The interfacial epidermal cells have toroidal structure and connect internal epithelial organs to the epidermis and are specialized cells forming an opening in the epithelium (White, 1988).

3.1.5.2.2. Gastrointestinal system

The gastrointestinal system (alimentary tract) is formed by a single tube. It consists of the bucal cavity, pharynx, intestine, rectum and anus.

3.1.5.2.2.1. Pharynx

The pharynx (the *C.elegans* foregut) is separated from the bucal cavity by a cuticular constriction. Its main function is to grind food (bacteria) and by rhythmic pumping push it into the gut.

The pharynx has six main cell types: epithelial, neuronal, muscular, gland, marginal, and valve cells (Albertson and Thompson, 1976). The pharynx consists of several regions: an anterior procorpus, a bulb-shaped metacorpus, an isthmus, a terminal bulb with the grinder, and a pharyngeal-intestinal valve (Fig. 4).

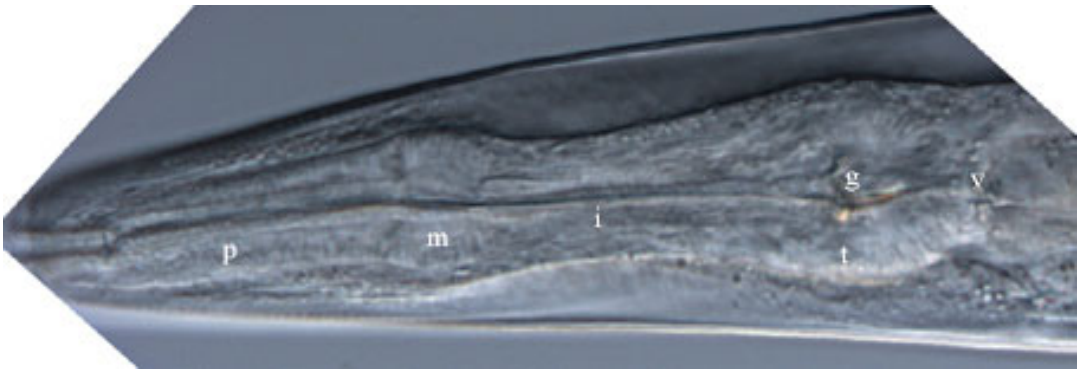

Fig. 4: The *C.elegans* foregut

A picture of *C.elegans* pharynx: procorpus (p), metacorpus (m), isthmus (i), terminal bulb (t) with the grinder (g) and pharyngeal-intestinal valve (v) are marked.

In the pharynx there are present two types of gland cells called g1 and g2. They differ in the amount of cell nuclei: g1 (two cells with three nuclei between them) and g2 (two cells with two nuclei between them).

Glands are located in the terminal bulb of the pharynx and are connected into the pharyngeal lumen. They secrete digestive enzymes during feeding and to help with degradation of chitin and cuticle during molting (Mango, 2007).Their innervation is from pharyngeal motoneurons. The connection between the pharynx and intestine is made by a pharyngeal-intestinal valve.

3.1.5.2.2.2. Intestine

The *C.elegans* intestine is formed from 20 large cells. They are bilaterally symmetric around the lumen, organized in pairs, which form a ring. Only the first anterior ring is formed by four cells. Intestinal cells are mononucleate at L1 stage. During postembryonic development intestinal nuclei undergo endoreduplication before each molt and they become

larger, binucleate and polyploidic (Hedgecock and White, 1985). The intestinal cells have many functions. Their main function is to secrete digestive enzymes into the lumen of the intestine, absorb nutrients and function as storage organs (White, 1988), and they are also involved in oxidative stress resistance (Onken B, 2010)

The connection between the intestine and rectum is made by a rectal valve.

3.1.5.2.2.3. Rectum

The part of the alimentary tract between the rectal valve and opening to the outside is called the rectum. The opening itself is called the anus.

There is a ring made by three large rectal gland cells - rect_D, rect_VL, rect_VR. These cells connect intestinal lumen to the rectal valve. One of their possible functions could be production or secretion of digestive enzymes into the caudal lumen of the intestine.

3.1.5.2.2.4. Male specific organs

The adult male tail consists of several somatic reproductive structures: the acellular fan, the rays, the sclerotic spicules and the gubernaculums, which is a specialized proctodeal chamber with a sclerotic roof and the hook. These structures are formed during the later half of the L4 larval stage. An anterior retraction of the cells of the tail region also occurs in this period (Sulston et al., 1977) (Figure 2).

During retraction the epidermal cells of the tail tip, hyp8-11, fuse (Nguyen et al., 1999) and more anterior hypodermal cells withdraw from the L4 cuticle and the intervening space is filled with fluid. At the same time the fan, which consists of the outer layer of the adult cuticle is formed and rays extend.

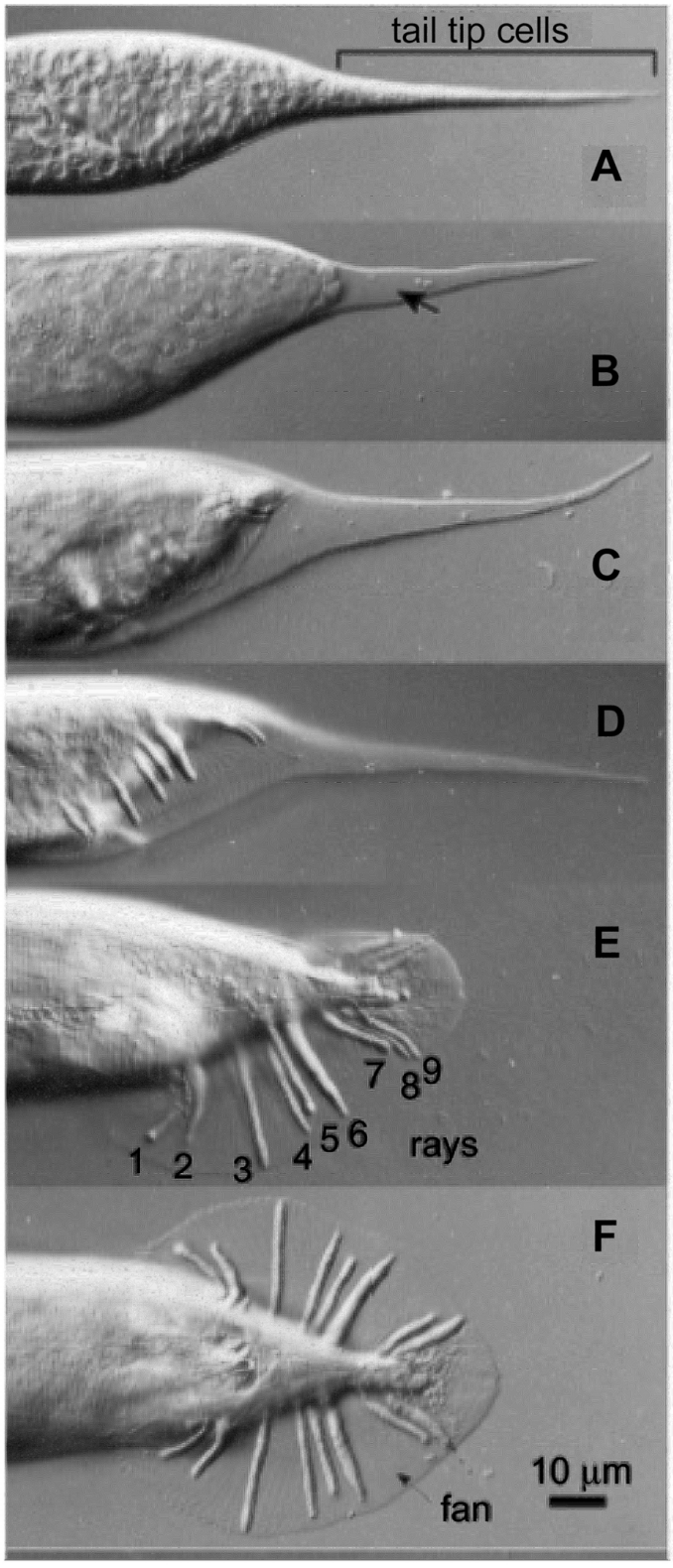

Fig.5: Formation of the fan and rays.

Nomarski picture of L4 male, the last 4 hours of the male tail retracting. (A) The male tail before retraction. (B) Hypodermal cells hyp 9 and 10 are the first cells to retract. The

arrow shows the growing fluid-filled cavity. (C) and (D) show L4 cuticle with the rays and fan extend. (E) and (F) adult male with rays, lateral and ventral views of the tail. Reprinted from *Developmental Biology*, Nguyen et al., 1999, Copyright (1999).

3.1.5.2.3. Neurons

C.elegans has a nervous system which represents the most complex organ of *C. elegans*. It is the organ with the most cells (302 neurons and 56 glial cells make up 37% of the somatic cells in a hermaphrodite) and the largest cellular diversity (at least 118 different neuron classes). Neurons share many different properties and lineage history of individual neurons is known.

Based on their anatomical features, ventral cord motor neurons can be divided into specific classes, which are diversified by the action of specific transcription factors. Members of the 118 neuron classes defined by White are distinguished by their differential anterior/posterior, dorsal/ventral and left/right position (White et al., 1988). For example, many head ganglia neuron classes are composed of a pair of two left/right symmetric cells Most neuron classes in the nervous system are composed of a pair of two bilaterally symmetric neurons (Sulston, 1983; Hobert et al., 2002).

3.2. Nuclear hormone receptors

3.2.1. Introduction

Nuclear hormone receptors (NHRs) are transcription factors with many specific functions. They are important for development, metabolism, cellular differentiation and defense against xenobiotics. NHRs consist of several domains described below.

Nuclear hormone receptors form a large superfamily of transcription factors which either activate or repress expression of specific genes (Beato et al., 1995; Robyr et al., 2000; Horwitz et al., 1996).

The number of NHRs found in different genomes varies from 18 in *Drosophila melanogaster*, 48 in the human genome, 268 in *C.briggsae* and more than 284 in *Caenorhabditis elegans* genome (Stein et al., 2003; Enmark and Gustafsson, 2001; Maglich et al., 2001; Van Gilst et al., 2002; Robinson-Rechavi et al., 2003; Gissendanner et al., 2004; King-Jones and Thummel, 2005; Antebi, 2006).

3.2.2. Structure of NHRs and functional domains

All NHRs have a conserved structure (Fig.6), which consists of five domains: A/B domain located at N-terminal region, a central DNA binding domain (DBD or C domain), a hinge region (D domain), a carboxy-terminal ligand binding domain (LBD, also called E domain) (Burris, 2008; Yen, 2001) and an F domain with unknown function located at C-terminal region (Laudet 1997, Aranda and Pascual, 2001). Each of these domains may sub-serve multiple functions, and thus their names may only reflect the first function ascribed to them (Yen, 2001).

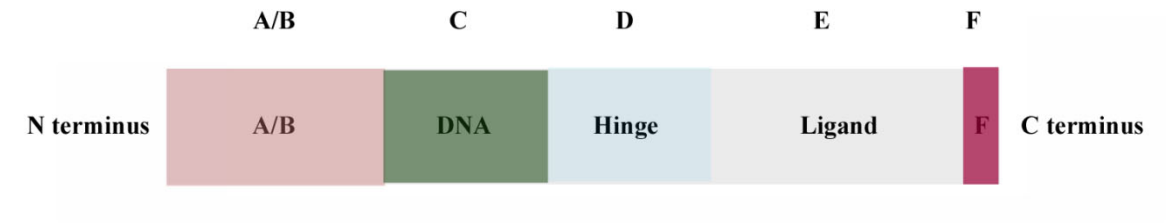

Fig. 6: A schema of NHR structure

NHRs consist of an A/B domain (A/B), a central DNA binding domain (C), a hinge region (D), carboxy-terminal LBD (E) and F region (F).

3.2.2.1. Amino-terminal (A/B) domain

N terminus consists of a region which is not conserved. The A/B region has variable length and sequence and is not conserved among the members of the super-family. The role of the amino-terminal domain has not been explained in detail yet. There is a specific AF-1 activation function-1 that contributes to interactions with transcriptional machinery (Robyr et al., 2000). This region can also interact with cell-specific (Giguere, 1999) and promoter-specific (Tora et al., 1988) transcription.

3.2.2.2. DNA binding domain

In the central part of the NHR is the DNA binding domain (DBD). This region consists of two zinc fingers motifs. There are highly conserved cysteine molecules coordinating binding of a zinc atom (Yen, 2001). This results in a formation of tertial

structure containing helixes that interact with DNA. Within the zinc finger, there is a “P box” sequence that is crucial in a sequence-specific recognition of hormone response elements (HRE) by different members of the nuclear hormone super-family (Wahli and Martinez,1991). The P box is involved in DNA binding and distinguishes subfamilies of NHRs. All NHRs may be divided into 6 main groups NR1 to NR6 (Ruau, 2004). Additionally, there are other important regions: “D box” and “A box”. “D box“contributes dimerization of NHRs and “A box”which is localized just downstream of the second zinc finger (Fig.7) contrivutes to binding to the HRE).

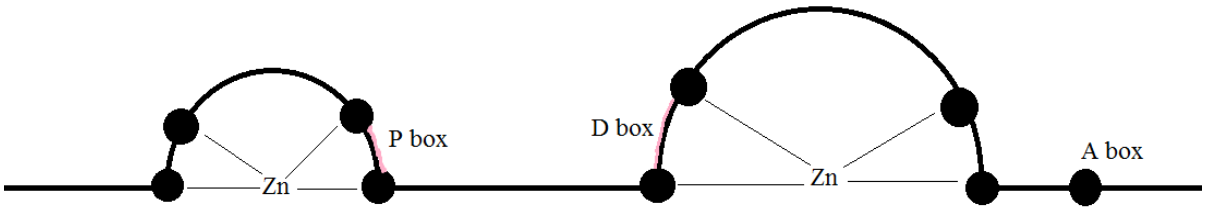

Fig. 7: DNA binding domain of nuclear hormone receptors

The picture shows a scheme of two “zinc fingers” in the DNA binding domain. The black dots represent all cysteins responsible for conformation.

3.2.2.3. Hinge region

In between DBD and LBD is a region called the Hinge region. This region contains an amino acid sequence that is associated with nuclear localization (Evans, 1988). This sequence allows DBD to rotate and enables some receptors to bind as a dimer to both direct and inverted HREs (Glass, 1994; Giguere, 1999).

3.2.2.4. Ligand binding domain

A ligand binding domain (LBD) is a highly conserved domain and is located at C terminus of the protein. It is responsible for dimerization, interaction with heat-shock proteins, transactivation and interaction with corepressors (Horwitz et al., 1996). The main function of LBD is binding ligands - hydrophobic molecules including steroid hormones, retinoids and lipid metabolites (Brelivet, 2004). The expression of target genes is dependent on cooperation and combination of binding of many transcription factors on gene promoters (Weinberg, 2007).

The LBD of nuclear hormone receptors consists of a conserved arrangement of helices (11-12 α-helices), sandwiching and participating in a ligand binding pocket (Yen, 2001, Mangelsdorf et al., 1995). At the carboxyterminus of the LBD is the AF-2 site responsible for activation function-2. The region mediates ligand dependent transactivation (Giguere,1999).

3.2.3. Regulation of transcription by nuclear hormone receptors

The gene regulation of transcription is a crucial event for development, growth, tissue maintenance and metabolism. First regulatory level is binding of NHRs to the target sequence and activation of gene expression or repression. In the 5´-flanking region of the target genes is usually sequence called the hormone response elements (HREs). Although the HREs are often found relatively close to the core promoter, in some cases HREs were found several kilobases upstream of the transcriptional initiation site (Aranda and Pascual, 2001).

Two main motifs have been identified for binding of NHRs – the sequence AGGTCA and AGAACA. First motif is recognized by NHR as thyroid and retinoid receptors (TR and RAR) with CxGCKGFFxR "P box" and second motif is recognized by mineralocorticoids, glucocorticoids, progesterone and androgen receptors (Umesono and Evans, 1989; Beato et al., 1995; Giguere, 1999).

Some monomeric receptors can bind HREs as homo- or heterodimers, typically of two core hexameric motifs. For dimeric HREs, the half-sites can be configured as palindromes (Pal), inverted palindromes (IPs), or direct repeats (DRs) (Aranda and Pascaul, 2001).

Steroid hormone receptors almost exclusively bind to the HREs as homodimers. Typical heterodimeric receptors such as TR, RAR, or VDR (vitamin D receptor) can bind to their response elements as hetorodimers and heterodimerization with RXR strongly increases the efficiency of DNA binding and transcriptional activity (Aranda and Pascaul, 2001).

The ligands could play a role in dimerization and binding to DNA. For example thyroid hormone inhibits binding of homodimers but not heterodimers to DNA, thus promoting formation of heterodimeric complexes on the HREs (Ribeiro et al., 1992).

3.2.3.1. Nuclear hormone receptor cofactors

In the absence of ligand thyroid receptors and retinoid receptors repress basal transcription. The unliganded receptors are bound to the HRE. This complex is associated with corepressors responsible for the silencing activity. When the lignad is bound,

conformation change causes dissociation of corepressors and the receptor is transcriptionaly active (Baniahmad et al., 1992). This active complex is responsible for transcriptional activation (Horwitz et al., 1996). This is applied to TR and RAR receptors, but not to the other receptors, such as the unliganded steroid receptors that do not bind corepressor proteins.

The coactivators and corepressors are very important in regulation of transcription. The first identified transcriptional corepressors were proteins named Nuclear Corepressor (NcoR) (Horlein et al., 1995) and Silencing Mediator of Retinoid and Thyroid Receptor (SMRT)(Chen and Evans, 1995). NCoR and SMRT (app. sizes are around 270 kDa) are related both structurally and functionally and are known to recruit a histone deacetylases (HDACs) through bridging molecules (Robyr et al., 2000).

3.2.4. Ligands

NHRs are activated by ligands, small hydrophobic molecules like steroid hormones, thyroid hormone, vitamin D, retinoids, farnesoids and related molecules. Ligands can be relatively efficiently transported through the cell membrane by diffusion. Bound ligand causes conformation changes of receptors with functional consequences (Mangelsdorf et al., 1995).

3.2.5. Orphan receptors

The nuclear hormone receptor family includes genes for which ligands are not known. These members are called orphan receptors. Orphan receptors can be found across the all animal species. Nuclear hormone receptors without ligands (orphans) can bind DNA with high affinity as monomers (Giguere, 1999). Orphan nuclear hormone receptors provide a unique and until now the largely unexploited resource to uncover regulatory systems that impact on health and human disease (Blumberg and Evans, 1998).

3.2.6. *C.elegans* NHRs

From 284 NHRs in *C.elegans* only 15 receptors are homologues to vertebrate and *Drosophila*. These genes are placed into five major subfamilies of NHRs (Sluder, 2001; Gissendanner, 2004).

The remaining 269 NHRs are now classified as supplementary nuclear hormone receptors (supnrs). These receptors seem to be specific for nematode species and are distantly related to hepatocyte nuclear factor 4 (HNF-4) (Robinson-Rechavi et al., 2005). Vertebrate

HNF-4 ((NR2A) in the Unified Nomenclature System for the Nuclear Receptors superfamily (http://www.enslyon.fr/LBMC/laudet/nurebase/nurebase.html (Duarte et al., 2002)) regulates glucose and lipids metabolism and differentiation of enterocytes and hepatocytes (Li et al., 2000; Hayhurst et al., 2001; Watt et al., 2003; Stegmann et al., 2006).

