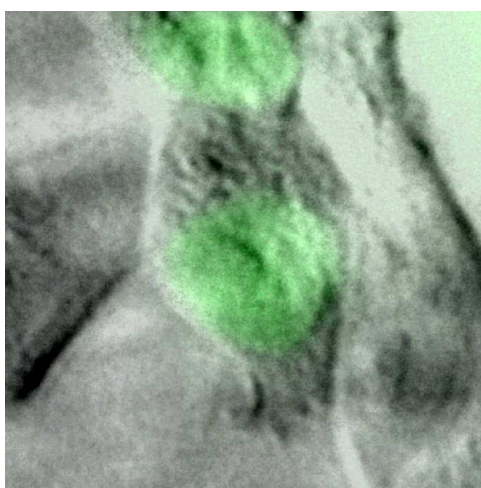




FACULTY OF SCIENCE
University of South Bohemia
České Budějovice

Master Thesis

**Attempts on chromatin immunoprecipitation
with *C. elegans* nuclear receptor NHR-25**



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Annotation

The aim of the work presented in this thesis was to establish chromatin immunoprecipitation method in our laboratory as a tool to study target genes of the nuclear receptor NHR-25 in *C. elegans*. Once the method is established, it will be also useful for studies of other DNA binding proteins. ChIP was performed in transiently transfected cells HEK293 and analyzed using PCR and qPCR. Although ChIP is typically used to find authentic target genes in the cell or in organisms, testing protein-DNA interactions by ChIP in transient transfection system (by transfecting both the expression vector of the protein of interest and a vector containing potential binding sequence/promoter of the protein) can be useful as it serves as a relatively quick tool to confirm the direct binding. Since the detection is by PCR, this method is sensitive yet less costly non radioactive method to analyze protein-DNA interaction. For the first step towards ChIP in *C. elegans*; pulling down tagged protein directly from the worm was also performed as a preparation for *in vivo* analysis of NHR-25 regulated genes.

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Prohlašuji, že jsem předloženou magisterskou práci vypracoval samostatně, pouze s použitím uvedené literatury.

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1. Introduction

1.1 *Caenorhabditis elegans* – tiny nematode with huge impact

Free living soil nematode, *Caenorhabditis elegans* (worm), is one of the most important model organisms. It shares characteristics with higher animals, while keeping simplicity to be easily studied and maintained in the laboratory. Worm developmental cycle, which lasts approximately 3 days in 20°C, consists of 4 larval stages (L1 to L4). Body of adult worm has only one millimeter in size and it consists of constant number of somatic cells – 959 for hermaphrodite and 1031 for male. Self-fertilizing hermaphrodites form vast majority of population, while males are rare in wild type population. Single worm produces about 300 eggs during its life cycle. In laboratory conditions, worms are cultured on agar or agarose plates with *Escherichia coli* as a food. Liquid cultures are also possible and are used for big scale production of worms. Under stress conditions, like low temperature or starving, developmental cycle is switched to alternative stage – dauer larva, which allows long term storage of worms in 15°C. Another option for storage of worm strains is freezing of L1 worms, which represents exceptional capability among metazoans.

C. elegans was introduced to research field in 1974 by Sydney Brenner, who established handling techniques and major genetic analyses in the worm (Brenner, 1974). Transparent worm body allowed John Sulston to trace entire cell lineage. Robert Horvitz identified cells that undergo apoptosis during *C. elegans* development (Sulston and Horvitz, 1977; Sulston et al., 1983). These three scientists, who established *C. elegans* as a model organism for modern research, were awarded Nobel Prize in Physiology or Medicine in 2002.

Another milestone was 1998, when *C. elegans* become first multicellular organism with whole genome sequenced (The *C. elegans* Sequencing Consortium, 1998). *C. elegans* research gained another two achievements. Andrew Fire and Craig Mello were awarded with Nobel Prize in Physiology or Medicine in 2006 for describing the mechanism of RNA interference in worms (Fire et al., 1998). And the third Nobel Prize in Chemistry in 2008 share Osamu Shimomura, Martin Chalfie and Roger Y. Tsien for the discovery and characterization of jellyfish *Aequorea victoria* green fluorescent protein (GFP), its introduction to *C. elegans* as a marker for gene expression and its improvement and color modifications (Shinomura et al., 1962; Chalfie et al., 1994; Heim et al., 1995; Shaner et al., 2005).

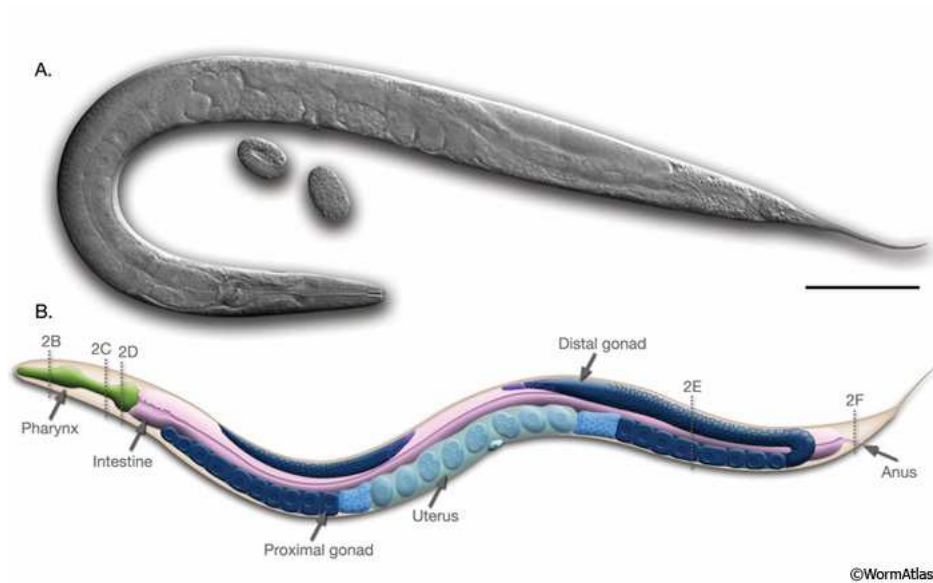


Fig. 1.1. *C. elegans* anatomy (DIC photo of living worm and illustration). Transparent worm body allows organs and even single cells observations offering unique tool for research (Altun & Hall, 2005).

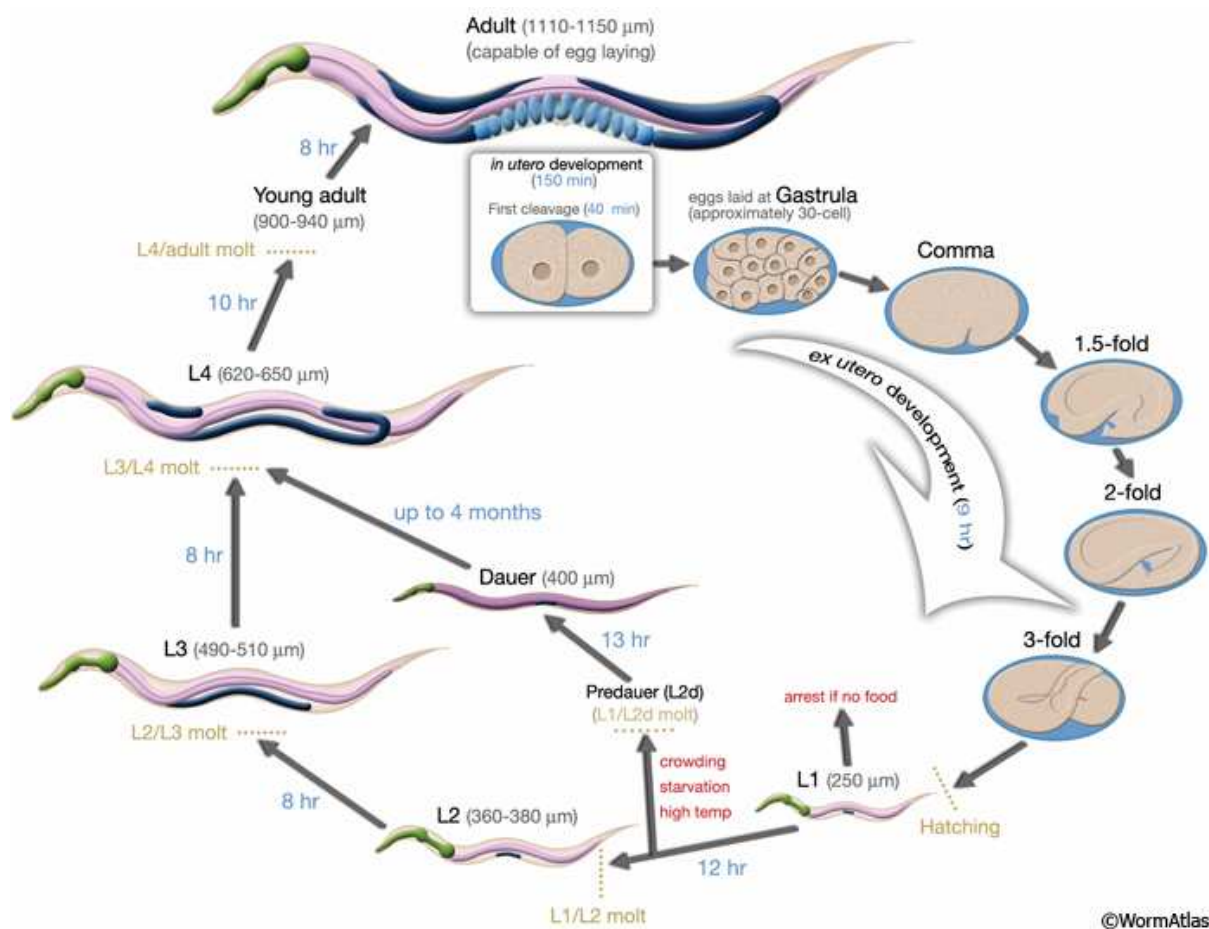


Fig. 1.2. *C. elegans* life cycle at 22°C. L1s hatch after approximately 14 hours of embryonic development and continue with following larval stages or enter dauer larva if under stress conditions. In optimal conditions adults start to lay eggs after approximately 3 days of development (Altun & Hall, 2005).

1.2 Nuclear Receptors

Nuclear receptors (NRs) are transcription factors occurring in metazoans. They are often regulated by small lipophilic molecules, such as steroids, retinoids, bile and fatty acids. Part of the NRs, called orphan receptors, has either no ligand or the ligand is unknown. These receptors play crucial role in physiology, development and reproduction.

Molecular structure of NRs is conserved and contains N-terminal DNA binding domain (DBD) consisting of two Cys4 zinc fingers. C-terminal ligand binding domain (LBD) serves for potential ligand binding and docks coactivators and corepressors, which couples NR to histone acetyltransferase and deacetylase complexes, respectively. Receptor homo- or heterodimerization is also mediated by LBD. Binding of ligand is followed by structural change in C-terminal trans-activation helix (AF-2) which interacts with LBD core. When corepressors are later replaced by coactivators, gene expression is triggered.

C. elegans genome contains genes encoding 284 NRs, compared to human (48) and fly (21), this family undergone great expansion in worms. Only about 20 of almost tree hundred of NRs have been genetically analyzed and 15 of them represent conserved homologues to other metazoans. These NRs regulate sex determination, molt cycle, dauer formation, xenobiotic response, metabolic control and neural development (reviewed in Antebi, 2006).

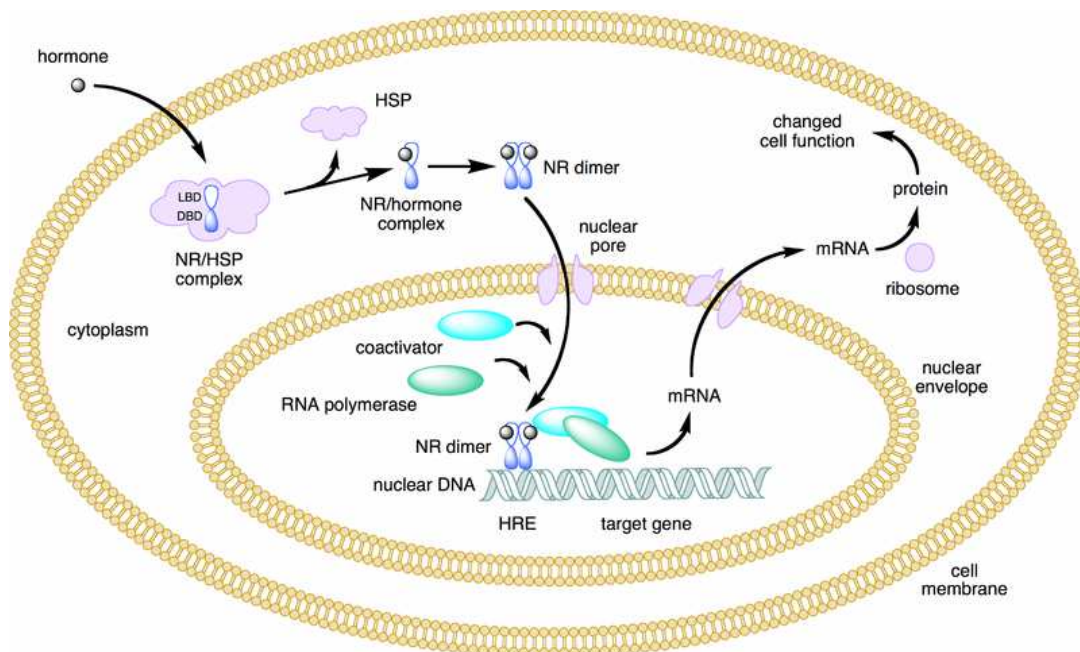


Fig. 1.3. Example of nuclear receptor regulatory mechanism. After binding of ligand NR is released from complex with heat shock proteins (HSP), dimerizes and is translocated to the nucleus. NR interacts with its coactivator, binds to the hormone response element (HRE) and triggers the transcription. (Wikipedia, 2008; http://en.wikipedia.org/wiki/File:Type_ii_nuclear_receptor_action.png)

1.3 NHR-25

Evolutionary conserved orphan nuclear receptor NHR-25 is one of the 20 genetically analyzed receptors in *C. elegans*. NHR-25 shares additional motif in DBD – so-called Ftz.F1 box with nuclear receptor subfamily 5 group A (NR5R). NHR-25 is ortholog of mammalian Steroidogenic factor 1 (SF-1) and Liver receptor homologue 1 (LRH-1) and *Drosophila* Fushi tarazu factor 1 (FTZ-F1). FTZ-F1 has two isoforms with different functions. α FTZ-F1 regulates embryonic segmentation via Hox gene fushi tarazu (*ftz*) and β FTZ-F1 plays role in molting and larval metamorphosis (Ueda et al., 1990; Guichet et al., 1997; Yu et al., 1997). Mammalian LHR regulates cholesterol homeostasis and bile acid metabolism (Fayard et al., 2004) and SF-1 controls differentiation of the gonad, as well as the adrenal gland, pituitary and hypothalamus, where it regulates steroidogenesis (Parker et al., 2002).

NHR-25 plays important role in the embryogenesis, molting and development of *C. elegans*. Null mutation in NHR-25 causes embryonic arrest and lethality. Hypomorph NHR-25 mutant or RNAi result in later developmental arrest and/or molting defect, sterility, egg-laying defect and failure in seam cells fusion (Asahina et al., 2000; Gissendanner & Sluder, 2000; Chen et al., 2004; Silhankova et al., 2005). NHR-25 cooperates with Wnt/ β -catenin pathway in regulation of T seam cell division and morphogenesis of the male sensory rays and loss of NHR-25 causes extra seam cells in adults, disrupted male tail morphology and defective T-seam cell polarity (Hajduskova et al., 2009). Interaction of NHR-25 with two β -catenins WRM-1 (suppressor) and SYS-1 (activator) affects the process of cell fate decision in the somatic gonad. Using luciferase reporter system and mammalian cell cultures interaction of NHR-25 with Ftz-F1/SF-1 consensus binding sites was demonstrated. NHR-25 activates promoter containing 2x sf-1 binding consensus site (2xTCA), while binding to mutated version of this consensus 2x sf-1* (2xTCT) was silent (Asahina et al., 2006).

1.4 Cyclin E

C. elegans cyclin E homolog (CYE-1) does not play typical role in the cell cycle G1 to S phase transition, but instead is required for embryogenesis and formation of vulva and gonad and regulates asymmetric division of somatic gonad precursor cells (Fay & Han, 2000; Brodigan et al., 2003; Fujita et al., 2007). Mutation in *cye-1* results in impaired cell fate decision during formation of vulva, phenotype similar to loss of function of NHR-25. In mammals cyclin E interacts with NHR-25 ortholog – LRH-1 (Botrugno et al., 2004). Two potential NHR-25 binding sites were found in *cye-1* promoter region. NHR-25 seems to activate wild-type *cye-1* promoter in luciferase reporter system in mammalian cell cultures,

whereas NHR-25 did not activate when one of the sites or both were mutated (Asahina, Korinek, Rynes, unpublished data).

1.5 Chromatin Immunoprecipitation

Since genomes of chosen model organisms are fully sequenced, research was focused on genome functional properties and the characterization of regulatory elements involved in controlling gene expression, gene function and genome stability. The regulation of genome functions is mostly mediated through strictly controlled, dynamic and transient protein-chromatin interactions with crucial role in physiology, homeostasis and development. Many diseases and cancer are caused by malfunction of DNA-binding proteins such as transcription factors, histone modifying enzymes or DNA repair proteins (reviewed in Wong & Wei, 2009). One of the techniques which serve for investigation of protein-DNA interactions that occur inside the nucleus of living cells is chromatin immunoprecipitation (ChIP). Because of its importance number of ChIP-performing laboratories has been rapidly growing past years.

In 1978 formaldehyde crosslinking was first used to demonstrate histone–DNA and histone–histone interactions in isolated nuclei (Jackson, 1978). Immunoprecipitation of protein-DNA complexes after crosslinking with formaldehyde or UV light and chromatin shearing was introduced several years later (Gilmour & Lis, 1984; Solomon et al., 1988). Further improvement was ensured by development of selective antibodies and introduction of polymerase chain reaction (PCR). Linking ChIP and microarray technique in ChIP-chip (also ChIP on chip) method represented expansion of analysis to genome-wide context (Ren et al., 2000; Iyer et al., 2001).

