Genetic Approach To Study Centromere Function In Vertebrate Cells: CENP-I is essential for centromere assembly in vertebrate cells.

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Abstract

Fundamental to all life processes is the ability of the cell to divide faithfully. Bipolar attachment of microtubules to kinetochores is essential for proper chromosome segregation in cell cycle. The centromere is an essential component of the chromosome that is required for accurate segregation of chromosomes during mitosis and meiosis. The centromere is not only the site of kinetochore formation; it is also directly involved in sister chromatid cohesion and separation, chromosome movement, and mitotic checkpoint control. Although we know that problems in chromosome segregation can lead to aneuploidy, cancer, and cell death, we do not fully understand the mechanism by which centromeres interact with microtubules of the spindle apparatus during cell division in any organism.

It has been difficult to apply genetic analyses to vertebrate centromeres because of the lack of suitable assays for mutations that affect the accuracy of chromosome segregation. Therefore, centromere proteins in vertebrate cells have been identified by immunological methods or as homologues of centromere-associated proteins in yeasts or fly. Of the centromere proteins in vertebrate cells, CENP-A and CENP-C are likely to be important for establishment of kinetochore function because they are essential for cell viability, and their functional homologs are present in yeast to human species. CENP-A is a histone H3 variant and is required for localization of CENP-C to the kinetochore. Fukagawa et al. have reported that CENP-H, a newly identified centromere component, is required for centromere targeting of CENP-C in vertebrate cells. CENP-A, -C, and -H play important roles in the establishment of centromere identity in higher vertebrates, and we hypothesized that there are other components involved in centromere function.

We are studying vertebrate centromere proteins with a DT40 cell line, which is a chicken B cell line and exhibits targeted integration with high efficiencies that are orders of magnitude higher than those of observed in mammalian cells. Systems for conditional gene disruption are established and DT40 cells exhibit invariant character in both karyotype and phenotype during extended periods of cell culture. Because of the relative tractability of these cells, DT40 cells are widely used for generation of conditional mutants.

In *Schizosaccharomyces pombe*, CENP-A requires functional Mis6 for the localization to the centromere. However, Ctf3p, the *Saccharomyces cerevisiae* homolog of Mis6, is not required for loading of a CENP-A homolog, Cse4p, into the centromere. Therefore, I attempted to isolate a functional vertebrate homolog of Mis6 to understand the establishment of centromere identity in higher vertebrate cells. In this thesis, I isolated a chicken homolog of Mis6 from DT40 cells. The isolated cDNA encodes 753 amino acids and 25% amino acid identity with *S. pombe* Mis6.

To determine the subcellular localization of the chicken Mis6, I constructed a plasmid encoding green fluorescent protein (GFP) tagged to the C terminus of the chicken Mis6 and expressed Mis6-GFP in DT40 cells. Simultaneous colocalization experiments with chicken Mis6-GFP and antibodies against several centromere proteins indicated that the chicken Mis6 is a constitutive component of the centromere that colocalizes with CENP-A, -C, and -H throughout the cell cycle in DT40 cells. Therefore, I have designated the chicken Mis6 centromere protein I (CENP-I).

To determine the precise function of CENP-I, conditional loss-of-function mutants of CENP-I controlled by a tetracycline (tet)-repressible promoter were generated. Western blot analysis showed a steady reduction in CENP-I protein with time in mutant cells incubated with tet. CENP-I protein was not detected by 12 h after addition of tet, indicating that CENP-I is actively turned over. Mutant cells stopped proliferating and went into apoptotic death after approximately 2.5 cell cycles in the presence of tet, indicating that CENP-I was essential for cell viability. Fluorescence-activated cell sorting (FACS) analysis showed that CENP-I-deficient cells began to accumulate in G2/M phase 24 h after the addition of tet and reached 60% at 36 h. Cells with 8N DNA content were observed at 48 and 60 h, and degradation of chromosomal DNA due to massive cell death was observed between 72 and 96 h. The mitotic index determined by cytological analysis increased at 24 h and reached 40% at 48 h after the addition of tet. No anaphase cells were observed at 48 h, indicating that CENP-I-deficient cells accumulate in M phase rather than G2 phase and that they delay prior to anaphase. In large populations of prometaphase-delayed cells, some chromosomes were highly condensed and appeared misaligned on the metaphase plate. I also detected cells with monopolar and multipolar spindles at 48 and 60 h after the addition of tet, and the most of cells underwent apoptotic death at 72 h. In *S. pombe*, mitotic defects in Mis6 mutants occur when cells progress into G1/S phase, suggesting that fission yeast Mis6 acts during G1/S phase. I then examined whether the mitotic defects in CENP-I-deficient DT40 cells occur during progression into G1/S. FACS analysis showed that CENP-I-deficient cells were delayed at M phase after passage through G1, but mitotic delay did not occur in cells that did not pass through G1, even at the same time point after addition of tet, indicating that G1 passage in CENP-I-deficient vertebrate cells, like Mis6 mutants of *S. pombe*, is required for mitotic delay.

FISH analysis showed highly condensed and abnormal numbers of painted chromosomes in CENP-I-deficient cells in the presence of tet. The proportion of these aneuploid cells gradually increased 24 h after the addition of tet, suggesting that CENP-I-deficiency induces chromosome missegregation due to a mitotic defect.

To visualize the aberrant chromosome behavior and microtubule movements in living cells, the human α-tubulin gene fused with GFP was integrated into the genome of conditional loss-of-

function mutant cells of CENP-I. Mutant cells expressing GFP-α-tubulin were stained with Hoechst 33342 and observed microscopically at 37°C. CENP-I-deficient cells delayed in prometaphase for 800 min. After prolonged delay, cells proceeded through the cell cycle without normal cell division and underwent apoptosis. These observations were consistent with data from FACS analysis in which cells with 8N DNA contents were observed between 48 and 60 h after addition of tet, and degradation of chromosomal DNA was observed between 72 and 96 h.

Immunofluorescence analysis of CENP-I-deficient cells showed that signals of BubR1 checkpoint protein were detected on some metaphase cells at 48 h after the addition of tet. However, the signal intensity became weak by 60 h, and weak or no signals were detected at 72 h after the addition of tet, suggesting that association of the checkpoint signaling proteins with the kinetochore structure is lost by 72 h.

To understand the role of CENP-I in formation of the prekinetochore structure, I performed immunofluorescence studies of a CENP-I-deficient cells. CENP-A antibody gave positive signals on interphase nuclei of CENP-I-deficient cells. However, the normal prominent, discrete CENP-C and CENP-H signals were absent. These results combined with our previous data suggested that CENP-I is necessary for localization of CENP-H and CENP-C but not CENP-A. CENP-I localization was lost in CENP-H-deficient cells, suggesting that CENP-I and CENP-H are interdependent for targeting to the prekinetochore structure and that both are necessary for CENP-C localization to centromeres. The data indicate that CENP-I plays an essential role in centromere assembly and function in vertebrate cells.

Contents

Chapter 1

Introduction

1.1 Cell division

Cells arise from other cells by cell division. The chromosomal complements of daughter cells are usually identical to that of the parental cell. These phenomena were first observed by light microscopy in the nineteenth century. In 1838, Schleiden and Schwann proposed the cell theory, and it was then discovered that cells usually contain a nucleus, which is surrounded by the nuclear membrane. In 1879, Fleming observed each nucleus enclosed a fixed number of chromosomes, and that each chromosome replicates to form two identical chromosomes before cell division (Wilson, 1925). One of each pair of daughter chromosomes moves into each daughter nucleus in a process termed mitosis. Mitosis has classically been divided into interphase (comprising G1 or Gap 1 phase, S or DNA-synthesis phase, and G2 or Gap 2 phase), prophase, prometaphase, metaphase, anaphase A and B, telophase, and cytokinesis (Choo, 1997). The first division of meiosis generally comprises stages and events similar to those of mitosis, with the obvious exception of an extended prophase that includes a period in which homologous chromosomes pair and undergo recombination.

While the detailed aspects of the various mitotic and meiotic phases may differ between organisms, three processes are shared universally (Koshland, 1994; Fuller, 1995). First, specialized structures necessary to mediate microtubule-dependent movements must be assembled. These structures include the spindle and the kinetochore. Second, the sister chromatids (in mitosis) or paternal and maternal homologues (in meiosis), assisted by correct pairing, must attach to microtubules extending from the opposite poles of the spindles. Third, the chromosomes unpair and then segregate toward their respective poles. In higher eukaryotes and fission yeast, chromosomes exist in a highly extended linear form during interphase, but they condense into much more compact bodies during prophase. In budding yeast, there is no visible chromosome condensation during mitosis, and there does not appear to be a G2 phase.

1.2 The spindle and microtubules

The spindle is a microtubule-based, bipolar structure. Microtubules are rigid polar polymers of α ß-tubulin dimmers and are highly dynamic and switch stochastically between phases of slow minus end change and fast plus end change (Hyman and Karsenti, 1996; Cassimeris, 1999; Wittmann et al., 2001; Heald and Nogales, 2002). This non-equilibrium behavior, known as

dynamic instability, is characterized by polymerization and depolymerization of the microtubule ends due to building and hydrolysis of GTP. The dynamics are further influenced by microtubule-stabilizing and -destabilizing factors, which function both spatially and temporally to generate different microtubule assemblies during the cell cycle. The primary roll of the mitotic spindle is to segregate chromosomes such that each complement of a replicated chromosome ends up at opposite spindle poles. Some factors, such as motor proteins CENP-E and Dynein in higher eukaryotes, act at kinetochores for chromosome movement (He et al., 2001; Howell et al., 2001). The mitotic spindle has at least three morphologically distinct sub-populations of microtubules: kinetochore microtubules, interpolar microtubules, and astral microtubules. Kinetochore microtubules interact with the outer region of the kinetochore and connect chromosomes to the spindle pole. Bipolar attachment of kinetochore microtubules to each chromosome occurs a single in the budding yeast *Saccharomyces cerevisiae* and up to 30 in mammals (Ding et al., 1993; Nicklas, 1997). Interpolar microtubules associate with chromosome arms and stabilize the bipolar nature of the spindle. Astral microtubules associate with centrosomes (the equivalent of spindle pole bodies in yeast), which appears to be important for spindle positioning in mammalian and yeast cells (Doxsey, 2001)

1.3 Cohesin and the spindle checkpoint

Sister chromatids remain connected to one another from S phase until the onset of anaphase. Cohesion between sister chromatids enables the cell to maintain the identity of chromatids as sister and possibly to align kinetochores of sister chromatids in a way that facilitates binding of microtubules from opposite spindle poles. In addition, sister chromatid cohesion counteracts the pulling forces of the spindle, thereby generating tension across the kinetochores and signaling the formation of stable bipolar attachments. Sister chromatid cohesion is mediated by an-evolutionarily conserved multi-subunit complex called cohesin (Natsmyth et al., 2000; Cohen-Fix, 2001). In budding yeasts, cohesin contains the Scc1/Mcd1, Scc3, Smc1, and Smc3 proteins during mitosis, but Rec8 replaces Scc1/Mcd1 in cells undergoing meiosis. In vertebrates, two cohesin complexes exist that differ in their Scc-3-like subunits. Cohesin binds to specific chromosomal loci (including centoromeres) for much of interphase, but it only establishes cohesion between sister chromatids during DNA replication.

Equally important to the establishment of cohesion is its subsequent dissolution. Scc1/Mcd1 is cleaved by a cysteine endopeptidase called separin or separase, and this cleavage at centromeres is required for the onset of anaphase (Amon, 2001). Separase activity and Scc1 cleavage must be tightly regulated to avoid premature initiation of anaphase. One of the regulators of this process is securin, which forms a complex with separase and inhibits its

activity. In addition, inhibitory phosphorylation of separase may account for the apparent normal separation of chromatids, and it is independent of securin activity (Stemmann et al., 2001). At the onset of anaphase, securin is degraded by ubiquitin-dependent proteolysis mediated by the anaphase-promoting complex (APC). APC-dependent proteolysis is important for separation of sister chromatid at the metaphase-anaphase transition and it participates in triggering the exit from mitosis by degrading mitotic cyclines. APC must also be regulated to prevent premature chromosome segregation. A surveillance system known as the spindle checkpoint stops cell cycle progression prior to sister chromatid separation triggered by APC (Amon, 1999). The spindle checkpoint can sense a multitude of spindle defects, ranging from the presence of a single unattached kinetochore to massive defects induced by a microtubule-depolymerizing drug such as nocodazole. Components of the spindle checkpoint, Mad1, Mad2, Mad3, Bub1, and Bub3, are highly conserved (Shah and Cleveland, 2000; Hoyat, 2001; Luo et al., 2002). The mammalian homologue of Mad3 is BubR1. Both localize specially to microtubule-free kinetochores during spindle assembly in normal cells and bind directly to Cdc20, an activator of APC, to inhibit of APC activity.

1.4 The centromere

The kinetochore is a complex of centromere DNA and associated proteins (Kitagawa and Hieter, 2001) and the site for interaction with kinetochore microtubules. Bipolar attachment of microtubules to kinetochores is essential for proper chromosome segregation and is sensed by the spindle checkpoint. The centromere is required for accurate segregation of chromosomes during mitosis and meiosis in eukaryotes. The centromere is not only the site of kinetochore formation, it is also directly involved in sister chromatid cohesion and separation, chromosome movement, and mitotic checkpoint control (Choo, 1997). Although problems in chromosome segregation can lead to aneuploidy, cancer, and cell death, the mechanism by which centromeres interact with microtubules of the spindle apparatus during cell division is not fully understood in any organism. Most eukaryotes have monocentric chromosomes, and kinetochores form on specific, limited regions of the chromosomes. However, organisms such as nematodes and crayfish have holocentric chromosomes, and the kinetochore forms along the entire length of the chromosomes, organisms such as centipedes have both types of chromosomes in the same nucleus (Pimpinelli and Goday, 1989; Sullivan et al., 2001). Centromeres are typically surrounded by or embedded in heterochromatin, a repeat-rich, gene-poor region of the genome that normally represses transcription of euchromatic genes. Although some functional and structural characteristics of centromeres are conserved, no specific DNA sequence or length

seems to be necessary or sufficient for centromeric function, it is presently unclear how centromere identity and propagation are determined (Tyler-Smith and Florida, 2000).

1.5 The centromere of the budding yeast *Saccharomyces cerevisiae*

Saccharomyces cerevisiae has proved to be a powerful experimental tool for the study of the eukaryotic centromere because it is relatively easy to manipulate genetically, molecularly, and biochemically. The genome of *S. cerevisiae* is divided among 16 chromosomes ranging in size from 260 kb to 3 Mb (Carle and Olson, 1985; Hyman, 1995). The total genome sequence of *S. cerevisiae* was determined (Goffeau et al., 1997). Centromeres of *S. cerevisiae* consist of an essential, 125-bp sequence that is sufficient for full centromere activity during both mitosis and meiosis (Cottarel et al., 1989). Introduction of functional centromere (*CEN*) to an *ARS* plasmid, which contains a functional replicator, provides an active mechanism for the segregation of the resulting minichromosome (Szostak and Blackburn, 1984). The sequence contains three elements: CDEI, II, and III. Single point mutations in CDEIII abolish centromere function during mitosis, indicating that CDEIII is essential for centromere activity (Murphy et al., 1991). CDEI is a short 8-bp DNA that contains a 6-bp palindrome extending from position 3 to 8. CDEI is the binding site for the chromatin protein Cbf1, which is involved in both kinetochore formation and transcriptional regulation (Wieland et al., 2001). CDEII, which is not conserved among chromosomes, is a 78 to 86-bp sequence with a high AT content. CDEII is thought to interact with Cse4, a histone H3 variant of *S. cerevisiae*, and it likely contributes to the structure of the kinetochore (Stoler et al., 1995). CDEIII is a highly conserved and imperfect palindrome with a 25-bp conserved core sequence that binds the CBF3 complex to the right. CBF3 contains four essential proteins, Ndc10, Cep3, Ctf13, and Skp1 that are encoded by genes identified previously through mutational studies (Lechner and Carbon, 1991; Doheny et al., 1993; Pidoux and Allshire, 2000). Many other proteins also interact with CBF3 or centromere DNA, and a growing number of these proteins have been identified through mutants, including the chromosome transmission fidelity *(ctf)*, chromosome loss *(chl)*, chromosome instability *(cin)*, chromosome segregation *(cse)*, minichromosome maintenance *(mcm)*, nuclear division cycle *(ndc)*, mitotic fidelity *(mif)*, and cell division cycle *(cdc)* mutants. These mutants possibly reflect abnormalities in novel kinetochore proteins that remain to be identified (Gardner et al., 2001; Measday et al., 2002).

Recently, the development of sensitive assays for detecting kinetochore association has allowed the identification of > 30 kinetochore proteins in *S. cerevisiae*. The organization of many yeast kinetochore proteins has been more completely established, due in a large part of to the identification of biochemically distinct subcomplexes within the kinetochore (Cheeseman et al. 2002a). The subcomplexes include Dam1 complex, Ndc80 complex, Ctf19 complex, and Ipl1

complex (Janke et al., 2002; Li et al., 2002; Wigge and Kilmartin, 2001; Cheeseman et al. 2002b), addition to Cse4 complex, CBF1 complex, and CBF3 complex.

1.6 The centromere of *Schizosaccharomyces pombe*

Like the budding yeast, fission yeast is an excellent model analysis of the eukaryotic cell division cycle. Mutants of *S. pombe* have been identified for various steps in the cell cycle that result in defective mitosis. In comparison to centromere DNAs of *S. cerevisiae*, centromere DNAs of *S. pombe* are significantly larger, and the mitotic cell cycle in *S. pombe* is more similar to that of higher eukaryotes. The genome of *S. pombe* has three chromosomes and total genome sequence was determined; their centromere DNAs (cen1, cen2, and cen3) ranges in size from 35 to 110 kb (Nakaseko et al., 1986; Chikashige et al., 1989; Pidoux and Allshire, 2000; Wood et al., 2002). Artificial linear or circular chromosomes containing these sequences segregate normally in both mitosis and meiosis (Hahnenberger et al., 1989; 1991; Clark and Baum, 1990; Matsumoto et al., 1990). Each centromere consists of a central 15-kb region that contains low copy number chromosome-specific sequences and 20 to 100 kb of surrounding sequences containing the highly repetitive motifs common to all chromosomes. The central region consists of an asymmetric inner sequence flanked by inverted repeats that are identical to each other. The central region is essential but not sufficient for centromere functions a portion of the outer repetitive region is also required (Takahashi et al, 1992). The larger outer region is necessary to ensure correct meiotic behavior. Screening of fission yeast mutants that have high rates of minichromosome loss at the permissive or semi-permissive temperature *(mis* mutants*)* has led to identification of proteins such as Mis6 and Mis12 that bind specifically to the central region of the centromere (Takahashi et al., 1994; Saitoh et al., 1997; Goshima et al., 1999). The outer region is associated with Chp1 and Swi6 (the homologue of mammalian and fly heterochromatin protein 1, HP1), and this association is dependent on Rik1 and Crl4 (homologue of fly and mammalian Su(var)39). Evolutionarily conserved centromere-specific proteins such as CENP-A and CENP-C are also present in the fission yeast centromere, and the centromere is also hypoacetylated on histones similar to heterochromatic regions in vertebrates (Takahashi et al., 2000; Pidoux and Allshire, 2000).

1.7 The centromere of vertebrates

In traditional electron micrographs, the kinetochore of higher eukaryotes resembles a trilaminar button-like structure that consists of the inner plate, outer plate, and inter zone (Fig. 1.1; Craig et al., 1999). Only the inner plate contains both proteins and DNA, whereas the other two plates appear to be composed largely of proteins (Cooke et al., 1993). Centromeric heterochromatin is

the region of a mitotic chromosome that remains condensed during interphase of the cell cycle. Constitutive heterochromatin is associated with relatively short DNA sequences that are highly repeated in long arrays, commonly referred to as satellite DNA. The primary repetitive DNA element of the human centromere is alpha-satellite DNA, which consists of a 171-bp monomer repeated for stretches100-kb to several megabases (Sullivan et al., 2001). The entire repetitive DNA array is unlikely to be involved in kinetochore assembly because antibodies to kinetochore proteins localize to only a portion of the repetitive array (Warburton et al., 1997; Spence et al., 2002). In addition, chromosomes where this DNA array has been naturally or artificially deleted can still assemble a kinetochore and segregate normally (du Sart et al., 1997; Yang et al., 2000). Controversy regarding the composition of vertebrate centromeric DNA has hindered identification of vertebrate centromere-binding proteins with biochemical methods. Centromere proteins in vertebrate cells have been identified primarily by immunological methods. Antibodies against centromere proteins have been isolated from patients with autoimmune diseases and from immunized animals (Compton et al., 1991), and this has led to the identification of several centromere-associated proteins known as CENPs (Moroi et al., 1980; Earnshaw and Rothfield, 1985; Pluta et al., 1995; Craig et al., 1999; Dobie et al., 1999; Choo, 2000; Tyler-Smith and Floridia, 2000; Sullivan, 2001; Sullivan et al., 2001). In addition, several centromere proteins in vertebrate cells have been identified as the homologues of CENPs in yeast or fly. For example, the vertebrate homologues of the checkpoint proteins localize to the centromere prior to association of the centromere with the spindle (Chen et al., 1996; Taylor and McKeon, 1997; Taylor et al., 1998; Amon, 1999). The *Drosophila melanogaster* mitotic checkpoint protein ZW10 has no known yeast homologue in but is conserved in vertebrates and *Caenorhabditis elegans* (Starr et al., 1997; Chan et al., 2000).

1.8 Constitutive centromere-associated proteins (CENPs)

Of the centromere proteins in vertebrate cells, CENP-A, -B, and -C are known to bind to the centromere constitutively throughout the cell cycle (Choo, 1997). CENP-A and CENP-C are thought to be important for the establishment of kinetochore function because they are essential for cell viabilities and their functional homologues are present in yeast to human species. CENP-A, a variant of histone H3, is present in budding and fission yeasts (Stoler et al., 1995; Meluh et al., 1998; Takahashi et al., 2000), *C. elegans* (Buchwitz et al., 1999), *D. melanogaster* (Henikoff et al., 2000; Blower and Karpen, 2001), and vertebrates (Palmer and Margolis, 1987; Shelby et al., 1997; Howman et al., 2000). Immunolocalization of CENP-A suggests a distinct nucleosome structure at the inner kinetochore plate of active centromeres (Vafa and Sullivan, 1997; Warburton et al., 1997). Nucleosomes can be assembled *in vitro* from purified CENP-A and

from histones H2A, H2B, and H4 (Yoda et al., 2000). Data from analyses of CENP-A knockout mice suggest that CENP-A plays a role in early organization of the centromeric chromatin during interphase (Howman et al., 2000). Therefore, it seems likely that a centromeric nucleosome containing a specific variant of H3 is a fundamental feature of eukaryotic kinetochore organization (Palmer and Margolis, 1987).

CENP-B binds to the 17-bp DNA motif known as the CENP-B box, which is present in human α-satellite and mouse minor satellite DNAs (Masumoto et al., 1989). CENP-B forms a complex with CENP-A and CENP-C through interactions with DNA (Ando et al., 2002); however, the precise function of CENP-B remains unclear because it binds to both active and non-active centromeres. CENP-B does not bind the Y chromosome in humans (Pluta et al., 1995), and CENP-B knockout mice are viable and appear to have normal centromeres (Kapoor et al., 1998; Perez-Castro et al., 1998; Hudson et al., 1998).

CENP-C, which is evolutionarily conserved, is also an important component in kinetochore function (Saitoh et al., 1992; Meluh and Koshland, 1995; Fukagawa and Brown, 1997; Kalitsis et al., 1998; Pidoux and Allshire, 2000; Moore and Roth, 2001). Earnshaw and colleagues originally used auto-antisera to identify CENP-C as a constitutive human centromere protein and cloned CENP-C gene in 1992 (Saitoh et al., 1992). They showed that CENP-C localized to the inner kinetochore plate with an immunoelectron microscopy. Mif2, which CENP-C homologue in *S. cerevisiae*, is an essential centromere protein and interacts with the CDE II element of the yeast centromere (Brown et al., 1993; Meluh and Koshland, 1995). Vertebrate CENP-C has been localized to the inner kinetochore plates adjacent to the centromeric DNA, and it is known to bind DNA directly (Saitoh et al., 1992; Yang et al., 1996). Inactivation of CENP-C causes in cell death due to kinetochore disruption, chromosome missegregation, and metaphase delay (Fukagawa and Brown, 1997; Kalitsis et al., 1998; Fukagawa et al., 1999a). Like CENP-A, CENP-C is found only at active centromeres and is needed to induce formation of a functional centromere (Earnshaw et al., 1989; Sullivan et al., 1995; Fukagawa et al., 1999).

CENP-H was initially identified as a component of the mouse centromere and constitutively localized in kinetochores throughout the cell cycle (Sugata et al., 1999). Human CENP-H colocalizes with CENP-A and CENP-C, and is found in neocentromeres, but not at inactive centromeres in stable dicentric chromosomes (Sugata et al., 2000). Although these localization data suggest that CENP-H may be a fundamental component of active centromere complexes, its precise function was unclear. Fukagawa and colleagues found that CENP-H is required for centromere targeting of CENP-C but not CENP-A and plays an important role mitotic cell division in vertebrate cells (Fukagawa et al., 2001). They also showed that inactivation of chicken CENP-H caused cell death, metaphase delay with kinetochore disruption, and

chromosome missegregation (Fukagawa et al., 2001). CENP-H homologues are found in chicken, mice, and humans, but not yeast, *C. elegans*, or *D. melanogaster*.