The function of some NHRs in *C.elegans* was found after the loss of their function (RNAi). There is a molting deffect (*nhr-23*, *nhr-25*, *nhr-67*) (Kostrouchova et al., 1998; Kostrouchova et al., 2001; Asahina et al., 2000; Duarte et al., 2002; Ruau et al., 2004;; Gissendanner and Sluder, 2000; Gissendanner et al., 2004), dauer formation (*nhr-41*, *nhr-85*), ovulation (*nhr-6*) (Gissendanner et al., 2004), epidermal cell development and differentiation (*nhr-25*) (Chen et al., 2004; Silhankova et al., 2005), vulval development and function (*nhr-67*, *nhr-85*), and toxin resistance (*nhr-8*) (Gissendanner et al., 2004). Genetic mutants in four of the conserved NHRs affect neuronal development (*fax-1*, *unc-55*), sex determination (*sex-1*), and dauer formation (*daf-12*) (Carmi et al., 1998; Zhou and Walthall, 1998; Antebi et al., 2000; Much et al., 2000;).

NHR-49 as one of the HNF4-related *C.elegans* receptors is an important regulator of fat metabolism involved in the nutritional response and fatty acid beta oxidation and is involved in functions supported by PPARs in vertebrates (Van Gilst et al., 2005a, b).

C.elegans NHRs could be divided according P box sequence into four classes (Van Gilst, 2002). TheP box which contains sequence CxGCKGFFxR belongs into the first class. This first class is similar to retinoid and thyroid receptors and can be further divided into seven groups of genes that include 60 genes. In this study we focused on two groups of receptors which contain P-box sequences **CNGCKTFFRR** and **CNGCKAFFRR.**

4. Materials and methods

4.1. Materials

4.1.1. *C.elegans* strains

N2 – wild type animals (var. Bristol)

Wild type *C.briggsae* (related nematode species) worms were kindly afforded by the CGC

4.1.2. *E. coli* strains

OP50 – used as a natural food for *C.elegans* and *C.briggsae*

HT115 - carry an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 RNA polymerase gene contained within a stable insertion of a modified lambda prophage λ DE3. The strain is deficient for RNase III, an enzyme that normally degrades a majority of dsRNAs in bacterial cells (Timmons et al., 2000)

DH5α - competent cells for transformation

TOP10 – competent cells for transformation

4.1.3. Vectors

pRF4 – vector containing the *rol-6*(su1006) mutant collagen gene. Its expression causes exhibition of a helically twisted body (Mello et al., 1991)

L4440 (pPD129.36) – vector containing two convergent T7 RNA polymerase promoters with opposite orientation separated by a multicloning site

pCR®4-TOPO® Cloning Vector (Invitrogen, Carlsbad, CA) – vector for the direct insertion of Taq polymerase-amplified PCR (polymerase chain reaction) products (using covalently bound topoisomerase I) containing T3 and T7 polymerase promoters

pCR®II (Invitrogen, Carlsbad, CA) TA Cloning Kits are designed for cloning Taq-amplified PCR products directly from a PCR reaction using an overnight ligation step. The kits use a pCR vector and yield greater than or equal to 80% recombinants with greater than or equal to 90% of the recombination

pPD95.67 – promoterless gfp vector with a nucleolar localization signal

4.1.4. Solutions and media

DNA lysis buffer: 0.1 M Tris pH 8.3, 50 mM EDTA, 200 mM NaCl

Worm lysis buffer: 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 0.45% Tween 20, 0.45% NP-40, 0.01% gelatin, 200 μg/ml Proteinase K

RNA lysis buffer: 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 5% 2-mercaptoethanol, 0.5% SDS

Lysis buffer: 50 mM Tris pH 8, 300 mM NaCl, 10 mM $MgCl_2$, 0.4% NP-40

Dilution buffer: 50 mM Tris pH 8, 0.4% NP-40, 2.5 μM $CaCl_2$, 1 μl of DNase I

Wash buffer: 50 mM Tris pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 0.4% NP-40

1x TTBS: 100 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween 20

10x TBE: 60.5 g of Trisbase, 30.85g of H_3BO_3, 3.72g of EDTA $2Na.2H_2O$, H_2O to 1l, autoclaved

10x PBS: 80 g of NaCl, 2 g of KCl, 14.4 g of Na_2HPO_4, 2.4 g of KH_2PO_4, H_2O to 1l, pH 7.4, autoclaved

LB medium: 10 g of Trypton, 5 g of Yeast Extract, 10 g of NaCl, 1 ml of 1 M NaOH, H_2O to 1l, autoclaved

LB agar: 10 g of Trypton, 5 g of Yeast Extract, 10 g of NaCl, 1 ml of 1 M NaOH, 15 g of Bacto Agar, H_2O to 1l, autoclaved

NGM: 17 g of Bacto Agar, 2.5 g of Bacto Pepton, 3 g of NaCl, 1 ml of Cholesterol (5 mg/ml in Ethanol), H_2O to 1l, autoclave, 25 ml of 1 M KH_2PO_4, 1 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$ added after autoclaving

M9 medium: 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 5 g of NaCl, 0.5 g of $MgSO_4$, H_2O to 1l, autoclaved

4.2. Methods

4.2.1. gDNA isolation

Animals were grown on 2% agarose plates with a layer of *E.coli* OP50. Mixed stages were washed with water and frozen in cryo-tubes at -80°C. 0,5 ml of DNA lysis buffer was added to thaw animals and the melted mix was transferred to falkon tubes. 4,5 ml of DNA lysis buffer was added. 0,5 ml of 10% SDS and 250 µl of Proteinase K (20 mg/ml) (Q-BIO gene, MP Biomedicals, Irvine, CA, USA) were added. The mixture was vortexed and heated for 30 min at 65°C. Phenol-chloroform extractions were performed two times (5 ml of phenol was added and the mixture was rocked for 30 min and then spinned down for 5 min at 5,000 RPM. The upper phase was transferred to a fresh tube. Then 5 ml of chlorophorm was added and rocked for 30 min. The upper layer was transferred into a new tube. DNA was precipitated by ethanol (2 volumes) with 3M NaOAc (0,1 volume of transferred sample volume). After gentle mixing of the solution, gDNA was wound on a glass pipette and was transferred to a fresh tube, air-dried and resuspended in 3 ml of water and was incubated with RNase A (ICN, MP Biomedicals, Irvine, CA, USA) (to the final concentration 10 µg/ml) for 30 min at 37°C. Next phenol-chloroform extraction and ethanol precipitation followed. The gDNA pellet was air-dried and resuspended in 20 µl of water.

4.2.2. Total RNA isolation

C.elegans and *C.briggsae* animals from all stages of development (mixed wild type worms) were grown on 2% agarose plates. Worms were washed with M9 medium and frozen at -80°C. Total RNA was prepared following the protocol described by Johnstone and Barry (Johnstone and Barry, 1996) with modifications (Kostrouchova et al., 2001). Briefly, the frozen pellets were re-suspended in 0.5 ml of re-suspension buffer (0.5% SDS; 5% 2-mercaptoethanol; 10 mM EDTA; 10 mM Tris/HCl (pH 7.5) with 12.5 µl of proteinase K (20 mg/ml), vortexed for 1 min. and incubated for 1 hour at 55°C. The samples were treated by phenol-chloroform extraction and ethanol precipitation and dried pellets were dissolved in water. Contaminating genomic DNA was eliminated by DNase treatment (Promega, Madison,WI), using 1 µl of solution containing 1 unit/µl per 1 µg total RNA and samples were incubated for 30 min. at 37°C. An additional phenol-chloroform extraction and ethanol precipitation step was included. Total RNA was dissolved in DEPC water and frozen at -80°C before further use.

4.2.3. RT-PCR

C.elegans and *C.briggsae* cDNA was prepared from total RNA by reverse transcription reaction using the Superscript II kit (Invitrogen, Carlsbad, CA). For reverse transcription, approximately equal amounts of RNA were used as assayed by a Nanodrop 2000c (Thermo Scientific, Wilmington, DE). One microgram of RNA was mixed with 100ng of random hexamers, heated for 5 min. at 65ºC and 4 µl of 1st strand buffer, 1 µl of 100mM DTT, 1 µl of RNasin, 1 µl of dNTPs and 1 µl of Superscript II were added. The mixture was incubated 10 min. at 25ºC, 50 min. at 42ºC and 15 min. at 70ºC. Prepared first strand cDNA was frozen at -20°C until was used for PCR.

4.2.4. PCR

All PCR reactions followed a standard protocol with gene specific primers and templates (N2 cDNA, N2 gDNA, or *C.briggsae* cDNA and gDNA) using BIO-X-ACT Short DNA polymerase (Bioline, London, UK) for short fragments up to 1,500 bp, BIO TAQ DNA polymerase (Bioline, London, UK) and Taq DNA polymerase (Invitrogen, Carlsbad,

California). For all PCR reactions PTC-100^{TM} and PTC-200^{TM} Peltier thermal cyclers (MJ Research Inc., Waltham, MA, USA) were used. To establish the expression of genes or their isoforms the following primers were used (table.1).

gene	sequence forward	sequence reverse	forward primer	reverse primer
nhr-206	gtcagaggaatcaaatagttttc	catcgaataaaggactgac	5214	5215
nhr-208	gatctagctgatattcttccggat	ggctaaccaagacaattgc	5194	5217
nhr-207	atgactagcgaagaatcaactagc	tcaggaactcattatctcatca	5190	5244
nhr-209	atgagctttttggtgcttccactg	ggcggatgtgagcattctcg	5306	5218
nhr-154	atgacaacatttgaagagtttgaa	ggagccattgcgagaatagttgcg	5307	5224
nhr-153	gactccaccacagaaatc	gacaatatcggaacaaacactgagcg	5225	5229
nhr-136	atgcttcctgactttattacagta	gctaattcgtgtgtatctaactgc	5309	5234

Table 1: Primers designed for amplification of full length cDNA

The table shows names of genes, sequences from 5´end to 3´end and their laboratory numbers

4.2.5. A single worm PCR (to confirm presence of the construct in the animal)

The worm was transferred to a PCR tube with 5 µl drop of worm lyses buffer and frozen for 10 min at –80°C. Then the tube was heated for 60 min at 60°C, 15 min at 95°C and finally stored at -20°C. 2.5 µl of prepared single worm DNA mixture served as a template for standard PCR reaction.

4.2.6. Cloning

T4 DNA ligase: vectors and PCR fragments were digested by restriction endonucleas enzymes. After purification, the DNA amplicons were ligated into the purified vectors by T4 DNA ligase (Invitrogen ,Carlsbad, CA or Fermentas, Burlington, Canada) for 1 hour at room temperature or overnight at 16°C.

TOPO TA Cloning: PCR amplified fragments (synthesized with Taq DNA polymerase) were cloned into the pCR® 4-TOPO vector using TOPO TA Cloning®Kit for Sequencing (Invitrogen, Carlsbad, CA).

4.2.7. Real-time PCR (RT-qPCR)

PCR products are measured as accumulation of dyes that fluorescence in the presence of DNA (e.g., SYBR Green I), or indirectly with florescence- labeled oligonucleotides known as fluorescent probes, which recognize and bind by base pairing to a specific region or site on one DNA strand. If a specific target DNA is present in a sample, the time it takes to generate a detectable signal depends on its initial concentration in the sample. Use of this cycle number information thus enables easy qualitative and quantitative gene analysis.

4.2.7.1. Real-time PCR: gene expression profile of individual genes using absolute quantification and SYBR Green I detection

Real-time PCR was performed in PTC0200 DNA EngineR Thermal Cycler equipped with ALS 0296 96-well Sample Block (MJ Research) using DyNAmoTM HS SYBR Green qPCR Kit (Finnzymes, Espoo, Finland) or on the LightCycler®1.2 with LightCycler® FastStart DNA MasterPLUS SYBR Green I (cat.n. 03515869001) chemistry.

When SYBR Green I dye intercalates into dsDNA, its fluorescence increases significantly. The background fluorescence from SYBR Green I when in solution as a free dye and stimulated by light of the appropriate wavelength is very low (App.note No. 18/2004) The double-stranded DNA product is formed. The DNA-dye complex results in a dramatic increase in fluorescence output, when properly illuminated, of roughly 2,000 times the initial, unbound fluorescent signal (Dorak M.T. 2006).

There are also other intercalating dyes (e.g. Resolight, LC green, Eva green) which are used either for quantification or for SNP (single nucleotide polymorphism) detection. They function in same principle but intercalate in every base pare.

The amplicons of genes (shown in the table 2a, and 2b) were amplified using RT-PCR from 5 µg of total *C.elegans* RNA (from mixed developmental stages). The amount of DNA was determined spectrophotometrically. Each sample was analyzed using **absolute quantification** in doublets and at least two times. Cp values of unknown samples were compared against those of standards with kown copy numbers. Purified PCR amplicons were measured spectrofotometricaly and diluted 10x. There was a series of dilution with concentration from 1 copy per µl to 1.10^9. These standards were used as a standard template. Transcribed cDNA was used as an unknown template and Cp values were compared with Cp

values of standards. Absolute quantification was performed on the ChromoIV instrument from Bio-Rad and used Opticon monitor™ version 3 software. Data and Cp values were exported into excel and the copy number was calculated manually there.

gene	forward	reverse	number forward	number reverse	length bp
nhr-206	gtcagaggaatcaaatagttttc	cagaaatcaatctgctcacttca	5214	5241	465
nhr-208	aaactgcaggatctagctgatattcttccggat	gatatactcagatcatcttccgctg	5194	5245	493
nhr-207	atgactagcgaagaatcaactagc	gctgaattcatcgtcgatact	5190	5243	436
nhr-209	atgttcatcaaatattgacttg	cgggatcccgctttccattcaagatagttctcc	5210	6031	445
nhr-154	cagtctccgttcgccgatgaaaag	caaatcttcctcggttactatc	5220	5254	410
nhr-153	gactccaccacagaaatc	gtatagctcattgacggtatttcc	5225	5249	583
nhr-136	gagccgaggaacatgtccatc	cttcaattccattactgtcagc	5230	5257	409

Table 2a., primers for gene expression study using SYBR Green I in *C.elegans*

The table shows the studied *C.elegans* genes, sequences of primers from 5´to 3´end, their laboratory numbers and length of amplicon.

	sequence	sequence	forward	reverse	length
gene	forward	reverse	primer	primer	bp
Cbr-nhr-209	atgagttttcttatgctacctttg	gcatcgagaaggaggcactcatca	5338	5339	700
Cbr-nhr-154	atgacaacatttgaagattttgaa	gctgtgcacatagtttgtgcatct	5342	5343	816
Cbr-nhr-153	atggactcatcttcgattctcagg	cttgagtggacaaagtttcgactacg	5346	5347	780
Cbr-nhr-136	atgcttcccgacatcataagtgtt	cgtcgtatcggagtatcattgggg	5350	5351	620

Table 2b., primers for gene expression study using SYBR Green I in *C.briggsae*

The table shows the studie *C.briggsae* genes, sequences of primers from 5´to 3´end, their laboratory numbers and length of amplicons.

Tm calling – confirmation of SYBR green I detection

To confirm amplicons detected by SYBR green I intercalating dye Tm calling analyses was performed for all studied genes. The following protocol was used. Pre-denaturation 95°C for 10 min, amplification 40 times 94°C for 10 sec, 58°C for 20 sec and 72°C for 20 sec. Fluorescence signal was caught in the end of a each 72°C step. Melting curve was measured with these parameters: 95°C for 0 sec, 65°C for 1 min and than continuously from 40°C to 95°C. (Fig.8) The melting temperature (Tm) of a sample is defined as the point at which half the probes (or dye) have melted off the DNA.

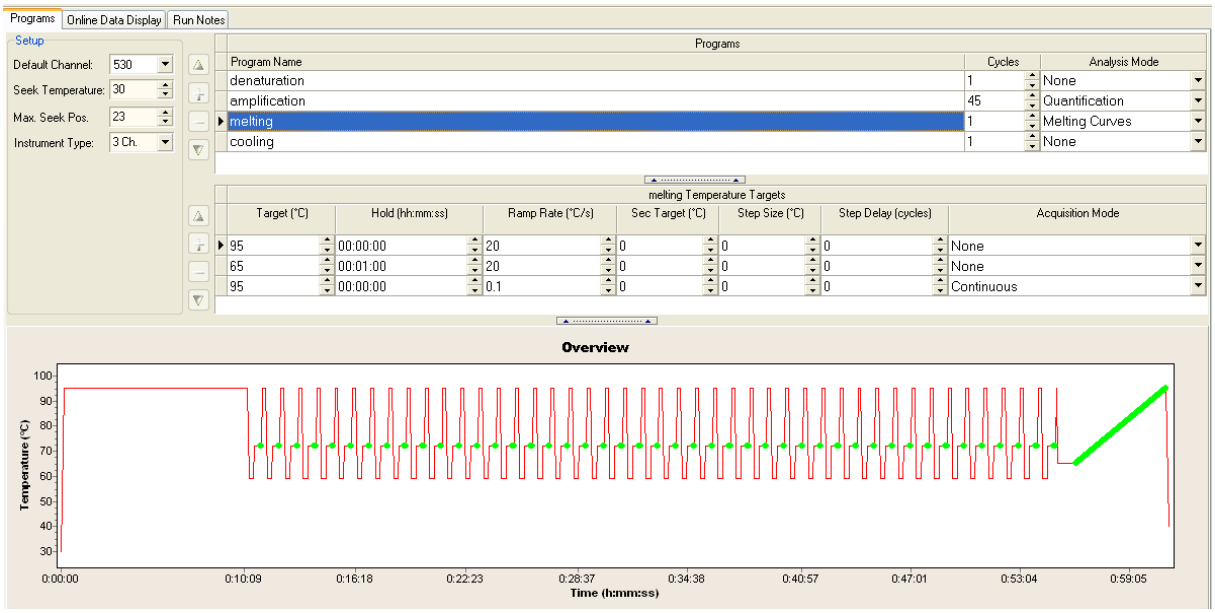

Fig.8 Melting curve analyses in the LightCycler® software

The picture shows the programming in the LightCycler® software. Denaturation, amplification and melting curve were performed on the LightCycler® instrument (Hoffman-La Roche, Basel, Switzerland).

Different PCR products melt at different temperatures providing an alternative to gel analysis. Instead of characterizing a PCR product by the size on a gel, the melting temperature (Tm) can be used for classification and identification. The appearance of a product at the correct Tm can be used to distinguish the presence or absence of that product, even down to a single copy (digital PCR) (Fig.9).

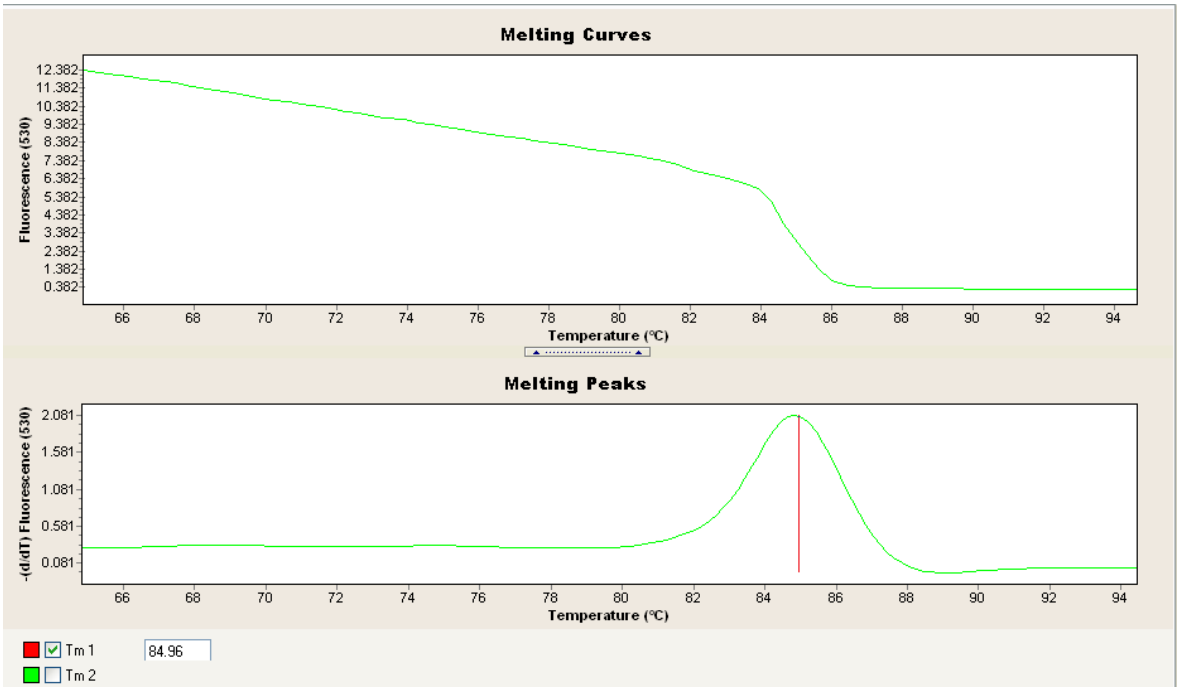

Fig.9 The melting curve analyses

The figure illustrates a melting curve chart of a *nhr-209* and a melting peak chart from a melting temperature analysis. The chart shows the downward curve in fluorescence for the samples as they melt. The analysis also displays a chart that plots the first negative derivative of the sample fluorescent curves. In this chart, the melting temperature of each sample appears as a peak. Displaying the melting temperatures as peaks makes it easier to distinguish each sample's characteristic melting profile and to show differences between samples.