ChIP was originally established for culture of *Drosophila* cells (Solomon et al., 1988) and other cell cultures were used later including mammalian lines. ChIP procedure is also possible for mammalian cell cultures transiently transfected with plasmids containing studied binding sites and/or gene encoding protein of interest (Luo et al., 1998; Wells & Farnham, 2002; Belyaev et al., 2004; Kobayashi et al., 2006). For *C. elegans* ChIP was performed with embryos (Ercan et al., 2007) and also with larvae or adult worms (Mukhopadhyay et al., 2008).

1.6 Detailed ChIP procedure

During ChIP procedure, transient protein-DNA interactions in living cells are captured using crosslinking agents. Isolated chromatin is then sheared and the fragmented DNA-protein complexes are selectively enriched by immunoprecipitation with specific antibodies. After reversal of the crosslinks, the enriched DNA is further analyzed.

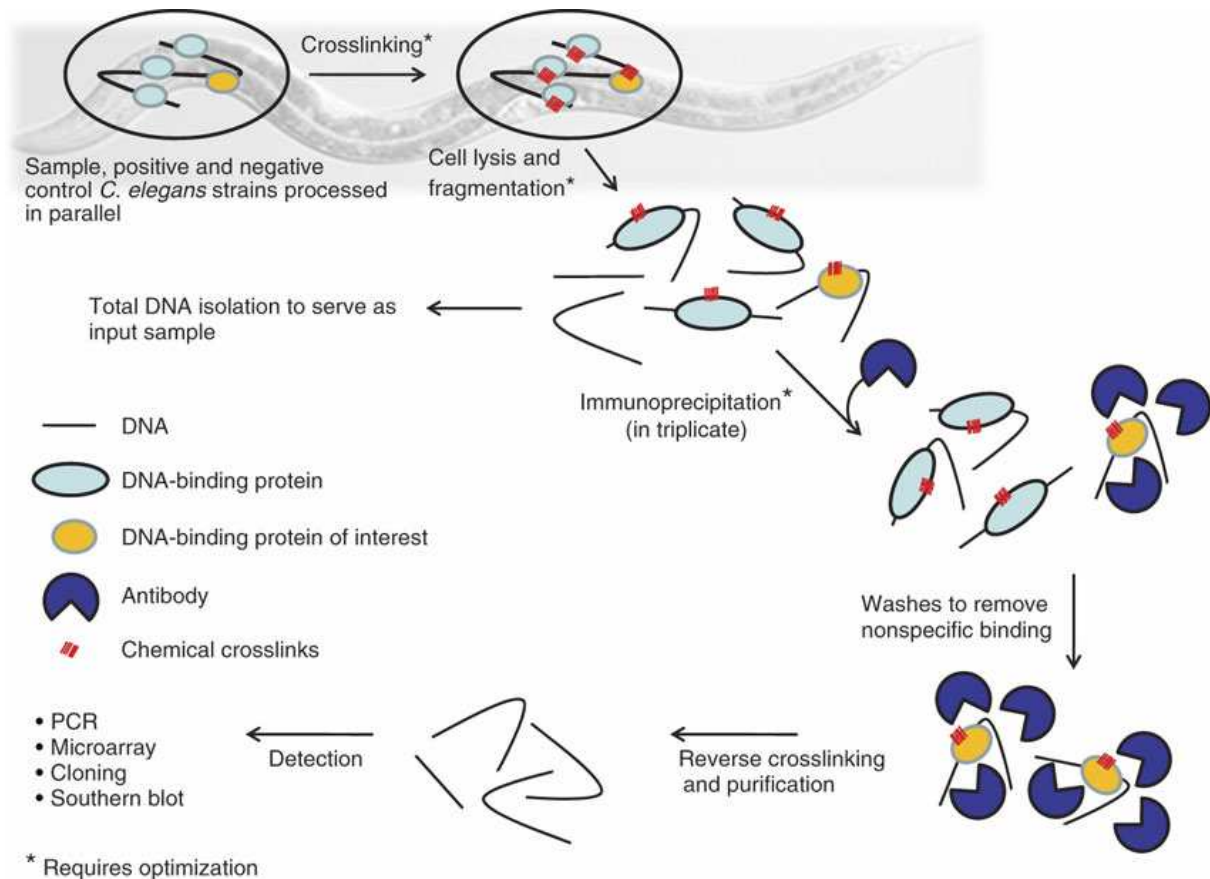


Fig. 1.4. Schematic diagram of chromatin immunoprecipitation procedure. ChIP starts with crosslinking protein-DNA interactions, after chromatin shearing samples are precipitated with highly specific antibody. Then crosslinks are reversed and DNA is investigated with different methods i.e. PCR, microarray hybridization (Mukhopadhyay et al., 2008).

Crosslinking: To preserve chromatin structure during isolation and ChIP procedure, crosslinking (usually with formaldehyde) is necessary (Solomon & Varshavsky, 1985; Solomon et al., 1988). Optimization of this method is important as over-crosslinking will restrict the ChIP procedure and insufficient crosslinking will not conserve the chromatin structure. Method and time of crosslinking highly depend on material used for ChIP experiment.

Shearing of chromatin: According to chromatin preparation and shearing ChIP can be divided to X-ChIP (shearing of crosslinked chromatin by sonication) and N-ChIP (digestion of native chromatin by nucleases). Both have their pros and cons (O'Neill & Turner, 2003). Optimal conditions for chromatin shearing can be verified by electrophoresis as chromatin is mostly sheared into fragments ranging from 250 to 750 bp in the ideal case. For X-ChIP low power sonication with several pulses and effective cooling of the sample should not result in foaming or overheating the sample and thus loss of the material (Clarkson et al., 1999). Digestion of chromatin with nuclease can significantly reduce variability of samples introduced with uneven sonication.

Immunoprecipitation of crosslinked and sheared chromatin: Antibody (Ab) is the most important factor in whole ChIP procedure. Successful use of selected antibody in other methods (i.e. Western blotting) does not guarantee its function in ChIP. Polyclonal Ab can help with enrichment of low abundance protein as it recognizes more epitopes than monoclonal Ab. Antibodies can vary in sensitivity to crosslinks or adjacent modifications (Hanlon & Lieb, 2004). ChIP reaction with use of no antibody (NoAb) is important control for estimation of background.

Analysis of precipitated chromatin: Most accessible and common methods for analysis of precipitated chromatin are conventional PCR and quantitative PCR (qPCR, real time PCR). Advantage of qPCR is detection of amount of DNA during the amplification instead of measurement the signal at the end of the reaction in the conventional PCR. Diluted chromatin which was not immunoprecipitated is used as a control for PCR (Input). More sophisticated and genome-wide methods are analysis by sequencing (ChIP-seq) and microarray (ChIP-chip, Ren et al., 2000). ChIP-chip procedure is similar to ChIP, but instead of PCR analysis, immunoprecipitated chromatin is fluorescently labeled and hybridized to DNA microarray. Input chromatin is labeled with different fluorescent label and is also hybridized to the array. From overlapping of the signals chromatin enrichment is calculated and target sequences are localized in genome. Sophisticated bioinformatics methods and normalizations are crucial point in analysis and interpretation of the results obtained with this high-throughput method (reviewed in Kirmizis & Farnham, 2004; Taverner et al., 2004; Hudson & Snyder, 2006; Massie & Mills, 2008; Haring et al., 2007). Advantage of ChIP-seq is its wide-range use compared to competing ChIP-chip, which is restricted to organisms for which microarrays are available. Microarrays also often cover only portion of the genome of the studied model (reviewed in Hoffman & Jones, 2009).

Because *C. elegans* cell cultures are very complicated to obtain and performing ChIP directly on worms would be difficult, time consuming and expensive, we decided to test DNA binding capacity of NHR-25 in mammalian cell culture by transfecting Human embryonic kidney cells (HEK293) which were available in our laboratory. Activation of sf-1 binding consensus site by NHR-25 was previously demonstrated (Asahina et al. 2006) and it served as a positive control to establish and optimize ChIP in our laboratory. No innate target of NHR-25 was known. Because we had good α GFP antibodies, fusion protein EGFP::NHR-25 was used for the ChIP.

2. Goals of the work

1. To establish chromatin immunoprecipitation (ChIP) method using NHR-25 and NHR-25/SF-1 binding consensus sequence in transiently transfected mammalian cell culture.
2. To analyze ChIP samples with conventional PCR and qPCR.
3. To pull down MBF-1::GFP fusion protein from a transgenic worm strain as a preparation for ChIP in *C. elegans*.

3. Materials and methods

3.1 *E. coli*

3.1.1 Preparation of competent cells

(Inoue et al., 1990).

Transformation buffer (TB): 10 mM piperazine-N,N'-bis(2-ethanesulfonic acid) - PIPES
15 mM CaCl₂
250 mM KCl
dissolve and set pH = 6.7 with KOH; autoclave
55 mM MnCl₂ as filter-sterilized 1 M stock

SOB medium: 20 g Bacto-tryptone
5 g Bacto-yeast extract
5 g NaCl
2.5 ml 1M KCl
ddH₂O to 1000 ml

1. Inoculate single colony of DH5 α cells into 3 ml of SOB, grow overnight at 25 or 37°C.
2. Add 1 ml of the culture into 240 ml SOB, grow with vigorous shaking at room temperature (RT) but no more than 25°C until OD₆₀₀ = 0.6. This may take overnight.
3. Cool 10 min on ice.
4. Spin at 2500 x g for 10 min at 4°C, resuspend in 80 ml of cold TB on ice.
5. Incubate 10 min on ice. Spin as in step 4.
6. Resuspend in 20 ml of cold TB. Add 1.5 ml of DMSO (7% final conc.) Mix well.
7. Incubate 10 min on ice.
8. Keeping the cells on ice, divide by 200 μ l aliquots into sterile 1.5 ml tubes on ice.
9. Close tubes and freeze in liquid nitrogen. Store at -80°C.

3.1.2 *E. coli* transformation by heat shock

LB medium: 10 g Bacto-Tryptone
5 g Bacto-yeast extract
10 g NaCl
ddH₂O to 1 l
adjust pH to 7.0 and autoclave to sterilize

LB plates: LB medium + 15 g Bacto-agar per 1 l

1. To 200 μ l of fresh competent cells on ice add completed ligation reaction or plasmid DNA (10 ng).
2. Mix and incubate on ice for 30 min.
3. Heat shock on 42 °C water bath for 90 sec, chill on ice for 2 min.

4. Add 0.8 ml of LB medium and incubate at 37°C for 45 min with shaking.
5. Dry LB-antibiotic plates at 37°C.
6. Plate 10 to 200 µl of transformed cells per 10 cm plate.
7. Incubate plates inversed at 37°C for 16 to 20 hours.

3.1.3 Plasmid preparation

Plasmid DNA was prepared with following kits: QIAGEN Plasmid Midi Kit, QIAprep Spin Miniprep Kit (Qiagen), PureYield™ Plasmid Midiprep System (Promega) according to manufacturer's instructions.

3.1.4 Glycerol stock of *E. coli* culture

1. Mix 850 µl of grown bacterial culture and 150 µl of sterile glycerol.
2. Snap freeze in liquid nitrogen. Replace to -80°C.
3. When recovering from frozen stock quickly scrape frozen cells (avoid melting of the whole stock), place on fresh LB plate with relevant antibiotics and grow overnight at 37°C.

3.2 Human Embryonic Kidney (HEK 293) cell culture

Manipulation based on manual for AD-293 Cells (Stratagene).

3.2.1 Media and reagents:

Growth Medium - Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing:

4.5 g/L glucose
 110 mg/L sodium pyruvate
 2 mM L-glutamine
 supplemented with 10% (v/v) heat-inactivated fetal bovine serum
 100 U of penicillin and 100 µg of streptomycin

Freezing Medium (100 ml):

50 ml DMEM
 40 ml heat-inactivated fetal bovine serum
 10 ml dimethylsulfoxide (DMSO)
 Filter sterilize

Phosphate-Buffered Saline (PBS):

137 mM NaCl
 2.6 mM KCl
 10 mM Na₂HPO₄
 1.8 mM KH₂PO₄
 Adjust the pH to 7.4 with HCl

Trypsin-EDTA Solution: 0.53 mM tetrasodium ethylenediamine-tetraacetic acid (EDTA)
 0.05% trypsin

3.2.2 Establishing culture from frozen cells

1. Place 10 ml of growth medium in a 15-ml conical tube.
2. Thaw the frozen cryovial of cells within 40–60 seconds by gentle agitation in a 37°C water bath. Remove the cryovial from the water bath and decontaminate the cryovial by immersing it in 70% (v/v) ethanol (at room temperature).
3. Transfer the thawed cell suspension to the conical tube containing 10 ml of growth medium.
4. Collect the cells by centrifugation at 200 x g for 5 minutes at room temperature. Remove the growth medium by aspiration.
5. Resuspend the cells in the conical tube in 5 ml of fresh growth medium.
6. Add 10 ml of growth medium to a 75-cm² tissue culture flask. Transfer the 5 ml of cell suspension to the same tissue culture flask. Place the cells in a 37°C incubator at 5% CO₂.
7. Monitor cell density daily. Cells should be passaged when the culture is >80% of confluence.

3.2.3 Preparation of an HEK293 Cell Liquid Nitrogen Stock

1. When growing cells for the production of an HEK293 liquid nitrogen stock, cultures should be maintained at ≤50% confluence.
2. Collect cells from a healthy, log-phase culture. Remove the culture medium by aspiration. Trypsinize cells for 1–3 minutes in 1.5-ml of Trypsin-EDTA solution. Incubate the cells in the Trypsin-EDTA solution for the minimum time required to release adherent cells from the flask. This process may be monitored using an inverted microscope. Excess trypsinization may damage or kill the cells.
3. Dilute the cells with 8.5 ml of growth medium. The serum in the medium inactivates the trypsin. Transfer the suspension to a 15-ml conical tube, and then collect the cells by centrifugation at 600 x g for 5 minutes at room temperature.
4. Remove the medium by aspiration. Resuspend the cell pellet in a minimal volume of growth medium (containing 10% fetal bovine serum). Count the cells present in an aliquot of the resuspension using a hemocytometer.

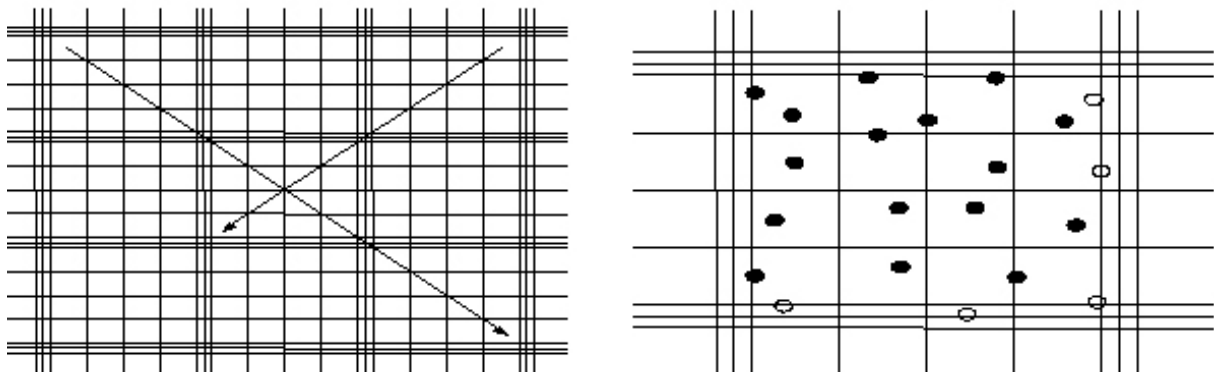
5. Dilute the cell suspension to 1×10^6 cells/ml in freezing medium and then dispense 1 ml aliquots of the suspension into 2-ml cryovials.
6. Freeze the cell aliquots gradually by placing the vials in a container and then placing the container in a -80°C freezer overnight.
7. Transfer the vials of frozen cells to liquid nitrogen for long-term storage.

3.2.4 Passaging of HEK293 Cells

When the cell monolayer is at $>80\%$ confluency, HEK293 cells should be split at a 1:10 ratio.

1. Remove the growth medium by aspiration. Wash cells once with 10 ml of phosphate-buffered saline.
2. Trypsinize cells for 1–3 minutes in 1.5 ml of Trypsin-EDTA solution.
3. Dilute the cells with 8.5 ml of growth medium to inactivate the trypsin.
4. Transfer 0.8 ml of the cell suspension to a fresh 25-cm^2 tissue culture flask and add 7.2 ml fresh growth medium. Place the cells in a 37°C incubator at $5\% \text{CO}_2$. Monitor cell density daily.

3.2.5 Counting cells with Bürker cell



(Lavens and Sorgeloos, 1996)

Fig. 3.1. Bürker cell - for counting directions follow arrow. For both, count the cells in the square and those which touch the top and left border (\bullet). Do not count the ones touching the right and lower border (\circ). The distance between the cover glass and the surface of the ruled area is 0.1 mm. The volume defined by a 1 square mm is thus 0.1 cubic mm.

3.2.6 Cell lysis and genomic DNA isolation for PCR

(Kontgen and Stewart, 1993; Sambrook and Russell, 2001)

The protocol is for 96-well plate (scale for bigger flasks/ plates).

PCR lysis solution B: 10 mM Tris-CI (pH 8.3)
50 mM KCl
2 mM MgCl₂
0.45% (v/v) Nonidet P-40 (NP-40)
0.45% (v/v) Tween-20
20 µg/ml proteinase K

Lysate buffer: 670 mM Tris-CI (pH 8.8)
166 mM ammonium sulfate
1 mg/ml bovine serum albumin

1. Remove the medium from cultures of cells growing in 96-well plates.
2. Deliver 50 µl of PCR lysis solution B into each well and lyse the cells by incubation overnight at 37°C, followed by 30-minute incubation at 95°C to inactivate the proteinase K.
3. To each lysate add an equal volume of a lysate buffer.
4. Use 10-50 µl aliquots of the DNA preparations as templates in PCRs.

3.2.7 Mycoplasma detection

HEK293 cell cultures were occasionally tested for *Mycoplasma sp.* contamination using Venor[®] GeM Mycoplasma Detection Kit for conventional PCR (Minerva Biolabs) according to manufacturer's instructions. Lower band 191 bp is positive control for PCR, upper band (270 bp) occurring only in positive control is *Mycoplasma* specific fragment (Fig. 3.2.). All cell cultures used for experiments were free of Mycoplasma contamination.

