Molekularbiologische und Röntgenmikroskopische Charakterisierung der Heterochromatinproteine des Nematoden *Caenorhabditis elegans*

Dissertation
zur Erlangung des Doktorgrades
der Mathematisch- Naturwissenschaftlichen Fakultäten
der Georg- August- Universität zu Göttingen

vorgelegt von

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SHIRAZ (IRAN)

Göttingen 2001

D7

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Tag der mündlichen Prüfung: 30.10.2001

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1 Introduction

Many interphase nuclei exhibit condensed and intensely stainable regions That have been designated heterochromatin. It has been described, that heterochromatin frequently retains its density throughout the cell cycle. and it appears in centromeres or telomeres. During cell division, the nucleus disappears and chromosomes are completely condensed. Concomitantly, the subnuclear structures of heterochromatin are seem as part of chromosomes and appears in centromere and telomere regions. At the beginning of interphase the chromosomes decondense and appear as the interphase chromatin of the daughter nuclei. However, the heterochromatin remains in a condensed form, which was first observed by the botanist Emil Heitz (1928) who followed the chromosomes throughout the cell cycle. The condensed form of heterochromatin in subnuclear structures remained visible in the light microscope until the onset of the next mitosis. Such structures could be traced and observed during the condensation of chromosomes, whereas this unraveling process appeared like other chromosomal material. These structures were located at homolog sites in the two homologs chromosomes. Subsequently, heterochromatin was recognized as a phenomenon in both, animals and plants (Heitz, 1930).

A heterochromatic phenotype depends on two components: DNA sequences and specific proteins (Csink *et al.*, 1997). Heterochromatin contains highly repetitive DNA sequences, which can easily be characterized by restriction endonucleases (Lica and Hamkalo, 1983). Heterochromatin in comparison to euchromatin, which is widely enriched with unique coding DNA sequences, contains largely repetitive DNA elements (Miklos and Costell, 1990). It has been suggested that such repetitive DNA sequences can induce specific topological structures (Dorer and Henikoff, 1994). Because of distinct localization of heterochromatin in the cell nucleus, it has been proposed to have crucial roles in chromosome segregation and inheritance of cell type identities.

Heterochromatin appears as highly condensed chromatin, which is not transcribed and is late replicating with described functions in epigenetic gene silencing and in the structural organization of centromeres and telomeres. But one part of heterochromatin is composed of almost entirely highly repetitive DNA sequences, which are severely underreplicated in chromosomes and therefore virtually invisible (Gall, 1973). On the other hand in a variety of cell types has been described, that the centromeric heterochromatin is localized in a distinct domain on the nuclear envelope during interphase (Brown, 1966; Franke *et al.*, 1981). Interestingly, there is a part of heterochromatin, which is composed of both, middle repetitive elements and a few genes of euchromatin (Miklos and Costell, 1990). It appears, that the molecular structure of heterochromatin in the genome, whether in centromeres, telomeres, or in silenced genes is very similar (James *et al.*, 1989). However, heterochromatin proteins in comparison to euchromatin proteins are not only composed of histones. For better understanding of heterochromatin, it becomes necessity to know about proteins which interact within.

Master copies of heterochromatin transposons have been identified in X-chromosomal heterochromatin that are required for the maintenance of telomerase in *Drosophila*, an example for a biological function of a heterochromatic chromosomal region (Biessmann *et al.*, 1992).

The influence of heterochromatin structure on gene expression has been explored in position effect variegation of *Drosophila*. Such experiments make use of genetic analysis and cytological observation. A position effect is observed when the vicinity of normally active genes to transcriptionally inactive condensed heterochromatin leads to its repression. This effect varies from cell to cell so that the phenotypic of the tissue is variegated. This gene silencing occurs in *Drosophila* at the transcriptional level, and is normally correlated with a more heterochromatin-like cytological appearance of the site in polytene chromosomes (Zhimulev, 1996).

Several models for heterochromatinization of euchromatin have been proposed. For example, Zuckerkandl (1974) has suggested, that some DNA sequences achieved a quaternary structure with invasive properties like protein forming new heterochromatin-related structures. Another model has suggested, that there are heterochromatin nucleation centers which are located in euchromatin. When these centers loop back into the chromocenter aggregation and stable inactivation occur (Dorer and Henikoff, 1994). Such classic heterochromatin is *e.g.* the centromere of *Schizosaccharomyces pombe* (Lorentz *et al.*, 1994). Furthermore, it has been suggested, that during transfer of epigenetic centromeric information an initial direct contact of a chromosomal region is required with an existing centromeric region (Karpen and Allshire, 1997). This would mean that the existence of such regions in centromere could induce the formation of neocentromeric sites.

A relocation of telomeric heterochromatin DNA and satellite DNA from heterochromatin in *Drosophila melanogaster* has been found (Koryakov *et al.*, 1999), which clearly shows that dynamic transitions can occur between the both states of chromatin. Furthermore, a telomeric-induced silencing of adjacent genes in *Saccharomyces cerevisiae* has been established (Gottschling *et al.*, 1990).

An extreme example of heterochromatin is the inactive X-chromosome in female mammals (Jeppesen and Turner, 1993). This heterochromatic component was observed by M. L. Barr (Barr & Bertram, 1949) in human female interphase cells, which was called sex chromatin (Barr body). This heterochromatic component is absent in interphase nuclei of males. “Heterochromatinization” yields one of the both X-chromosomes in human females inactive. The silencing of this chromosome is caused by XARs (X-activation regulators), which are produced during early development (Penny *et al.*, 1996). XARs are required to

bind cooperatively to a regulatory site on the X-chromosome and that they determine X-chromosome remains active in subsequent cell generations.

Interestingly, heterochromatin elimination has been observed in the presumptive soma cells of several species of crustacean copepods. It occurs in *Cyclops divulsus* at the 5th cleavage, in *Cyclops furcifer* at the 6th and 7th , and in *Cyclops strenuus* as early as the 4th cleavage (Beermann, 1977). Furthermore, this diminution mechanism is obviously involved in site-specific recombination. Subsequently, this mechanism causes a release of circular DNA (Beermann, 1984). It has been suggested, that this mechanism may be analogous to that of prokaryotic DNA excision.

The virtually higher compact organization of heterochromatin led likely to imagine, that it has somehow a different organization in comparison to the euchromatin. However, electronmicroscopy images do not show any differences between high order chromatin in euchromatin and heterochromatin fibers (Traut, 1991).

The protein-DNA interaction was an intriguing topic which was investigated to elucidate the general features of chromatin structure (van Holde, 1989). Based on studies in the seventies, the DNA and nuclear proteins compose a chromatin complex, whereas the histone proteins are the major components of the complex. There are two types of histones, the core -and the linker histones. The core particle consists of histone octamer (Kornberg, 1974, Kornberg and klug, 1981). The DNA is coiled around a protein core containing the core particle. The structure of the histone octamer (H2A, H2B, H3 and H4)$_2$ around which DNA is wrapped, has been investigated as the nucleosome (Eickbush and Moudrianakis, 1978). The structural studies has shown the structural features of DNA associated with the core histones (Luger *et al.*, 1997). The core histones, around which the DNA is wrapped, and these are in two turns of a superhelix which has been called chromatosome (Simpson, 1978). For examination of

binding sites of both amino- and carboxy-terminal domain with DNA was used hydroxyl radical cleavage (Dong *et al.*, 1990; Hayes *et al.*, 1991). The protein-DNA cross-linking studies have included the presence of histone H4 interactions with DNA (Mirzabekov *et al.*, 1989; Pruss and Wolffe, 1993). Furthermore, a second feature of histone-DNA interaction was shown inside the nucleosome with histone H3 cross-linking (Bavykin *et al.*, 1990).

The histone H2A and H2B interact also with the DNA (Pruss and Wolffe, 1993). It has been shown, that the carboxy-terminal tail of H2A binds to DNA around the nucleosome (Gushchin *et al.*, 1991). Interestingly, it has been shown, that a higher concentration of a subtype of histone H2A, macroH2A, in the Barr body is correlate with the higher nucleosome density (Perche *et al.*, 2000). The core histones are strongly conserved in their sequences (Isenberg, 1979) and are present in every eukaryotic cell. A very intriguing interaction between histone H3 in mammalian was shown recently (Lachner *et al.*, 2001). Furthermore, it has been shown a histone-H3-like protein in *Caenorhabditis elegans* (Buchwitz *et al.*, 1999), which is necessary for the segregation of chromosomes during the mitosis. Furthermore, they identified the histone-H3-like protein as an identification for its requirement in centromeres of holocentric chromosome in *C. elegans*.

An additional characterization of protein associated with DNA in discrete histone-DNA complexes during digestion of chromatin with micrococcal nuclease revealed a fifth histone type the linker histone H1. Linker histones are highly variable in their primary structures, however, it has been shown, that there is possible to recognize conserved motifs by sequence analysis. But, there are distinct variants of linker histones during development of several the organisms including vertebrates (Poccia, 1986). The linker histone H1 of the eukaryote has three domains: amino- and carboxy-terminal domains are flanking a central globular domain. The globular domain protects the linker DNA around the nucleosome. The terminal domains of histone H1 have an interaction with

the linker DNA, but for high order DNA organization in the nucleosomes requires all three domains (Allan *et al.*, 1980). The further interaction of linker histones with other linker histones within adjacent nucleosomes led highly ordered chromatin fibers (Thoma *et al.*, 1979). It was shown, that individual chromosomes contain core histones that contain distinct types of linker histones (Mohr *et al.*, 1989; Schulze *et al.*, 1993; Grossbach, U., 1995; Wisniewski and Grossbach, 1996). Furthermore, it has been shown, that the histone H1 variants distinguish in their distribution in chromatin and interphase chromosome (Hoyer-Fender and Grossbach, 1988). More recently it was shown, that a single histone H1 isoform (H1.1) in *C. elegans* is essential for chromatin silencing and germline development (Jedrusik and Schulze, 2001).

The heterochromatin-associated protein1 (HP1) of *Drosophila* was initially described as a protein associated with the chromocenter of polytene chromosomes in larval salivary glands (James and Elgin, 1986). A molecular genetic study has revealed that HP1 is encoded by a dominant suppressor of position effect variegation (PEV), *Su(var)2-5* (Wustmann *et al.*, 1989). *Su(var)2-5* fulfils the genetic criteria of dosage dependency of structural protein of heterochromatin (Locke *et al.*, 1988). It functions as haplo-insufficient suppressor and triplo-abnormal enhancer of PEV in *Drosophila* (Eissenberg and Elgin, 2000). Another locus which was identified as a dominant suppressor of heterochromatic PEV is *Su(var)3-7*, which is also involved in gene silencing of *Drosophila* (Cleard *et al.*, 1997). The SU(VAR)3-7 protein contains seven zinc-finger motifs which possibly support a protein-DNA binding activity (Reuter *et al.*, 1990). It has been suggested that SU(VAR)3-7 and HP1 cooperate in building the genomic silencing apparatus associated with the heterochromatin (Cleard *et al.*, 1997), because they colocalize cytologically.

HP1 homologs have been identified in different species of the animal and plant kingdoms (Lorentz *et al.*, 1994; Huang *et al.*, 1999; Epstein *et al.*, 1992; Wreggett *et al.*, 1994; Singh *et al.*, 1991; Saunders *et al.*, 1993). Recently, Motzkus *et al.* (1999) has shown, that the mammalian HP1 homolog, murine-HP1 (M31), has a novel function in mammalian spermatogenesis.

The HP1 family proteins are relatively small proteins with molecular weights of 15-35 kDa (Eissenberg and Elgin, 2000). HP1 contains a carboxy-terminal chromo domain and a structurally related carboxy-terminal motif, the "chromo shadow" domain (Aasland and Stewart, 1995; Smothers and Henikoff, 2000). A hinge region between these motifs contains a conserved sequence block within the hinge contains an invariant sequence (KRK) and a nuclear localization motif (Smothers and Henikoff, 2000).

The chromo domain (*chromatin organization modifier domain*) has been identified as a region of 37 amino acids residues (Paro and Hogness, 1991). A chromo domain is present also in Polycomb, a protein required for maintaining homeotic genes repressed in regions of the *Drosophila* embryo where their expression would interfere with normal development. A molecular analysis has shown that chromo domain facilitates an interaction between the human HP1 homolog and the *inner centromere protein* (INCENP) (Ainsztein *et al.*, 1998). INCENP has been identified as a component of the mitotic chromosome scaffold, which is associated with the centromere in early metaphase but moves progressively to the spindle fibers and the plasma membrane at the cleavage furrow (Ainsztein *et al.*, 1998).

Three-dimensional structural analysis of murine HP1 showed that HP1 has a remarkable similarity to the histone-like archeobacterial proteins Sac7d and Sso7d but lacks the surface charge necessary for DNA binding (Ball *et al.*, 1997; Brasher *et al.*, 2000). According to this study, the chromo domain of the

murine HP1 homolog has an overall negative surface charge distribution and appears to be better suited for protein-protein interaction than for protein-nucleic acid interaction (Ball *et al.*, 1997). Furthermore, it has been shown that a mammalian HP1 homolog has a dosage-dependent effect on position effect variegation in transgenic mice (Festenstein *et al.*, 1999). On the other hand the human homolog of *Drosophila* HP1 is also a DNA-binding protein and contains a DNA-binding motif (Sugimoto *et al.*, 1996). It has been reported that HP1 family proteins undergo self-association (Epstein *et al.*, 1992; Le Douarin *et al.*, 1996; Ye and Worman, 1996) and that the chromo shadow domain is mostly involved in these interactions. It has been reported that the *Drosophila* HP1 can be multiply phosphorylated by serine/threonine kinases one of which is casein kinase II (CKII) (Zhao and Eissenberg, 1999). Finally, it has been suggested, that HP1 protein act as a bifunctional cross-linker which perhaps organizes a higher order chromatin structure by linking or anchoring chromatin subunits (Eissenberg and Elgin, 2000).

Interactions of proteins of the HP1 protein family with other nuclear proteins have been shown (Huang *et al.*, 1998; Pak *et al.*, 1997; Ainsztein *et al.*, 1998; Lachner *et al.*, 2001; Pyrpasopoulou *et al.*, 1996, Ye and Worman, 1996). The origin recognition complex protein (*orc2*) is associated with HP1 and affects HP1 expression in higher eukaryotes (Huang *et al.*, 1998; Pak *et al.*, 1997). HP1 localization in the heterochromatin of nuclei of diploid cells in *Drosophila* was disrupted in mutants (k43) of the ORC2 subunit (Huang *et al.*, 1998).

In vitro binding experiments have shown a physical interaction between the inner centromeric protein (INCENP) and the mammalian HP1 protein (Hsalpha), (Ainsztein *et al.*, 1998). M31, a mammalian HP1 homolog also has a specific biding site for the SET domain protein SU(VAR)3-9 (Aagaard *et al.*, 1999; Lachner *et al.*, 2001). *Su(var)3-9* is a suppressor of position effect

variegation (Wustmann *et al.*, 1989). SU(VAR)3-9 contains a chromo domain and a SET domain (Tschiersch *et al.*, 1994). They have demonstrated that, the SU(VAR)3-9, methylates histone H3 lysine 9 and thereby creates a binding site for HP1 proteins (Lachner *et al.*, 2001). These interactions have consequences for the interaction of the SU(VAR)3-9 HP1 complex with DNA and may plays a role in higher order chromatin.

Su(var)3-7, another modifier of PEV (Reuter *et al.*, 1990), may also interact with HP1 (see above). Finally, the lamin B receptor an integral membrane protein which binds B-type lamins and double-stranded DNA, interacts with human HP1 family proteins (Pyrpasopoulou *et al.*, 1996; Ye and Worman, 1996). The human HP1 could possibly serve as a linker, connecting peripheral heterochromatin to the inner nuclear membrane and mediate nuclear envelope reassembly at the end of mitosis (Ye *et al.*, 1997).

What is the spatial distribution of HP1.1 in the nucleus, and what is its role during cell division? To answer these questions interphase and mitotic nuclei have to be carefully analyzed in living cells. HP1 in *Drosophila* is associated with the heterochromatin at the chromocenter of polytene nuclei (James an Elgin, 1986).

The lethal phenotype of *Su(var)205* includes defects in chromosome morphology and segregation (Kellum and Alberts, 1995). Furthermore, HP1 is colocalized with the centromeric heterochromatin of embryonic nuclei in interphase chromosomes (Kellum *et al.*, 1995), whereas its homolog in *S. pombe*, Swi6, localizes at telomeres, in the *silent mating-type* locus and in the centromeres. Subsequently, mutation of the *swi6* locus results a high increase of the rates of chromosome loss (Ekwall *et al.*, 1995).

The HP1 in *Drosophila* plays an important role in the silencing of genes located next to heterochromatin (see above). Notably, the HP1 homolog in the ciliated protozoan *Tetrahymena thermophila* is missing from transcriptionally silent micronuclei but, is enriched in heterochromatin-like chromatin bodies that

presumably comprise repressed chromatin in macronuclei (Huang *et al.*, 1999). These findings provide evidence that HP1-like proteins are not exclusively associated with permanently silent chromosomal domains (Huang *et al.*, 1999).

In my thesis, the aim was to get further insight into the functions of HP1 by studying the nematode, *Caenorhabditis elegans*, and its HP1 homologs, HP1.1. *C. elegans* is a model organism of molecular and developmental biology (Riddle *et al.*, 1997) and was the first multicellular organisms the genome of which was completely sequenced (The *C. elegans* Sequencing Consortium, 1998). A sequence alignment within over project revealed three HP1 homologs in the genome of *C. elegans*.

Until now, there have been no cytological data on heterochromatin in *C. elegans* or on the distribution of any proteins that are associated to heterochromatin in other organisms. I have therefore analyzed, on a cytological level, the expression of an HP1 protein in embryogenesis and its location in distinct nuclear structures of interphase nuclei. For this purpose, an anti HP1 antibody was used, and HP1::GFP constructs were cloned and expressed in embryonic cells. Furthermore, a dynamic distribution of HP1 during the cell cycle was observed by concomitantly localizing HP1 and histone H1 by Yellow Fluorescent -and Cyan Fluorescent Protein, respectively.

Transiently knock-out of HP1 by the RNAi method yielded mutant phenotypes. Finally, it was tried to find interaction of HP1 with other nucleus proteins in *C. elegans* by injecting ds-RNAs of selected nuclear proteins. For these studies, a confocal laser scanning microscope (Zeiss LSM 510) was used. In parallel, techniques were modified for immuno-microscopic analysis on a level beyond the resolution of the light microscope, and the X-ray microscope constructed by the group of G. Schmahl (Göttingen) was used.

2 Materials and Methods

2.1 Materials

2.1.1 cDNA and cosmid clones

The following cDNAs and cosmid clones for this work were obtained from the Sanger Center, Hinxton Hall, Cambridge, provided by Dr. A. Coulson, and from Japanese National Institute of Genetics obtained from Dr. Y. Kohara. These clones were used as a template for amplification of genes via PCR for cloning of genes for reverse genetic, and also making dsRNA for reverse genetic experiments.

Table I-1 These cDNAs and cosmids were used for this work in *C. elegans*.

Protein name	predicted protein	cDNA	cosmid
HP1.1	K08H2.6	Yk432c11	K08H2
HP1.2	K01G5.2a	Yk470a11	K01G5
HP1.3	K01G5.2	Yk106f2	K01G5
LBR	B0250.7		B0250
ORC2	F59E10.1	Yk236f8	F59E10
SU(VAR) 3-9	C41G7.4		C41G7
SET domain	C15H11.5	Yk701e8	C15H11

2.1.2 Cloning vectors

The following vectors were used for genetic engineering in bacteria and construct of reporter gene to obtain transgenic worms in this work.

Table I-2 These vectors have been used for genetic manipulation in bacteria, and creation of fluorescent protein fusions in *C. elegans*.

Vector	**feature**
M 13-helper phage	Stratagene, CA, USA, Alting-Mees and Short, 1994
λ ZAP II	Stratagene, CA, USA, Short *et al.*, 1988
pBluescript (SK+)	Stratagene, La Jolla, USA
PECFP-N1	Clontech, CA, USA
PEGFP-N1	Clontech, CA, USA
PEYFP-N1	Clontech, CA, USA
PGEM-T	Promega, Wisconsin, USA
PUC18	Norrander *et al.*, 1983

2.1.3 *Caenorhabditis elegans* strains and bacterial strains

The following *C. elegans* strains and bacterial strains were used for this work.

Table I-3 These *C. elegans* strains have been used for this work. Most of these strains, EC001 to EC014, have been generated by myself.

Strain	**genotype**	**outcrossed**
N2, variation Bristol	wild-type, Brenner, 1974	
EC001	*hp1.1::gfp* extrachromosomal array;*rol-6*(su*1006*)	
EC002	*hp1.1::s::gfp* extrachromosomal array;*rol-6*(*su1006*	
EC003	*hp1.1::gfp::sIIc* extrachromosomal array	
EC004	*hp1.1::gfp* integrated array;*rol-6*(*su1006*)	
EC005	*hp1.1::gfp* integrated array;*rol-6*(*su1006*)	*him*-8(*e1489*)
EC006	*hp1.1::gfp* integrated array;*rol-6*(*su1006*); *him*-8(*e1489*)	CB1370
EC007	*hp1.1::yfp* extrachromosomal array	
EC008	*his-24::cfp* extrachromosomal array	
EC009	*his-24::cfp* integrated array	
EC010	*hp1.1::yfp and his-24::cfp* extrachromosomal array	
EC011	*hp1.1::gfp* integrated array;*rol-6*(*su1006*); *him*-8(*e1489*)	E009
EC012	*hp1.1::gfp* integrated array;*rol-6*(*su1006*); *him*-8(*e1489*)	*mes-3*(*bn21*
EC013	CB1370	*him-8*(*e1489*)
EC014	*hp1.1::gfp* integrated array;*rol-6*(*su1006*)	EC 013
GE24	*pha*-1(*e2123*), Granato *et al.*, 1994,	
SS222	*mes-3*(*bn21*), Paulsen *et al.*, 1995	
CB1489	*him-8*(*e1489*), CGC	
CB1370	*daf*-2(*e1370*), CGC	

Table I-4 These *E. coli* strains have been used in this work. Most of these strains were used for manipulation in bacteria, whereas OP50 was only used for food source for *C. elegans*.

Strain	**feature**
Escherichia coli DH5α	Hanahan, 1985
E. coli OP50	Brenner, 1974
E. coli XL1-Blue MRF‘	Jerpseth *et al.*, 1992
E. coli SOLR	Hay and Short, 1992

2.1.4 Primers for Polymerase Chain Reactions (PCRs)

The following primers were used for the amplification of genes from cDNAs, cosmids or plasmids. These have been designed with PCGENE (IntelliGenetics Inc.) and purchased commercially.

Table I-5 These primers have been used for amplification of genes in the present work.

Primer	**sequence**
ESMG57:	GGGGTACCTCAATAAAGCGACGACAGATGTAAACA
ESMG59	CGGGATCCGCGCTCATTCCTCCTGGGATGGTTGG
ESAD06	CTAGTGGGGCCCGGGATCCATGGTCTCATCCACAGTTTGAGA AA
ESAD07	GATCTTTCTCAAACTGTGGATGAGACCATGGATCCCGGGCCCC A
MBMG01	GCTGCAGAGTTCTCTCCCTAGATGCTCGTGATACACT
MBMG02	GGGTACCCCTGAGTTTCTTGGGAACAAGAGACTGTCATCAT
MB_LBRT7for1	CGCGCGTAATACGACTCACTATAGGGCATGGGTCCCTTC CATTCC CGCCTCTCCGG
MB_T7LBRbac1	CGCGCGTAATACGACTCACTATAGGGCTCATCTCTTCTCACGC GGCTTAGGAGCTG
MB_Su(var)39for1	CGCGCGTAATACGACTCACTATAGGGCATGAGGATGTGAAAG GCACAATGATGC
MB_Su(var)39bac1	CGCGCGTAATACGACTCACTATAGGGCCGTTGCTCCGCCAAA TGAAGTCTCC
ESMG67	CGCGCGTAATACGACTCACTATAGGGCGAATTGCC CTCACTAAAGGGA
T7-Stratagene	GTAATACGACTCACTATAGGGC

2.1.5 Reagent for antibody staining of *C. elegans* embryos.

2.1.5.1 Primary antibodies

For the labeling of the nuclear structures were the following antibodies performed for immunofluorescence in this work. For detection one of the mammalian HP1 homolog proteins in *C. elegans* lysate were used the Mac (0, N) antibodies. K76 and OICID4 were used to staining of P-granules in embryos as a control in comparison to latter antibody staining. For HP1.1::GFP staining were anti-GFP (rabbit serum) used.

Table I-6 The primary antibodies, which were performed in immunostaining of nuclear structures in this work.

antibody	**source**	**concentration**
K76	Strome and Wood, 1982; 1983	50 µg/ml
OIC1D4	Strome, 1986	46 µg/ml
Mac (0, N)	Wreggett *et al.*, 1994	
anti-GFP rabbit serum		1 mg/ml

2.1.5.2 Secondary antibodies, and horseradish peroxidase (HRP) conjugated Strep-Tactin

The following secondary antibodies were used for decoration of interest labeled epitopes in the immunostaining for both fluorescence.

Table I-7 These secondary antibodies have been used for indirect immunostaining in this work. * This phototope conjugated secondary antibody was performed for detection of HP1 homolog protein of *C. elegans* on western blot.

antibody	chromophore	concentration	source
Goat anti-Mouse IgG, H&L	Cy2-conjugated	750 µg/ml	Jackson ImmunoResearch
Goat anti-Rabbit IgG (H&L)	Cy2-conjugated	500 µg/ml	Jackson ImmunoResearch
Goat anti-Rat IgG (H&L)	Cy3 labeled	1 mg/ml	Nycomed Amersham
Goat anti-Rabbit IgG (H&L)	1-nm gold Cy2-conjugated		British Biocell
IgG (H&L)*	HRP	Molar ratio (IgG:HRP) 1.0:1.3	Sigma

Table I-8 These reagents have been used for this work. The Strep-Tactin was performed for protein-protein recognizing of HP1.1::GFP::SIIc with Strep-Tactin in *C. elegans*. LI-Silver Kit was used for enhancement of gold particles in the nano-gold immunostaining for X-ray microscopy.

reagent	phototope	concentration	source
Strep-Tactin	HRP conjugated		IBA, Göttingen
LI-Silver Kit			Nanoprobes, USA

2.1.6 Data bank, Software, and *C. elegans*

The Sanger center provides a *C. elegans* sequence data base in a web accessible form of ACeDB. For analysis of protein and nucleic acids were used several different data bases and programs. With PCGENE (IntelliGenetics Inc., version IGI 3064) were preformed a lot of gene and protein analysis. The multiple alignment of all HP1 and HP1 like proteins of *C. elegans* and few other organisms were generated with the program ClustalW (Thompson *et al.*, 1994, version 1.5b).

The laser scanning microscope (LSM510) was equipped by a special program (version 2.50.0929) developed by Zeiss. The Spot-Camera

(DIAGNOSTIC, instruments, inc.) has its program (version 3.0 for Windows), too.

For analysis of protein and nucleic acids were used several different data bank and programs. PCGENE (IntelliGenetics Inc., version IGI 3064) were preformed for gene and protein analysis. Subsequently, were performed some programs of Microsoft like, Microsoft Excel (version 97), Microsoft Power-Point (version 97), Microsoft Word (version 97), and Paint. For photo processing was used XnView for windows (version1.19), CorelDraw (version 7.468).

2.1.7 Equipments of fluorescence light microscopy

The documentation of most photomicrographs were performed with a Zeiss Axioplan 2 microscope equipped with Zeiss confocal laser scanning module Zeiss 510 (Jena, Germany). It is equipped with three laser excitation systems, confocal optics. The acquisition of data is through an 12-bit 1024/1024 frame buffer. Image processing was carried out an image analysis software developed by Zeiss. Therefore, conventional and confocal light microscopy of Nomarski differential interference contrast (Nomarski-DIC) and epifluorescence of specimen were performed with Zeiss 510. The Axioplan 2 is equipped with a set of several lenses with magnification of 10 x Plan-Neofluar (NA of 0.3), 20 x Plan-Neofluar (NA of 0.5), 40 x Neofluar (NA 1.3, oil), 63 x Plan-Apofluar (NA of 1.4 oil, and DIC), and 100x Plan-Neofluar (NA of 1.3, oil). I preferred to use the 40 x Neofluar for my recorded micrographs. Series of up to 30 optical sections were recorded. Some other images were captured on a Spotcamera with a charge coupled digital (CCD) camera (Diagnostic Instruments, Sterling Height, MI).

For looking at the specimen on the slides were used a mercury high pressure lamp (HBO 103 W/2, Osram) of LSM 510. The samples were excited

with a Laser 351 nm and 364 nm for DNA staining, 488 nm *e.g.* for GFP and Cy2, 458 nm for CFP, 514 nm for YFP, and 543 nm for Cy3 and conventional Nomarski-DIC light photo. To taking of micrographs were used the appropriate filter of the following filterset (see below).

Table I-8 The spectrum of chromophores from fluorescent proteins and dyes with the appropriate band-pass set of confocal laser scanning microscope 510.

fluorochrome	**excitation(λ), nm**	**band-pass (λ), nm**	**emission(λ), nm**
H33342	364	385-470	465
Cy2	489	505-530	505
CFP	433	505-530	475
GFP	488	505-530	509
YFP	513	505-550	527
Cy3	575	560-615	605

For screening of the *C. elegans* culture plates were used a coaxial fluorescence attachment (dissection) stereo-microscope SZX-RFL2 from Olympus (Tokyo Japan) which is equipped with a mercury high pressure lamp (HBO USH-102D, Ushio, Japan) and filters for observation of CFP, GFP, and YFP expression pattern in worm.

Table I-9 The filter set of the stereo-microscope SZX-RFL2 for observation of fluorescent proteins in transgenic animals in the present work.

fluorescent protein	**band-pass, λ nm**	**band-pass, λ nm**
CFP (Haas *et al.*, 1996, Yang *et al.*, 1996)	460-490	BA510-550
GFP(Chalfie *et al.*, 1994)	460-560	590
YFP (Ormö *et al.*, 1996)	540-580	BA610

For screening of different obvious phenotype at the culture plates for everyday purpose a dissecting stereo-microscope equipped with a transmitted

light source was used. I used such standard stereo-microscope of Wild-Heerbrugg model (Switzerland) with 10x eyepieces and a lens revolver ranging from 5 x to 50 x magnification.

2.1.8 Equipments for X-ray microscopy

For X-ray transmission microscopy special foils have been developed, that were constructed by mounting of 400-nm-thick polypropylene foils on a 100-µm-thick stainless steel ring, which has an inner and outer diameter of 13 mm and 25 mm respectively. The middle of such foils were a 3 mm-diameter hole, onto which a patch of 125-nm-thick polyimide foil placed.

This polyimide part, which was coated with 20-nm silicon and 20-nm silicon dioxide to protect the evaporation of water through the foil. The silicon layer were used to reflect the visible light, that also served to visualization of the specimen in the incident light microscope. It was also used for adjustment of the specimen for the X-ray microscope. The silicon dioxide layer served for the attachment of the cells onto the polyimide foils.

For protection of the evaporation of water in cover foils were these coated with a transparent 50-nm-thick aluminum oxide layer. For easy finding of the favorite cells on the specimen were used a finder grid, which were developed in Institut für Röntgenphysik. It were a grid mask from electron microscopy placed onto the polyimide foils before evaporating of silicon layer. This mask were removed from the polyimide foil, and a finder grid pattern were imprinted on it. The special specimen chamber (Niemann *et al.*, 1994) were used for room temperature X-ray microscopy. The X-ray micrograph were recorded in the X-ray micrographs at the electron storage ring BESSYI in Berlin. The Göttingen transmission X-ray microscope was equipped with an objective with 40 nm outermost zone width (Weiss *et al.*, 1988), a X-ray condensor with 54 nm outermost zone width (Hettwer and Rudolph, 1998), and recorded with a

backside-illuminated slow-scan CCD camera AT200L from Photometrics (Munich, Germany).