4.2.7.2. Real-time PCR: relative quantification and Universal probe library detection

UPL experiments were performed on the LightCycler® 1.2 instrument. Ready to use master mix LightCycler® TaqMan® Master (cat.n. 04535286001) was used. Into this master mix individual primers (shown in the table) were added and Universal Probe library probes followed by first strand cDNA. Universal probe library probes are hydrolysis probe assays, conventionally called TaqMan® assays and can be described as homogenous 5´nuclease assays, which are cleaved during PCR amplification.

During PCR, the 5´nuclase activity of the polymerase cleaves the hydrolysis probe, separating the reporter and quencher. In the cleaved probe the reporter is no longer quenched and can emit a fluorescence signal when excited (Fig.10).

The following protocol was used. Pre-denaturation 95°C for 10 minutes, amplification 45 times, denaturation 95°C for 15 sec, 60°C UPL experiments were performed on the LightCycler® 1.2 instrument. Ready to use master mix LightCycler® TaqMan® Master (cat.n. 04535286001) was used. Into this master mix individual primers (shown in the table) were added and Universal Probe libraty probes followed by first strand cDNA.

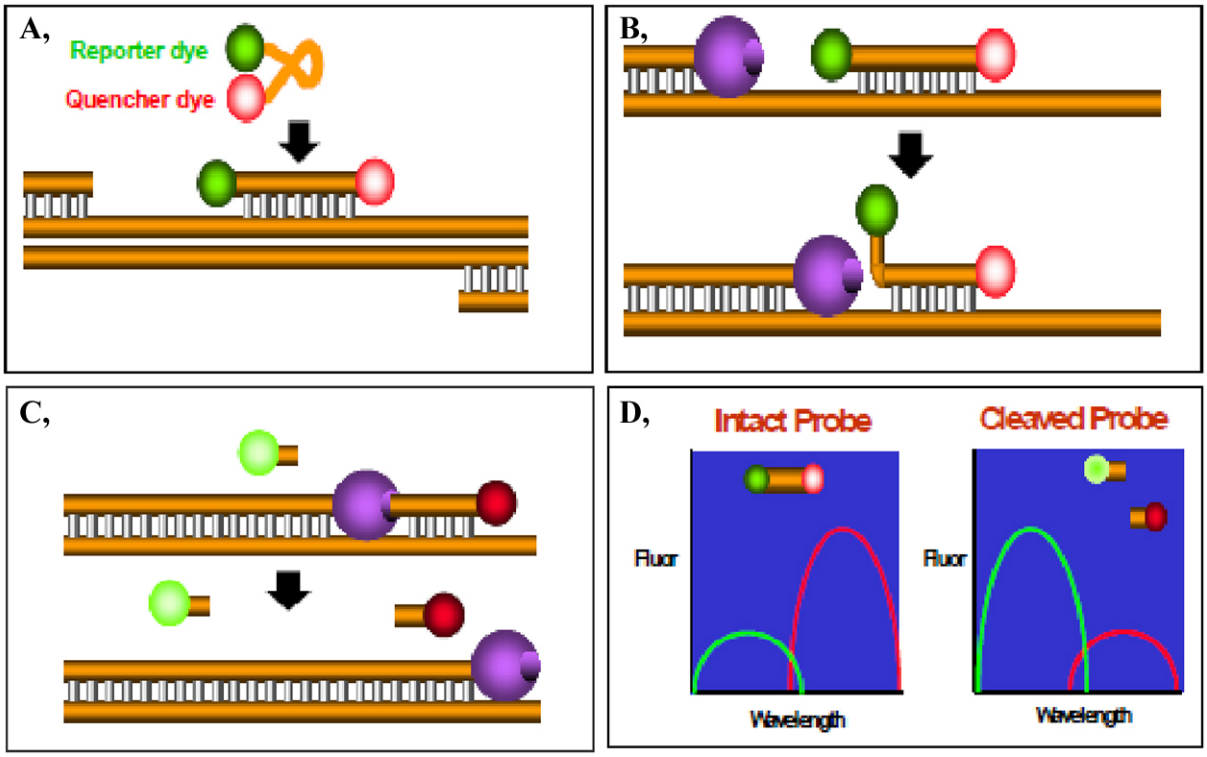

Fig.10. Princip of hydrolysis probes:

A, 5´nuclease assay, fluorescein (reporter) dye is quenched by the quencher; B, Polymerase collidates with TaqMan® Probes; C, Cleavage of TaqMan® probe, 5´end exonuclease activity of Taq polymerase cleaves the probe; D, Increased fluorescence activity due to the cleaved probe

Primers and probes were designed by Probe Finder software (www.roche-applied-science.com F. Hoffmann-La Roche Ltd, Switzerland). Gene *Cbr-rpb-1* is a homolog of *ama-1* in *C.briggsae.* Universal ProbeLibrary Probe follows established real-time PCR protocols using the LightCycler® TaqMan® Master on the LightCycler® System. All UPL experiments were performed on a LightCycler®1.2 with software LightCycler®4.1.

Gene	forward	reverse	number forward	number reverse	length	probe number or sequence
nhr-206	ggagattggagaacactttcaga	tccagctagttcagcattcattt	7176	7177	111	135
nhr-208	atctagctgatattcttccggatatt	tctaggacacttgatcgatgtagaa	7178	7179	98	142
nhr-207	tccaatggcaattaaatctgaa	gctgaattcatcgtcgatagc	7180	7181	127	154
nhr-209	aagccatactaatgagaaatccatc	tccgttcattttcaatgataagc	7182	7183	75	54
nhr-154	tgaaccattacatcgttctgga	gaatttcttccaattttcttatgtctc	7184	7185	65	79
nhr-153	tcagatacacttttatacccagatggt	tcaatcaaaggaccatttttcc	7186	7187	71	49
nhr-136	tcgacgatcaattctggatg	acatgcgcctagacaagtca	7188	7189	95	124
acs-2	aagaaggcttgtaagagaggaatg	gatttcgaatttcttgacttttcc	7190	7191	114	136
fat-7	caaacggccgtcttctca	ggtgtggttgccttgtatga	7196	7197	104	54
nhr-49	gctgctcattgagaatatgacact	aactcggagagcagagaatcc	7201	7202	72	53
ama-1	gagtcatagatcgtcgtggaagatg	tgagaagccgtccgcagtag	ama-1 F	ama-1 R	202	cccgtatttcacattggattccctcacaa
Cbr-nhr-209	cgctctccgaaattcttcaa	ggtgtgagattttgaattttgtacg	7229	7230	64	93
Cbr-nhr-154	tcaatgaatgaaatgaaatcagatg	ttgtgcattggtccactaatagat	7231	7232	68	58
Cbr-nhr-153	tcaacatgggacactgaacg	tggttttcgagaacattgcat	7233	7234	72	137
Cbr-nhr-136	atcttgccgatttgaacagtg	catctgcttgaatcgctgac	7235	7236	62	75
Cbr-rpb-1	ggacccgaggagattaaacgaa	agcacatgtcatacatcttccacg	CBG05355 F	CBG05355 R	137	aggtctacgagaacggaaaacccaaaat

Table 3: Combination of primers and probes designed for UPL probe based real-time PCR assays

The table shows studied genes, primer sequences and laboratory primer numbers, amplicons lenth and the UPL probe number. There are two sequences for *ama-1* gene in *C.elegans* and *C.briggrase* homolog of *ama-1 Cbr-rpb-1*, which are labeled with DYXL dye. These two probes are not a part of UPL probes.

All UPL probes ((Hoffman-La Roche, Basel, Switzerland) are LNA modified (locked nucleic acid). LNA probes are hydrolysis probes that are much shorter due to the higher binding affinity of locked nucleic acid based to standard DNA (Goldenberg at al., 2005). LNAs are bicyclical DNA or RNA analogues (Fig.11). For this reason, LNA-based probes need to be only 8-9 bases long to achieve a Tm of 68-69°C under standard assay conditions. The combination of software Probe Finder and 165 pre-validated probes enables the design of UPL assays which are intron-spanning, amplicons are very short (up to 110 bp). The first design success is more than 96%.

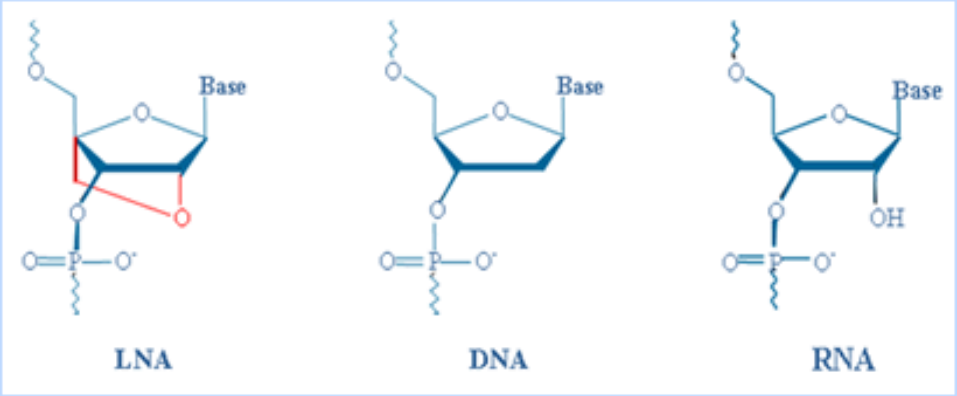

Fig.11: Locked Nucleic Acids.

LNA is a class of nucleic acids analogues, where the ribose ring is "locked" with a methylene bridge connecting the 2'-O atom with the 4'-C atom (see structure above). LNA nucleosides containing the six common nucleobases (T, C, G, A, U and mC) that appear in DNA and RNA are able to form base-pairs with their complementary nucleosides according to the standard Watson-Crick base pairing rules. (www.roche-applied-science.com F. Hoffmann-La Roche Ltd, Switzerland).

Probe base technology was used for relative quantification comparison of two different target sequences in a single sample (target gene of interest and a reference gene). There is a final result shown as a ratio of these gene levels. The second, or reference, gene is a constitutively expressed gene (housekeeping gene) that is found in constant copy numbers under all tested conditions. This reference gene, also known as an endogenous control, provides a basis for normalizing sample-to-sample differences. (Tellmann G. 2006)

In this study relative quantification was used to show expression changes in L1 larvae in a starving experiment. We used *ama-1* (F36A4.7 a large subunit of RNA polymerase II) as a reference gene where the same expression level was observed. Three more controls were included in a starving experiment *nhr-49* as the positive control with the same expression level, *asc-2* as an up-regulated and *fat-7* as a down-regulated gene (Aarnio V, 2010). There is very high association with accurate normalization. The choice of internal standard is critical in order to obtain reliable and consistent results. There are many publications commonly using reference genes such as *act-1* and *ama-1*and others (Tab.4)

gene	cosmid num.	function
act-1	T04C12.6	Cytoskeletal structural protein
ama-1	F36A4.7	RNA polymerase II
cdc-42	R07G3.1	RHO GTPase
csq-1	F40E10.3	Calcium ion binding protein
eif-3.C	T23D8.4	Translation initiation factor
mdh-1	F20H11.3	Malate dehydrogenase
gpd-2	K10B3.8	Glyceraldehyde-3-phosphate dehydrogenase
pmp-3	C54G10.3	Acyl-CoA transporter
tba-1	F26E4.8	Alpha tubulin

Table 4. Commonly used reference genes

The table shows names of reference genes, their cosmid number and their function.

Because PCR amplification is an enzymatic reaction, it may vary in quality. For that purpose we have included efficiency correction and we performed several analyses to get efficiency values for each gene and their amplicons.

If target and reference PCR efficiencies are identical, method ΔΔ Ct can be used. In this calculation efficiency of both PCRs is assumed to be 2, which represents a doubling of molecules in each cycler. In this study there were different efficiency values of individual studied genes, therefore ΔΔ Ct could not be used. Advance analyses developed by Roche Applied Science, called E-Method, was used. This method can compensate differences in the target and reference gene amplification efficiency during the experiment or between experiments (Tellmann G. 2006).

4.2.8. Constructs

Primers used for PCR amplification of DNA fragments of our interest, the sizes of amplified fragments, used DNA templates and vectors are mentioned later in this section.

4.2.8.1. Constructs for GFP expression (the preparation of transcriptional fusion genes)

All constructs were prepared by PCR amplification of genomic DNA using primers with appropriate restriction sites (Table 5). The PCR products were digested and cloned in frame to the corresponding restriction sites in the vector pPD95.67, containing a gene for green fluorescent protein and a nuclear localization signal (NLS).

Transgenic lines were prepared from N2 wild type worms as described [Mello, 1995]. In general, the cloning strategy was designed to keep the first exon, the first intron and a part of the second exon in the transgene. Two different length of putative promoters were chosen for promoter analysis using gfp (green fluorescent protein)-fusion transgenes, the short, with the length of approximately 500 to 600 bp and the longer, with the length from 1000 to 2000 bp of putative promoter regions. In general, the short promoter regions included only intergenic sequences, but long promoter sequences partially overlap with the coding region of the preceding gene.

Each promoter was analyzed with a minimum of three independent lines prepared from short and long promoter regions. The exception was *nhr-153*, for which only one size of promoter sequence was used, 968 bp.

All transgenic lines were prepared by co-injection of plasmid DNA containing pPD95.67 vector with cloned inserts (at the concentration 50 ng/µl) and a marker, pRF4 plasmid (50ng/µl) carrying mutated *rol-6* gene (Su1006). The mixture was injected into gonads of young adult hermaphrodites. The progeny of injected mothers were screened for a rolling phenotype and lines were kept in continual cultures and stocks frozen in liquid nitrogen.

	sequence	sequence	forward	reverse	promoter	amplicon	coding
gene	forward	reverse	primer	primer	length bp	length bp	region
nhr-206	aactgcaggccgttgtggttttcacccacaca	cgggatcctgataaccgacagcagggtttccgc	5259	6025	511	880	369
nhr-206	aactgcagctgcatctctccacaatttgtagc	cgggatcctgataaccgacagcagggtttccgc	6024	6025	1839	2208	369
nhr-208	aactgcacggagtagttgcatgtactgtgtgtc	gctctagagccttacatccattgcatgatgcg	6026	6028	501	750	249
nhr-208	aactgcaggcggaaacctgctgtcggttatc	gctctagagccttacatccattgcatgatgcg	6027	6028	1973	2222	249
nhr-207	aactgcaggatcaatgatgacgagcagaagatc	cgggatccagtcttccagttattacagttctccg	5195	5196	858	1176	318
nhr-207	aaactgcaggatctagctgatattcttccggat	cgggatccagtcttccagttattacagttctccg	5194	5196	1851	2169	318
nhr-209	cgggatccacagtttgacagctgtgaatactgtg	cgggatcccgctttccattcaagatagttctcc	6030	6031	530	896	366
nhr-209	acgcgtcgacccgggtaccagattatgtggatc	cgggatcccgctttccattcaagatagttctcc	6029	6031	1834	2200	366
nhr-154	gctctagatcaaatcggcatgctcgacacatg	cgggatcccttcccgtgataattgttcgacgg	5235	6033	1594	1905	311
nhr-154	aactgcagccctggtctcacattggatgatca	cgggatcccttcccgtgataattgttcgacgg	6032	6033	1912	2223	311
nhr-153	aactgcaggtggacgtttcatgtggtgctgaag	cgggatccctccttcatcagtctttgcttctgc	5236	5237	968	1917	949
nhr-136	aactgcagcaggccaaaacgaaggagtcgaaac	gctctagatcaaatcggcatgctcgacacatg	6034	6035	1951	2200	249
nhr-136	aactgcaggtggaatttgccagtacttcagc	gctctagagcgtcagtggttttcatgtcggcttg	5238	5299	984	1233	249

Table 5., GFP primers and promoter lengths

The table shows names of the genes, sequences from 5´-3´end, laboratory numbers, sizes of promoter regions, amplicon length and numbers of bp in coding regions.

4.2.9. Transformation

TOPO TA cloning or ligation was followed by transformation into competent cells. The ligation mixture was incubated with suitable competent cells for 10 min on wet ice. Heat-shock was done at 41°C for 1 min and returned to ice water for two minutes. 500 µl of LB medium was added. Incubation at 37°C folowed with shaking for 45 min. The culture was spread on LB agar plates with Ampicillin (ICN, MP Biomedicals, Irvine, CA, USA) (100 µg/ml) or Kanamycin (50 µg/ml) (Serva, Heidelberg, Germany) and grown overnight at 37°C. DNA constructs were isolated from *E.coli* using JETQUICK plasmid Miniprep Spin Kit (GENOMED, Lőhne, Germany) or High pure plasmid mini kit (F. Hoffmann-La Roche Ltd, Switzerland).

The cloning of DNA fragments into the vectors was confirmed using a control digestion with appropriate restriction enzymes with nucleic acid electrophoresis and sequencing.

Constructs and PCR products were sequenced on ABI Prism 3100-Avant sequencer (Applied Biosystems, Foster City, CA, USA) kindly performed by Helena Trešlová (Institute of Inherited Metabolic Disorders, Prague).

4.2.10. *In vitro* transcription

The constructs for in vitro transcription were prepared by cDNA amplification of *C.elegans* with appropriate primers and subsequent ligation into L4440 vector or TOPO TA cloning into pCR®4-TOPO vector. All constructs were linearised at the ends of inserted DNA using restriction sites. 500 ng of each linearised construct was mixed with 20 µl of rNTP´s, 10 µl of 5x transcription reaction buffer, 1 µl of 100 mM DTT, 1 µl of RNase Inhibitor, 4 µl of T7 polymerase (Promega, Madison, WI) (2.5 µl for the first hour, next 1.5 µl for the second hour) and water up to the final volume of 50 µl. Equal molar mixture (confirmed by electrophoresis) for sense ssRNA and antisense ssRNA were mixed together and were incubated for 10 min at 70°C followed by 30 min at room temperature to anneal dsRNA followed by phenol-chloroform extraction and ethanol precipitation.

4.2.11. RACE

Trans-splicing confirmation of the gene *nhr-209* was done by 5' RACE Kit, 2nd Generation (Hoffman-La Roche, Basel, Switzerland). 5´end was amplified by PCR. Tailed cDNA was amplified by PCR using a gene-specific primer and the oligo dT-anchor primer. The obtained cDNA was further amplified by a second PCR using a nested specific primer and the PCR anchor primer. The RACE products were further sequenced. Qualitative control of transcription was included into this experiment using primers neo2/rev and neo3/for to obtain a 157-bp PCR product.

4.2.12. Agarose gel detection

PCR products were detected on 1% agarose gel. PCR fragments were loaded into the gel and run in an electric field of 100 Volts for 1,5 hour. Ethidium bromide was intercalated in to the double stranded DNA and gel was analyzed under the UV light.