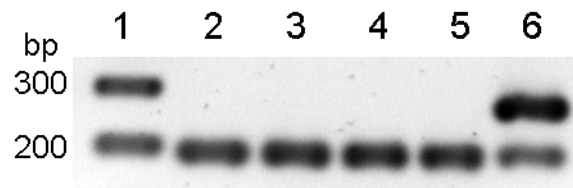


Fig. 3.2. PCR analysis to test Mycoplasma contamination in the cell culture media

lane 1, DNA Marker; lane 2, Negative control; lanes 3-5, independent cell culture samples; lane 6, positive control

3.2.8 Transfection using Polyethylenimine (PEI)

(based on V. Bryja's protocol, Masaryk University Brno)

PEI: Aldrich cat. no. 40,872-7, stock solution pH 7.4

Protocol for 10 cm plate (can be scaled down in the same ratios, tab. 3.1)

1. Mix 400 μ l of serum free media (DMEM) with 5 μ g of plasmid DNA and 12.5 μ l of PEI (1:2.5 ratio). (You can go up to 12 μ g DNA/10 cm plate if you need to increase efficiency). Technically is the best to pipette DNA into tube and add the mixture of DMEM/PEI (mastermix).
2. Mix by vortexing and let the complexes to form for 15 min RT.
3. Pipette it on sub confluent cells in complete media.
4. (optional) Spin cells 10 min @ 300g.
5. Let cells transfect for 4 hours.
6. Change to fresh media (DMEM with 10% serum) after 4 hours (because of PEI toxicity) - collect in 24 or 48 hours after the transfection for analysis.

dish	area	Medium [μ l]	DNA [μ g]	PEI [μ l]
10 cm plate	60 cm ²	400	5 (12)	12.5(30)
6-well plate	10 cm ²	80	1 (2,4)	2,5 (6)
24-well plate	2 cm ²	16	0,2 (0,5)	0,5 (1,25)

Tab. 3.1. Scales for the most commonly used plates.

3.2.9 Transfection using Lipofectamine™ 2000

Cells were transfected according to Lipofectamine™ 2000 Transfection Reagent manual (Invitrogen).

3.3 *C. elegans* culture

3.3.1 Handling of worms

Rich nematode growth medium (RNGM) plates: 3 g NaCl
 7.5 g Bacto Peptone
 20 g Agarose/Agar
 5 μ g cholesterol
 1 mM CaCl₂
 1 mM MgSO₄
 25 mM K-phosphate (pH 6.0)
 deionized water to 1 l

S-basal gelatin composition 100 mM NaCl
 50 mM potassium phosphate (pH 6)
 5 μ g cholesterol
 0.05% gelatin

C. elegans worms were cultured on RNGM plates fed on *E. coli* strain OP50 at 20°C as described (Brenner, 1974) and transferred to new plates individually with platinum picker or by washing with S-basal gelatin buffer. Worm stocks were kept at 15°C.

3.3.2 Bleaching

Technique used for synchronization of worm culture or for cleaning the culture from contamination. Another way of synchronization of the culture is placing 10 adult worms on fresh plate.

Bleach mixture: 285 µl bleach (Savo)
180 µl 4 M NaOH
535 µl deionized water

1. Collect the eggs from RNGM plate using 2x 800 µl S-basal gelatin buffer and scraper.
2. Spin at 1500 rpm for 1 min, discard the part of the supernatant and leave 100 µl without disturbing worm pellet.
3. Add bleach mixture to worm pellet, observe for approximately 4 min until worms start to lyse.
4. Vortex, spin at 1500 rpm for 1 min, discard the supernatant, wash 2x with S-basal gelatin, discard most of the supernatant without disturbing the egg pellet.
5. Place eggs on fresh plates.

3.3.3 1st strand cDNA making from worms

(Protocol by M. Asahina)

RNA extraction (total RNA using TRIzol)

1. Harvest worms from 2-3 plates using S-basal gelatin.
2. Wash worms with diethylpyrocarbonate water (DEPC-H₂O) and discard as much water as possible.
3. Add 500 µl of TRIzol (Invitrogen), total volume is about 600 µl, vortex and freeze in -80°C.
4. Incubate at 65°C for 15 min, vortex vigorously.
5. Add 1/5 volume (~120 µl) of chloroform, vortex.
6. Incubate for 3 min at RT, spin not more than 12000 x g for 15 min at 4°C.
7. Transfer supernatant to a fresh tube.
8. Precipitate with 50% volume (~ 100 µl) isopropanol, vortex.
9. Incubate for 10 min at RT, spin not more than 12000 x g for 10 min at 4°C.

10. Wash the pellet with 500 μ l ethanol (75%, -30°C), vortex.
11. Spin at 7500 x g for 5min at 4°C, take out all liquid, air-dry for 5 min at RT.
12. Resuspend in 16 μ l RNase-free H₂O, on ice.

First strand cDNA synthesis (20 μ l reaction, in 0.5 ml tube)

1. Mix RNA template and 1 μ l oligo d(T) primers.
2. Add 1 μ l dNTPs (10 mM mix).
3. Add sterile water up to 12 μ l if necessary.
4. Heat 65°C for 5 min, Put on ice for 1 min, spin briefly.
5. Add 7 μ l cocktail: 4 μ l 5x buffer
2 μ l 0.1 M dithiothreitol (DTT)
1 μ l RNaseOUT (Invitrogen)
6. Incubate at 42°C (water bath) for 5 min.
7. Add 1 μ l SuperScript II RT 200 U/ μ l (Invitrogen).
8. Incubate at 42°C for 50 min.
9. Incubate at 70°C for 15 min.
10. Put it on ice, spin briefly.
11. Add 1 μ l RNaseH @37°C for 20 min (for >1 kb PCR targets).
12. Use 5 μ l in 50 μ l PCR reaction.

3.3.4 Microinjections

(Adapted from M. Koelle, Mello & Fire, 1995 and M. Asahina)

10X microinjection buffer: 20 % polyethylene glycol, molecular weight 6000-8000
200 mM potassium phosphate, pH 7.5
30 mM potassium citrate, pH 7.5

Agarose pads

1. Heat 2% agarose in deionized water by autoclaving or in microwave.
2. While keeping the agarose warm on thermal plate drop approximately 90 μ l on each 24x50 mm coverslip. Immediately after drop place a second coverslip on top, which will flatten the agarose into a thin pad (avoid air bubbles).
3. When the agarose has hardened slide off the top coverslip, mark the side with agarose.
4. Place the coverslips in a box, cover with aluminum and bake 20 min at 100 °C.
5. Dried injection pads can be stored for a long term in coverslip box.

Preparation of worms

1. Synchronize worms by bleaching and culture them to young adults on RNGM plates with OP50 in 20 °C.
2. Before injection replace worms to new plate without OP50 to get them cleaned from bacteria.

Microinjection procedure

1. Pull the needle (GDC-1, Narishige) on vertical needle puller (Narishige).
2. Prepare 20 μ l of injection mix consisting of 2 μ g plasmid DNA mixture in 1x injection buffer.
3. Filter the injection mix just before injection through Ultrafree-MC column (0.1 μ m, Millipore).
4. Load about 1.5 μ l of injection mix to the tip of the injection needle.
5. Put the clean worm on the agarose pad with drop of mineral oil (Sigma).
6. Place the pad under the injection microscope (Olympus), using the lenses 4x and 40x adjust the needle tip and gonadal arm into one focal plane.
7. Inject one or both gonadal arms as fast as possible.
8. Drop S-basal gelatin on injection pad and replace worm to fresh RNGM plate with OP50.
9. Next day separate worms to new RNGM plates with OP50.

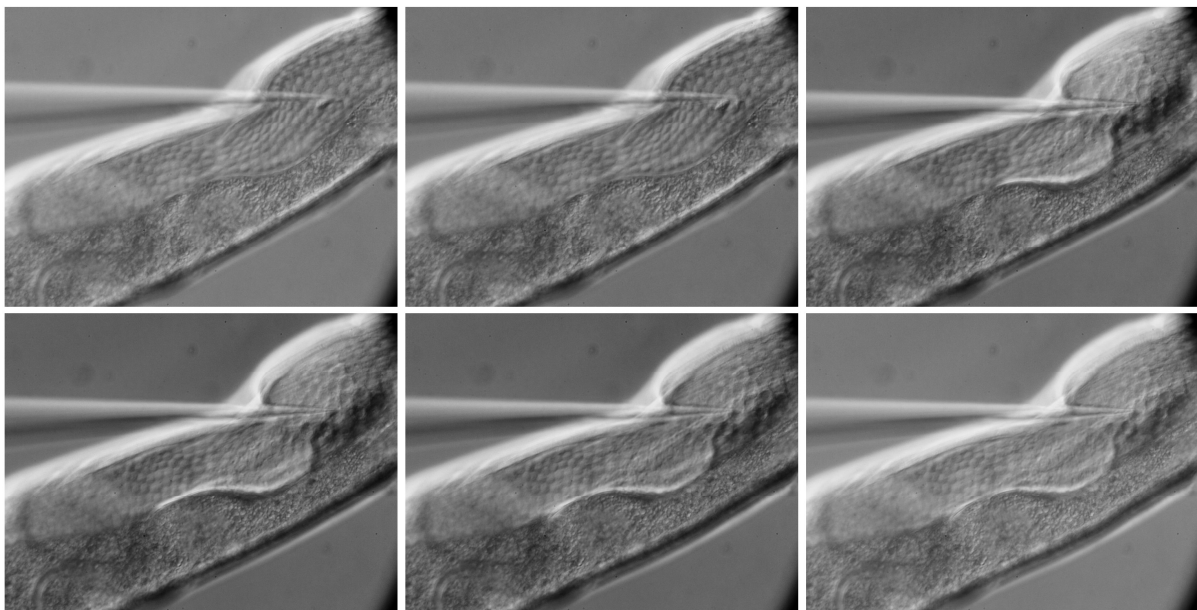


Fig. 3.3. Injection into gonadal arm. Swelling of the arm is visible after injection of DNA mixture. Olympus microscope, DIC, 40x lens. Note: do not overload the gonad or it will be damaged.

3.4.3 Real time PCR

Reaction using iQ SYBR kit (BioRad)

Single reaction (20 µl; flat-top 0.2-ml PCR tubes):

	Stock	Volume (µl)	Final concentration
Sterile distilled water		5.0	
iQ SYBR	2x	10	1x
Primer A	10 µM	0.5	0.25 µM
Primer B	10 µM	0.5	0.25 µM
DNA		4.0	

Program:

Initial denaturation of DNA, polymerase activation	hold 1: 95 °C	5 min
Denaturation	94 °C	45 sec
Annealing	62 °C	45 sec
Extension and first READ (acquire)	72 °C	30 sec
Second READ (acquire)	83 °C	15 sec
	Repeat cycle 40 times	
	Hold 2: 50 °C	2 min
	Melting analysis	

Primer sequences and details are in Supplementary information (8.2 – 8.4).

1. Set the dilution row of standards (DNA template of known concentration) for calculating the standard curve, prepare fresh each time (DNA sticking to the tube cause significant change in low concentration samples).
2. For each specific primer set, always prepare a master mix containing all reagents except DNA to minimize pipetting errors. Mix well and return on ice.
3. Dispense mastermix to chilled tubes by 16 µl.
4. Add 4 µl of DNA samples, close tubes, vortex gently and spin, return on ice.
5. Place all tubes in the chilled rotor, screw in tightly to machine (Rotor Gene 3000, Corbett).
6. Start the program.

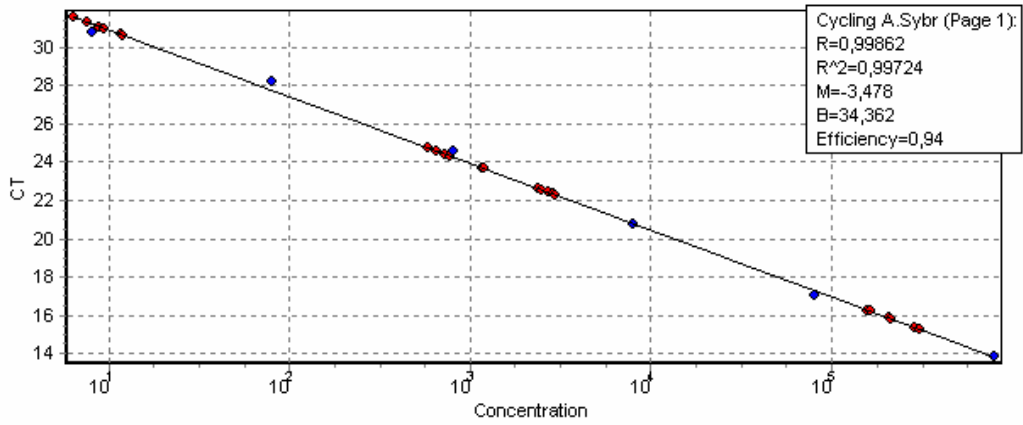


Fig. 3.4. Standard curve with reaction efficiency value.

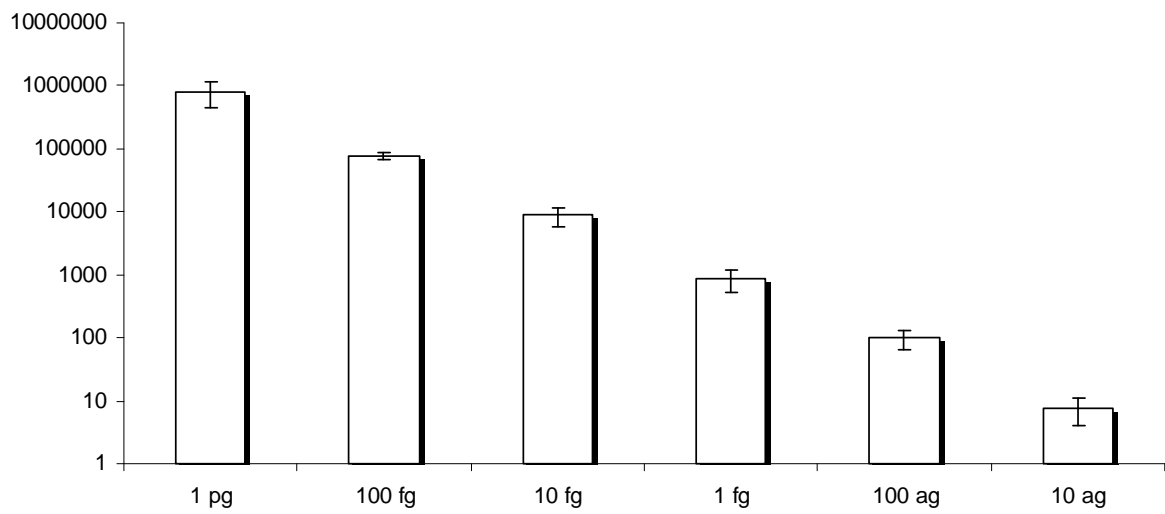


Fig. 3.5. Copy number of dilution row samples used for calculation of standard curve.

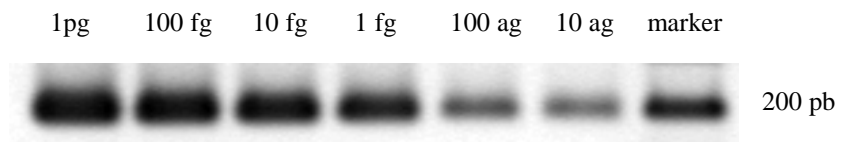


Fig.3. 6. Electrophoretic separation of fragments amplified with qPCR.

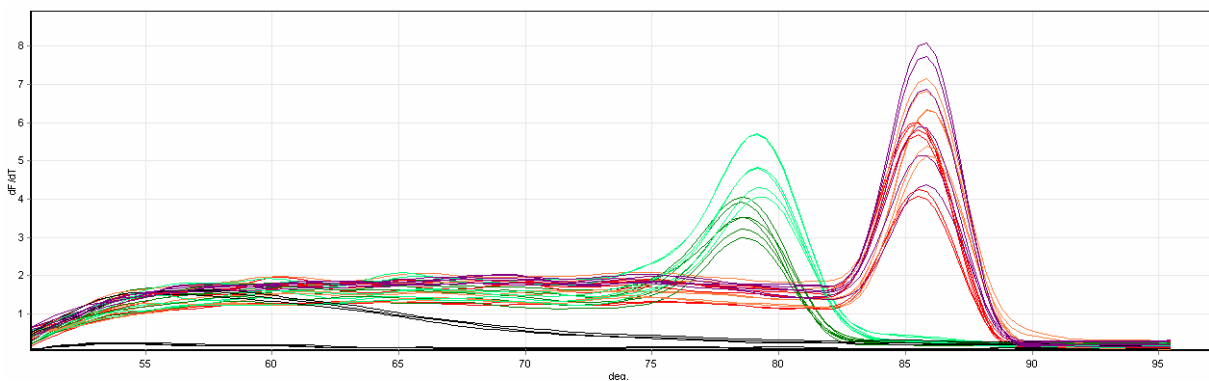


Fig. 3.7. Typical melting curve obtained for qPCR samples.

3.4.4 Cutting DNA with Restriction Endonucleases

Reaction mixture: 2 µl 10x enzyme reaction buffer
1 µg DNA
0,5 µl restriction endonuclease (A)
(0,5 µl restriction endonuclease B if cutting with two enzymes in one reaction)
Deionized water to 20 µl

Incubate according to enzyme manufacturer's instructions (usually 1 hour at 37 °C), terminate the reaction by heating at 70 °C for 15 min.

3.4.5 Phenol chlorophorm extraction, ethanol precipitation

This method also serves as inactivation of Phusion polymerase after PCR.

1. Mix PCR products with equal amount of phenol/chlorophorm/isoamylalcohol mixture (ratios 25:24:1), vortex 1 min, spin 8 min at full speed (all centrifugations are performed in pre-chilled centrifuge).
2. Take the water phase. Mix with equal amount of chlorophorm, vortex and spin 8 min at full speed.
3. Take the water phase, add 1/10 V 3 M sodium acetate, 2.5 V ethanol, 1/40 V glycogen, precipitate at least 15 min at – 80 °C (or 2 hours at – 20 °C).
4. Spin 15 min at full speed.
5. Wash with 500 µl of 70% ethanol, spin 5 min at full speed, remove the liquid without disturbing the pellet and dry.
6. Resuspend in desired amount (10-20 µl) of deionized water, measure the DNA concentration by spectrophotometer.

3.4.6 Ligation in low melting point agarose gel

(Based on Inoue et al., 1990).