2.1.9 Chemicals, enzymes and other equipment's

The chemicals, enzymes, and other equipment were purchased from Abimed Analysen-Technik (Langenfeld, Germany), Ambion (Frankfurt, Germany), Amersham (Freiburg, Germany), Applichem (Darmstadt, Germany), Biolabs (Frankfurt, Germany), Biometra (Göttingen, Germany), Biomol (Ilvesheim, Germany), Boehringer (Mannheim, Germany), Dako Diagnostka (Hamburg, Germany), Dianova, (Hamburg, Germany), Eppendorf (Hamburg, Germany), IBA (Göttingen, Germany), ICN Biomedicals (Eschwege, Germany), Life Technologies (Karlsruhe, Germany), Macherey-Nagel (Düren, Germany), MBI Fermentas (St. Leon-Rot, Germany), Merk (Darmstadt, Germany), MWG-Biotech (Ebersberg, Germany), NEN (Cologne, Germany), peqlab (Erlangen, Germany), Pharmacia (Freiburg, Germany), Promega (Mannheim, Germany), Qiagen (Duesseldorf, Germany), Roche (Mannheim), Roth (Karlsruhe, Germany), Sarstedt (Nuembrecht, Germany), Sigma and Sigma-Aldrich (Munich, Germany), Stratagene Europe (Amsterdam, The Netherlands), Sued-Laborbedarf (Munich, Germany), and Zeiss (Germany).

2.1.9.1 Enzymes

Table I-10 These enzymes have been performed in experiments of the present work.

nomenclature	enzyme
EC3.1.3.1	Alkaline phosphatase
EC3.2.1.14	Chitinase
EC3.4.21.1	Chymotrypsin
EC3.1.21.1	DNaseI, RNase-free
EC2.7.7.49	Proteinase K
EC2.7.7.6	RNase
EC6.5.1.1	T4-DNA-Ligase
EC2.7.7.7	T4-DNA-Polymerase
EC2.7.7.7	T7-DNA-Polymerase
EC2.7.7.6	T3-RNA-Polymerase
EC2.7.7.6	T7-RNA-Polymerase
EC2.7.7.7	Taq-DNA-Polymerase
EC2.7.7.7	Expand long template *Taq*
EC2.7.7.7	Expand high fidelity *Taq*

2.1.9.2 Solutions and bacteria growth medium

Table I-11 These are solutions and buffers which have been used in experiments of the present work.

solution	Contents I
anode buffer-1	300 mM Tris pH 10.4, 20% methanol, 0.1% SDS
anode buffer-2	25 mM Tris pH 10.4, 20% methanol, 0.1% SDS
buffer B^+	10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, and 100 µg/ml BSA
buffer G^+	10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and 100 µg/ml BSA
buffer O^+	50 mM Tris-HCl pH adjusted to 7.5, 10 mM $MgCl_2$, 100 mM NaCl, and 100 µg/ml BSA
buffer PI	400 µl/ml RNase A, 10 mM Tris-HCl, 10 mM ETDA, pH 8.0
buffer PII	200 mM NaOH, 1% SDS
buffer PIII	2.5 M KAc, pH 4.88

solution	Contents II
buffer R^+	10 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 100 mM KCl, and 100 µg/ml BSA
buffer Y^+	33 mM Tris-Ac pH 7.9, 10 mM Mg-Ac, 66 mM K-Ac, and 100 µg/ml BSA
bleaching solution	900 µl of 3% NaOCl diluted in egg-salt, and 100 µl of 500 mM NaOH
destaining solution	70 ml MilliQ water, 20 ml methanol and 10 ml acetic acid
cathode buffer	25 mM Tris pH 9.4; 40 mM aminocapron acid; 20% methanol; and 0.1% SDS
Coomassie Blue	1 mg/ml Coomassie Blue G-250, 50% methanol and 10% acetic acid
egg-salt	118 mM NaCl, 48 mM KCl, ddH_2O for solutions, and autoclaved
electrode buffer	250 mM Tris-HCl, 1.92 M glycine, and 0.1% SDS
extraction buffer	Tris-HCl 20 mM pH 6.8, glycerin 4 mM, SDS 1.66 mM, bromophenol blue 29.9 µM
LB	Laura-Bertani (LB) medium, 5 g NaCl, 10 g peptone, 5 g yeast extract, pH 7.2, and autoclaved
LB-Agar	LB medium, 5 g NaCl, 1 g peptone, 0.5 g yeast extract, 15 g agar, pH 7.2, Miller, 1972
LB-Amp	LB medium, 5 g NaCl, 10 g peptone, 5 g yeast extract, pH 7.2, and autoclaved ,100 µg/ml ampicillin
LB-Kana	LB medium, 5 g NaCl, 1 g peptone, 5 g yeast extract, pH 7.2, and autoclaved, 70 µg/ml kanamycin
LB-X-Gal	LB medium, 5 g NaCl, 10 g peptone, 5 g yeast extract, pH 7.2, and autoclaved, 100 µg/ml ampicillin,1 mM IPTG, 48.95 nM X-Gal staining (in dimethyl formamide stored at -20°C)
Loading buffer	0.25% xylene cyanol, 0.25% bromophenol blue, approximately 30% ficoll, 500 mM EDTA
Lysis buffer	200 mM NaCl, 100 mM Tris-HCl, pH 8.5, 50 mM EDTA, 0.5% SDS
M9 solution	3 g KH_2PO_4, 6g Na_2HPO_4, 5 g NaCl, 1 ml 1 m $MgSO_4$, and added dH_2O to 1 liter and autoclaved
PBS	140 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2, and autoclaved
PBS1	1.4 M NaCl, 27 mM KCl, 43 mM Na_2HPO_4, 14.7 mM KH_2PO_4, pH to 7.2, and autoclaved
PCR buffer 10x	500 mM Tris-HCl pH 9.2, 17.5 mM $MgCl_2$, 160 mM $(NH_4)_2SO_4$
reaction buffer10x	400 mM Tris-HCl pH 8.0, 60 mM $MgCl_2$, 100 mM Dithiotreitol (DTT), 20 mM spermidin

solution	Contents III
sample buffer	20 mM Tris-HCl, pH 6.8, 480 ng/ml SDS; (1:250, v/v) glycerol; 5 µg/ml bromophenol blue and twentieth part of β-mercaptoethanol fresh made
SM buffer	100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 14.5 mM $MgSO_4*H_2O$, and 0.01% gelatine, and autoclaved
T4ligation buffer	10x T4-ligation buffer, 400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8
TAE	2 M Tris, 2 M Acetic acid, 50 mM EDTA, and autoclaved
TBS	10 mM Tris-HCl, 150 mM NaCl, pH 7.4, and autoclaved
TBS2	20 mM Tris-HCl, 136 mM NaCl, pH 7.6, and autoclaved
TBS2T	TBS2, and 0.1% Tween X20
transfer buffer	25 mM Tris-HCl, 192 mM glycine, 20% methanol, 0.1% SDS at pH 8.3

2.2 Methods

2.2.1 Molecular biological methods of bacteria

2.2.1.1 PCR as a tool for amplification of genes

For the amplification of the gene from cDNAs, cosmids, and genomic DNA, used the polymerase chain reaction (PCR) (Saiki, *et al.*, 1985) as an important tool. For amplification of *hp1.1* from cosmid K08H2, it was used an individual hot-start protocol. In a 500 µl reaction tube on ice were combined quickly the following reagents:

1.0 µl of template DNA (10 ng/µl)
5.0 µl of 10x PCR buffer
8.0 µl of 2 mM dATP, dCTP, dGTP, and dTTP each
2.0 µl 25 mM $MgCl_2$
0.5 µl of forward primer ESMG57 100 pmol/µl
0.5 µl of reverse primer ESMG59 100 pmol/µl
33 µl of ddH_2O
1 U Expand and high fidelity Taq polymerase
add 33 µl of sterile (autoclaved) glycerol

Before adding the enzyme, this mixture was stirred well, and the PCR reaction was carried out 31 cycles in a 3 block TRIO-Thermocycler from Biometra (Göttingen, Germany) as followed: 94°C, 2 min; 65°C, 2 min; 68°C, 5 min. After a final extension for 5 min at 72°C, carefully took the reaction mixture from under the glycerin. The PCR product was then precipitated with 1 volume of phenol/chloroform (50:50) solution (pH 7.6-8.0), after powerful hand-shaking and on Vortex-VF2 from W.Krannich (Göttingen, Germany) for 1 min at room temperature, the suspension wan centrifuged for 2 min at 12000 x g (approximately 14000 r.p.m.) in EBA12 table-centrifuge. The supernatant was carefully taken and removed into a new sterile reaction tube, then added 1 volume chloroform/isoamylalcohol, the mixture was for 1 min vortexed, and were centrifuged for 2 min at 12000 x g. In the end were the last step as well as once again repeated. The supernatant was removed in a new sterile reaction tube, added to this tenth part of volume of NaAc (3 M, pH 5.2), and 2 volumes of absolute ethanol, and kept for 30 min at -20°C. Subsequently, the DNA was harvested for 10 min at 12000 x g, the supernatant was removed, DNA was washed with ice-cold 70% ethanol, dried in a speedvac Hetovac from Hettich (Hannover, Germany) and resuspended in 25 µl ddH_2O. For the first use, this DNA were incubated for 2 hours at room temperature, and later stored at -20°C.

The concentration of the PCR-product was determined in a spectrophotometer Kontron Instruments (Milan, Italy) at A260 nm. In order to this, an OD of 1 corresponds to approximately 50 µg/ml of dsDNA. The molecular size fractionation of DNA was carried out in an agarose gel using agarose powder, which was dissolved in the appropriate concentration (usually at 0.7% in our lab) in TAE buffer. Subsequently, followed an analysis on an agarose gel. 100 ng of PCR-product were digested with 1U of *Bam*HI in a sterile reaction tube with 1 µl of buffer G^+ and *Kpn*I (with 1 µl of buffer B^+), and 8.0 µl ddH_2O. After analysis of PCR-product, we loaded this on a low melting point

agarose gel, cut the favorite band out, and used it after digestion with and for the ligation into a pUC18 vector.

2.2.1.2 Restrictions enzyme analysis, and agarose-gel electrophoresis

It is very important to have a pure plasmid-DNA for digestion, and because of that plasmid-DNA midi preparation was done before. It was 1 µg of favorite DNA placed into a sterile reaction tube, and 1 µl of the appropriate 10 x restriction enzyme buffer was added and normally 1 U of restriction enzyme in a final volume of 10 µl. It's very important to take care about the buffer which could be used for each enzyme, *e.g.* has *Bam*HI a favorable activity at 100% in 1 x buffer G^+ and Y^+. This steps should be done on ice and afterwards incubation was done at the appropriate temperature for 1 to 2 hours.

The DNA size fractionation was performed in a low melting point agarose-gel (normally at 0.7% in our lab) for about 30 min, 100 mV at room temperature. Usually, low melting point agarose powder (peqlab, Erlangen, Germany) was dissolved in the right concentration in TAE, and cooked in the microwave until entirely dissolved. Subsequently, the solution cooled to 50°C, then added 500nl of ethidium bromide (Roth, Karlsruhe, Germany) with the concentration of 10 mg/ml, and then it was casted in a mold on which was a comb placed in one of the poles side. Finally, the gel submerged in a electrophoresis chamber containing TAE buffer, the DNA was mixed with loading buffer and applied to the slots created by the comb.

As a DNA marker were used self-made ladder out of λ-DNA (300 µg/ml), which is a dsDNA with a molecular weight of $31.5*10^6$ daltons and contains 48502 bp. It was mixed 90 µl λ-DNA, 10 µl buffer R^+, 4 µl *Hind*III, 4 µl *Eco*RI, digested for 2-3 hours at 37°C, and stopped to the end concentration of 1 mM

EDTA. For every agarose gel 2 µl of this ladder marker was used and applied to one slot in the corner of the gel. Subsequently, was the chamber submitted to an electric field about 10 V/cm in such a way that the DNA molecules migrated towards the anode. After about 30 min the gel was examined in a 258 nm UV light lamp and photographed firstly. It should be mentioned that the dye ethidium bromide, which bind to the DNA via "intercalation", fluoresces at 310 nm. Secondly the favorite DNA bands for ligation reaction were cut out of the low melting point agarose gel and brought to a new sterile reaction tube (Dretzen *et al.*, 1981). This method was used for example for cloning of *hp1.1* into the pEGFP-N1 vector, which was performed for generation of transgenic worms.

2.2.1.3 Ligation of DNA for transformation into the competent *E. coli* cells

For one ligation reaction firstly incubated the reaction tube containing with cut low melting point agarose gel pieces for 5 min at 65°C to dissolve the agarose gel. Secondly, 3 µl of the vector gel and 6 µl of the insert gel were taken after bringing the temperature into 37°C, and put it into a new sterile reaction tube, which was placed with 2 µl 10 x T4-ligation buffer, 7 µl ddH_2O, and at least 2 µl T4 DNA ligase. Last step might be done very fast because of freezing the low melting point agarose gel pieces. Subsequently, the reaction was incubated for 1 min on ice, the tubes brought into a RM20-Lauda water bath (Germany) over night at 14°C. Finally, for the transformation into self-made competent cells (DH5α) the ligation reaction should be brought into a Julbo-water-bath (W.Krannich, Göttingen, Germany) for 5 min at 65°C to dissolve the gel pieces, cooled down in the hand and put it into an aliquot of previously thawed DH5α cells. This method was used for every gene manipulation in the bacteria.

2.2.1.4 Transformation of the DNA into competent *E. coli* cells

For the bacterial transformation I needed some competent cell necessary, which should be prepared firstly. I used our self made competent cells in our laboratory in the following way. At first it would be to make one over night culture of DH5α cells in 5 ml LB medium. Finally were took 1 ml of this culture and inoculated into an Erlenmeyer flask with 50 ml of LB medium and grown to an optical density, OD_{600} of 0.3. Then the cells were harvested at 4°C, 3600 g, 7 min and incubated in ice-cold , sterile 50 mM $CaCl_2$ for 15 min. Repeatedly were cells centrifuged and resuspended in 20 ml 50 mM $CaCl_2$ with additionally 20% glycerol, an aliquot into reaction tube each 250 µl and frozen at -80°C.

For the transformation of plasmid-DNA was firstly one aliquot of cells thawed on ice, and the favorite DNA was added to them, followed by one incubation for 40 min on ice. In the next step were the cells shortly submitted for a heat-shock treatment (exactly 2 min at 42°C and no longer!), returned to the room temperature for 10 min and then 250 µl of LB medium was added. Subsequently, the cells were then transferred to a Heraeus-incubator (Hannover, Germany) at 37°C for 1 hour and plated on LB plates containing the require antibiotic.

The competence of the cells was measured by counting colonies of transformed plates, and it should be 10^6 colony forming units (CFU) per 1 µg transformed DNA. It is very important to take a control sample for each transformation. For the control sample was taken instead of insert gel the same volume of distilled water. Single clones on the plates were used for making plasmid DNA mini preparation. The molecular size fractionation of DNA was carried out in agarose gels using agarose powder, which was dissolved in the appropriate concentration (usually at 0.7% in our lab) in TAE. This method was used for every bacterial DNA transformation for this work.

2.2.1.5 Isolation of plasmids from transformed *E. coli* cells

This technique for the preparation of the small scale plasmid DNA was performed to the alkaline lysis method after Birnboim and Doly (1979). According to this experiments a single bacterial colony was from a agar plate, transferred to 5 ml of LB with the appropriate antibiotic and incubated over night on the rotor Certomat (B.Braun, Melsungen, Germany) at 220 r.p.m. and 37°C.

The culture was transferred to a reaction tube and centrifuged in a table Labofuge 6000 at 12000 x g for 1 min. The supernatant was removed and the pellet was resuspended in 300 µl of buffer PI and incubated for 5 min at 4°C. In the next step 300 ml of buffer PII (fresh made) was added and 5 min at the room temperature incubated. 300 µl of buffer PIII was added after mixing, centrifuged in a Labofuge 6000 (Heraeus, Hannover, Germany) at 12000 x g. The supernatant was transferred to a sterile reaction tube with 750 µl isopropanol. The DNA was precipitated by 12000 x g for 15 min, and washed with 70% ethanol, and resuspended in 25 µl autoclaved MilliQ-water (ddH_2O). Finally were 1 µl of the DNA was taken for the restriction analysis to elucidated the quality of it on the 0.7% agarose gel.

For higher amount of plasmid DNA the preparation was used the Qiagen DNA midi preparation Kit and „Nucleobond PC 100 Kit“ from Macherey-Nagel (Düren, Germany) according to their manufacturer manuscript. The harvested DNA were washed with 70% ethanol, dried in a speedvac Hetovac from Hettich (Hannover, Germany) and resuspended in 50 µl ddH_2O. The concentration of the Plasmid DNA was determined by using a spectrophotometer of Kontron Instruments (Milan, Italy) at a wave-length of A260 nm. For the determining of protein amount was also measured at 280 nm, respectively. For the quality of the plasmid-DNA should be the ratio between the both wave lengths higher than 2.

2.2.2 Molecular biological methods of *C. elegans*

2.2.2.1 HP1 homolog genes in the genome of *C. elegans*

The cDNA phage clones yk432c11, yk106f2, and yk470a11 were transferred through a Zap reaction into the *E. coli* strain XL1-Blue MRF' as followed. Into a sterile tube with 5 ml LB medium were added 100 µl of a 10% maltose solution (0.2% v/v) and 50 µl of a 1 M $MgSO_4$ (10 mM end concentration), and inoculated with *E. coli* strain XL1-Blue MRF', and incubated for 2-5 hours in a Certomat incubator (Braun, Melsungen, Germany) at 220 r.p.m. and 37°C. Cells were harvested by spinning down for 10 min at 2000 r.p.m., the supernatant removed, and the pellet in 10 mM $MgSO_4$ solution resuspended. By using a spectrometer from Kontron instruments the optical density (OD_{600}) was adjusted to 0.5, taking plastic cuvettes from Sarstedt (Nuembrecht, Germany). The phage clones, that containing in SM buffer were used for a dilution series (1:10, 1:100, 1:1000, and 1:10000) in 200 µl of adjusted XL1-Blue MRF' cells, and incubated by gently shaking for 15 min at 37°C. The sample was subsequently added to 1.5 ml of liquid LB-Agar, of 48°C, and then inoculated onto LB-Agar Petri dishes and incubated over night at 37°C. Finally, the phages were harvested from each plate with SM buffer. Now it is ready to make an *in vivo* excision of the plasmid as followed.

Grow an overnight culture of XL1-Blue MRF′ and SOLR cells in LB broth at 30°C. A 1/100 dilution of the cells, 250 µl of the overnight culture and 25 ml of LB broth and have been grown at 37°C for 2-3 hours to mid-log phase (*e.g.* OD_{600} of 0.3). Gently spin-down the XL1-Blue MRF′ cells at 2000 r.p.m., and resuspended to an OD_{600} of 1 for single-clone excision. In a 15 ml conical tube combine 200 µl of these XL1-Blue MRF′ cells with 250 µl of each clones of phage stock (containing more than 1 x 10^5 phage particle), and add 1.0 µl Exassist helper phage (containing more than 1 x 10^6 phage particle). The

mixture were incubated at 37°C for 15 min. Add 3 ml of LB broth and incubate for 2.5 hours at 37°C by gently shaking. Spin cells down for 15 min at 2000 r.p.m., and transfer the supernatant to an autoclaved reaction tube. Heat the tube at 70°C for 15 min, and then spin again for 15 min at 4000 r.p.m. Decant the supernatant, which contains the excised phagemid pBluescript packaged as filamentous phage particle, into a sterile reaction tube.

To plate the rescued phagemids of each clones, add 200 µl of freshly grown SOLR cells (OD_{600} of 1) to two reaction tubes. Add 100 µl of the excised phage stock above to one tube and 10 µl of the excised to the other tube. Incubated the tubes at 37°C for 15 min, and plate 20 and 50 µl from each tube on LB-ampicillin Petri dishes (100 µg/ml) and incubate over night at 37°C. From this strains were picked single clones for making DNA mini preparation. After analysis of DNA, it was made a DNA midi preparation. This DNA were used for PCR reaction as a template, for further cloning steps.

2.2.2.2 Isolation of genomic DNA from *C. elegans* N2 strain

Isolation of genomic DNA of *C. elegans* was accomplished preferably using a protocol from Plasterk's laboratory which was modified in below. From 3 or 4 NGM medium-seized (60 mm diameter) NGM culture plates seeded with OP50, grown worm of all stages were washed with ice-cold M9 buffer down. After three wash-step the suspension was centrifuged for 2 min at 3000 x g. The supernatant was removed, added 300 µl lysis buffer, incubated for 30 min at 65°C, and the reaction tube have been shaken every 5 min during the incubation time. Subsequently added 5 µl of Proteinase K with a concentration between 5 to 10 µg/µl, after that followed incubation for 30 min at 37°C. In the next step was added 10 µl of a RNase A solution and it was incubated for 30 min at 37°C.

The genomic DNA was then precipitated with 500 µl phenol buffered solution (pH 7.6-8.0), powerful shaking, incubated for 30 min at room

temperature, and lastly was the solution centrifuged for 7 min at 12000 x g. The supernatant was carefully transferred into a new sterile reaction tube, added 500 µl phenol/chloroform/isoamylalcohol solution (v/v 25:24:1), the mixture has been powerfully shaken for 30 min on the shaker KS250basic (IKA Labortechnik, Germany). Subsequently, the solution has been centrifuged for 5 min at 12000 x g. The latter step was repeated as well as once again. The supernatant was transferred into a new sterile reaction tube, than added to this 500 µl chloroform, and repeatedly centrifuged. Finally, the supernatant was placed into a new sterile reaction tube, added 1 ml 96% ethanol solution, and after mixing the solution was incubated over night at room temperature.

The genomic DNA was for 15 min at 12000 x g spinned down, the supernatant removed, than washed with 500 µl ice-cold 70% ethanol and resuspended in 100 µl ddH_2O. The concentration of the genomic was determined by using a spectrophotometer of Kontron Instruments (Milan, Italy) at a wave-length of A260 nm. For the determining of protein amount, it was also measured at 280 nm, respectively. According to the quality of genomic DNA the ratio between the both wave-lengths should be higher than 2. Accordingly, an OD of 1 corresponds to approximately 50 µg ml of dsDNA.

2.2.2.3 Creating of transgenic animals of *C. elegans* (Fire, 1986)

The nematode *C. elegans* was kept at 16°C on NGM culture plates, which were inoculated with the *E. coli* bacteria strain OP50 and were stored at 4°C. For creating of transgene animals we loaded DNA in a Femtotip II (Eppendorf, Hamburg, Germany) by using a microloader. For reducing of the DNase activity one should take the DNA on some ice. In the case of HP1.1 it was a 4.6 kb fragment containing the promoter region and coding sequence, which was genetically fusionized into the pEGFP-N1. This was injected at 10 ng/µl along with pRF4, which contains *rol*-6(*su1006*), at 80 ng/µl to obtain the first extrachromosomal transgenic line for this work. By injecting mixtures of

genetically marked DNA molecules, it can be show that large extrachromosomal arrays assemble directly from the injected molecules and that homologous recombination drives array assembly. Appropriately placed double-strand breaks stimulated homologous recombination during array formation. The size of the assembled transgenic structures determines whether or not they will be maintained extrachromosomally or lost. The low copy number extrachromosomaltransformation can be achieved by adjusting the relative concentration of DNA molecules in the microinjection procedure. For microinjection was young hermaphrodites at the dissecting microscope Wild-Heerbrugg model (Switzerland) with 10 x eyepieces and a lens revolver ranging from 5 x to 50 x magnification, chosen. Each animals was transferred with a worm-pick, which was sterilized with 70% ethanol, onto a 0.075 to 0.15% agarose pad, and mounted with a drop of mineral oil.

From now you should undertake the following operation very fast because otherwise the animal do not have any more chances to be alive. The axiovert microscope (Zeiss, Germany) was adjusted with the 10 x objective, any animal was brought in the middle of the site field, change the objective on 40 x, the Femtotip needle was carefully injected with a micormanipulator PMZ 20 with mother-steering (Zeiss, Germany) into one of the both gonad, and with a pressure of 2-3 hPa was made the injection of a little volume of DNA-solution into one of the both gonad arms. It is very important to do not unnecessary injure the worms.

After successfully microinjection one should give 1 µl M9 solution to protect it of dry up and to detach the worm from the agarose pad, transfer it onto a fresh NGM plate with OP50, and finally placed into the 20°C. It followed some other lines with pECFP-N1, and pEYFP-N1 without pRF4 because it was not practical for mating experiments. For the maintaining of strains which wear

the pRF4, we picked Roller phenotype but it should be take care about them, because some times they lose the fusion genes.

The vectors pEGFP-N1, pECFP-N1, and pEYFP-N1 (Stratagene, Ca, USA) were used for transferring the favorite gene into the L4 or young hermaphrodite animals of N2 (variation Bristol, Brenner, 1974) strain, which is usually used for fostering embryos, as a source for transgenic worms. The plates should be observed after at least three days for seeing the F1 generation, and judgement if there are some transgenic embryos on the plates. Subsequently, should the next generation, F2, if possible to give the favorite extrachromosomal array stable line.

2.2.2.4 Preparation of Nematode Growth Medium (NGM) culture plates and seeding with *E. coli* strain OP50 as a food

This medium were prepared out of components as follows: Briefly, 2.5 g peptone out of casein digestion, (Roth, Karlsruhe, Germany) powder, 17 g agar (Bacteriology grade, Applichem, Darmstadt, Germany) powder, and 3 g NaCl (Roth, Karlsruhe, Germany) were dissolved in 975 ml of dH_2O. The pH were adjusted to 7.2, and were sterilized by autoclaving for 20 min at 121°C.

This solution was cooled down to 55°C, then added the following sterile solutions 1 ml cholesterol (5 mg/ml dissolved in absolute ethanol, stock solution), 25 ml KH_2PO_4 (stock solution 1 M, pH 6), 1 ml $MgSO_4$ (1 M stock solution), and mixed well. This mixture were dispensed under sterile conditions onto small-seized (35 mm diameter) culture plates 2.5 ml, medium-sized (60 mm diameter) Petri plates 5 ml, or large-sized (100 mm diameter) Petri dishes 10 ml each under sterile condition, and let to get rigid for 15 min at room temperature.

The plates should be at first cooled to room temperature before adding the worms seed, OP50. *C. elegans* culture plates stored in an airtight container for several days at 4°C. For inoculating of NGM plates were used 50 µl of a diluted

suspension of *E. coli* strain OP50 out of fresh culture in a 5 ml LB medium, and spread with a sterile glass rod. OP50 is used in the laboratory as food source for *C. elegans* (Brenner, 1974). This strain is an uracil auxotrophic strain whose growth is limited on NGM plates and this is well desirable for observation, and was obtained from CGC. These culture plates were incubated for 8 hours at room temperature, stored at 4°C until they used.

2.2.2.5 Designing of the reporter gene *hp1.1::gfp*

Green fluorescence protein (GFP) which was originally isolated from the jellyfish *Aequorea victoria*, is a protein of 238 amino acids residues, that was used as vital marker to localize proteins of interest in *C. elegans* (Chalfie *et al.*, 1994). The wild-type protein absorb blue light at 395 nm (with a minor peak at 470 nm) and emitted light at 509 nm (with a shoulder at 540 nm). The great advantage of this marker is that it does not require any exogenous substrates to monitor the expression of interest genes in the living worm. This is possible if GFP was under the control of an appropriate promotor, *e.g.* HP1.1. to follow the cytological events in the interphase cells. Furthermore GFP is a powerful tool for screening of mutation that alter specific gene expression pattern in the living worms. In this order we used not only GFP but also and YFP (Yellow Fluorescent Protein) as powerful living marker in this project. YFP absorb green light at 513 nm and emitted light at 527 nm.

For amplification of the gene HP1.1 from the cosmid K08H2 were used PCR taking the forward primer ESMG57, and ESMG59 as a reverse primer. The analysis of PCR product was performed in 0.7% agarose gel. The DNA migration on the gel was examined in a 310 nm UV lamp because of the ethidium bromide, which binds to DNA and fluoresces at this wavelength. After this step a low melting point agarose gel was prepared, the 4.6 kbp band of HP1.1 was cut (*Bam*HI/*Kpn*I), and transferred into a sterile reaction tube. HP1.1

was ligated with pUC18 vector. It followed a transformation into competent *E. coli* (DH5α) cells. Subsequently, an aliquot of these cells was inoculated onto LB-agar plates containing ampicillin (100 µg/ml). Few single colonies containing *hp1.1::gfp* were picked from an agar plate, transferred each to 5 ml of LB medium with ampicillin (100 µg/ml) and incubated over night at 37°C with shaking for DNA mini preparation. After analyzing of the harvested DNA 1 single colony was chosen to make DNA midi preparation.

The HP1.1 was cut out at the restriction sites *Kpn*I (using buffer B^+ as a reaction buffer) and *Bam*HI (using buffer G^+ as a reaction buffer) from pUC18 vector. At the same time was pEGFP-N1 also digested with *Kpn*I and *Bam*HI. It followed a second ligation. After transformation, an aliquot of the bacteria was inoculated onto LB-agar plate containing kanamycin (70 µg/ml), and incubated at 37°C over night. Few single clonies were chosen for DNA mini preparation. It followed a DNA midi preparation. After an analyzing step on an agarose gel the sequence was confirmed by Seqlab (Göttingen, Germany) of obtained *hp1.1::gfp*. Second, this DNA was used for the microinjection with a concentration of 20 ng/µl with 80 ng/µl Roller marker *rol*-6(*su1006*) into the gonad arm of healthy young hermaphrodites N2 strains to generate transgenic animals (see above). For YFP was performed the same procedure, as well as pEYFP-N1 as vector.

2.2.2.6 Generation of the reporter genes *hp1.1:: s:: gfp c* and *hp1.1::gfp::sIIc* in *C. elegans* for X-ray microscopy, and as a novel tool for protein purification

The Strep-tag was described as a selected nine-amino acid peptide (AWRHPQFGG) (Schmidt *et al.*, 1996), which displays intrinsic binding affinity towards streptavidin. Furthermore, Strep-tag has been used as an affinity tag for recombinant proteins. In order to this method, we wanted to elucidate the

sub cellular structures of HP1.1 in the nuclear region. This sequence is as a widespread part of conjugates and other affinity reagent, and because of this property could streptavidin constitutes a binding bridges to HP1.1.::GFP for detection purpose. Furthermore, this method should be as an important tool for individual biochemical properties constitutes of HP1 protein family in protein purification.

An amount of *hp1.1*, pEGFP-N1 (100 ng) and both oligo nucleotides ESAD06 and ESAD07 were digested with *Apa*I (using buffer B^+ as a reaction buffer) and *Bam*HI (using buffer G^+ as a reaction buffer) for 2-3 hours at 37°C. Subsequently, it followed a ligation reaction over night at 14°C in a RM20-Lauda water bath (Germany), and the DNA was transformed into the competent *E. coli* (DH5α) cells. After transformation, an aliquot of these cell was plated onto LB plates containing (70 µg/ml) kanamycin.

Few single bacteria colonies containing HP1.1::S::GFP were picked from an agar plates, transferred each to 5 ml of LB medium with kanamycin (70 µg/ml) and incubated over night at 37°C for DNA mini preparation. After analyzing of the harvested DNA 1 single colony was chosen to make DNA midi preparation. The latter DNA was first sequenced to confirm the DNA sequence. Second, the DNA was used for the microinjection with a concentration of 20 ng/µl with 10 ng/µl Roller marker *rol*-6(*su1006*) into the gonad arm of healthy young hermaphrodites N2 strains to generate transgenic animals (see above).

For localization of HP1.1 in subnuclear structures in nuclei with concomitant Strep-tag was the *hp1.1::gfp::sIIc* construct designed. Order to this, 100ng of *hp1.1*, and *gfp::sIIc* were digested with *Kpn*I (using buffer B^+ as a reaction buffer) and *Bam*HI for 2-3 hours at 37°C. It followed a ligation of digested HP1.1 and GFP::SIIc in a sterile reaction tube over night at 14°C in a RM20-Lauda water bath (Germany), and the DNA was transformed into the

competent *E. coli* (DH5α) cells. After transformation, an aliquot of these cells was plated on LB plates containing kanamycin (70 µg/ml). Few single bacterial colonies carrying *hp1.1::gfp::sIIc* were picked from an agar plate, transferred each to 5 ml of LB medium containing kanamycin (70 µg/ml) and incubated over night with shaking at 37°C for making DNA mini preparation. After analyzing the harvested DNA, we have chosen 1 single colony to make DNA midi preparation. The latter DNA was sequenced at first to elucidate the real sequence of fusion gene. Furthermore, the DNA was used for the microinjection with a concentration of 20 ng/µl into the gonad arm of healthy young hermaphrodites wild-type strain to generate transgenic animals.