4.2.13. Splice leaders

In order to characterize the mRNAs transcribed from this cluster, we prepared cDNAs from mixed cultures of *C.elegans* wild type (N2) worms and performed polymerase chain reactions (PCR) with primers derived from each gene as identified by the GeneFinder program. To capture the 5' mRNA ends, we included the SL1 and SL2 splice leader primers or used rapid amplification of cDNA ends (RACE). PCR or RACE products were cloned into pCR4 and/or PCRII vectors and sequenced. This strategy confirmed the expression of mRNAs for all seven genes in the cluster with exon-intron boundaries as depicted in WormBase (WS207).

4.2.14. Worm cultivation

C.elegans are maintained in the laboratory on nematode growth medium (NGM) agar, which had been aseptically poured in Petri dishes (Brenner, 1974). NGM agar plates are seeded with OP50 *E. coli* lawn. Worms could be transferred on a new plate using a chunk of agar from an old plate. Worms could be also picked individually using a platinum wire with a hook at the end, which could be easily sterilized in a flame.

4.2.15. Worm culture synchronization

C.elegans plates with gravid hermaphrodites were washed with water. Worms were collected in 12 ml centrifuge tubes and washed with water several times. 2 ml of 5 M NaOH and 1 ml of bleach (SAVO) (Bochemie, Bohumín, Czech Republic) were added to 7 ml of water with worms. The mixture was shaken for 6 min until worms disappeared and only embryos were visible. Embryos were washed 3x-6x with water. Then the solution was divided into two tubes, water was added. Centrifugation (1,000 rpm for 5 min) followed.

The pellet of released embryos was resuspended in 10 ml of M9 and incubated overnight at room temperature, shaking. Next day, the worm culture was synchronized in L1 stage.

4.2.16. Preparation of transgenic *C.elegans* strains

The plasmid DNA at the concentration of 50 ng/µl was co-injected with pRF4 plasmid which contains *rol-6* (su1006) mutant collagen gene (50 ng/µl) into the ovarial syncytium of young adult N2 hermaphrodites (Olympus Inverted System Microscope IX70 equipped with Narishige Micromanipulator) (Olympus, Tokyo, Japan), kindly performed by Hana Prouzová (Prague, Institute of Inherited Metabolic Disorders). The progeny of microinjected animals was screened to pick worms rolling due to expression of *rol-6*. The presence of transgenic construct in rolling animals was confirmed using single worm PCR.

4.2.17. Determination of GFP expression

Transgenic worms carrying specific GFP-transcriptional constructs were observed using Olympus SZX12 Stereomicroscope System or Olympus BX60 System Microscope both equipped with a light fluorescence attachment. BX60 Microscope is equipped also with Olympus DP30BW camera allowing taking pictures of observed objects (Olympus, Tokyo, Japan). SZX12 microscope allows observing worms directly on NGM plates. To observe worms using BX60 microscope, worms must be put into a drop of water on a 2% agarose pad on the slide.

4.2.18. RNA interference

For RNA interference, selected regions of cDNA clones (excluding DNA binding domains) were used as templates. Primers used for amplification of selected regions are listed in Table 6. PCR products were cloned to pCR4 and or pCRII vectors (Invitrogen, Carlsbad, CA) and sequenced. dsRNA was prepared by in vitro transcription using bacterial polymerases T7 or T3 or SP6 from linearized plasmids (Promega, Madison, WI). The resulting ssRNAs were checked by agarose electrophoresis for quality, annealed with an equal molar amount of the corresponding opposite ssRNA, and purified by phenol/chloroform extraction and ethanol precipitation. Purified dsRNA was used for RNAi by microinjections to the gonad of young adult N2 worms at a concentration 2 mg/ml using a Narishige (Olympus, Tokyo, Japan) system coupled with the Olympus IX 70 (Olympus, Tokyo, Japan) inverted microscope.

To check a possible non-specific effect of prepared dsRNA a control dsRNA, which was prepared from the (non-coding) promoter region of *nhr-60*, was used (Simeckova et al., 2007).

4.2.19. Densitometry

Densitometry is a tool to measure optical density of a light sensitive material due to exposure to light. Optical density gives results of the darkness of developed pictures. Results can be expressed in absolute numbers of dark spots or relative values expressed in a scale.

For the densitometric analysis of expression of gfp fusion genes, the worms were started as small synchronized cultures and all worms that expressed any GFP in the particular culture were photographed at constant settings (objective 40x, exposure 100 ms or 50 in case of *nhr-206*). The pictures were analyzed using Image J program (Abramoff, 2004). Total areas of body containing the gut without the pharynx or pharynx areas were densitometrically analyzed with subtraction of the background that was calculated as a mean of three measurements taken outside the body of analyzed worms. Results were analyzed using Excel computer program.

5. Results

5.1. Characterization of the cosmids R07B7.13 and C13C4.3

The genomic region, which contains this locus on chromosome V, is included in two cosmids, R07B7.13 and C13C4.3 and spans 17 kb (V: 12 092 022 – 12 109 114, WS207). The genes localized at this region are in close proximity to each other and include the following nhr genes starting from 5'end: *nhr-206, nhr-208, nhr-207, nhr-209, nhr-154, nhr-153* and *nhr-136*.

In order to characterize the cDNAs transcribed from this cluster, we prepared cDNAs from mixed cultures of *C.elegans* N2 worms and performed PCR reactions with primers derived from genes identified by the GeneFinder program including the SL1 and SL2 splice leader primers. PCR products were cloned into pCR4 and or PCRII vectors and sequenced. This strategy confirmed the expression of mRNAs of all seven genes in the cluster as predicted by the WormBase (WS 207 data release).

The alignment of amino acid sequences of all receptors from this region was analyzed by the Clustal program [Larkin, 2007] (Fig.12). The analysis shows the strongest homology between receptors in the DNA binding domain and at the C terminal parts of the receptors. In the remaining regions, the receptors show striking sequence diversity despite that the Blast search identifies genes of this cluster as the closest homologues in the *C.elegans* genome that is in agreement with recent origin of this cluster by successive duplications, however with a significant sequence diversification. The phylogenic and cladistic analyses indicate that the genes in the cluster can be divided to two subgroups, first containing the genes that show phylogenic relation with *nhr-207* and the second clustering with a common ancestor of *nhr-153* and *nhr-136* (Fig. 13A). The analysis also indicates that the later cluster is more ancient and the genes *nhr-206*, *nhr-208* and *nhr-207* were formed by more recent duplications.

All seven receptors belong to the Class I of *C.elegans* nuclear hormone receptors based on their P-box sequences [Van Gilst, 2002]. Their classification according to the P-box sequence keeps with the division based on the overall homology estimated by the Blast program: the first three receptors, NHR-206, NHR-208 and NHR-207, have P box sequence CNGCKA and form a small subgroup of receptors in the subgroup 12 [Van Gilst, 2002 #32] and four receptors NHR-209, NHR-154, NHR-153 and NHR-136, which have the P box sequence CNGCKT form the subgroup 8 [Van Gilst, 2002 #32].

Next, we compared the sequences of the clustered *C.elegans* receptors with their closest homologues in *C.briggsae* and found that the receptors with P box sequence CNGCKT are conserved in *C.briggsae* and have also the same P-box sequence. Comparison of the *C.elegans* and *C.briggsae* genomic sequences indicates the following relationships: the orthologue of NHR-209 is CBP 20617 (CBG 23379), the orthologue of NHR-154 is CBP 24460 (CBG23380), the orthologue of NHR-153 is CBP24461 (CBG23381) and the orthologue of NHR-136 is CBP 24462 (CBG23383). These genes are named as *Cbr-nhr-209*, *Cbr- nhr-154*, *Cbr-nhr-153* and *Cbr-nhr-136*. The amino acid sequences of NHR-209, NHR-154, NHR-153 and NHR-136 in *C.elegans* are 66% to 76% identical to the receptors in *C.briggsae* (NHR-209, 66%; NHR-154, 76%; NHR-153, 69 %, NHR-136, 75%); we also found conservation in sizes of exons between these two species (Fig. 13B). The sequences and sizes of the introns are not conserved, as well as the sequences in intergenic regions. In contrary, the first three receptors in the cluster, NHR-206, NHR-208 and NHR-207 do not have orthologues in *C.briggsae*. We have found only one isoform for each gene.

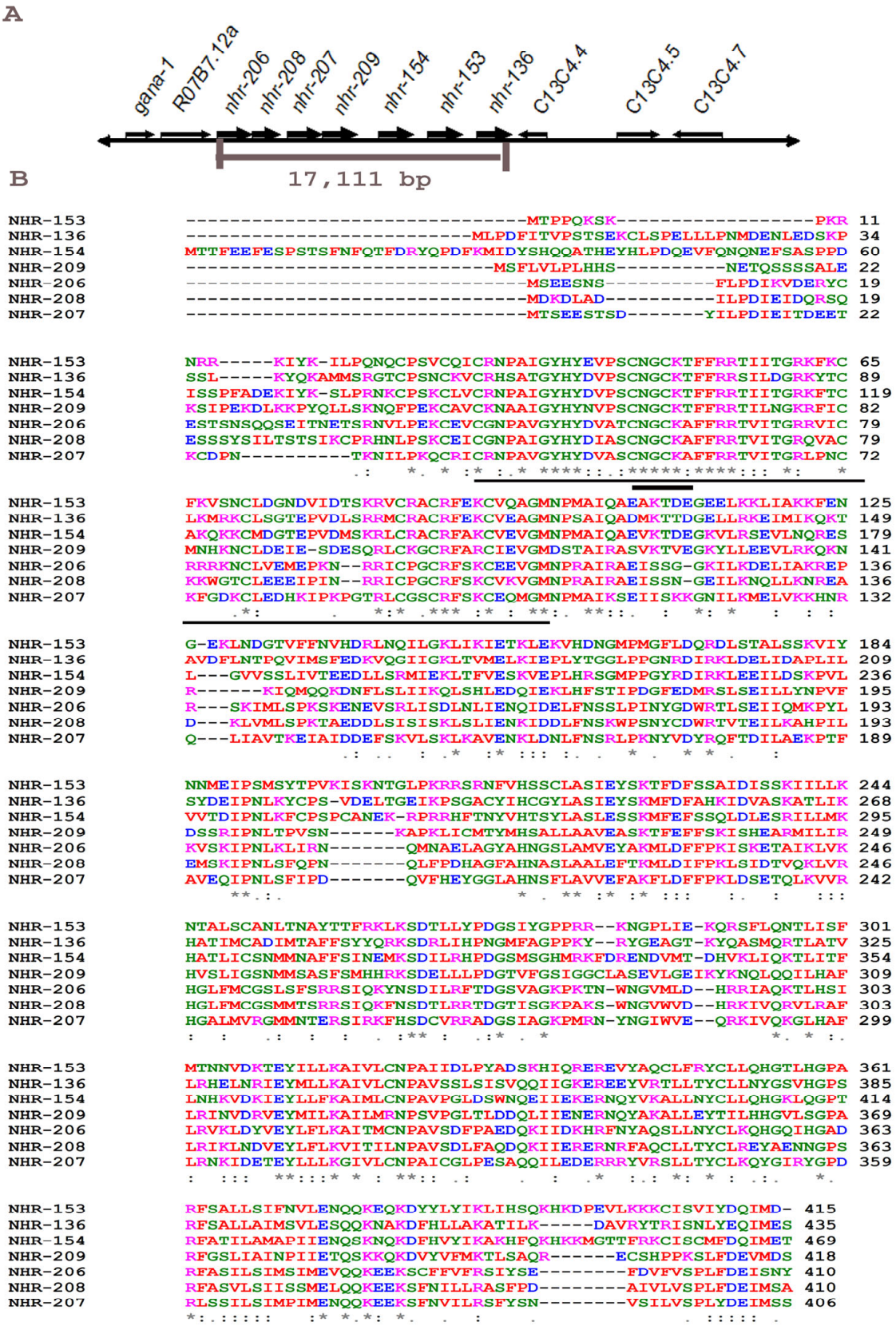

Figure 12. Genomic organization and sequence similarity of seven clustered NHRs.

A) A schematic diagram of genes localized in a region of Chromosome V. A cluster of seven *nhr* genes (thick back arrows) spanning 17 kb are bracketed by the unrelated genes *R07B7.12a* at the 5' end *C13C4.4* at the 3' end.

B) The Clustal analysis of the amino acid sequences corresponding to the seven clustered nuclear hormone receptors: NHR-206, NHR-208, NHR-207, NHR-209, NHR-154, NHR-153 and NHR-136. The alignment shows regions of high sequence conservation and regions that have substantially diversified. The highly conserved DNA binding domain (DBD, thin underline) and the sub domain involved in the contact of nuclear hormone receptors with DNA response elements (P box, thick underline). The ligand binding domains constituting the majority of approximate C- terminal halves of receptors show substantial diversification (visualized using standard color parameters, representing small and hydrophobic amino acids in red, acidic amino acids in blue, basic in magenta and hydroxyl- or amine- group containing amino acids in green color and the remaining amino acids in grey color).

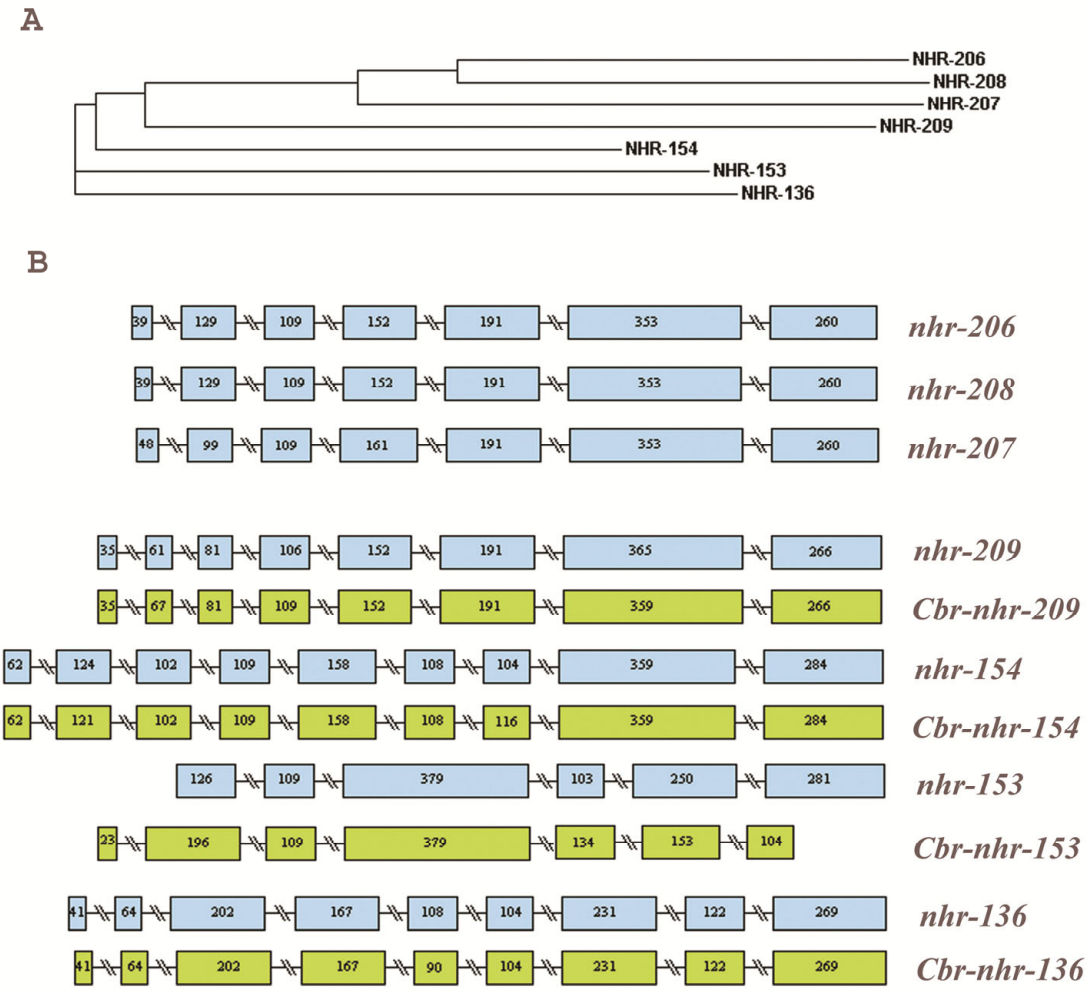

Figure 13. Evolutionary relatedness and intron-exon similarities among the seven clustered *nhrs*.

A) The phylogram of the seven clustered NHRs, as calculated from protein sequences using the Clustal program, indicated that NHR-209, NHR-54, NHR-153 and NHR-136 group together while NHR-206, NHR-208 and NHR-207 represent more recent duplications and are most closely related to NHR-209.
B) The coding region organization of the seven clustered *C.elegans nhr* genes and their closest homologs in *C.briggsae.* The diagram shows differences between recently duplicated genes present only in *C.elegans* and genes conserved between *C.elegans* and *C.briggsae*. While the recently duplicated genes have almost identical exon sizes, the conserved genes show wider diversity of exon sizes between genes in the particular species but are conserved between these closest corresponding *C.elegans* and *C.briggsae* homologues.

5.2. *nhr-207* and *nhr- 209* are organized in an operon

The intergenic regions between genes of the studied cluster vary from 270 to 1277 bp. A 270 bp long region of a putative promoter or an intergenic region is upstream of *nhr-206*. Preceeding *nhr-206*, Wormbase identifies a pseudogene R07B7.12. The other regions have sizes of 651, 338, 542, 1277, 1141, 1156 bp. This suggests that some genes of the cluster may be organized in operons.

In order to distinguish between individually organized genes and genes expressed from operons, we prepared cDNA from mixed stages of N2 animals and performed multiple PCRs with primers specific for splice leader 1 (SL1) and splice leader 2 (SL2) together with gene specific reverse primers. This strategy identified trans-splicing with SL1 primer in the case of *nhr-154* and its 13 bp long 5'UTR (tatagtggcagcc). The trans-splicing with SL2 splice leader was detected in the case of *nhr-209* along with the 257bp long 5'UTR. Surprisingly, trans-splicing was not detected in the remaining genes of the studied cluster, despite that the expression of predicted mRNAs was readily amplified using gene specific primers derived from the predicted sequences. Keeping with our results, WormBase lists SL1 splice leader for *nhr-154*.

The same region and splice leader SL2 for *nhr-209* was also found by the RACE method.

5.3 Expression of studied genes

We have found that all genes are expressed during all developmental stages at a similar and relatively stable expression level accept *nhr-154.* We started with semi-quantitative PCR. The expression level was confirmed by real-time PCR (see later).

5.3.1. Qualitative end-point PCR expression of genes

End-point PCR followed by 1% agarose gel electrophoreses showed that all genes are expressed (Fig14). PCR reaction had been stopped after 30 cycles to ensure that all products were in exponential phase.