TCM buffer: 10 mM Tris-HCl
10 mM MgCl
10 mM CaCl₂

1. Prepare 1% agarose gel, cut the inner part and leave the scaffold for low melting point agarose gel (0,7-2,5%). this helps manipulation with the gel.
2. Separate DNA fragments with electrophoresis and digested vector DNA using the TAE buffer system.
3. Stain with gel ethidium bromide.

4. Cut out the DNA bands with razor blade, try to minimize excessive amount of the gel, use a long-wave UV lamp to prevent damage of DNA.
5. Melt the gel slices for 10 min on 65-67°C water bath in sterile tubes.
6. Prepare two sets of small sterile tubes. In one set, mix appropriate combinations of melted gel containing insert (e.g. 5 µl) and vector (e.g. 2 µl) DNA. Set the volume to about 13 µl with sterile deionized water, mix well and return to 65-67°C for 5 to 10 min.
7. Work on ice, prepare 10 µl of 2x concentrated ligase buffer containing ATP and 1 µl of T4 DNA ligase (Promega) per each reaction, to the other set of tubes add 10 µl of the ligase mix.
8. Add 10 µl of the melted gel mixture into the appropriate tube with ligase mix on ice (when pipetting the hot gel, wait 3 seconds before mixing with ligase not to kill the enzyme), immediately and thoroughly mix by pipetting up and down before the gel solidifies.
9. Incubate ligation reactions for 12 to 16 h at 16°C.
10. For transformation, melt the completed reaction mix for 15 min on 65-67°C water bath, add 40-80 µl of sterile distilled water or TCM buffer to prevent gelling.

3.4.7 Sequencing

Using the ABI Big-Dye-Terminators v.1.1 (Perkin Elmer), based on the manufacturers instructions.

Reaction composition:

2.5x sequencing reaction buffer	4 µl
Big-Dye Terminator kit (1.1)	4 µl
Primer	3.2 pmol (3.2 µl of 1µM stock)
H ₂ O	up to 8 µl

Mix DNA (1 µg of plasmid DNA gives good result) and H₂O to 12 µl and add 8 µl of premix.
Total reaction volume: 20 µl

Procedure:

1. Run the reaction.
2. Clean the product using G-50 column.
3. Dry using speedvac (30-40 min) at RT.
4. Submit samples for sequencing on ABI 310 automatic sequencer.

5. Process the sequences using Chromas (Technelysium) and DNASTar (Lasergene) software.

3.4.8 Gel image analysis

Using Image Quant™ and Image Quant™ TL software (Molecular Dynamics) according to manufacturer's instructions.

1. Preparation: determine the optimal amount of PCR reaction cycles for set of samples (try to avoid saturation of reaction), run PCR, separate fragments on gel (3% agarose gel in TBE gives nice results for small fragments about 100-200 bp), stain with ethidium bromide, take photo on UV transilluminator with ideal exposure time and aperture (avoid loosing information in strong bands by long exposure).
2. Open image in Image Quant software.
3. Make a selection of areas of interest.
4. Choose appropriate background correction method – crucial step (fig 3.8 - 3.10)
 - a. None: no background subtraction
 - b. Local Average: counts background from the value of pixels underneath the line trace of each individual analyzed object (gives mean average), it is used when background is not even, avoid the perimeter of selected object overlay part of another band or dust
 - c. Local Median: similar to Local average, but instead of taking a mean average value, it takes a median, which reduces the effect of speckles on the object perimeter
 - d. Object Average: value chosen background rectangle is subtracted from the volume, it is used when background is even but bands are too close to use Local Average or Local Median
 - e. Histogram Peak: whole lane of the band that is used to produce the histogram and the most often occurring value is chosen as a background, be aware of another bands or artifacts above or below the object
5. Perform volume report analysis.
6. Export data to Microsoft Excel.

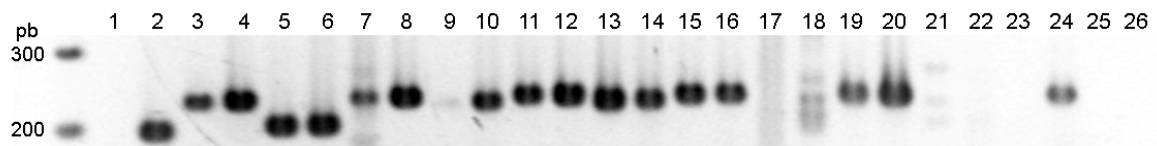


Fig. 3.8. Gel image that was used in Image Quant software for band volume calculations.

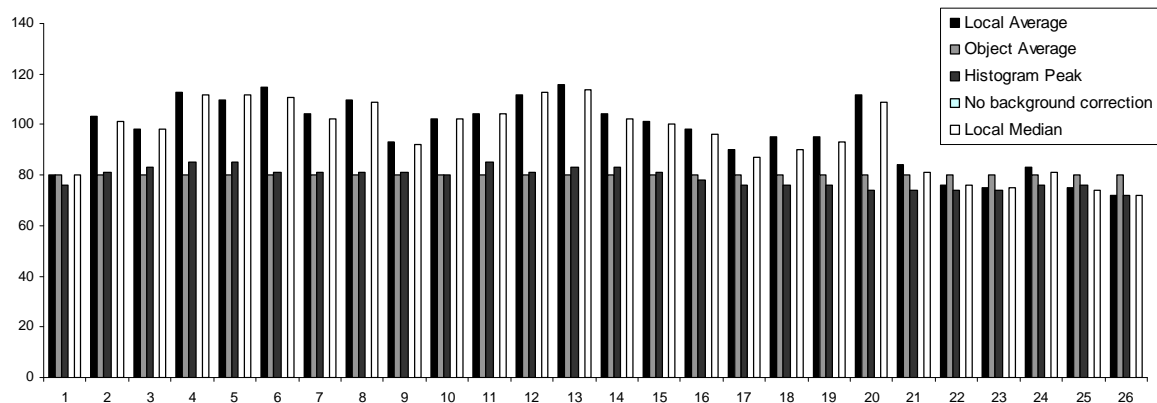


Fig. 3.9. Amount of background calculated with different amounts of background correction.

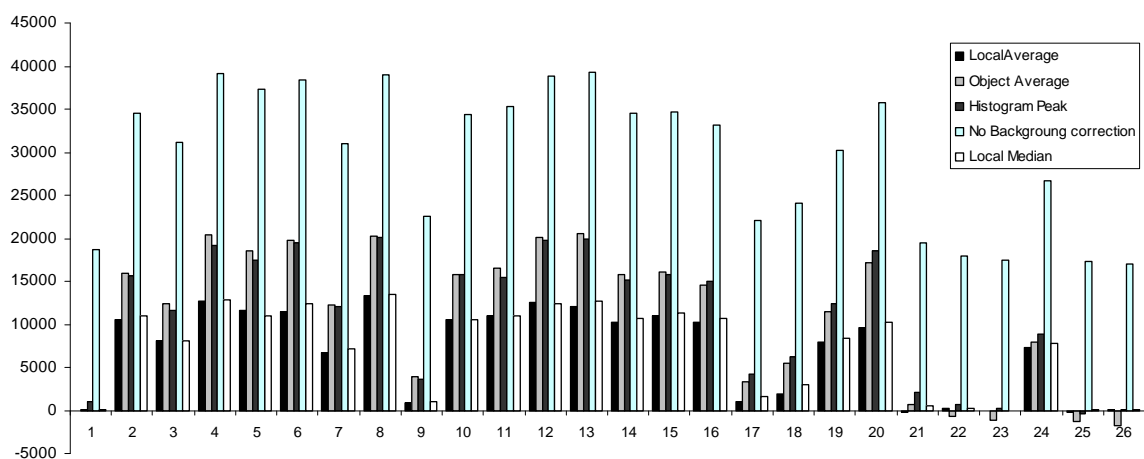


Fig. 3.10. Band volume levels corrected with different methods of background correction.

3.4.9 Chromatin immunoprecipitation (ChIP)

Using ChIP-IT™ Express Enzymatic kit (Active Motif) and manufacturer's instructions.
Solutions and reagents provided in the kit: 10x PBS, 10x Glycine buffer, 100 mM PMSF, Protease inhibitor cocktail (PIC), Enzymatic shearing cocktail (2×10^4 U/ml), 0.5 M EDTA, Protein G Magnetic Beads, ChIP buffer I, ChIP buffer II

Preparation of cells, fixation, chromatin shearing

Fixation solution: 1.62 ml 37 % formaldehyde
 60 ml minimal cell culture medium (DMEM)
 Mix and leave at RT

1x PBS: 7ml 10x PBS to 63 ml of dH₂O, place on ice

Glycine stop-fix solution: 3 ml 10x Glycine buffer
3 ml 10x PBS
24 ml dH₂O
Mix and leave at RT

Cell scraping solution: 600 µl 10x PBS
5.4 ml dH₂O
Mix and place on ice, just before use add 30 µl 100 mM
phenylmethanesulphonylfluoride (PMSF)

1. Transfect one or two 75 cm² flasks with PEI or Lipofectamine™ 2000 as described above for one of plasmid constructs set.
2. Grow cells to 70-80 % confluency.
3. Before harvesting cells prepare solutions above (amount for three 15 cm plates or six 75 cm² flasks).
4. Pour medium off the cultured cells, add 10 ml of Fixation solution per 75 cm² flask and incubate on a shaking platform for 10 min at RT.
5. Pour Fixation solution off the plates and wash each flask with 5 ml ice cold 1x PBS, rock the plate for 5 sec, pour off the PBS.
6. Stop the fixation reaction by adding 5 ml Glycine stop-fix solution to each of the flasks, rock 5 min at RT.
7. Pour off the Glycine stop-fix solution; wash with 5 ml ice cold 1x PBS, rock for 5 sec and pour off the PBS.
8. Add PMSF to Cell scraping solution, add 1 ml of this solution to each flask and scrape cells with cell scraper, collect the cells to a 15 ml conical tube on ice.
9. Spin the cells at 720 x g for 10 min at 4°C.
10. Remove the supernatant and discard, pellet can be frozen at this point, if freezing add 1 µl 100 mM PMSF and 1 µl PIC and freeze at -80°C.
11. Thaw the pellet (if necessary), resuspend cells in 250 µl ice-cold Lysis buffer (with 1.25 µl PIC and 1,25 µl PMSF), incubate on ice for 30 min.
12. Transfer the cells to an ice-cold dounce homogenizer, gently dounce on ice with 10 strokes to aid in nuclei release, transfer cells to a 1.7 ml microcentrifuge tube and centrifuge at 2400 x g for 10 min at 4°C to pellet the nuclei.
13. Carefully remove the supernatant and discard, resuspend the nuclei pellet in 250 µl Digestion buffer (with 1.25 µl PIC and 1.25 µl PMSF), pre-warm the solution at 37°C for 5 min.

14. Prepare a working stock of Enzymatic shearing cocktail (200 U/ml) by diluting the supplied Enzymatic shearing cocktail (2×10^4 U/ml) 1:100 with 50 % glycerol in dH₂O. This stock is stable for 1-2 weeks at 4 °C.
15. Add 12.5 µl of working stock of Enzymatic shearing cocktail to pre-warmed nuclei and vortex to mix, incubate at 37°C for 10 min, vortex the tube periodically during the incubation.
16. Stop the reaction by adding 5 µl of ice-cold 0,5 M EDTA to each tube, chill on ice 10 min.
17. Spin the sheared DNA samples at 10 000 – 15 000 rpm at 4°C for 10 min, collect the supernatant, which contains sheared chromatin, use or store at -80°C, remove 25 µl for use in checking the DNA shearing efficiency and DNA concentration, split the rest to several aliquots.

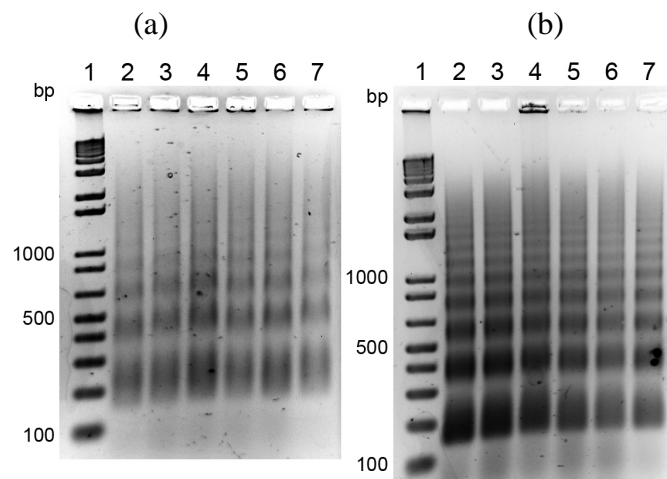


Fig. 3.11. Enzymatic shearing of chromatin isolated from cross-linked HEK293 cells. In the beginning of ChIP procedure after the transfection, cells were first chemically cross-linked. Then, chromatin was isolated and enzymatically sheared by incubating for 10 min at 37 °C in enzymatic shearing cocktail supplied in ChIP-IT™ Express Enzymatic kit. Shared DNA was checked on an agarose gel. Image shows shared DNA before phenol-chloroform extraction and ethanol precipitation (a) and after (b) for each samples. Cells were transfected with various DNAs (lane 2, 2 x sf-1* + pEGFP:nhr-25; lane 3, 2 x sf-1 + pEGFP:nhr-25; lane 4; 4 x sf-1* + pEGFP:nhr-25; lane 5, 4 x sf-1 + pEGFP:nhr-25; lane 6, pEGFP:nhr-25; lane 7, untransfected cells) and chromatin was isolated from each transfection and shared. In all samples, chromatin was efficiently sheared into 200 to 1500 bp fragments.

Capture chromatin on magnetic beads

1. Thaw the chromatin, separate 10 µl as the Input DNA for PCR control, store at 4°C if used within 6 hours or at -20 °C.
2. Setup the ChIP reaction in 1.7 ml siliconized microcentrifuge tubes:

Protein G Magnetic Beads	25 µl (fully resuspended by mixing or vortexing)
ChIP buffer I	10 µl
Sheared chromatin	15 – 100 µl

PIC	1 μ l
Antibody	1-3 μ g (1-20 μ l rabbit polyclonal α GFP) – the final component added to the reaction
dH2O	to 100 μ l (or to 200 μ l if more than 60 μ l of chromatin is used)

3. Cap tubes and mix thoroughly.
4. Incubate on a rolling shaker for 4 hours at 4°C, sensitivity might be improved if the incubation is performed overnight.
5. Spin briefly to collect liquid from the inside of the cap.
6. Place tube in magnetic stand to pellet beads on the tube side.
7. Carefully remove and discard the supernatant.

Wash Magnetic Beads

1. Wash beads two times with 800 μ l ChIP buffer I – after addition of wash buffer incubate samples for several min with gentle agitation before removing the buffer (increases specificity).
2. Wash beads two times with 800 μ l ChIP buffer II, after final wash remove as much supernatant as possible.

Elute chromatin, reverse cross-links and treat with Proteinase K

Procedure was performed according to ChIP-IT manual.

PCR analysis

Performed according to ChIP-IT manual and protocols above.

PCR data normalization

Summary of controls and normalization types in processing ChIP data:

Controls	Primers sets to test	Purpose
(a) <i>Controls for ChIP</i>		
Input Sample	All primer sets	Positive control for presence of chromatin, used to calculate %IP
No Antibody control	All primer sets	Determines background signal level
(b) <i>Controls for QPCR</i>		
Calibration line	All primer sets	Positive control for QPCR, used for quantification of QPCR signals
Melting curve	All primer sets	Tests for amplification of the correct fragment
No-template PCR	All primer sets	Negative control for QPCR, indicates primer artifacts

Tab. 3.2. Important controls for qPCR and ChIP (Haring et al., 2007)

Normalization strategy	Normalization method	Points of interest
No normalization	ChIP data is not normalized, equal amounts of input chromatin are used in different experiments	Differences in chromatin purity may cause differences in ChIP signal
Background subtraction	The no-antibody signal is subtracted from the ChIP signal	Background signal levels in the NoAb control samples may be different from those in the ChIP samples, depending on the used primer set, chromatin purity and sample handling. The normalization will be strongly influenced by fluctuations in the background signal
Fold enrichment % of input	Normalization to background signal Normalization to the amount of input chromatin	See <i>Background subtraction</i> Differences in sample handling between input and ChIP samples affect the normalization
Relative to control genes	Normalization to the ChIP signal obtained at a control sequence	Control sequences need to be developed. Control ChIP signals may differ between different tissues and developmental stages
Relative to nucleosome density	Normalization to the ChIP signal obtained with an antibody for unmodified histone protein	ChIP signals obtained with different antibodies are difficult to compare quantitatively. Regions with low nucleosome density can yield incorrect normalization

Tab. 3.3. ChIP – qPCR data normalization methods (Haring et al., 2007)

3.4.10 Protein pull down

Preparation, cross-linking and sonication of worms

(Based on Mukhopadhyay et al., 2008)

HLB: 50 mM HEPES-KOH, pH 7.5
150 mM NaCl
1 mM EDTA
0.1% (wt/vol) sodium deoxycholate
1% (vol/vol) Triton X-100
0.1% (wt/vol) SDS
1 mM PMSF and diluted protease inhibitor cocktail (10 µl /ml)

CB: 1x PBS containing 1% formaldehyde (vol/vol), this solution is made fresh each time and discarded after use. Maintain at RT.

1. Harvest worms by centrifugation at 650 x g for 2 min, discard the supernatant. About 0.4 ml of pellet (50000 or more gravid adults and 80000 or more L4/young adults) gives best results.
2. Wash the worm pellet three times with 30 ml 1x PBS to remove bacteria.
3. Wash the worm pellet once with 3 ml of CB, quickly spin down for 30 s at 650 x g and then resuspend in 3 ml of fresh CB.
4. Use a glass Dounce homogenizer to lyse the worms partially and allow the fixative to penetrate the worms. Use eight strokes with a 1/3 turn after each stroke.
5. Transfer the worm suspension to a 15-ml tube. Wash the homogenizer with an additional 1 ml of CB and add to the suspension in the 15-ml tube. Incubate at RT on a shaker (25 rocking actions per minute) for 20 min (for ChIP crosslinking time should be determined experimentally)

6. Add 200 ml of 2.5 M Glycine to the same tube and incubate on the shaker for an additional 15–20 min. The Glycine quenches the formaldehyde and stops the crosslinking reaction.
7. Pellet worms by centrifugation at 650 x g for 2 min.
8. Wash the pellet four times with 15 ml of 1x PBS containing protease inhibitor cocktail and snap freeze in liquid nitrogen, (crosslinked worms may be stored indefinitely at -80°C).
9. Thaw worm pellet from Step 8 on ice and add 2 ml of freshly prepared HLB containing protease inhibitor cocktail.
10. Pellet worms by centrifugation at 650 x g for 2 min (4°C), remove the supernatant and resuspend the pellet in 800 µl of HLB.
11. Incubate on ice for 10 min.
12. Using the M5 73 probe sonicate the worms 4 times with program settings: 10s, cycle: 5 (5 strokes), output 50%, between the sonications chill the tube at least 2 min on ice (dry ice with ethanol or ice chilled with liquid nitrogen) to prevent overheating and frothing, which results in loss of sample.
13. Centrifuge the lysate (16000 x g, 4°C, 15 min) to pellet the debris. Transfer the supernatant to a fresh tube carefully, avoiding floating debris.