2.2.2.7 Designing of the reporter gene *his-24*::*cfp* as a DNA marker in *C. elegans*

The full length histone H1 was used as a specifically DNA-marker which allows the cell nuclei to be visualized in the in-vivo observation. 100 ng of DNA was digested with *Eco*RI (using buffer O^+) and *Stu*I (using buffer B^+) for 2-3 hours at 37°C. At the same time was 100ng of pECFP-N1 vector digested with *Sma*I (using buffer Y^+) for 2-3 hours at 25°C, and transferred for 2-3 hours into 37°C for digestion with *Eco*RI (using buffer O^+). The size fractioning of digested DNA was performed in 0.7% low melting agarose gel, when the right band cut out and transferred into a sterile reaction tube. The ligation reaction of digested pECFP-N1 and *his-24* was made over night at 14°C. Finally followed the transformation into *E. coli* (DH5α) cells. An aliquot of these cells was plated onto LB plates containing kanamycin (70 µg/ml), incubated over night at 37°C for getting single colonies to making DNA mini preparation. After analyzing of the obtained DNA, 1 single colony was chosen to prepare DNA with higher quality, also for making DNA midi preparation. This DNA was first sequenced to know, whether the fusion gene has the right sequence. Second, the DNA was

used for the microinjection with a concentration of 20 ng/µl into the gonad arm of healthy young hermaphrodites of N2 strains to generate transgenic animals.

2.2.2.8 Integration of the reporter gene *hp1.1::gfp*;*rol*-6(*su1006*) and *his-24::cfp* into the genome of *C. elegans*

It is well recommended to generate an integration of the interest gene into the genome of *C. elegans* as a stable element. The entire extrachromosomal array can be incorporated into the genome as a single element and it is very stable for a vast of experiments. Regarding to this it were recommend to generation of such stable lines. In order to this, for integration of an extrachromosomal array was used a protocol of Michael Koelle's laboratory which was modified for integration of the *hp1.1::gfp* co-injected with *rol-6*(*su1006*) into the genome of *C. elegans*. Therefore were picked 70 L4 healthy hermaphrodite larvae, which were grown on a NGM Petri plate at 16°C, and placed on a new NGM culture plate subjected to X-ray irradiation at 25 Gy, and 100 keV. A 1 mm thick copper-blade was used as filter.

These animals were then placed onto individual plates at room temperature, and allowed the plates to see the F1, picked about 500 individual animals, placed onto new fresh individual plates, and after few days were plates scored for the presence of Roller phenotypes and non-Rollers. From each plates with more than 70% Roller phenotype were picked about 1000 F2 individual animals and brought onto new fresh NGM culture plates. I looked for any plates with all Roller phenotypes, and followed them for an integrated line. From this plates were finally took some individual hermaphrodites and placed onto new individual fresh plates and scored a few days later for 100% Roller phenotype descendants.

For the integration of *his-24* were used animals, which were grown on one NGM Petri-plate at 20°C. 35 L4 larvae were picked and placed on a new NGM plate, and were subjected to X-ray irradiation in a STIPLAN X-ray-cell of

Siemens (Munich, Germany) at 200 keV, and 38 Gy. A 500 µm thick copper-blade was used as filter.

The animals were placed on NGM Petri dishes each individually and were incubated into a Heraeus BK-600 incubator at 20°C. Subsequently, were observed until the F2 generation. 300 individual F2 animals were picked on new NGM Petri plates each animal onto one individual plate, and incubated at 20°C. The culture plates were continually observed, after a couple of days (waiting for F2 progeny) were picked several individual L4 hermaphrodite larvae and placed onto new NGM Petri dishes each animal.

2.2.2.9 Freezing and recovery of *C. elegans* strains using the liquid freezing solution

For keeping the *C. elegans* cultures were these frozen and stored in freezing solution with an addition of glycerol, and a gradual cooling *i.e.* 1°C per min in a Styrofoam box to -80°C. Mostly fresh starved young larvae at stages of L1-L2 survive freezing best. In this order, it was a medium-seized (60 mm diameter) NGM culture plates with higher amount of L1-L2 larvae chosen, and washed with 500 µl M9 down, removed into a sterile reaction tube. It was added an equal volume of freezing solution. This solution were mixed under sterile condition out of 129 ml of 50 mM K_2HPO_4 and KH_2PO_4, 100 mM NaCl in 1 liter dH_2O, (pH 6.0, and autoclaved) and added 30% sterile glycerol (v/v). The reaction tubes were packed in a small Styrofoam box with slots for holding such cups, and placed in a -80°C freezer. To examine the viability of worms, it was thawed on vial as a control.

2.2.2.10 Cleaning of contaminated *C. elegans* strains

Contaminated *C. elegans* stocks with other bacteria, yeast , or mould should be cleaned because it is possible that they affect the worms. Furthermore, it is easier to score phenotypes. To clean contaminated worm culture plates were

bleached adult hermaphrodites with bleaching solution, which were made fresh just before use. This solution kill the contaminant and all worms. Only the embryos survived this treatment, which are clean, and were used for cultivation.

2.2.2.11 Reproduction, maintenance, scoring, and crossing of *C. elegans* strains culture

Hermaphrodites of *C. elegans* are able to reproduce either by self-fertilization or by cross-fertilization with males, and these are not able cross-fertilize each other. Every hermaphrodite are able to lay approximately about 300 embryos. Regarding to this, for keeping the *C. elegans* strains is depend on the stability of the mutants. It means that the *e.g.* heterozygous (-/+) of a such strains will eliminate it's activity, and this genotype will rapidly be taken over by wild-type (wt) homozygous (+/+).

In a number of experiment, *e.g.* RNAi microinjection or crossing of two strains of *C. elegans,* it was necessary to know about the progeny of worms. For scoring of offspring in experiments were the hermaphrodites transferred to new NGM plates, and scored the progeny. Transgene extrachromosomal arrays like *hp1.1::gfp;rol-6*(*su1006*), *hp1.1::s::gfp*, *hp1.1::gfp::sIIc*, *hp1.1::yfp*, *his-24::cfp* are not stable. For experiments with these strains were transferred several worms bearing transgene array to keep the strain alive. Maintaining of an integrated strains like *hp1.1::gfp;rol-6*(*su1006*) or *his-24::cfp* are very easy. One single healthy hermaphrodite could keep the stain alive.

N2 strain (wild type) has normally 0.2% males which arise spontaneously in hermaphrodites (Hodgkin, 1999). For cross experiments were used males out of *him*-8(*e1489*) strain which results in male frequencies above 30%. The strain HP1.1::GFP;*rol*-6(*su1006*) integrated array has rarely males. For cross experiments we crossed first this strain with *him*-8(*e1489*). Because of Roller phenotype was cross experiment not always so efficiently like *him*-8 strain. *hp1.1::gfp;rol-6*(*su1006*) were for determination of chromatin desilencing in the

germline crossed with *mes*-3(*bn21*) strain. Furthermore, *hp1.1::gfp;rol-6*(*su1006*) was crossed for *hp1.1::gfp* in the dauer larvae with CB1370.

2.2.2.12 RNA interference (RNAi) in *C. elegans*

2.2.2.12.1 Preparation of agarose coated coverslips (pads) using for microinjection

An agarose (ultra-quality, Roth, Germany) solution (0.075 to 0.15%) in dH_2O was boiled in a microwave, a drop of it was dispensed onto a coverslip (24 x 40 mm, Menzel-Gläser, Germany), and air-dried over night at room temperature. Before every use, the agarose-coated coverslip should be moisturized by breathing over the surface. The latter makes it obvious, which side of the coverslip the agarose pad is fixed to.

2.2.2.12.2 RNAi as a reverse genetic tool

Reverse genetics as a tool, to distinguish the phenotype of gene suppression, generated with RNA-intermediate interference (RNAi), which has been described previously (Fire *et al.*, 1998). First, the full-length cDNA (HP1.1, yk432c11; HP1.2 yk470a11; yk106f2) templates were subcloned from ZAP-II phage into pBluescript II SK (-) from Stratagene. For *in vitro* transcription of these were used specifically designed forward and reverse primers in single experiments (ESMG67/T7, for HP1.1; MBMG01/ MBMG02 for HP1.2). In order to this were used the solution of Ambion (Frankfurt, Germany) MEGAscript T7-Kit. For preparation of dsRNA was used diethyl pyrocarbonate (DEPC), which should protect RNA of enzymatic digestion through RNase. The solution and pipette tips were prepared with a concentration of 0.1% (v/v) DEPC in dH_2O, incubated several hours at room temperature, and autoclaved for 20 min at 121°C. The dsRNA of each gene was normally made

by synthesizing of both the sense and antisense from a cDNA using T7 RNA polymerases.

1 µg of midi preparation-made linearized template DNA of each gene was transferred to a sterile and DEPC rinsed reaction tube with 2 µl 10 x reaction buffer, 2 µl nucleotides ATP, CTP, GTP, and UTP each, Nuclease-free H_2O, and was added 2 µl of enzyme T7 RNA-polymerase to a final volume of 20 µl after all other components, and mixed well. This mixture was than incubated for 6 hours at 37°C. Remove the template DNA using 1 µl of DNase, mixed well and incubated for 15 min at 37°C. The RNA was recovered as followed. 115 µl nuclease-free water and 15 µl NH_4Ac stop solution mixed thoroughly, extract with an equal volume of phenol (pH 4.0)/chloroform and then with an equal volume of chloroform. The RNA was precipitated by adding 1 volume of isopropanol and mixed gently well, chilled for at least 15 min at -20°C. The solution was centrifuged 15 min at 4°C at 14000 r.p.m. for harvesting of RNA. The supernatant were carefully removed, and the RNA pellet was resuspended with RNase-free water. The concentration of HP1.1dsRNA (8.6 µg/µl) was determined by UV spectrophotometer and confirmed by ethidium bromide staining in the 0.7% agarose gel. The HP1.1dsRNA solution was stored at -20°C.

In this order were the dsRNA from HP1.1 injected into the gonad arms (germline) of healthy L4 or young hermaphrodites of N2, BK48 (Kelly and Fire, 1998) and EC107 (Jedrusik and Schulze, 2001) strain, and *hp1.1::gfp*;*rol-6*(*su1006*) integrated line, led to the epigenetic inactivation of the gene in the F1 offspring. The first 12 hours harvested embryos were removed and the adult worms were then transferred to new fresh NGM culture plates where they were allowed to lay embryos for 24 to 36 hours after microinjection. Every 8 hours were the injected worms transferred to new culture plates, and scored the offspring for new phenotypes. Regarding to that, subsequently were scored the progeny, noticed every deviation in comparison to the control injected worms.

dsRNA out of K08H2.6 were produced from the cosmid K08H2 using the forward primer ESMG67, and reverse primer T7 (Stratagene). The HP1.1dsRNA was injected into wild-type, BK48, and *mes*3(*bn21*) strain.

Furthermore, was made the dsRNA of C41G7.4 using forward primer MB_Su(var)39for1, and MB_Su(var)39bac1 as a reverse primer; C15H11.5 and F59E10.1 from the cDNA clone yk236f8 using the forward primer ESMG67, and T7 as a reverse primer; and B0250.7 (4.7 µg/µl) out of the cosmid B0250 taking the forward primer MB_LBRT7for1 and the reverse primer MB_T7LBRbac1 for possible interaction with HP1.1.

The concentration of these RNAi with lamin B receptor, B0250.7RNA (4.7 µg/µl and 5.8 µg/µl); with SET domain, C41G7.4 (5.7 µg/µl); SET domain, C15H11.5 (5.0 µg/µl); and origin recognition complex ORC2, F59E10.1 (8.1 µg/µl) were determine in a spectrophotometer Kontron Instruments (Milan, Italy) at A260 nm. In order to this, is an OD of 1 corresponds to approximately 40 µg per 1 ml of dsRNA. This results were confirmed by ethidium bromide staining by UV light at 310 nm on the gel.

2.2.3 Microscopy

2.2.3.1 Preparation of agarose coated slides for fluorescence light microscopy use of *C. elegans*

An appropriate agarose powder (3-5%) were solved in M9 and boiled in the microwave, placed a drop of it on a pre cleaned and ready-to-use cut edges microscope slide (76 x 26 mm, Menzel-Gläser, Germany), kept a second slide on it so, that the agarose were spread as a thin layer, and waited for 1-3 min to get solidify. The second slide were popped off with a quick twist of wrist, and placed 20 µl sterile M9 buffer onto it to avoid getting dry. Worms and embryos could be removed from a NGM culture plates onto such slide and ready to use

for examined in a microscope. Such made slides were useful every time fresh, if it would be need.

2.2.3.2 Preparation of poly-L-lysine coated slides for immunostaining for fluorescence light microscopy (Cole and Schierenberg, 1986)

200 ml of dH_2O were heated to the temperature of 60°C, it added 400 mg of gelatine (end concentration of 0.2%), after that the temperature was cooled by gently heating at 40°C, it added 40 mg of $CrK(SO_4)_2*12H_2O$) at the end concentration of 0.02% under agitation. To each ml of this solution were added 1 mg of poly-L-lysine (Sigma P9011, MW >300 kDa) and mixed well. 20 µl of this mixture was rubbed onto a circle shape in the middle of each slides (76 x 26 mm, Menzel-Gläser, Germany), which was scratched with a electric diamond-pen, transferred on a Thermoblock RCTbasic from IKA Labortechnik (Germany) at 60-80°C, and it kept without dust at 4°C.

2.2.3.3 Embryo preparation of *C. elegans* for immunostaining use in fluorescence light microscopy (Sulston & Horvitz, 1977)

A typical healthy NGM culture plate with *C. elegans* strain shows a lot of embryos and a thin layer of OP50 on it. *C. elegans* embryos develop with a chitinous shell, which functioned as a barrier, and for immunocytological use should be destroyed this layer. For the analysis of embryonic development were embryos obtained from 10 to 20 young healthy gravid adult hermaphrodites from N2 strain on NGM Petri plates.

These worms were transferred with an eyelash and placed into a watchglass with a drop of M9 buffer. After washing, worms were transferred onto the labeled area of a poly-L-lysine-coated slide with 20 µl M9 buffer. Worms were cut with a syringe needle so, that the embryos come out of the worms. The slides were covered with a 18 x 18 mm (Menzel-Gläser, Germany) coverslip, were loaded into a head-cut-Falcon-tube, and immerse in a pool of

liquid nitrogen using a long forceps for 1 min. After freezing, wedge the tip of a razorblade under one corner of the 18 x 18 mm coverslip (Menzel-Gläser, Germany) and popped it off with a very quick twist of wrist.

For antibody staining were the mounted embryos fixed in an ice-cold (-20°C) absolute ethanol for 20 min, for another 10 min in an ice-cold (-20°C) acetone, and led they drying for a few minutes at room temperature. Unfortunately, for antibody staining of HP1.1::GFP does not work this method. In generally it is very important to keep the minimize of both solutions carryover and the wet surface.

For blocking were the specimen incubated with a 5% nonimmune goat serum for 1 hour at room temperature. The slides were laid horizontally in a sealed humidity chamber. Afterwards the blocking reagent was removed and the excess solution wicked off, the first antibodies solutions anti-HP1 and anti-P-granules were added to the required dilution (1:100) and (1:400) on the marked area, were covered over the previously marked area of the slides, and incubated over night at 4°C. The slides were transferred to a Coplin jar containing TBS1 and washed for 5 min. This step were repeated for another 2 times. Subsequently, the slides was shaken to remove of the TBS1 buffer, the remaining excess buffer was then with a twisted corner of Kimberly-Clark kerchief wiped off.

The secondary antibodies, Cy3-conjugated goat anti-rat (Nycomed Amersham) antibody and Cy2-conjugated goat anti-mouse (Jackson ImmunoResearch) was covered with the dilution (1:400) onto the labeled area, incubated in a sealed humidity chamber, whose cover were wrapped with a thin layer of aluminum foil, for 1 hour in a Heraeus-incubator (Hannover, Germany) at 37°C. From this step onward the slides were protected against exposure of the light.

The DNA was stained with the fluorochrome Hoechst 33342 at a final concentration of 1.6 µM for 8 min at room temperature. Normally a

concentrated stock solution of 1 mM of Hoechst 33342 in ddH_2O, and it kept in a sterile reaction tube at 4°C for long-time storage in the darkness. The slides were washed 6 times each 5 min with changes of TBS1 in Coplin jars at room temperature. Slides were shaken to remove the TBS1 buffer, and carefully wipe off with a twisted corner of paper towel as much solution from the slides as possible without permitting it to dry out.

Finally, a small drop of mounting medium (2-5% propylgallate solution in TBS1 and 90% glycerol) for protection chromophores of bleaching reaction (Giloh and Sedat, 1982), was spread on the slides. Subsequently, covered the specimen with a coverglass carefully without any air bubble and sideways movement. The edges of the coverslip were sealed with a clear nail varnish. Lastly, slides should be protected to light, and transferred them for storage in a slide boxes in the darkness at 4°C. Unfortunately, the freeze-cracking method did not work for the HP1.1::GFP, because the subnuclear structures were destroyed.

2.2.3.4 Preparation of embryonic single cells of *C. elegans* for fluorescence light microscopy, and immuno-gold staining in X-ray microscopy

C. elegans embryos have a chitinous shell, which functioned as a barrier and protected it from environment, and for immunocytological use, it should be destroyed. For this analysis of embryonic development were embryos obtained from healthy young gravid adult hermaphrodites out of a *hp1.1::gfp* extra chromosomal array on NGM plates. These worms were washed with 1 to 2 ml of M9 to rinse them from the plates. After additional washing steps to remove the *E. coli* OP50 were worms transferred into a reaction tube and collected at 2000-2500 r.p.m. for 1 min.

The supernatant was removed very quickly because of living worms, and lastly to keeping a small amount of volume. In the next step were this suspension treated with a bleaching solution (900 µl of 3% NaOCl diluted in

egg-salt, and 100 µl of 500 mM NaOH) for 3 min, washing 3 times for 1 min with PBS1, centrifuged at 3000 r.p.m. for 1 min. Embryos were washed 3 times for 1 min each in egg-salt.

To crash the vitelline membrane embryos were underwent a treatment of chitinase (from *Serratia marcescens*, Sigma C7809) for 8-10 min at 37°C. After 3 wash-step with PBS1 and egg-salts 1 min each, the cells collected in a small volume. 5 to 10 µl of cell suspension were placed on to poly-L-lysine coated slides, let few minutes to "sticking" the cells at room temperature, than they were fixed with 2% formaldehyde (or glutaraldehyde) for 5-10 min. Every time were after washes with TBS tried firmly dry the area around the specimen, so that the small volumes of solution saturated the specimen directly and were not spread over a large area of the marked slides.

The cells were permeabilized with 1% Triton for 5 min, blocked with 5% nonimmune goat serum and NH_4Cl (500 µM) for 30 min. After 3 wash-steps, *i.e.* rinsed the slides in 1 x TBS1 for 5 min each in a solution-filled Coplin jar, mounted the anti-GFP (rabbit serum) antibody solution in favorite dilution (*i.e.* 1:500) on the marked area, and incubated the slides horizontally in a sealed humidity chamber at 37°C for 1 hour.

The samples were rinsed in Coplin jar in TBS1 and washed 3 times for 5 min each, and with a twisted Kimberly-Clark kerchief were carefully wipe off the excess buffer from around the specimen, and added the Cy2 conjugated goat anti-rabbit (Jackson ImmunoResearch) secondary antibody (1:500) and incubated in a sealed humidity chamber for 45 min at 37°C. After that, the slides were washed 3 times in TBS1.

The DNA was stained with Hoechst 33342 fluorochrome at a final concentration of 1.6 µM for 8 min at room temperature. The slides were washed 6 times each 5 min with changes of TBS1 in Coplin jars at room temperature. From slides were the TBS1 buffer removed, and carefully wiped off with a

twisted corner of paper towel as much solution from the slides as possible without permitting it to dry out.

Specimen for X-ray microscopy were made on a special foils as described in 2.1.8. The procedure of preparation of single cells, and primary antibody staining was performed like above, in this section. Afterward, it was performed as followed

The foils were rinsed in Coplin jar in TBS1 and washed 3 times for 5 min each, and with a twisted Kimberly-Clark kerchief were carefully wipe off the excess buffer from around the specimen, and added the 1-nm gold-conjugated secondary antibody (1:500, from British Biocell) and incubated in a sealed humidity chamber for 10 hours at 4°C. The DNA was stained with Hoechst 33342 fluorochrome at a final concentration of 1.6 μM for 8 min at room temperature. The foils were washed 6 times each 5 min with changes of TBS1 in Coplin jars at room temperature. From foils were the TBS1 buffer removed, and carefully wiped off with a twisted corner of paper towel as much solution from the slides as possible without permitting it to dry out.

Finally, foils were fixed for 1 hour in 0.5-1% glutaraldehyde buffered with TBS1 at room temperature. Afterward, were the foils were washed several times in double-distilled water. It followed a silver enhancement using a Kit (LI Silver, Nanoprobes Inc., Stony Brook) for 20-25 min at room temperature. In this step should the foils be protected to exposure of the light, and were the specimen transferred in the darkness. The reaction was stopped using double-distilled water. After several wash-steps in distilled water were the samples fixed with a fresh made 1% glutaraldehyde solution for 1 hour at 4°C to reduce the shrinkage. Finally, the foils after a few wash-steps were contained in double-distilled water at 4°C before recording the X-ray micrographs at BESSYI.

Every foil was used only once in a special chamber of X-ray microscope. A small drop of double-distilled water was spread on every foil, fixed in a special X-ray chamber, covered the specimen with a cover foil carefully without

any air bubble and sideways movement. The edges of the foils were screwed together. Subsequently, it was recorded micrograph in the Göttingen transmission X-ray microscope.

2.2.3.5 Fluorescence microscopy of *C. elegans* specimen using Laser Scanning Microscope 510 (LSM 510)

To analyze the phenotype of favorite worm or embryos is the microscope the suitable tool because of the perfect dimensions of embryo (50 x 90 μm) and adult approximately 1 mm. Thus, subcellular details of the cell architecture of the nucleus can be seen and followed during the cell division through the microscope. I am specially interested in the mitosis events and therefore is such microscope very well suitable. Subsequently, for observation of antibody staining and transgenic lines with a living-fluorescence proteins just like EGFP, EYFP, and ECFP, were characterized using the Laser scanning microscope 510.

Before using the microscope were checked the Köhler illumination system, which provides homogeneously illuminated images and permitted an increase in the resolving power due to the use of a condensor.

One slide were mounted with a droplet of halogen free immersions oil (Zeiss, Germany), and put in the appropriate place on the microscope table. Subsequently, were check up the specimen on the slide, and chose a typical embryo or a single cell. In the next step were the main switch of laser-scan microscope turned on, and the program started. Depended on the recording micrograph started the appropriate laser line. For recording micrographs was firstly chosen an embryo or cell on the slide, and then started with the scanning. For detection signals from HP1.1::GFP was first the filter set UV/488 as a main color splitter HFT. The NFT490 was chosen as a color splitter, and the filter set with the band-pass 505-530 nm was used for harvesting signals between 505 nm to 530 nm for example from GFP. NFT 490 passes emission light wave-lengths greater than 490. The confocal laser scanning microscope provides argon laser

lines that are ideal for discrete excitation of CFP (458 nm) and YFP (514 nm). Finally, the images were taken with filter combinations bp 560-615 (YFP) and, bp 505-550 (CFP) respectively.

2.2.3.6 Isolation of a total *C. elegans* lysate

The isolation of proteins from *C. elegans* strain N2 were carried out at a deep temperature in liquid nitrogen. The worms were homogenized using a mortar and pestle which contained extraction buffer. The tissue to buffer ratio was 1.5 g/ml. The solution was dispensed in 100 µl aliquots, and stored at -20°C. The separation of the proteins was performed via SDS polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli, 1970).

The 12% separation gel which contained 18.7 ml acrylamide solution, 29.1% w/v acrylamide and 0.9% biscaryamide dissolved in ddH_2O, 18.1 ml of ddH_2O, 9.3 ml of a 1.875 M Tris-HCl pH 8.8 under stirring condition in vacuum the solution was degassed for 5 min at room temperature. Furthermore, for polymerization of the gel 466 µl 10% SDS, 23.3 µl TEMED, and 49 µl 30% APS were mixed gently and bubble free. This should be pour between two glass plates, which were washed with ddH_2O and 70% ethanol, dried with paper towel, fixed into the gel box.

To avoid the oxidation condition of the top of gel, it was poured with additional butanol saturated ddH_2O onto it, respectively. After polymerization of the separation gel were prepared the stacking gel. The stacking gel 5% was mixed 2.4 ml acrylamide solution,10.8 ml ddH_2O, 1.5 ml of 1.25 M Tris-HCl pH 6.8 under stirring for 5 min in vacuum was the solution degassed at room temperature. It was added 150 µl 10% SDS, 15 µl TEMED, 15 µl 30% APS. One comb was very carefully put into the top of separation gel without touching it, and poured the ready mixed solution onto the top of separation gel and let polymerized it. The comb was carefully pulled out of the gel.

The slots of the stacking gel were three times washed with electrode buffer (Laemmli, 1970). The total protein sample of *C. elegans* (12 µl droplets) and Promega's Mid-Range protein molecular weight marker (8 µl droplets) were mixed with an equally volume of loading buffer including 10 µl β-mercaptoethanol, molecular biology grade, from Applichem (Darmstadt, Germany), and incubated for 3 min in a DAGLEF-PATZ water-bath (Holstein, Germany) at 95-100°C. After quickly centrifugation were the samples placed on ice for 1 min and the transfer into slots of gel carrying the Hamilton-Bonaduz microliter syringe, the sample was sucked into the microliter syringe, delivered into the slot on top of the stacking gel, and overlaid with electrode buffer.

The assembly was put into electrophoretic chamber SE 600 from Hoefer Scientific Instruments (San Francisco, USA). Electrophoresis was performed at 50V and 20 mA during the stacking gel for more than 1.5 hours, and 250 V and 40 mA for 4-5 hours during separation gel. Gels were removed from the gel plate, and the protein were stained on a KS250basic-shaker from W.Krannich (Göttingen, Germany) with 50 r.p.m. for 1 hour in 1 mg/ml Coomassie Blue G-250 (Serva, Heidelberg, Germany) solution, 50% methanol and 10% acetic acid). The discoloring procedure of the gel followed in a destaining solution overnight at room temperature. Gels were photographed through an Polaroid camera.

2.2.3.7 Western blot analysis of total *C. elegans* lysate

Polypeptide was obtained by preparation of SDS polyacrylamide gel electrophoresis (Laemmli, 1970). Transfer of the protein to nitrocellulose sheet BA85, Schleicher & Schuell (Göttingen, Germany) was performed under definitive condition as described before (Towbin *et al.*, 1979). On a plastic grid which was wetted with transfer buffer laid a pad (*e.g.* foam material) and a sheet of nitrocellulose.

The polyacrylamide gel was put on the nitrocellulose sheet and the slots were labeled for indication of the right place each sample. It is important to working without any air bubbles. A second pad and plastic grid were laid on the gel. The grids were pushed together which pressed the gel against the nitrocellulose sheet. The transfer buffer was put in the electroblot chamber TE 62 from Hoefer Scientific Instruments (San Francisco, USA). The grids were quickly without any bubble put in the electroblot chamber. The transfer of polypeptide was performed at 1 A and 4°C for 1 hour.

2.2.3.8 Detection of HP1 homologs of *C. elegans* using a monoclonal anti-murine-HP1 antibody via chemoluminescence

The elicitation of rat monoclonal antibody directed against heterochromatin protein (HP1) of mouse has been described in the earlier (Singh *et al.*, 1991). The polyclonal rabbit antibody horseradish peroxidase conjugated anti-rat IgG (whole molecule) was purchased from Sigma-Aldrich (Steinheim, Germany).

The membrane was rinsed 4 times 5 min each in TBS2 for 20 min at room temperature, and for the blocking of non specific binding was preincubated with 10% non-fat dry milk in TBS2T for 1 hour. The membrane was washed 3 times in TBS2 and incubated with primary antibody rat anti-mouse (Singh *et al.*, 1991) at a dilution of 1:1000 in blocking solution over night at 4°C. The membrane was washed 4 times 5 min each in TBS2T. The anti-rat-IgG linked horseradish peroxidase (HRP) from Sigma-Aldrich (Steinheim, Germany) at a dilution 1:30000 in blocking solution was used as a secondary antibody, incubated for 1 hour at room temperature.

Repeatedly 4 times in TBS2T and 2 times 5 min each in TBS2 washed the membrane and visualized via Chemoluminescence, Renaissance Western-Blot Chemoluminescence Reagent (NEN, Boston, MA). An equal volume of

enhanced luminol reagent and oxidizing reagent were mixed and incubated on the membrane for 1 min at room temperature.

With a layer of Whatman-paper were the membrane dried, enveloped it quickly in plastic sheet and put in a cassette. A Kodak X-Omat Blue XB-1 (Tokyo, Japan) film was placed on the plastic sheet and exposition 10 min at room temperature. The film was 4 min in the X-ray fixing solution (Roentgensuperfix) from Tetanal-Photowerk (Norderstedt, Germany), after short washing, 4 min in the developer solution (Roentgenliquid) of Tetanal-Photowerk, washed again at room temperature and get to dry.

2.2.3.9 Protein-isolation and Semi-dry electroblotting from *hp1.1::gfp..sIIc* strain of *C. elegans*

The isolation of proteins from *C. elegans* strain *hp1.1::gfp..sIIc* were carried out at room temperature in M9 buffer from a medium-sized (60 mm diameter) culture plate. The worms were washed for 3 times, centrifuged for 1 min at 3000 r.p.m., the supernatant removed quickly, and added equal volume of sample buffer, and incubated for 3 min in a DAGLEF-PATZ water-bath (Holstein, Germany) at 95-100°C. The sample was centrifuged for 1 min at 12000 x g, and the separation of the proteins was performed via SDS polyacrylamide gel electrophoresis (see above).

Stacking gel and separation gel as a midget-gel contained 5% and 12%, respectively. The slots of the stacking gel was three times washed with electrode buffer (Laemmli, 1970). The total protein sample of *C. elegans* strain *hp1.1::gfp..sIIc* (20 µl droplets), strain *hp1.1::s::gfp* (20 µl droplets), *gfp::sIIc* (10 µl droplets) as a positive control, and 2 µl Promega's Mid-Range protein molecular weight marker (10 µl droplets including 8 µl sample buffer) were transferred into slots of gel carrying Hamilton-Bonaduz microliter syringe. The samples were separately sucked into the microlitre syringe, delivered into the slot on top of the stacking gel, and overlaid with electrode buffer.

The assembly was put into electrophoretic chamber Semi-Dry Electroblotting with Sartorius's Sartoblot II (Göttingen, Germany). Electrophoresis was performed at 50 V and 20 mA during the stacking gel for more than 15 min, and 200 V and 20 mA for 1 hour during separation gel. Gels were removed from the gel plate, and one of them were stained for 2 hours in Coomassie Blue G-250 solution on shaker-KS250basic (IKA Labortechnik, Germany) with 50 r.p.m. The discoloring procedure of the gel followed in a destaining solution over night at room temperature. Gels were photographed through an CCD camera.

The second gel was used for semi-dry electroblotting. Polypeptides, which were obtained by preparation of SDS polyacrylamide gel electrophoresis (Laemmli, 1970) should be transferred onto a nitrocellulose sheet to detect the proteins using HRP-conjugated Strep-Tactin. The transfer of the proteins onto a nitrocellulose sheet BA85 from Schleicher & Schuell (Göttingen, Germany) was performed under definite condition for semi-dry electroblotting with Sartorius's Sartoblot II as described below. It is necessary to wear gloves during the experiment. For layer of Whatman paper exactly as large as gel shape were cut for the transfer. Both graphite plates should be washed with dH_2O. Two layer of Whatman papers as a pad which was wetted with cathode buffer, was placed onto cathode graphite-plate (the bottom plate).

The polyacrylamide gel was rinsed in cathode buffer and put onto this, the nitrocellulose sheet, which eventually rinsed in the cathode buffer was carefully placed on the gel. It's very important to work very precisely because of "smeary bands", and it is not allowed to change the position, if the nitrocellulose sheet have been placed on gel. One layer Whatman paper were submerged into the anode buffer-1, that was placed onto the nitrocellulose sheet.