CENP-A, -C, and -H play important roles in the establishment of centromere identity in cells of higher vertebrates, and we hypothesized that there are other proteins involved in centromere function. Therefore, one of the goals of our research is to identify and characterize potential centromere components so as to clarify the pathway of vertebrate kinetochore assembly.

1.9 A chicken B cell line, DT40

The chicken B cell line, DT40, transformed with an avian leukosis virus (Baba et al., 1985), continuously undergoes the immunoglobulin (Ig) gene conversion, diversification of the Ig variable (IgV) gene through homologous DNA recombination (HR) (Reynaud et al., 1985; Reynaud et al., 1987), during in vitro culture (Buerstedde et al., 1990). Remarkably, targeted integration occurs at essentially all loci in these cells with efficiencies that are orders of magnitude higher than those observed in mammalian cells (Buerstedde et al., 1991). Efficient gene targeting appears to reflect an intrinsic character of chicken B lymphocytes because this extraordinary capability is observed in most chicken B lymphocyte lines analyzed. The molecular mechanism responsible for the high targeting efficiencies in chicken B lymphocyte line is not clear. Presumably, a mechanism that is involved in the Ig gene conversion also enhances gene targeting efficiencies, because both processes are mediated by HR and observed only chicken B lymphocyte lines.

The chicken karyotype consists of 39 chromosomes, which include 38 autosomal chromosome pairs and the Z/W sex chromosomes. Chromosomes 1-6 are considered macrochromosomes and are relatively AT-rich and (CA)n microsatellite repeat-rich. Chromosomes 7-39 are classified as microchromosomes and contain GC-rich DNA, are enriched for repetitive sequences, and contain heterochromatin as determined by C banding (Mcqueen et al., 1998). It has been suggested that the structural organization of the human genome is closer to that of chicken than that of mouse (Burt et al., 1999).

Besides efficient gene targeting, DT40 cells possess a number of advantages as a tool for reverse genetic studies. First, DT40 cells exhibit a relatively invariant character, both karyotype and phenotype, during extended periods of cell culture. This invariant character makes it possible to sequentially target multiple genes, employing seven selectable marker genes (neo, his, bsr, hyg, puro, ecogypt, and zeocin). Second, the rapid growth rate of DT40 cells, with a doubling time of 8-12 hours, allows for the performance of colony survival assays without changing culture media. Third, since cloning efficiency of wild-type cells is nearly 100%, isolation of stably transfected cells as well as subcloning of cells is easy done. Forth, the absence of

functional p53, which induces apoptosis and cell cycle arrest upon DNA damage, allows us to isolate gene disrupted clones that exhibit genome instability (Takano et al., 1999). Lastly, a new EST database of nearly 312,000 chicken ESTs from multiple tissues and several developmental stages is now available, greatly facilitating identification of chicken genes (Hudson et al., 2002).

DT40 is widely used for studies of cell cycle including chromosome segregation (Buerstedde and Takeda, 1991; Fukagawa et al., 1999a, 1999b, 2001; Sonoda et al., 2001; Sudo et al., 2001). It has been difficult to apply genetic analyses to vertebrate centromeres because of the lack of suitable assays for mutations that affect the accuracy of chromosome segregation. Therefore, centromere proteins in vertebrate cells have been identified by immunological methods or as homologues of centromere-associated proteins in yeasts or fly. DT40 cells enable the genetic analysis of cell-autonomous functions in vertebrate. In this thesis, I describe the characterization of a novel component of vertebrate centromeres with DT40 cells.

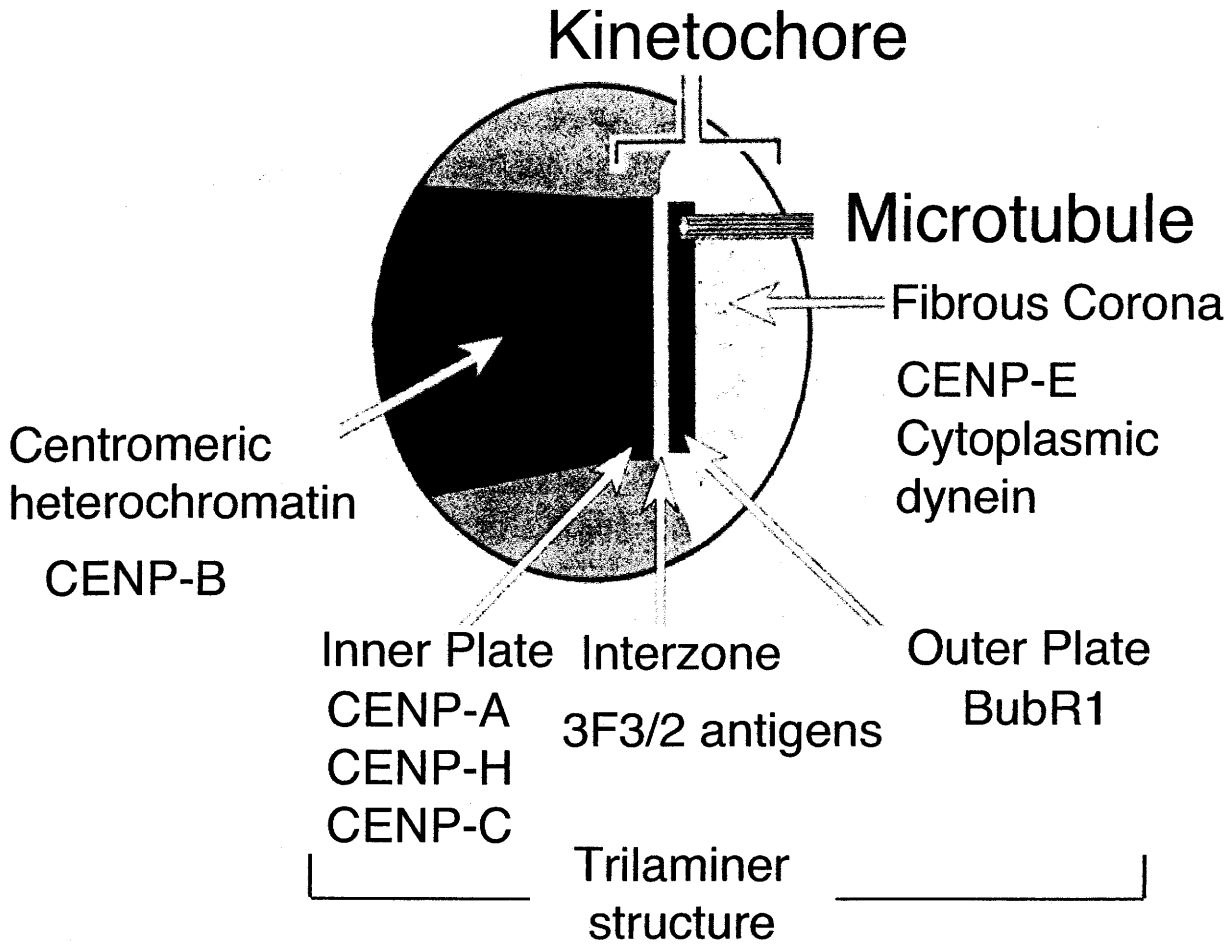


Fig. 1.1 Organization of the mammalian centromere

The condensed centromeric heterochromatin, which functions as a structural support for the kinetochore and which may have a role in regulating sister chromatid cohesion, is rich in α-satellite DNA and its binding protein CENP-B. The inner kinetochore plate, which has an essential role in kinetochore assembly, contains the outermost DNA present in the kinetochore as well as CENPs, CENP-A, C, and H. The outer kinetochore plate, which may be both a microtubule binding and a mitotic checkpoint signaling structure, contains such as the cell cycle signaling kinase homologue BubR1. The interzone between the two plates contains phosphoantigens recognized by the 3F3/2 monoclonal antibody. These phosphoantigens, which are detected only on chromosomes that have not yet achieved a bipolar spindle attachment, are thought to reflect the activity of the mitotic checkpoint kinases (Campbell and Gorbsky, 1995). The fibrous corona contains microtubule motor proteins, including kinesin CENP-E and cytoplasmic dynein.

Chapter 2

Results

2.1 Generation of a DT40 cell line that expresses CENP-H-GFP

It is difficult to stain centromres by an immunofluorescence. Therefore, it is very useful to generate a cell line in which centromeres are marked. Then I generated a DT40 cell line that expressed CENP-H-GFP. CENP-H was discovered as a constitutive component of the centromere that co-localizes with CENP-A and CENP-C throughout the cell cycle (Sugata et al., 1999; 2000), It would be useful for centromere study in DT40 cells if a cell line that expresses CENP-H-GFP. I used a chicken CENP-H cDNA to isolate genomic clones that covered the entire CENP-H locus. Limited sequencing revealed the genomic organization of the CENP-H gene, which spans approximately 7-kb and contains 9 exons (Fig. 2.1A). Southern blot analysis of chicken genomic DNA showed a single band (Fukagawa et al., 2001), indicating that there is a single CENP-H gene in the chicken genome. DT40 cells are very useful for analyses of CENP-H functions because the CENP-H gene is a single allele and the native promoter regulates a replaced gene by a target event. I constructed a plasmid which green fluorescent protein (GFP) was tagged to the C terminus of the chicken CENP-H cDNA (Cenp-H-GFP plasmid). In the construct, the start codon of GFP was mutated so that GFP expression would be dependent on translation of the upstream CENP-H sequences. This ensured that GFP alone was not expressed as a result of internal translation initiation. The fused gene was integrated in a DT40 cell line; thus, the single CENP-H gene was replaced with a gene encoding CENP-H-GFP. The replacement-targeting strategy is illustrated in Fig. 2.1A. The replacement-targeting construct was transfected into wild-type DT40 cells, and G418-resistant clones were screened by Southern hybridization of BamHI digests with a probe located beyond the 5'-end of the targeting construct. Successful targeting was expected to replace the 12-kb cognate fragment with a 3.5-kb fragment (Fig. 2.1A). I identified several clones in which CENP-H was replaced by CENP-H-GFP (Fig. 2.1B). Because these cell line proliferates like wild-type DT40, the CENP-H-GFP fusion protein appears to be functionally identical to CENP-H protein.

I used these cell line for immunostaining with anti-CENP-C antibody. CENP-C was used as a centromeric marker because it localizes to centromeres throughout the cell cycle (Saitoh et al., 1992; Fukagawa et al., 1999a). CENP-H-GFP signals and anti-CENP-C signals throughout the cell cycle are shown in Figure 2.1C. During interphase, CENP-H-GFP was co-localized with CENP-C as discrete signals in the nucleus. Co-localization of CENP-H-GFP with CENP-C was

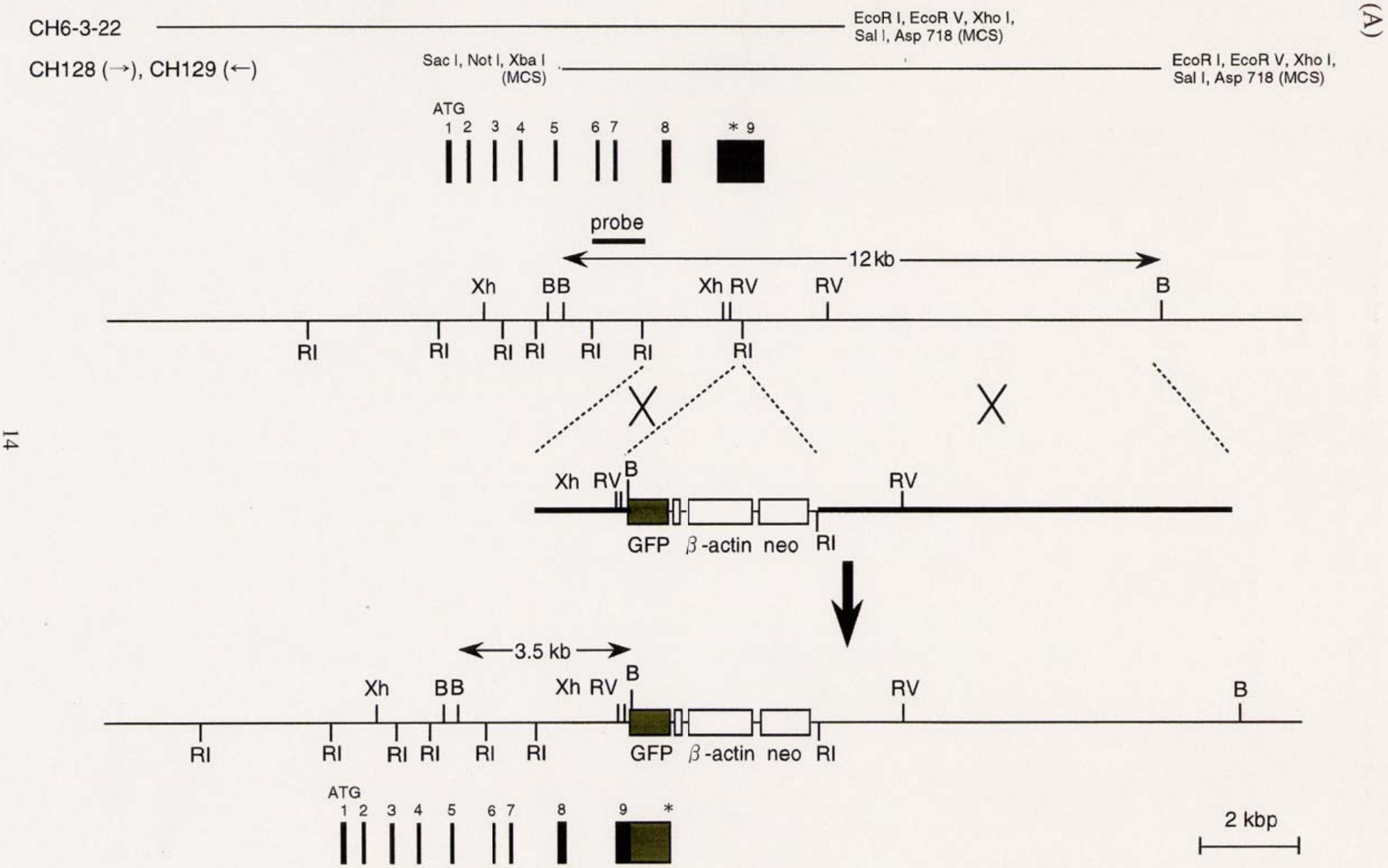
also observed during prophase, metaphase, and anaphase of mitosis. This cell line that express CENP-H-GFP is useful for centromere studies (Sonoda et al., 2001; Spence et al., 2002).

Fig. 2.1 Chicken CENP-H localizes to centromeres throughout the cell cycle

(A) Restriction endonuclease maps of the CENP-H gene, the CENP-H-GFP replacement construct, and the configuration of the targeted locus. Black boxes indicate the positions of exons. Exon 9, which contains the stop codon, was replaced with a portion of exon 9 fused to a GFP tag, the neomycin-resistance gene (neo), and 3' homology region. Relevant restriction enzyme sites shown are as follows: RI, EcoRI; Xh, XhoI; B, BamHI; RV, EcoRV. The position of the probe used for Southern hybridization is indicated. If targeted integration of the replacement construct occurs, a novel 3.5-kb fragment is detected.

(B) Restriction analysis of the CENP-H-GFP allele. Genomic DNAs from wild-type DT40 cells (lane 1) and targeted clones (lanes 2 and 3) were digested with BamHI, size fractionated by 0.7% agarose gel electrophoresis, transferred to a nylon filter and hybridized with the probe indicated in (A). A novel 3.5-kb fragments due to targeted integrations of the constructs, replaced the 12.5-kb wild-type fragments in targeted clones (lanes 2 and 3).

(C) Localization of CENP-H-GFP at progressive stages of the cell cycle in DT40 cells. #5-5 cells carrying the integrated CENP-H-GFP construct were fixed with PFA and stained with anti-CENP-C antibody. Green signals are specific for CENP-H-GFP. CENP-C signals were detected with Cy3-conjugated secondary antibodies (red). Nuclei and chromosomes were visualized by counterstaining with DAPI (blue). The scale bar corresponds to 10 μm. As shown in the merged images, CENP-H-GFP signals are co-localized with CENP-C signals throughout the cell cycle.


CH6-3-22
EcoR I, EcoR V, Xho I,
Sal I, Asp 718 (MCS)
CH128 (→), CH129 (←)
Sac I, Not I, Xba I
(MCS)
EcoR I, EcoR V, Xho I,
Sal I, Asp 718 (MCS)
ATG
1 2 3 4 5 6 7 8 * 9
probe
12 kb
Xh BB Xh RV RV B
RI RI RI RI RI RI RI
Xh RV B RV
GFP β-actin neo RI
3.5 kb
Xh BB Xh RV B RV B
RI RI RI RI RI RI
GFP β-actin neo RI
ATG
1 2 3 4 5 6 7 8 9 *
2 kbp

(A)

(B)


1
2
3
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12.0 kb
9.4 kb
6.6 kb
4.4 kb
3.5 kb
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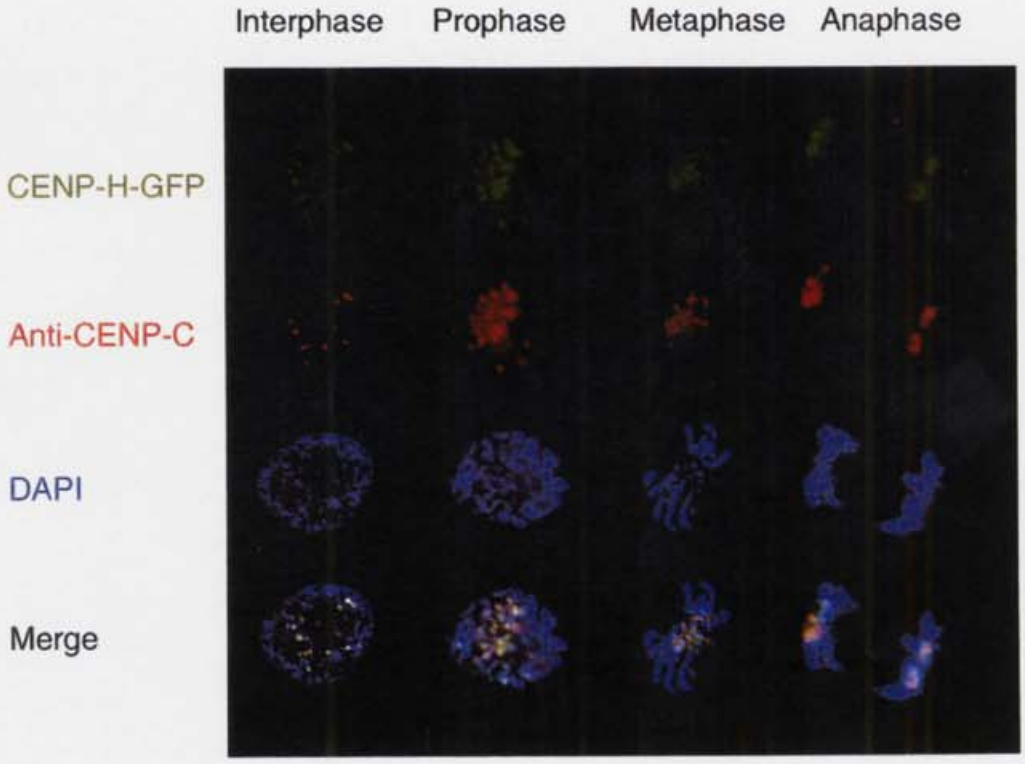
2.0 kb

(C)


Interphase
Prophase
Metaphase
Anaphase
CENP-H-GFP
Anti-CENP-C
DAPI
Merge

2.2 Isolation of a chicken gene homologous to the *S. pombe* Mis6 gene

Mis6 was previously identified as an essential centromere protein in *S. pombe*. A *mis6* mutant shows missegregation of chromosomes with loss of viability at permissive temperature, and Mis6 protein appears to be important for centromere specific chromosome (Saitoh et al., 1997). CENP-A homolog in *S. pombe* requires functional Mis6 for the localization to the inner centromere (Takahashi et al., 2000). A potential vertebrate homolog of Mis6, LRPR1 that is leucine-rich and regulated by follicle-stimulating hormone are found in rats and humans (Slegtenhorst-Eegdeman et al., 1995, 1998). However, the function of this early response protein and its relation to the centromere are unknown. Therefore, I attempted to isolate a chicken homolog of Mis6 to characterize the gene product in DT40 cells.

I used a region highly conserved between the human and rat LRPR1 genes to design degenerate primers for isolation of a short stretch of chicken cDNA by RT-PCR. This RT-PCR product was used as a probe to screen a chicken cDNA library and isolate several cDNA clones that contained the entire coding sequence. The translation initiation site was confirmed by the 5'-RACE methods and the full-length sequence was inserted into pBluescript (Stratagene; Mis6-cDNA plasmid). The cDNA encodes a 753-aa polypeptide with a predicted molecular mass of 86.9 kDa (Fig. 2.2A). The deduced chicken Mis6 protein has 56% amino acid sequence identity with human LRPR1, 52% amino acid identity with rat LRPR1, and 25% amino acid identity with *S. pombe* Mis6 (Fig. 2.2B; detail sequence information for the chicken Mis6 is available at DDBJ/EMBL/NCBI, Accession No. AB073426). Like fission yeast Mis6, the chicken Mis6 is basic (pI, 7.90) and relatively rich (45%) in hydrophobic amino acids. 177G, which is 135G in *S. pombe* Mis6 that is mutation site in *mis6-302* mutant, is also conserved in the chicken Mis6 as rat and human LRP1.

Fig. 2.2 Chicken Mis6 gene and amino acid sequence

(A) Nucleotide sequence of the chicken Mis6 and the predicted amino acid sequence are shown. The chicken Mis6 gene encodes a 753 aa polypeptide with a predicted molecular mass of 86.9 kDa. The stop codon is indicated with an asterisk.

(B) Amino acid sequence similarity among the chicken Mis6, *S. pombe* Mis6, and LRP1 from rat, and human. Identical amino acids are boxed; similar ones are shaded. The deduced amino acid sequence of the chicken Mis6 has 56% identity with human LRPR1, 52% identity with rat LRPR1, and 25% amino acid identity with *S. pombe* Mis6.