Immunoprecipitation with Dynabeads-protein G

TBS: 50 mM Tris-HCl (pH 7.8)
 150 mM NaCl
 1 mM EDTA (pH 8)

TBS/N: TBS + 0.01% NP-40

Lysis buffer: 25 mM Hepes pH 7.4
 60 mM NaCl
 1% Triton X-100
 1 mM EDTA pH 8
 1,5 mM MgCl₂
 Roche complete tablet Protease inhibitors 25x diluted or 1 tablet Roche miniEDTA free for 10 ml Lysis buffer

Tris: 50 mM Tris-HCl, pH 7.5
 Tris/SDS: 50 mM Tris-HCl pH 6.8 + 2% SDS

1. Resuspend the beads in the vial by vortexing (1-2 min).
2. Transfer 20 µl beads to 1.5 ml Eppendorf tube, place the tube for 1 min in the

magnetic stand and than pipette off the fluid.

3. Wash 3x 1 min in 1 ml TBS – 1 min washing on the rotating (or rocking) device, then 1 min in magnetic stand, discard the fluid.
4. Resuspend he beads in 80 μ l of TBS/N + antibody; incubate for 1 hour at RT on rotator.
5. Collect beads for 1 min in the magnetic stand, discard the fluid.
6. Wash 3x 5 min in 1 ml TBS/N – 5 min washing on rotator, 1 min in magnetic stand, then discard the fluid.
7. Add protein lysate to the beads – do not exceed 5x the volume (100 μ l) of beads originally pipetted from the stock, save an aliquot 1 for SDS-PAGE.
8. Incubate 4 hours at 4°C on the rocking platform or rotator.
9. Collect the beads for 1 min in the magnetic stand, discard the liquid, but save an aliquot 2 for SDS-PAGE.
10. Wash 3x 10min in 1ml Lysis buffer (with protease inhibitor cocktail) at 4°C on rocking platform.
11. Wash 2x 10 min in 1 ml TBS/N at 4°C on rocking platform.
12. Resuspend in 25 μ l of Tris/SDS, leave for 15 min at RT, mix occasionally.
13. Collect the beads for 1 min in magnetic stand, save the supernatant for SDS-PAGE and discard the beads.

SDS-PAGE and western blotting

5x Running buffer: 15,1 g Tris base

94 g glycine (electrophoresis grade)

25 ml 20% SDS

Distilled water to 1000 ml

Transfer buffer: 5.72 g sodium borate per 1000 ml od distilled water

10x Ponceau: 30 g trichloroacetic acid

30 g sulphosalicylic acid

2 g Ponceau reagents

Distilled water to 100 ml

Sample buffer: 50 mM Tris-HCl (pH 6,8)

10% glycerol

2% SDS

100 mM DTT

Distilled water to 1 ml

Bromphenol blue

1. Boil samples in sample buffer for 10 min, spin briefly at maximum speed.

2. Load 10 or 15 μg of total protein in sample buffer on 10% SDS-PAGE minigel with stacking gel on top.
3. Run under 100-120V at RT for 90 min or until bromphenol blue reaches the edge of gel.
4. Remove gels, cut off the stacking gel and mark the orientation by cutting one corner.
5. Soak for several minutes in transfer buffer along with 3 MM filter paper and components of the BioRad transfer apparatus.
6. Setup transfer using the Protran (Schleicher and Schuell) nitrocellulose.
7. Transfer with stirring for 2 hours at RT under 200 mA constant current per each transfer unit.
8. Check quality of the transfer and equal loading by staining with 1x Ponceau reagent; shake for 5 min in Ponceau, then rinse with several changes of distilled water.
9. Store filters sealed in a plastic bag refrigerated

Immunoblotting

1x TBST buffer: 50 mM Tris-HCl (pH 7.8)
 150 mM NaCl
 1 mM EDTA (pH 8)
 0.1% Tween-20
 (10x TBST can be made)

Blocking buffer: 1x TBST
 5% BSA (fraction V, Sigma)
 5% nonfat dry milk
 (Keep refrigerated, use within 24 hours)

1. Seal up to two filters in a Seal-a-meal bag with 6 ml of blocking buffer
2. Block overnight on rocking platform at 4 °C.
3. Drain most of the blocking buffer into a clean tube and add the appropriate dilution of primary antibody to this tube, mix well, then return to the bag, re-seal and shake well.
4. Incubate for 2 h at RT with shaking.
5. Discard the primary antibody solution and wash the filters 5 times for 10 min per each change with shaking in fair amount (150 ml) of TBST at RT
6. Seal filters in a fresh bag with 5 ml of blocking buffer containing peroxidase conjugated secondary antibody
7. Incubate for 1 hour at RT with shaking
8. Wash 5 times in TBST as in step 5.

Detection with chemiluminiscence (ECL)

Frozen aliquots: Luminol (Sigma A8511): 250 mM in DMSO
p-coumaric acid (Sigma C9008): 90 mM in DMSO

Solution 1: 4.5 ml water
500 µl 1 M Tris-HCl (pH 8,5)
50 µl 250 mM luminol
22 µl 90 mM p-coumaric acid

Solution 2: 4.5 ml water
500 µl 1 M Tris-HCl (pH 8.5)
3 µl 30% H₂O₂

1. Combine solutions 1 and 2 just before use.
2. Incubate the membrane for 2-5 min and immediately wrap and expose (the signal rapidly decreases and is completely dead after 30-45 min).
3. Save the photo using luminometer Fujifilm LAS 3000.

Stripping the membrane after western blotting

Stripping buffer: 2% SDS
65.5 mM Tris-HCl pH 6,8
100m M Mercaptoethanol (142.5x dilution)

1. Seal in bag with 5 ml of stripping buffer, incubate 30 min at 70°C in water bath, stir the water.
2. Block again in blocking buffer overnight before incubation with another primary antibody.

3.5. Worm strains

The wild type *C. elegans* Bristol strain (N2) and the following transgenic and mutant strains were maintained according to the standard protocol (Brenner, 1974) at 20°C. N2 was used for microinjections. Strain MH1955 *nhr-25(ku217)* (Chen et al., 2004) was used for 1st strand cDNA synthesis of *nhr-25(ku217)* mutant allele. *C. elegans* B4 strain carrying *jmEx2[mbf-1promoter::MBF-1::gfp+pRF4]* (Asahina, unpublished data) was used for GFP pull down experiment.

3.6 Antibodies

Following antibodies were used for immunoblotting, ChIP and protein pull down.

Primary antibodies:

Polyclonal Rabbit α GFP (Lukas et al., 2009)

Monoclonal Mouse α GFP (Sigma cat.n. G 6539)

Polyclonal Rabbit anti-*dm*MBF-1 (Jindra et al., 2004)

Rabbit antiserum α NHR-25 (Asahina et al., 2000)

Rabbit polyclonal anti-*tc*MET (Jindra, unpublished)

Secondary antibodies:

Rabbit anti-Mouse HRP (Sigma)

Goat anti-Rabbit HRP (Jackson Immuno Research cat.n. 111-035-144).

4. Results

We used human embryonic kidney cell line (HEK293) as it is commonly used for cell transfection assays and chromatin immunoprecipitation (ChIP) method to study DNA-binding capabilities of the *Caenorhabditis elegans* nuclear hormone receptor NHR-25. To date there is no *C. elegans* cell line available and it has been tested that NHR-25 can be active in HEK293 on luciferase reporter assay (Asahina et al., 2006). The work presented here also serves as a preparation for ChIP directly from *C. elegans*.

4.1 Testing ChIP-IT kit

For ChIP assay, we used commercially available ChIP-IT™ Express Enzymatic kit from Active Motif®. First, ChIP-IT® Control Kit - Human was used for technical control on HEK293 cells. Chromatin was isolated from two 75 cm² flasks of non-transfected HEK293 cells, sheared and precipitated with α RNA Pol II and α IgG antibodies. After immunoprecipitation, chromatin was analyzed by PCR with primers for GAPDH promoter region (36 cycles) and separated by electrophoresis on 3% agarose gel. Transcriptionally active GAPDH promoter was immunoprecipitated and enriched with anti-RNAPol II antibody whereas there was no enrichment detected using anti-IgG antibody (Fig. 4.1b and 4.1c).

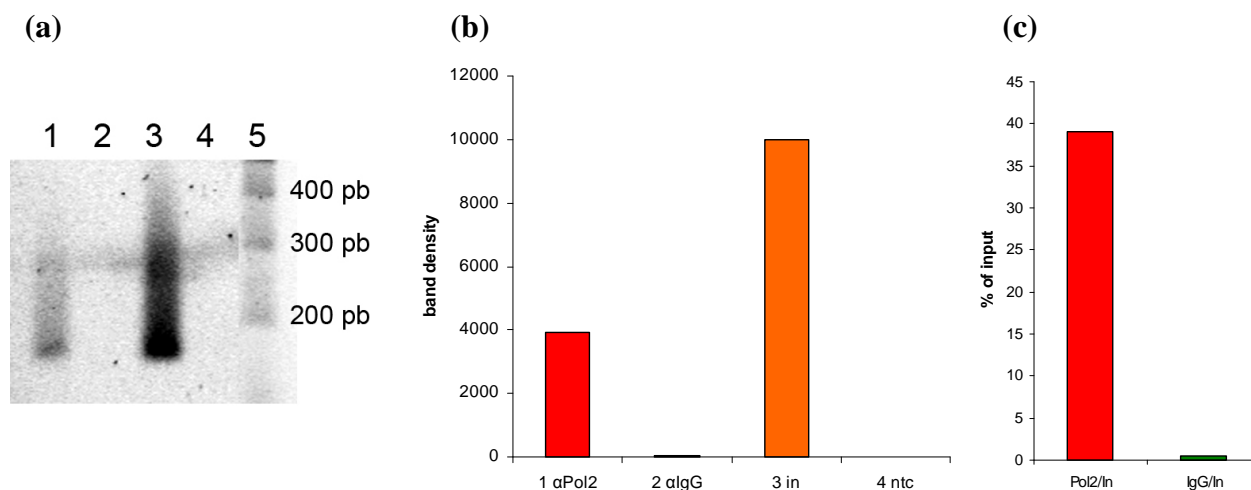


Fig. 4.1. ChIP with ChIP-IT® Control Kit – Human.

(a) Gel electrophoresis of PCR amplified samples. lane 1, ChIP with α RNA Pol II antibody (positive control); lane 2, ChIP with α IgG antibody (negative control); lane 3, input chromatin; lane 4, no template control for PCR; lane 5, DNA ladder; (b) ImageQuant™ TL analysis of the electrophoresis image (band density, Object average background correction). Lanes 1-4 correspond to lanes in (a); (c) Ratio of immunoprecipitated DNA over input DNA (%). input samples were diluted 1:10. Pol2/In, ChIP with α RNA Pol II antibody; IgG/In, ChIP with α IgG antibody.

4.2 Transfection of HEK 293 cells

HEK293 cells were routinely transfected with plasmid constructs using polyethylenimine (PEI) or Lipofectamine 2000™. Expression of the fusion protein EGFP::NHR-25 was monitored under the fluorescent microscope to check the transfection efficiency and the localization of the protein. HEK293 cells were transfected with approximately 50% efficiency (Fig. 4.2) and the fusion protein EGFP::NHR-25 was localized in the nucleus (Fig. 4.3) as we expected. Expression of recombinant fusion protein EGFP::NHR-25 from the cell lysate was also verified with specific antibodies namely monoclonal mouse α GFP and polyclonal rabbit α NHR-25 antibodies on western blotting. While there was no band detected when pK-Myc expression vector was transfected, specific bands (size approx. 95 kDa) were detected with the lysate from pEGFP::nhr-25 transfected cells (Fig. 4.4).

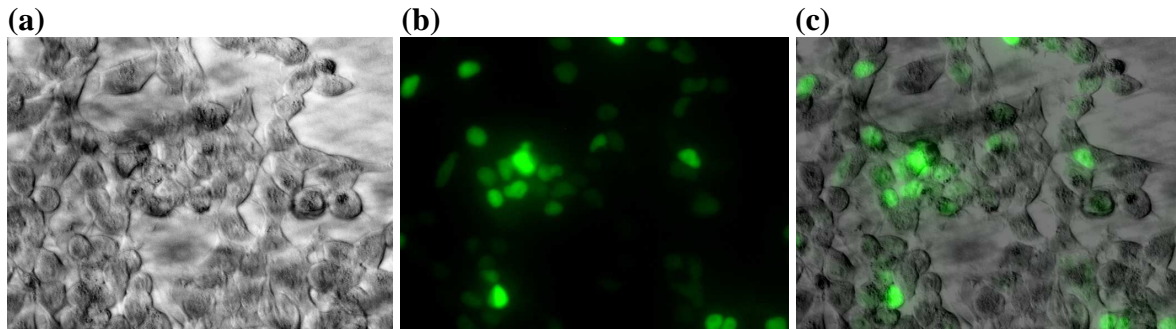


Fig. 4. 2. Visualization of transfection efficiency in HEK293 cells
 (a) DIC image with 40x objective; (b) Expression of GFP; (c) merged

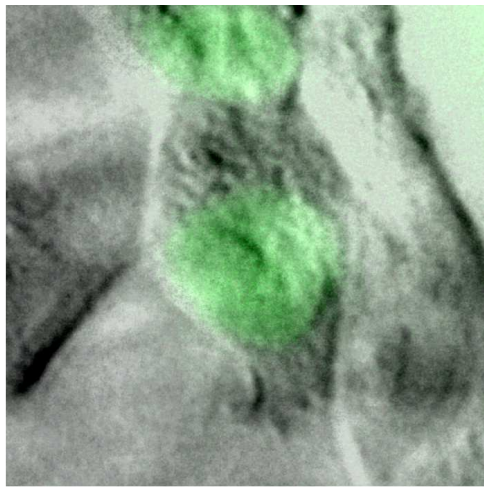


Fig. 4.3. Nuclear localization of EGFP::NHR-25
 DIC image was merged with a picture taken with GFP filter under a fluorescent microscope.

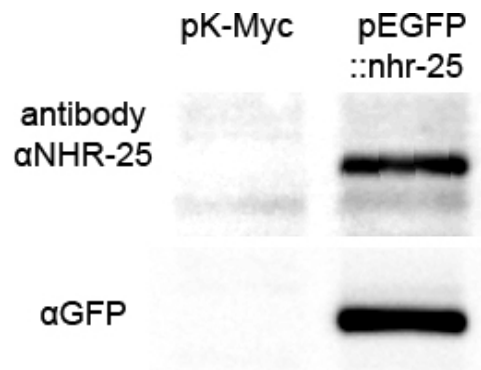


Fig. 4.4. Detection of the fusion protein EGFP::NHR-25 with antibodies against NHR-25 or GFP

(right lane) Cells transfected with construct pEGFP::nhr-25. (left lane) Cells transfected with pK-Myc vector as a negative control.

4.3 Chromatin immunoprecipitation of NHR-25

4.3.1 Conventional PCR analysis of ChIP I samples

HEK293 cells were transfected with two plasmid constructs using PEI. One was the plasmid construct contained two or four repeats of SF-1 binding consensus sequence, which was previously found to be activated by NHR-25 in Luciferase transfection assay (Asahina et al., 2006), or their mutated versions which should serve as a negative control for binding. The other plasmid was the expression construct carrying sequence encoding EGFP::NHR-25 fusion protein. Untransfected cells or cells transfected only with one of those constructs served as controls for estimation of background level of ChIP. For ChIP, set of two 75 cm²

flasks of 50 % confluent cells was transfected with total amount of 30 μ g DNA (15 μ g of each construct or with 30 μ g of one construct). ChIP was performed from 100 μ l of sheared chromatin and the sample was incubated with 2 μ g of mouse monoclonal α GFP antibody for 16 hours. Input chromatin was diluted 10 times for PCR analysis. Samples were amplified by PCR with sf-1 binding sequence specific primers (ChIPFw2, ChIPBw2) or specific control primers (NHR-25FW7, Nhr25full-R) for 36 cycles and then separated on 3% agarose gel. Bands were quantified with ImageQuantTM TL software (Fig. 4.5).

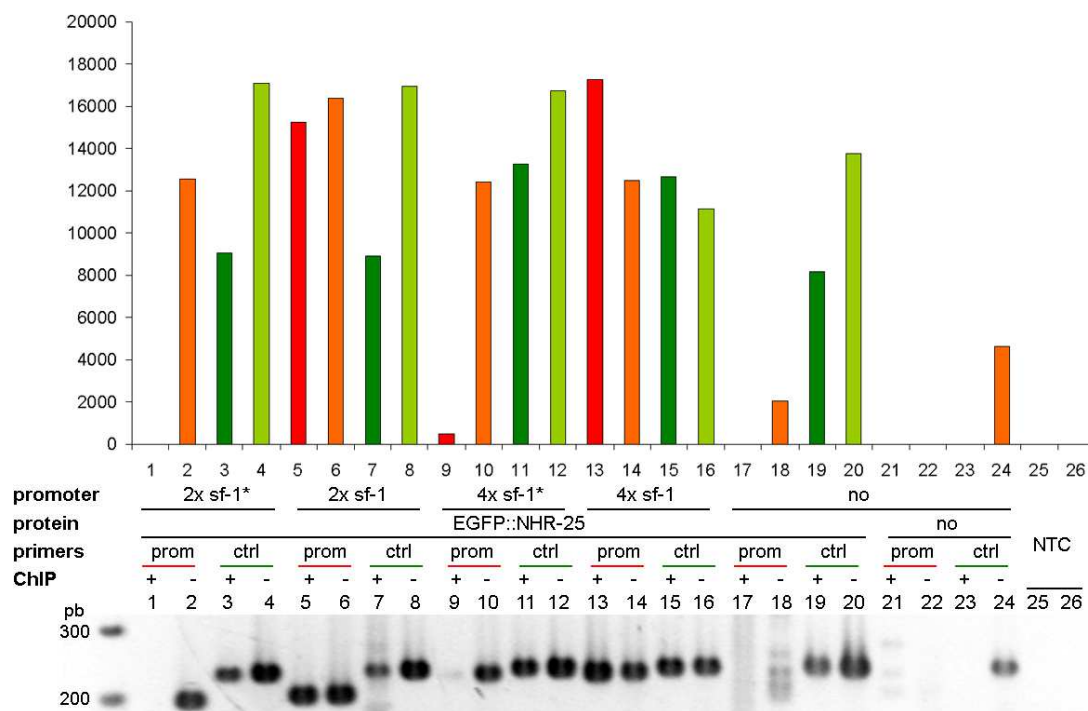


Fig. 4.5. PCR and electrophoresis gel analysis with ImageQuantTM TL software for ChIP I.