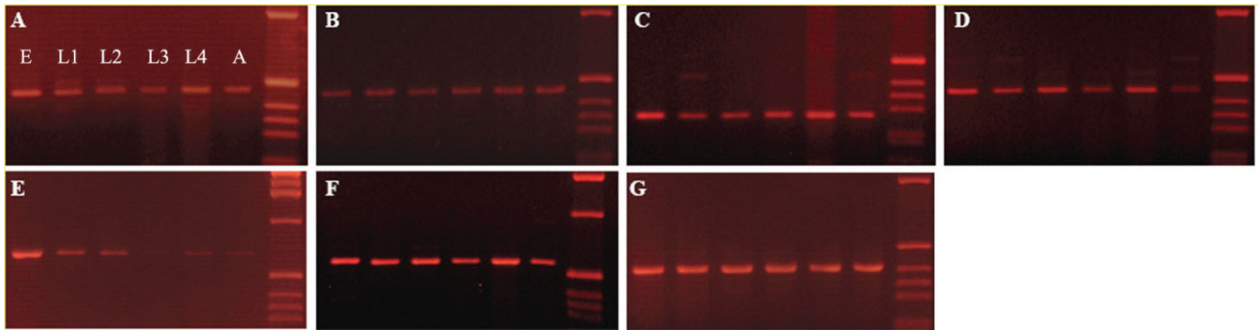

Fig.14 Agarose gel electrophoreses of qualitative PCR of all genes.
Panel A, shows *nhr-206*; B, *nhr-208*, C, *nhr-207*; D, *nhr-209*; E, *nhr-154*; F, *nhr-153*; G, *nhr-136* during all developmental stages from embryo to adult

5.3.2. Real-time PCR

We confirmed that *ama-1* could be used as a reference gene in our study. Fig15 shows relative changes through developmental stages, different sample collection and different time of RNA isolation. There were treated worms (stressed by limitation of food) and control N2 worms. We have found that the confidential interval and standard deviation is close to one (no expression changes). After this confirmation we were sure that ama-1 can be used as a reference (housekeeping) gene in this study.

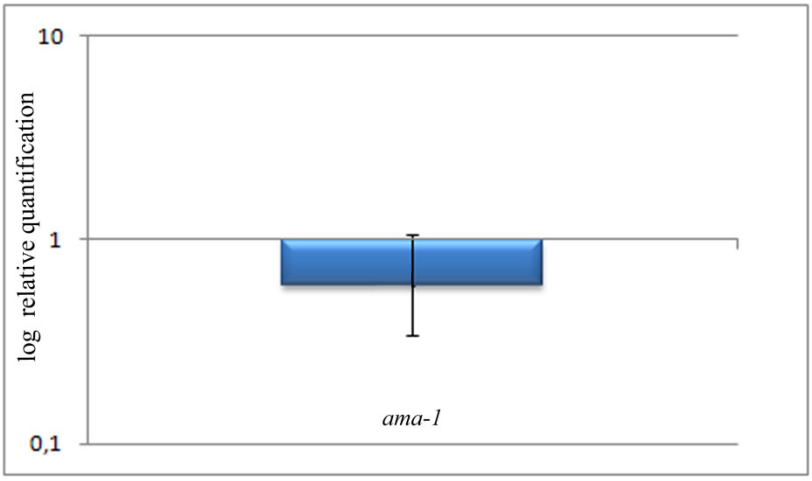

Fig.15 Relative quantification of reference gene *ama-1*

Relative expression of stressed and N2 control worms. The picture shows relative ratio averages of fed and hungry N2 worms. There were more than 30 different measurements compared. SD are shown. The Student T test shows $P<0.05$ compared with null hypothesis.

PCR amplification for each amplicon was measured and calculated using the formula $N=10^{-1/slope}$. For the actual calculations, the base of the exponential amplification function was used (e.g. 1.96 means 96% amplification efficiency). Table 6 shows how easy or dificult is to amplify amplicons, bordered by primers shown in table 3. The formula of regression was calculated automatically by the LighCycler® software. Each specific amplicon was diluted ten times. This diluent was used as a template for real-time PCR. To visualize Cp values absolute quantification using 2nd derivation maximum was used. Cp values were used to create curve, formula of regresion and efficiency value. (Figure 16).

gene	efficiency		gene	efficiency
nhr-206	1,715		*Cbr-nhr-209*	1,989
nhr-208	1,859		*Cbr-nhr-154*	1,858
nhr-207	1,879		*Cbr-nhr-153*	2,041
nhr-209	1,875		*Cbr-nhr-136*	1,898
nhr-154	1,88		*Cbr-rpb-1*	1,895
nhr-153	1,774		*asc-2*	1,898
nhr-136	1,777		*fed-7*	1,816
ama-1	1,99		*nhr-49*	1,918

Table 5: Efficiency values of the genes

The table shows names of genes and efficiency values counted from the standard curves.

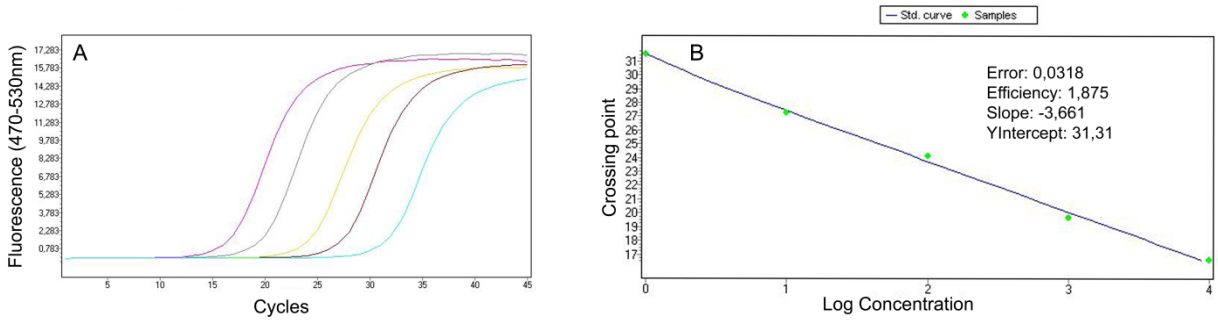

Fig.16 Amplification of diluted standards and standard curve

A PCR amplicon of *nhr-209* was used as a standard and was 10 times diluted. Standards with the concentration from 10 copies to 10^5 copies were used. Cp values and concentration were used to calculate standard curve, slope and efficiency.

Quantitative PCR has confirmed that the expression level of all genes is relatively stable contrary to *nhr-154* which was expressed at a high level in embryos and L1 larvae and than the expression decreased. High levels of expression of *nhr-154* were observed again in young adults (**Fig. 17**). Using qPCR we have found that expression level of *nhr-206*, *nhr-208* and *nhr-207* is expressed in hundreds of copies in contrary to *nhr-209*, *nhr-154*, *nhr-153* and *nhr-136* were the expression is in thousands of copies (expressed in absolute values). The values detected for *C. brigssae* orthologues *Cbr-nhr-209*, *Cbr-nhr-154*, *Cbr-nhr-153* and *Cbr-nhr-136* were similarly large **(Fig. 18)** as in the case of *C.elegans* orthologues suggesting that the expression of conserved genes at standard laboratory conditions is bigger in comparison with the expression of genes that arouse as new members of the family the in *C.elegans* genome.

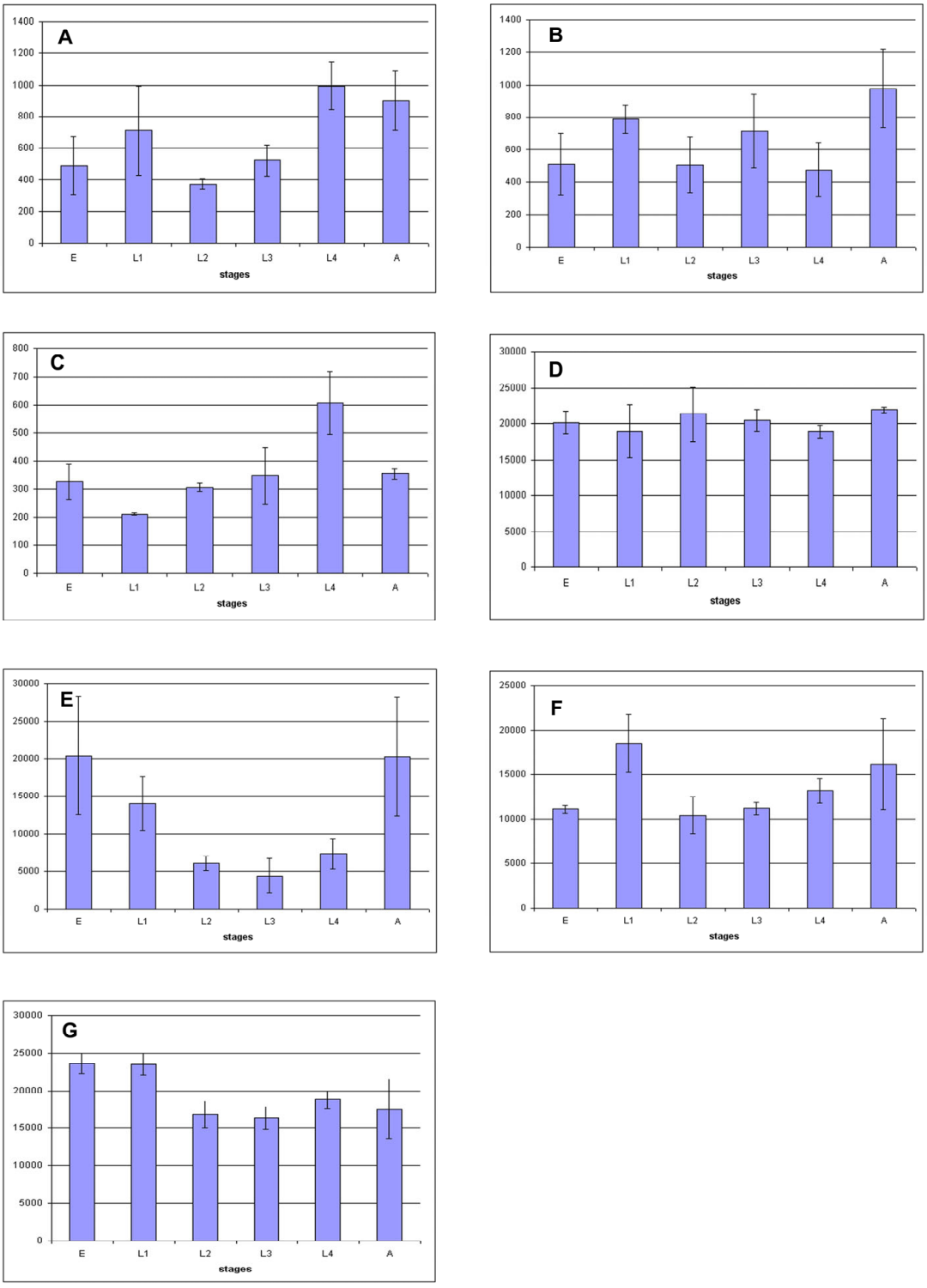

Fig.17 The expression level of *nhr-206, nhr-208, nhr-207, nhr-209, nhr-154, nhr-153, nhr-136*

The picture shows the expression level in individual larval stages and studied genes. The expression of the seven clustered *C.elegans* nhr genes during development was analyzed by RT-qPCR revealing that all genes are expressed under standard laboratory growth conditions. The recently duplicated genes within the cluster (*nhr-206*, *nhr-208* and *nhr-207*) are

expressed at substantially lower levels than the genes that are conserved between *C.elegans* and *C.briggsae*. All genes, with the exception of *nhr-154*, are expressed at relatively constant levels throughout development in *C.elegans* . The expression of *nhr-154* decreases in mid-larval stages with the lowest levels detected at the L3 stage.

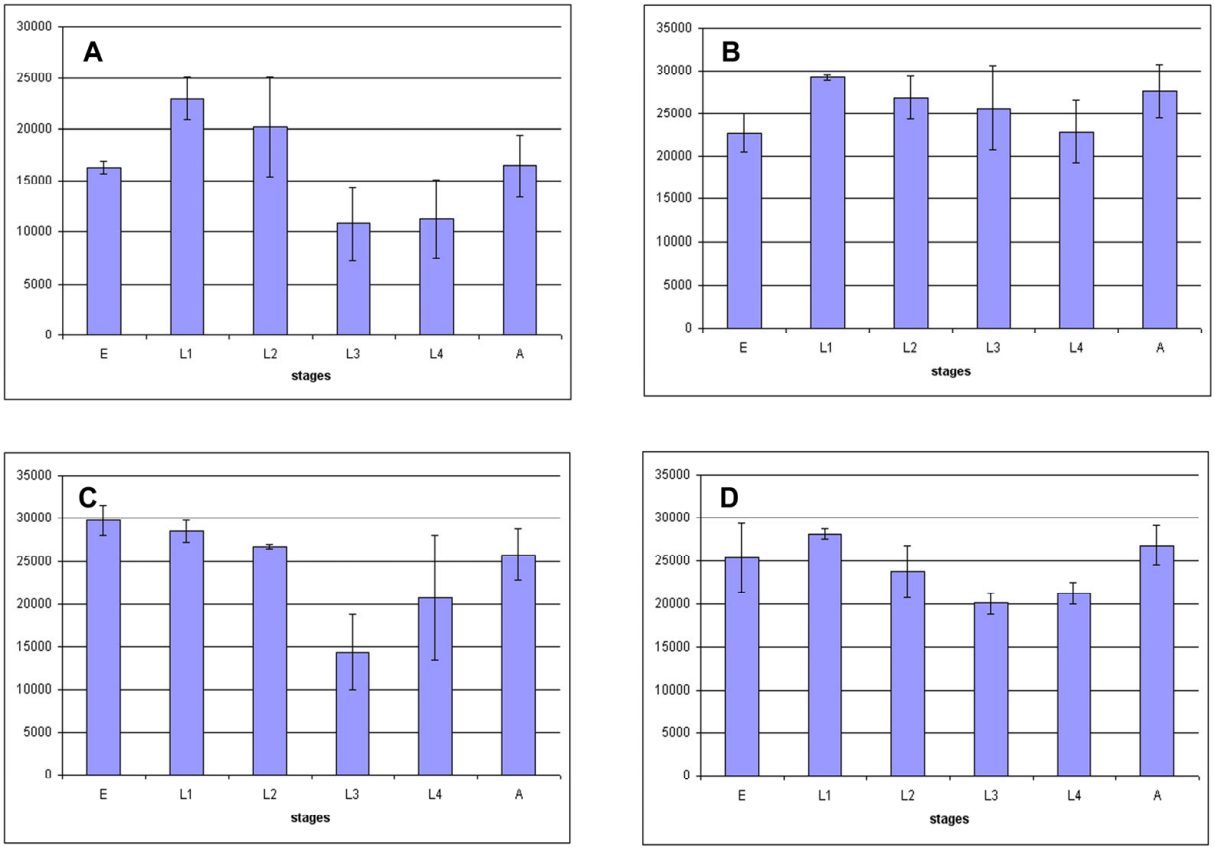

Fig.18 Gene expression of *Cbr-nhr-209, Cbr-nhr-154, Cbr-nhr-153* and *Cbr-nhr-136*
Analysis of the expression of *C.briggsae* nhr cluster orthologs by RT-qPCR. The expression of Cbr-*nhr-209*, Cbr-*nhr-154*, Cbr-*nhr-153*, Cbr-*nhr-136* is relatively stable during development and shows levels comparable to their *C.elegans* orthologs.

5.4 GFP expression

In order to determine expression patterns for nhr studied genes, we prepared various transgenic lines expressing GFP as transcriptional fusion constructs. Our strategy was to have two different promoter lenthts. The shorter promoter region was located upstream of the gene but only in the intergenic region. The longer promoter region was approximately 1-2 kb and usually took a 3´end of the previous gene. Fig.19 shows the organization of the studied genes on chromosome V, their orientation and according to our strategy promoter regions.

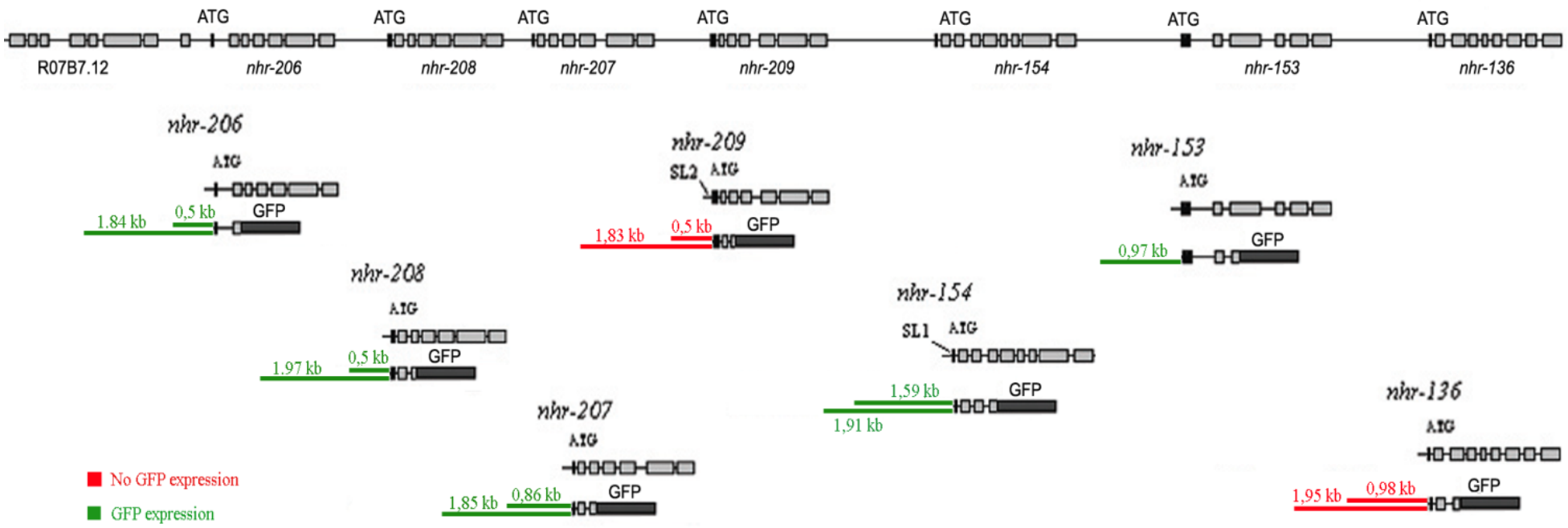

Fig.19 Organization on chromosome V and GFP promoters

The picture shows organization, gene orientation, gene and intergenic sizes. There are promoter regions which were tested using GFP constructs. Green and red colors show if GFP expression was visible.

5.4.1. The expression of *nhr-206*::GFP

Since we did not find trans-splicing with the SL2 splice leader in the case of *nhr-206*, that would show possible expression from an operon with the preceding gene, we used as a putative promoter the 270 bp long sequence that is in front of *nhr-206* together with two differently long regions included in R07B7.12. Both strategies (putative promoters with the length of 511 bp and 1839 bp) lead to functional promoters with an identical expression pattern.