(A)

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1     GAATTCTGAAAAGATGCAACGAAGACAGAGTTCTAAGCACTCCAAGCGACCCCTGCAAGT  60
                M  Q  R  R  Q  S  S  K  H  S  K  R  P  L  Q  V
61    TCACCACAGCAATCAGACTGATCTCTCTGCGTGGCGAAAAGGAGGGACAGTTGATACTGA  120
      H  H  S  N  Q  T  D  L  S  A  W  R  K  G  G  T  V  D  T  E
121   AAAAAGTGCCCAGAATCGTCAATCTCTGAGTGATCAGAAAAATGACAACGAGCAAGACTC  180
      K  S  A  Q  N  R  Q  S  L  S  D  Q  K  N  D  N  E  Q  D  S
181   CCTTGAGCAAGCTCTGAGCTACTTTGAGAAAATTCAAGACCGAGTTTCACTGAAAAAGAG  240
      L  E  Q  A  L  S  Y  F  E  K  I  Q  D  R  V  S  L  K  K  S
241   TGAAGTTCTGCAGAAACATTTGTCTACTATGGAAAGCATTGCACTGAAAAGAGGTTTACC  300
      E  V  L  Q  K  H  L  S  T  M  E  S  I  A  L  K  R  G  L  P
301   CCCTGAAGGATTTGATGTATTGCTAGATGTGGCACTCAGTGGCAAACTTGCTGATACAGT  360
      P  E  G  F  D  V  L  L  D  V  A  L  S  G  K  L  A  D  T  V
361   GAATACTCGTTTACTGAAGAGCCTGATCCCAGCCTCAGCAATACCAGAAAGTTCTATTGT  420
      N  T  R  L  L  K  S  L  I  P  A  S  A  I  P  E  S  S  I  V
421   TTCTTCTGTATCTTGGTTCTGTGTCAGCAAATGTTCAAGTAACATCCAGCTGCTTTTTCT  480
      S  S  V  S  W  F  C  V  S  K  C  S  S  N  I  Q  L  L  F  L
481   AAGATGGCTGATCACAATGTTTGACTTCATTGATCACAAGGAACAAGTTCATGCCCTCTA  540
      R  W  L  I  T  M  F  D  F  I  D  H  K  E  Q  V  H  A  L  Y
541   TGGCATCTTCTTTTCCTTCCTGAATGATGAGAAGTTGTGTCCCTACATCTGCCATGTGCT  600
      G  I  F  F  S  F  L  N  D  E  K  L  C  P  Y  I  C  H  V  L
601   CTATCTGCTGACCAGGAAAGAAAATGTCAAGCCTTTTCGGGTCAGGCGACTCCTTGATCT  660
      Y  L  L  T  R  K  E  N  V  K  P  F  R  V  R  R  L  L  D  L
661   CCAATCAAAAATGGGTATGCAACCTCATCTACAGGCTCTGCTATCACTTTATAAGCTTTT  720
      Q  S  K  M  G  M  Q  P  H  L  Q  A  L  L  S  L  Y  K  L  F
721   CTGTCCTGAGCTGGTATCCATAACCCTTCCTCAGAAGATGAAGACTTATTTCAAGAATGC  780
      C  P  E  L  V  S  I  T  L  P  Q  K  M  K  T  Y  F  K  N  A
781   AGACGGCCCTTGGAAAGCAGCAATCAACGCTGTGAGGCAAAGAAACCAGGCCAATTCTAC  840
      D  G  P  W  K  A  A  I  N  A  V  R  Q  R  N  Q  A  N  S  T
841   AGTGCCCCAACCACTGCTTTTAGGCACAGCTCAACCTCACTCACGAAAAAGAAAATGGAA  900
      V  P  Q  P  L  L  L  G  T  A  Q  P  H  S  R  K  R  K  W  N
901   TACCCAGTTGATTGTACCTGCAAGCAGTGCCAACGCACAGAATTTAGTAGTGGGTGGGAA  960
      T  Q  L  I  V  P  A  S  S  A  N  A  Q  N  L  V  V  G  G  K
961   AATGAGCCGTGCTGATTCATACAGTGCTAATGAATCTTTTCCAGTGGAGCAGCTGCGGAC  1020
      M  S  R  A  D  S  Y  S  A  N  E  S  F  P  V  E  Q  L  R  T
1021  CTTTCCCCAACTCCTACAAAACATCCACCGCCTGGAGTTTCCTTCCCAGATGGGCTCAGT  1080
      F  P  Q  L  L  Q  N  I  H  R  L  E  F  P  S  Q  M  G  S  V
1081  GCTAACAAACCCATTATTGCTTCACTACATGAACTGCAGCAAAGATGAATCTGTTTATCT  1140
      L  T  N  P  L  L  L  H  Y  M  N  C  S  K  D  E  S  V  Y  L
1141  GAGGCTCTACTATTGGATGGGACAGACTCTTCAGGAAGAGTGCACCTGGTGTGTAGTTGA  1200
      R  L  Y  Y  W  M  G  Q  T  L  Q  E  E  C  T  W  C  V  V  D
1201  TAATAACCAGTATGAAGAAGAATTCAGAGGCTTCCTGGAAACTGTCTACAAGGCAGAATG  1260
      N  N  Q  Y  E  E  E  F  R  G  F  L  E  T  V  Y  K  A  E  C
1261  CTTCTTGCAGGAGGGATTTCCTTCCTGTGAGGAGTTCCTGTACAGGAGTCTTCCTCTCTG  1320
      F  L  Q  E  G  F  P  S  C  E  E  F  L  Y  R  S  L  P  L  W
1321  GGATGGTGTTTCCTGCCGATCGCAAATCCTCCAACTCGTGAGTTGGATCCCCCTCAGTAC  1380
      D  G  V  S  C  R  S  Q  I  L  Q  L  V  S  W  I  P  L  S  T
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1381  CTTCTCTGAAATGAAGTCACAACTCTGTGATCCCCTGGCACAGCTCTTCTTCACATCGTC  1440
        F   S   E   M   K   S   Q   L   C   D   P   L   A   Q   L   F   F   T   S   S
1441  CCTTTACTTTAAGTGCAGTGTTCTGGAGAGTCTGAAAGAGCTGTTACAGAACTGGTTAAA  1500
        L   Y   F   K   C   S   V   L   E   S   L   K   E   L   L   Q   N   W   L   N
1501  CTGGCACGTGGTTCAGCTGGATTCAGAATCAGATTCTCAATTCAGTTCTTTGAATACCAC  1560
        W   H   V   V   Q   L   D   S   E   S   D   S   Q   F   S   S   L   N   T   T
1561  CCTTTCTGGACTAGTGAATGGAGTGGCCGAACTGATCAACTTTGTGGGACGGATTTCTAC  1620
        L   S   G   L   V   N   G   V   A   E   L   I   N   F   V   G   R   I   S   T
1621  TGCTGCATTGCACTTGGAAAAGAGTCATACCTTCTTGCTGTACTTCATCCTGGATTTCTA  1680
        A   A   L   H   L   E   K   S   H   T   F   L   L   Y   F   I   L   D   F   Y
1681  CGAGACTGTGTGTGACATATATCTGAAGTACAAACTGCCGTTGCTGATAATGCCTCCTGC  1740
        E   T   V   C   D   I   Y   L   K   Y   K   L   P   L   L   I   M   P   P   A
1741  TGGAGTTTTCTACCCAGCATTGCTCAGCATGGATTCTGTCAACTTAAATCAGCTCTGCTA  1800
        G   V   F   Y   P   A   L   L   S   M   D   S   V   N   L   N   Q   L   C   Y
1801  CATCATGTACAGGTATAGAACCAACTTGATAGCTGCAAAAGAGAACGAGATGAGTAAAAA  1860
        I   M   Y   R   Y   R   T   N   L   I   A   A   K   E   N   E   M   S   K   K
1861  GAAAATACAGCAATTCAAGTTCAGTAGCCAGACATATCAAGAGTATAACCAGTACATAAT  1920
        K   I   Q   Q   F   K   F   S   S   Q   T   Y   Q   E   Y   N   Q   Y   I   I
1921  AGCCATGGTGGGTTGTCTGTGGACATCTAGTGCATTCCAGAAAGATAATCATCCTGAGGG  1980
        A   M   V   G   C   L   W   T   S   S   A   F   Q   K   D   N   H   P   E   G
1981  CATTCGTCTGGATGATGAGTTGCTGAAGAAAACTGGAGTGCGGGAATACAAGAACAGCTT  2040
        I   R   L   D   D   E   L   L   K   K   T   G   V   R   E   Y   K   N   S   F
2041  CAATATTGTCTACCACCCAGCTCTGATGTGCTATGCTGTTGACTTCCTGCAGCAGGCCTG  2100
        N   I   V   Y   H   P   A   L   M   C   Y   A   V   D   F   L   Q   Q   A   W
2101  GCCAGATGATACCACCTTCAACTTCAATCTAATTAAGGGAAAGAAGTGGAACTGGTATCT  2160
        P   D   D   T   T   F   N   F   N   L   I   K   G   K   K   W   N   W   Y   L
2161  GAGATATCTCTACGGGCAAGGTTTAGAGGGCCTGAAGCTCTTCATTGAAAGCAGTATCAA  2220
        R   Y   L   Y   G   Q   G   L   E   G   L   K   L   F   I   E   S   S   I   N
2221  TCGTGTTTCCAAGGCCTCTCAAAGTAAAGCAGAGGATGAGGATGAGAAAGTGTGACTCCA  2280
        R   V   S   K   A   S   Q   S   K   A   E   D   E   D   E   K   V   *
2281  GGACTTGTTGGAGCCTTTCTGTCATCTGGAATAGGGAAACAGAAGATTAACAGACTGTGT  2340
2341  CAGTAGTTCTTCCTTCCTCAGAGGAAAGAAGCAGAGCAATTATGCTTGCCTGCATTTTCA  2400
2401  TGCTGGCATCTGTGTTAGGAAAGCAAAAATAGAGAAGGAACGTTTAGGCTGTTTTGGTTC  2460
2461  ACCAGCACTGAATCCCCTTATTTCCTGCTGAACTTTTTTAGCCCGAGGCTGCTGAGCTGA  2520
2521  TGTGATATGTAACATCCCTGTTTTCCATCCTCATTTTAATTTGCTGGAGTATCTGAGGGG  2580
2581  AAAAAACATTCTCCATCTCGAAGCTGCGGTGAGAAGTGCTGCCTCATTTGGAGGACATGC  2640
2641  TCATATGGCCCTCCTCTTTCTTCCCTGACAACTTACACAGTACCGTGAGCAACTGATTTT  2700
2701  GTGCCACACTGGAGGAGTACATATTTCCCAGATCCCTGTTGTGCCATATACACGCTGGCT  2760
2761  CATAACAGGATGGCCAAACTGACCCTCCTGAACTTCGCTTCTGTTACGAGTCTTCCAGAA  2820
2821  GACTTTGTGATACCTTTGCTTATAATGTCCTTGTAATGCAGTTGAAGAACTTTGTGCGCT  2880
2881  GCTGGTTTGCTGGTGACCTTCAACGGCTGAACAGCATTGCCACTGGCTCACCACTTCCTA  2940
2941  TTGAGAAGCTTTCCTTTTTGTGTTTTTAGTTCTATAATGCTGTATATTAAATGTGTACAT  3000
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3061  TTAAAAAAAAAAAAAAAAAAAAACTCGAG  3089
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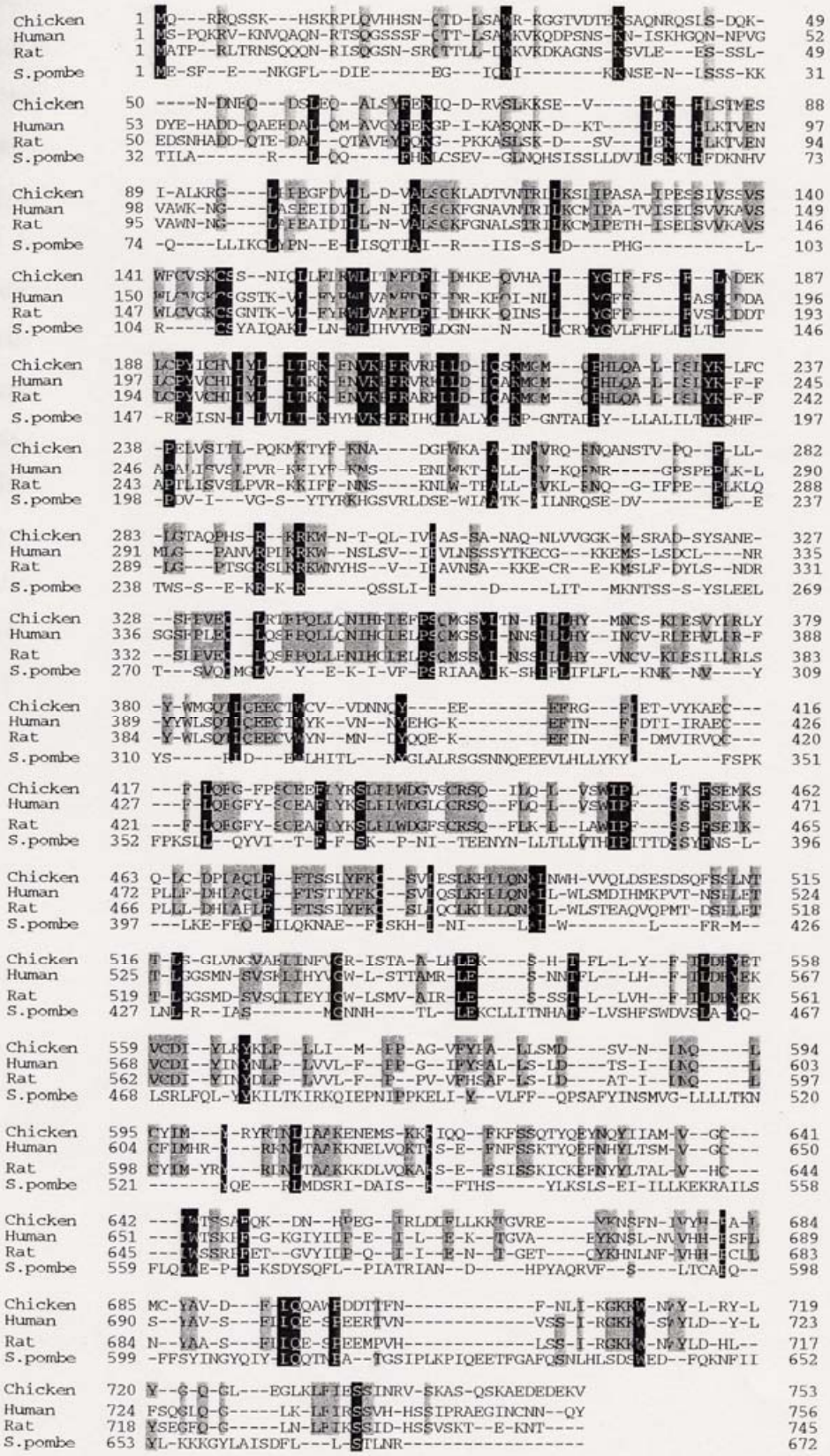
(B)



3 Chicken Mis6 associates with centromeres and is designated as CENP-I

:loned the chicken Mis6, but its localization and relation to the centromere were unknown. To termine the subcellular localization of the chicken Mis6, I constructed a plasmid with GFP gged to the C terminus of the chicken Mis6 (Mis6-GFP plasmid) and integrated and expressed is construct in DT40 cells (Mis6-3rd-GFP cell in Chapter 4). The fused gene was co-nsfected with a plasmid containing a hygromycin (hyg)-resistance cassette or blasticidin last)-resistance cassette to select integrated cells. A portion of selection-resistant clones was ed with paraformaldehyde (PFA) and GFP signals were confirmed by fluorescence icroscopy. Several Mis6-3rd-GFP cell lines were isolated.

The fusion protein was functional and able to fully suppress the phenotype of chicken Mis6 ockout cells (described below). Subcellular localization of chicken Mis6-GFP was observed fluorescence microscopy in combination with immunostaining with anti-CENP-C antibody. ENP-C was used as a centromeric marker because it localizes to centromeres throughout the ll cycle (Saitoh et al., 1992; Fukagawa et al., 1999a). Chicken Mis6-GFP signals and anti-ENP-C signals throughout the cell cycle are shown in Fig. 2.3A. During interphase, Mis6-GFP as co-localized with CENP-C as discrete signals in the nucleus. Co-localization of Mis6-GFP ith CENP-C was also observed during prophase, metaphase, and anaphase of mitosis. I also nerated a polyclonal antibody against the central region of chicken Mis6 and examined the stribution of this protein in a cell line in which the CENP-H gene had been replaced with a ne encoding CENP-H-GFP (described in 2.1). CENP-H-GFP signals also co-localized with ti-Mis6 signals throughout the cell cycle (Fig. 2.3B). Our results indicate that the chicken Mis6 localized to the centromere throughout the cell cycle in vertebrate cells. Therefore, I have signated the chicken Mis6 centromere protein I (CENP-I).

CENP-I-GFP signals on metaphase chromosomes in DT40 cells showed a typical paired-dot ttern at every centromere, but detailed localization of CENP-I-GFP with respect to the ntromere was unknown. To determine the precise localization of CENP-I on metaphase romosomes, I stained CENP-I-GFP-marked chromosomes with anti-BubR1 and anti-ZW10 tibodies. BubR1 is localized in the outer kinetochore plate (Jablonski et al., 1998), and ZW10 pears to be localized in the fibrous corona (Starr et al., 1998). Our results indicate that CENP-I located internal to BubR1 and ZW10 (Fig. 2.3C and D). These data together with our finding at CENP-C and CENP-I-GFP colocalize (Fig. 2.3E) suggest that CENP-I is localized in or near e inner kinetochore plate.

Fig. 2.3 Chicken CENP-I localizes to centromeres throughout the cell cycle and is located in or near the inner kinetochore

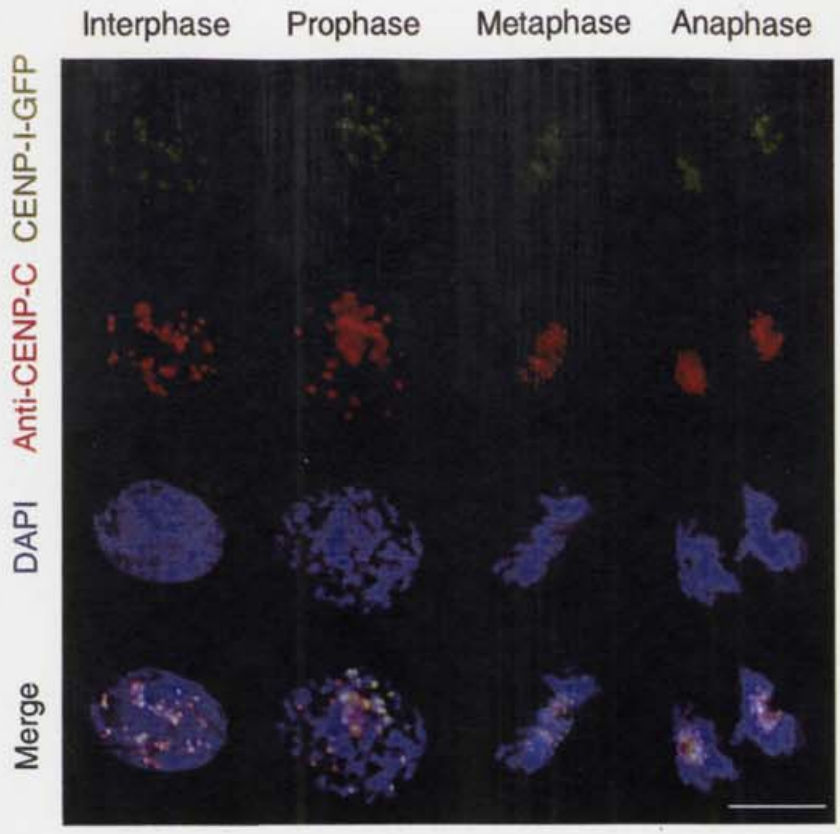
(A) Localization of CENP-I-GFP at various stages of the cell cycle in DT40 cells. Cells were fixed with PFA and stained with anti-CENP-C antibody. Green signals are specific for CENP-I-GFP. Immunoreaction with CENP-C was detected with Cy3-conjugated secondary antibody (red). Nuclei and chromosomes were visualized by counterstaining with DAPI (blue). The scale bar corresponds to 10 μm. As shown in the merged images, CENP-I-GFP signals are colocalized with CENP-C signals throughout the cell cycle.

(B) CENP-I signals are colocalized CENP-H-GFP signals. Cells expressing CENP-H-GFP were fixed with MeOH and stained with anti-CENP-I antibody. Green signals are specific for CENP-H-GFP, and immunoreactions with CENP-I were detected with Cy3-conjugated secondary antibody (red). DNA was counterstained with DAPI (blue). As shown in the merged images, CENP-I signals (red) are colcalized with CENP-H-GFP-signals (green) in both interphase and metaphase cells.

(C) CENP-I localizes in or near the inner kinetochore. Cells expressing CENP-I-GFP were fixed with MeOH and stained with anti-BubR1 antibody (upper) or anti-ZW10 antibody (lower). Green signals are specific for CENP-I-GFP. BubR1 and ZW10 immunoreactions were detected with Cy3-conjugated secondary antibodies (red). DNA was counterstained with DAPI (blue). As shown in the merged images, BubR1 signals (red; upper) and ZW10 signals (red; lower) are external to CENP-I-GFP-signals (green) on metaphase chromosomes (blue). The scale bar corresponds to 10 μm.

(D) CENP-C signals are colocalized with CENP-I-GFP-signals (green) on metaphase chromosomes (blue). Cells were treated with colcemid, fixed with MeOH, and stained with anti-CENP-C antibody to determine localization of CENP-C and CENP-I signals on metaphase chromosome more precisely than shown in (A). Antibody signals were detected with Cy3-conjugated secondary antibodies (red).

(A)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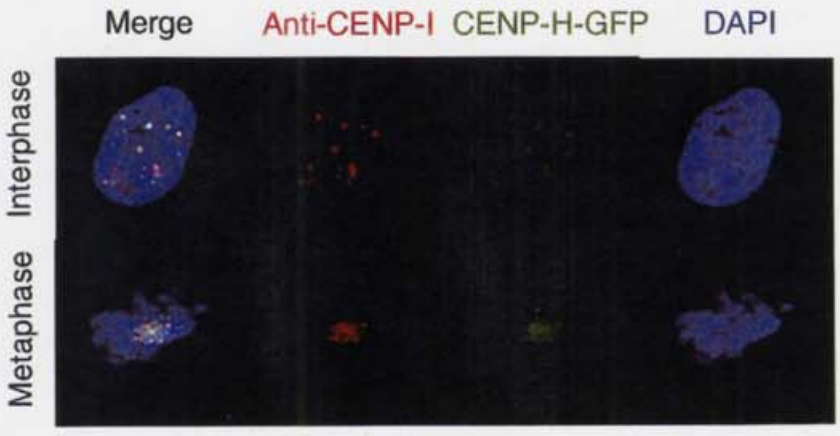
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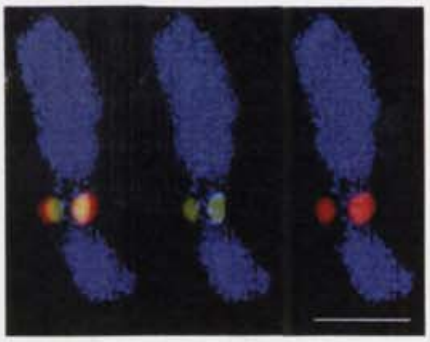
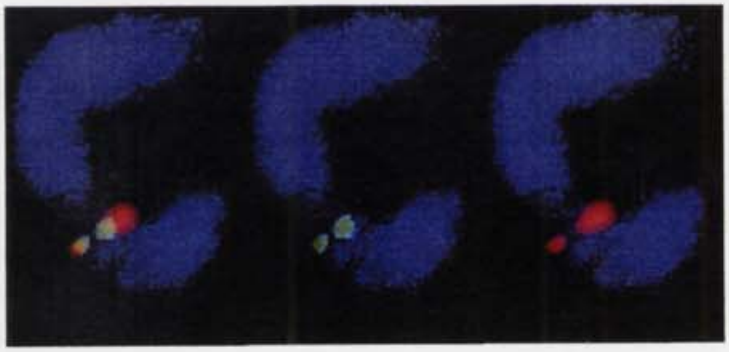
CENP-H-GFP
DAPI
Interphase
Metaphase

(C)

Merge CENP-I-GFP Anti-BubR1

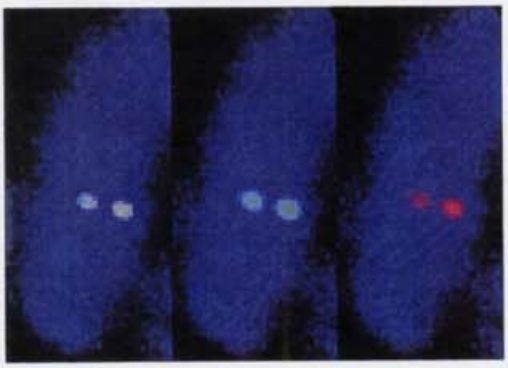


Merge CENP-I-GFP Anti-ZW10



(D)

Marge CENP-I-GFP Anti-CENP-C



2.4 Generation of conditional mutants of CENP-I in DT40 cells

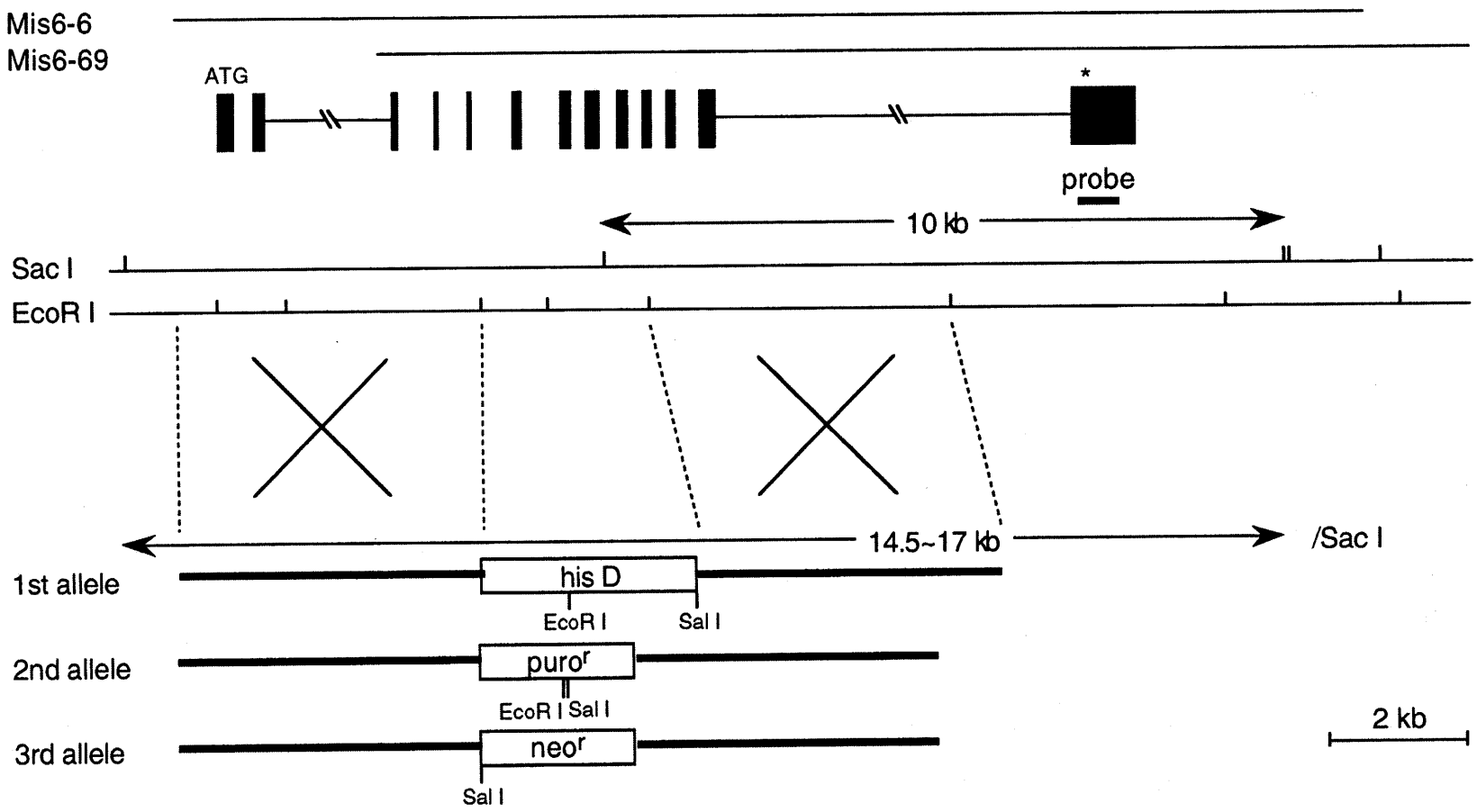
Our data suggest that CENP-I is localized in or near the inner kinetochore plate. In *S. pombe*, Mis6 is essential for viability, but it was unknown whether CENP-I was essential for viability or when CENP-I functioned in cell cycle progression. To investigate the role of CENP-I in higher vertebrate cells, I generated conditional mutants of CENP-I in which cells with disruption of the CENP-I gene were sustained by expression of CENP-I cDNA under control of a tetracycline (tet)-repressible promoter (Gossen and Bujard, 1992). A CENP-I deletion construct was generated such that the 2.5-kb genomic fragment encoding amino acids 221-402 was replaced with one of several selection cassettes. Three CENP-I alleles are present in the DT40 genome (described in Fig. 2.4B). I sequentially transfected CENP-I disruption constructs containing either the histidinol- or puromycin-resistance cassettes (Mis6-his plasmid and Mis6-puro plasmid, respectively) into DT40 cells (Fig. 2.4A). Targeted events were confirmed by Southern hybridization analyses, and CENP-I+/-/- clones were isolated (Fig. 2.4B, 2nd). One CENP-I+/-/- clone was co-transfected with a chicken CENP-I transgene under the control of a tet-repressible promoter (Mis6/pUHD10-3 plasmid) and a tet-repressible transactivator containing a zeocin (zeo)-resistance cassette (tTAbleo plasmid). I selected zeo-resistant colonies and identified several clones carrying these constructs integrated at random sites in the genome (CENP-I+/-/-/CENP-I transgene, Fig. 2.4B, 2nd+cDNA). Four clones with the CENP-I+/-/-/CENP-I transgene genotype were transfected with the neomycin CENP-I disruption construct (Mis6-neo plasmid) to disrupt the remaining CENP-I allele (Fig. 2.4B, 3rd+cDNA). I obtained two clones with the CENP-I-/-/-/CENP-I transgene genotype, and one clone, M90, was chosen for further analysis. Western blot analysis showed a steady reduction in CENP-I protein with time in M90 cells incubated with tet (Fig. 2.4C). CENP-I protein was not detected by 12 h after the addition of tet, indicating that CENP-I is actively turned over. After the addition of tet to the medium, I examined cell growth and viability.