Each lane of the gel picture corresponds to the bar graph above. 2x sf-1, 4x sf-1: promoters containing 2 times or 4 times SF-1 binding consensus respectively; sf-1*: mutated SF-1 binding consensus as a negative control. Chromatin was amplified with either primers for SF-1 binding consensus sequences (prom, red and orange bars) or for pEGFP::NHR-25 vector (ctrl, dark or light green bars). NTC: no template negative control. (+):ChIP samples; (-): input chromatin. Background correction method: Object average.

PCR products were amplified over 8000 unit level with control primers (amplify a vector region of pEGFP::NHR-25) in all samples (see bars in green) except in no pEGFP::NHR-25 transfected samples (Fig. 4.5, lanes 21-24). When cells were transfected with vectors carrying either 2x or 4x of intact or mutated SF-1 binding sites (2x sf-1, 4x sf-1 or 2x sf-1*, 4x sf-1* respectively) in their promoter regions, PCR products with specific primers that amplify SF-1 binding consensus region were amplified with over 12000 unit level (input samples, see orange bars). When the samples were immunoprecipitated with anti-

GFP antibody (red bars), the products were enriched in the samples with 2x sf-1 and 4x sf-1 (lanes 5 and 13 respectively) while there was no enrichment seen in the samples with mutated SF-1 binding sites (lanes 1 and 9). The ratio of ChIP over input samples were shown in Fig. 4.6. The enrichment is evident in samples transfected with intact SF-1 binding sites (lanes 3 and 7). No difference in enrichment between 2x sf-1 and 4x sf-1 was seen (Fig. 4.7).

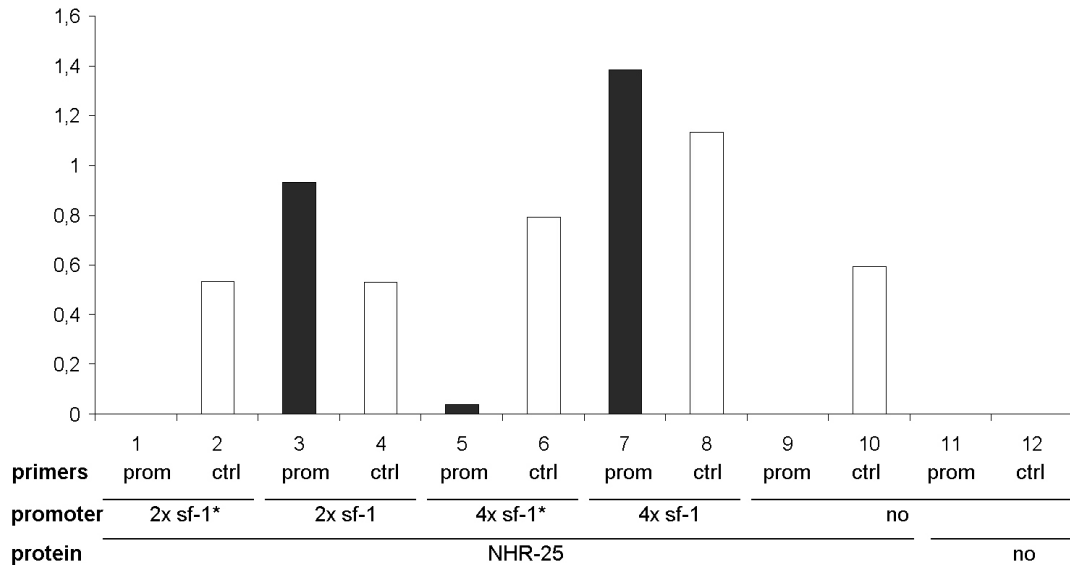


Fig. 4.6. Enrichment of promoters carrying 2x sf-1 and 4x sf-1 by NHR-25. Each ChIP sample shown in Fig. 6 was normalized against its input sample and the ratio is indicated as Y-axis.

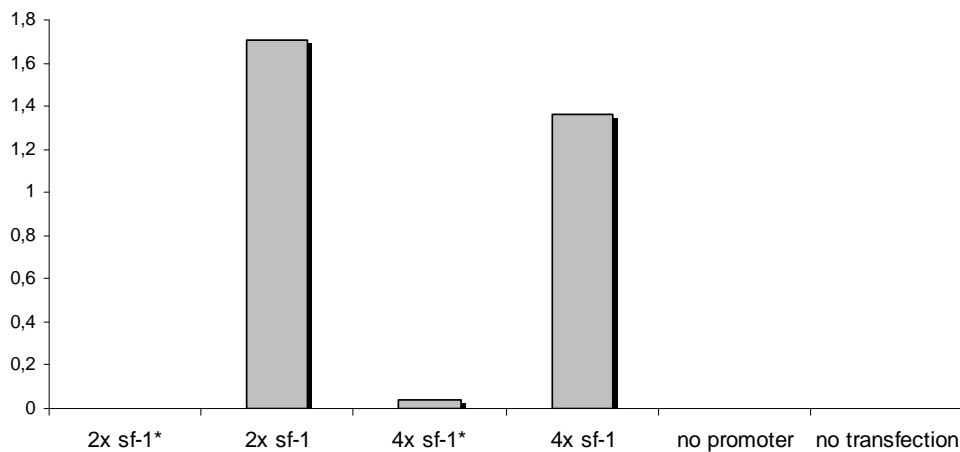


Fig. 4.7. Fold of enrichment after normalizing against control-primer data PCR data from ChIP for target promoter sequence were divided by data for non-specific target promoter (control) sequence.

4.3.2 Multiplex PCR analyses of ChIP I and II

Another round of ChIP, ChIP II, was performed. The procedure was similar to ChIP I except total amount of transfected DNA for each of two 75 cm² flasks was 12.5 µg. Chromatin from

both ChIP I and II was then analyzed by multiplex PCR for 28 cycles using specific primer sets for SF-1 binding consensus region (ChIPFw2, ChIPBw2) and negative control sequence (EGFPC2CtrlFw1, EGFPC2CtrlBw1, amplify a vector region of pEGFP::NHR-25) in one reaction. Input chromatin was diluted 1:1000 for ChIP I and 1:100 for ChIP II for the PCR reaction. We have noticed that the sequence in the construct containing four repeats of mutated SF-1 binding consensus (4x sf-1*) creates perfect palindrome and therefore significantly lowers the efficiency of PCR amplification. Therefore this construct was excluded for further analyses.

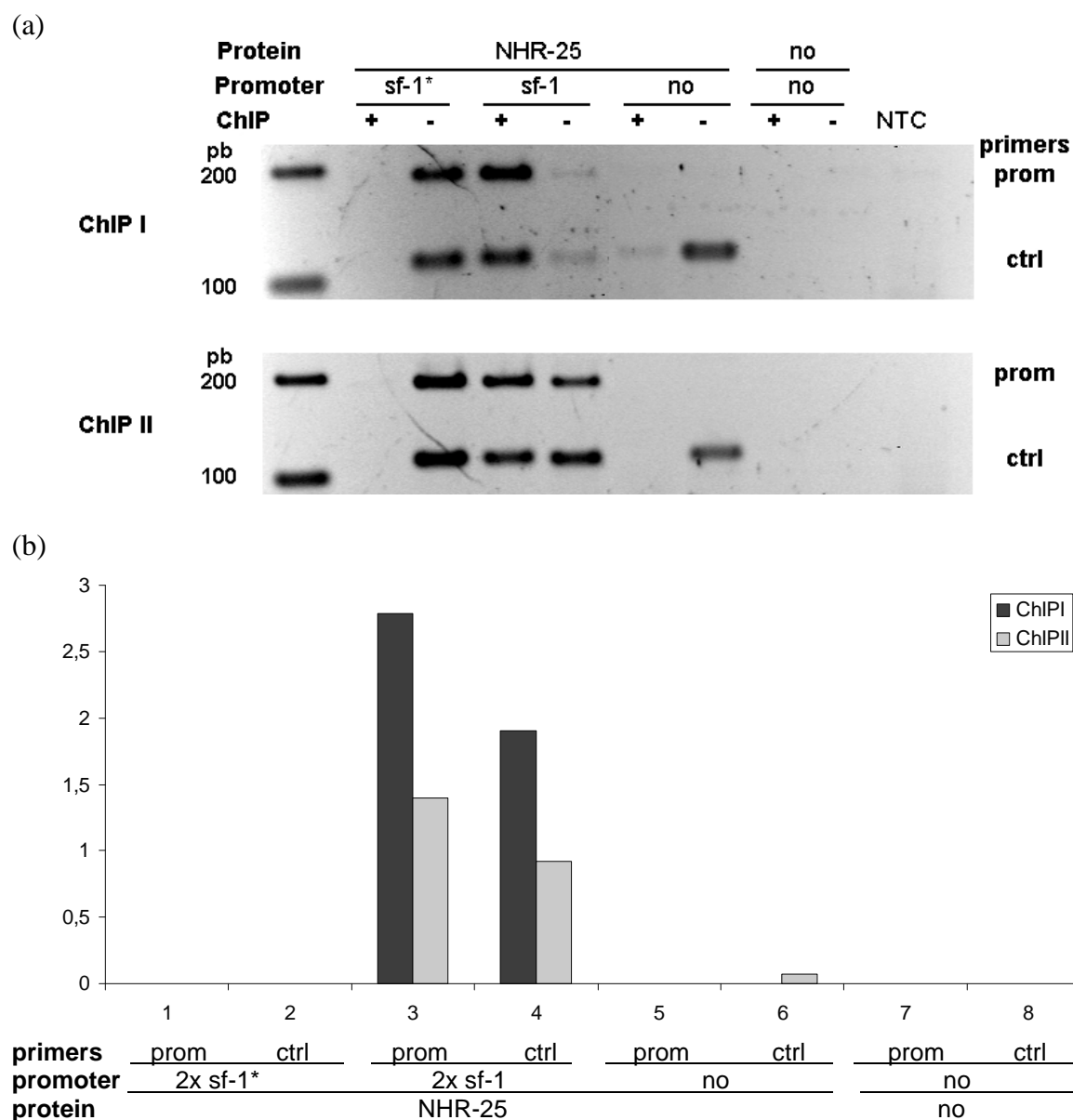


Fig. 4.8. Multiplex PCR analysis on two independent ChIPs

(a) Gel electrophoresis of PCR amplified samples from ChIP I and ChIP II. Top band corresponds to the product detecting a promoter carrying SF-1 binding sites (prom) and the bottom band corresponds to the product of non-specific primer (ctrl, detecting pEGFP::NHR-25 vector). (b) Ratio of enrichment normalized against input DNA. Quantification was made using ImageQuant™ TL software. (+): ChIP sample, (-): input sample.

There were no product specific to the promoter carrying SF-1 binding sites amplified when neither pEGFP::

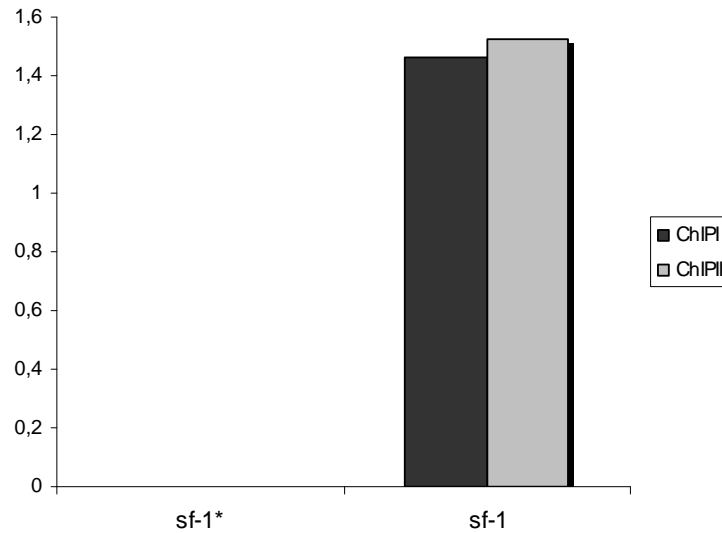


Fig. 4.9. Fold of enrichment of sf-1* or sf-1 binding consensus sequence after normalization against negative control sequence (pEGFP::

4.3.3 Real-time PCR analysis of ChIP III samples

To have more precise results, we decided to confirm data with real time PCR. For this, another round of ChIP was performed (ChIP III). HEK293 cells were transfected in a similar way as ChIP I and ChIP II experiments except Lipofectamine 2000TM was used as a transfection reagent and only one 75 cm² flask per set was transfected with 30 µg of total DNA. The ratio of transfected plasmid constructs was 1:3 for plasmid containing target sf-1 or sf-1* binding consensus sequence to NHR-25 expression vector (encoding EGFP::f, GAPDHreal_r) region was used as a negative control. Data

obtained from qPCR were analyzed in Rotor-Gene 6 software, normalized with NoAb sample, with input DNA and with negative control. Standard deviation reflects differences in parallels in qPCR.

In the experiment with monoclonal α GFP antibody, the enrichment of mutated sf-1* consensus sequence was stronger than intact sf-1 samples. However enrichment of non-specific sequence (ctrl) was also seen in the sf-1* sample while there was no such background in the sample with intact SF-1 binding sites (Fig. 4.10). When the chromatin samples were precipitated with three different concentrations of polyclonal α GFP antibody (1, 3 or 20 μ l of the antibody), each showed different results (Fig. 4.11). Nonetheless when these data were normalized with control samples, the enrichment of promoter containing intact SF-1 binding sites was seen in all samples (Fig. 4.12). Among three concentrations of polyclonal antibody testes, 3 μ l gave the best result.

ChIP III using mouse monoclonal α GFP antibody

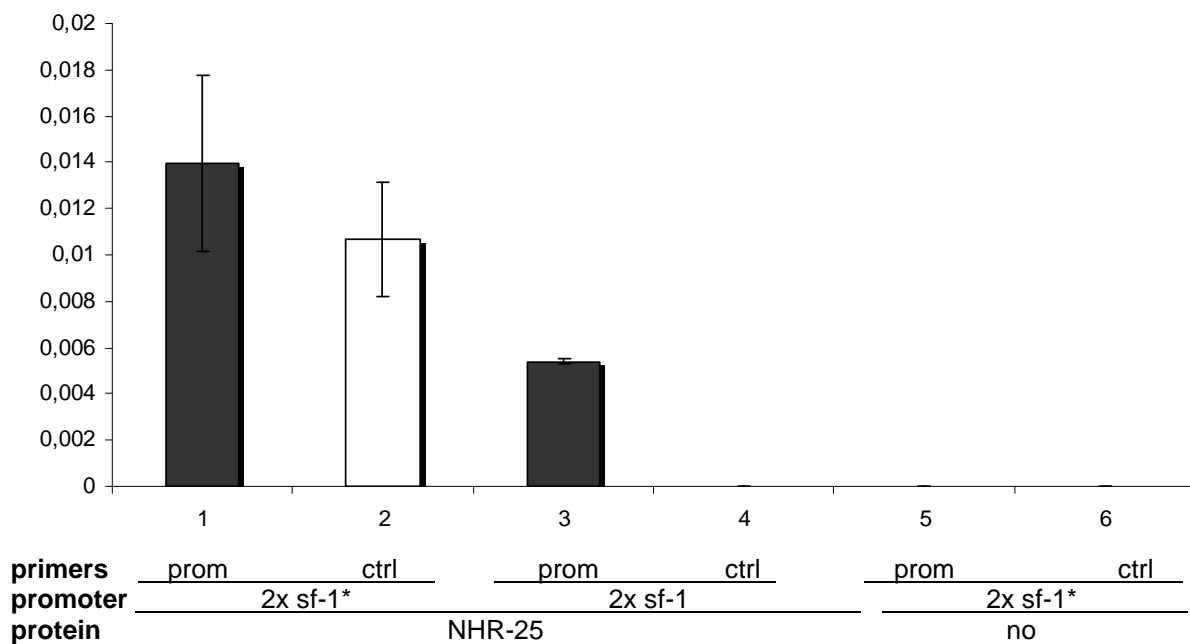


Fig. 4.10. qPCR analysis on ChIP III (IP with monoclonal α GFP antibody) samples after normalization against input DNA Chromatin was amplified with either primers for SF-1 binding consensus sequences (prom) or for GAPDH promoter region (ctrl). 2x sf-1: promoter containing two intact SF-1 binding sites, 2x sf-1*: promoter containing two mutated SF-1 binding sites, NHR-25: transfected with pEGFP::NHR-25 plasmid