Subsequently, the second layer Whatman paper which were rinsed with anode buffer-2 was placed: The superfluous buffers were picked up with a twisted corner of paper towel. The top plate, anode graphite-plate was put on

this “sandwich” and was started. The transfer of polypeptide was performed at 0.8-1.2 mA/cm^2 (constant current) for 1 hour at room temperature, and the current should not be higher than 200 mA. The graphite plates should be washed carefully with water and let dry.

2.2.3.10 Detection of HP1.1::GFP::SIIc with Strep-Tactin as a protein-protein interaction tool of *C. elegans* proteins

For detection of HP1.1::GFP::SIIc on the blot was performed with the phototope horseradish peroxidase (HRP) which was conjugated to Strep-Tactin. The Strep-Tactin-HRP was purchase from IBA (Göttingen, Germany).

After electroblotting was the cellulose (membrane) sheet washed with dH_2O. To examine of the protein bands were preformed a reversible membrane staining via Ponceau S8 (Salinovich and Montelaro, 1986). The membrane was washed 2 times with ddH_2O, 1 min in 5% Ponceau S8 solution (Salinovich and Montelaro, 1986) in acetic acid. Subsequently, the membrane was washed with ddH_2O until visualization of the protein bands. After examining of the bands on blot, was performed the neutralization reaction, using 20 mM NaOH.

The membrane was rinsed 4 times in TBS2 for 5 min at room temperature, and for the blocking of non specific binding sites was preincubated with 3% BSA in TBS2 with 0,2% Tween-X20 (TBS2T), and chicken avidin in TBS2 (10 µg/ml) for 20 hours. The membrane were washed 3 times in PBST at room temperature, and incubated with Strep-Tactin-conjugated to HRP at a dilution of 1:5000 in 3% BSA in PBS with 0.5% Tween-X20 for 1 hour at room temperature. The membrane was washed 2 times in TBS2T and 2 times with TBS2.

Finally, the bands on the membrane were visualized in Lumiglo solution (400 µl of 20 x concentrated Lumiglo reagent A of Cellsignaling (LifeTechnology, Karlsruhe, Germany) and 400 µl 20 x concentrated H_2O_2

reagent B of Cellsignaling of LifeTechnology (Karlsruhe, Germany), and 9.4 ml ddH_2O). The membrane was shaking for 1 min at room temperature. It was dried using Whatman paper, and was quickly wrapped in a plastic sheet, and fixed it in the cassette. Subsequently, was quickly exposed a Kodak's blue X-Omat, XB1-film 18 x 24 cm (Tokyo, Japan) in the darkness for 10 to 20 sec. The film was 4 min in the X-ray fixing solution (Roentgensuperfix) from Tetanal-Photowerk (Norderstedt, Germany), after short washing, 4 min in the developer solution (Roentgenliquid) of Tetanal-Photowerk, washed again at room temperature and get to dry.

3 Results

3.1 HP1 in *C. elegans* and the aim of the present investigation

The heterochromatin-binding protein1 (HP1) of *Drosophila* was initially described as a protein associated with the chromocenter of polytene chromosomes in larval salivary glands (James and Elgin, 1986). HP1 is encoded by a dominant suppressor of position effect variegation (PEV), *Su(var)2-5* (Wustmann *et al.*, 1989). A molecular genetic study has revealed that *Su(var)2-5* fulfils the criteria of dosage dependency of structural protein of heterochromatin (Locke *et al.*, 1988). HP1 homologs have been identified in different species from yeast to human (James and Elgin, 1986; Lorentz *et al.*, 1994; Huang *et al.*, 1999; Epstein *et al.*, 1992; Wreggett *et al.*, 1994; Singh *et al.*, 1991; Saunders *et al.*, 1993). Recently, Motzkus *et al.* (1999) have shown that the mammalian HP1 homolog, mouse-HP1 (M31), has a novel function in mammalian spermatogenesis.

The HP1 family proteins are relatively small proteins with molecular weights of 15-35 kDa (Eissenberg and Elgin, 2000). HP1 homologs contains a carboxy-terminal chromo domain and a structurally related carboxy-terminal motif, the chromo shadow domain (Aasland and Stewart, 1995; Smothers and Henikoff, 2000). The chromo domain has been defined as a region of 50 amino acids residues in HP1 and Polycomb (Pc). A chromo shadow domain is present also in HP1 (Aasland and Stewart, 1995) which is absent in Polycomb. The chromo domain of human HP1 homolog facilitates an interaction with INCENP (Ainsztein *et al.*, 1998). The chromo domain of Pc targets Pc to specific euchromatic sites on polytene chromatin (Messmer *et al.*, 1992) whereas the HP1 chromo domain is dispensable for the targeting of HP1 to heterochromatin (Powers and Eissenberg, 1993). Chromo domain and chromo shadow domain

can apparently function independently to mediate specific localization in chromatin (Messmer *et al.*, 1992, Powers and Eissenberg, 1993). The chromo shadow domain is 64 amino acids residues in HP1 (Aasland and Stewart, 1995). The *S. pombe* SWI6 protein which is involved in repression of the silent mating-type locus, is a chromo shadow domain proteins. The chromo shadow domain occurs in proteins from yeast to higher eukaryotes. It has been reported that HP1 family proteins undergo self-association (Epstein *et al.*, 1992; Le Douarin *et al.*, 1996; Ye and Worman, 1996) and that the chromo shadow domain is mostly involved in these interactions. A hinge region between these motifs contains a conserved sequence block within which it contains an invariant sequence (KRK) and a nuclear localization motif (Smothers and Henikoff, 2000).

The structural analysis of mouse HP1 showed that HP1 has a remarkable similarity to the nuclear proteins but lacks the surface charge necessary for DNA binding (Ball *et al.*, 1997; Brasher *et al.*, 2000). According to this study, the chromo domain of the mouse HP1 homolog has an overall negative surface charge distribution and appears to be better suited for protein-protein interaction than for protein-nucleic acid interaction (Ball *et al.*, 1997). Mammalian HP1 homolog has a dosage-dependent effect on position effect variegation in transgenic mice (Festenstein *et al.*, 1999). On the other hand, it has been shown that the human homolog of *Drosophila* HP1 is also a DNA-binding protein and contains a DNA-binding motif (Sugimoto *et al.*, 1996). *Drosophila* HP1 can be multiply phosphorylated by serine/threonine kinases one of which is casein kinase II (Zhao and Eissenberg, 1999). Finally, it has been suggested that HP1 protein acts as a bifunctional cross-linker which perhaps organizes a higher order chromatin structure by linking or anchoring chromatin subunits (Eissenberg and Elgin, 2000). HP1 family proteins have been shown to interact with other nuclear proteins (Huang *et al.*, 1998; Pak *et al.*, 1997; Ainsztein *et al.*, 1998; Lachner *et al.*, 2001; Pyrpasopoulou *et al.*, 1996, Ye and Worman, 1996).

For the first time, in this study the nematode *C. elegans* was used to study HP1 and its homologs in order to get further insight into their functions. This organism, *C. elegans*, was the first multicellular organisms whose genome was completely sequenced (The *C. elegans* Sequencing Consortium, 1998). A sequence alignment within our project revealed three HP1 homologs, HP1.1, HP1.2, and HP1.3 in the genome of *C. elegans*. The *C. elegans* HP1 homolog proteins share both chromo - and chromo shadow domain motifs (Fig. 3-0). Whereas HP1.1 and HP1.2 are relatively small proteins with a molecular weight of 20,884, and 20,427 kDa, the HP1.3 is a large protein with a molecular weight of 39,630 kDa. HP1.3 has in comparison to the HP1.1 and HP1.2 a long carboxy-terminal region (Fig. 3-1).

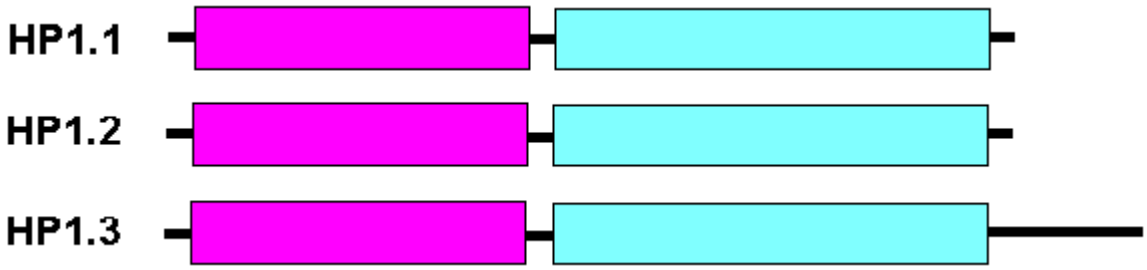

Fig. 3-0 The three putative HP1 proteins of *C. elegans*. The chromo domain motif of each protein is shown in violet, and the chromo shadow domain is depicted in blue. HP1.3 has in comparison to HP1.1 and HP1.2 a large tail region. *hp1.1* encode HP1.1, whereas HP1.2 and HP1.3 are a splice variant of the gene *hp1.2*.

Three HP1 proteins in *C. elegans* were studied to get more insight into the function of HP1 in chromatin. Transgenic animals were made that carry extra copies of *hp1.1* fused to GFP either as extrachromosomal or as integrated arrays. Furthermore, the RNAi technique was applied to knock-out transiently HP1.1 and HP1.2 and to analyze the phenotypes. Furthermore, a dynamic distribution of HP1 during the cell cycle was observed by concomitantly localizing HP1 and histone H1 by Yellow Fluorescent -and Cyan Fluorescent Protein, respectively.

Until now, there have been no cytological data on heterochromatin in *C. elegans*, and this is the first work on heterochromatin proteins in *C. elegans*. A confocal laser scanning microscope (Zeiss 510) was used for these analyses.

Because of the about 5 times higher resolution (Vogt *et al.*, 2000), it was also tried to use X-ray microscopy for analysis of immuno-gold stained nuclear structures of embryonic nuclei in single cells of *C. elegans*. For this purpose, an anti GFP antibody was used.

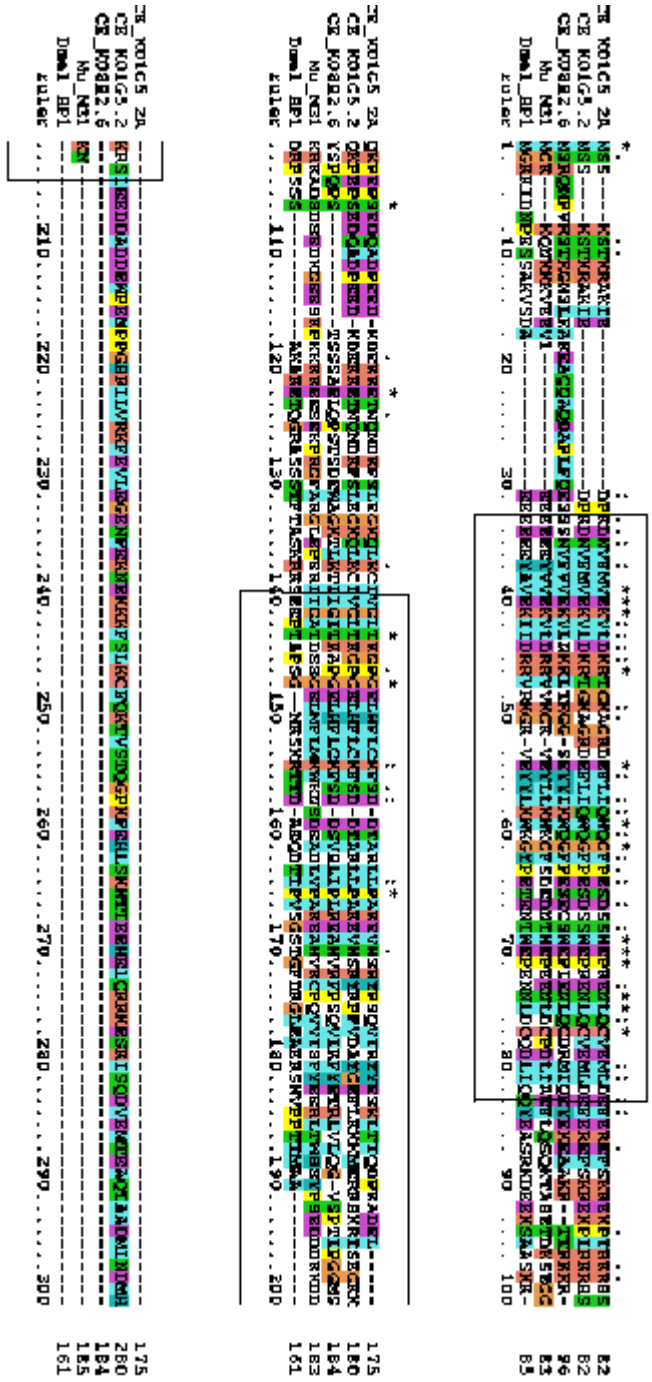

Fig. 3-1 Alignment of three putative HP1 proteins of *C. elegans* with their homologs in mouse (M31) and *Drosophila* (HP1). The alignment was generated with the program ClustalW (Thompson *et al.*, 1994). The accession numbers of the *C elegans* cDNAs are AF056580 (K08H2.6), AF123574 (K01G5.2A), and AF123573 (K01G5.2). A consensus common to both chromo and chromo shadow domains is shown: yellow, strongly conserved hydrophobicity; violet, conserved acidic residues; green and brown, semi-conserved hydrophobicity; orange, conserved basic residues.

3.2 Expression of HP1 in embryonic development

3.2.1 An antibody against the mouse homolog of HP1, M31, reacts with single protein in a lysate of *C. elegans*

For the identification and characterization of HP1 homologs in *C. elegans* a rat monoclonal antibody directed against the mouse protein M31, a homolog of HP1 (Singh *et al.*, 1991) was used which was obtained from the laboratory of P. B. Singh. A Western blot performed with this antibody on a total lysate of *C. elegans* exhibited a single band, suggesting that only a single protein in the total lysate of *C. elegans* was recognized by the antibody (Fig. 3-2).

This protein has an apparent molecular size of approximately 35 kDa. The three putative HP1 proteins encoded by the genome of *C. elegans* (Fig. 3-1) have molecular weights of 20,884, 20,427, and 39,630 kDa. The apparent molecular weight of 35 kDa appears to make the largest HP1 protein of *C. elegans*, HP1.3, a good candidate for the observed reaction on the Western blot. Because of their much smaller sizes, the two other putative HP1 proteins of *C. elegans*, HP1.1 and HP1.2, are very probably not contained in the antibody-decorated fraction on the Western blot. Presumably, these proteins are not detected by the antibody applied. Furthermore, the large HP1 protein, HP1.3, shares a peptide with the mouse protein M31 against which the antibody was rised (Singh *et al.*, 1991, Schulze personal communication). I conclude therefore that the antibody probably recognized one of three putative HP1 proteins, HP1.3, in the total lysate of *C. elegans*.

Also, the antibody did probably not crossreact with other proteins in the lysate of *C. elegans* that are not related to HP1 homologs. The anti-mouse-HP1 antibody was therefore applied in indirect immunofluorescence preparations to follow the expression of HP1 in the embryonic development of *C. elegans*.

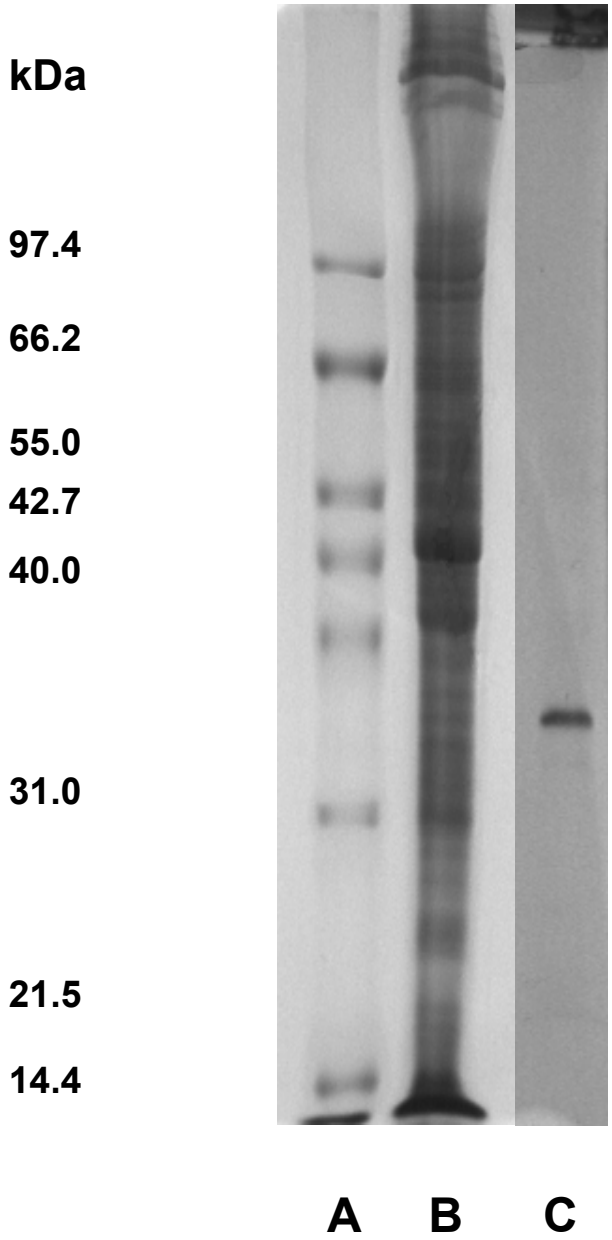

Fig. 3-2 Western blot analysis of total *C. elegans* lysate with an antibody against a mouse homolog of the HP1 protein. A and B: Coomassie Blue-stained gel, while C: Western blot. The protein were separated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose (Towbin *et al.*, 1979). The protein band on the blot was visualized using a chemoluminescence Kit. B shows the total protein lysate of *C. elegans*, whereas A gives the protein marker.

3.2.2 HP1 is expressed in peripheral anterior cells of the 100-200-cell stage

When the different stages in embryonic development were examined by indirect immunofluorescence with the anti-mouse HP1 antibody, the earliest stages that exhibited immunofluorescence signals had approximately between 100 and 200 cells. Furthermore, there were only a few embryonic cells that were decorated by this antibody (Fig. 3-3). These cells were localized at the anterior side at the periphery of the embryo.

Fig. 3-3 shows an embryo at the stage of approximately 200 cells in which four cells in the anterior part are immunodecorated (red). The posterior germ line cells, Z2 and Z3, were stained with an antibody against their P-granules (green). Because of the large number of cells it has not yet been possible to identify the cells in which the HP1 protein is expressed. Presumably, they derive from the AB cell that gives rise to many cells in the anterior periphery of the later embryo.

The expression of an HP1 protein, probably HP1.3, in a few specific cells of a specific embryonic stage indicates that this protein may play a role in development, presumably as part of a regulatory mechanism in chromatin.

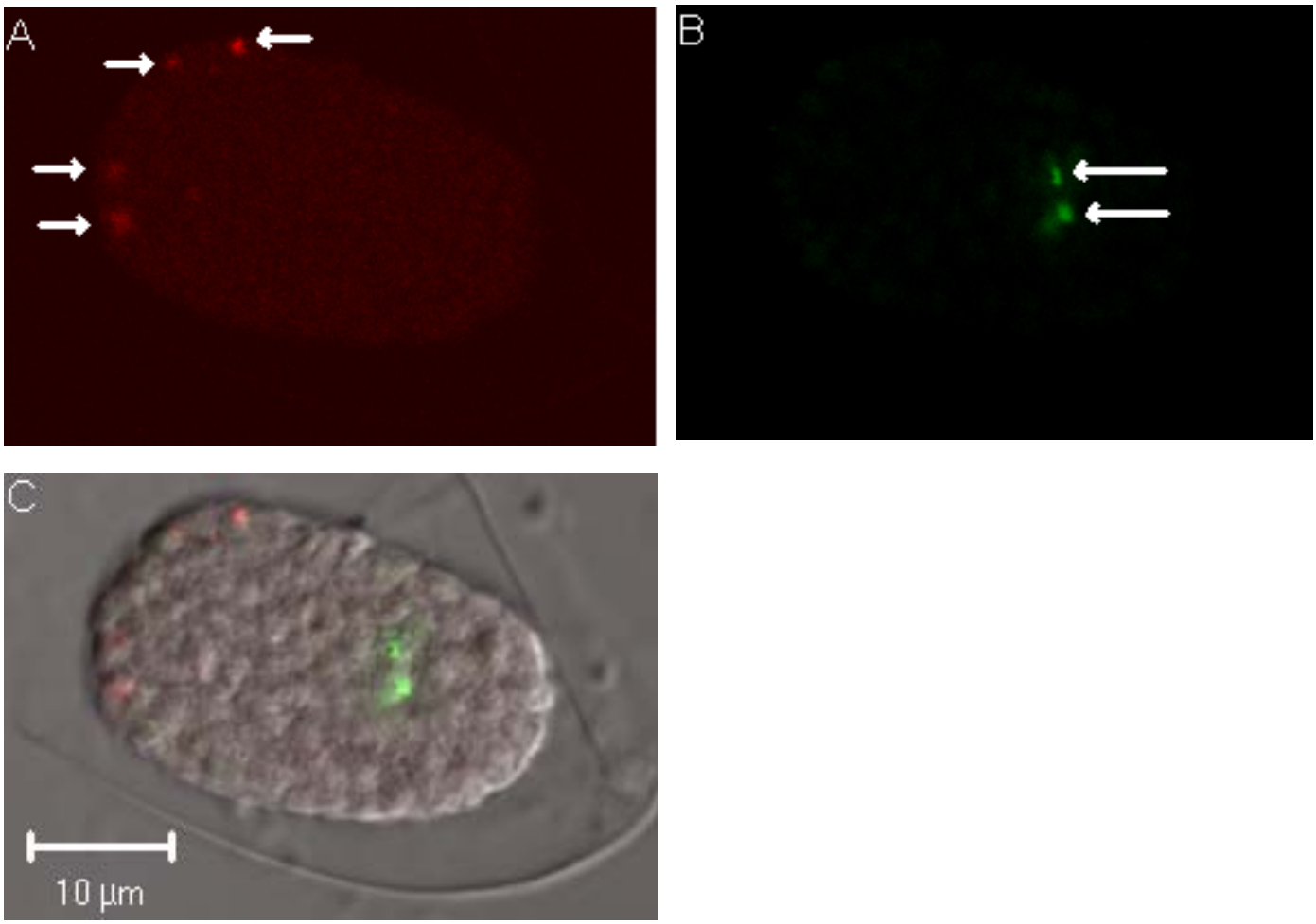

Fig. 3-3 HP1 expression in an embryo of *C. elegans*. An embryo at the stage of approximately 200 cells was indirectly stained with an antibody against HP1 (A) and an antibody against a component of the P granules (B), respectively. Both images were superimposed onto the Nomarski image of the embryo (C). Four cells in the anterior periphery are seen to express an HP1 protein (red), probably HP1.3 (see text). The germline cells Z2 and Z3 in the posterior part of the embryo were stained for orientation (green).

3.3 Localization of *C. elegans* HP1.1 within the nucleus by the GFP-constructs

To elucidate the expression pattern of HP1.1, an extrachromosomal array was constructed in which the *C. elegans* HP1.1 protein in full length was fused in frame to the sequence encoding the GFP protein (Clontech). This array was established in a wild-type background. GFP under the control of an appropriate promoter is widely used as an indicator of specific gene expression (Chalfie *et al.*, 1994). To monitor transfection, plasmids carrying HP1.1::GFP constructs *rol-6*(*su1006*) (Kramer *et al.*, 1990) were coinjected with the *hp1.1::gfp* constructs. *rol-6*(*su1006*) is a dominant mutation that can easily be identified because of the aberrant motions of the worms (Roller Phenotype).

Both plasmids were mixed and microinjected into the gonad arms of young healthy hermaphrodites as circular DNA. The worms were placed on a new NGM plate and incubated for 3 days at 20°C. Transgenic F1 animals were selected under a binocular microscope and their progeny isolated for further analysis. In these animals carrying extrachromosomal arrays, the expression of the reporter gene fusion *hp1.1::gfp* was found to be often mosaic. Therefore, the determination of an expression pattern required the observation of many animals.

The onset of HP1.1 expression was found to occur in an stage with approximately 60 cells in embryogenesis. The HP1.1::GFP was expressed in a large number of cells. Fig. 3-4 shows the GFP-fluorescence in the nuclei of an embryo at the stage of approximately 70 cells. Most of the fluorescence was concentrated to distinct nuclei (Fig. 3-4, see below).

Because of the mosaic expression, the extrachromosomal arrays of *hp1.1::gfp*;*rol-6*(*su1006*) were stabilized by a mutagenesis-induced integration into the genome of *C. elegans*. This integration of *hp1.1::gfp*;*rol-6*(*su1006*) was performed using an X-ray source (at 25 Gy, and 100 keV) to induce

chromosomal breaks. Fortunately, no unrelated mutant phenotype was found associated with the integration of *hp1.1::gfp*;*rol-6*(*su1006*), which was conceivable. For genetic crossing experiments it was desirable to clean up the background from mutation, which had been occurred by generation of this integrated stable line. Subsequently, this integrated *hp1.1::gfp*;*rol-6*(*su1006*) line was backcrossed repeatedly with the *him*-8(*e1489*) strain (CB1489), which is also convenient for the replacement of integrated array through the recombination into the wild-type background. The integrated *hp1.1::gfp* was found to be expressed in most tissues. Fig. 3-5 shows the GFP fluorescence in an L3 larva. A large number of cells especially in the anterior part and in the tail region express the HP1.1::GFP construct. Most conspicuous in this respect are the intestine cells. The number of subnuclear structures in the intestine cells are higher in comparison to those of other tissues. This is because these cells are polyploid. The expression of the integrated *hp1.1::gfp* construct could be observed during development in the living embryos.

Unfortunately, the Rol phenotype which was used for the initial identification of transfected worms has a disadvantage. In the genetic cross experiments, the males could not mate efficiently which rendered such experiments rather difficult, but not impossible.

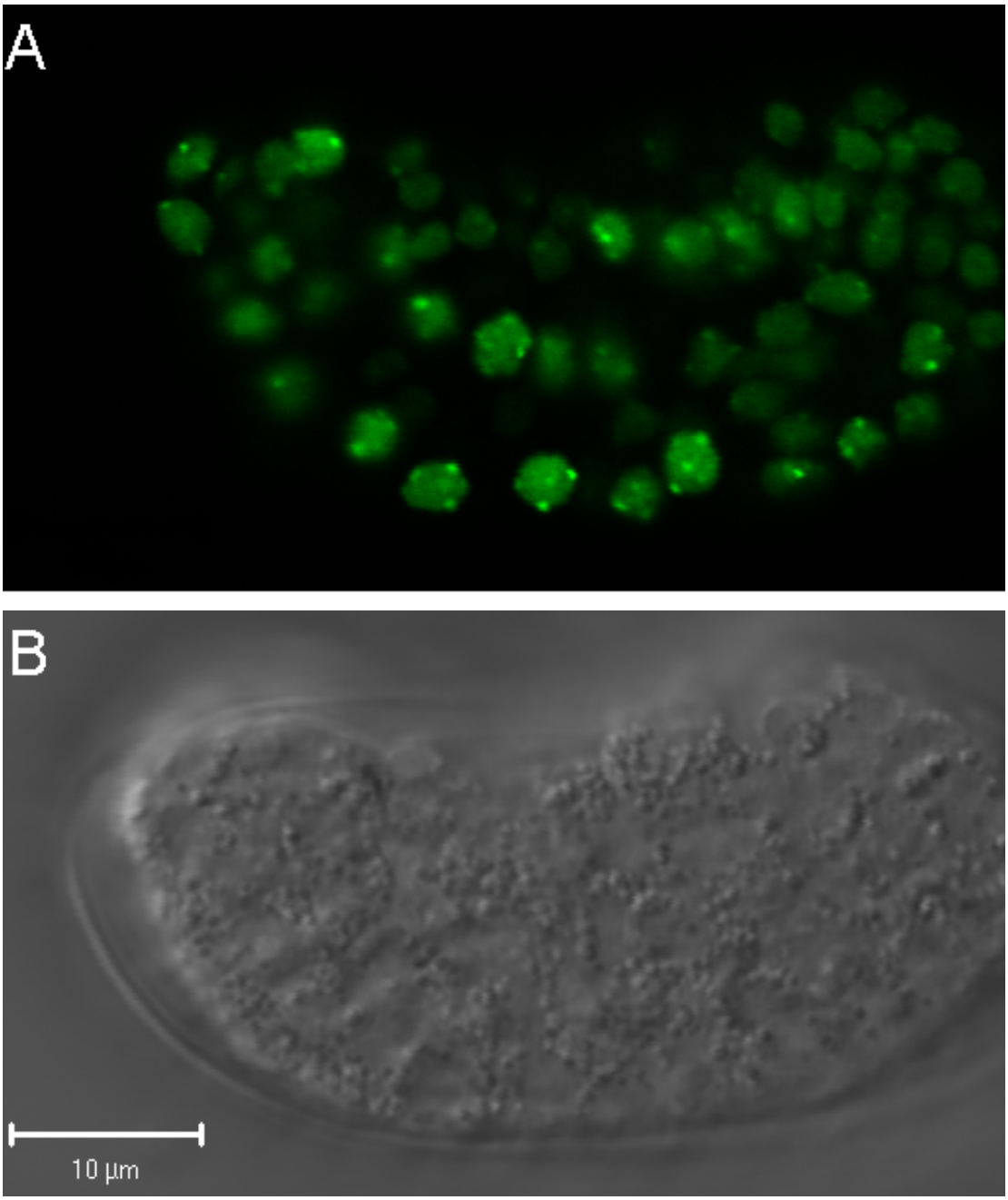

Fig. 3-4 Expression integrated of the *hp1.1::gfp*;*rol*-6(*su1006*) extrachromosomal array in a *C. elegans* embryo. Distinct spot-like structures of HP1.1::GFP, which are located in chromatin new to the nuclear envelope in the embryonic cells are very well visible (A). The micrograph shows an additionally Nomarski illumination (B). This embryo is in the beginning of Komma stage.

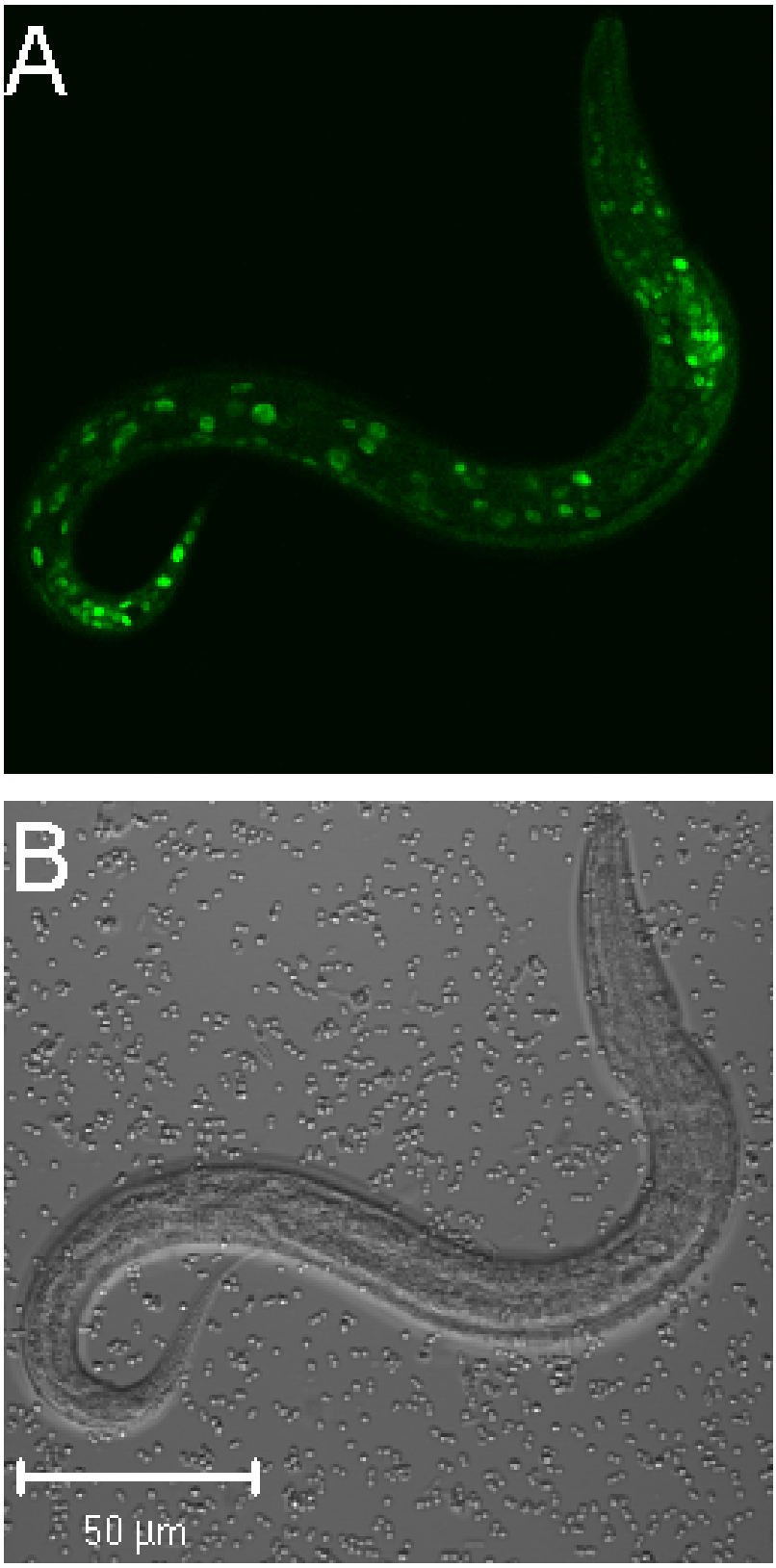

Fig. 3-5 An L3 larva of *C. elegans* with the *hp1.1::gfp* construct in the N2 wild type genome. Expression pattern of HP1.1::GFP (A), Nomarski illumination (B). Many tissues of the larva express the HP1.1::GFP construct. Among these, the polyploid intestine cells are especially conspicuous.