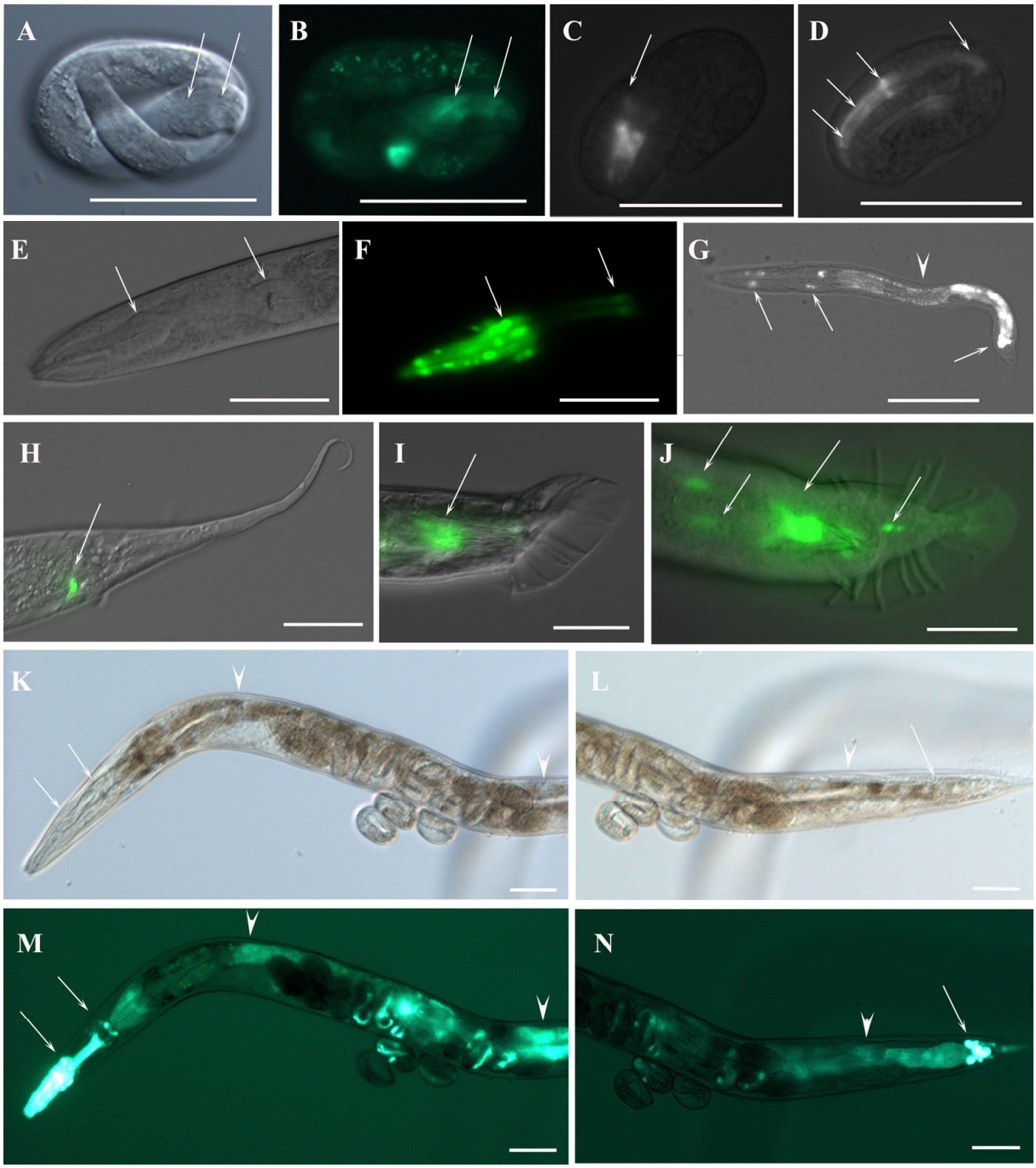

Fig. 20. The detection of *nhr-206*::GFP

The picture shows GFP expression during development. The expression is localized in the head region in pharynx (B,C,D,F,G,M) then in tail area in the sphincter (G,H,L,N,), rectal gland cells (G,L,N) and neurons of the tail (I,J). Weak expression is in the intestine (G,M,N); A,B, Nomarski and GFP image od 3-fold embryo; C,D nomarski and GFP combination of embryos; E,F, Nomarski and GFP image of L3 hermaphrodite head; G, Nomarski picture of L2 hermaphrodite; H, combination of Nomarski and GFP of hermaphrodite, expression is visible in the anal sphincter; I,J Combination of Nomarski and GFP in male tail, expression is localized in male specific organs, epidermal cells, neurons of the rays cells; K,L,M,N Nomarski and GFP expression in adult mother with eggs. Scale bar 40 μm.

The expression of *nhr-206* GFP reporter transgenes starts during the comma stage of embryogenesis, and is initially seen in four unidentified cells localized in the head region. By the 2-fold stage, embryonic expression is observed in the pharynx with weaker expression in the intestine. This expression pattern continues throughout all larval stages and in adults with pronounced anterior pharyngeal expression. The reporter genes were also strongly expressed in rectal gland cells, the anal sphincter, and in epidermal cells in the tail. Weaker expression was also observed in the vulva and spermatheca. In males, expression was visible in male specific neurons of the tail and rays. The pattern of expression is very similar to that described by Reece-Hoyes and coworkers in their high throughput screen (Reece-Hoyes et al., 2007).

5.4.2. The expression of *nhr-208*::GFP

The expression of *nhr-208* GFP reporters started in embryos at the 1.5-fold stage within the pharynx, intestinal sphincter, and epidermal cells in the tail. By the 3-fold stage of embryogenesis, additional expression was observed in rectal gland and surrounding cells. During all larval stages, strong expression of the transgenes was visible in the pharyngeal and unidentified head neurons, the pharyngeal-intestinal valve cell, the posterior part of the intestine, the intestinal sphincter, two rectal gland cells, the intestinal-rectal valve cell, and the epidermal hyp10 cell (Fig. 21E,J,M). In males, the expression was seen in several rays (6-8) and other male specific neurons (Fig. 21L). This pattern was similar to that described by Reece-Hoyes and coworkers (using a 653 bp long promoter), although our reporters did not result in expression in the excretory cell and vulva.

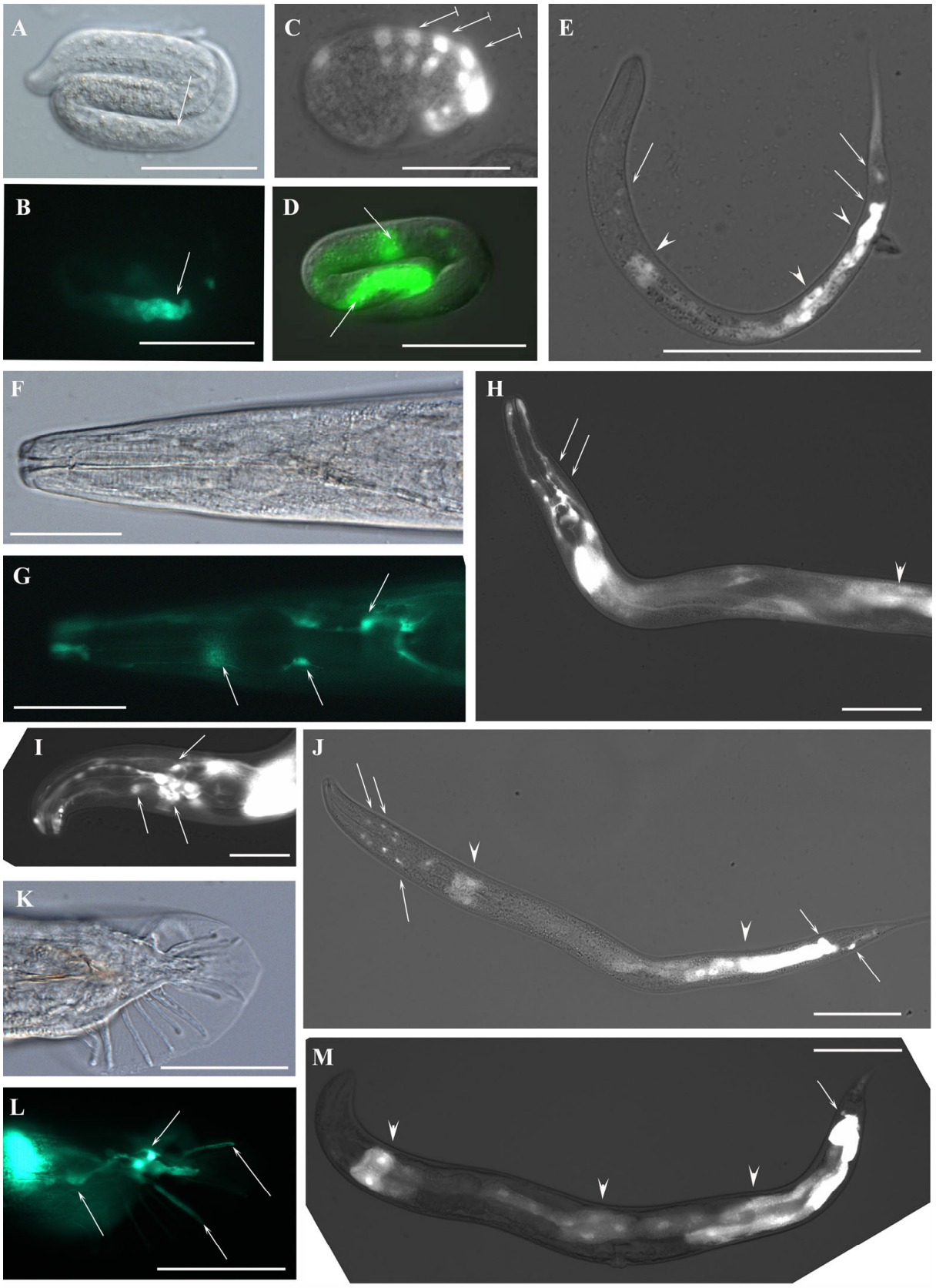

Fig. 21. The detection of *nhr-208::*GFP

The picture shows GFP expression of *nhr-208* during development. The expression of *nhr-208* is localized in head neurons in the pharynx and pharyngeal-intestinal valve cells

(D,E,H,G,I,J). In the tail area in intestinal sphincter(B,E,J,M), epidermal cells (C,E,H,J), rectal gland cells, intestine-rectal gland cells, the intestine-rectal valve cell (E,J,M) and epidermal hyp10 cell (E,J). Two rays cells (6 and 8) and male specific neurons were observed (L).

A,B Nomarski and GFP picture of 3-fold embryo; C – GFP expression of 1,5-fold embryo; D – GFP expression of 3-fold stage; E – L2 stage GFP picture; F,G – Nomarski and GFP picture of the head area; H,I – GFP of L4 and adult head area; J – GFP picture of L3; K,L – Nomarski and GFP picture of the male tail area; M – GFP picture of adult; Scale bar 40μm.

5.4.3. The expression of *nhr-207*::GFP

The expression of *nhr-207* GFP reporters began in 1.5-fold embryos in pharyngeal and epidermal cells. In 3-fold stage embryos, expression was observed in pharyngeal neurons, intestinal cells, the intestinal-rectal valve, and the sphincter. This pattern of expression was present during all larval stages and in adults (Fig. 22). In larvae and in adults, additional expression was observed in the pharyngeal-intestinal valve (Fig. 22K) and spermathecae. In males, the expression was seen in male specific neurons, including rays (Fig. 22N,O,P). Our results overlap those reported by Reece-Hoyes and coworkers (using a 340 bp long promoter).

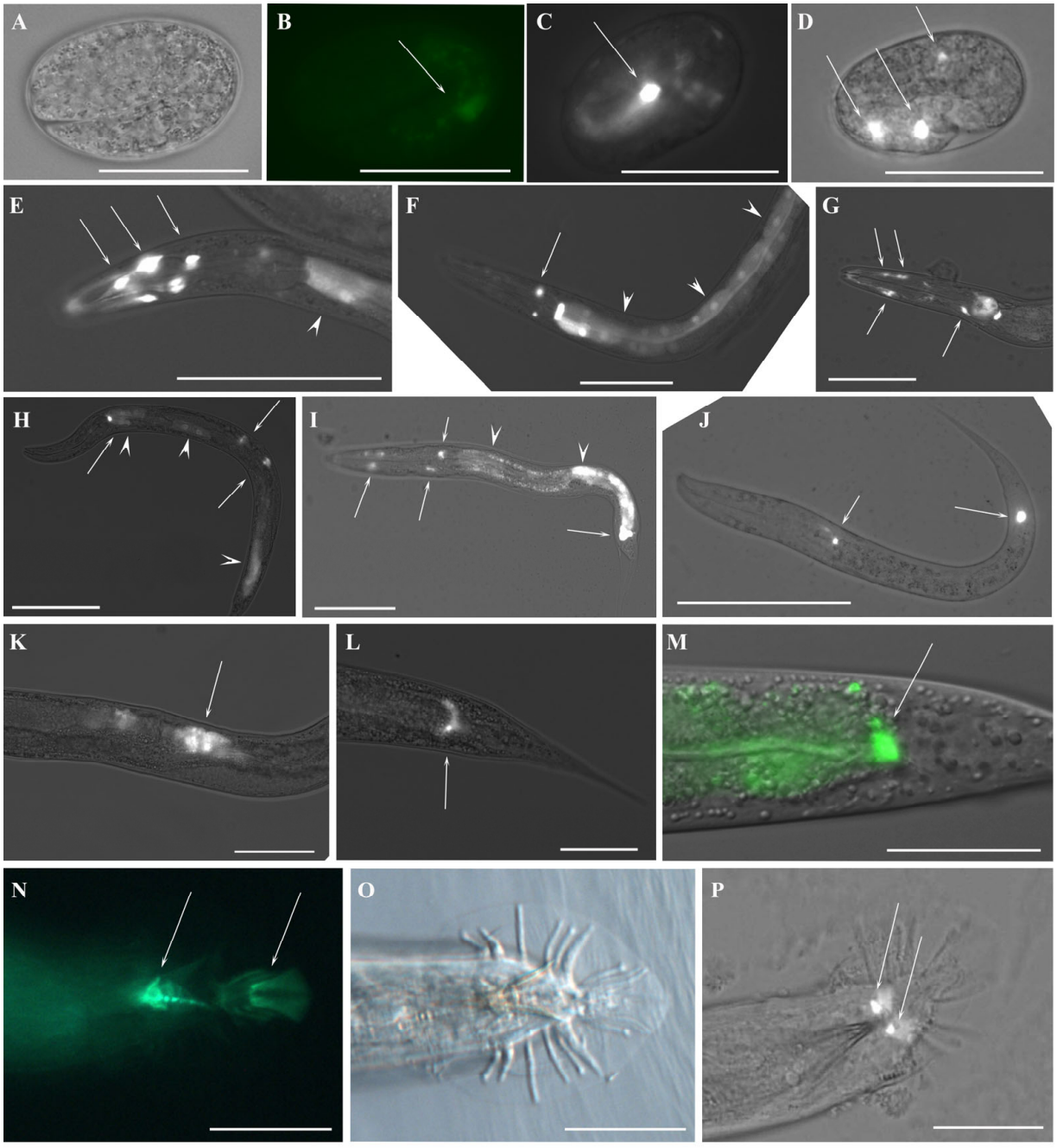

Fig. 22 The expression of *nhr-207*::GFP

Picture shows expression in pharyngeal neurons in the head (D,E,F,G,H,I,J). In the tail area, there is an expression in the intestinal cells, the intestinal-rectal valve, and in the sphincter(B,C,D,H,I,J,L,M). Males have expression in male specific neurons – rays (N,P). In larvae and in adults there is also expression in pharyngeal-intestinal valve (L) and spermathecae (K).

A,B – Nomarski and GFP picture of the 1,5-fold embryo; C,D – GFP expression of 3-fold embryo; E,F,G – GFP picture of the head area of a L2 larvae; H – GFP picture of adult; I,J – GFP expression of L3 and L2; K- GFP picture of adult sperathecae; L, – GFP expression of

intestinal rectal valve in adult hermaphrodite; M – GFP expression of sphincter in adult hermaphrodite; N,O,P – GFP and Nomarski picture of male tail; Scale bar 40μm.

5.4.4. The expression of *nhr-209*::GFP

In search of a putative promoter of *nhr-209*, we prepared six transgenic lines with a construct made from the short upstream region (530 bp) and four lines with a construct made from a long upstream region of ***nhr-209*** (1834 bp), but we did not see any GFP expression. We also attempted to create a construct with the promoter of *nhr-207*, the complete genomic sequence of *nhr-207*, the intergenic region between *nhr-207* and *nhr-209* and fusion of *gfp* gene with the second exon of *nhr-209* (by overlapping PCR products injected with a linearized pRF4plasmid) but we did not observe GFP expression in the progeny of injected mothers.

5.4.5. The expression of *nhr-154*::GFP

The expression of *nhr-154* GFP reporter transgenes was first detected in the 2-fold stage embryo within the developing pharynx and in precursors of several unidentified head neurons. By the three-fold stage of embryogenesis, expression was seen in the pharynx and throughout the intestine, a pattern reminiscent of the developmental transcription factor pha-4 (Smith and Mango, 2007; Updike and Mango, 2006) (Fig. 23). In the L1 and L2 stages, the reporter gene expression was strong in the pharyngeal muscles (anterior and posterior bulbs, predominantly), in unidentified head neurons, the intestine, and in the intestinal-rectal valve or sphincter cell. Consistent with our RTqPCR analysis, GFP reporter gene expression decreased in subsequent larval stages (L3, L4) so that by the adult stage only pharyngeal expression persistence was reproducibly observed. This suggested that the peak of expression in adults detected by RT-qPCR might be due to the embryos inside gravid adults in these samples. No obvious differences in pattern were seen between the two promoter lengths tested.

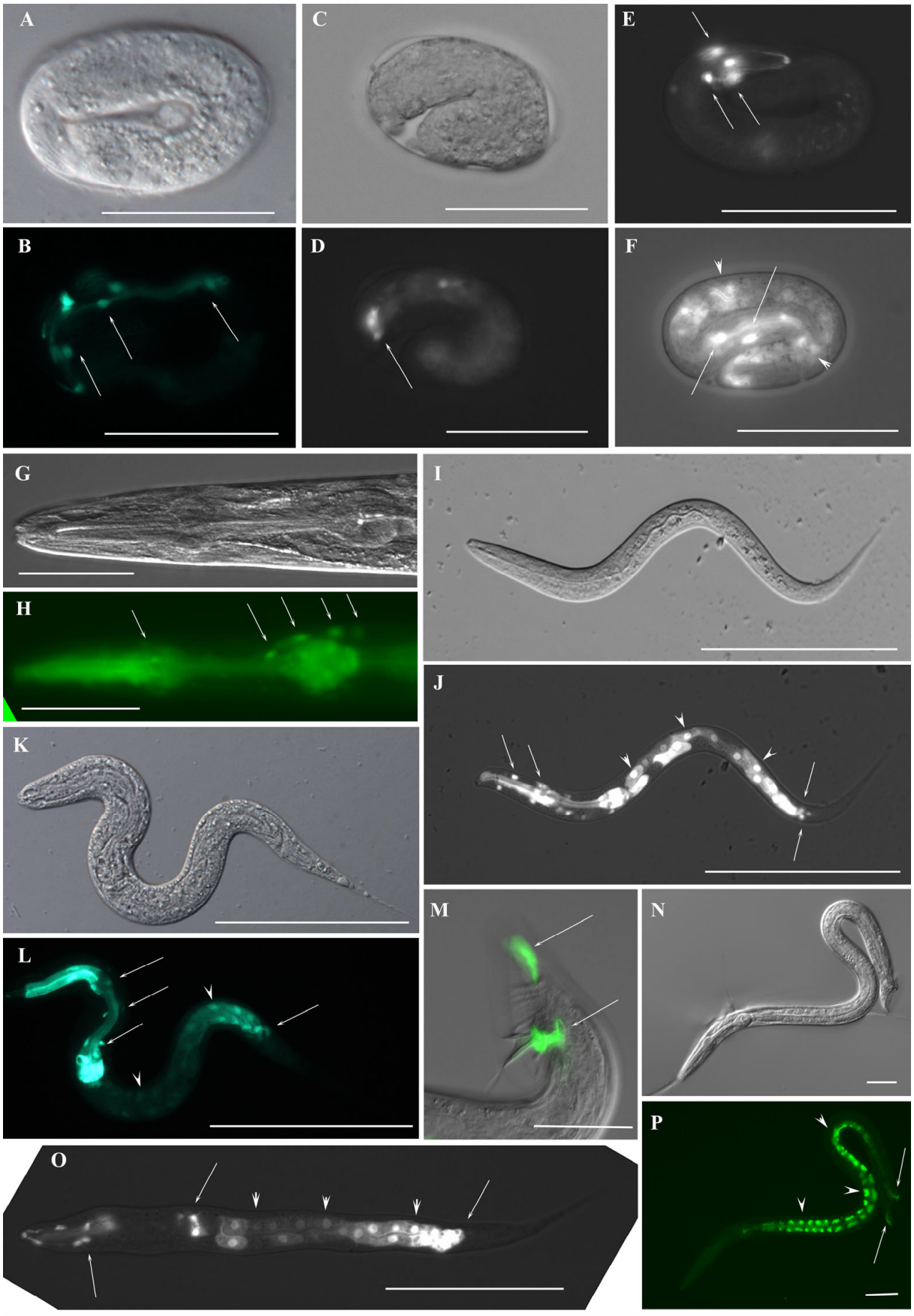

Fig. 23 The expression of *nhr-154*::GFP

The picture shows expression in the head area in pharyngeal neurons and pharyngeal muscle cells (B,D,E,F,H,J,L,O). In the body, there is an expression in intestinal cells, in the intestinal-rectal valve or sphincter (D,F,J,L,O). Males have expression in male specific neurons – rays(M,P) and in the intestine.
A,B – Nomarski and GFP picture of 3-fold embryo; C,D – Nomarski and GFP expression of 1,5-fold embryo; E,F, – GFP expression of 3-fold embryo; G,H Nomarski and GFP picture of head area adult hermaphrodite; I,J Nomarski and GFP picture of L1 larva; K,L – Nomarski and GFP picture of L1 larva; M – GFP expression of the male tail; N,P – Nomarski and GFP adult male tail; O – GFP picture of L1 worm; Scale bar 40μm.