'ig. 2.4 Generation of a CENP-I -/-/- clone carrying a chicken CENP-I transgene under the control of a tet-repressible promoter

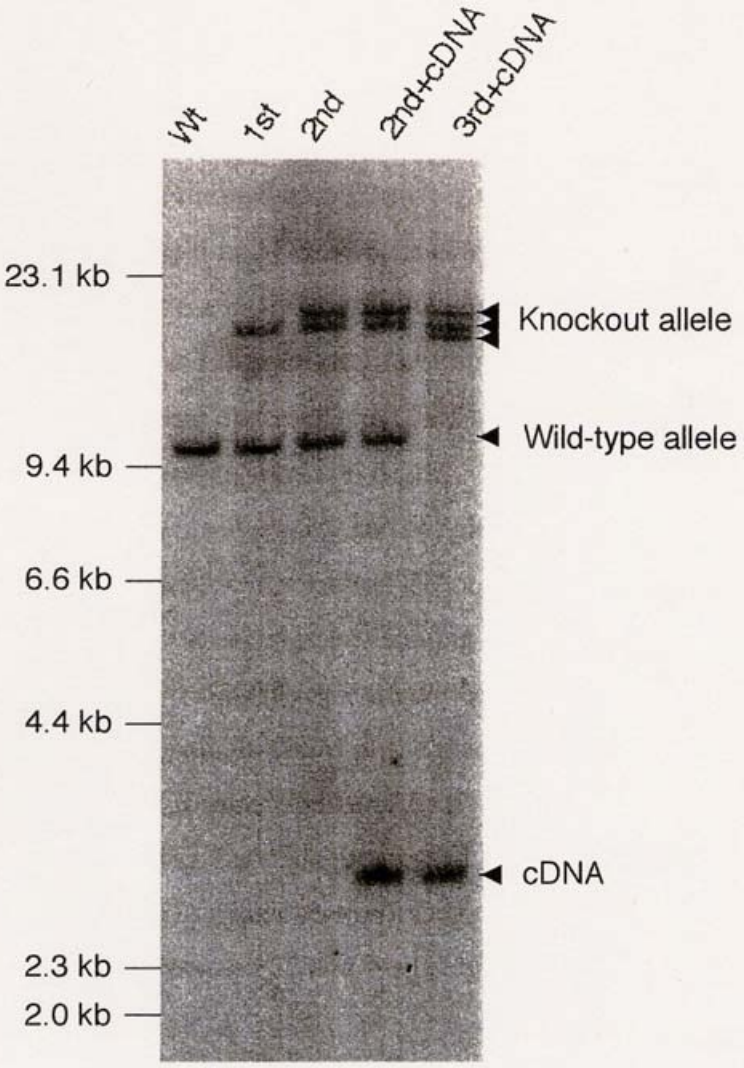
A) Restriction maps of the CENP-I locus, the gene disruption constructs, and the targeted allele. Black boxes indicate the positions of exons, and appropriate targeting is expected to disrupt four exons. EcoRI and SacI restriction sites are shown. The position of the probe used for Southern hybridization is indicated. Novel 14.5–17-kb SacI fragments hybridize to the probe if targeted integrations of the various constructs occur. The cDNA probe was used to confirm the presence of a randomly integrated chicken CENP-I transgene under the control of a tet-repressible promoter. A novel 1.2-kb EcoRI fragment hybridizes to the probe if this construct integrates into the genome (Data not shown). #6-6 and #6-69 are plasmids cloned from a DT40 genomic library, the lines in this map indicate the genomic region contained in these plasmids.

B) Restriction analysis of targeted disruption of the CENP-I locus. Genomic DNAs from wild-type DT40 cells (Wt), a clone after first round targeting (+/+/-, 1st), a clone after second round targeting (+/-/-, 2nd), a clone after second round targeting and random integration of the CENP-I transgene (+/-/- CENP-I+, 2nd+cDNA), and a clone after third round targeting (-/-/- CENP-I+, 3rd+cDNA) were digested with SacI, size fractionated by 0.7% agarose gel electrophoresis, transferred to a nylon filter, and hybridized with the probe indicated in (A). Knockout alleles indicate novel 14.5-17-kb SacI fragments due to targeted integrations of the constructs, which replaced the 10-kb wild-type fragments.

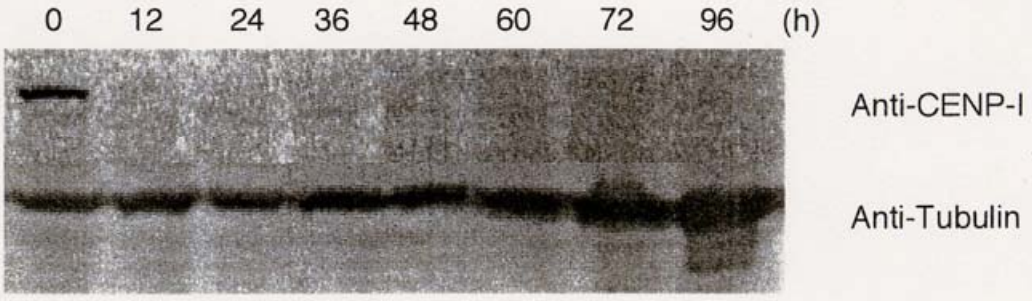
C) Western blot analysis of M90 whole cell extracts with anti-CENP-I antibody at the indicated times following addition of tet. Protein contents of extracts were measured with the DC protein assay (Bio-Rad) and were separated by 5-20% gradient acrylamide SDS-PAGE. Equal amounts of each extract were also separated and analyzed by Western blot with anti-tubulin antibody as an internal control.


Mis6-6
Mis6-69
ATG
*
probe
10 kb
Sac I
EcoR I
14.5~17 kb
/Sac I
1st allele
his D
EcoR I
Sal I
2nd allele
puro^r
EcoR I Sal I
3rd allele
neo^r
Sal I
2 kb

(B)


Wt
1st
2nd
2nd+cDNA
3rd+cDNA
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
Knockout allele
Wild-type allele
cDNA

(C)


0
12
24
36
48
60
72
96
(h)
Anti-CENP-I
Anti-Tubulin

2.5 Deletion of CENP-I results in accumulation of cells in G2/M and subsequent cell death

The proliferative properties of wild type and CENP-I-/-/- cells were monitored by growth curves and cell cycle analysis. Viable and dead cells were assessed by trypan blue exclusion. The growth curve of M90 cells (CENP-I-/-/- CENP-I+, tet-) was indistinguishable from that of wild-type DT40 cells (Fig. 2.5A and B), indicating that the integrated CENP-I cDNA was functional. The total numbers of cells at each time point are shown in Fig. 2.5B. CENP-I+ M90 cells (tet-) and wild-type DT40 cells divided approximately nine times during the 96-h culture, which indicates that the doubling time of these cells is approximately 10.6 h (Fig. 2.5A and B). I then examined cell proliferation and viability following the addition of tet to the medium. M90 cells (CENP-I-/-/- CENP-I-, tet+) stopped proliferating approximately 2.5 cell cycles after the addition of tet (Fig. 2.5A and B), and most cells had died by 96 h, indicating that depletion of CENP-I causes growth arrest and subsequent cell death.

I then examined the delayed growth and cell death following CENP-I depletion in M90 cells. For this purpose, I measured both cellular DNA content and DNA synthesis by fluorescence-activated cell sorting (FACS) after pulse-labeling with BrdU (Fig. 2.5C). Cell cycle analysis showed that CENP-I- M90 cells (tet+) started to accumulate in G2/M phase 24 h after addition of tet, and the proportion of cells in G2/M phase reached 60% by 36 h (Fig. 2.5C). After a prolonged delay in G2/M, further uptake of BrdU without cell division occurred between 48 and 60 h (Fig. 2.5C), suggesting that cells progressed to the next cycle without undergoing cytokinesis (details given in a later section). Degradation of chromosomal DNA due to massive cell death was observed between 72 and 96 h (Fig. 2.5C and 2.6B).

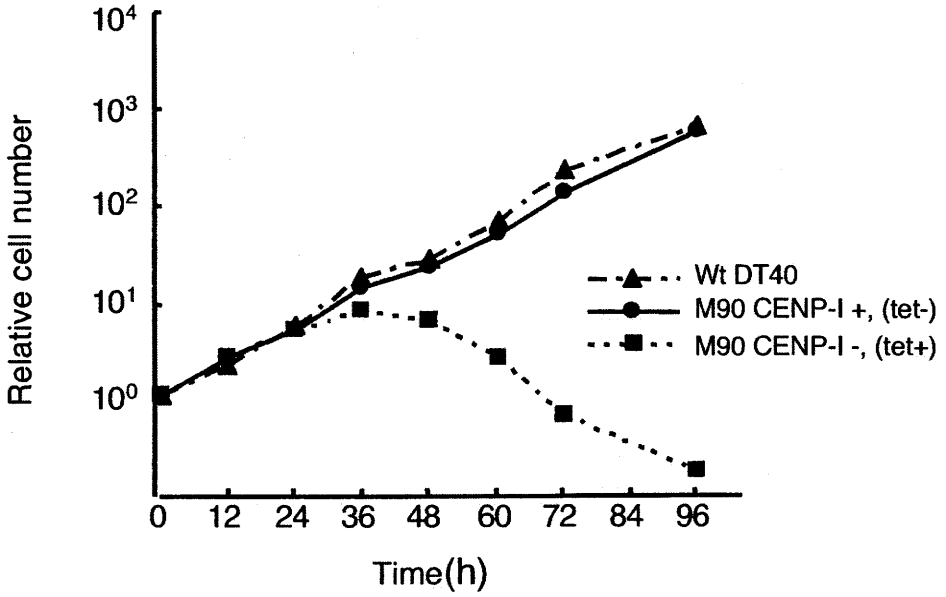
Fig. 2.5 CENP-I is essential for normal progression of the cell cycle

(A) Representative growth curves for the indicated cell cultures. Tet was added at time 0 to the M90 CENP-I-deficient cell tet (+) culture, and the number of cells not stained with trypan blue was counted with a hemacytometer. Each experiment was performed twice, and each time point was examined in duplicate.

(B) Total cell number plotted in (A). The growth of CENP-I+ M90 cells (tet-) was similar to that of wild-type DT40 cells; however, CENP-I- M90 cells (tet+) delayed and stopped proliferating at approximately 25 h after addition of tet, which was estimated to be approximately 2.5 cell cycles.

(C) Cell-cycle distribution of M90 cells following inhibition of CENP-I transgene expression by addition of tet at time 0 [tet (+) culture above]. Cells were stained with FITC-anti-BrdU (y-axis, log scale) to detect BrdU incorporation (DNA replication) and with propidum iodide to detect total DNA (x-axis, linear scale). The lower-left box represents G1-phase cells, the upper box represents S-phase cells and the lower-right box represents G2/M-phase cells. The numbers given in the boxes indicate the percentage of gated events. Arrows at 48 h and 60 h indicate further BrdU uptake by cells with 4N DNA content. CENP-I- M90 cells started to accumulate in G2/M phase 24 h after addition of tet; cells with 8N DNA content were observed at 48, 60 and 72h.

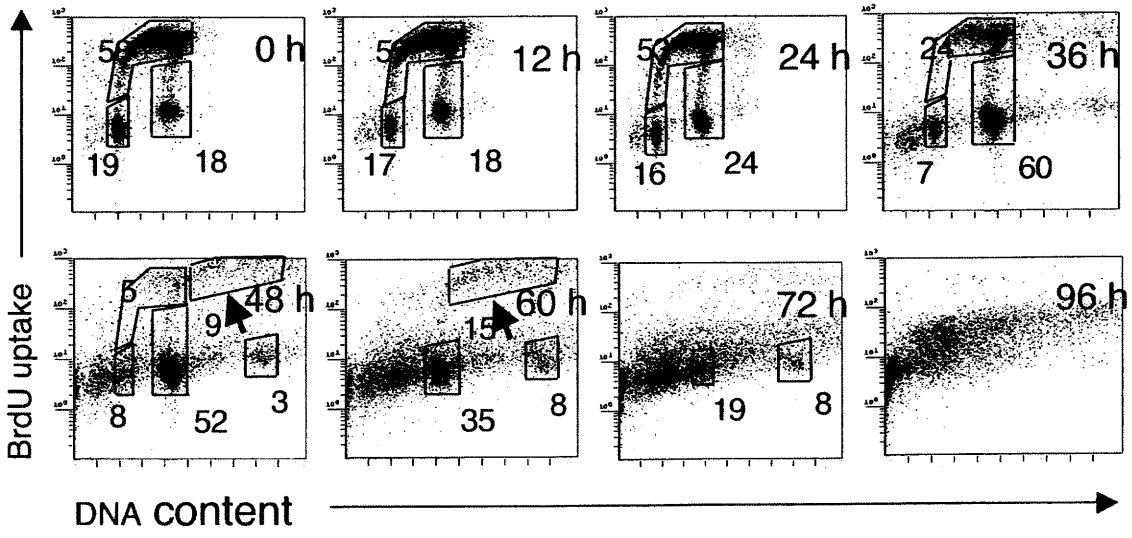
(A)



(B)

	0 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h
+tet	$3.6x10^5$	$8.8x10^5$	$1.7x10^6$	$2.7x10^6$	$2.2x10^6$	$8.8x10^5$	$2.2x10^5$	$5.6x10^4$
-tet	$3.6x10^5$	$8.7x10^5$	$1.7x10^6$	$4.7x10^6$	$7.6x10^6$	$1.7x10^7$	$4.3x10^7$	$1.8x10^8$
Wild	$2.6x10^5$	$7.4x10^5$	$1.9x10^6$	$6.1x10^6$	$9.3x10^6$	$2.2x10^7$	$7.5x10^7$	$2.1x10^8$

(C)



2.6 Deletion of CENP-I causes chromosome aberrations and leads to chromosome missegregation

FACS analysis showed that M90 cells accumulated in G2/M phase in the presence of tet (Fig. 2.5C), but I could not distinguish the exact stage of G2/M phase by FACS analysis. To determine the exact nature of mitotic delay, I used DNA staining and immunocytochemical staining of microtubules to examine the time course of events following the depletion of CENP-I (Fig. 2.6A and B). The mitotic index began to increase at 24 h and reached 36% at 48 h after the addition of tet, and no anaphase cells were observed at 48 h (Fig. 2.6B). By considering the FACS data together with data from cytological analyses, I estimated that 16% of cells are in G2 phase at 48 h (52%; G2/M at 48 h in Fig. 2.5C -36%; metaphase at 48 h in Fig. 2.6B). In control cultures (0 h), 3 to 4% of cells are in mitosis (Fig. 2.6B), 18% of cells are in G2/M as determined by FACS (Fig. 2.5C), and 14 to 15% of cells are in G2 phase. These data indicate that CENP-I-deficient cells accumulate in M phase rather than G2 phase and that they are delayed prior to anaphase.

In the course of cytological analysis of CENP-I-deficient cells, I observed many abnormal mitotic cells (Fig. 2.6A and B). In large populations of prometaphase-delayed cells, some chromosomes were highly condensed and appeared misaligned on the metaphase plate (Fig. 2.6A; 24, 48, and 72 h). Eventually, the cells with highly condensed chromosomes underwent apoptotic death (Fig. 2.6A; 72 h). In controls, chromosomes appeared ordered and aligned on the metaphase plate (Fig. 2.6A; 0 h, metaphase).

Control cells cultured in the absence of tet showed well-ordered bipolar spindles during both metaphase and anaphase (Fig.2.6A; 0 h). At 48 h, most cells cultured in the presence of tet appeared to have normal microtubule spindle assemblies, but some contained multi- or monopolar spindles (Fig. 2.6A and C). This might account for the abnormal chromosome alignments described above; however, even in the 70% (48 h after addition of tet) and 30% (72 h after addition of tet) of mutant cells that had proper bipolar spindle poles, some chromosomes failed to achieve a proper alignment on the metaphase plate (Fig. 2.6A and C). In other cells, chromosome alignment was completely disordered. I observed that several chromosomes had not congressed, although many had aligned at the midzone in CENP-I-deficient cells (Fig. 2.6A). It was, therefore, of interest to investigate the attachments between microtubules and chromosomes in CENP-I-deficient cells.

M90 cells at 0 h and 48 h after addition of tet were stained with anti-tubulin and anti-CENP-A antibodies and observed by fluorescence microscopy. Double staining of these cells revealed that congressed chromosomes appeared attached to microtubules, whereas misaligned chromosomes were not attached (Fig. 2.6D). These results indicate that CENP-I, like CENP-H (Fukagawa et al.,

2001), is required either directly or indirectly for congression and/or maintenance of stable chromosome alignment and, ultimately, for progression from metaphase to anaphase.

Fig. 2.6 CENP-I-deficient cells show metaphase delay associated with aberrant chromosomes and spindles that lead to chromosome missegregation

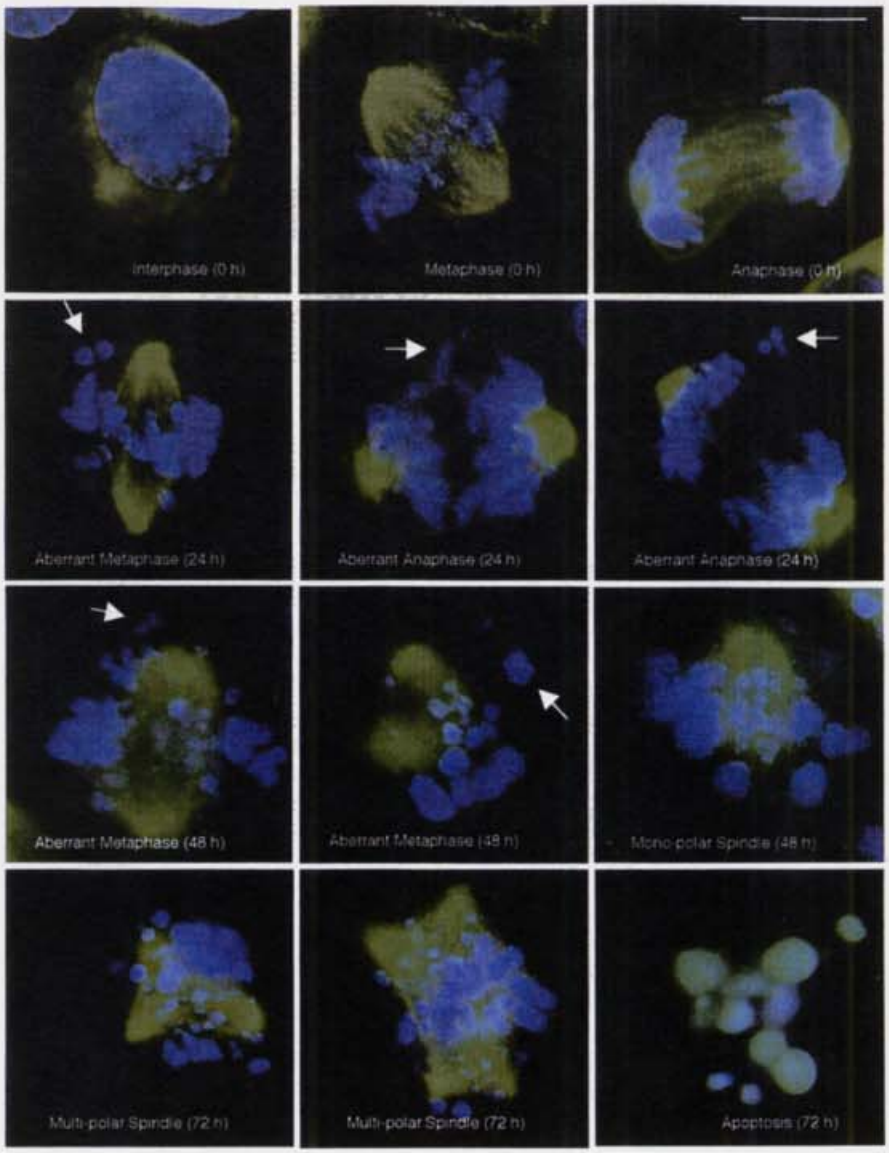
(A) Chromosome morphology and α-tubulin staining (green) in the absence or presence of tet. M90 Cells were fixed with PFA and stained with FITC-conjugated anti-α-tubulin antibody. DNA was counterstained with DAPI (blue). In the absence of tet, cells show the normal staining pattern for α-tubulin (upper three panels). In the presence of tet, chromosomes were not aligned at the metaphase plate. Arrows indicate misaligned chromosomes at the metaphase plate or lagging chromosomes during anaphase at 24 or 48 h after addition of tet. Apoptotic cells were observed at 72 h after addition of tet. An apoptotic cell was detected by TUNEL assay (green). I also detected cells with monopolar and multipolar spindles. The scale bar corresponds to 10 μm.

(B) Quantitation of aberrant M90 cells following inhibition of CENP-I transgene expression by addition of tet at time 0. I scored the number of interphase cells, normal metaphase cells, aberrant metaphase cells described in (A), anaphase cells, and apoptotic cells at the indicated time points. I scored approximately 3000 cells for each time point. Apoptotic cells were detected by TUNEL assay. At 48 h after addition of tet, no anaphase cells were observed, and aberrant metaphase cells represented approximately about 30% of the total cell population.

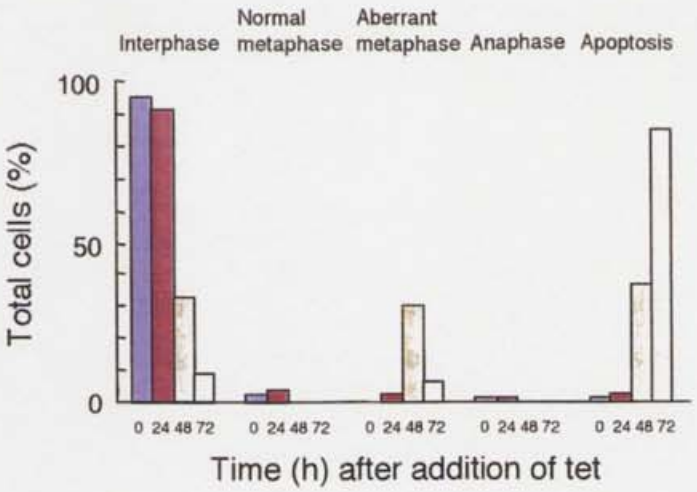
(C) Quantitation of cells with misaligned chromosomes after addition of tet at time 0. Mis-aligned chromosomes were classified as bipolar spindle with misaligned chromosomes, monopolar spindle, or multispindle. At 48 h after addition of tet, most cells showed abnormality in spindle bipolarity and/or chromosome alignment.