3.3.1.1 HP1.1::GFP is preferentially located in distinct nuclear structures in the interphase nuclei of embryos

In the interphase nuclei of embryonic cells, subnuclear spots are visible, that exhibit especially high GFP fluorescence and therefore most probably contain especially high amounts of HP1.1::GFP (Fig. 3-4). These spots appear in different numbers (often six) per nucleus and are located in the chromatin at the periphery near the nuclear envelope. Fig 3-6 shows another example, an embryo at the stage of about 80 cells. To find out which structures correspond to these centers of HP1.1 concentration laser scanning microscopy was used for the observation of living embryos through the cell cycle. Time series of recorded images allowed to follow the localization of HP1.1 at all stages during the cell cycle by recording the fluorescence signals of the nuclei with the confocal laser scanning microscope.

Embryos of transgenic animals were used that carried the *hp1.1::gfp* construct either as extrachromosomal arrays or integrated into the genome. The nuclear GFP fluorescence was detected in embryogenesis from the 60-cell stage on. This stage is active in proliferation and thus well suited for observation. Most nuclei of the embryos exhibited several especially prominent subnuclear spots of intense GFP-fluorescence in the chromatin that were located close to the nuclear envelope. GFP-fluorescence at lower intensity was observed in the total nuclear area in all interphase nuclei (Fig. 3-6).

The distribution of the GFP-fluorescence and thus the location of the HP1.1::GFP protein construct was analyzed during cell division in the living embryos.

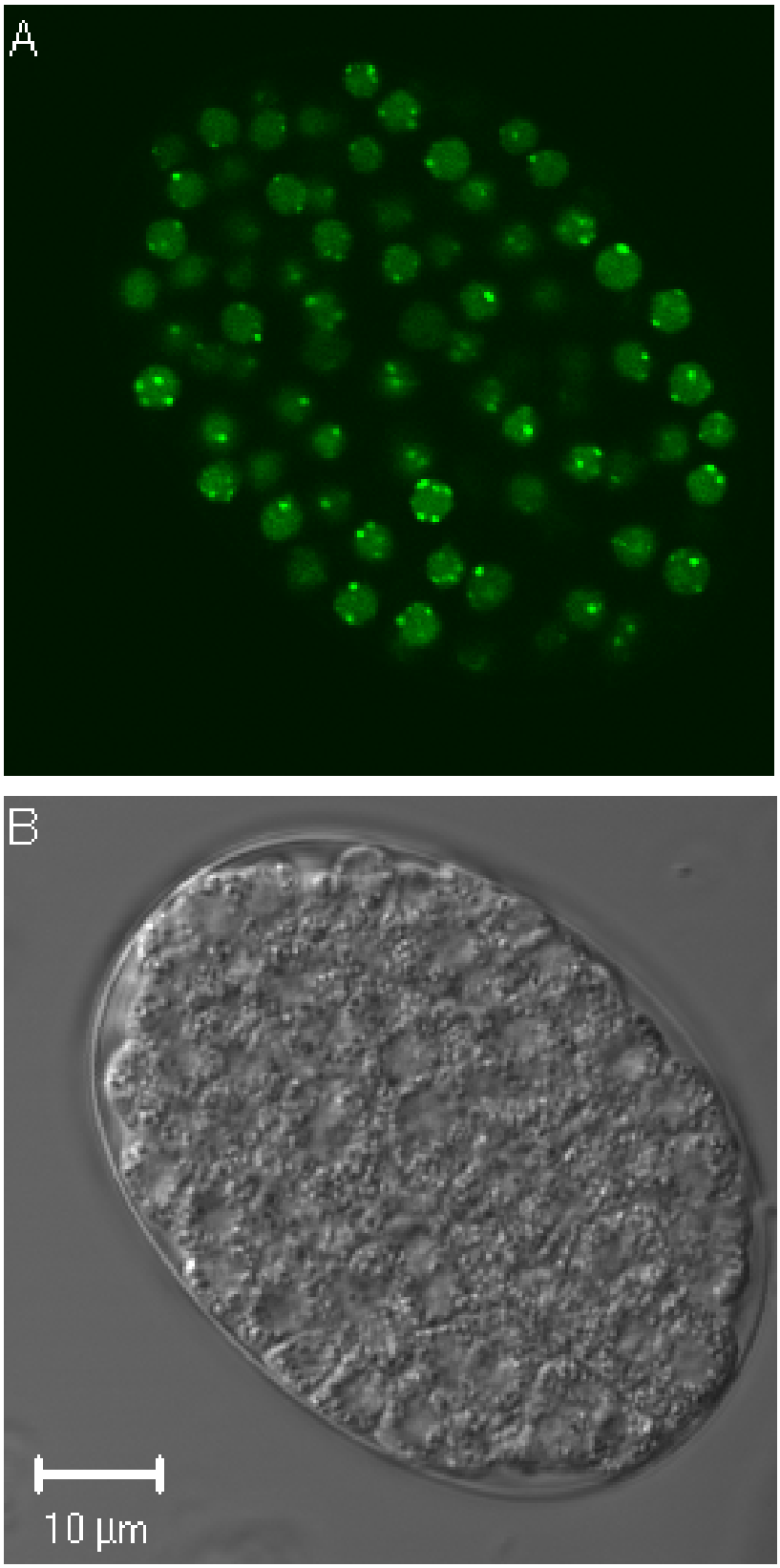

Fig. 3-6 Distribution of HP1.1::GFP in the nuclei of an embryo of *C. elegans* that carries arrays of *hp1.1::gfp* integrated into the genome. The prominent structures of high concentration of HP1.1::GFP, which are located in chromatin at the nuclear periphery in the embryonic cells (A) are well visible. Micrograph shows additionally a Nomarski-DIC (B). These images were recorded with a laser scanning confocal microscope (Zeiss 510).

Fig. 3-7 shows a series of images of an embryonic nucleus in different subsequent stages of the cell cycle. In interphase, the HP1.1 protein is concentrated in distinct regions of the nucleus located close to the nuclear envelope (Fig. 7 A). A later stage, shortly before the onset of mitosis, shows that the positions of structures in the nucleus have dynamically changed (B). In prophase, when the nuclear envelope breaks down, the distinct spot-like structures disappear (C). In metaphase (D), HP1.1 appears in the metaphase plate, with little or no differences of intensity.

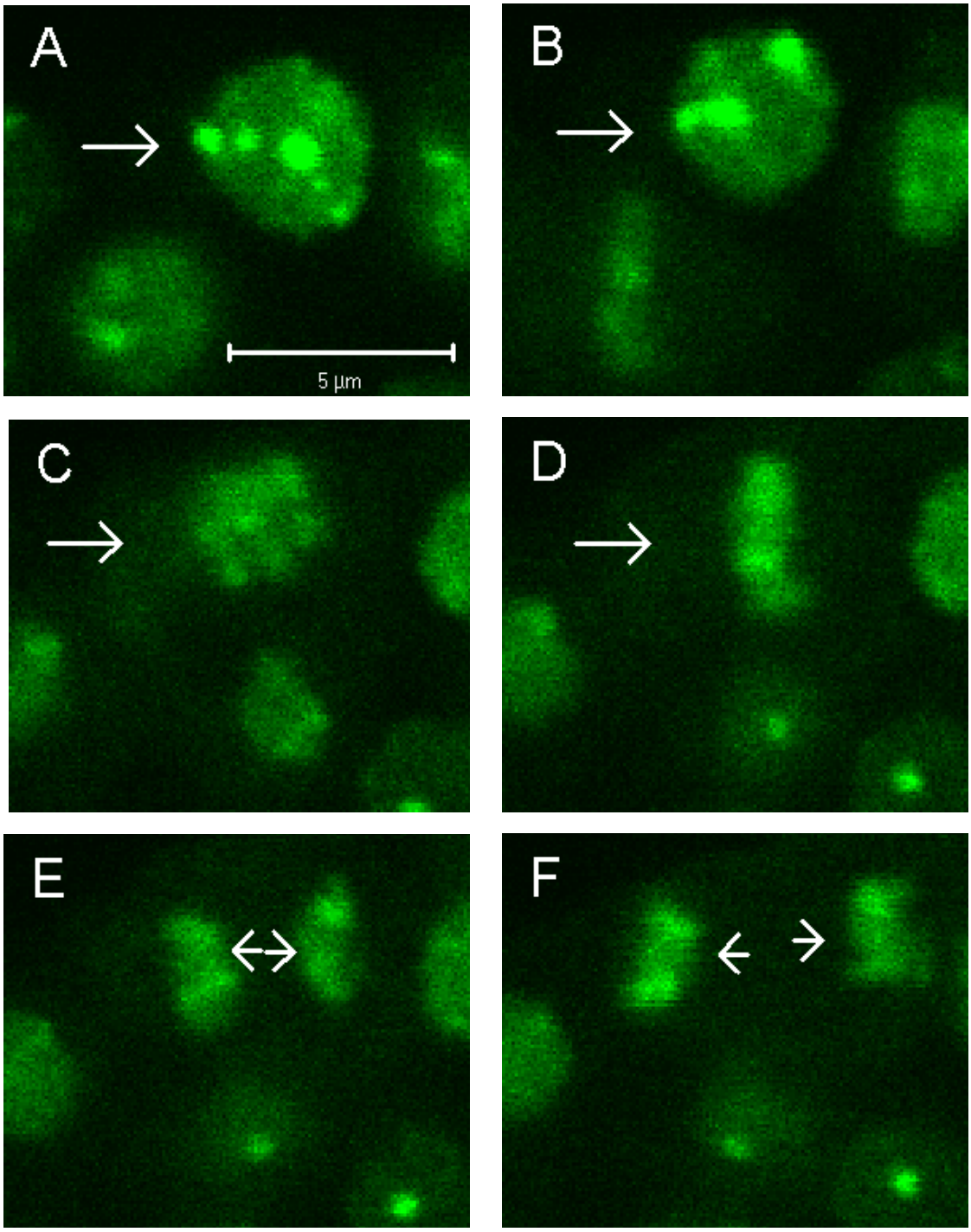

Fig. 3-7 Distribution of HP1.1::GFP in a living embryonic cell nucleus during the cell cycle. A: Interphase (0 sec); B : before onset of prophase (240 sec); C: prophase (540 sec); D: metaphase (660 sec); E: anaphase (720 sec); F: telophase (780). The green fluorescence of the HP1.1::GFP construct shows the dynamic changes of its distribution (for details see text). The strain used carried the *hp1.1::gfp* gene construct integrated into the chromosomal genome. Images were taken every 60 sec with a confocal laser scanning microscope.

As compared to the GFP-fluorescence of the interphase subnuclear spot-likes structures, the GFP fluorescence intensity at the metaphase plate appeared less intense. When the chromatids separate at anaphase, the distribution of GFP-fluorescence and thus that of HP1.1 still appears uniform (E). In late telophase (F), the distinct spot-like structures of high GFP-fluorescence begin to reappear. A small fraction of HP1.1::GFP appeared in the cytoplasm during mitosis (see below).

An antibody against M31, the murine homolog of HP1, localized to bodies of condensed chromatin in mouse interphase nuclei, and to the centromeres of both mouse and human chromosomes in metaphase (Wreggett *et al.*, 1994). The authors conclude that the M31 protein is a component of constitutive heterochromatin. Centromeres cannot be observed within the tiny chromosomes of *C. elegans*. Such structures may also be absent because the *C. elegans* chromosomes are holocentric (Nigon, 1949a; Albertson and Thomson, 1982). Concerning the spots of high concentration of HP1.1 in interphase nuclei, it is tempting to speculate that they correspond to some type of heterochromatin, which cannot be detected during mitosis because of the overall condensation of the chromosomes.

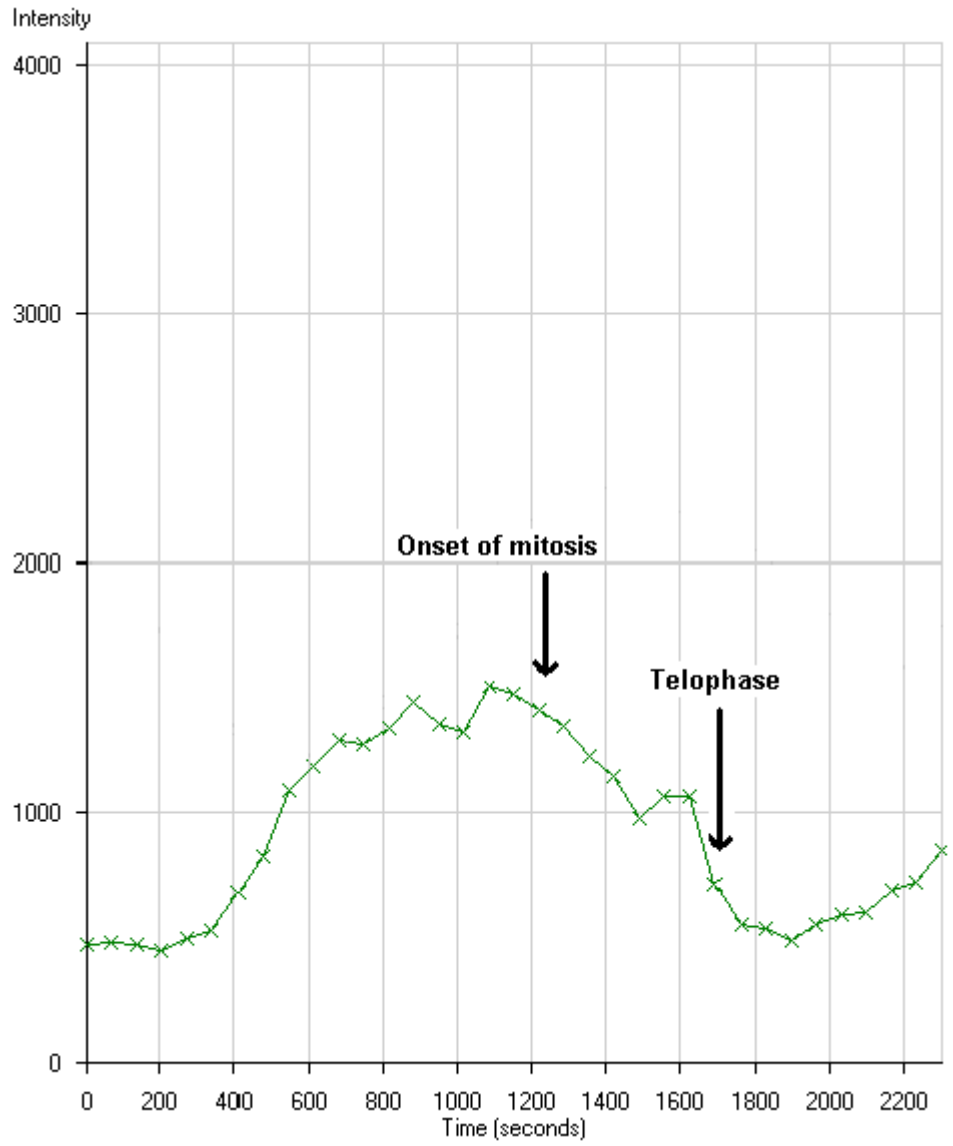

Fig. 3-8 The plot curve of GFP-fluorescence signal intensity in dependent of time from the high concentration spot like structures of HP1.1::GFP construct of a single embryonic nucleus of *C. elegans* that carries arrays of *hp1.1::gfp* integrated into the genome during the cell cycle. The GFP-fluorescence signal intensity of the HP1.1::GFP increase throughout the cell cycle. However, the signal intensity decrease during mitosis. Arrows show the onset of mitosis, and at late telophase. The GFP-fluorescence signal intensity curve is from images which were taken every 60 sec with a confocal laser scanning microscope (Zeiss 510).

In the plot curve is shown the same nucleus with fluorescence intensity in dependency of time (Fig. 3-8). The GFP-fluorescence signal intensity of HP1.1::GFP increases during the cell cycle until the start of mitosis. The signal intensity curve shows a peak (at 1080 sec) before the onset of mitosis (Fig. 3-8 arrow). The HP1.1::GFP signal intensity decreases with onset of mitosis and also throughout mitosis. Telophase is shown at too (Fig. 3-8 arrow).

Interestingly, a small fraction of HP1.1::GFP appears in the cytoplasm during mitosis. This can clearly be demonstrated when the images are overexposed (Fig. 3-9). This dispersed protein reflects a temporal dissociation of a fraction of protein from the chromatin during mitosis. The HP1.1::GFP fraction relocates into the nucleus within the late telophase. The cytoplasm of daughter cells do not display any GFP-fluorescence signals more.

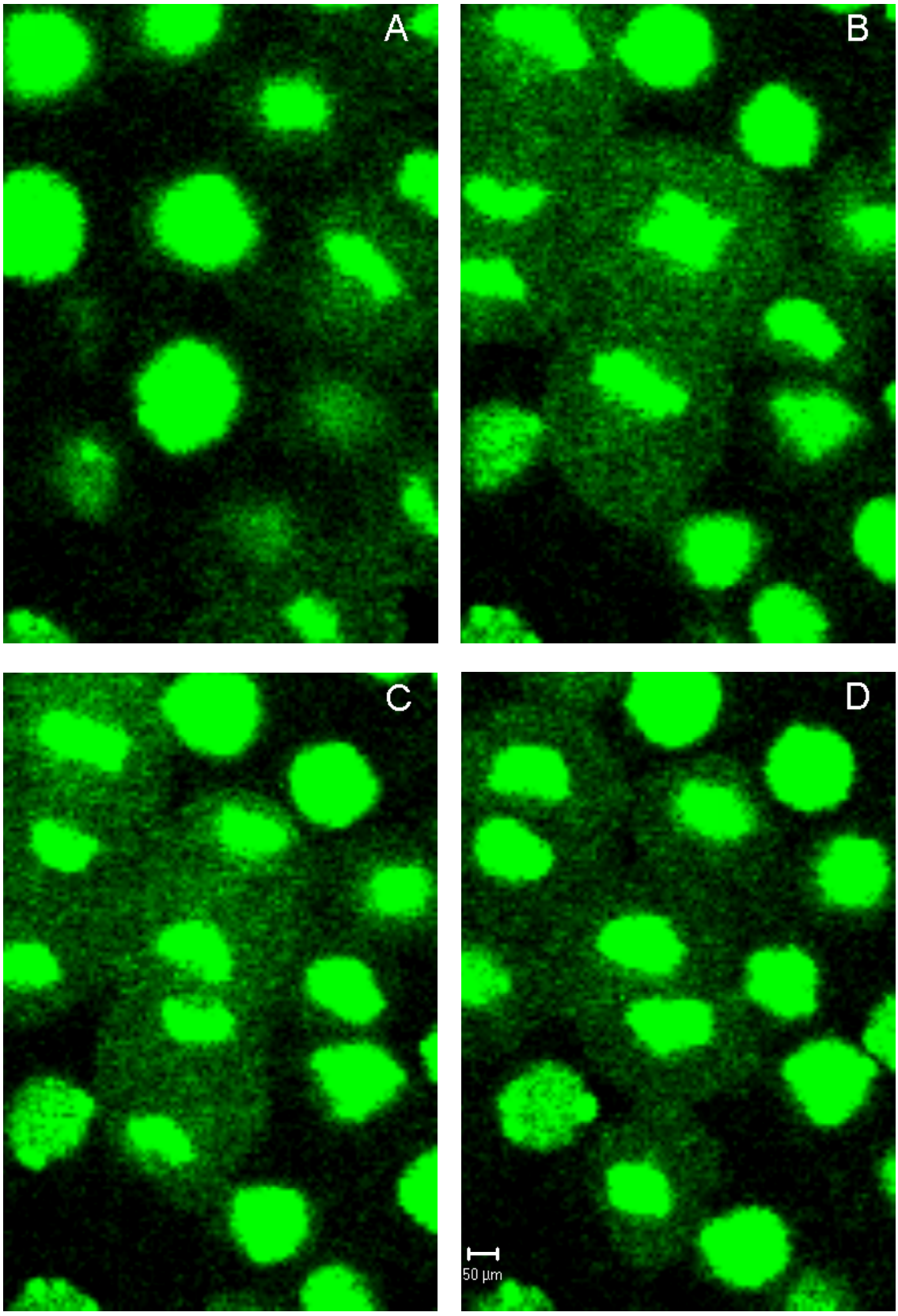

Fig. 3-9 HP1.1 in embryonic cells of *C. elegans* enters the cytoplasm during mitosis. Time series of micrographs of a living embryo shows the two cells in the center at interphase (A; 0 sec), metaphase (B, 200 sec), anaphase (C, 260 sec), and telophase (440 sec). The HP1.1::GFP protein construct expressed by the cells is localized in the nuclei at interphase but enters the cytoplasm in part during mitosis.

3.3.1.2 What are the interphase nuclear structures that contain high concentrations of HP1.1?

Several spots of high GFP-fluorescence are regularly in the interphase nuclei of embryos of *C. elegans* that carry copies of an *hp1.1::gfp* gene construct, either as extrachromosomal arrays or integrated into the chromosomal genome (Figs. 3-4, 3-5, 3-6, 3-7, 3-9). These spots must represent intrachromosomal structures that contain high concentrations of HP1.1. These structures are located near the periphery of the nuclei close to the nuclear envelope. There is at present no evidence on the physical nature of these structures. It is also not clear whether their number is constant or varies from nucleus to nucleus. However, as a number of six HP1.1-rich structures per nucleus is often observed and more than this in a nucleus have not been noticed, it may be justified to speculate that there might be one of these structures associated with every of the six chromosomes pairs. It appears conceivable that such structures are "heterochromatic" in a very general sense, given the association of HP1 homolog proteins in other organisms with centromere (*Drosophila*; Wreggett *et al.*, 1994) or centromere and telomere (*Schizosaccharomyces*, Lorentz *et al.*, 1994) heterochromatin. The observation, that the HP1.1-rich chromatin structures become part of the condensing chromosomes in early mitosis, just as has been noticed in the classical studies of heterochromatin (Heitz, 1928), points in the same direction. However, as the mitotic chromosomes of *C. elegans* are holocentric (Nigon, 1949a; Albertson and Thomson, 1982), it is difficult to imagine that HP1.1-rich interphase structures could represent centromeres or pairs of centromere regions (As to the location of HP1.1 in relation to chromatin during mitosis, see chapter 3.4). Other proteins have been identified that are associated with centromeres and show different localization (Dernburg, 2001; Howe *et al.*, 2001; Moore and Roth, 2001; Oegema *et al.*, 2001).

3.3.2 X-ray microscopy of HP1.1::GFP containing structures

3.3.2.1 Principal advantages of X-ray microscopy

X-ray microscopy allows the analysis of whole cell mounts in an aqueous environment at a resolution five times higher than the resolution of the light microscope (Vogt *et al.*, 2000). Aqueous preparations are subjected to vitrification. Briefly, proteins are a source of carbon, and water a source of oxygen. They have an absorption wavelength region, carbon (4.38 nm; 238 eV) and oxygen (2.34 nm; 531 eV), in the K absorption edges. A natural absorption contrast is known between carbon, the primary X-ray absorbing atom in proteins, and oxygen, the primary X-ray absorbing atom in water (Wolter, 1952). At these energy stages proteins absorb X-rays about an order of magnitude stronger than water, which is suitable for high-amplitude contrast for protein structures in water (Vogt *et al.*, 2000). Biological specimen like cells need to be stabilized against radiation damage. Therefore, the chemical fixation such as glutaraldehyde stabilizes cells for radiation doses of 10^5-10^6 Gy (1 Gy = 1 J/kg). This corresponds to a single image at about 50 nm resolution in an X-ray microscope (Gilbert *et al.*, 1992).

The depth of specimen can be in the range of several micrometers, which allows the analysis of structures in entire cells. Methods for tomographic analysis have been developed and are available (Weiß, 2000). Recently, a specific nuclear structures, the X-chromosome of male *Drosophila* interphase cells, has been analyzed by X-ray microscopy with novel techniques of immunodecoration (Vogt *et al.*, 2000). I have therefore used X-ray microscopy and appropriate immunological techniques, in collaboration with the laboratory of G. Schmahl, Forschungseinrichtung Röntgenmikroskopie der Universität Göttingen, for an analysis of HP1.1-rich structures on a level beyond the resolution of the high microscope.

3.3.2.2 Decoration of embryonic cells that express HP1.1::GFP protein with an anti-GFP-antibody

Preliminary experiments for the X-ray microscopy were performed in which the cells were subjected to an indirect immunofluorescence staining. Single embryonic cells, such as are needed for X-ray microscopy, were prepared from embryos. *C. elegans* embryos have a shell with a chitinous layer, which functions as a barrier to the environment. This shell was removed by enzymatic digestion of the vitelline membrane with chitinase from *Serratia marcescens* (Edgar, 1995) to obtain single cells. After harvesting embryos of an *hp1.1::gfp* strain from NGM plates, cells were visualized by their GFP-fluorescence, fixed with formaldehyde or paraformaldehyde, and stained with a rabbit anti-GFP antibody. The confocal laser scanning was used for recording of fluorescence images. Fluorescence of a secondary antibody, conjugated with Cy2 fluorochrome, was recorded with the laser line 488 nm. Differential interference contrast (DIC) Nomarski images and DNA staining images were recorded in parallel.

Indirect immunofluorescence of the HP1.1::GFP protein with the anti-GFP antibody in isolated embryonic cells is shown in Fig. 3-10. The Hoechst 33342 DNA fluorochrome was used to visualize the cell nuclei. The immunodecorated regions were located inside the nuclei, however, the distinct spots of high HP1.1 concentration could not be detected. As these structures are regularly observed by GFP-fluorescence in living embryos (see Figs. 3-4, 3-6, 3-7), they have probably been destroyed during the isolation of the cells or in the immunostaining procedure. Furthermore, a comparison to the distribution of DNA (Fig. 3-10 C) shows that the indirect immunofluorescence of the HP1.1::GFP fusion protein occurred only in part of the nuclear area and

appeared to have spread into the cytoplasm. This may indicate that part of the protein was dissolved or redistributed during fixation.

In order to find more appropriate conditions for the fixing of *C. elegans* embryonic cells, several protocols with different reagents such as formaldehyde or glutaraldehyde and with different incubation times (Harlow and Lane, 1988) were used, but all yielded results similar to these shown in Fig. 3-10. The distinct spots of high HP1.1 concentration could not be identified after the fixing procedures probably because they were destroyed by this fixing treatment. It is well known that proteins in cells are often dissolved and or redistributed during fixation. Noticeably, when Figs. 3-10 A and 3-10 C are compared, it appears that part of the nuclear area still contains HP1.1::GFP, whereas the protein has disappeared from other nuclear regions.

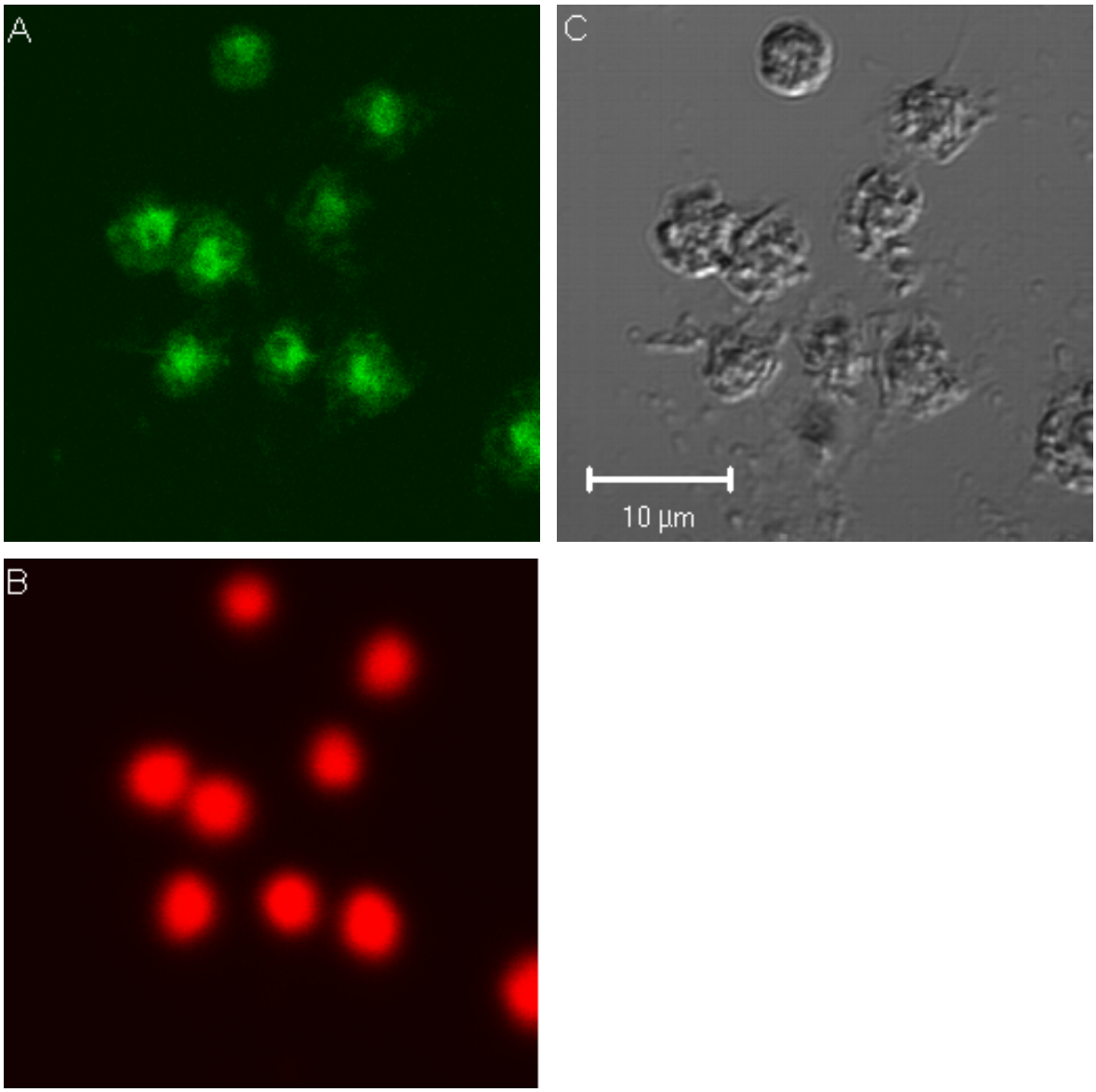

Fig. 3-10 Indirect immunofluorescence detection of HP1.1::GFP in single isolated embryonic cells of *C. elegans* that express extrachromosomal arrays of *hp1.1::gfp*. A: Indirect immunofluorescence of HP1.1::GFP with an anti-GFP antibody. B: DNA staining by Hoechst 33342. C: Nomarski illumination. Structures of high HP1.1 concentration could not be detected by this procedure. The images were recorded with a confocal laser scanning microscope (Zeiss LSM 510) and were processed using artificial computer colors.