ChIP III using rabbit polyclonal α GFP antibody

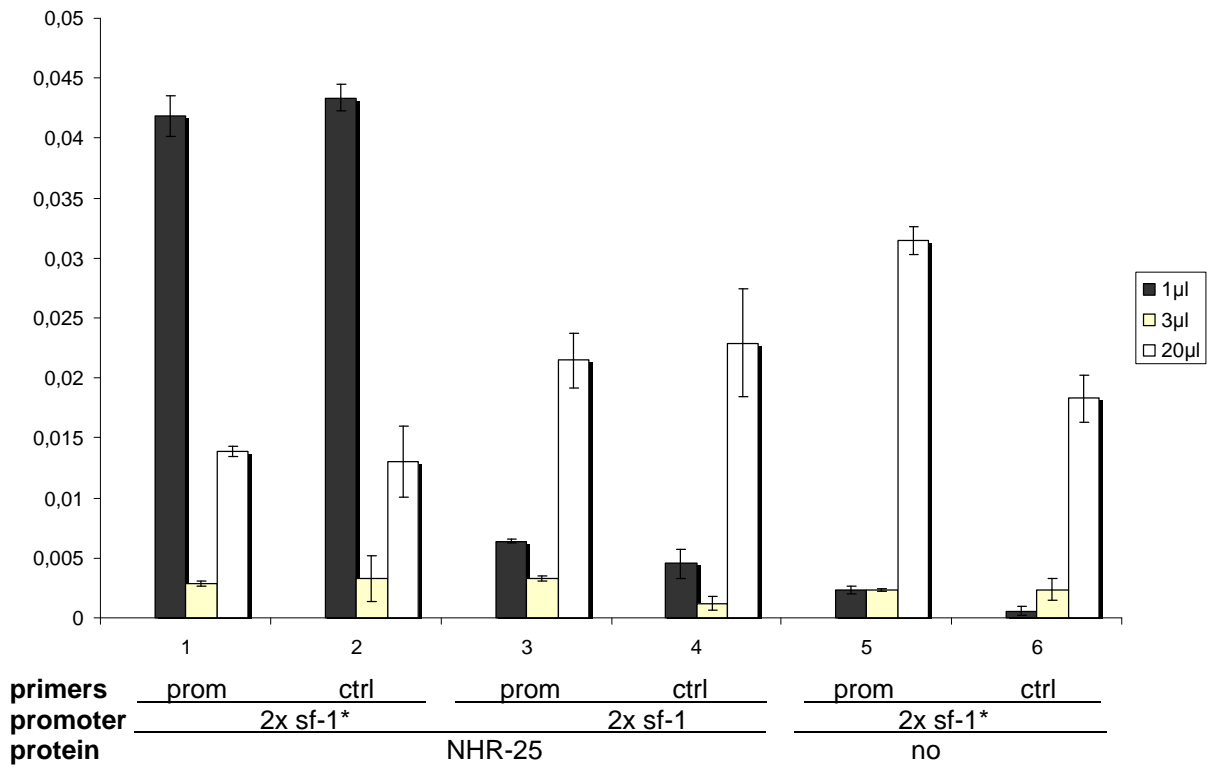


Fig. 4.11. ChIP with three different concentrations of rabbit polyclonal α GFP antibody Chromatin sample was incubated with either 1, 3 or 20 μ l of polyclonal α GFP antibody. ChIP samples were amplified with either primers for sf-1 binding consensus sequences (prom) or for control GAPDH promoter region (ctrl). qPCR data was normalized against input DNA for each sample. 2x sf-1: promoter containing two intact SF-1 binding sites, 2x sf-1*: promoter containing two mutated SF-1 binding sites, NHR-25: transfected with pEGFP::NHR-25 plasmid

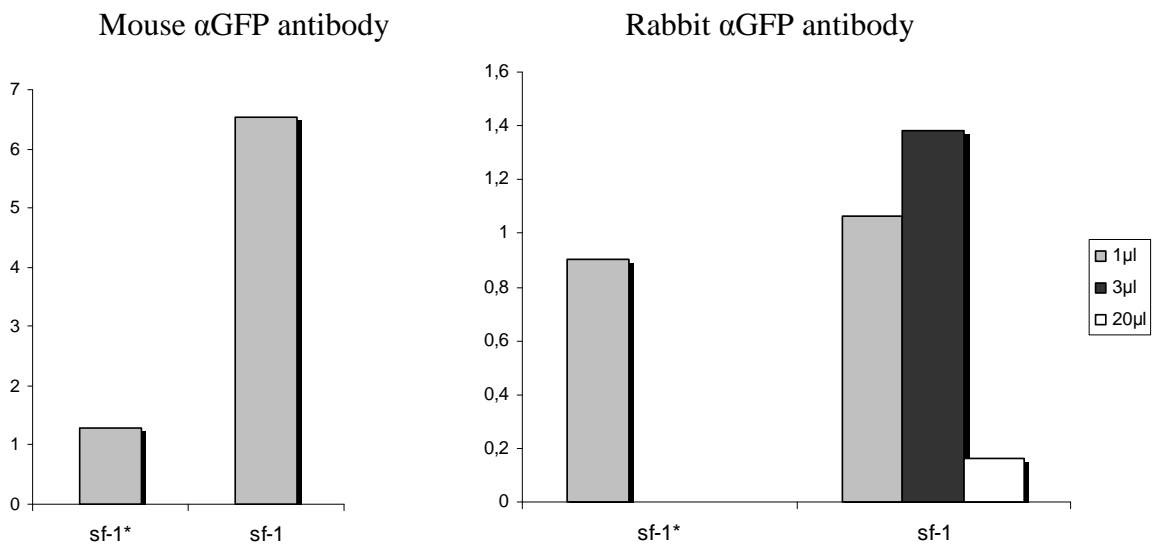


Fig. 4.12. Summary of ChIP III. Double normalization against input and negative control sequence was used.

4.3.4 Cyclin E

Based on preliminary data, Cyclin E might be the possible target of NHR-25 (Asahina, Rynes, Korinek, unpublished data). Therefore two possible binding sites for NHR-25 in Cyclin E promoter region were tested in ChIP reaction. Either a plasmid carrying intact NHR-25 binding sites (pJR102) or mutated sites (pJR111) were co-transfected with pEGFP::GFP plasmid. Cyclin E ChIP procedure was similar to ChIP II mentioned above and was immunoprecipitated with mouse monoclonal α GFP antibody. Chromatin sample was then amplified with specific primers for cyclin E promoter (CycEpromFw1, CycEpromBw1) and control sequence (GAPDHrealf, GAPDHrealr) in real time PCR reaction as described above. Data were then normalized against input DNA.

There seems to be a higher enrichment with intact cyclin E promoter (2x bs) compared to promoter containing mutated binding sites (2x bs*). However the similar enrichment was seen with control primers (Fig 4.13). Therefore this result is not conclusive.

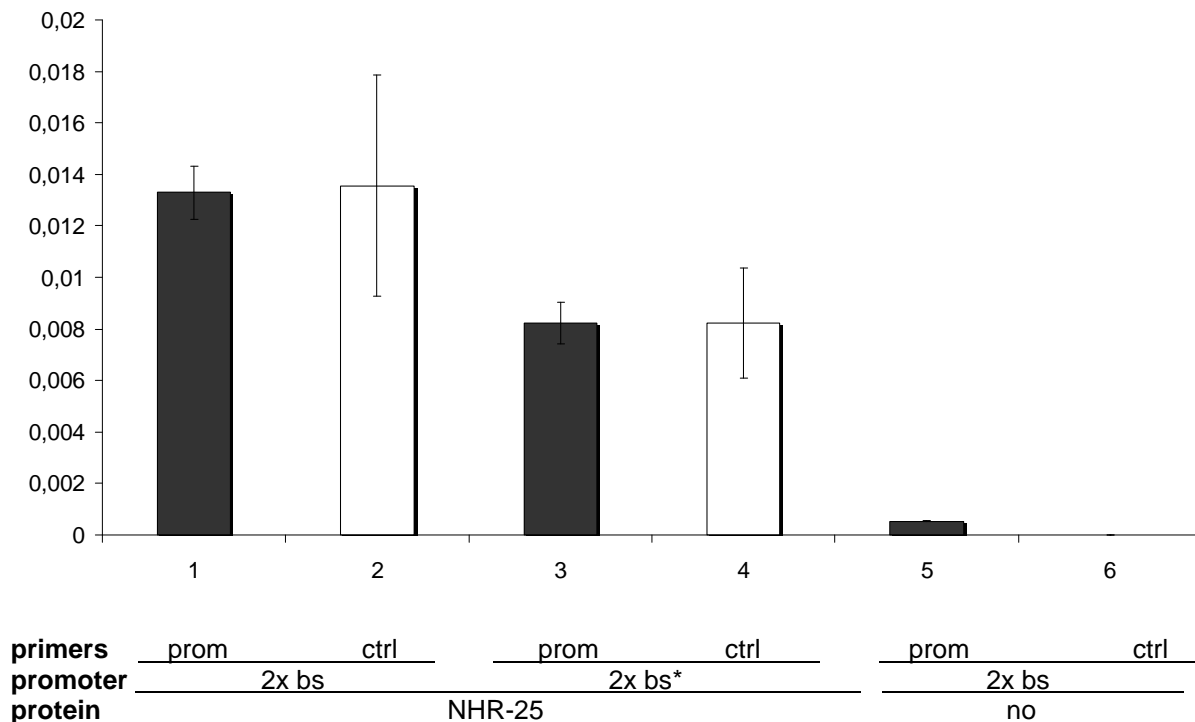


Fig. 4.13. ChIP for cyclin E promoter analyzed with qPCR

Chromatin sample was amplified with either primers for potential NHR-25 binding sites sequences (prom) or for non-specific region in pEGFP::NHR-25 vector (ctrl). Cells were transfected either with plasmid construct containing both potential binding sites (2x bs) or with potential binding sites mutated (2x bs*).

4.4 GFP protein pull down from transgenic *C. elegans* strain.

As a preparation for CHIP directly in *C. elegans*, we first tried to test whether GFP expressed in the transgenic worms can be pulled down with α GFP antibody. We have utilized the transgenic worm strain B4 containing *mbf-1::gfp* under ubiquitous *mbf-1* promoter. Worms were grown on approximately twenty 10cm plates and harvested. Worms were first cross-linked with formaldehyde and sonicated. For one set of protein pull down, 100 μ l of worm lysate was used. For the second one, 50 μ l diluted lysate in 50 μ l of deionized water was used. GFP protein was pulled down from the lysate using rabbit polyclonal α GFP antibody. Pull down with unrelated rabbit polyclonal α Met (*Tribolium castaneum*) antibody was performed as a negative control. Proteins were separated by SDS-page and processed on western blotting. Membranes were incubated with antibodies and detected using enhanced chemiluminescence technique. Incubation only with secondary antibody was served as a control. Polyclonal (rabbit) α MBF-1 antibody against *Drosophila melanogaster* protein was used for verification of specificity.

GFP protein from transgenic *C. elegans* strain was successfully pulled down with rabbit polyclonal α GFP antibody (Fig. 4.14). Mouse monoclonal α GFP antibody detected two bands [left membranes in (a) and (b)]. These bands seem to be specific as there were no band seen when the same membrane (after stripping) was incubated with secondary antibody, α Mouse HRP without incubation with primary antibody [right membrane in (a)]. Lower band corresponds with size to fusion protein MBF-1::GFP and heavier band probably represents dimer of this fusion protein. These bands are weaker in samples precipitated with α Met antibody, but they are still visible probably because of some nonspecificity of this antibody. Incubation with secondary α Rabbit antibody shows heavy chain of rabbit antibody used for protein pull down [middle membranes in (a) and (b)]. α MBF-1 antibody made against *Drosophila* MBF-1 protein did not seem to recognize MBF-1 from *C. elegans*.

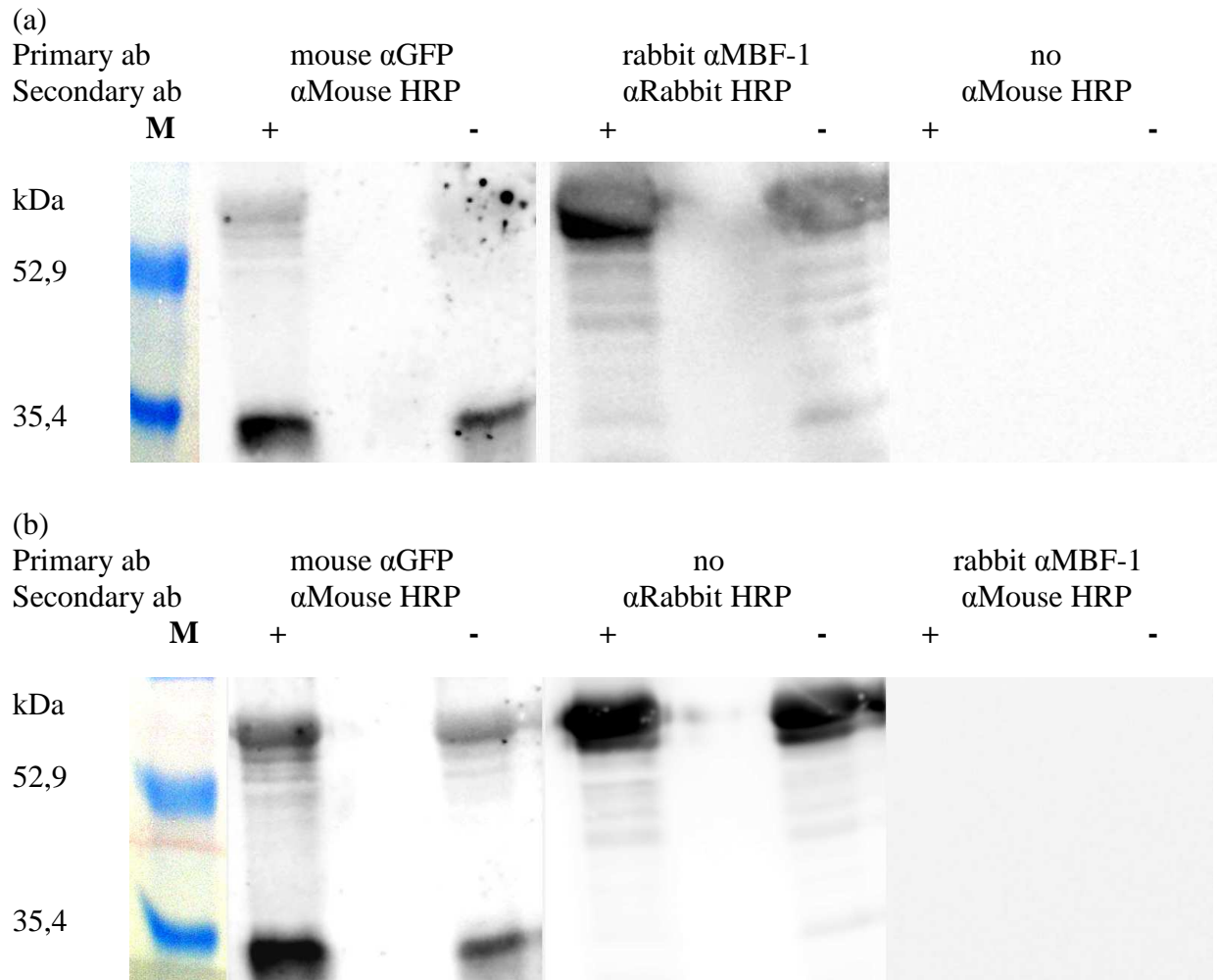


Fig. 4.14. Pull down of GFP from worms

GFP protein was pulled down from transgenic worms carrying MBF-1::GFP. (a) 100 μ l of worm lysate (b) 50 μ l of worm lysate diluted in 50 μ l of deionized water. M: protein marker, +: protein pull down with rabbit polyclonal α GFP antibody, -: control, protein pull down with unrelated rabbit polyclonal α Met (*Tribolium castaneum*) antibody.

5. Discussion

Nuclear receptors regulate vitally important processes in metazoans, thus our deep knowledge of physiology and development depends on identifying their target genes. Chromatin immunoprecipitation is rapidly spreading method for investigation of protein-DNA interactions often used for identification of nuclear receptor target genes (reviewed in Deblois & Giguere, 1999; Tavera-Mendoza et al., 2006).

In this study we performed several ChIP experiments with fusion protein EGFP::NHR-25 and SF-1 consensus binding sites in mammalian cell culture (HEK293) environment. Using cell cultures transiently transfected with both investigated target sequences and protein of interest is rare technique which requires many optimization steps (Luo et al., 1998; Wells & Farnham, 2002; Belyaev et al., 2004; Kobayashi, 2006). Model situation of NHR-25 binding to 2x sf-1 sequence with mutated constructs 2x sf-1* as the negative control (Asahina et al., 2006) served for investigation of suitable antibodies and establishment of ChIP in our laboratory. Fusion protein EGFP::NHR-25 was selected because of good available α GFP antibodies. Rabbit antiserum against a portion of ligand binding domain of NHR-25 (Asahina et al., 2000) was not tested for ChIP as there were problem with high background when this antibody was used in western blotting (data not shown). For ChIP we tried both Mouse monoclonal α GFP Ab (Sigma, G 6539) and Rabbit polyclonal Ab (Lukas et al., 2009). Two methods of HEK293 cells transfection were optimized.

Transfection with PEI is very cheap and efficient method of plasmid DNA delivery into mammalian cells (Fig. 4.2) (reviewed in Lungwitz et al., 2005). After selection of desired PEI type, the most efficient pH of working solution and PEI to DNA ratio must be experimentally found for each PEI batch (reviewed in Guillem & Alino, 2004; Bryja V., personal communication). Transfection with LipofectamineTM 2000 is more expensive, but simple and efficient. After optimization of transfections, similar efficiencies for PEI and LipofectamineTM use were observed (Fig. 4.2; data for LipofectamineTM not shown). For establishment of new method in our laboratory we decided to perform ChIP experiments using ChIP-IT[®] Express Enzymatic kit (Active Motif). Enzymatic shearing of chromatin (N-ChIP) was selected over X-ChIP to avoid uneven processing of samples and losing material during sonication. Advantage of optimized procedure of ChIP-IT[®] Express Enzymatic kit is omitting phenol-chlorophorm extraction and ethanol precipitation before PCR analysis of

DNA, as this procedure can also unequally alter amount of DNA in different samples. ChIP-IT[®] Control Kit – Human is also available for testing of ChIP procedure. Control kit is supplied with α RNA Pol II Ab and α IgG Ab which serves as a negative control. RNA Polymerase II is widely used positive control for ChIP (Sandoval et al., 2004). Control primers in this kit are designed for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter region, which is housekeeping gene with constitutive expression and thus is bound by RNA Pol II (Foss et al., 1994). GAPDH region was significantly enriched with α RNA Pol II, while there was no enrichment by control Ab as IgG does not bind DNA (Fig. 4.1).

5.1 ChIP

Conventional PCR was used for first analysis of ChIP samples (Fig. 4.5). After two most common types of data normalization were used (Haring et al., 2007) we got promising result showing binding of NHR-25 to 2x sf-1 and 4x sf-1 binding sites (Fig. 4.6 and 4.7). Level of negative control sequence immunoprecipitated during ChIP procedure was also higher than had been expected (Fig. 4.5 and 4.6). One possible explanation for negative control signal is nonspecific enrichment of highly abundant sequence, which is connected with amount of transfected plasmid DNA (Bruce A., personal communication). It is also possible, that high background volume is connected with usage of PEI as a transfection reagent. PEI-DNA complexes are stable at least for 4 hours after the transfection (Godbey et al., 1999) and while the precise mechanism of plasmid DNA transport after transfection with PEI remains unclear (Lungwitz et al., 2005) there is possibility that DNA is still in complex in the time of cell fixation (36-48 hours from transfection). In that case transfected plasmids can become fixed together and negative control DNA could be precipitated together with specifically enriched binding sites. This hypothesis is supported by the fact that we usually don't see negative control band without the enrichment of binding sites.