(D) Double staining of M90 cells at 0 h (-tet) and 48 h (+tet) after addition of tet with FITC-conjugated anti-α-tubulin (green) and anti-CENP-A antibodies. Cells were fixed with PFA and stained. CENP-A signals were detected with Cy3-conjugated secondary antibodies (red). Arrows indicate misaligned chromosomes that do not appear to be attached to microtubules, although many chromosomes appear to have formed microtubule attachments. The scale bar corresponds to 10 μm.

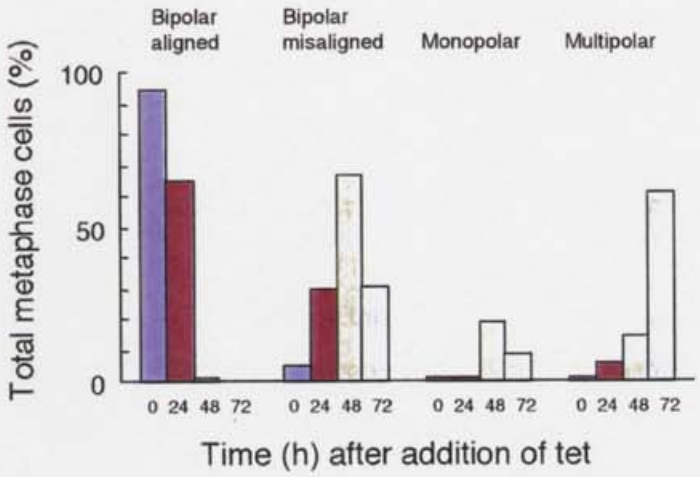
(A)


Interphase (0 h)
Metaphase (0 h)
Anaphase (0 h)
Aberrant Metaphase (24 h)
Aberrant Anaphase (24 h)
Aberrant Anaphase (24 h)
Aberrant Metaphase (48 h)
Aberrant Metaphase (48 h)
Mono-polar Spindle (48 h)
Multi-polar Spindle (72 h)
Multi-polar Spindle (72 h)
Apoptosis (72 h)

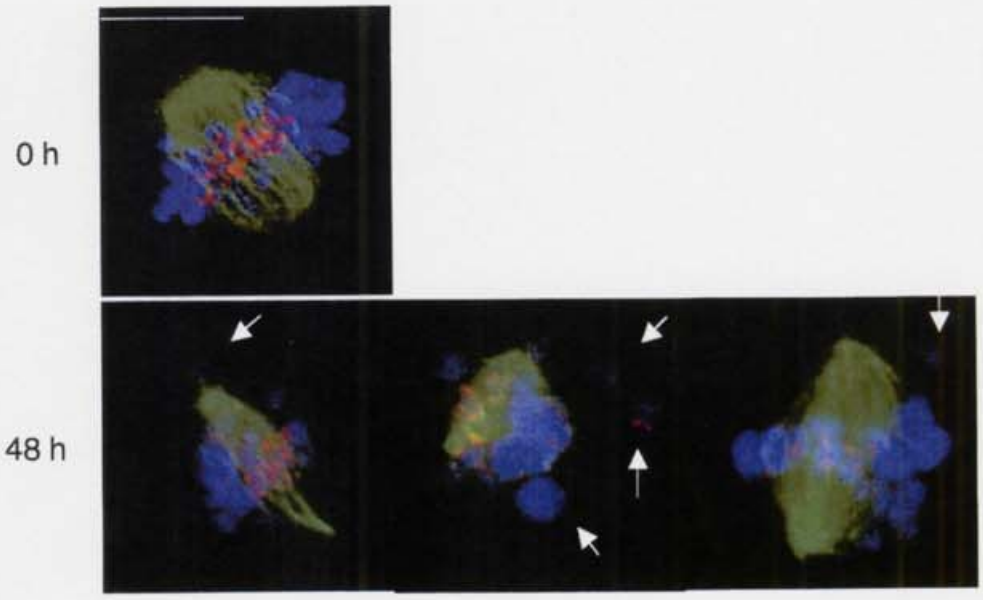
(B)


Interphase
Normal metaphase
Aberrant metaphase
Anaphase
Apoptosis
100
50
0
Total cells (%)
0 24 48 72
Time (h) after addition of tet

(C)


Bipolar aligned
Bipolar misaligned
Monopolar
Multipolar
100
50
0
Total metaphase cells (%)
0 24 48 72
Time (h) after addition of tet

(D)


0 h
48 h

2.7 Passage into G1/S is associated with mitotic defects in CENP-I-deficient DT40 cells

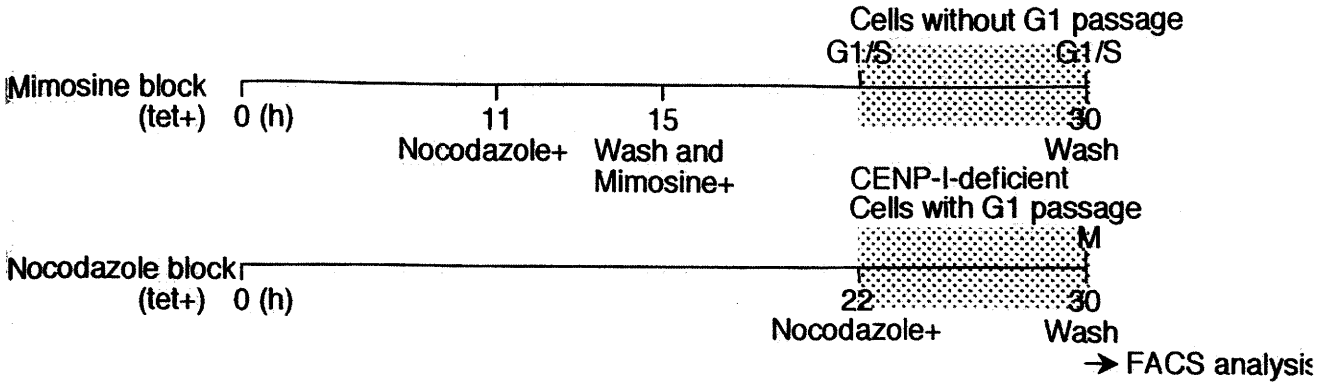
In *S. pombe*, mitotic defects in Mis6 mutants occur when cells progress into G1/S phase, suggesting that Mis6 acts during G1/S phase (Saitoh et al., 1997). I then examined whether the mitotic defects in CENP-I-deficient DT40 cells occur during progression into G1/S. M90 cells were synchronized at the G1/S transition or M phase. Because the effect of CENP-I-deficiency was observed from 22-24 h after the addition of tet (Fig. 2.5A), I prepared two populations of cells: one that did not pass G1 between 22 and 30 h after the addition of tet and one did (Fig. 2.7A). In the first population, cells were cultured in medium with tet for 11 h. Nocodazole was added to the medium after 11 h, and cells were incubated for 4 h to synchronize cells gently before addition of mimosine. Cells were then washed and incubated with mimosine and tet for 15 h. Because mimosine arrests cells in G1/S transition, cells are blocked in G1/S transition before 22 h after addition of tet and do not pass through G1 between 22-30 h (Fig. 2.7A, upper line). In the second population, cells were cultured with tet for 22 h. Nocodazole was then added and cells were incubated for 8 h. In this population, because cells at all points in the cell cycle were present at 22 h, many cells passed through G1 between 22-30 h and were blocked at M phase (Fig. 2.7A, lower line). Cells were then washed to release the block and cultured with tet for up to 42 h for analysis of cell cycle progression (Fig. 2.7B). In the second population, cells were delayed at M phase after passage through G1, but mitotic delay did not occur in first population of cells that did not pass through G1, even at the same time point after addition of tet (Fig. 2.7B), indicating that G1 passage in CENP-I-deficient vertebrate cells, like Mis6 mutants of *S. pombe*, is required for mitotic delay.

Fig. 2.7 G1 passage in CENP-I-deficient cells is required for mitotic delay

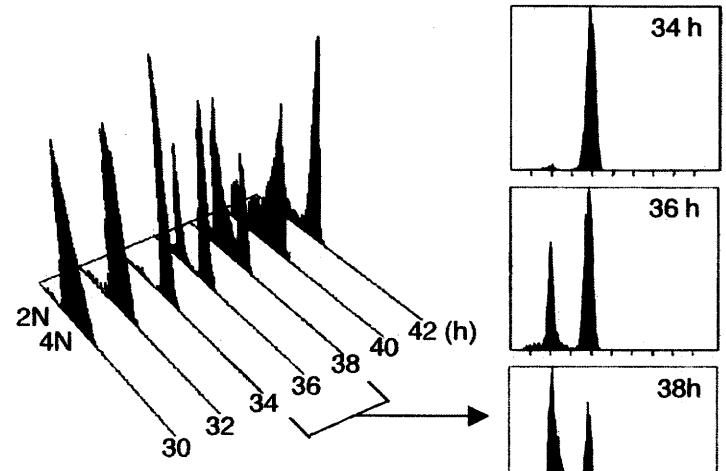
(A) Schematic representation of synchronization process. In one population (mimosine block), cells do not pass through G1 between 22 and 30 h after addition of tet. In another population (nocodazole block), cells pass through G1 between 22 and 30 h after addition of tet before cells delay at M phase. At 30 h, cells were free from blocking by wash and incubated with tet. An aliquot of cells was harvested every 2 h up to 42 h.

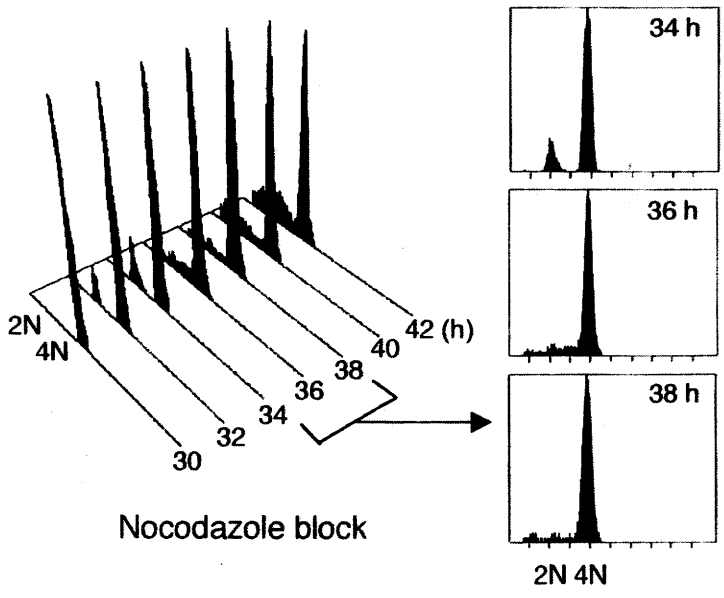
(B) Flow cytometric analysis of two kinds of synchronized cell cultures. The positions of cell populations with 2N and 4N DNA contents are indicated. The time points indicate time after addition of tet. Mitotic delay occurred in nocodazole block but not in mimosine block at 36 h and 38 h after addition of tet.

(A)


Cells without G1 passage
G1/S
G1/S
Mimosine block
(tet+)
0 (h)
11
Nocodazole+
15
Wash and
Mimosine+
30
Wash
CENP-I-deficient
Cells with G1 passage
M
Nocodazole block
(tet+)
0 (h)
22
Nocodazole+
30
Wash
FACS analysis

(B)


34 h
36 h
38h
2N
4N
30
32
34
36
38
40
42 (h)
Mimosine block
2N 4N


34 h
36 h
38 h
2N
4N
30
32
34
36
38
40
42 (h)
Nocodazole block
2N 4N

2.8 Depletion of CENP-I increases number of aneuploid cells with condensed chromosomes

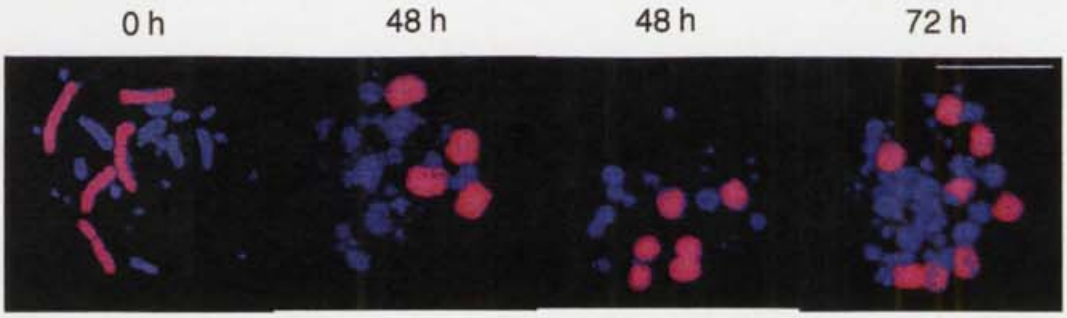
I next examined whether mitotic defects caused by CENP-I depletion were associated with the induction of aneuploidy. CENP-I-deficient M90 cells were analyzed by FISH metaphase spreads with chromosome painting probes specific for chicken chromosomes 1 and 2. Because DT40 cells contain three copies of chromosome 2, five fluorescent signals can be observed for each wild-type cell (Fig. 2.8.A, 0 h). As shown in Fig. 2.8A, I observed abnormal numbers of painted chromosomes in CENP-I-deficient M90 cells in the presence of tet. The proportion of these aneuploid cells gradually increased after 24 h (Fig. 2.8B), suggesting that CENP-I-deficiency induces chromosome missegregation due to a mitotic defect. Condensed chromosomes were also increased after 24 h even though five fluorescent signals were observed. This is consistent with the increase of highly condensed chromosomes in CENP-I deficient cells stained with DAPI and shown in Fig. 2.6A.

Fig. 2.8 Mitotic defects caused by CENP-I depletion were associated with the induction of aneuploidy

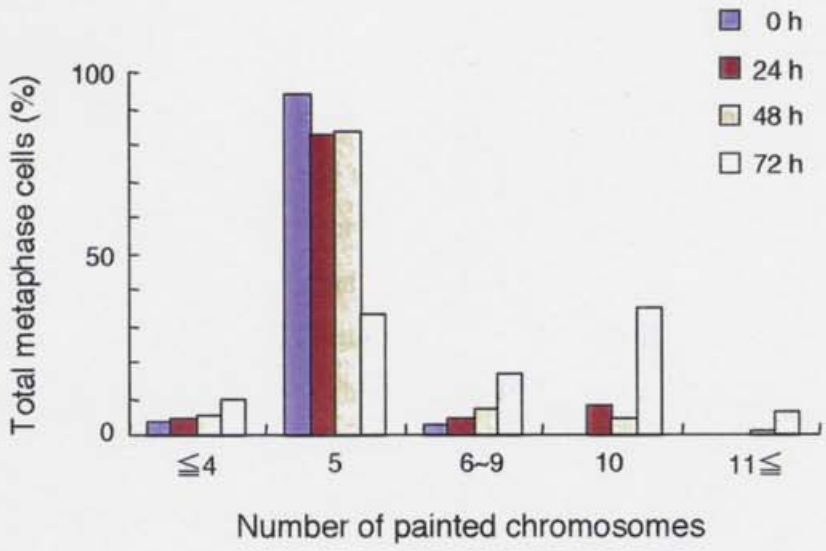
(A) To examine chromosome loss, I used FISH analysis with chromosome-specific painting probes. I used painting probes for chicken chromosomes 1 and 2. The painting probes were labeled with biotin, and signals were detected with Cy3-labeled avidin (red). Because DT40 cells have three copies of chromosome 2, I observed five painted chromosomes in normal cells (0 h). M90 cells with reduced numbers of chromosomes (48 h; right) and increased numbers of chromosomes (48 h; right and 72 h) were detected after addition of tet. The scale bar corresponds to 10 μm.

(B) Distribution of the number of painted chromosomes per cell. M90 cells were cultured with tet. At the indicated times, cells were treated with colcemid for 3 h. The number of painted chromosomes was scored in approximately 1000 metaphase cells, and both normal chromosomes and highly condensed chromosomes, which appeared to be caused by CENP-I depletion, were visible.

(A)


0 h
48 h
48 h
72 h

(B)


0 h
24 h
48 h
72 h
Total metaphase cells (%)
100
50
0
≦4
5
6~9
10
11≦
Number of painted chromosomes

2.9 Dynamic behavior of chromosomes and microtubules in CENP-I-deficient cells

I analyzed the cellular effects of CENP-I-deficiency at various time points. In addition to performing such analyses, it is important to observe the dynamic behavior of individual living cells after suppression of CENP-I expression. To visualize microtubules in living cells, human α-tubulin gene fused with GFP (pEGFP-Tub, Clontech) was integrated into the genome of M90 cells (Mis6-3rd-Tub cell in Chapter 4, described as M90-Tub below). The fused gene was co-tansfected with a plasmid containing a hygromycin (hyg)-resistance cassette (plasmid #257, Fukagawa) to select integrated cells. A portion of hygromycin-resistant cells was fixed with PFA and the expression of α-tubulin-GFP was confirmed by fluorescence microscopy. Several M90-Tub cell lines were isolated. I then stained the chromosomes of living M90-Tub cells expressing GFP-tubulin with Hoechst 33342 and observed the cells microscopically at 37°C. Images were obtained at intervals of 2 min. Chromosomes and microtubules were observed in individual cells throughout an entire mitotic cell cycle. An example of the time-lapse data for M90 cells in the absence of tet (control) is shown in Fig. 2.9A. In all color images in Fig. 2.9, chromosomes are displayed in red, whereas microtubules are stained green. I was able to observe a well-ordered metaphase plate and the bipolar spindle in control cells (6 –18 min). Chromosomes began segregating as the central spindle elongated (22 min). Cells then underwent cytokinesis, the mitotic spindle disassembled (24 min), and the chromosomes decondensed (34 min). Phototoxicity induced by UV light irradiation is known to cause mitotic delay. To avoid this, I used an ND filter and did not observe cells delayed at mitosis in control experiments. Control cells (n=10) required approximately 34 min to progress from prophase to telophase. After addition of tet to M90-Tub cells, I observed abnormal mitotic behavior (Fig. 2.9B). Time point 0 in Fig. 2.9B corresponds to 40 h after addition of tet to cultures of M90-Tub cells. The single cell shown in Fig. 2.9B entered mitosis (2 min) as condensed individual chromosomes appeared and the mitotic spindle formed. However, chromosome congression to the metaphase plate and subsequent anaphase were not observed. Instead, the cell remained delayed in prometaphase with hyper-condensed chromosomes for 800 min. During this prolonged mitotic delay, I observed strange microtubule behavior. I observed that microtubules bind several chromosomes but that they do not bind to chromosomes without metaphase alignment (Fig. 2.6D). Early after mitotic entry (Fig. 2.9B, 2-22 min) a normal-looking bipolar mitotic spindle was formed; however, several chromosomes did not align properly at the metaphase plate. I subsequently observed fragmentation of the spindle poles (Fig. 2.9B, 42 min) and formation of a multipolar spindle (Fig. 2.9B, 257-533 min). This observation shows that multiplication of centrosomes or spindles occurs independent of chromosome behavior in CENP-I-deficient cells. After prolonged mitotic delay, the chromosomes decondensed, and the mitotic spindle disassembled without

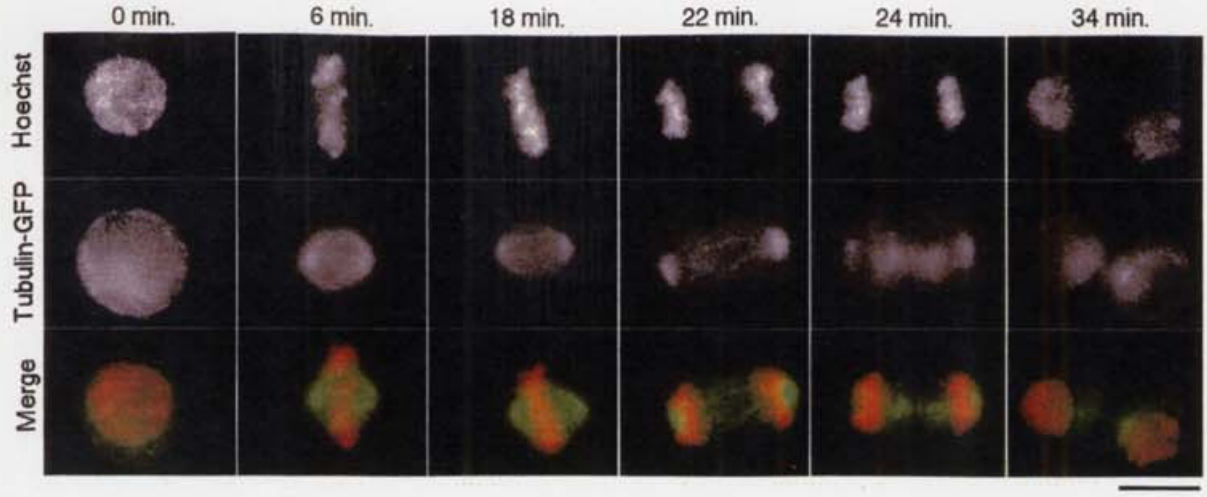
chromosome segregation (859–906 min). These a living cell observations were consistent with data from FACS (Fig. 2.5C) in which additional BrdU uptake without cell division occurred between 48 and 60 h after the addition of tet to M90 cell cultures. After the unusual interphase stage, the cell underwent apoptosis (Fig. 2.9B, 978 min).

Fig. 2.9 Dynamics of chromosomes and microtubules in CENP-I-deficient cells by time-lapse observation.

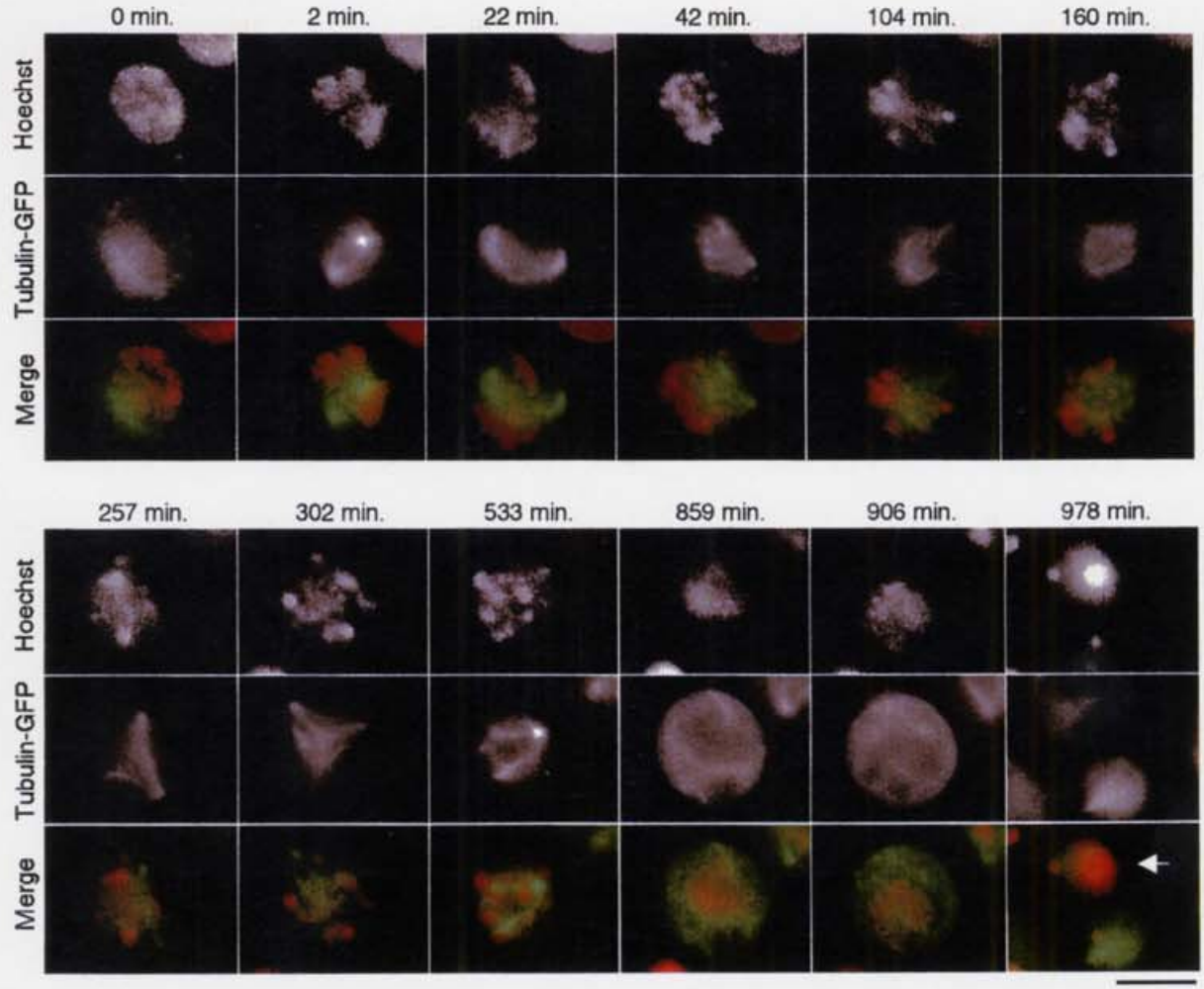
(A) Selected images of chromosomes (upper) and microtubules (middle) from prophase to telophase in M90-Tub cells (-tet). Cells expressed α–tubulin-GFP (green) and were stained with Hoechst 33342 (red) to detect DNA. In the superimposed images (lower), chromosomes and microtubules are displayed in red and green, respectively. The numbers at the top of each image represent the time from telophase in minutes. The scale bar corresponds to 10 μm. Control cells required approximately 34 min to progress from prophase to telophase.