As there was no other choice to prepare single embryonic cells of *C. elegans* for the immunodecoration necessary to show the distribution of GFP in the X-ray microscope, the procedure described for light microscopy was used

for the nano-gold staining for X-ray microscopy. A rabbit anti-GFP antibody was used as a primary antibody. The secondary antibody was 1-nm colloidal gold-conjugated goat $F(ab')_2$ anti-rabbit IgG. The 1-nm gold particles are too small to be detected in the X-ray microscope, silver enhancement was applied (see Methods). Fig. 3-11 shows such an X-ray image of an embryonic cell obtained by this procedure. Notably, Fig. 3-11 appears that nucleus region is immunodecorated, probably because it contains HP1.1::GFP, but the distinct structures of high HP1.1 concentration could not be identified (dark region of high contrast). Probably the fixing procedures structures (see Methods) destroyed these structures. It is well known that proteins in cells are often dissolved and or redistributed during fixation. Furthermore, the micrograph show in part of the cell X-ray absorption typical of carbon-containing (gray region surrounding the nucleus).

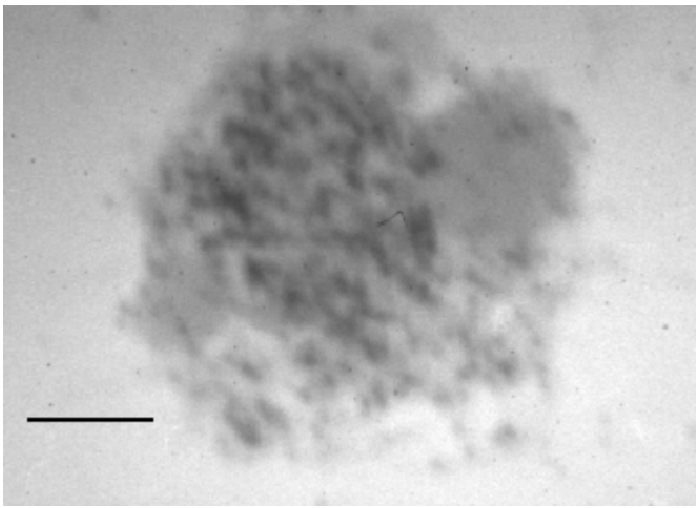

Fig. 3-11 X-ray microscope image of an isolated embryonic cell of *C. elegans*, decorated with an anti-GFP antibody and a colloidal gold-conjugated secondary antibody. In the cell extrachromosomal arrays of *hp1.1::gfp* are expressed. The distribution of HP1.1::GFP was made visible by silver enhancement of the gold label (dark areas). Scale bar: 1 µm. The image was taken with the X-ray microscope of the Forschungseinrichtung Röntgenmikroskopie der Universität Göttingen at the electron storage ring BESSYI (Berlin).

3.3.2.3 Application of the streptavidin technique for the X-ray microscopy of nuclear structures containing HP1.1::GFP

A novel strategy for protein tags is provided by a short eight-amino acid peptide (NH_2-WSHPQFEK-COOH) (Schmidt *et al.*, 1996) which exhibits binding affinity towards streptavidin. This peptide has been used as an affinity tag for recombinant proteins. The Strep-tag could be used not only for *in vitro* experiments but *in vivo* after genetically fusing it into the GFP reporter. Such *in vivo* experiments could simplify the detection of proteins in cells. In Strep-tag experiments, samples were incubated after fixing, with the Cy5 conjugated Strep-Tactin (Voss and Skerra, 1997). In this work the Strep-tag technique was therefore, applied to try to monitor the expression of HP1.1 in *C. elegans* cells and its intranuclear distribution by X-ray microscopy. This tag could also be a powerful tool for HP1.1 protein isolation and purification, *e.g.* for the characterization of HP1.1 binding partners in *C. elegans*.

3.3.2.3.1 Construction of *hp1.1::gfp::strep-tag* gene fusions and their expression on blots

First, I generated the construct *hp1.1::s::gfp*, which genetically fused the Strep-tag to the carboxy-terminus of HP1.1. s encodes the protein tag binding to streptavidin whose sequence is contained in ESAD05 and ESAD06 with an overhang, and was fused between carboxy-terminal region of *hp1.1* and amino-terminal region of *gfp* (see Methods). Unfortunately, it was not possible to see any signal of HP1.1::S::GFP using Cy5 conjugated Strep-Tactin, whereas the DNA sequence has confirmed the sequence identity. Because the cytological experiments also did not reveal any indication of an expression of HP1.1::S::GFP (not shown), we decided to work with another construct, GFP::SIIc (kindly provided by Dr. T. Schmidt), where the Strep-tag is fused to the carboxy-terminus of GFP. HP1.1 was fused to this construct.

HP1.1::GFP::SIIc was also found to react with Strep-Tactin (Voss and Skerra, 1997) from IBA (Göttingen, Germany).

A blot was performed with this protein tag, which is based only on protein-protein interaction. Therefore, a total lysate of a *hp1.1::gfp::sIIc* strain of *C. elegans* was prepared. Proteins were separated by SDS-PAGE (Laemmli, 1970) and a blot (Towbin *et al.*, 1979) performed. The HP1.1::GFP::SIIc was detected by the Strep-Tactin reagent, which had been conjugated to horseradish peroxidase. Only one protein band in the total lysate of *C. elegans* (Fig. 3-12) was visible that had an apparent size of 58 kDa. The construct size is in fact 58 kDa, of which 27 kDa are accounted for by GFP, 21 kDa by HP1.1, and 10 kDa by streptavidin.

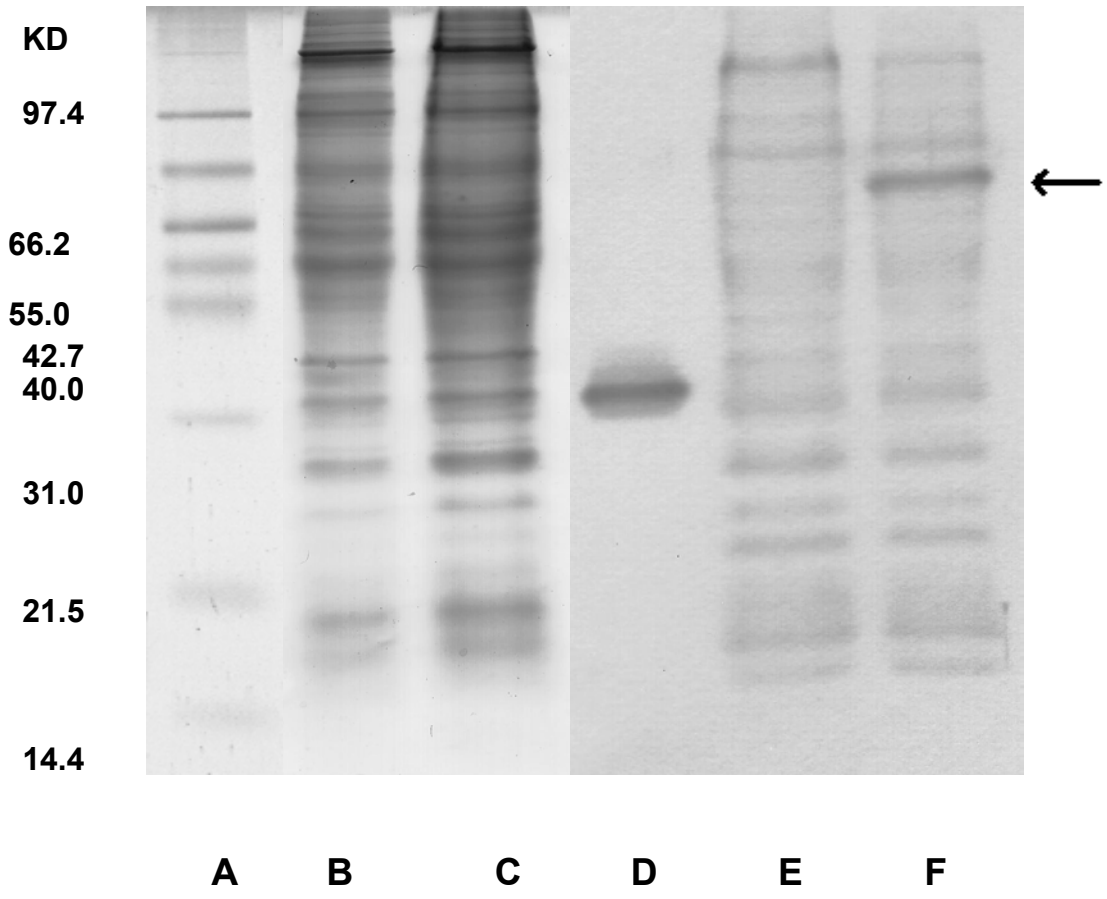

Fig. 3-12 Blot analysis of the binding of horseradish peroxidase conjugated Strep-Tactin to a streptavidin conjugated protein construct, HP1.1::GFP, expressed in *C. elegans*. A-C: Coomassie protein staining. D-E gel blot exposed to binding of Strep-Tactin. A: Molecular weight marker. B: Total lysate of *C. elegans* strain that express the HP1.1::S::GFP construct. C: Total lysate of a *C. elegans* strain that express the HP1.1::GFP::SIIc construct. D:

GFP::SIIc. E: blot of B. F: blot of C. Strep-Tactin interacted with a protein band of 58 kDa (arrow) which corresponds to the construct.

I conclude from this result that this protein tag is specifically reactive with Strep-Tactin, and that Strep-Tactin did not react with other proteins on the blot. Therefore, the Strep-Tactin obviously bound to the HP1.1::GFP::SIIc protein construct expressed in the *C. elegans* cells.

3.3.2.3.2 Use of the streptavidin technique to labeled HP1.1::GFP containing nuclear structures was not successful

The next step was to try to detected the designed protein construct in immunocytological experiments with *C. elegans* using Cy5-conjugated Strep-Tactin. Unfortunately, it was not possible to show the protein in the embryos. GFP::SIIc was used for a test on nitrocellulose sheets to confirm its reactivity. When the Cy5-conjugated Strep-Tactin was tested in a dot-blot experiment in which equal quantities of Strep-tag were used, it was reactive in different intensities, which was conspicuous (not shown). It is conceivable that the binding sites of Strep-tag are not available for Strep-Tactin. A possible explanation would be that this Cy5-conjugated Strep-Tactin could not interact with Streptavidin because of conformational changes in the HP1.1::GFP::SIIc. It was thus not possible to use the streptavidin technique to analyze the distribution of HP1.1 in embryonic cells by X-ray microscopy.

3.4 Dynamic alterations in the distribution of HP1.during the cell cycle

For an understanding of the functions of HP1.1, a more detail analysis of its distribution during the cell cycle would be very useful. This was performed by double-labeling living embryonic cells of *C. elegans* with two different fluorescent protein vectors, the cyan fluorescence protein (ECFP) vector (Haas

et al., 1996, Yang *et al.*, 1996) and the yellow fluorescence protein (YFP) vector (Ormö *et al.*, 1996). To follow the distribution of "core" chromatin through the cell cycle, an histone H1 gene of *C. elegans* was fused in frame to the *cfp* gene. The histone H1 gene (*his-24*) including the promoter region was used, and the construct *his-24::cfp* was microinjected into the wild type strain N2. The expression of the generated extrachromosomal arrays of *his-24::cfp* was examined in the dissection stereo-microscope in UV light. Extrachromosomal arrays of the *his-24::cfp* were also integrated into the genome of *C. elegans*.

To enable the localization of HP1.1 in relation to the H1-containing "core" chromatin throughout the cell cycle, the *hp1.1* gene was fused in frame to the *yfp* gene, the construct was microinjected into the wild type strain N2, and the expression of the extrachromosomal arrays in the offspring was checked in UV light. Both constructs were found to be expressed. The GFP variants cyan fluorescence and yellow fluorescence protein with their different spectral characteristics thus offer an enormous potential for the double-labeling of cells. Double-labeled transgenic worms, in which both histone H1 and HP1.1 could be localized *in vivo*, were obtained by coinjecting both the *his-24::cfp* and the *hp1.1::yfp* construct into the N2 strain. These transgenic animals yielded embryonic cells that exhibited both the CFP and the YFP fluorescence and thus permitted the concomitant localization of the histone H1 and the HP1.1 constructs (Fig. 3-13).

Fig. 3-13 shows a living embryo that carries extrachromosomal arrays of both *hp1.1::yfp* and *his-24::cfp* and exhibits cyan fluorescence (histone H1 construct, B) and yellow fluorescence (HP1.1 construct, A). The spots of high concentration of HP1.1 that have been described earlier (see chapter 3.3, 3.3.1, 3.3.1.2) are clearly visible (Fig. 3-13 A). They appear to be enriched in H1 too (arrows) and thus might be areas of higher concentration of interphase chromatin.

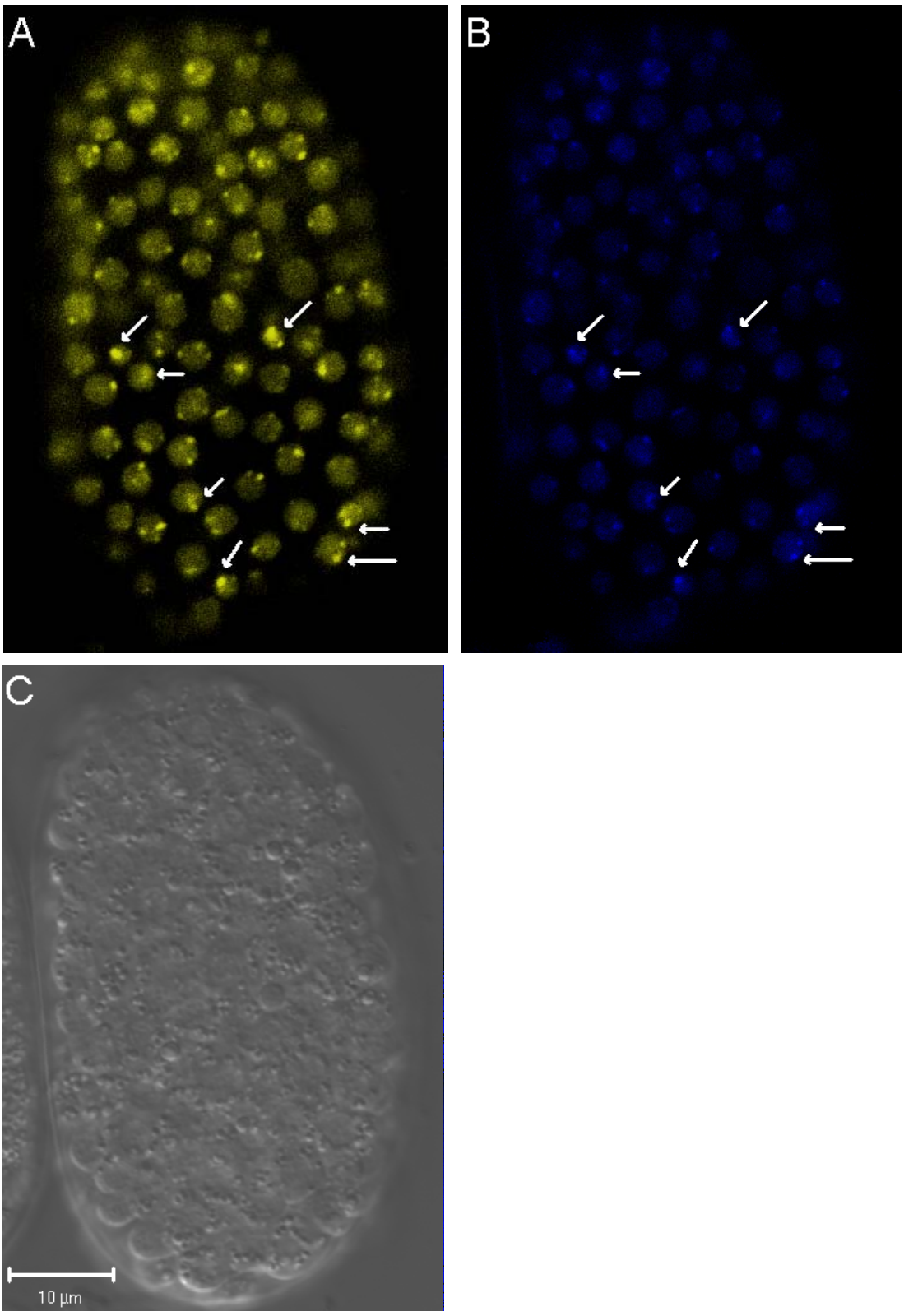

Fig. 3-13 Living embryo of *C. elegans* that carries extrachromosomal arrays of two types of gene constructs containing different fluorescent protein genes. An HP1.1 gene fused to a Yellow Fluorescent Protein gene allowed the localization of HP1.1 by yellow fluorescence (A). An histone H1 gene fused to a Cyan Fluorescent Protein gene allowed the localization of histone H1 by the cyan fluorescence of the protein construct (B). The images were taken with

a confocal laser scanning microscope (Zeiss 510) with filter combinations bp 560-615 (A) and, bp 505-550 (B) respectively. C: Nomarski illumination.

The localization of HP1.1 and of histone H1 in the cell nucleus was followed throughout the cell cycle in embryonic cells that express both the HP1.1::YFP and the H1.1::CFP protein constructs, in series of images of nuclei. Figures 3-14 and 3-15 show two such series that have been recorded in great detail. Artificial computer colors were chosen to record the distribution of HP1.1::YFP in red and that of H1::CFP in green. Both HP1.1 and histone H1 were found to be present in the embryonic nuclei throughout the cell cycle. The two proteins are colocalized at many regions of the nucleus that appear in yellow. However, the localization of HP1.1 in relation to that of histone H1 was found to change during the cell cycle. In interphase, the spots of high HP1.1 concentration partially colocalize with histone H1 (Fig. 3-14 A). When the nuclear envelope breaks down, HP1.1 moves onto the condensing chromosomes, and this location is conspicuous at prophase (Fig. 3-14 B). In prometaphase, HP1.1 is seen separated from the condensed chromosomes (Fig. 3-14 D, Fig. 3-15 B). Subsequently, HP1.1 relocates to the chromosomes at metaphase, and there is a nearly total colocalization of HP1.1 and histone H1 at late metaphase (Fig. 3-14 F, Fig. 3-15 C). During anaphase, HP1.1 is in part dispersed outside the chromosomes and occupies the regions where the spindle-fibers binding sites of the holocentric chromosomes should be located (Fig. 3-14 F, G; Fig. 3-15 D, E). Therefore, it may be suggested that HP1.1 may be part of the outer kinetochores of the chromosomes. Finally, at late telophase, when the nuclear envelope reforms, HP1.1 becomes located in the spot-like structures (Fig. 3-14 H, Fig. 3-15 F).

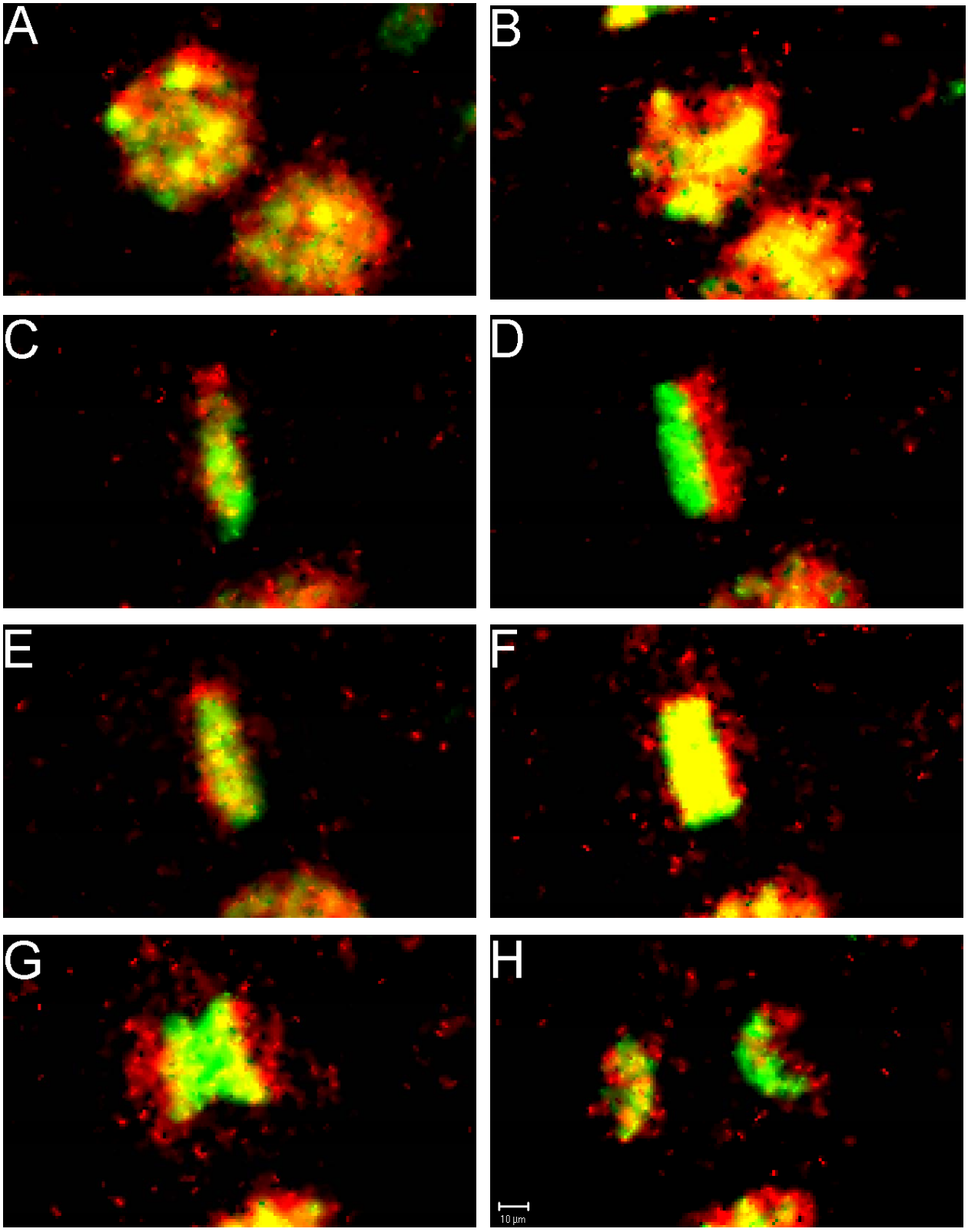

Fig. 3-14 Localization of HP1.1 and "core" chromatin throughout the cell cycle of an embryonic cell of *C. elegans*. *hp1.1* genes fused to Yellow Fluorescent Protein (YFP) genes and histone H1 genes fused to Cyan Fluorescent Protein (CFP) genes were expressed from extrachromosomal arrays, and the localization of the protein constructs in the living cells was monitored with a confocal laser scanning microscope. The images were processed by consecutive steps of light filter combinations to prevent "bleeding" and to select for 560-615

nm (YFP) and 505-550 nm (CFP); for details see Methods. Artificial computer colors were chosen to show the distributions of the HP1.1 (red) and the histone H1 (green) construct. A (0 sec): interphase. B (180 sec): break-down of nuclear envelope. C (330 sec): early prometaphase. D (420 sec): prometaphase; E (450 sec); late prometaphase ; F (570 sec): late metaphase; G (600 sec): anaphase; H: (630 sec) telophase. As can be seen, the location of HP1.1 (red) is dynamically altered during the cell cycle and does not coincide with the location of chromosomal histone H1 (green) in prometaphase (D) and anaphase (G).

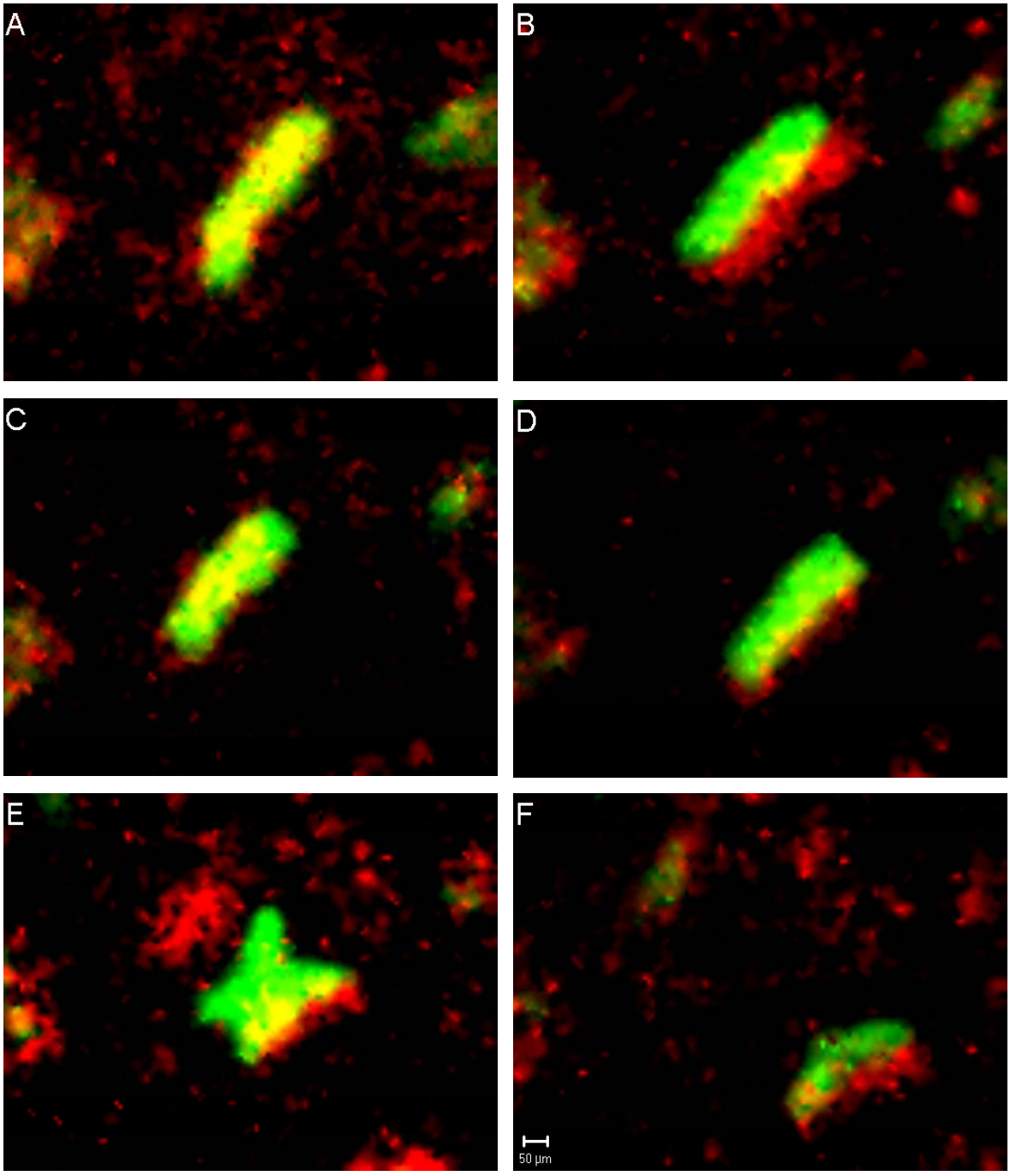

Fig. 3-15 Another example of the localization of HP1.1 and "core" chromatin throughout the cell cycle of an embryonic cell of *C. elegans*. *hp1.1* genes fused to Yellow Fluorescent Protein (YFP) genes and histone H1 genes fused to Cyan Fluorescent Protein (CFP) genes

were expressed from extrachromosomal arrays, and the localization of the protein constructs in the living cells was monitored with a confocal laser scanning microscope. The images were processed by consecutive steps of light filter combinations to prevent "bleeding" and to select for 560-615 nm (YFP) and 505-550 nm (CFP); for details see Methods. Artificial computer colors were chosen to show the distributions of the HP1.1 (red) and the histone H1 (green) construct. A: Late prophase (0 sec); B: early prometaphase (30 sec); C: metaphase (60 sec); D: late metaphase (90 sec); E; anaphase (120 sec); F:, telophase (150 sec). As can be seen, the location of HP1.1 (red) is dynamically altered during the cell cycle and does not coincide with the location of chromosomal histone H1 (green) in prometaphase (B) and anaphase (E).

3.5 Transient knock-out of HP1.1 by RNA interference

3.5.1 Injection of HP1.1-dsRNA resulted in suppression of HP1.1 expression in an *hp1.1::gfp* strain

The dsRNA of full length HP1.1-cDNA was microinjected into the gonads of young hermaphrodites of a strain carrying *hp1.1::gfp* constructs integrated into the genome. Embryos of the F1 progeny were screened for GFP expression to find out whether dsRNA could abolish the whole GFP expression. The results of the injection of the HP1.1 dsRNA are given in Table I-12. Interestingly, 95% of the F1 progeny of the injected animals did not exhibit any visible GFP signal from the nuclei (Fig. 3-16). Furthermore, there were 12.3% (94 out of 766) dead embryos observed in the F1 progeny. The results indicate that injection of dsHP1.1RNA did inhibit HP1.1 expression in F1 embryos.

Table I-12 Percentage of dead embryos and of GFP fluorescence loss after injection of HP1.1 dsRNA. The numbers in parentheses give the total number of animals screened in each experiment; n, the number of injected hermaphrodites; m, the number of dead embryos, *, integrated array.

RNAi	Strain	%Dead embryos	% loss of GFP signal from nuclei
HP1.1	*hp1.1::gfp**;*him*-8(*e1489*)	12.3 (766, n=91,m=94)	94.9 (727, n=91)

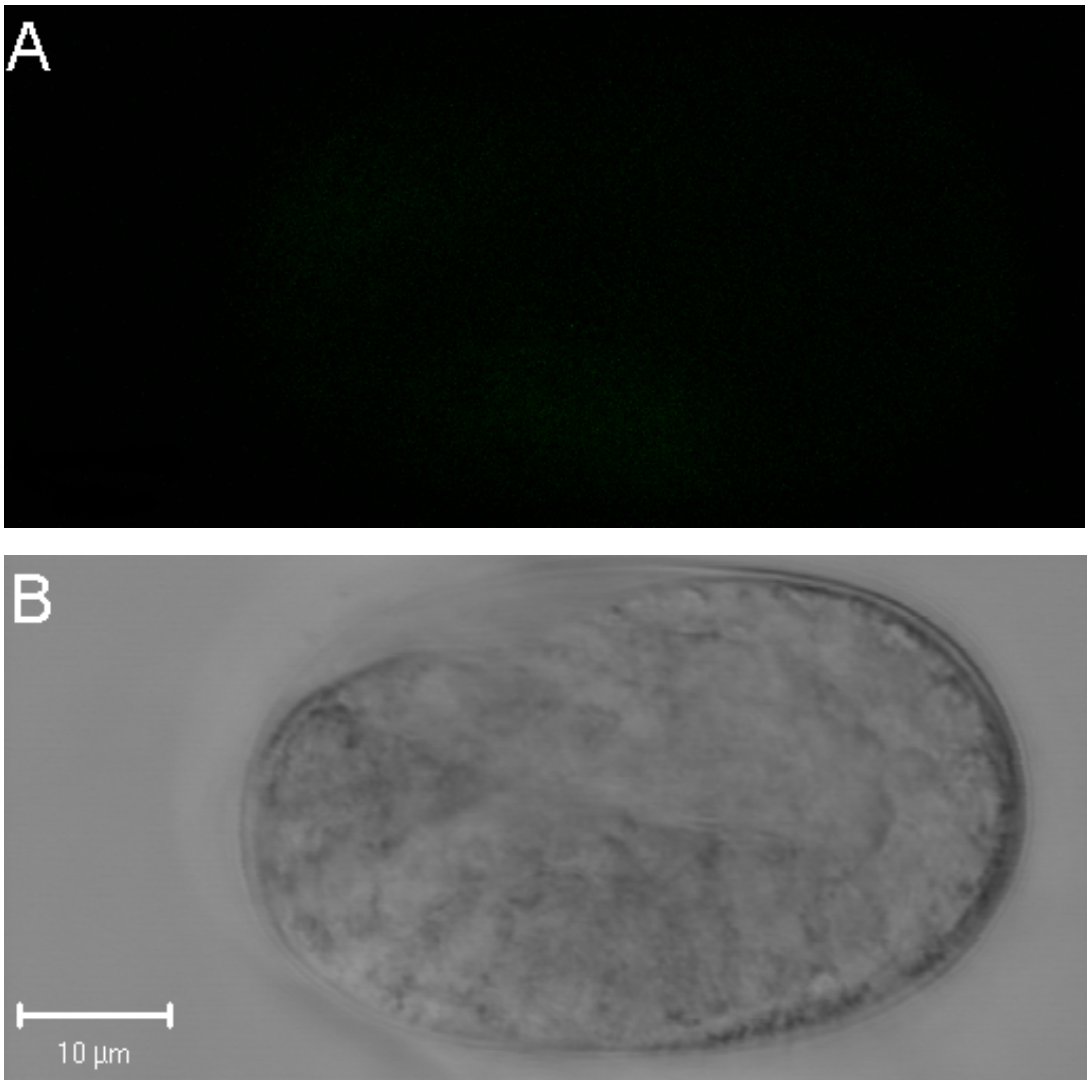

Fig. 3-16 Suppression of HP1.1::GFP expression by HP1.1 dsRNA. dsRNA corresponding to the total length of in *hp1.1* cDNA was injected into a *C. elegans* strain, that carries *hp1.1::gfp* in its genome, and yielded a total loss of the GFP fluorescence (A). B: Nomarski illumination.