To lower unspecific binding of abundant sequence, we lowered amount of DNA transfected to the cells for ChIP II. To decrease number of total PCR reactions performed, we established multiplex PCR with two pairs of primers (promoter and control) in one reaction (Fig. 4.8a). Number of cycles was lowered to 28 and input DNA was diluted 1:100 or 1:1000 to avoid saturation of the PCR reaction. After careful control of sequences we have find out, that construct containing 4x sf-1* creates perfect palindrome and its PCR amplification rate is significantly lowered. Therefore we excluded 4 times repeats of SF-1 binding sites from further experiments. After normalization of band densitometry data (Fig. 4.8a) we confirmed

binding of NHR-25 to 2x sf-1 compared to mutant version 2x sf-1* where no binding was observed (Fig. 4.8b and 4.9). However presence of high background level persisted.

Therefore we decided to switch to Lipofectamine transfection method (ChIP III). In further experiments, DNA samples were analyzed with qPCR and genomic GAPDH region was used as a negative control sequence. In ChIP III set of immunoprecipitations both Mouse monoclonal α GFP Ab and Rabbit polyclonal α GFP Ab was used because monoclonal and polyclonal antibodies may significantly vary in recognition of target epitope or epitopes of protein of interest crosslinked to chromatin. Important control - NoAb sample was also introduced for estimation of immunoprecipitation background level. All qPCR data obtained with specific antibodies were lowered with corresponding NoAb values. After qPCR analysis of ChIP with mouse Ab and data normalization with against input DNA, enrichment of mutated 2x sf-1* binding sites was higher than for 2x sf-1 binding sites. In mutated binding sites sample also negative control was high while 2x sf-1 signal, although lower, was cleaner with no background (Fig 4.10).

Different amounts of rabbit antibody used in following precipitations showed inconsistent data while qPCR data normalized only against input (Fig. 4.11). With increasing amount of rabbit antibody nonspecific immunoprecipitation was growing and signal was the highest in control cells with no NHR-25, therefore background level exceeded specific signal. One microliter of Ab wasn't probably enough for specific recognition of signal. When we used double normalization (against input and against negative control sequence) enrichment of untouched 2x sf-1 sequence compared to mutated 2x sf-1* was seen for all immunoprecipitations with both monoclonal and polyclonal antibodies (Fig. 4.12). Middle concentration of rabbit polyclonal antibody (3 μ l) gave the best signal to noise ratio.

ChIP with Cyclin E promoter seems to be showing enrichment of sequence with 2x bs compared to lower signal in mutated 2x bs*. Together with target sequence is also enriched negative control sequence (Fig. 4.13). This is again supporting the hypothesis about persisting complexes of transfected constructs as cells for Cyclin E ChIP were transfected using PEI. For further investigation of NHR-25 and cye1 promoter in transfected cells Lipofectamine can give better result. If this interaction, previously shown in luciferase reporter assay (Asahina, Rynes, Korinek, unpublished data) is verified with ChIP it would be first evidence of NHR-25 gene target.

5.2 GFP pull down

As a first step of preparation for ChIP directly in worms GFP protein pull down from transgenic *C. elegans* strain was performed (Fig. 4.14). We used transgenic worm strain expressing MBF-1::GFP fusion protein under *mbf1* promoter. Protocol for ChIP directly in worms was followed until the immunoprecipitation step and contained formaldehyde crosslinking (Lee et al., 2006). Crosslinking step not only alters recognition of target protein by antibodies, but also makes worm lysis more difficult. Pull down of MBF-1::GFP fusion protein with rabbit polyclonal α GFP antibody was successful and with mouse monoclonal α GFP we detected two specific bands on western blotting. Light band corresponds with size to MBF-1::GFP fusion protein (17 kDa + 27 kDa) and because protein-protein interactions can be crosslinked with formaldehyde (Jackson, 1978) and GFP dimerizes, heavy band probably represents dimer of this fusion protein (Yang et al., 1996). Presence of these bands in negative control pull down can be explained with nonspecificity to highly abundant protein as *mbf1* promoter is highly active (Asahina, unpublished data).

Following step for ChIP on worms is cloning of *egfp::nhr-25* with appropriate promoter and preparation of transgenic worms. Promoter selection for ectopic *nhr-25* expression in *C. elegans* is crucial step because overexpression of this gene is lethal (Asahina et al., 2000). First attempts of worm microinjections to manage the technique were already done (Fig. 2.3).

In this study we established ChIP with fusion protein EGFP::NHR-25 and SF-1 binding consensus sequences in transiently transfected HEK293 cells. Immunoprecipitation with both monoclonal and polyclonal antibody was successful, although further optimizations of ChIP procedure are necessary and once optimal conditions are found, transfections, ChIP and qPCR should be performed in triplicates. As a control of NHR-25 binding in ChIP we have prepared construct for expression in mammalian cells containing mutant version *nhr-25(ku217)*. Allele of *nhr-25(ku217)* was obtained through mRNA isolation from mutant worm strain and RT-PCR and cloned. Mutation *ku217* affect DBD of NHR-25 and disrupts its DNA binding capability (Chen et al., 2004).

For genome-wide study of NHR-25 gene targets performing ChIP on chip or ChIP-seq in collaboration with ChIP-experienced laboratory would be the best choice.

6. Conclusions

1. Chromatin immunoprecipitation with NHR-25 and SF-1 binding consensus sequence in transiently transfected mammalian cell culture was established.
2. ChIP samples (ChIP I and II) were amplified by conventional PCR and multiplex PCR and analyzed by gel band densitometry. ChIP III samples were also analyzed with qPCR. Binding of NHR-25 to 2x sf-1 binding sites was higher than binding to mutated 2x sf-1* version. Further optimization is needed for lowering the background level in control samples.
3. Fusion protein MBF-1::GFP pull down from transgenic worm strain was successful fulfilling the first step for preparation of ChIP in *C. elegans*.

7. References

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8. Supplementary information

8.1 Plasmid constructs

Luciferase constructs containing 2 or 4 sf-1 binding sites or its mutated versions sf-1* were used for CHIP of NHR-25 (a gift from M. Van Gilst, Fred Hutchinson Cancer Research Center, Seattle, WA; Van Gilst et al., 2002; Asahina et al., 2006).

The pEGFP-C2 plasmid (Clontech) was used for expression of full-length NHR-25 N-terminally fused with EGFP (Asahina et al., 2006).

Cye-1 promoter was cloned into the vector pGL3basic (Promega), construct pJR102 contains 2 potential NHR-25 binding sites and construct pJR111 contains mutation in both sites (Rynes, J., unpublished data).

Vector pK-Myc-C2 was obtained from V. Korinek (IMG, Prague).

8.2 Primers used for CHIP analyses

Negative control sequence primers (ctrl)

GAPDHreal_f (FhGAPDH#1198real): 5'- **CACCACACTGAATCTCCCCT** -3'

GAPDHreal_r (RhGAPDH#1252real): 5'- **CCCCTCTTCAAGGGGTCTAC** -3'

Amplicon size: 54 bp

(Sequence provided by Fafilek B., IMG, Prague)

EGFPC2CtrlFw1: 5'- **CGATTCCACCGCCGCCTTC** -3'

EGFPC2CtrlBw1: 5'- **CTAGGGTGGGCGAAGAACTC** -3'

Amplicon size: 114 bp

NHR-25FW7: 5'- **CGCTTACGACTCACTACGGTA** -3'

Nhr25full-R: 5'- **TTGGATCCTTATGATGCCATGTACGGCACA** -3'

Amplicon size: 225 bp

Target sequence primers (prom)

sf-1 binding consensus sites

ChIPFw2: 5'- **TTGTAAAACGACGGCCAGTG** -3'

ChIPBw2: 5'- **CGGAATGCCAAGCTACTCG** -3'

Amplicon size: 218 bp (4x sf-1); 195 bp (2x sf-1)

cye-1 promoter

CycEpromFw1: 5'- TCTAGAGACCATATGACGGAG -3'

CycEpromBw1: 5'- GCACGATACTTTCTATGCTTG -3'

Amplicon size: 181pb

8.3 Sequences of binding sites

sf-1 bindind sites

2xTCT (2xsf-1*) CCA**TGACCTA GATTATTAAT GACCTAGA**GA TCT----- -----
 GCTG

2xTGA (2xsf-1) CCA**TGACCTT CATTATTAAT GACCTTCA**GA TCT----- -----
 GCTG

4xTCT (4xsf-1*) CCA**TGACCTA GATTATTAAT GACCTAGA**GA TC**TCTAGGTC ATTAATAATC**
TAGGTCACTG

4xTGA (2xsf-1) CCA**TGACCTT CATTATTAAT GACCTTCA**GA TC**TCAAGGTC ATTAATAATC**
AAGGTCACTG

various versions of sf-1 binding site

PCR Fragment amplified with ChIPFw2 and ChIPBw2 primer pair

ChIPFw2 →

TTGTAAACG ACGGCCAGTG CCAAGCTATA GCGGCCA**TGA CCTAGA**TTAT TAA**TGACCTA**
GAGATCTCTA GGTCATTAAT AAT**TCTAGGTC A**CTGCAGGTC GACTCTAGAG GATCTCGGTA
 TTTAAGGAGC TCGAAGGTC CAAGTCATGC ATTATTGTCT CAGTCGCAGT TGTCAGTTGC
 AGTTAGCAGA CGGGCTA**CG AGTAGCTTGG CATTCCG**

← ChIPBw2

cye-1 promoter

1 CGCGTCGATT GCCTGCGTCT CTGTGTCAAG AGGCACCCTC TCATCTCTCT GGAGACGGCA
 61 GAGAAACATG AGATCGTGGT AAGACCATCA GGGAGGACGG CGGCAAATCG GAGGGAACGC

CycEpromFw1 →

121 GCGAAATGAG CGCGCGATTT GAA**TCTAGAG ACCATATGAC GGAG**GTTGTC TGTAGT**TGAC**
 181 **CTT**TTTTCGGT TTTATCTGAA CTTTCACAGA TTGATTGCGT AATGTCCACT AGACATTCGT
 241 TATGGTATAA TTGATATTTT TTTATTAATT TTCATTAAAT ATCAGATATC AATCTCTAAA

← CycEpromBw1

301 TTT**CAAGCAT AGAAAGTATC GTGC**AAACAT GAACAGTTTC ATGAGCTCAG TTTATATTTT
 361 AAATTCACAC CTAATTCAA TCTTAAATAA TTTTAAATGG AATAACTGCA AATCTAAATC
 421 TAAAACCAA AC**GACCTT**CA TTCACTTGTA CCAAATCTTA TCTGATTTTA TGAATTTAAA

481 ATCTTATTCT TCTTCACCTC TGACTGATAG TTTATGTTCT CCCTATTGTG TACACTCAA

NHR-25 binding site

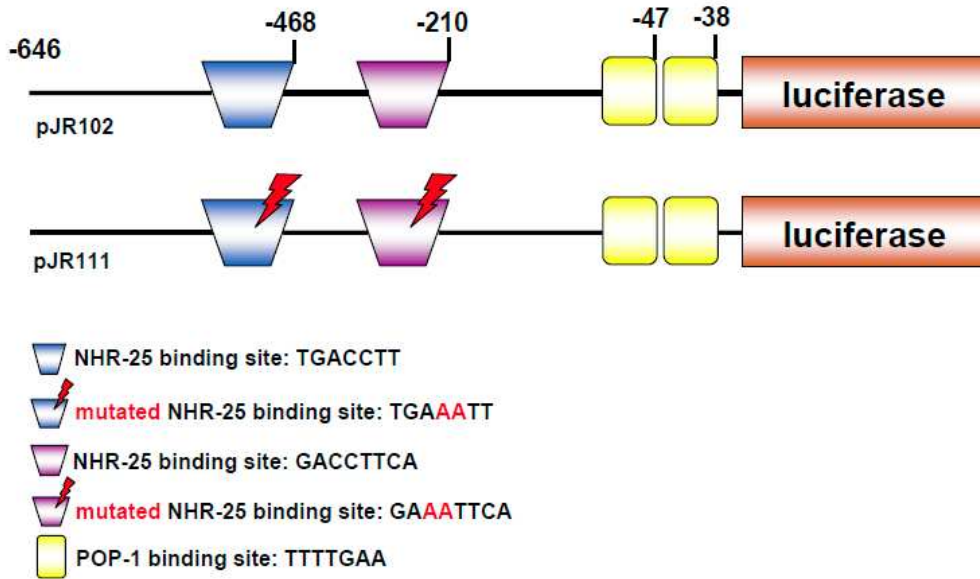


Fig. 8.1 Cye-1 promoter construct in pGL3basic vector. (fig. adapted from Hajduskova, M., 2009)

8.4 Sequence of pEGFP::NHR-25 construct with marked position of control primers

tagttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccg
 cgttacataacttacggtaaattggcccgctggctgaccgccaacgacccccgccatt
 gacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtca
 atgggtggagatatttacggtaaactgcccaacttggcagtagatcaagtgtatcatatgcc
 aagtacgccccctattgacgtcaatgacggtaaattggcccgctggcattatgccagta
 catgacctatgggactttcctacttggcagtagatcaatggggcgtggatagcgggttgactcacggy
 atttccaagtctccaccctattgacgtcaatggggagttgttttggcaccacaaatcaacg
 ggactttccaaaatgtcgtacaactccgccccattgacgcaaatgggcggtaggcgtgt
 acgggtgggaggtctatataagcagagctgggttagtgaaccgtcagatccgctagcgtca

EGFP

ccggctgccaccatgggtgagcaagggcgaggagctgttcaccgggggtggtgcccatcctg
 P V A T M V S K G E E L F T G V V P I L
 gtcgagctggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggc
 V E L D G D V N G H K F S V S G E G E G
 gatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtg
 D A T Y G K L T L K F I C T T G K L P V
 ccctggccccaccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctacccc
 P W P T L V T T L T Y G V Q C F S R Y P
 gaccacatgaagcagcagcagcttcttcaagtcgcccattgcccgaaggctacgtccaggag
 D H M K Q H D F F K S A M P E G Y V Q E
 cgcaccatcttcttcaaggacgacggcaactacaagaccgcccggagggtgaagttcgag
 R T I F F K D D G N Y K T R A E V K F E
 ggcgacaccctgggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaac
 G D T L V N R I E L K G I D F K E D G N
 atcctggggcacaagctggagtagaactacaacagccacaacgtctatatcatggccgac
 I L G H K L E Y N Y N S H N V Y I M A D

aagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagc
K Q K N G I K V N F K I R H N I E D G S
gtgcagctcgccgaccactaccagcagaacacccccatcgggcagcggccccgtgctgctg
V Q L A D H Y Q Q N T P I G D G P V L L
cccgacaaccactacctgagcaccagtcggccctgagcaaagaccccacgagaagcgc
P D N H Y L S T Q S A L S K D P N E K R
gatcacatgggtcctgctggagttcgtgaccgcccgggatcactctcggcatggacgag
D H M V L L E F V T A A G I T L G M D E

NHR-25

ctgtacaagtcggccggactcagatctcgagctcaagcttcgaattcatgactgacgtc
L Y K S G R T Q I S S S S F E F M T D V
gagaggatggttctgcgaccgaatcatgaaggcgagatgtgccgggtttgtggatgacga
E R M V L R P N H E G E M C P V C G D R
gtctctggatatcactacggccttctgacgtgtgaaagttgcaagggcttcttcaaagcgc
V S G Y H Y G L L T C E S C K G F F K R
actgttcaaaacaagaagcaatatcaatgctctgccgaagccaactgccacgtggatcgt
T V Q N K K Q Y Q C S A E A N C H V D R
acttgccggaagaggtgtccaagctgtcgattccagaagtgccctcacaatgggaatgaaa
T C R K R C P S C R F Q K C L T M G M K
atggaagcgggtgcgtgccgataggatgagaggtggacgaaacaagttcggatcgttttat
M E A V R A D R M R G G R N K F G S F Y
aagaaggatcgggctcatcgtatgcagagaaacgcaatgagagtgagcacagtgcaagtt
K K D R A H R M Q R N A M R V S T V Q V
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P A V L G A Q S Q A Q T F Y Q P P E H Q
gtttcaagcagcacaactgaccagaacaaccaaactcaactattttgaccaaactaaaatc
V S S S T T D Q N N Q I N Y F D Q T K I
aaaactgaatatacaaaactgagtatgatgcacatctccagagtccaacattgtccagt
K T E Y I K T E Y D A H L Q S P T L S S
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S T N Q Q L S V S D F I M R P G Y L V D
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P Q D S L A V L L G S T I D D P L L R H
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T F P A A Y Q L N E V K Q E P F D Y S E
cagttcattcatcactcattgcacgactatccaacttataccagcaacactaccaattat
Q F I H H S L H D Y P T Y T S N T T N Y
gctacaatgatgccaatgacaactgtttccagcacacaatccttggtcacttcgacttct
A T M M P M T T V S S T Q S L V T S T S
tctacaacaactggccgaatgacagaagctagcagcacatcaccaattctcccactttgc
S T T T G R M T E A S S T S P I L P L C
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P A P T E K T V D H F Y N S S I A E M C
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K T L P D D A Q I A R I F T S V K G T S
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K P E K H A F S I Q V A E E N L K D I V
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I W A K N D Q L F S K L S L D D Q M I L
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L Q T S W T T V H I V D I T N A M V H G
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N L L S Q Y K M S N G D E V P V G L V A
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L L G N Q T F V S S W N D V V I R L R N
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M G F T N F D Y C A F R F L A L F D Q S
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M D S F P A V S T A R S R V L Q S W R E

gtgagatgtactaccgcttttctggaattttcgaacaaatcagacgactcgcttacgac
V R C T T A F L E I F E Q I R R L A Y D
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S L R Y L W N L H S N C P T N W E Q F F
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P E A S L V L E M I R T T V N R S A S S
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S V T A I T Q V P A I Q L P T P Q A T Y

← Nhr25full-R

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