(B) Selected images of M90-Tub cells (+tet). From 40 h after addition of tet, one prophase cell was observed at 2-min intervals for time-lapse analysis. Selected frames are shown. Once the cell entered mitosis, it was delayed at prometaphase for more than 800 min. Fragmentation of the spindle poles was observed at 42 min and formation of a multipolar spindle was observed at 257-533 min. At 859 min, the cell proceeded to the next cell cycle without normal cell division. The cell showed interphase-type microtubules between 859-906 min and then underwent apoptosis as shown at 978 min.

(A)


0 min.
6 min.
18 min.
22 min.
24 min.
34 min.
Hoechst
Tubulin-GFP
Merge

B)


0 min.
2 min.
22 min.
42 min.
104 min.
160 min.
257 min.
302 min.
533 min.
859 min.
906 min.
978 min.
Hoechst
Tubulin-GFP
Merge

2.10 CENP-I-deficient cells proceed through the cell cycle without normal cytokinesis

In the living-cell (Fig. 2.9B) and BrdU-uptake (Fig. 2.5C) analyses, I observed that CENP-I-deficient cells progressed to the next cell cycle after a lengthy prometaphase delay without cytokinesis. I then confirmed that M90 cells proceeded through the cell cycle without normal chromosome segregation by FACS analysis (Fig. 2.10A). As mentioned above, cells accumulated at 4N DNA content between 36 h and 48 h after addition of tet (Figs. 2.5C and 2.10A). After delay in prometaphase, cells progressed to the next cell cycle without normal cytokinesis, and these cells completed further DNA replication with 8N DNA content at 72 h after the addition of tet (Fig. 2.10A). These findings suggest that the mitotic checkpoint is inactivated by 72 h after the addition of tet. To investigate the localization of checkpoint protein in CENP-I-deficient cells, I then stained prometaphase-delayed cells with antibody against the BubR1 checkpoint protein (Cahill et al., 1998; Jablonski et al., 1998; Chan et al., 1999). In controls, the metaphase chromosomes of M90 cells (tet-, CENP-I+) delayed with colcemid were stained, and I observed strong centromere staining with BubR1 (Fig. 2.10B, -tet). I then stained chromosomes of M90 cells with anti-BubR1 after addition of tet. BubR1 signals were detected on some metaphase cells at 48 h after the addition of tet (Fig 2.10B and C). However, the signal intensity became weak by 60 h, and weak or no signals were detected at 72 h after addition of tet (Fig. 2.10B and C), suggesting that association of the checkpoint signaling proteins with the kinetochore structure is lost by 72 h. In control experiments, if wild-type cells were arrested with nocodazole for 60 h, the majority of cells died, although 5 to 6% of cells progressed to the next cell cycle.

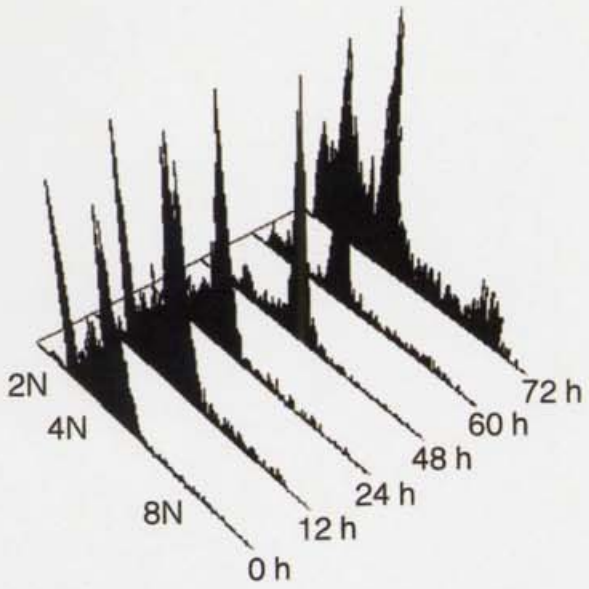
Fig. 2.10 CENP-I-deficient cells proceed through the cell cycle without cell division dependent of disruption of mitotic checkpoint

(A) FACS analysis of the cell cycle pattern of M90 cells after addition of tet. The positions of cell populations with 2N, 4N, and 8N DNA content are illustrated. At 72 h, a peak of 8N DNA content is observed.

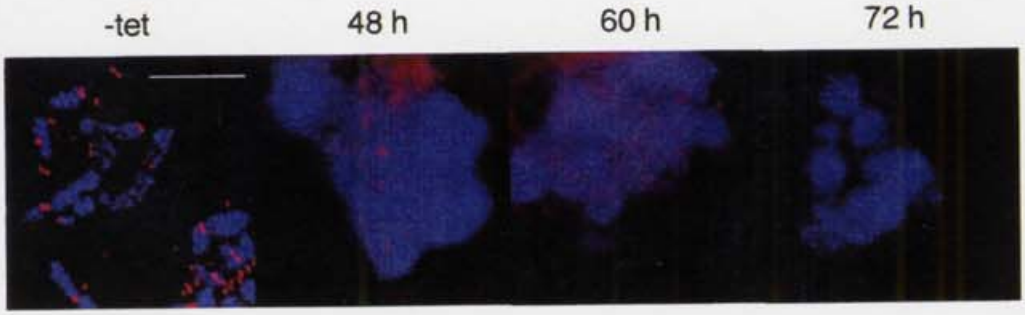
(B) Immunofluorescence analysis of (pro)metaphase M90 cells at 0 h, 48 h, 60 h, and 72 h after addition of tet with antibody against checkpoint protein BubR1. Cells were fixed with PFA and stained with anti-BubR1 antibody. Antibody signals were detected with Cy3-conjugated secondary antibodies (red). DNA was counterstained with DAPI (blue). In control (-tet), cells were treated with colcemid for 3 h before staining. The scale bar corresponds to 10 μm. BubR1 signals were detected on some metaphase chromosomes at 48 h after addition of tet.

(C) Quantitation of (pro)metaphase chromosomes with strong, weak or no BubR1 signals. The numbers of (pro)metaphase chromosomes with strong BubR1 signals decreased following addition of tet to M90 cells. I scored approximately 1000 cells for each time point. Approximately 60% of cells showed relatively strong BubR1 signals at 48 h after addition of tet, but the signal intensity was lost by 60 h and 72 h.

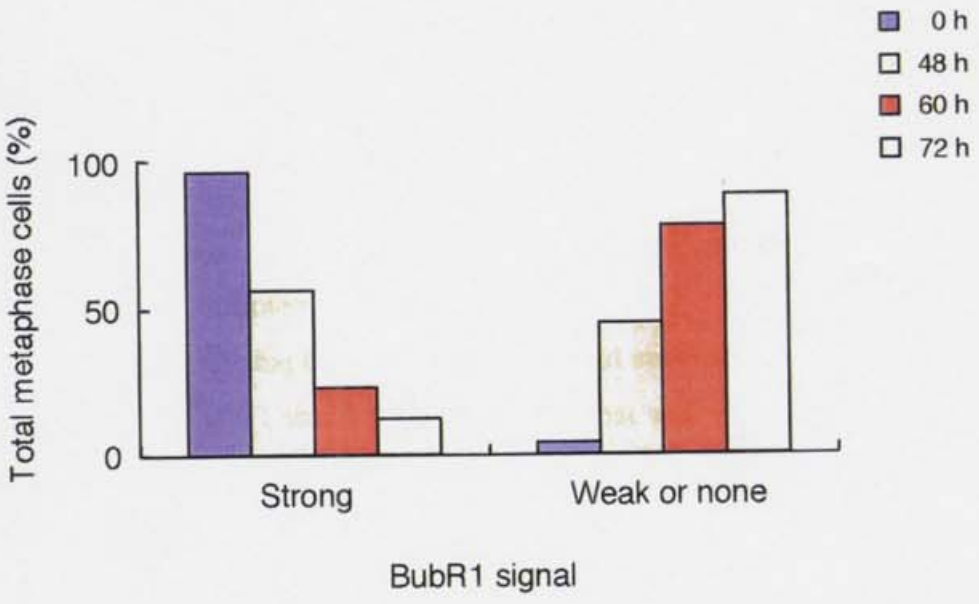
(A)


2N
4N
8N
0 h
12 h
24 h
48 h
60 h
72 h

(B)


-tet
48 h
60 h
72 h

(C)


0 h
48 h
60 h
72 h
Total metaphase cells (%)
100
50
0
Strong
Weak or none
BubR1 signal

2.11 Localization of other centromere proteins in CENP-I-deficient cells

I found perfect co-localization of chicken CENP-C, -H, and -I in the present study (Fig. 2.3). CENP-A, -C, and -H are thought to form a "prekinetochore structure" that exists throughout the cell cycle and marks the location of the centromere on the chromosome in vertebrate cells. To understand the role of CENP-I in formation of the prekinetochore structure, I performed immunofluorescence studies of a CENP-I-deficient mutant. Interphase nuclei of M90 cells were fixed with MeOH and analyzed with antibodies against chicken CENP-A (Fukagawa et al., 2001), CENP-C (Fukagawa et al., 1999a), CENP-H (Fukagawa et al., 2001), and CENP-I in the presence or absence of tet. Typical results are shown in Fig. 2.11A. In the absence of tet, all four antibodies gave strong, discrete signals on interphase nuclei of M90 cells (Fig. 2.11A, upper panel). At 48 h after the addition of tet, I could not detect CENP-I signals in interphase nuclei of M90 cells consistent with the complete depletion of CENP-I (Fig. 2.11A, lower left panel). CENP-A antibody gave positive signals on interphase nuclei of CENP-I-deficient cells (Fig. 2.11A, lower panel); however, the normal prominent, discrete CENP-C and CENP-H signals (Fig. 2.11A, upper panels) were absent. Instead, diffuse or weak signals for CENP-C and CENP-H were observed (Fig. 2.11A, lower panels). I counted interphase cells that showed diffuse or weak signals (Fig. 2.11B) and found that after the addition of tet approximately 83% and 98% of interphase cells displayed diffuse CENP-C and CENP-H signals, respectively (Fig. 2.11B).

Immunocytochemical analysis of CENP-I-deficient cells showed that CENP-I is necessary for localization of CENP-H to the centromere. It was, therefore, of interest to determine whether CENP-H was necessary for CENP-I localization to the centromere. To address this question, immunofluorescence analyses of the #5-5 cell line, which is a conditional loss-of-function mutant of CENP-H (Fukagawa et al., 2001), were performed. We previously showed that CENP-H signals were lost and that CENP-C signals were diffuse in #5-5 cells 48 h after the addition of tet (Fukagawa et al., 2001). I then analyzed localization of CENP-I and found that CENP-I signals were also lost (Fig. 2.11C and D) in CENP-H-deficient cells. These results combined with our previous data (Fukagawa et al., 2001) suggest that CENP-I and CENP-H are mutually interdependent for targeting to the prekinetochore structure and that both are necessary for CENP-C localization to centromeres.

Although our data showed that CENP-A localization did not change in CENP-I-deficient cells (Fig. 2.11A), it is possible that I observed only CENP-A that was targeted to the centromere before CENP-I deficiency and that newly synthesized CENP-A is not targeted to centromeres. To address this question, CENP-A-3HA was transfected transiently into M90 cells at 24 h after the addition of tet. The transfected cells were fixed with MeOH, stained with anti-HA antibody, and observed by fluorescence microscopy (Fig. 2.11E). HA signals were detected at 48 h after

the addition of tet in CENP-I+M90 cells (tet-). It appears that newly synthesized CENP-A-3HA is targeted to the centromere in CENP-I-deficient cells.

Fig. 2.11 CENP-I is essential for centromere assembly in vertebrate cells

(A) Immunofluorescence analysis of M90 cells at 0 h (-tet) or 48 h (+tet) after addition of tet. Interphase cells were fixed with MeOH, stained with anti-CENP-I, anti-CENP-A, anti-CENP-C, and anti-CENP-H antibodies. Antibody signals were detected with Cy3-conjugated secondary antibodies (red). DNA was counterstained with DAPI (blue). Scale bars correspond to 10 μm. At 48 h (+tet) after addition of tet, no CENP-I signal was observed.

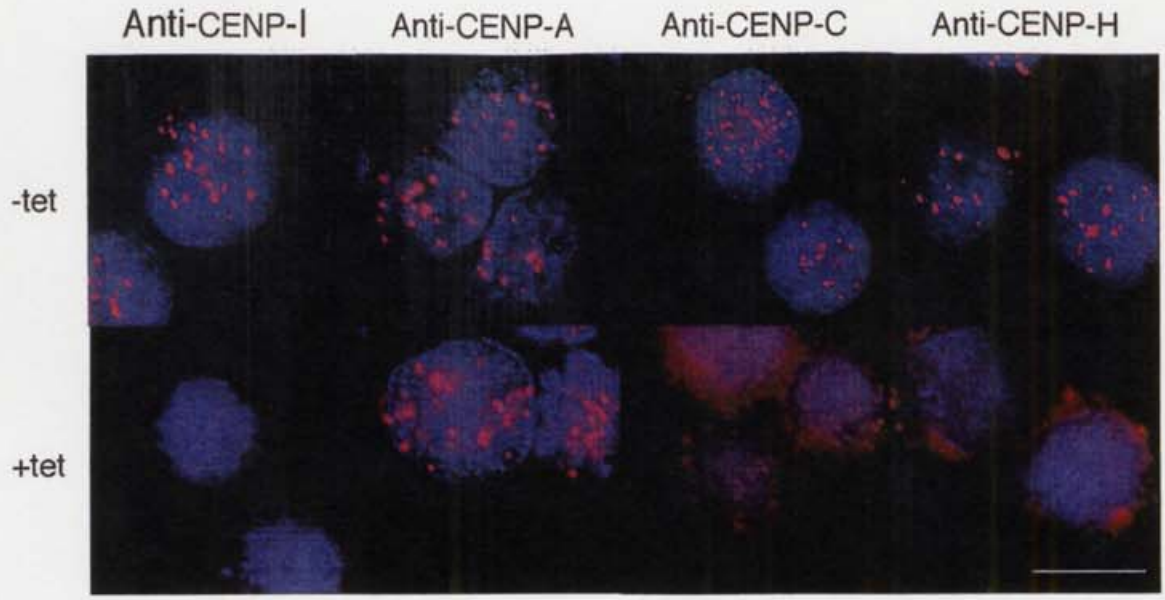
(B) Quantitation of M90 interphase cells with diffuse CENP-A, CENP-C, and CENP-H signals. As shown in (A), many M90 interphase cells displayed diffuse or weak CENP-C and CENP-H signals at 48 h after addition of tet, whereas discrete CENP-C and CENP-H signals were observed in the absence of tet. The graphs provide a quantitative representation of the data. I scored approximately 2000 interphase cells.

(C) Immunofluorescence analysis of #5-5 (Δ/CENP-H, CENP-H transgene) cells grown in the absence (-tet) or presence (+tet, restrictive conditions) of tet for 48 h. Rabbit antibodies to CENP-H or CENP-I were applied to interphase cells and detected with Cy3-conjugated secondary antibodies (red). DNA was counterstained with DAPI (blue). The scale bar corresponds to 10 μm. CENP-I signals were lost in +tet cells.

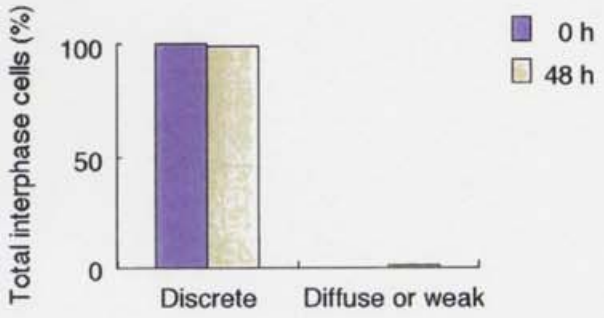
(D) Quantitation of #5-5 interphase cells with diffuse or weak CENP-I signals. I scored approximately 2000 interphase cells.

(E) Immunofluorescence analysis of M90 cells transiently expressing CENP-A-3HA. M90 cells were cultured for 24 h with tet and transfected with a CENP-A-3HA construct. At 48 h after addition of tet, cells were fixed with MeOH and stained with anti-HA antibody. Antibody signals were detected with Cy3-conjugated secondary antibodies (red). DNA was counterstained with DAPI (blue). Discrete signals were observed in the transfected cells at 48 h after addition of tet.

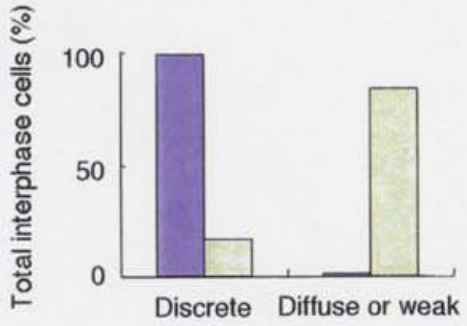
(A)



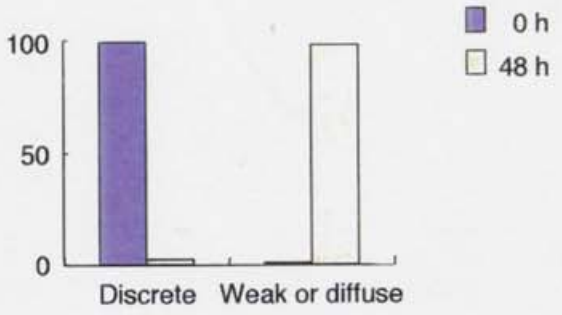
(B)



CENP-A signal

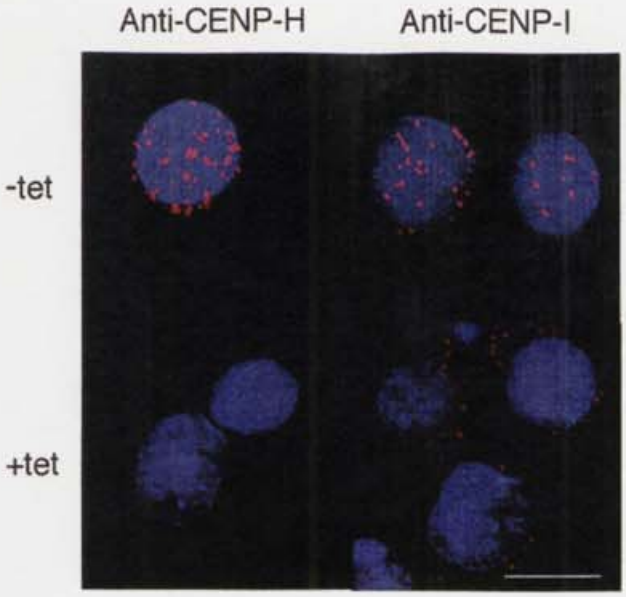


CENP-C signal

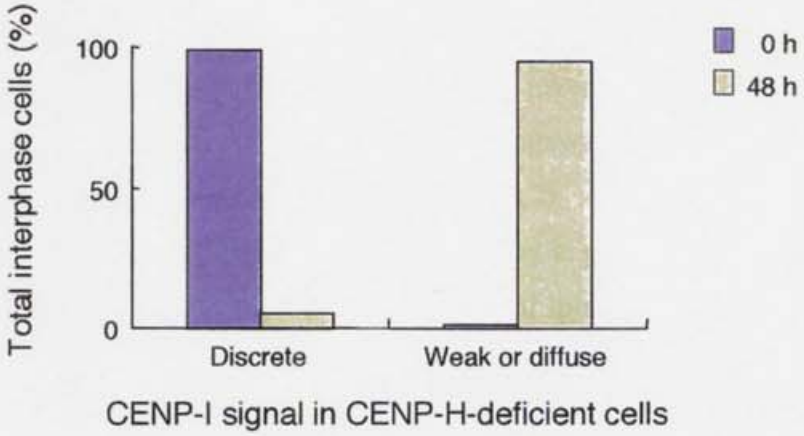


CENP-H signal

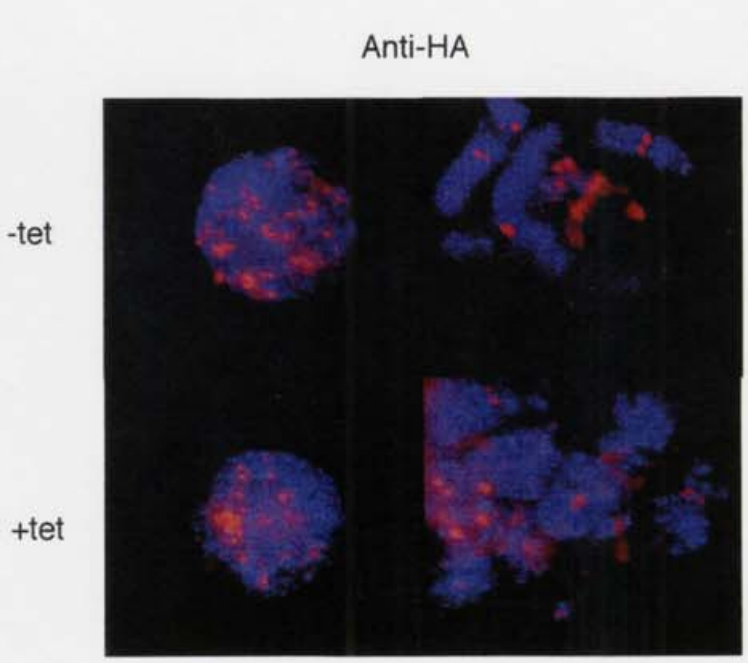
(C)


Anti-CENP-H
Anti-CENP-I
-tet
+tet

(D)


Total interphase cells (%)
100
50
0
Discrete
Weak or diffuse
0 h
48 h
CENP-I signal in CENP-H-deficient cells

(E)


Anti-HA
-tet
+tet

Chapter 3

Discussion

3.1 CENP-I is required for centromere function during mitosis

The centromere is essential for faithful chromosome segregation. Despite its crucial role in the cell cycle, relatively little is known about how the kinetochore assembles on the centromeric DNA and the nature of the kinetochore structure. *S. pombe* Mis6 play a critical role in the fidelity of chromosome segregation and contributes to construction of the centromere-specific chromatin structure (Saitoh et al., 1997; Takahashi et al., 2000). Rat and human LRP1 have sequence similarities with Mis6, but it was unknown whether the vertebrate homologs of Mis6 localize at the centromere and function in the cell cycle. In the present study, I identified a chicken homolog of Mis6 and showed that chicken Mis6 localizes to the centromere throughout the cell cycle in DT40 cells (Fig. 2.3). Therefore, I named it CENP-I. Colocalization experiments with CENP-I-GFP and antibodies against several centromere proteins revealed that CENP-I, like CENP-A, -C, and –H (Saitoh et al., 1992 Warburton et al., 1997; Fukagawa et al., 2001), localizes in or near the inner kinetochore plate, not in the outer plate (Fig. 2.2).

CENP-I-deficient cells were subject to a lengthy delay in prometaphase, characterized by the presence of hypercondensed chromosomes and abnormal spindle structures (Fig. 2.6 and Fig. 2.9). The cells eventually exited mitosis following loss of spindle checkpoint components from kinetochores, replicated the DNAs again and ultimately underwent apoptosis. Lengthy delays in prometaphase had previously been observed in cells with targeted disruptions of CENP-C and CENP-H (Fukagawa and Brown, 1997; Fukagawa et al., 2001), suggesting that this phenotype is characteristic of the loss of inner kinetochore plate function in vertebrate cells.

S. pombe Mis6 acts at the G1/S transition (Saitoh et al., 1997). Mitotic defects caused by CENP-I deficiency also occur after passage through G1 phase (Fig. 2.7), suggesting that a functional "centromere chromatin" structure is formed during interphase. This was also the conclusion of researchers who performed experiments in which the effects of microinjection of anti-centromere antibodies on the cell cycle were examined (Bernat et al., 1990; 1991).

3.2 Role of CENP-I in assembly of the inner kinetochore

Recent data suggest that centromere-specific nucleosome structures, including CENP-A, may determine centromere identity (Karpen and Allshire, 1997; Ahmad and Henikoff, 2001; Choo, 2001; Lo et al., 2001). CENP-A defines active centromere regions by forming centromere-specific nucleosomes and serves functions different from those of H3. CENP-A has a highly

conserved C-terminal domain that is believed to be very similar to that of H3 on the basis of greater than 40% amino acid identity and analysis of reconstituted nucleosomes (Henikoff et al., 2001, Yoda et al., 2000). However, the N-termini of these proteins, which vary in size from 20 to 200 aa, can not be aligned across different eukaryotic lineages. Malik et al. (2002) reported that conserved sequences that resemble minor groove DNA binding motifs found in various histone tails are present in both the histone-fold domain and the N-terminal tail of *Drosophila* Cids and mouse and human CENP-A. This suggests that the N-terminal tail has a packing function, which results in a unique chromatin organization at the primary construction, a cytological marker of centromeres. In *S. cerevisiae* and mammalian cells, the N-terminal tail of CENP-A appears to be important for recruitment and interaction of kinetochore components (Chen et al., 2000; Van Hooser et al., 2001). However, the mechanism by which CENP-A is loaded into centromeric nucleosomes and which proteins are involved with the CENP-A containing structure in vertebrate cells are not known. In fission yeast, CENP-A localization is altered in Mis6 mutants, suggesting that Mis6 may be involved in CENP-A localization to the centromere (Takahashi et al., 2000). The situation in vertebrate cells appears to be different. I found that depletion of CENP-I does not alter the discrete, typical centromeric signals of CENP-A in CENP-I-deficient cells (Fig. 2.11). Thus, CENP-I is not required for centromere targeting of CENP-A. A similar conclusion has been reached from experiments involving microinjection with anti-Mis6 antibody (Tim Yen, personal communication). Measday et al. (2002) reported that Ctf3p, the *S. cerevisiae* homologue of Mis6, is not required for loading of a CENP-A homolog, Cse4p, into the CEN sequence. Ctf3p associates with CEN DNA in a Ctf19p dependent manner, but the function is unknown. As described below (3.3, P.59), our results suggest that vertebrate kinetochores may not be assembled in a simple linear pathway.