3.5.2 Injection of HP1.1-dsRNA resulted in mutant phenotypes

The dsRNA of full-length cDNA of HP1.1 was microinjected into the gonads of young hermaphrodite worms of the wild type strain N2, and the embryos of the F1 offspring and their phenotypes were screened. In other experiments, dsRNA corresponding to the full-length cDNA of HP1.2 was injected separately or in combination with HP1.1 dsRNA.

Table I-13 Percentage of dead embryos in F1 after injection of dsRNA of *hp1* genes. The numbers in parentheses give the total number of animals screened in each experiment; n, the number of injected hermaphrodites.

RNAi	Strain	%Dead embryos
HP1.1	N2	13.52(1769, n=116)
HP1.2	N2	10.05 (209, n=26)
HP1.1 & HP1.2	N2	11.84 (380, n=56)

Control injection	Strain	% Dead embryos
M9	N2	0.92 (1200, n=97)
M9	N2	0.9 (782, n=35)

A number of dead embryos was observed in the F1 progeny. 13.5% dead embryos were observed in the injection series with HP1.1 dsRNA, 10% with HP1.2 dsRNA, and 11.8% when both types of dsRNA had been injected (Table I-13). Approximately 5% of the F1 animals screened after injection of HP1.1 dsRNA alone or in combination with HP1.2 dsRNA (for the total numbers of animals screened see Table I-13) showed abnormal development. These embryos were smaller than those of the N2 wild type strain and had a different morphology. When they grew up, their larvae had a different body shape in comparison to wild-type larvae. Mostly they had abnormal tail regions. Fig. 3-17 shows a larva with this phenotype. These animals have a lumpy, dumbbell or twisted body, did not grow normally, and were arrested in their development. They can be compared with *vab* mutants, specifically *vab*-6 (*variable abnormal morphology*) mutants. Usually the larvae could not move freely around on the NGM plates as N2 animals do but kept within a very small area. Other larvae grew and looked like dumpy mutants. Of the dumpy-like mutants, some had progeny whereas others were arrested in their development.

Fig. 3-17 Abnormal phenotype of an *C. elegans* larva due to transient knock-out of *hp1.1*. HP1.1 dsRNA was microinjected into young hermaphrodites of the N2 wild type strain, and the F1 was screened for embryonic lethality and for new phenotypes. The micrograph (Nomarski-DIC) shows severe defects in the tail region. Image recorded with a confocal laser scanning microscope (Zeiss 510).

3.5.3 dsRNA-mediated interference with HP1.1 expression did not show interaction with gene silencing in the germline of *C. elegans*

A germline specific reporter system (Kelly and Fire, 1998) was used in order to check whether HP1.1 is involved in chromatin silencing in the germline of *C. elegans*. In this system, a GFP-tagged gene (*let*-858) is established as a repetitive extrachromosomal transgenic array in a genetic background (*pha*-1, Granato *et al.*, 1994), which allows positive selection for the transgene at the temperature of 25°C. The LET-858::GFP reporter was established as a

replicating transgene. The endogenous *let-858* promoter is active in the germline, whereas LET-858::GFP expression as consequence is lost after a number of animal generation. The loss of expression of the reporter gene in the germline was used to indicate the status of gene silencing. HP1.1 dsRNA was injected into the gonads of healthy young hermaphrodite worms, that carry the reporter transgene as extrachromosomal arrays, and the gonads of adult F1 animals were screened for LET-858::GFP fluorescence.

No desilencing effect of the HP1.1 dsRNA injections was observed in F1 hermaphrodites and in male progeny (not shown). If HP1.1 played a role in gene silencing, one would expect to see the GFP fluorescence signal in the germline of the F1 progeny (adult hermaphrodites) of microinjected young hermaphrodites, because of the suppression of HP1.1. However, no indication of any fluorescence signal in any of the animals was observed that might have indicated germline desilencing.

In additional experiments, the SS222 (*mes*-3(*bn21*) I) strain was crossed with a strain that carried the *hp1.1::gfp* construct as integrated arrays, and the F1 progeny was screened for HP1.1::GFP expression in the gonad arms. The hermaphrodite gonads in *mes-3* animals are deprived of mitotic nuclei as well as of differentiated germ cells. It was checked whether HP1.1 is involved in chromatin silencing in the germline using the *mes-3* strain. Only the abnormally enlarged germ nuclei with prominent nucleoli were observed, that are a trait of the *mes*-3 strain. It must be concluded that the HP1.1::GFP expression pattern did not cause any noticeable changes in the *mes*-3 strain comparable to *let-858* (Kelly and Fire, 1998).

Taken together, the experiments performed did not indicate that HP1.1 is involved in the remarkable chromatin silencing occurring in the germline of *C. elegans*.

3.6 Interaction of HP1.1 with structural chromatin proteins in *C. elegans*

It was tried to identify possible partners of HP1.1 in *C. elegans*. Based on HP1 interactions with other nuclear proteins that are known from other organisms, four proteins were selected: the lamin B receptor (LBR), the origin recognition complex (ORC2), and two SET domain proteins.

The human lamin B receptor (LBR), which binds to B-type lamins, contains a nucleoplasmic amino-terminal domain of ~200 amino acids residues length and a hydrophobic domain with eight putative transmembrane segments (Worman *et al.*, 1988). LBR has sequence similarity with yeast and plant sterol reductases. It interacts (Pyrpasopoulou *et al.*, 1996; Ye *et al.*, 1997; Kourmouli *et al.*, 2000) with the three HP1 homologs that have been described in humans (Saunders *et al.*, 1993).

The Origin recognition complex is a complex of six subunits, which is required for eukaryotic DNA replication initiation (Pflumm and Botchan, 2001). Furthermore, this protein is involved in silencing of the heterochromatic mating type loci in *Saccharomyces cerevisiae* (Fox and Rine, 1996). It has been shown that mutants of subunit 2 of the *Drosophila* origin recognition complex (ORC2) affect HP1 expression in comparison to *Drosophila* wild-type (Landis *et al.*, 1997).

The SET domain was initially characterized as a common motif in the *Drosophila* proteins, position effect variegation modifier SU(VAR)3-9, the Polycomb-group protein enhancer of zest E(Z), and the trithorax-group protein TRX (Rea *et al.*, 2000). These proteins have a chromo domain and a SET domain. The mammalian homolog of *Drosophila Su(var)3-9* encodes a specific methyltransferase which methylates histone H3 in *in vitro* experiments (Rea *et*

al., 2000). The methylated histone H3 is then able to bind to HP1 (Nielsen *et al.*, 2001).

The *C. elegans* homologs of these proteins have been identified using sequence analysis in Wormbase and the EST data base of Y. Kohara. The homolog of LBR is the sequence B250.7 and that of ORC2 is the sequence F59E10.1. Two SET domain protein homologs, C41G7.4 and C15H11.5, were also selected, of which the former is a *C. elegans* homolog of SU(VAR)3-9. dsRNAs were prepared according to these sequences, the integrity of which was determined by agarose gel electrophoresis.

Each dsRNA was then injected into the gonad arms of healthy young hermaphrodites that carried copies of the *hp1.1::gfp* construct in their genomes and had several times been backcrossed with *him*-8(*e1489*). Subsequently, living embryos of the F1 offspring were collected and screened for GFP fluorescence that indicated normal expression and location of HP1.1. It was hoped that the results would help to find possible interaction partners of HP1.1.

Fig. 3-18 shows that RNA interference by dsRNA of the lamin B receptor homolog B250.7 resulted in an altered appearance of the GFP fluorescence of HP1.1::GFP in interphase chromatin in comparison to the not injected embryos. The fluorescence was reduced, and the spots of high HP1.1::GFP concentration in the periphery of interphase chromatin were completely lacking. Probably therefore, the lamin B receptor homolog B250.7 interacts with HP1.1 in *C. elegans*.

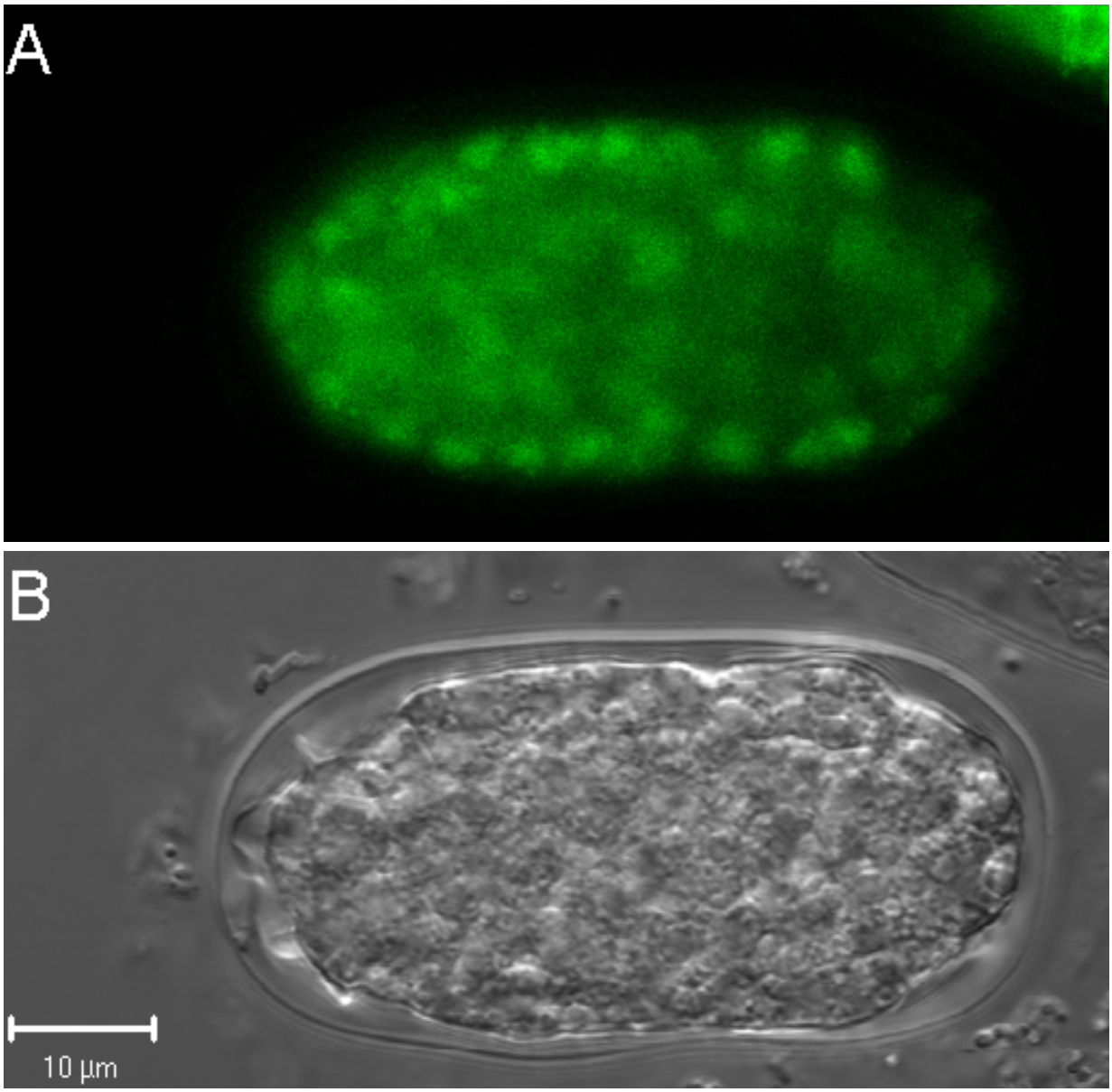

Fig. 3-18 Abnormal distribution and reduced amount of HP1.1 after a transient knock-out of the Lamin B Receptor (LBR) homolog in *C. elegans*. Young hermaphrodites were injected with dsRNA of B250.7, the *C. elegans* homolog of LBR, and the F1 embryos, which carry copies of the *hp1.1::gfp* construct, were screened for GFP fluorescence (A). B: Nomarski illumination.

dsRNA constructed according the homolog of subunit 2 of the *Drosophila* origin recognition complex of *C. elegans*, F59E10.1, also clearly affected the distribution of HP1.1::GFP in the interphase nuclei of the F1 progeny (Fig. 3-19). The intensity of the GFP fluorescence was greatly reduced and the peripheral spots of high concentration of the HP1.1::GFP construct were not detectable.

This means that the expression and distribution of HP1.1 was very different in comparison to embryos not microinjected with dsRNA. In many of the cells of the embryos, no HP1.1::GFP expression was visible (Fig. 3-19). Probably therefore, the *C. elegans* homolog of *Drosophila* ORC2 interacts with HP1.1. Results comparable to this observation have been obtained in *Drosophila* (Huang *et al.*, 1998).

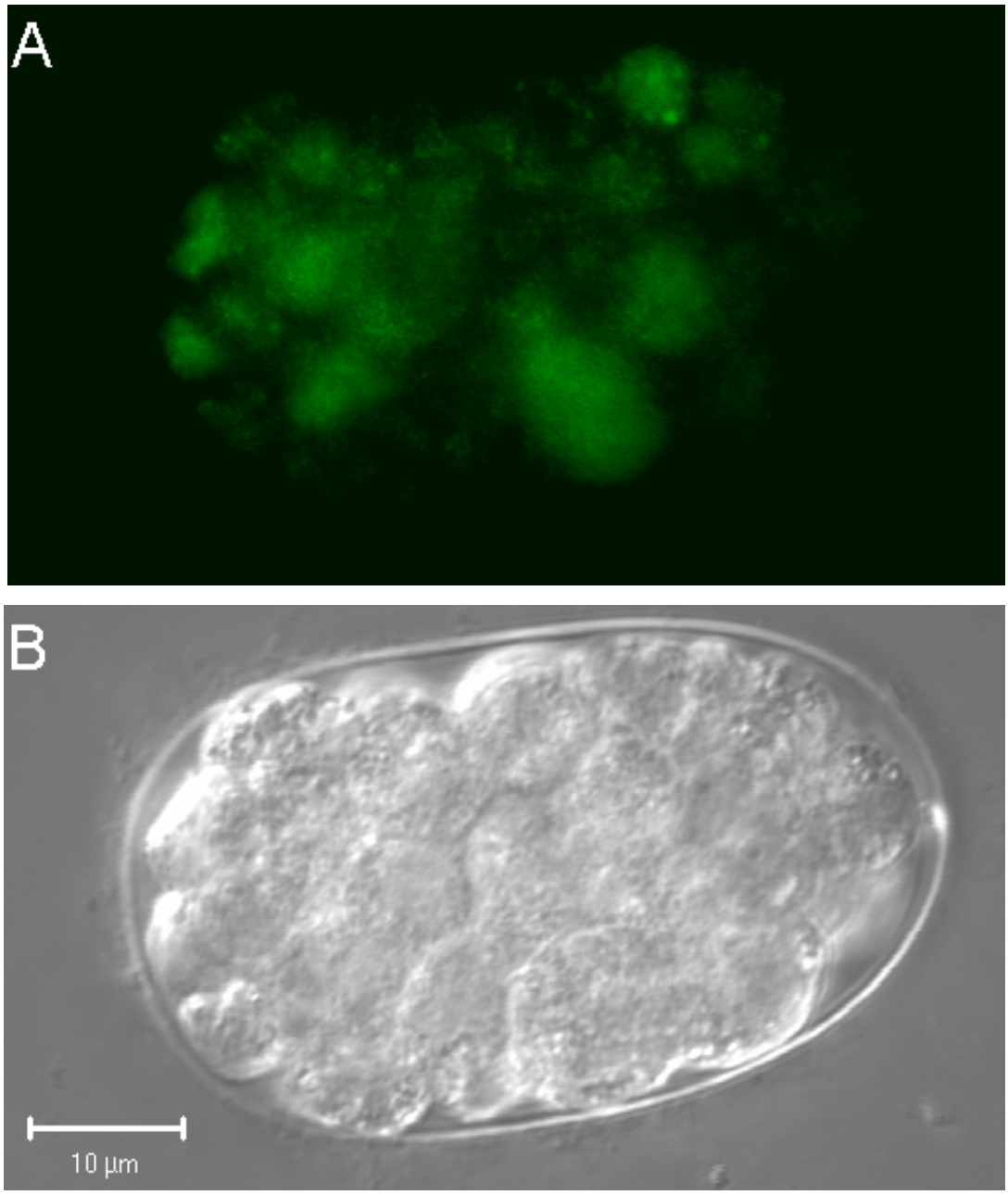

Fig. 3-19 Abnormal distribution and reduced amount of HP1.1 after a transient knock-out of the Origin Recognition Complex subunit 2 (ORC2) homolog in *C. elegans*. Young hermaphrodites were injected with dsRNA of F59E10.1, the *C. elegans* homolog of ORC2, and the F1 embryos, which carry copies of the *hp1.1::gfp* construct, were screened for GFP fluorescence (A). B: Nomarski illumination.

The *C. elegans* protein homolog to SU(VAR)3-9 was also checked for interaction with HP1.1. C41G7.4 dsRNA was injected and yielded in the F1 progeny a dramatic change in the embryonic cells (Fig. 3-20). The nuclear structures in the periphery containing high concentrations of HP1.1::GFP were completely lacking. Furthermore, the HP1.1 was relocated into the cytoplasm. The SU(VAR)3-9 homolog should thus interact with HP1.1 in *C. elegans*.

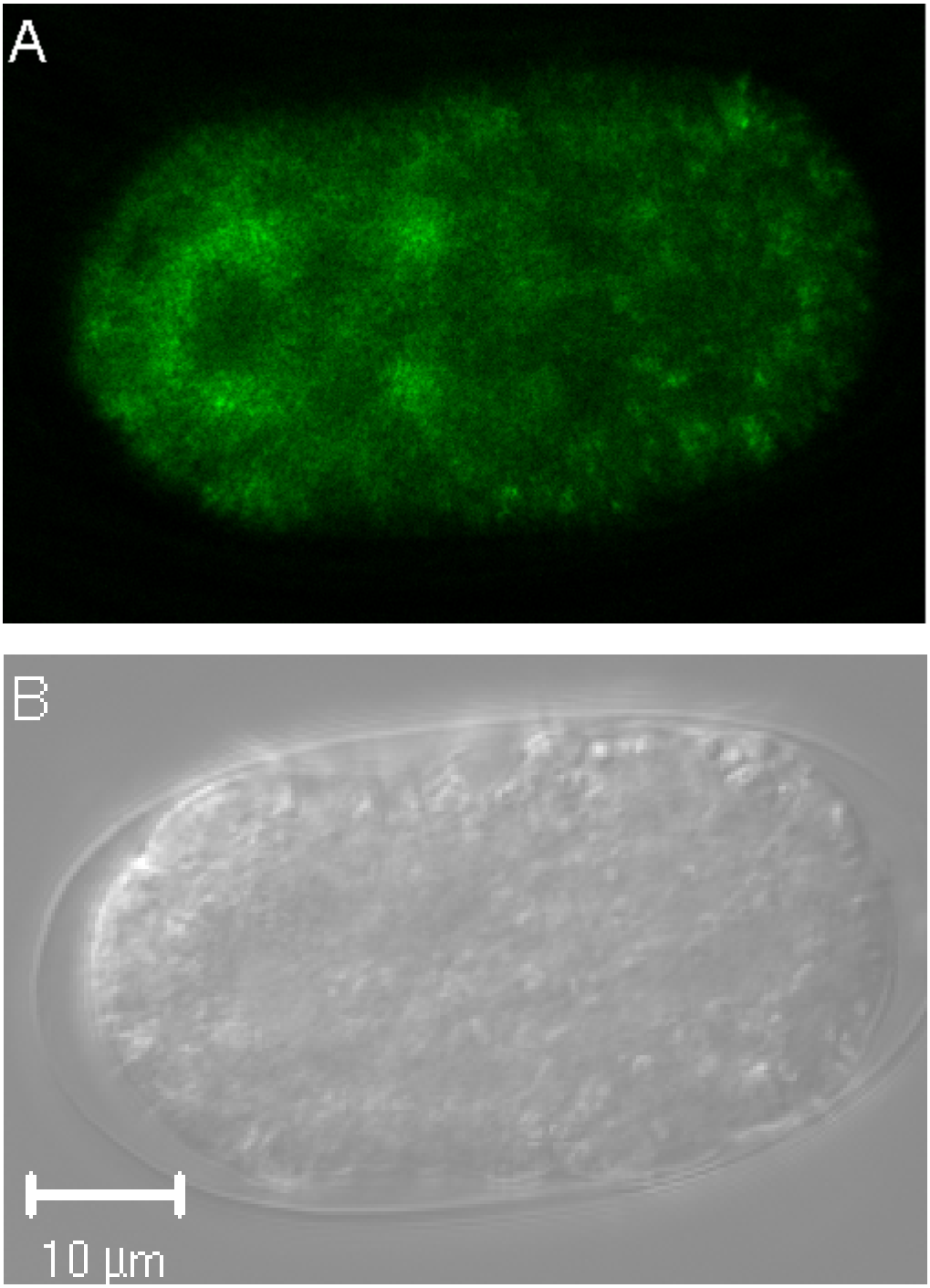

Fig. 3-20 Abnormal distribution and reduced amount of HP1.1 after a transient knock-out of the SU(VAR)3-9 homolog in *C. elegans*. Young hermaphrodites were injected with dsRNA of C41G7.4, the *C. elegans* homolog of SU(VAR)3-9, and the F1 embryos, which carry copies of the *hp1.1::gfp* construct, were screened for GFP fluorescence (A). B: Nomarski illumination.

Finally, another SET domain protein in *C. elegans* C15H11.5, was checked for possible interactions with HP1.1. Also in this case, the RNA technique that should transiently knock out its expression, led to an abnormal

distribution of HP1.1 and the disappearance of the peripheral spots of high HP1.1 concentration (Fig. 3-21), a result similar to that with C41G7.4 dsRNA. Also this SET domain protein may therefore interact with HP1.1 and its localization in the cell.

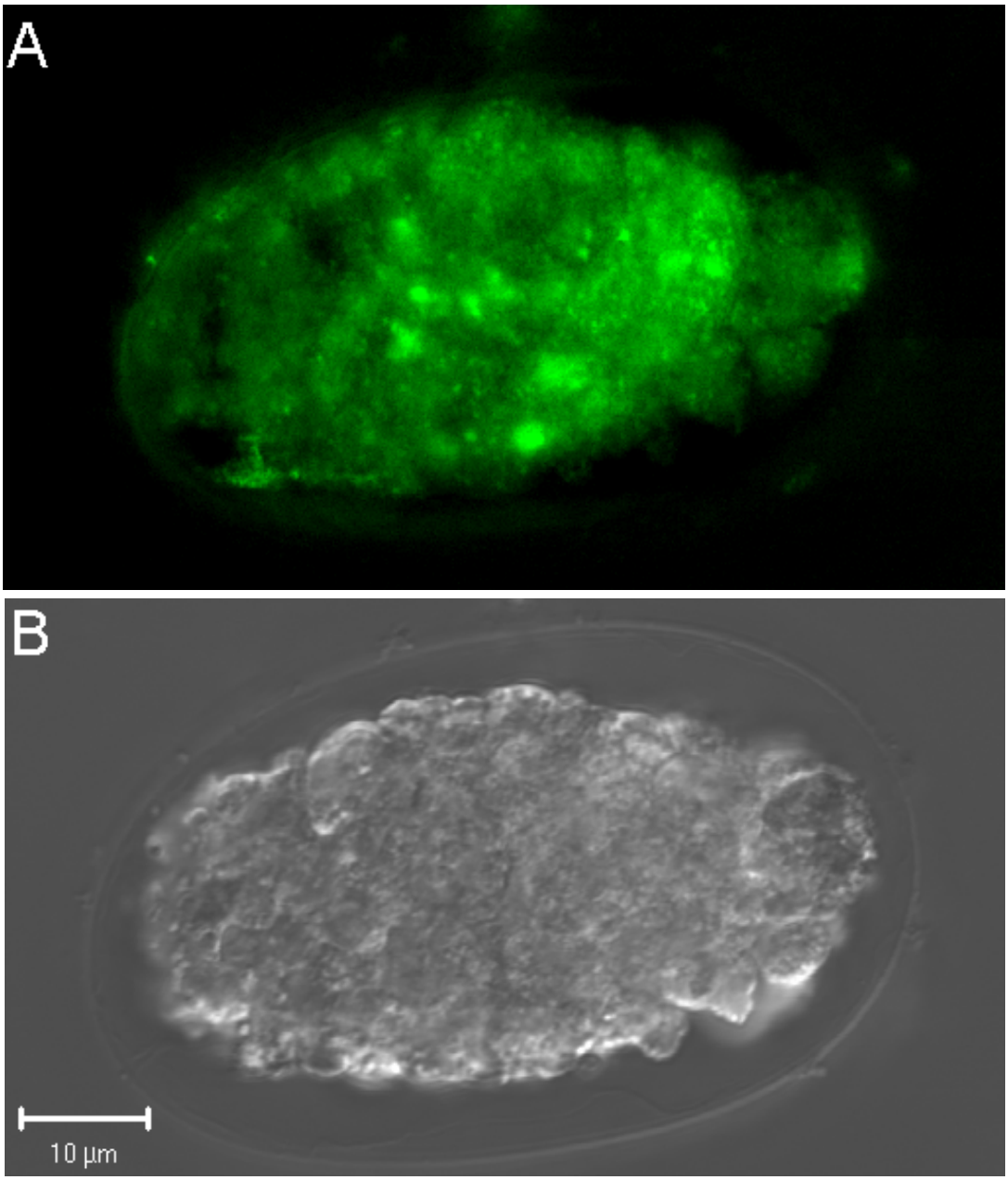

Fig. 3-21 Abnormal distribution and reduced amount of HP1.1 after a transient knock-out of a SET domain chromatin protein in *C. elegans*. Young hermaphrodites were injected with dsRNA of a sequence, C15H11.5, that encodes the protein, and the F1 embryos, which carry copies of the *hp1.1::gfp* construct, were screened for GFP fluorescence (A). B: Nomarski illumination.

4 Discussion

The nematode *C. elegans*, is the first multicellular organisms whose genome was completely sequenced (The *C. elegans* Sequencing Consortium, 1998), and is an important model organism for developmental, cytological, molecular and biochemical studies. The heterochromatin associated protein 1 of *Drosophila* was initially described as a protein associated with the chromocenter of polytene chromosomes in larval salivary glands (James and Elgin, 1986). In contrast to other organisms, there are no data on the cytological functions of the conserved HP1 homologous proteins in *C. elegans*, HP1.1, HP1.2 and HP1.3. Based on the analysis of HP1 in other organisms (Lorentz *et al.*, 1994; Huang *et al.*, 1999; Epstein *et al.*, 1992; Wreggett *et al.*, 1994; Singh *et al.*, 1991; Saunders *et al.*, 1993), it should be possible to investigate HP1 homologs in *C. elegans*. Database search and sequence alignment have shown that *C. elegans* contains two HP1 homologous genes (*hp1.1* and *hp1.2*). The *hp1.2* encodes two proteins because of alternative splicing processes. HP1 homologs share sequence homology with *Drosophila* HP1 and murine HP1 homologs (Fig. 3-1). The *C. elegans* HP1 homolog proteins contains an amino-terminal chromo domain. The experimental approach of the this work shows that HP1.1 in *C. elegans* is a functional HP1 homolog of *Drosophila*. In this work I am focusing on the distinct nuclear structures in the chromatin of holocentric chromosomes of embryonic cells in *C. elegans*. For the visualization of heterochromatin structures, there were two different approaches, which could be used. Indirect immunofluorescence staining for the visualization of structures which was used for recognizing the HP1.1 in *C. elegans*.

This work presents the cytological characterization of HP1.1 and HP1.3, from *C. elegans*. First I demonstrate protein analysis on a western blot of total *C. elegans* lysate, which shows a single protein band with a molecular weight of

35 kDa, designated as HP1.3 (Fig. 3-2). No other *C. elegans* proteins were reactive with this antibody. To confirm these results, it was shown, that the antibody recognize HP1.3 in indirect immunofluorescence staining *C. elegans*. These experiments have shown that the HP1.3 is present in only a few cells in the anterior periphery of a developing embryo (Fig. 3-3).

This new approach was used, which allows the *in vivo* visualization of heterochromatin of *C. elegans*. For the localization of HP1.1 in living embryos of the nematode *C. elegans,* it were used fluorescent protein fusions (Chalfie *et al.*, 1994). HP1.1::GFP expression exhibits subnuclear structures in the chromatin of interphase nuclei. In contrast to fixed martial it can be followed throughout the cell cycle because fluorescently labeled proteins enable the direct observation of mitotic nuclei and their nuclear structures. Regarding to this order, a *hp1.1::gfp* reporter genes was created as an extrachromosomal array in the genome of *C. elegans*. Direct *in vivo* visualization has the advantage that there is no need for fixing of the living material. The ability to visualize this dynamic process has been crucial for dissecting the molecular mechanisms underlying chromosome segregation. Using a laser scanning microscope (Zeiss 510), I showed that HP1.1 in the embryonic nuclei can be observed directly over a time scale of seconds or minutes.

HP1.1::GFP was integrated into the genome of *C. elegans* as a stable array (Fig. 3-6). Such an integrated array has advantages comparable to the extrachromosomal arrays. This is convenient for a variety of experiments *e.g.* reverse genetic. For antibody staining and protein extraction were such strains also well recommended. Extrachromosomal arrays changes their properties over time, because of transmission frequency, which is very variable. Furthermore, the expression of genes from such extrachromosomal arrays therefore potentially suffers from this kind of variation. For time series images from living

embryos from *hp1.1::gfp* integrated strain was used a laser scanning microscope, which allows us to follow the cell division.

It could be shown, that HP1.1 is enriched in spot-like structures in the nuclear region very close to the nuclear envelope, where in other organisms the heterochromatin is located during interphase (Fig. 3-4, 3-6, 3-7). There were mostly six spot-like structures in chromatin very close to the nuclear envelope of embryonic cells, which disappear dynamically during mitosis. These structures become conspicuous at late telophase. It is conceivable that these structures leave their location throughout mitosis, and relocate after mitosis. *C. elegans* has five pairs of autosomes and one pair of X chromosome in hermaphrodites, and in males it has five pairs of autosomes and a single X chromosome, based on Feulgen staining and light microscopy (Nigon, 1949a; Albertson and Thomson, 1982).

It is well conceivable that HP1.1 is enriched in distinct nuclear structure because of well known HP1 homologs which are in the architecture in repressed chromatin in the nuclei. The visualization of decondensed interphase chromosomes has remained technically challenge in all organisms. Basic question of interphase subnuclear structures as a result have relied largely on static images from fixed material and have been plagued by concern over specimen preparation artificial induced by fixation. This means, this approach has now been greatly enhanced by the application of GFP. Not only can specific nuclear structures be visualized using GFP, but it is also possible to observe dynamics throughout the cell cycle (Fig. 3-7). Subsequently, direct observation of chromosome dynamics in eukaryotic cells have been crucial in formulating mechanistic models of mitotic chromosome segregation. This work demonstrates that the subnuclear structures can be followed in the natural environment of living embryonic cells. It was to be proven whether the subnuclear structures can be shown in a double labeled nuclei in living

embryonic nuclei. Fortunately, there was possible a combination of more than one reporter gene, which also could be very well recommend for investigation.