5.4.6. The expression of *nhr-153*::GFP

The expression of *nhr-153* GFP reporter transgenes was first detected in the 2-fold stage embryo in the pharyngeal and intestinal cells (Fig. 24). In all larval stages, the reporter gene expression was very strong in the posterior bulb of the pharynx and in all intestinal cells as well as the intestinal-rectal valve or rectal gland cells. Expression was also seen in several unidentified neurons near the posterior pharyngeal bulb and in the tail (Fig. 24 E,H,I,J,M). In males, very strong reporter expression was observed in some of the ray-associated neurons (Fig. 24 K,L,M).

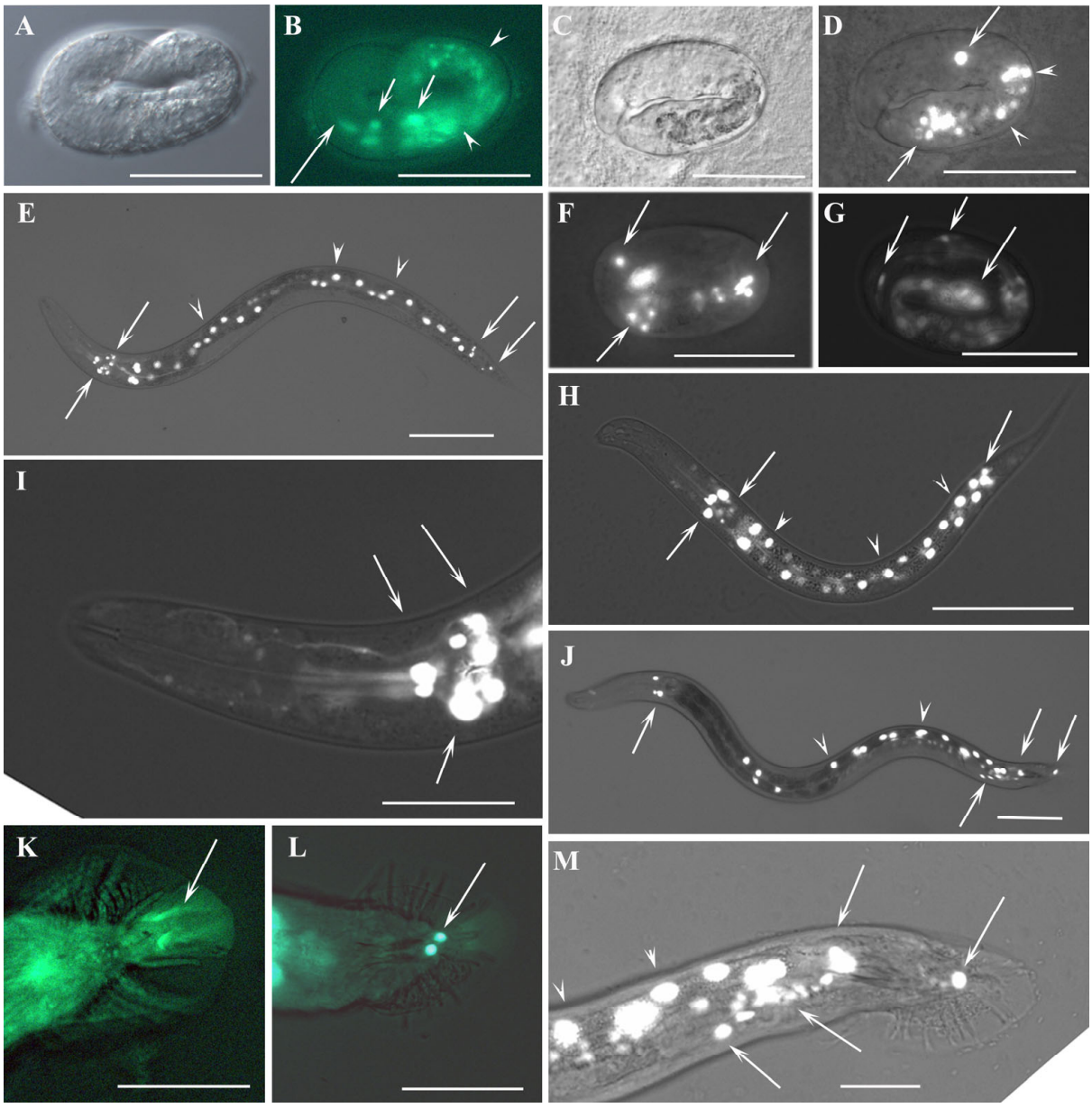

Fig. 24 The expression of *nhr-153*::GFP

GFP expression of *nhr-153* GFP is detected in the head area in neurons near the posterior pharyngeal bulb (B,D,E,F,G,H,I,J). In the body the expression is localized in all intestinal cells as well as the intestinal-rectal valve or rectal gland cells and some neurons (D,E,G,H,J,M). In males expression was observed in some rays (K) and some of the ray-associated neurons (J,L,M).

A,B – Nomarski and GFP picture of the 3-fold embryo; C,D – Nomarski and GFP picture of 2-fold embryo; E, GFP picture of L3 larva; F,G, GFP expression of 3-fold embryo; H, GFP picture of L1 larva; I, GFP image of the head area of an adult hermaphrodite; J, GFP image of whole adult male; K,L,M, GFP pictures of the tail area of an adult male; Scale bar 40μm.

5.4.7. The expression of *nhr-136*::GFP

The construct ***nhr-136::gfp*** was prepared in the same way as other constructs and we prepared 12 transgenic lines, with promoter lengths 951 and 1900 bp, but we did not observe GFP expression despite that the transgene was detected in transgenic animals by PCR and the integrity of the construct was confirmed by sequencing.

Summarized results of reporter gene expression for *C.elegans* clustered nhrs. The intensity of the expression was scored from low (+) to high (+++) in each cell or tissue type. Absence of expression is indicated (-). If specific developmental stages are not reported, expression was observed in all larval stages and in adults.

Reporter Gene	*nhr-206*	*nhr-208*	*nhr-207*	*nhr-209*	*nhr-154*	*nhr-153*	*nhr-136*
Start of emb. expression	Comma	1.5-fold	1.5-fold	-	2-fold	2-fold	-
Phar. Muscles	+++	-	+	-	+++	-	-
Head Neurons	+++	+++	++	-	++	+++	-
Phar. – Intest. Valve	-	-	+++	-	-	-	-
Intestine	+	+	+	-	+++ L1, L2, L3	+++	-
Int.- Rectal Valve	-	+	++	-	+	+	-
Rectal Glands	+++	+++	+	-	+	+	-
Int. Sphincter	+	+	+	-	+	-	-
Tail Neurons	-	+	-	-	-	+	-
Epidermal cells in head	++	+	-	-	+	-	-
Epidermal cells in tail	++	++	-	-	-	+	-
Spermatheca	+++	-	+++	-	-	-	-
Vulva	++	-	++	-	-	-	-
Rays	+ R6	+ R6, 7, 8	+ R6	-	-	-	-
Ray Neurons	+	+	+	-	-	+	-
Male Specific Neurons	++	-	++	-	-	-	-

5.5. The expression of clustered nuclear hormone receptors responds to fasting

We performed 6 hour and 54 hour fasting experiments in synchronized larvae L1 of *C.elegans* and *C.briggsae* and assayed the expression of clustered *nhr*s by reverse transcription – quantitative PCR. This strategy revealed that the expression of *nhr-206, nhr-*

208, nhr-207, nhr-153 and *nhr-136* was higher in starving animals. The expression of *ama-1* was not affected by six-hour or 54 hour starvation (Fig.15).

Analysis of the expression of *C.briggsae* orthologues, the Cbr-*nhr-209, Cbr-nhr-154, Cbr-nhr-153*, and *Cbr-nhr-136* showed that the first gene in *the C.briggsae* cluster, the *Cbr-nhr-209* was also elevated, approximately twice, in starving larvae (Fig. 26).

In order to see if the up-regulation of gene expression at fasting conditions is not caused by errors in the experimental setting, we assayed the expression of genes that are known to be affected by feeding status. We have chosen acs-2, fat-7 and nhr-49 that are known to be elevated, decreased and not affected by fasting, respectively [Van Gilst, 2005 #29]. As expected, acs-2 was up-regulated approximately 2-fold by fasting, fat-7 decreased approximately 5 to 10 times and the expression of nhr-49 did not change (Fig. 25).

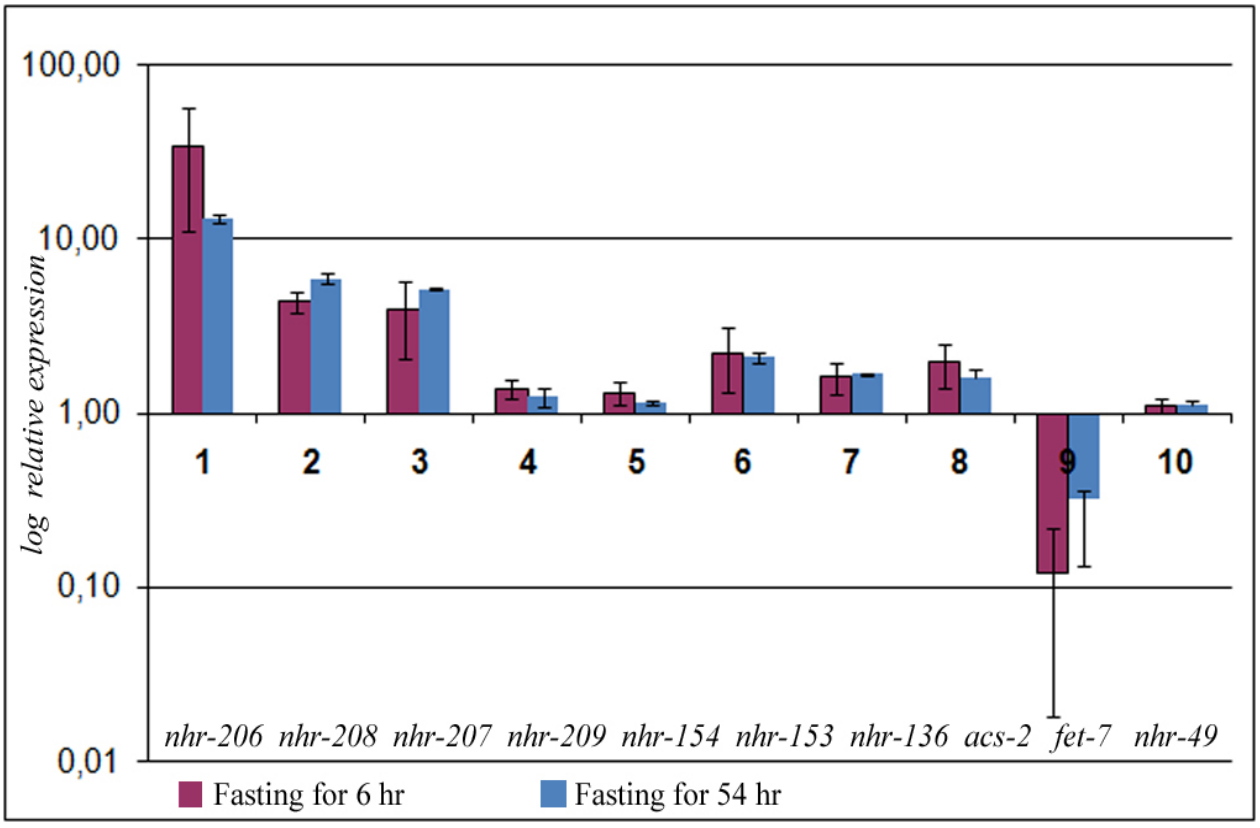

Figure 25. The fasting experiment of *C.elegans*

The picture shows the expression of seven clustered *C.elegans nhrs* during fasting and analyzed by RT-qPCR. Expression of the recently duplicated *C.elegans* genes *nhr-206, nhr-208 and nhr-207* is strongly induced in animals after either 6 hours (violet columns) or 54 hours (blue columns) of fasting. The results are shown in a logarithmic scale where the value

of one is equal to unchanged expression compared to fed control animals. Values greater than one indicate up-regulation and levels less than one represent down-regulation during fasting conditions. The expression of genes reported previously to be affected by fasting (Van Gilst et al., 2005b), *acs-2* (increased), *fat-7* (decreased) and *nhr-49* (unchanged) were included as additional controls for these experiments.

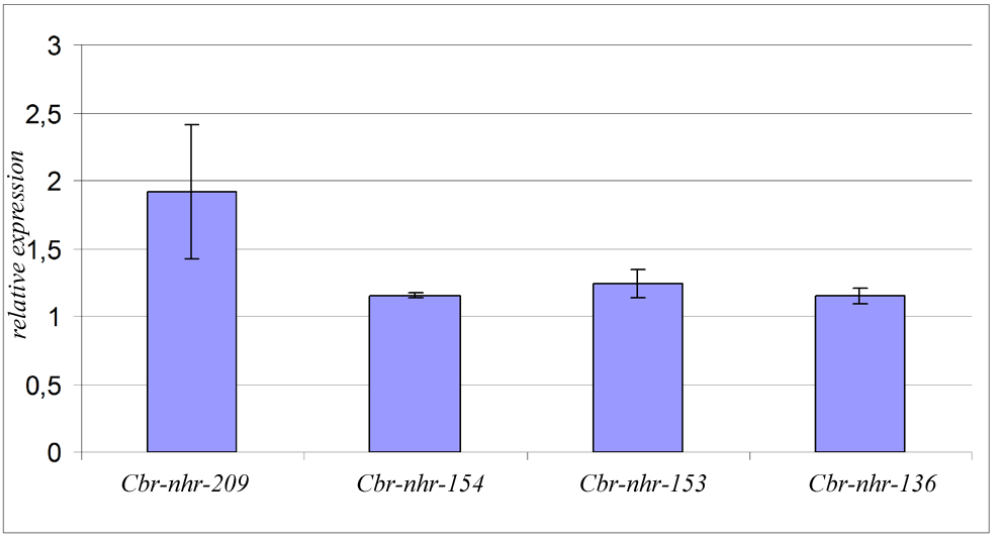

Figure 26. The fasting experiment of *C.briggsae*

The picture shows the expression of *C.briggsae* homologues *Cbr-nhr-209*, *Cbr-nhr-154*, *Cbr-nhr-153* and *Cbr-nhr-136.* The expression was analyzed by RT-qPCR in animals after 6 hours of fasting. Only *Cbr-nhr-209* showed an increase of expression (2-fold) during fasting.

Although we did not perform fully calibrated experiments that would take in account the possible differences in the efficiency of reverse transcription of individual genes and our results were normalized against *ama-1*, the results showed consistently higher reading values for the expression of genes conserved between *C.elegans* and *C.briggsae*. The values of conserved genes were 5 to 10 times higher than the values obtained in the cases of more recently duplicated genes.

5.6. Densitometric analyses

Since the up-regulation of gene expression of certain clustered *nhrs* detected by RT-qPCR was substantial, we wanted to see if the effect of starvation may be detected also by gfp fusion transgenes. The elevated expression of GFP fusion trasgenes was clearly visible in

intestinal cells in case of *nhr-206*, *nhr-208* and *nhr-207* (Fig. 20,21,22). To confirm this, we performed densitometric analyses of sets of pictures taken randomly at constant setting. The densitometric analyses confirmed the elevated expression in *gfp* fusion genes in intestinal cells of transgenic animals expressing *nhr-206*, *nhr-208* and *nhr-207* and in pharynx of animals expressing gfp fusion genes of *nhr-153* and *nhr-154* (Fig. 27).

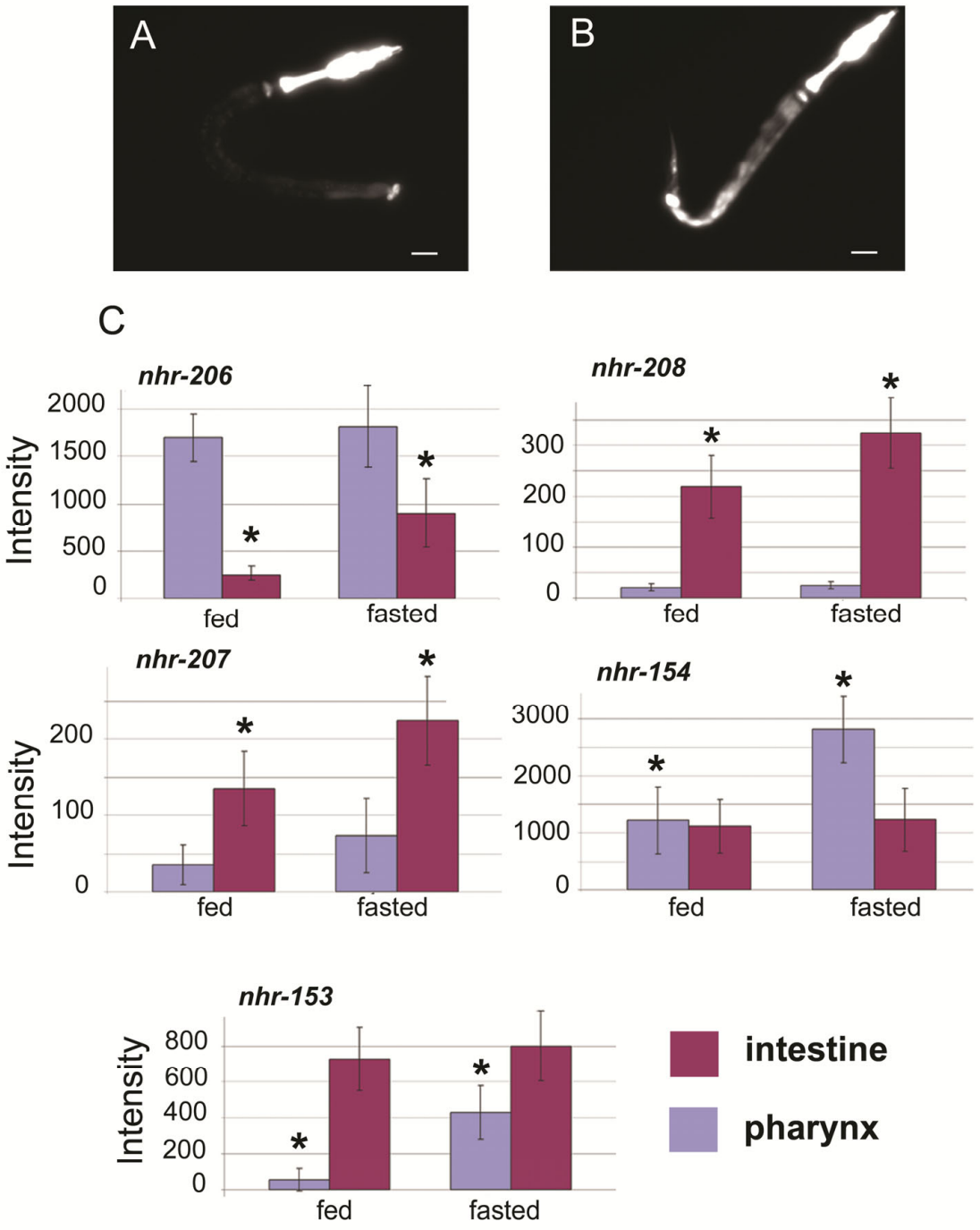

Figure 27. Responses of GFP reporter genes to fasting and feeding conditions.