If CENP-I is not involved in CENP-A loading into the centromeres of higher vertebrate cells, how is CENP-A targeted to centromeres? It was recently reported that synthesis of CENP-A is uncoupled from replication of centromeric DNA in human cells (Shelby et al., 2000) and *Drosophila* cells (Sullivan and Karpen, 2001). It was proposed that CENP-A deposition requires a CENP-A-specific chromatin assembly factor that adds CENP-A directly to vacant nucleosomal sites or replaces existing histone H3 with CENP-A. One candidate for this CENP-A-specific chromatin assembly factor is CAF1-like complex. CAF1 is a chromatin assembly factor for histones H3 and H4 and comprising three polypeptides, p48, p60 and p150 (Verreault et al., 1996). Change of one subunit of CAF1 could make the complex specific for CENP-A. It is interesting to observe the phenotypes with gene disruption of each subunits of CAF1 in DT40. Clarification of the mechanisms underlying formation of centromeres will contribute to our understanding of mitosis in higher vertebrate cells. The DNA and protein components specific to

centromeric chromatin evolve rapidly, although the chromosome segregation machinery is highly conserved (Henikoff et al., 2001). Incompatibilities between rapidly evolving centromeric components may be responsible for variations in the organization of centromeric regions and the reproductive isolation of new species.

Discrete centromeric CENP-C and CENP-H signals are lost in CENP-I-deficient cells (Fig. 2.11), and the normally discrete CENP-I signals are diffuse in CENP-H-deficient cells, indicating that CENP-H and CENP-I are mutually interdependent for targeting to centromeres. A preliminary *E. coli* two-hybrid analysis with CENP-H and CENP-I revealed that the N-terminal region of CENP-H interacts with the N-terminus of CENP-I. Our present data together with our previous results showing that CENP-H is necessary for recruitment of CENP-C to form a functional centromere (Fukagawa et al., 2001) suggest that formation of CENP-A-containing nucleosomes occurs first, and CENP-H and CENP-I are then targeted to these structures, possibly as a macromolecular complex. CENP-C then interacts with the CENP-A/CENP-H/CENP-I complex to form the kinetochore plate structure. During mitosis, the centromere chromatins consist of an inner plate. Centromere proteins at the interzone, outer plate and fibrous corona associate with the inner plate to form a complete centromere structure that facilitates microtubule attachment and faithful chromosome segregation during mitosis. CENP-C binds the α-satellite DNA similar to CENP-B, although only CENP-C binds active centromeres (Politi et al., 2002). CENP-A-containing non-centromeric chromatin can associate with CENP-C (Van Hooser et al., 2001), suggesting that the CENP-A/CENP-H/CENP-I chromatin may contribute to the generation of a particular DNA structure or modify upon specific DNA sequences that allow CENP-C to be recruited and assembled into the centromere chromatin.

3.3 Chromosome and microtubule aberrations in CENP-I-deficient cells

Many proteins related to cell cycle progression are essential for cell development and viability. Because CENP-I is an essential gene, a conditional knockout system is necessary for detailed analysis of the cell cycle. The DT40 cell line is suitable for such analysis because it is relatively easy to disrupt genes and generate conditional knockout mutants with tetracycline-responsive promoters. In the present study, I observed temporal accumulation of CENP-I-conditional mutant cells by two-dimensional FACS and microscopic analysis. Furthermore, I observed the abnormal behavior of single CENP-I-deficient cell in real time by living-cell analysis (Fig. 2.9). CENP-I-deficient cells were delayed in prometaphase for more than 10 h and showed aberrant mitotic structures after addition of tet. This phenotype resembled that of CENP-H-deficient cells (Fukagawa et al., 2001) and cells injected with anti-CENP-C antibody (Tomkiel et al., 1994). Inactivation of CENP-C was previously shown to alter or disrupt the kinetochore structure in

affected cells (Tomkiel et al., 1994). Earnshaw and colleagues showed that cells with disrupted kinetochores confirmed by electron microscopy could bind to microtubules and in some cases congress normally to a metaphase plate (Tomkiel et al., 1994; Bernat et al., 1991). The affected cells eventually executed a highly aberrant exit from mitosis following an extensive delay in prometaphase. Van Hooser et al. (2001) reported that experimentally mistargeted CENP-A assembles CENP-C but not the microtubule-associated factors CENP-E and ZW10. I concluded from these studies that assembly of an ordered trilaminar kinetochore structure is not required for microtubule binding but may be required for the mitotic checkpoint to be inactivated following chromosome attachment to the spindle (Bernat et al., 1991).

Our cytological and living cell analyses revealed that many chromosomes in CENP-I-deficient cells attach to the microtubules and congress normally, although I observed that in each cell several chromosomes fail to align at the metaphase plate. The similarity of this phenotype to those in our previous studies of CENP-C- and CENP-H-disrupted cells suggests that CENP-I-deficient cells lack proper kinetochore function and that the kinetochore structure may be disrupted. The metaphase delay of CENP-I-deficient cells could be explained by disruption of functional kinetochore structures. Even if microtubules attach to the kinetochores in CENP-I-deficient cells, this attachment does not appear to be strong enough to ensure proper chromosome segregation. It is also possible that metaphase delay in CENP-I-deficient cells is due to activation of a mitotic checkpoint. I observed many signals for the checkpoint protein BubR1 in prometaphase-delayed M90 cells 48 h after addition of tet. Although the kinetochore structure of M90 cells is thought be weak and unstable at 48 h after addition of tet, many chromosomes still formed attachments to microtubules (Fig. 2.6). Kinetochores that form improper or unstable microtubule attachments may continue to transmit inhibitory signals even after the chromosomes have achieved a bipolar orientation and have congressed to the spindle equator. It is likely that the prolonged mitotic delay observed in CENP-I-deficient cells is due to the combined effects of improper kinetochore structure and activation of a mitotic checkpoint. These result may consistent that the spindle checkpoint protein Mad2 localized to the metaphase chromosomes and bipolar spindle appeared to form normally in cohesin subunit Scc1-deficient DT40 cells, which show a delay in M phase with chromosome segregation defects but normal localization of CENP-H and CENP-C (Sonoda et al., 2001). In *S. cerevisiae*, mutants in several kinetochore proteins, including Ndc10p, Ctf13p, Cep3p, Spc24p, and Spc25p are defective for mitotic checkpoints, indicating that these proteins provide mitotic checkpoint signals (Cheeseman et al., 2002a). Further studies to identify the signal proteins or complexes associated with the mitotic checkpoint in vertebrates are needed.

The fact that microtubule binding and mitotic checkpoint signaling can proceed even though a number of essential components of the inner kinetochore plate are absent indicates that kinetochore assembly does not follow a simple linearly ordered assembly pathway in vertebrates. Partial outer kinetochore function is observed even when assembly of the inner kinetochore structure is disrupted. Furthermore, these results suggest that one function of the inner kinetochore plate may be to create a foundation that enables checkpoint proteins in the outer kinetochore to detect proper attachment of the chromosome to the spindle.

The lengthy delay in mitosis observed in CENP-I-deficient cells eventually ends, and the cells exit mitosis without undergoing cytokinesis. I also observed cells with 8N DNA contents by FACS analysis (Fig. 2.5 and 2.10). This phenotype likely reflects kinetochore structure and function. Although kinetochore function in M90 cells is weak and unstable at 48 h after the addition of tet, the cells still have partial kinetochore function by which checkpoint proteins are assembled. However, kinetochore function, including mitotic checkpoint function, appears to be disrupted completely by 72 h. I consistently observed immunofluorescence signals for the BubR1 checkpoint protein at 48 h after the addition of tet but BubR1 signals were not observed at 72 h after its addition (Fig. 2.10), suggesting that the kinetochore structure is disrupted completely by 72 h. Cells delayed in metaphase finally lose mitotic checkpoint function and proceed through the cell cycle without normal chromosome segregation (Fig. 2.9, 859-905 min). The cells then form a nuclear membrane around the near-tetraploid genome and eventually undergo apoptosis.

I observed dynamic behaviors of the chromosomes and the microtubules in real time during the prolonged metaphase delay of CENP-I-deficient cells (Fig. 2.9). If mitotic defects occur in cells, the cell cycle is arrested at mitosis by activation of a mitotic checkpoint (Amon, 1999). The observed duplication of centrosomes and spindles during prometaphase delay of CENP-I-deficient cells suggest that the behavior of the mitotic spindle is not influenced by the mitotic or cell cycle checkpoint for monitoring centrosome integrity and that function is disrupted in CENP-I-deficient cells. Recent studies suggest that the centrosome plays an active role not only in the regulation microtubule nucleation activity, but also in the coordination of centrosome duplication with cell cycle progression, during stress responses and cell cycle checkpoint control (Lange, 2002). In budding yeast, two spindle checkpoint proteins, Bfa1p and Bub2p, are associated with the spindle pole and bind a Ras-like GTPase, Tem1p, which is involved in regulation of mitotic exit (Pereira et al., 2000). Bfa1p and Bub2p are members of a spindle checkpoint pathway that differs from the conserved spindle checkpoint, which consists of Bub1p, Bub3p, Mad1p, Mad2p, and Mad3p. CENP-I-deficient vertebrate cells may reflect these cell cycle checkpoint proteins at the centrosome.

Centromere defects can read to genetic instability, with consequences in fertility (e.g., in wheat; Martinez-Perez et al., 2001), human disease (e.g., Roverts syndrome; Van Den Berg and Francke, 1993), and cancer (Cahill et al., 1998; Fodde et al., 2001; Kaplan et al., 2001; Michel et al., 2001). By identifying key molecules involved in centromere defects, research could lead to new diagnostic tools and treatments. Furthermore, a more complete understanding of centromere and chromosome inheritance in multicellular eukaryotes may lead to efficient, controlled used of artificial chromosome cloning vectors, and the ability to transfer intact, functional chromosomes into animal and plant cells would be useful for engineering livestock and crops and for developing gene therapy vectors for use in humans. The accumulation of knowledge in centromere biology would contribute to development of both basic biology and medical science.

Chapter 4

Materials and method

4.1 Abbreviations and acronyms

In addition to standard abbreviations for metric measurements (e.g., ml) and chemical symbols (e.g., HCl), the abbreviations and acronyms below are used throughout this study.

BrdU: 5-bromodeoxyuridine

cDNA: complementary DNA

C. elegans: *Caenorhabditis elegans*

CS: chicken serum

DAPI: 4', 6-diamidino-2-phenylindole

dATP: deoxyadenosine triphosphate

dCTP: deoxycytosine triphosphate

ddH_2O: double-distilled water

DMSO: dimethyl sulfoxide

dNTP: deoxynucleoside triphosphate

dTTP: deoxythymidine triphosphate

dUTP: deoxyuridine triphosphate

EDTA: ethylenediaminetetraacetic acid

EtOH: ethanol

FBS: fetal bovine serum

FISH: fluorescence in situ hybridization

FITC: fluorescein isothiocyanate

GFP: green fluorescent protein

HA: hemagglutinin antigen

HEPES: *N*- (2-hydroxyethyl) piperazine-*N'*- (2-ethanesulfonic acid)

Hoechst 33258: (2'[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'bi-1H)-benzimidazole

IgG: immunoglobulin G

IPTG: isopropylthio-ß-D-galactoside

LB: Luria broth

LB broth: Luria-Bertani broth

MeOH: methanol

NP-40: Nonidet P-40

OD: optical density

ORF: open reading frame

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffer saline

PCR: polymerase chain reaction

PEG: polyethylene glycol

PFA: paraformaldehyde

pfu: plaque-forming unit

PI: propidium iodide

RACE: rapid amplification of cDNA ends

RNase: ribonuclease

rpm: revolutions per minute

RT-PCR: reverse transcription followed by PCR

S. cerevisiae: *Saccharomyces cerevisiae*

SDS: sodium dodecyl sulfate

S. pombe: *Schizosaccharomyces pombe*

SSC: standard saline citrate

SV40: simian virus 40

TAE: Tris-acetate-EDTA

Taq DNA polymerase: DNA polymerase from *Thermus aquaticus*

TBE: Tris-borate/ EDTA

TE: Tris/ EDTA buffer

TEMED: N, N, N', N'-tetramethylethylenediamine

*T*m: melting temperature

UV: ultraviolet

v/v: volume/volume

w/v: weight/volume

X-gal: 5-bromo-4-chloro-3-indoyl-ß-D-galactoside

4.2 Materials

Table 4.1 Kits and devices used in this study

See detail manufacturer's protocol.

Manipulation	Kit
Excision of phagemid from λphage	ExAssist Interference-Resistant Helper Phage (Stratagene, #200253)
Removal of protruding 3' termini to blunt-ended	Blunting kit (Takara, 6025)
Ligation reaction of DNA fragments	DNA Ligation kit ver.2 (Takara, 6022)
E. coli competent cell for general transformation	DH5α (TOYOBO, DNA-903) JM109 (TOYOBO, DNA-900)
Maxi prep of DNA from λphage	QIAGEN Lambda Maxi Kit (QIAGEN, 12562)
Maxi prep of plasmid DNA from E. coli	QIAGEN Plasmid Maxi Kit (QIAGEN, 12263)
Dideoxy-mediated cycle sequencing of DNA	ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, 4303153)
Purification PCR products from sequencing reactions	SigmaSpin Post-Reaction Purification Columns (SIGMA, S-5059)
Purification of DNA fragment from gel	QIAEX II Gel Extraction Kit (QIAGEN, 20021)
Purification of DNA fragment from gel or PCR products	GFX PCR DNA and Gel Band Purification Kit (Amersham, 27-9602-01)
Labeling of DNA with ^{32}P	Megaprime DNA labeling systems (Amersham, RPN1604)
Quantification of protein	Bio-Rad *DC* Protein Assay (Bio-Rad, 71555)
Detection of specific antigens conjugated to HRP labeled antibodies	ECL Plus Western blotting detection reagents (Amersham, RPN2131)
Mini prep of genomic DNA from cells	DNeasy Tissue Kit (QIAGEN, 69506)
Maxi prep of genomic DNA from cells	QIAGEN Blood & Cell Culture DNA Maxi Kit (QIAGEN, 13362)

Detection of apoptotic cells	*In Situ* Cell Death Detection Kit, Fluorescein (Roche, 1 684 795)

Manipulation	Devices
Gel electrophoresis of DNA	Mupid (Advance), Horizontal gel electrophoresis units (Marisoru)
Mini prep of plasmid DNA from E. coli	Automatic DNA Isolation System (Kurabo)
Thermal cycler	GeneAmp PCR system 9700 (Applied Biosystems)
Analysis of DNA sequencing	ABI PRISM 377 DNA Sequencer (Applied Biosystems)
SDS-PAGE	Slab gel electrophoresis system (Bio Craft)
Western transfer	Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad)
Imaging of labeled samples in Southern and Western blotting	Storm imaging system (Molecular Dynamics)
Flow cytometry analysis	EPICS ALTRA (Beckman Coulter)
Electropolation	GENE PULSER II (Bio-Rad)

Table 4.2 Primers for CENP-I cDNA used in this study

Arrows indicate the forward primer (→) or the reverse primer (←). Small letters indicate added sequence for subcloning, underlines indicate the site of restriction enzyme.

Name	ORF No. (1=ATG)	Length (mer)	Sequence (5'-3')
mis6-6a	-7~15	→ 30	cggaattcTGAAAAGATGCAACGAAGACAG
mis6-75	-7~60	→ 75	cggaattcTGAAAAGATGCAACGAAGACAGA
			GTTCTAAGCACTCCAAGCGACCCCTGCAA
			GTTCACCACAGCAAT
rMis6-2	30~50	← 21	TGAACTTGCAGGGGTCGCTTG
rMis6-1	54~75	← 22	AGAGAGATCAGTCTGATTGCTG
rMis6-4	115~135	← 21	CAGAGATTGACGATTCTGGGC
rMis6-3	153~174	← 22	CTCAAGGGAGTCTTGCTCGTTG
mis6-h	339~356	→ 18	TGATACAGTGAATACTCG
mis6-6b	339~361	→ 23	TGATACAGTGAATACTCGTTTAC
mis6-6f	502~522	← 21	GGCATGAACTTGTTCCTTGTG
mis6-I	639~661	← 18	TTTGATTGGAGATCAAGG
mis6-6c	711~731	→ 21	TCCTGAGCTGGTATCCATAAC
mis6-6e	850~870	← 20	TGAGTGAGGTTGAGCTGTGC
mis6-6d	1207~1229	← 23	GTTTCCAGGAAGCCTCTGAATTC
mis6-g	2085~2103	→ 19	CTGGCCAGATGATACCACC
mis6-EV	2143~2168	→ 26	TATCTGAGATATCTCTACGGGCAAGG
mis6+Xba	2242~2259	← 26	gctctagaCACTTTCTCATCCTCATC
			(connected XbaI site next to the stop codon)
mis6+Xho	2241~2259	← 30	ccgctcgagtcCACTTTCTCATCCTCATCC
			(connected XhoI site next to the stop codon)
M6-8	334~351	← 29	cggaattctcaATTCACTGTATCAGCAAG
M6-9	352~370	→ 27	cggaattcACTCGTTTACTGAAGAGCC
M6-10	897~915	← 30	cggaattctcaACTGCTTGCAGGTACAATC
M6-11	916~934	→ 27	tccccggGCCAACGCACAGAATTTAG
M6-12	1498~1515	← 30	tccccgggtcaTTCTGAATCCAGCTGAAC
M6-13	1516~1533	→ 26	cggaattcTCAGATTCTCAATTCAGT

M6-14	2245~2262	← 26	cggaattcTCACACTTTCTCATCCTC
M6-15	1061~1078	→ 18	GCTCAGTGCTAACAAACC
M6-16	1656~1673	→ 18	CCTGGATTTCTACGAGAC
M6-17	2125~2142	← 18	CCAGTTCCACTTCTTTCC

4.2.1 Constructed plasmids for this study

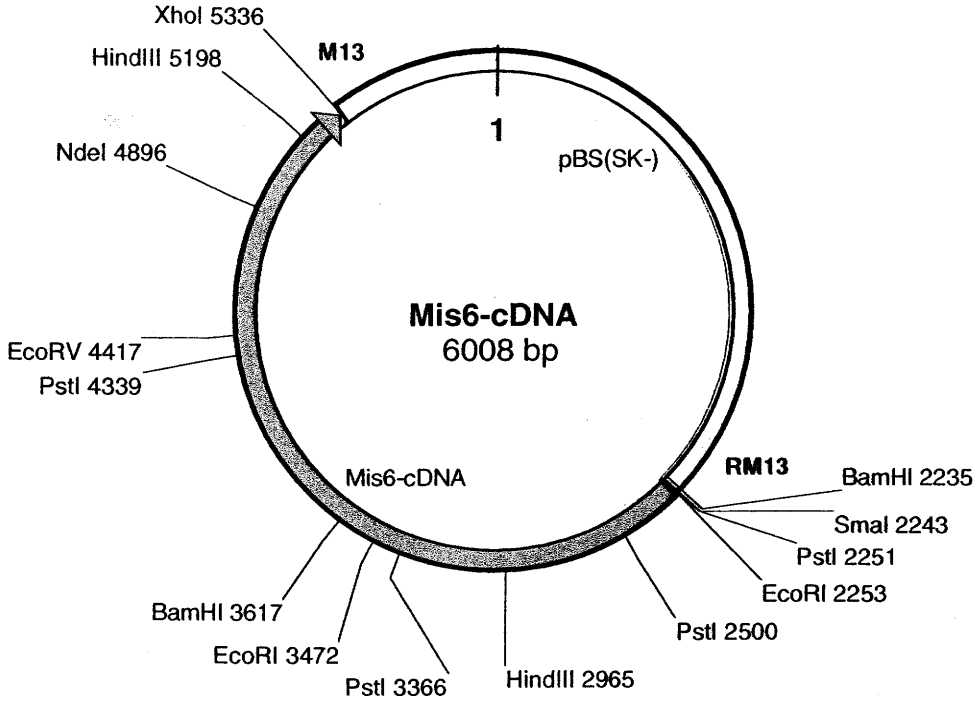


Fig. 4.1 Restriction map of Mis6-cDNA plasmid

Full-length CENP-I cDNA (described as Mis6 cDNA in this figure) is cloned into the EcoRI site (2253) and the XhoI site (5336) of pBluescript (SK-). The ORF of CENP-I is from 2266 to 4527, 753aa.

1) Mis6-37 plasmid was digested with EcoRI, dephosphorylated 5'- phosphate residues with SAP and then purified.
2) A 1.2-kb fragment (2253-3472 in this figure) was amplified with Mis6-37 plasmid as a template, mis6-75 primer, and mis6-6d primer. Then the product was digested with EcoRI, and then purified.
3) These fragments were connected by ligase reaction, then transformed and purified.

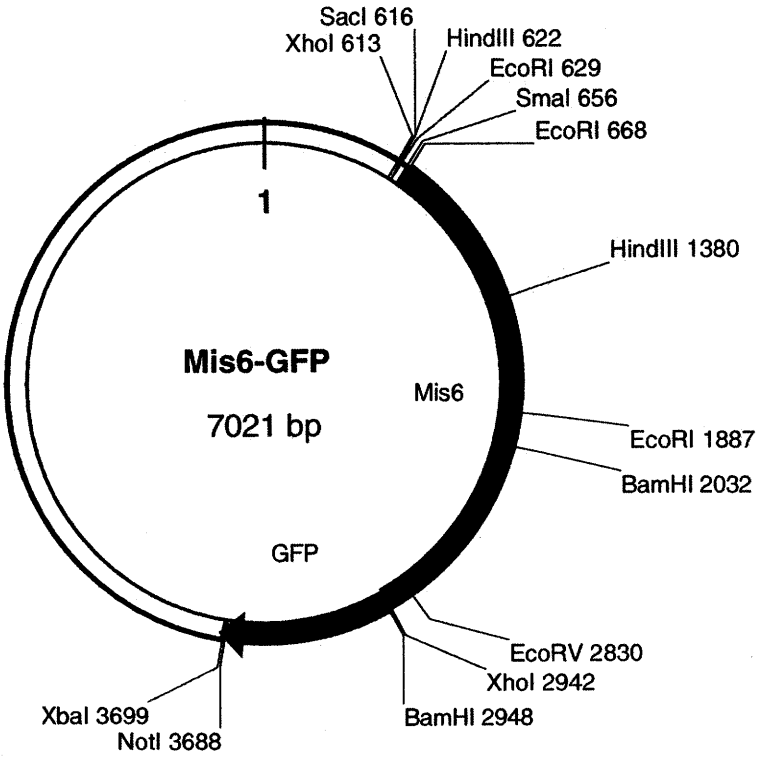


Fig. 4.2 Restriction map of Mis6-GFP plasmid

CENP-I cDNA (described as Mis6 cDNA in this figure) is cloned into the SmaI site (656) of pEGFP-N1 (Clontech). CENP-I expresses as a fusion protein with a red-shifted variant of wild-type GFP in DT40 cells.

1) Mis6-cDNA plasmid was digested with EcoRV and XhoI, purified the 5.1-kb fragment.
2) A 110-bp fragment was amplified with Mis6-cDNA plasmid as a template, mis6-EV primer and mis6+XhoI primer. The product was digested with EcoRV and XhoI, and then purified.
3) These fragments were connected by ligase reaction, then transformed and purified. The subclone was digested with SmaI and XhoI, filled protruding 5' termini with the Klenow fragment, and purified 2.3-kb fragment.
4) pEGFP-N1 was digested with SmaI, dephosphorylated 5'- phosphate residues with SAP and purified.
5) These fragments were connected by ligase reaction, then transformed and purified.