The distinct structural properties of heterochromatin accommodate a diverse group of vital chromosome functions on both chromosome types, monocentric and holocentric. In comparison to the monocentric chromosomes the holocentric chromosomes are differently organized. The nematode *C. elegans* has monocentric chromosomes in the meiotic cells and holocentric chromosomes in the diploid cells.

During the cell cycle, HP1.1::GFP-labeled structures dynamically underwent a translocation from the nuclear periphery of the interphase nuclei into the chromocenter of chromosomes at the metaphase plates. Beside of these, there were the chromosomal ends, which remain still conspicuous in this stages, respectively. In this work I determined the intracellular locations of HP1.1 in the embryonic cell cycles and investigated its presence on mitotic chromosomes in embryonic cells.

HP1.1, cycles between condensed and decondensed states during the cell cycle in the embryonic nucleus. The onset of HP1.1 expression occurs at approximately the 60 cell stage, because we observed at this stage the appearance of *hp1.1::gfp* expression as a spatially distinct nuclear structure within interphase nuclei. In the embryonic interphase heterochromatin is characteristically localized at the periphery of the nucleus as a spot-like subnuclear structure. The early embryonic development in *C. elegans* is characterized by a period of rapid cell cycles. To explore this molecularly, proteins which might interact with HP1.1 were tested. This work should achieve more information about the molecular function of HP1.1 of the diploid nucleus in the distinct structures, which are visible by HP1.1::GFP. The recent application of this method focuses particularly on an example, that should

provide new insights into chromatin structures of interphase nuclei and their dynamic during cell division in living embryos of *C. elegans*.

The interphase nucleus is now believed to be inhomogeneous with many subnuclear structures. In particular, considerable structures in a most prominent nuclear region, heterochromatin, are organized either in a distinct chromosomal region. Previously, subnuclear structures have been difficult to visualize in fixed material. Moreover, it was not possible to show the movement of these interphase structures dynamically throughout the cell cycle. Considerably nothing is known about the dynamics of the subnuclear structures of heterochromatin of interphase nuclei throughout the cell cycle. The dynamic localization has been the subject of interest for at least two reasons. First, this should reflect and influence important aspects of their organization throughout mitosis. Second, it should provide information about their functions. More extended observation has revealed that these structures, located in chromatin at the periphery of nuclear envelope, show dynamic alterations within mitosis. First, it was shown that these structures change their position without any decreasing signal. But with the onset of mitosis they disappear dynamically, which causes a dramatically change of the HP1.1::GFP signal in the living embryonic nuclei. This can be shown in a plot curve of the HP1.1::GFP signal intensity in dependence of time (Fig. 3-8). The GFP signal increases with the ongoing of the cell cycle, an it peaks short before the onset of mitosis. There was a change of GFP signal, which decrease throughout mitosis. This signal decreasing can be caused by break down of the nuclear envelope, which is concomitant with chromosome segregation. The plot curve shows an increasing of HP1::GFP signal in every stage of the cell cycle until the onset of prophase. The decrease of signal intensity throughout mitosis, and remains approximately on the same level of the start of cell cycle. The solid appearance of these spot-

like structures led to an imagination that the heterochromatin packing within "regions" would limit in distinct macromolecular complexes in *C. elegans*.

In order to minimize possible interference of laser light microscopy with the dynamic of subnuclear structures of interphase nuclei throughout the cell cycle, a highly sensitive optical detection system was used and very low light level were applied to achieve a valid recording of light optical sections. Direct *in vivo* visualization showed that with the onset of mitosis the subnuclear structures in interphase nuclei changed their position dynamically, and moved onto the chromosomes following a single nucleus throughout the cell cycle.

Interestingly, it could be shown that a small fraction of *C. elegans* HP1.1 becomes cytoplasmic, which is visible by comparison of mitotic nuclei with those which are at the interphase (Fig. 3-9). Kellum *et al.* (1995) have shown that HP1 in *Drosophila* tissue culture cells is dispersed throughout the cytoplasm during mitosis. Moreover, they could not show HP1 on the mitotic chromosomes of these cells using antibody staining. However, they were able to detect HP1 in *Drosophila* tissue culture cells without fixation. It could be possible that the dispersed HP1.1 fraction reflects a dissociation of a protein fraction from heterochromatin during mitosis. This assumption can be supported because at the late telophase the cytoplasmic fraction of HP1.1 disappears. It seems, that it relocates to the nucleus. Using time series images, it could be shown, that the cytoplasmic fraction of HP1.1 is conspicuous throughout mitosis (Fig. 3-9).

The condensation of interphase chromatin to package the DNA for segregation during mitosis is one of the more dramatic events in the cell cycle. Chromosomes can be visualized by a DNA dye, Hoechst 33342 or DAPI. Here we describe the successful use of two fluorescence protein CFP (Haas *et al.*,

1996, Yang *et al.*, 1996) and YFP (Ormö *et al.*, 1996) for double labeling in living *C. elegans*. For concomitant visualization of HP1 and DNA the double-label was used as a vital reporter for monitoring localization and dynamics of HP1.1 and histone H1. Histone H1, which facilitates generally higher order chromatin structure was used as an indirect DNA marker. I generated double-labeled transgenic animals using *hp1.1::gfp* and *his-24::cfp* reporter genes (Fig. 3-13). Time series recorded of double-labeled embryonic nuclei showed details of HP1.1 dynamics in the cell cycle (Figs. 3-14, 3-15). It was very astonishing to see that during the prometaphase HP1.1 in living embryos was not localized exclusively on the chromosomes but was localized as a stripe in an area beside the chromosomes. The sudden accumulation of HP1.1 beside the chromosomes during prometaphase may play a role for the rebuilding of the outer kinetochore.

HP1.1 relocates to the chromosomes, and it was totally colocalized with histone H1 at the late metaphase. Interestingly, HP1.1 occupied the spindle fibers binding sites as a layer during anaphase. It is obviously the outer kinetochore of *C. elegans* holocentric chromosomes. HP1.1 was localized again in the spot-like structures with histone H1 at the late telophase, when the nuclear envelope reforms and chromosomes decondensed.

Because of this, it could be conceivable that every two homolog chromosomes are connected to one spot-like structure, respectively. It has been shown that, during mitosis the spindle attachments can be observed along the entire length of the holocentric chromosome of *C. elegans* (Albertson and Thomson, 1982). The centromeric heterochromatin in a number of cell types is localized in a distinct region on the nuclear envelope during interphase (Brown, 1966). It is interesting to know, whether these distinct structures correspond to centromeric heterochromatin or the heterochromatin in telomeres of *C. elegans*. The human and murine HP1 homologs have been shown to be associated with centromeric heterochromatin (Wreggett *et al.*, 1994). Such finding suggest that

the HP1.1 could also have a centromeric function. The *S. pombe* HP1 homolog (SWI6) is localized in telomeric and centromeric regions. However, because of time series micrographs it is more obvious, that HP1.1 in *C. elegans* binds to the outer kinetochore of holocentric chromosomes.

HP1.1 is located in *C. elegans* chromatin at the periphery in nuclei. Because of this topology, it could be possible that these structures are formed by the pairing of DNA repeats (Dorer and Henikoff, 1994) or by a specific histone modification, such as H3, which is methylated a lysine 9, which generates a binding site for HP1 homologs (Jenuwein, 2001). Furthermore, such peripheral location would confirm the role of HP1.1 in the higher organization of heterochromatin within the interphase nucleus. Csink and Henikoff (1996) have characterized the heterochromatin of bw^{D} ($brown^{Dominant}$) locus in diploid cells by fluorescence *in situ* hybridization (FISH). Because in this procedure the cells have to be fixed, this is unfortunately not possible to be used it for HP1.1::GFP. But it is conceivable that the sequence of telomeric or centromeric satellites could be used for the detection of these regions.

Normally, the embryos formed a tough shell around itself, which consists of an inner vitelline membrane as a biological barrier to solutions, a middle chitinous layer, and an outer layer consisting of lipids and cross-linked proteins (Chitwood and Chitwood, 1974). For indirect immunofluorescence staining the freeze crack method can be used. Because of the distinct structures in chromatin of the *hp1.1::gfp* strain, it could be possible to show these structures with X-ray microscopy which shows details comparable to light microscopy with 5 times higher resolution (Vogt *et al.*, 2000). For localization of distinct structures embryo were used for the freeze-crack method. Using this method, however, it was not possible to recognize the distinct structures of interphase nuclei on chromatin in these cells in comparison to the living images. Embryonic single cells of *C. elegans* were prepared using an enzymatic method. The harvested

cells were incubated onto glass slides, and subsequently fixed with glutaraldehyde or formaldehyde, respectively (Fig. 3-10). However, it was impossible to detect these structures in a suitable with the X-ray microscope (Fig. 3-11).

Wreggett *et al.* (1994) have shown, that the murine homolog of HP1 can be localized at the centromeres of metaphase chromosomes when these were not fixed. Kellum *et al.* (1995) have used unfixed mitotic chromosomes of *Drosophila* Schneider tissue culture cells for antibody staining with anti-HP1 antibodies, because HP1 could not be confined to the nuclear region after fixation. This indicates that HP1.1 was affected during the fixation procedure for antibody staining of *C. elegans* embryonic cells for its localization.

A second attempt for the investigation of the spot-like structures was performed using the Strep-tag system. The *hp1.1* was inserted in frame into the pEGFP::SIIc vector. Transgenic animals were generated, which express the fusion protein HP1.1::GFP::SIIc. A protein analysis by western blot of a total *C. elegans* lysate from a *hp1.1::gfp::sIIc* strain shows a single protein band with a molecular weight of 58 kDa (Fig. 3-12). This band consists of the constructed HP1.1::GFP::SIIc which has in fact 58 kDa, of which 27 kDa are accounted for by GFP, 21 kDa by HP1.1, and 10 kDa by streptavidin. Strep-tag has been introduced as a possible novel technique for analyzing the distribution of HP1.1 protein in *C. elegans* embryonic cells by X-ray microscopy. A GFP-fluorescence has been observed of both the *hp1.1::s::gfp*, and the *hp1.1::gfp::sIIc* constructs. Unfortunately, it could not be labeled using Cy5 conjugated Strep-Tactin. It is well conceivable that the binding sites of Strep-tag are not available for Strep-Tactin. A plausible explanation would be that this Cy5-conjugated Strep-Tactin could not interact with Streptavidin because of conformational changes in the HP1.1::GFP::SIIc. Subsequently, it was thus not possible to use this technique to

analyze the distribution of HP1.1 in embryonic cells by X-ray microscopy. Unfortunately, the cytological detection reaction did not work. The efficacy of streptavidin is derived from its extremely high affinity to the vitamin biotin, and therefore it can be used in a variety of biological applications. Thus, this procedure was formerly developed for the production of recombinant core streptavidin in *E. coli* and its purification (Schmidt and Skerra, 1994). Based on this study, HP1.1::GFP::SIIc could be used to identifying HP1.1 and its interacting partners by complex location and mass spectroscopy.

Taken together, HP1.3 protein in *C. elegans* could be shown only in a few cells, whereas HP1.1::GFP is expressed in a huge number of cells in most tissues. Based on these observations, both proteins have a clearly distinguishable expression pattern. Because HP1.3 is expressed only in a few cells in the periphery of the embryo, it could possibly play a role in embryonic development. Unfortunately, it was not possible to identify these cells exactly, but most cells of the embryonic periphery are inherited from the AB founder cell comparable to cell lineage (Sulston *et al.*, 1988). The expression pattern of this protein is very different in contrast to the HP1.1::GFP expression.

When either the sense or antisense transcript from a gene (*par*-1) were injected into the germline, this let to epigenetic inactivation of the gene in the resulting progeny (Guo and Kemphues, 1995). This phenomenon was extended to other genes. Subsequently, it has been shown that, the phenomenon is strongest with double-stranded RNA (Fire *et al.*, 1998). RNA-mediated interference (RNAi) with HP1.1 in *C. elegans* was used to suppress the HP1.1 expression. HP1.1 dsRNA resulted in the *hp1.1::gfp* integrated strain of 95% of F1 progeny which lost their GFP-fluorescence signal. The HP1.1 dsRNA in the wild-type animals showed multiple and variable defects. There were about 13% dead embryos, and approximately 5% animals, which had defects in their

development (Fig. 3-17). These animals have a different morphology in their embryonic development as wild-type embryos. This means, that they buildup larval arrest in comparison to the N2 embryos. Because their phenotype they can be comparable with *vab* mutants specially *vab*-6 (*variable abnormal morphology*) mutants.

HP1.2 dsRNA shows 10% dead embryos. It could not be observed such phenotypic effects as with HP1.1 dsRNA. But a mixture of both dsRNAs shows the F1 progeny with abnormal development. The interpretation of this result can be done in the following way. If an embryo has a higher amount of HP1.1 dsRNA, this would lead to its dead. Otherwise, if the dosage of HP1.1 dsRNA is not high enough, it cause defects in their development. This result is comparable to the dosage dependence of HP1 in *Drosophila*. Position-effect variegation was observed when euchromatic genes have been transposed into vicinity of heterochromatic chromosome regions by chromosomal rearrangement (Reuter and Wolff, 1981). HP1 was first described in *Drosophila* (James and Elgin, 1986), that suppresses the dominant *Drosophila* modifier of position-effect-variegation (PEV) *Su(var)3-9* (Reuter *et al.*, 1982; Eissenberg *et al.*, 1990).

Drosophila HP1 is encoded by *Su(var)205*, one of the PEV modifier genes, which is known as the best-studied structural protein associated with heterochromatin (Eissenberg *et al.*, 1992). PEV has been shown in flies in the following manner: a loss-of-function mutation in *Su(var)205* in *Drosophila* leads to increased expression of euchromatic genes that suffer from position effect, *i.e. White*, while overexpression of HP1 results in decreased expression of these genes (Eissenberg *et al.*, 1992). Whereas, the opposite results in *Drosophila* have been obtained for genes closely associated with heterochromatin, *i.e. Light*, (Hearn *et al.*, 1991). Further investigation has shown that overexpression of HP1 leads to enhanced PEV (Eissenberg *et al.*, 1992). When the duplication of the allele *E(var)39A* is present, three copys of HP1 are present and PEV is enhanced (Locke *et al.*, 1988). HP1 is a haplo-insufficient

suppressor and a triplo-abnormal enhancer of PEV (Eissenberg and Elgin, 2000) in *Drosophila*. Finally, the PEV is formally analogous to the regulation of the mating type in budding yeast. One mating type gene is maintained in a repressed state at a pair of silent loci and become transcriptionally competent when moved to an active mating type locus (Laurenson and Rine, 1992). It seems that the HP1.2 dsRNA does not show such a dosage effect.

The analysis of the prominent chromatin silencing in the germline of *C. elegans* HP1.1 dsRNA was injected into the animals carrying the *let*-858::*gfp* in the *pha*-1 genetic background (Granato *et al.*, 1994). There was no GFP-fluorescence signals out of the germline in F1 adult hermaphrodites. To confirm these results, *hp1.1::gfp*;*him*-8(*e1489*) integrated strain was crossed with *mes*-3 (Paulsen *et al.*, 1995). There were no differences in HP1.1::GFP expression observed in the germline of this animals, too. Because of these results, it should be concluded that, the HP1.1 is not involved in the prominent chromatin silencing in the germline of *C. elegans*. HP1.1 is not involved in the germline silencing because it is not expressed in the germline.

Two primordial germ cells, Z2 and Z3, produced in the early embryo of *C. elegans* (Sulston *et al.*, 1983) are distinguishable in comparison to the rapidly dividing and differentiating somatic cells. These cells can be identified in the earlier embryo development performing immunofluorescence staining, using P granules antibody (Strome and Wood, 1982). The chromatin of germ cells play not only an important role for creating of transgenes in *C. elegans*. But also silences repetitive transgenes (Kelly *et al.*, 1997).

However, desilencing by HPL-2 RNAi (HP1-like protein or HP1.2) has been shown in the germline of *C. elegans* (Personal communication F. Palladino). Furthermore, the onset of HP1.2 expression is with the beginning of

30-cell stage in the embryogenesis (Personal communication F. Palladino). Based on this, suggesting that the HP1.1 is a soma specific protein, it is very interesting to know, what does HP1.1 in the somatic cells? Notably, the HP1 homolog in *Tetrahymena* is missing from transcriptionally silent micronuclei but is enriched in heterochromatin-like chromatin bodies that presumably comprise repressed chromatin in macronuclei. This findings specially provide an evidence that HP1-like proteins are not exclusively associated with permanently silent chromosomal domains (Huang *et al.*, 1999).

For the characterization of HP1.1 in distinct spot-like structures dsRNA of four *C. elegans* nuclear proteins were injected into the gonads of *hp1.1::gfp* integrated strain. The lamin B receptor was chosen (LBR, B250.7), the subunit 2 of origin recognition complex (ORC2, F59E10.1), SU(VAR)3-9, C41G7.4 and another SET domain protein, C15H11.5.

Human LBR is an integral protein of the inner nuclear membrane. This protein has an amino-terminal domain of approximately 200 amino acids residues and a carboxy-terminal domain similar in sequence to yeast and plant sterol reductases. The amino-terminal part of the LBR domain contains an nuclear localization signal and contributes to the attachment of the membrane to chromatin. The nuclear lamina is involved in nuclear organization, cell cycle regulation, and differentiation (Goldberg *et al.*, 1999): The dsRNA with *C. elegans* LBR homolog let to a diffuse intranuclear distribution of HP1.1::GFP in the spot-like structures of embryonic nuclei so, that these were destroyed (Fig. 3-18). This observation shows that the *C. elegans* LBR homolog (B250.7) interacts with HP1.1. Interestingly, LBR is a member of a family of lamina-associated proteins whose prominent member is emerin. A mutation in emerin causes Emery-Dreifuss muscular dystrophy (EDMD) (Manilal *et al.*, 1999). It means, that Lamin B is also almost absent from skeletal muscle nuclei. The

dynamics of mammalian HP1 proteins was studied using a quantitative *in vitro* assay. In order to this, the interaction of mammalian HP1 is potently inhibited by using soluble factors present in mitotic and interphase cytosol (Kourmouli *et al.*, 2000).

Initially ORC was described as a multi-protein complex, which binds and initiates DNA replication from autonomous replicating sequence elements distributed throughout the yeast genome (Bell and Stillman, 1992). *Drosophila* mutant in subunit 2 of origin recognition complex (ORC2) showed a different HP1 expression pattern (Huang *et al.*, 1998). The HP1 localization in heterochromatin is disrupted in such *Drosophila* mutants. With respect to this experiment, it was interesting to show, whether the ORC2 homolog in *C. elegans* has a functional interaction with HP1.1. The RNAi with the *C. elegans* ORC2 homolog (F59E10.1) in the *hp1.1::gfp* integrated strain showed a misregulation of the quantity of HP1.1::GFP in the nuclear structures. It has been demonstrated that heterochromatin plays a role in mitotic chromosome condensation and organizing the nuclear architecture (Hochstrasser *et al.*, 1986; Kellum and Alberts, 1995; Csink and Henikoff, 1996; Dernburg *et al.*, 1996a). The F59E10.1 dsRNA in *C. elegans* shows that the ORC2 homolog interacts with HP1.1. This result also confirms the suggestion, that the HP1 proteins in *C. elegans* could possibly interact in a complex with ORC2. Finally, the interaction of HP1.1 with ORC2 in *C. elegans* could be studied in molecular details, *e.g.* to identify the binding sites. In eukaryotes ORC serves as the platform upon which the replication initiation complexes assembles (Lee and Bell, 2000). Thus the distribution of ORC along the DNA determines, in part, the sites at which replication may start. Furthermore, It was demonstrated, that the yeast ORC plays a discrete role in forming heterochromatin at the HMR (silent mating-type genes) locus through recruitment of Sir1 (Fox and Rine, 1996). The aim in this work concentrated on HP1.1 of embryonic cells, and this experiment showed, that *C. elegans* ORC2 interacts with HP1.1 (Fig. 3-19).

The *Drosophila suppressors of position effect variegation* [*Su(var)3-9*] (Tschiersch *et al.*, 1994) is related to the *S. pombe* silencing factor *clr4*, *cryptic loci regulator*, (Ivanova *et al.*, 1998). These proteins influence centromeric silencing. These have been identified by genetic screens both in *S. pombe* (Allshire *et al.*, 1995) and in *Drosophila* (Reuter and Spierer, 1992). SET domains are a protein family which are involved in chromatin regulation (Jenuwein *et al.*, 1998). The chromo domain has a protein motif that directs heterochromatic or euchromatic associations (Messmer *et al.*, 1992). It has been implicated that SET domains act as a target in phosphorylation-dependent signaling pathways that trigger proliferation or differentiation (Cui *et al.*, 1998). A mostly cysteine-rich SET domain protein has been described before (Huang *et al.*, 1998). SET domain could be a target for phosphorylation-dependent signals (Cui *et al.*, 1998) that may trigger dynamic transition in chromatin structure.

Recently, Jenuwein and coworker (Rea *et al.*, 2000) have shown that the mammalian homolog of *Drosophila* SU(VAR)3-9, a SET domain protein, that is rich in cysteine and histidine residues methylates histone H3, which then binds to mammalian HP1 homologs. Based on these facts we examined the SU(VAR)3-9 homolog of *C. elegans* to find out, whether this protein has any interaction with HP1.1. The C41G7.4 RNAi was performed in the *hp1.1::gfp* integrated array and images were recorded (Fig. 3-20). The spot-like structures of HP1.1::GFP in the interphase nuclei were destroyed. It can be suggested that both proteins act as two members of a protein complex in concert of a network of higher order of chromatin structure.

Another SET domain homolog protein was used for a RNAi study, C15H11.5 of *C. elegans* (Fig. 3-21). The result of C15H11.5 RNAi in the *hp1.1::gfp* strain shows results similar to C41G7.4 in *C. elegans*. This can be interpreted that both SET domain protein C41G7.4, and the SU(VAR)3-9

homolog have an interaction with HP1.1 in *C. elegans*. These proteins are also involved in chromatin remodeling. Whether *C. elegans* SET domain proteins bind in the same manner like *Drosophila* and human homologs remains to be investigated in the future. The assembly of higher order chromatin structures has been linked to the covalent modifications of histone tails. Both the conserved chromo-and SET domains of Clr4 are required for histone H3 Lys 9 methylation *in vivo*. Whether *C. elegans* SET domain homolog protein also binds to histone H3 remain to investigated in the future. Histone amino-terminal modification cause, epigenetic effects which play a role in development.

Taken together, these four nuclear proteins are well known from other organisms as HP1 interacting proteins. The aim of these experiments were, whether these proteins also show any interaction with HP1.1 in *C. elegans*. The dsRNA with these four nuclear architectural protein showed a deviation of HP1.1::GFP distribution in the nucleus. These results have given rise to interpretations that there may be a number of different complexes of HP1.1 in the chromatin. Additionally HP1.1 is an important member of the outer kinetochores of the holocentric chromosomes in *C. elegans*.

5 Abstract

Heterochromatin binding protein homologs of *Caenorhabditis elegans*

The nematode *C. elegans* was the first multicellular organisms whose genome was completely sequenced (The *C. elegans* Sequencing Consortium, 1998). This model organism is highly eligible for a variety of approaches in developmental biology, genetics and biochemistry.

For the first time, in this study the nematode *C. elegans* was used to investigate heterochromatin binding protein 1 (HP1) homologs in order to analyze their molecular functions. A sequence alignment revealed three HP1 homologs, HP1.1, HP1.2, and HP1.3 in the genome of *C. elegans*. HP1.3 was detected with an anti mouse-HP1 antibody. The nematode *C. elegans* is a model organism which can be used for genetical and biochemical approaches. The aim of this work was to explore the role of heterochromatin protein homologs in *C. elegans*. Extrachromosomal and integrated arrays expressions fluorescent fusion proteins of living specimen by HP1.1::GFP were created, which allowed the cytological observation with a confocal laser scanning microscope. The dynamics of the HP1.1 distribution throughout the cell cycle has been documented.

The expression of HP1.1::GFP begins with the 60-cell stage in embryogenesis. HP1.1 is present in a very high number of cells in most of the tissues. Furthermore, there are mostly six spot-like subnuclear structures in the chromatin near to the nuclear envelope of interphase nuclei. These subnuclear structures disappear dynamically with the onset of mitosis, when the nuclear envelope breaks down. HP1.1 separates from chromosomes in the prometaphase completely, and relocates to chromosomes at late metaphase. At the anaphase HP1.1 occupies binding sites of the spindle-fibers as a layer. For concomitant visualization of HP1 and DNA a double-label experiment was used, in which H1.1::CFP serves as a indirect marker for DNA. I show that HP1.1 is part of the

outer kinetochore of *C. elegans* holocentric chromosomes using images recorded with a confocal laser scanning microscope.

dsRNA with HP1.1 expression showed multiple and variable defects including embryonic death, slow growth, dumpy-like animals, and larval arrest. The molecular mechanisms of HP1.1 localization to the spot-like structures in interphase were analyzed by dsRNA with established HP1 interacting proteins in the *hp1.1::gfp* reporter strain. dsRNA with the SET domain proteins SU(VAR)3-9 (C41G7.4), and another SET domain (C15H11.5) relocated HP1.1::GFP to the cytoplasm. dsRNA with the lamin B receptor (B0250.7) led to a diffuse intranuclear distribution of HP1.1::GFP in the nuclei. dsRNA with ORC2 (F59E10.1) expression resulted in misregulation of the quantity of HP1.1 expression and a modified intranuclear distribution. These results have given rise to interpretations that HP1.1 is involved in a number of different chromatin protein complexes.

HP1.1 is not involved in the chromatin silencing in the germ line of *C. elegans* as shown by HP1.1 dsRNA experiments in a germline silenced reporter strain. This is additional and confirmed by the analysis of HP1.1 expression patterns. HP1.1 is involved in a number of different chromatin complexes which show that HP1.1 is a strictly somatic protein. The observations in this work indicate that at the molecular level HP1.1 in *C. elegans* functions in contrast to organisms which contain visible amounts of heterochromatin.

6 Outlook

The generation of HP1.1::GFP expression strains of *C. elegans* will allow to analyze the quantitation chromatin bindings protein of HP1.1 complex by the technique of fluorescence recovery after photobleaching (FRAP).This FRAP technique (Axelrod *et al.*, 1976) can be used to investigate more details of the molecular mechanism. Briefly, an intense laser pulse is used to bleach (render nonfluorescence) many of the fluorescence molecules in a small subregion of HP1.1::GFP in subnuclear structures in living embryonic nuclei. The return of fluorescence to this subregion is then monitored by normal confocal fluorescence microscopy. FRAP can be used for the quantitative study of the fluorescence recovery of the bleached area in spot-like structures *in vivo*, which almost to determine diffusion constants.

Additionally, a Strep-tag transgenic line expressing HP1.1::GFP::SIIc reporter has been created which could be useful for the biochemical characterization of HP1.1. This reporter can be used for the identification, isolation and purification of HP1.1 containing protein complexes, using Strep-Tactin coated columns. By this way, it might be possible to identify further HP1.1 binding proteins using mass spectroscopy.

7 Official statement

I want to confirm, that I made the present work by myself. I did not adopt foreign ways.

8 Abbreviations

A	absorbency, ampere, and aperture
Amp	ampicillin, α-aminobenzylpenicillin
APS	ammonium persulfate
ATP	adenosine triphosphate
BESSYI	electron storage ring in Berlin (Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung m.b.H)
bp	base pairs
bp	band-pass
BSA	bovine serum albumin
CCD	charge coupled digital
cDNA	complementary DNA
°C	degrees Celsius
C. elegans	*Caenorhabditis elegans*
CFP	cyan fluorescence protein
CGC	Caenorhabditis Genetic Center
clr4	*cryptic loci regulator*
cm	centimeters
Cy2	Cyanine Cy2
Cy3	Cyanine Cy3
daf	*dauer formation*
DAPI	4´,6-diamidino-2-phenylindol
dATP	desoxyadenosine triphosphate
dCTP	desoxycitosine triphosphate
ddH_2O	double-distilled water
dGTP	desoxyguanosine triphosphate
dH_2O	distilled water
DIC	differential interference contrast
DMF	dimethylformade
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double-stranded
DTT	dithiotreitol

dTTP	desoxythymidin triphosphate
E	Embryo
EC	Enzyme Commission (number)
EDTA	ethylenediaminetetraacetic acid
e.g.	for example (Lat. *exempli gratia*)
EST	expressed sequence tag
et al.	et alii, and others
F1	first filial generation
F2	second filial generation
Fig.	figure
g	gravitation units for centrifugation, and gram for weight
GFP	green fluorescence protein
Gy	Gray, energy dose, (1 Gy = 1 J/kg)
H33342	Hoechst day, (2`-[4-Ethoxyphenyl-5-[4-methyl-1-piperazinyl]-2,5´-bi-1H bezimidazole)
HCl	Hydrochloric acid
him	*high incidence of males*
HP1	heterochromatin binding protein
HRP	horse radish peroxidase
Hz	hertz
IPTG	isopropyl-1-thio-β-D-galactopyranoside and 5-Bromo-4-chloro-3-indolyl-β -D-glactopyranoside
1 J	1 Joule=1 Wattsecond=1 Newtometer=1 $kg*m^2*s^{-2}$
Kan	kanamycin, from *Streptomyces kanamyceticus*
kb	kilobase
LB	Laura-Bertani medium
LBR	lamin B receptor
LSM 510	laser scanning microscope 510
µ	micro
m	milli
mA	milli ampere
M	Molar
mes	*maternal effects sterile*
min	minute & minutes

mRNA	messenger RNA
MW	molecular weight
n	nano
NA	numerical aperture
NaCl	sodium chloride
NaOH	sodium hydroxide
NGM	nematode growth medium
NH_4Ac	ammonium acetate
Nomarski-DIC	Nomarski differential interference contrast
N-Terminal	amino-terminal
OD	optical density
PAGE	polyacrylamide gel electrophoresis
par-1	*embryonic partitioning abnormal*
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
propylgallate	3,4,5-Trihydroxybezoic acid
RNA	ribonucleic acid
RNAi	RNA-mediated interference
RNase	ribonuclease
r.p.m.	rotation per minute
s	seconds
S	Streptavidin
SIIc	Streptavidin, second vector type
SDS	sodium dodecyl sulfate
Su(var)3-9	*suppressors of position effect variegation*
TAE	tris acetate EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl)-aminomethane
U	units
UV	ultra-violet
v	volume
vab	*variable abnormal morphology*
V	volts
VA	volt-ampere

X-Gal	5-Bromo-4-chloro-3-indolyl--β-D-galactoside
YFP	yellow fluorescence protein
-/-	homozygous mutant
+/-	heterozygous mutant
+/+	wild type animal

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10 Publication and Meetings

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Petersen, C., Aumüller, G., Bahrami, M., and Hoyer-Fender, S. (2001): Molecular cloning of *odf3* encoding a novel coiled-coil protein of sperm tail outer dense fibers. Mol. Reprod. Dev. **9999**, 1-11.

11 Acknowledgments

I would gratefully thank to Dr. U. Grossbach, who gave me the opportunity, conditions and support to develop the present work. I thank Dr. W. Engel for undertaking assessment of my work.

Specially I would like to thank Dr. G. Schmahl and Dr. D. Rudolph, Research Unit X-ray Physic for their financial support of this work.

I thank Dr. E. Schulze for supervising of the present work.

I am thankful for the providing of some cDNA clones to Dr. Y. Kohara at Japanese National Institute of Genetics; for providing of cosmid clones to Dr. A. Coulson at the Sanger Center; and for providing of *C. elegans* strains to T. Stiernagle from Caenorhabditis Genetic Center.

I would thank Mr. P. B. Singh for providing of mammalian anti murine-HP1 antibody, and Dr. T. Schmidt for providing of *gfp::sIIc* vector.

I thank S. Fröde for critics and discussion, tips and helps, and P. Guttmann for helps for recording X-ray micrographs. Finally, I would thank all those people from both institutes, Third Department of Zoology-Developmental Biology-, and Research Unit X-ray Physic of University of Göttingen, who helped me in any kind for this work during my Ph.D. time in Göttingen.

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