The picture shows changes in reporter gene expression in response to feeding (A) or fasting (B) and could be easily visualized like in this representative image for *nhr-206::gfp*. The change in GFP expression in either the pharynx or intestine was quantified by densitometric analysis during fed or fasting conditions for the reporters *nhr-206::gfp*, *nhr-208::gfp*, *nhr-207::gfp, nhr-154::gfp, nhr-153::gfp*. Calculations were based on capturing the anterior area of animals coving the pharynx (gray columns) or the whole body and intestine, excluding the pharynx (dark columns); note that the intensity scale was adjusted for each reporter gene. The data revealed that *nhr-206*, *nhr-208,* and *nhr-207* reporters each showed significant (2- to 4-fold), intestinal-specific up-regulation of expression in response to fasting. In contrast, *nhr-154* and *nhr-153* reporters showed significant (2- to 8-fold), pharyngeal-specific increases in expression in response to fasting. Standard deviations are indicated in each column. An asterisk (*) indicates the probability of the result in the Student T test at $P<0.05$ compared with null hypothesis. Scale: 20 μm. The picture shows densitometry changes during the starving experiment. Two areas (pharyng and gut) were compared by the densitometry method. Panel A: *nhr-206*::GFP; B: *nhr-208*::GFP; C: *nhr-207*::GFP; D: *nhr-154*::GFP; E: *nhr-153*::GFP

5.7. Study of function

The function of all studied nuclear hormon receptors in *C.elegans* was studied by several approaches. We blocked the natural function of the gene by RNA interference. We prepared gene specific dsRNA in vitro and introduced it into the animals and then processed into 21-24 bp long RNA duplexes (small interfering RNAs – siRNAs) by the RNaseIII enzyme Dicer. After unwinding, siRNA single strands were incorporated into the multi-subunit RNA-induced silencing complex (RISC) and lead the complex to cleave naturally produced RNA with a sequence identical to the siRNAs (Fire et al., 1991; Timmons and Fire, 1995, Voinnet, 2005). dsRNA was introduced into worms either by soaking, feeding or by microinjecting. To increase RNAi effect in this study cluster (R07B7 and C13C4.3) we blocked one, two, three and in the R07B7 cluster all four genes.

5.7.1. RNA mediated interference

PCR products (a part of cDNAs located in the ligand binding domain) were cloned to pCR4 and or pCRII vectors (Invitrogen), and sequenced. Primers´ number and their sequences

which amplify for this purpose are shown in table 6. dsRNA was prepared by in vitro transcription using bacterial polymerases T7 and T3 or SP6 from linearized plasmids (Promega, Madison, WI). The resulting ssRNAs were controlled by agarose electrophoresis for the quality, annealed with the corresponding opposite ssRNA and purified by phenol/chloroform extraction and ethanol precipitation. Purified dsRNA was used for RNAi using microinjections to the ovarial syncytium of young adult N2 worms at a concentration of 2 mg/ml. The microinjection system from Narishige (Tritech Research, Inc., Los Angeles) coupled with the Olympus IX 70 (Olympus America Inc., Center Halley) inverted microscope was used.

gene	sequence forward	sequence reverse	forward primer	reverse primer
nhr-206	attcgagcagaaatžttcatcaggcg	gatcagcaccgtgaatctgtccatg	4957	4958
nhr-208	ccaagagcaattagagctgaaatc	cagaagacattgagcaaatctgttc	4961	4962
nhr-207	atagctatcgacgatgaattcagc	tcgtcttccagaatttgctgtgctg	4959	4960
nhr-209	ctgcaattcgtgcatcagtgaaaac	caagtagagcttttgcatattgg	4963	4964
nhr-154	gtggaaagtaaggttgaacc	ggagccattgcgagaatagttgcg	5222	5224
nhr-153	catgataatggaatgccaatgg	gacaatatcggaacaaacactgagcg	5227	5229
nhr-136	caggaaattggacgagttgattg	gctaattcgtgtgtatctaactgc	5232	5234

Table 6. RNAi primers

The table shows names of genes, their sequences and laboratory numbers.

Double stranded RNA was also used for induction of RNA interference by the soaking method (at concentration 2 mg/ml). Hermaphrodites were soaked in dsRNA and transferred every12 hours to new plates for a total of a 4 day period. The embryonic lethality and changes of larval phenotype were recorded at least twice a day on individual plates kept at 22ºC.

To check a possible non-specific effect of prepared dsRNA a control dsRNA, which was prepared from the (non-coding) promoter region of *nhr-60* was used (Simeckova, 2007 #21).

For feeding, the same constructs were transformed into bacteria E. coli HT 115. After the induction, bacteria produced gene specific dsRNA. Worms were grown on these bacteria.

All methods (soaking, feeding, microinjecting) have had very similar results. We did not observed any morphological phenotypes as a result of RNAi but a marginal increase of embryonic lethality was found when more genes were inhibited simultaneously (Table 7).

RNAi results								
A. RNAi of individual genes made by soaking								
nhr gene	*206*	*208*	*207*	*209*	*154*	*153*	*136*	control water
Soaked Hermaphrodites	11	12	5	4	6	18	14	19
Progeny Scored	1227	1275	571	587	1467	2485	2027	2418
Embryonic Arrest	0	34 (3%)	0	0	20 (1.4%)	36 (1.4%)	10 (0.5%)	20 (0.8%)

B. RNAi of individual genes made by microinjection								
nhr gene	*206*	*208*	*207*	*209*	*154*	*153*	*136*	Control P*nhr-60*
Injected Hermaphrodites	9	21	18	14	6	9	10	18
Progeny Scored	1252	4536	2993	3175	1467	921	1370	2815
Embryonic Arrest	39 (3%)	37 (0.8%)	77 (2.6%)	119 (3.8%)	20 (1.3%)	9 (1%)	40 (2.9%)	26 (0.9%)

C. RNAi of combinations of two genes by microinjections					
Combinations of *nhr* genes	*206 + 208*	*207 + 209*	*154 + 153*	*154 + 136*	*153 + 136*
Injected Hermaphrodites	ND	ND	9	7	10
Progeny Scored	ND	ND	844	579	1481
Embryonic Arrest	ND	ND	14 (1.6%)	12 (2%)	18 (1.2%)

D. RNAi of combinations of three, four and seven genes by microinjections				
Combinations of *nhr* genes	*154+153+ 136*	*206+208+207+ 209*	*209+154+153+ 136*	*206+208+207+ 209+154+153+136*
Injected Hermaphrodites	8	25	32	31
Progeny Scored	662	4664	4910	5148
Embryonic Arrest	32 (4.8%)	75 (1.6%)	90 (1.8%)	129 (2.5%)

Table 7. RNAi results

The table shows summarized results of RNA-mediated inhibition (RNAi) of individual genes by soaking in solutions containing double stranded RNA (Part A), microinjection of dsRNA (Part B), by microinjection of combinations of dsRNA for two selected genes (Part C), or by microinjection of combinations of dsRNA for three, four and all seven genes (Part D). No developmental defects were observed in larvae and adult animals, but slight increase (approximately 5%) in embryonic lethality was observed when a combination of dsRNA for three conserved genes was used for microinjections.

6. Discussion

The presented thesis focused on functional study aimed at elucidation of biological role of seven nuclear hormone receptors that are found in *C. elegans* genome on chromosome V in the region 12,092,022-12,109,114; WS207. The genomes of Rhabditidae species that were sequenced to date contain unexpectedly large number of sequences that are recognized by sequence comparison as potential NHRs. The genome of *C. elegans* contains 284 potential NHRs and the genome of *C. briggsae* and *C. romanei* contain 232 respectively 256 potential NHRs (Anteby 2006; Haerty 2008).

This large number of NHRs in nematode species contrasts to only 18 NHRs in the genome of *Drosophila* and mammals have 48 to 50 such genes. The gene cluster that was chosen for this study has several interesting features. Although several clusters of candidate NHRs can be found on the chromosome V, the cluster V: 12,092,022-12,109,114; WS207 seems to be unusually condensed, contains genes with the same orientation and bears characteristics of a recent origin by gene duplication. In this work we hoped that the expressional and functional analysis of this cluster may yield data that may be related to biological role of recently duplicated NHRs and shed some light on the evolutionary mechanisms and selection pressure that is connected to the process of enlargement of NHR family of genes in Rhabditidae.

The data found in this study indicated, that the first three receptors in the locus are likely to be the most recently duplicated and are not conserved in the closest nematode *C. briggsae*. There is of course also a possibility, that the genes in *C. briggsae* were lost during the separate evolution. Although this seems to be unlikely, large deep-in sequencing employing the new sequencing methods that are powered to provide a more complete and detailed whole genome sequencing of a bigger number of Rhabditidae species may answer to this question.

In our work, we found that the closest homologue of *C. briggsae* genes located on the corresponding chromosomal location in *C. elegans* genome is NHR-136. The other four receptors have orthologs in *C. briggsae*. These receptors, which are present in *C. elegans* and in *C. briggsae* (NHR-209, NHR-154, NHR-153, and NHR-136), despite a pronounced diversification of their coding sequence have almost identical sizes of exons. This supports the conservation of the overall structure of these receptors and their functionality as classical NHRs.

We have found that each gene in the *C. elegans* cluster is expressed in one isoform. The same result was found in case of conserved members of the studied gene cluster. This contrasts with many *C. elegans* NHRs and with vertebrate NHRs (the data can be retrieved from www.receptors.org/NR for all NHRs and www.wormbase.org for *C.elegans* and is in agreement with the possibility that the recently duplicated receptors are compared to the later named receptors, which are functionally more restricted and specialized.

The tight organization of studied genes in the cluster suggested that several receptors could be organized in operons (Blumental 2002, 2005). Contrary to the prediction, only two studied genes were found to be organized in the operon. The intergenic region separating *nhr-207* and *nhr-209* is quite small (270 bp) to contain a functional promoter if the sequence that is already in the coding region of *nhr-207* or some more distant sequence is not included in the formation of the functional promoter for *nhr-209* . While we have found the SL1 in front of *nhr-154* (the same result is stated by WormBase), we found the SL2 in front of *nhr-209*. This proves that *nhr-207* and *nhr-209* are transcribed from same mRNA and indicates that they have, at least for the operon base expression the same expression pattern.

The data presented in this thesis characterized the stage specific as well as the cell and tissue specific expression pattern for all genes of the studied cluster. We have found that the expression level of all *C. elegans* genes (*nhr-206, nhr-208, nhr-207, nhr-209, nhr-154, nhr-153* and *nhr-136*) starts in embryogenesis and is present in all larval stages to adulthood. The exception is *nhr-154*, which expression is high in embryos and early larval stages and later decreases. Interestingly the expression in conserved genes in *C. elegans* and *C. briggsae* (*Cbr-nhr-209, Cbr-nhr-154, Cbr-nhr-153* and *Cbr-nhr-136*) is app. 10x higher at standard laboratory conditions in comparison with the expression of genes that arouse as new members of the family in the *C. elegans* genome.

The expression of *nhr-206* is similar to that described by Reece-Hoyes and coworkers in their high throughput screen (Reece-Hoyes et al., 2007). For *nhr-208* we found similar expression profile, although our reporters did not result in expression in the excretory cell and vulva and *nhr-207* our results overlap those reported by Reece-Hoyes and coworkers (Reece-Hoyes et al., 2007). From conserved genes, we prepared transgenic lines only for two genes *nhr-154* and *nhr-153*. Their expression is mainly in intestine, but varies during the development (the expression of *nhr-154*::GFP lines decreases from the middle larval stages to adulthood and we confirmed the expression found by RT Q PCR). We were not successful in preparation of transgenic lines for *nhr-209* and *nhr-136*. In the case of *nhr-209*, the localization of this receptor in an operon with upstream gene *nhr-207* suggests that the

expression could be the same as it is in case of *nhr-207*::GFP. The transgenes that contained the intergenic region preceding the ATG of *nhr-209* did not yield any GFP expression. Similarly, transgenes that contained in addition to the intergenic sequence also large part of the preceding *nhr-207* did not show expression of GFP as they do not contain functional promoter. This further supports the expression of *nhr-209* from the operon with *nhr-207*.

The second gene for which we were not able to prepare the GFP expressing transgenic lines was *nhr-136*. This gene is localized in the end of studied locus and in front of ATG has region which could be sufficient to function as promoter for about 2000bp. We prepared many transgenic lines, which contained the experimental transgenes, but we did not see the GFP expression. By PCR from single worms (rollers) we confirmed that not expressing transgenic lines carry the promoter region of *nhr-136*. This indicates that other distant factors and chromosomal arrangement are important for expression of this gene. This also suggests that chromosomal arrangement is at least partially important for functionality of multiplied *nhr* genes in Rhabditidae.

To study the function of clustered genes, we decided to inhibit them by RNA interference (Timmons,L 1998, 2003). We prepared double stranded RNA for all seven genes and attempted to inhibit the gene function of individual genes. Since we did not see any observable phenotype or changes of development, we also inhibited combinations of clustered genes simultaneously in order to see if there is a functional redundancy of clustered genes. The combinations of two, three, four and all seven genes did not show visible phenotypical changes. We found only slightly higher embryonic lethality in case of some combinations of inhibited genes (Table 7).

In our previous work on NHR-40, we found that the penetrance of its developmental phenotype was affected by feeding status and environmental conditions such as feeding and temperature (Brozova 2006, Pohludka, 2008). Since the clustered *nhrs* belong to the same subgroup of NHRs, we searched if their expression and loss of function are affected by feeding status.

As a metabolic stress, we used fasting implemented in two separate protocols. The first protocol consisted of short fasting which was done after synchronization of two paired cultures overnight and fasting was done for 6 hours while the control group of worms was on plate with food (Gilst 2005). Long fasting was done by keeping the synchronized worms 2 days (48 hours) without food and than one culture was subjected to fasting for the next 6 hours while the control group of the worms was kept on plates with food during the same period of time.

The expression of the conserved genes during fasting experiment was 5 to 10 times lower than the values obtained in case of more recently duplicated genes.

Interestingly, fasting lead to recording of values of the more recently duplicated genes that were reaching levels observed for conserved genes both during fasting and feeding. Although it can not be ruled out that some metabolites that are elevated during fasting, such as metabolites of beta-oxidation may cause activation of gene expression of fasting-up-regulated genes, it seems more likely that the more recently duplicated genes are repressed at feeding status and a de-repression is bringing the expression of fasting-responsive genes to the level seen for conserved genes.

Moreover we found that the expression of genes at fasting changed their expressional patterns. All three recently duplicated genes (*nhr-206, nhr-208, nhr-207*) which were strongly expressed in pharynx and only slightly expressed in intestine after fasting had much higher expression in intestine while the expression in pharynx was not changed. The expression of *nhr-154* and *nhr-153* genes from conserved group had slightly increased expression after fasting, their expression in intestine did not change, but the expression in pharynx was increased. The expression of *nhr-209* and *nhr-136* did not change. This agreement between the data obtained by RT Q PCR and expression in transgenes supports the validity of the data obtained by isolated genes expressed as transgenes and indicates that the diversified promoters constitute the target of the functional evolutional pressure. The second support of this concept is the fact, that the multiplied genes show overlapping (although partially diversified) expression patterns despite the sequence dissimilarity which did not allow identifying regulatory elements by pattern recognition computer programs.

Together the data presented in this thesis support the concept that the multiplied nhrs in Rhabditidae are functional genes that have specific regulatory functions. Our data support the concept that NHRs in Rhabditidae constitute a very dynamic end evolutionally flexible gene family, where individual members may accept new functions, most likely in tuning and orchestrating the metabolic and developmental needs.

7. Conclusions

The goal of this study was to determine if the members of the superfamily of nuclear receptors that are predicted based on sequence homology in the *C. elegans* genome are functional genes and if so, to characterize their functions.

For this study, we selected a gene cluster consisting of seven genes arranged as tandem repeats.

Since the tandem arrangement of genes indicated recent and successive gene duplications, we hypothesized that the expressional and functional data may shed light at the biological mechanisms that project to the multiplication of this gene family.

The presented work:

proved that the selected recently duplicated nuclear receptors are functional genes

characterized the pattern of their tissue specific expression

established that receptors acquired species specific metabolism dependent expression regulation

The data presented in this thesis contributed to understanding of the biological role of selected members of the multiplied superfamily of nuclear receptors in Rhabditidae by narrowing their function towards involvement of this genes in metabolism.

Our data indicate that the duplicated members of NHR family acquire new species specific functions.

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9 Appendix

9.1 The list of figures

9.2 The list of tables

9.3 The list of author's publications and presentations

Impact factor: 2,076

Vohanka J, Simecková K, Machalová E, Behenský F, Krause MW, Kostrouch Z, Kostrouchová M.

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M. Pohludka, **J.Vohanka**, M. Kostrouchova, Z. Kostrouch: Analysis of the proteome phenotype of *nhr-23* RNAi by two dimensional chromatography. Poster Presentaion. 16th International *C. elegans* Meeting, Los Angeles, USA, June 27 - July 1, 2007.

E. Brozova, M. Pohludka, **J. Vohanka**, P. Yilma, M. Kostrouchova, Z. Kostrouch Characterization of *C. elegans* mutant L1 larvae (*nhr-40*, RB840) on the proteome level by comparative two dimensional chromatography: chromatographic focusing followed in line

by reversed phase chromatography and mass spectroscopy. Poster Presentaion. 16th International *C. elegans* Meeting, Los Angeles, USA, June 27 - July 1, 2007.

K. Simeckova, E. Brozova, **J. Vohanka**, M. Pohludka, Z. Kostrouch, M. Kostrouchova NHR-60 regulates development of seam cells downstream of NHR-23 (CHR3). Poster Presentaion. 16th International *C. elegans* Meeting, Los Angeles, USA, June 27 - July 1, 2007.

D. Kostrouch, P. Liby, M. Kostrouchova, M. Pohludka, **J. Vohanka**, E. Brozova, M. Kostrouchova, Z. Kostrouch: Analysis of BIR-1 function using comparative DNA microarrays and 2D chromatography. Poster Presentation. 16th International *C. elegans* Meeting, Los Angeles, USA, June 27 - July 1, 2007.

J. Sikora, M. Pohludka, L. Dvořáková, Z. Vernerová, P. Přikryl, **J. Vohánka**, P. Novák, L. Stolnaja, I. Rychlík, J. Vlasák, Z. Kostrouch and M. Elleder: Is there a primary (hereditary) SAA amyloidosis? Clinical, histopathologic, proteomic and genetic study in a family with amyloidosis. Oral presentation. 3. sympózium & workshop Molekulární patologie: proteomika, genomika, Olomouc, Czech Republic, May 04 – May 05, 2007.

M. Pohludka, **J. Vohánka**, P. Novák, M. Kostrouchová, P. Yilma, P. Libý, Z. Kostrouch: Použití microarrays a komparativní dvoudimensionální chromatografie pro analýzu exprese mRNA a proteinů v prvním larválním stádiu *Caenorhabditis elegans* v závislosti na potlačení exprese CHR-3 (*nhr-23*). Oral presentation. Proteo 2006, Liběchov, Czech Republic, Sep 26 – Sep 27, 2006.

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K. Simeckova, E. Brozova, **J. Vohanka**, M. Pohludka, Z. Kostrouch, M. Kostrouchova.: NHR-60, a *Caenorhabditis elegans* Supplementary Nuclear Receptor residing at the Nuclear Periphery, Regulates Embryonic Development in Connection with Acyl-Coenzyme a Binding Protein. Poster presentation. European Worm Meeting, Hersonissos, Greece, April 29 – May 3, 2006.

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