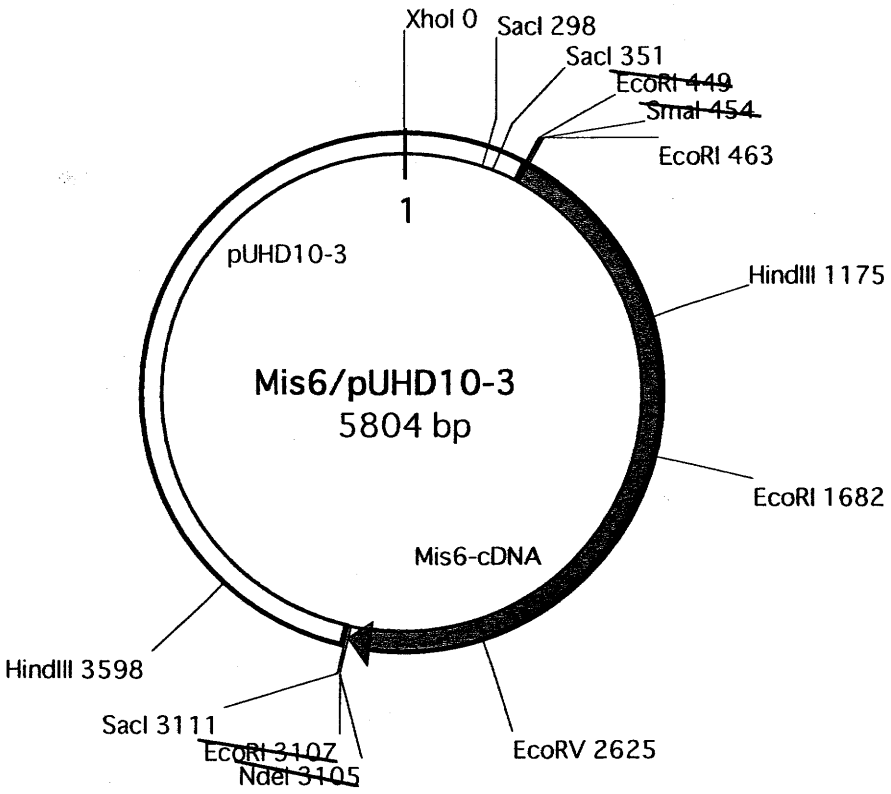


Fig. 4.3 Restriction map of Mis6/pUHD10-3 plasmid

CENP-I cDNA (described as Mis6 cDNA in this figure) is cloned into the EcoRI site of pUHD10-3 (Gossen and Bujard, 1992).

1) pUHD10-3 was digested with EcoRI, dephosphorylated 5'- phosphate residues with SAP (Roche, Phosphatase alkaline shrimp, 1 758 250), filled protruding 5' termini with the Klenow fragment and purified 3.15-kb fragment.
2) Mis6-cDNA plasmid was digested with SmaI and NdeI, filled protruding 5' termini with the Klenow fragment (Nippon gene, 312-00814) , and purified 2.65-kb fragment.
3) These fragments were connected by ligase reaction, then transformed and purified.

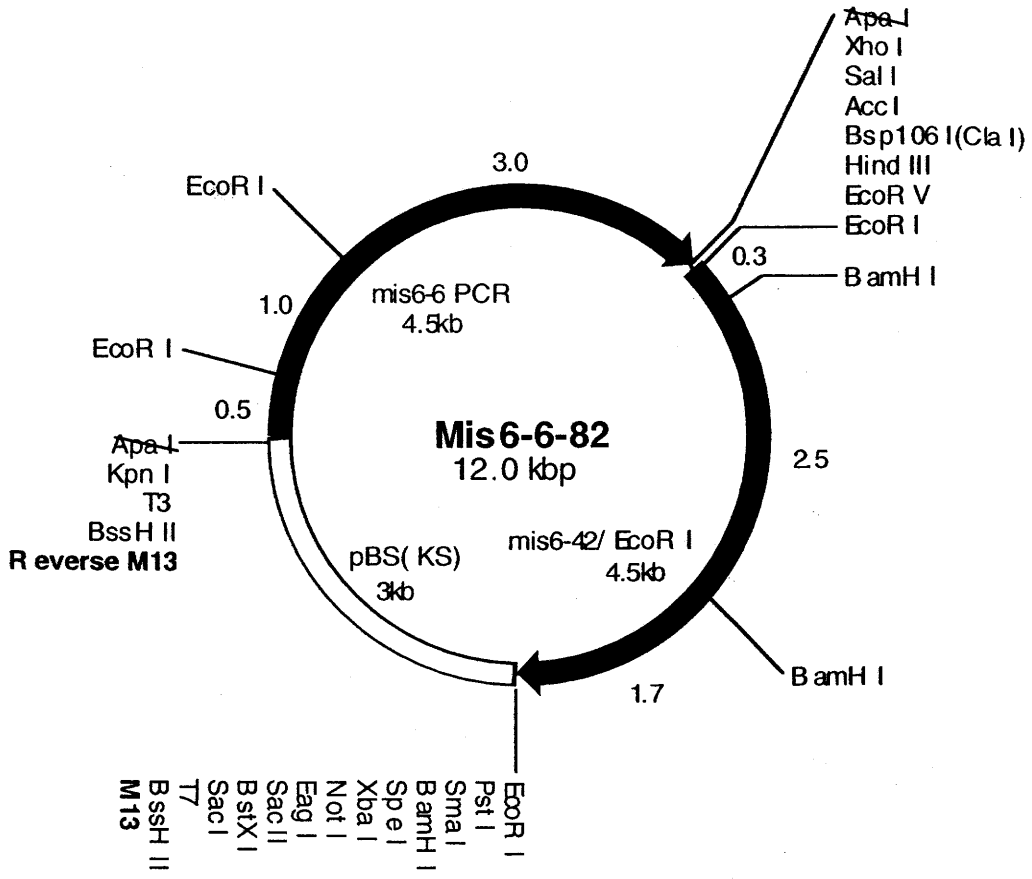


Fig. 4.4 Restriction map of Mis6-82 plasmid

The left (4.5-kb) and right (4.5-kb) arms of CENP-I for the disruption constructs are cloned sequentially into pBluescript (Stratagene). This plasmid was used for construction of Mis6-his, Mis6-puro, Mis6-neo plasmid.

1) pBluescript (KS) was digested with EcoRI, dephosphorylated 5'- phosphate residues with SAP and purified.
2) Mis6-42 plasmid was digested with EcoRI, purified a 4.5-kb fragment. These fragments were connected by ligase reaction, then transformed and purified.
3) The subclone was digested with ApaI, removed 3' protruding termini with Blunting kit (Takara), dephosphorylated 5'- phosphate residues with SAP and purified.
4) A 4.5-kb fragment was amplified with Mis6-6 plasmid as a template, Reverse M13 primer, Mis6+X primer. The product was digested with SmaI and XhoI, filled protruding 5' termini with the Klenow fragment and then purified.
5) The fragments in step 3) and 4) are connected by ligase reaction, then transformed and purified.

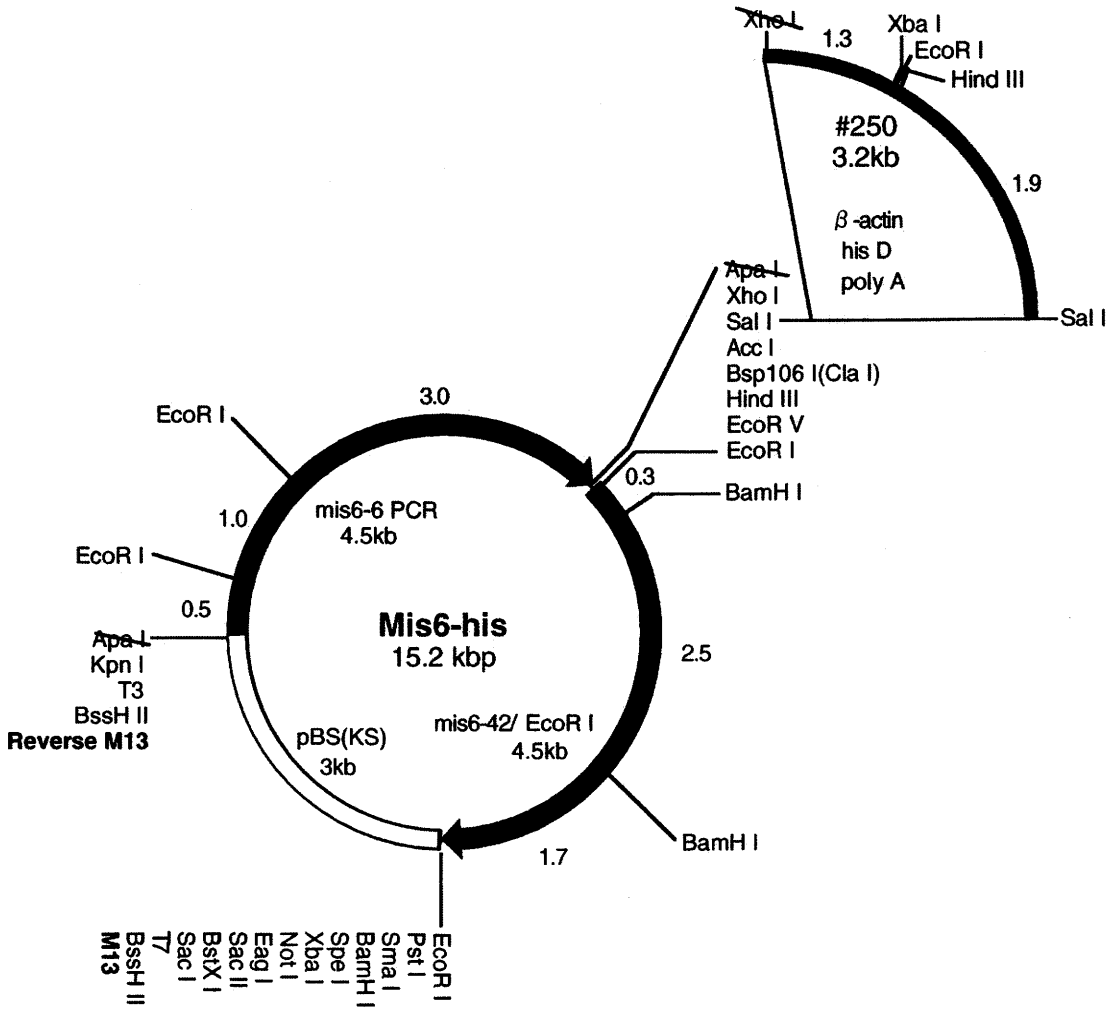


Fig. 4.5 Restriction map of Mis6-his plasmid

Histidinol-resistance cassette under control of the ß -actin promoter is inserted into the SalI site of Mis6-6-82 plasmid.

1) Mis6-6-82 plasmid was digested with SalI, dephosphorylated 5'- phosphate residues with SAP, filled protruding 5' termini with the Klenow fragment and purified.
2) #250 plasmid was digested with XhoI and SalI, filled protruding 5' termini with the Klenow fragment and purified a 3.2-kb fragment.
3) These fragments were connected by ligase reaction, then transformed and purified.

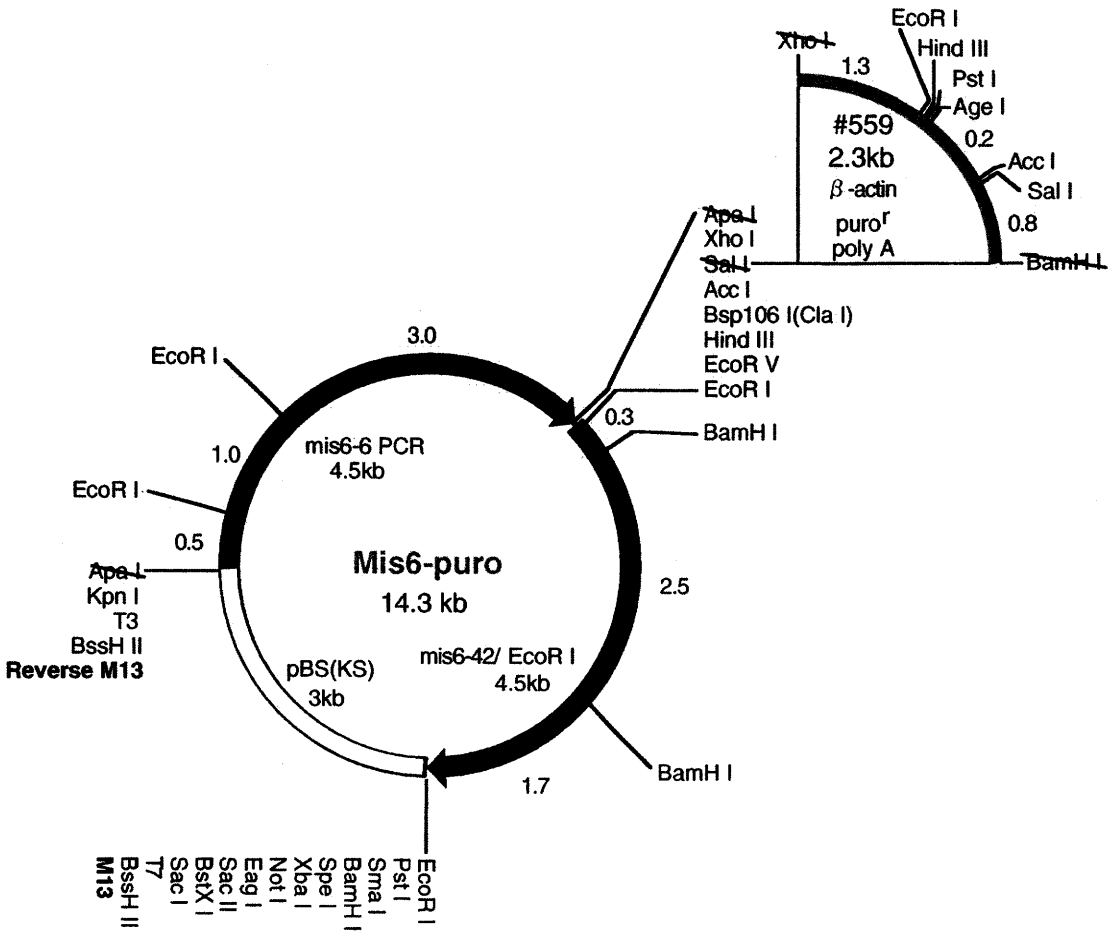


Fig. 4.6 Restriction map of Mis6-puro plasmid

Puromycin-resistance cassette under control of the ß -actin promoter is inserted into the SalI site of Mis6-6-82 plasmid.

1) Mis6-6-82 plasmid was digested with SalI, dephosphorylated 5'- phosphate residues with SAP, filled protruding 5' termini with the Klenow fragment and purified.
2) #559 plasmid was digested with XhoI and BamHI, filled protruding 5' termini with the Klenow fragment and purified a 2.3-kb fragment.
3) These fragments were connected by ligase reaction, then transformed and purified.

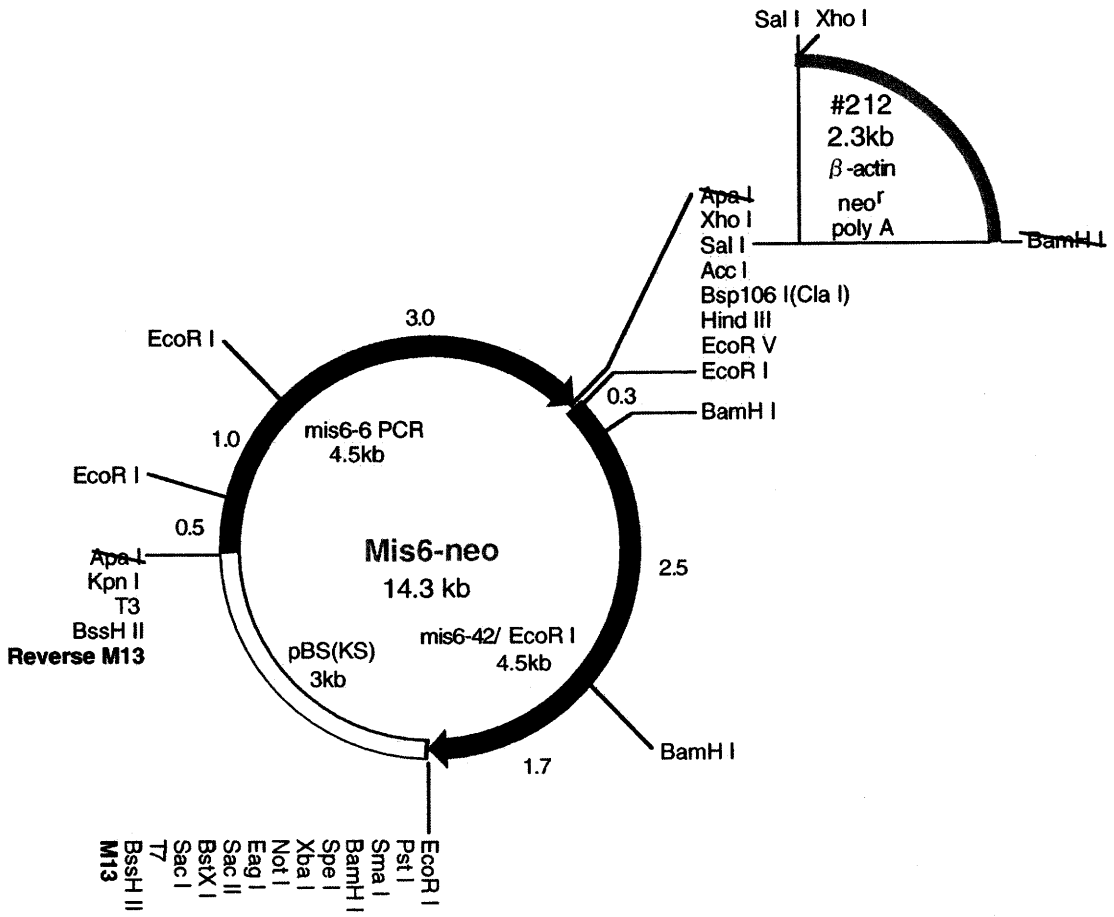


Fig. 4.7 Restriction map of Mis6-neo plasmid

Neomycin-resistance cassette under control of the ß -actin promoter is inserted into the SalI site of Mis6-6-82 plasmid.

1) Mis6-6-82 plasmid was digested with SalI, dephosphorylated 5'- phosphate residues with SAP, filled protruding 5' termini with the Klenow fragment and purified.
2) #212 plasmid was digested with SalI and BamHI, filled protruding 5' termini with the Klenow fragment and purified a 2.3-kb fragment.
3) These fragments were connected by ligase reaction, then transformed and purified.

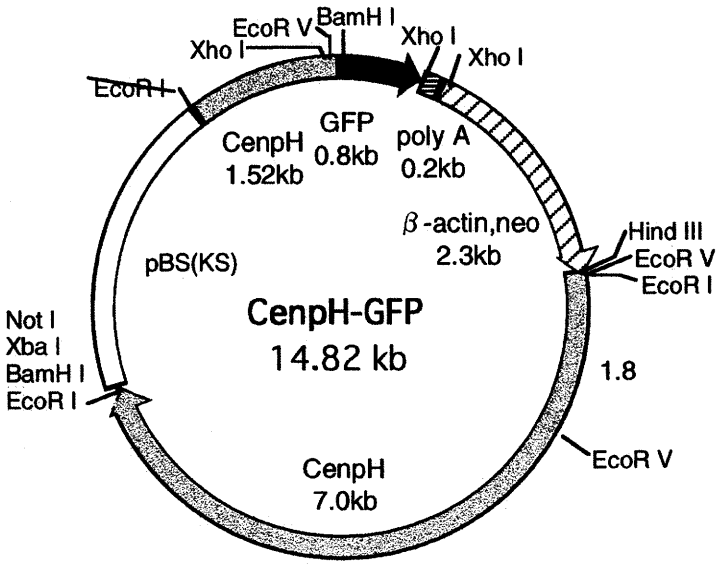


Fig. 4.8 Restriction map of CenpH-GFP plasmid

A-10kb fragment containing the last exon of CENP-H into pBluescript and replace the stop codon in the last exon with a GFP sequence followed by neomycin-resistance cassette driven by the ß -actin promoter.

1) CH128-8-58-40 plasmid (described below) was digested with BamHI and NotI, purified a 4.5-kb fragment.
2) pEGFP-N1 (Clontech) plasmid was digested with BamHI and NotI, purified a 0.8-kb fragment. These fragments were connected by ligase reaction, then transformed and purified (CH 128-8-58-40-2 plasmid).
3) #212-19 plasmid (described below) was digested with Asp718 (KpnI), dephosphorylated 5'-phosphate residues with SAP, filled protruding 5' termini with the Klenow fragment, and purified.
4) CH 128-8-58-40-2 plasmid was digested with EcoRI and NotI, filled protruding 5' termini with the Klenow fragment, and purified a 2.3-kb fragment. These fragments were connected by ligase reaction, then transformed and purified (#212-19-16 plasmid).
5) #212-19-16 plasmid was digested with EcoRI, dephosphorylated 5'- phosphate residues with SAP and purified.
6) CH 128 plasmid was digested with EcoRI, purified a 7.0-kb fragment.
7) These fragments were connected by ligase reaction, then transformed and purified.

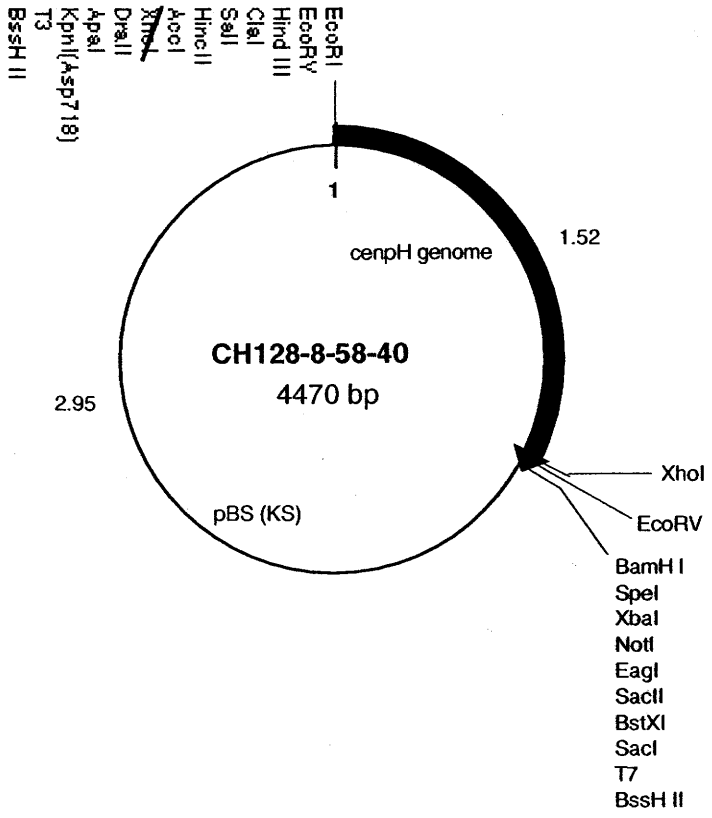


Fig. 4.9 Restriction map of CH128-8-58-40 plasmid

CENP-H genome including from the first exon to the just before the stop codon in the last exon, was inserted into pBluescript. Thiis plasmid was used for construction of Cenp-H-GFP plasmid.

1) pBluescript (KS, Stratagene) was digested with EcoRI, dephosphorylated 5'- phosphate residues with SAP and purified.
2) CH128 was digested with EcoRI, purified a 2.0-kb fragment. These fragments were connected by ligase reaction, then transformed and purified (CH 128-8 plasmid).
3) CH 128-8 plasmid was digested with XhoI partially, filled protruding 5' termini with the Klenow fragment, and purified. This fragment was circulated by ligase reaction, then transformed and purified (CH 128-8-58 plasmid; only the XhoI site in multi cloning site was broken).
4) CH 128-8-58 plasmid was digested with XhoI and BamHI, purified a 4.5-kb fragment.
5) A 32-bp fragment amplified by PCR with CH 128-8 plasmid as a template, CenpH gfp-1 primer and CenpH gfp-2 primer. The product was digested with XhoI and BamHI, purified. These fragments were connected by ligase reaction, then transformed and purified.

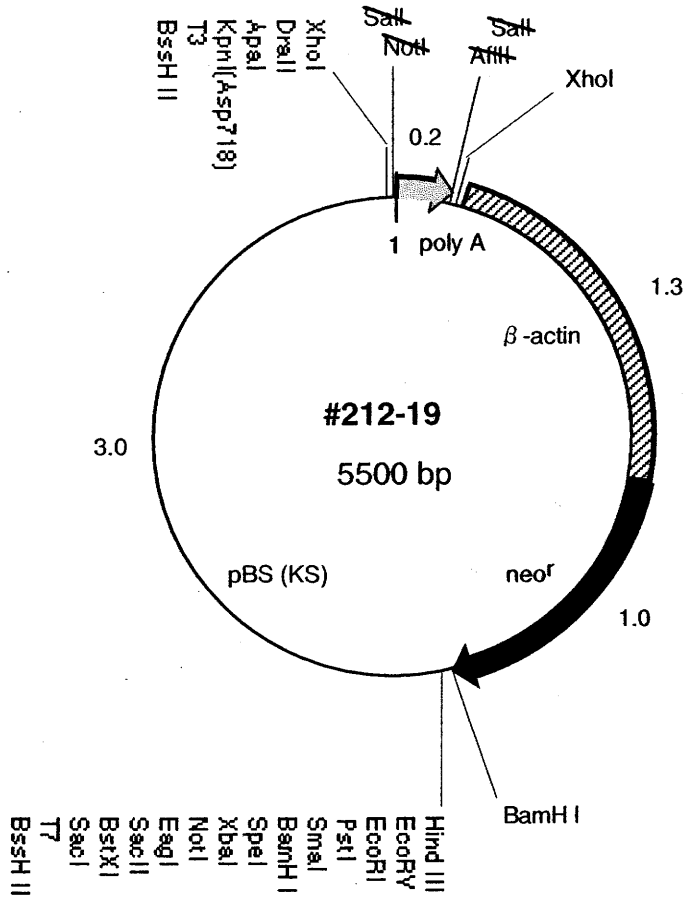


Fig. 4.10 Restriction map of #212-19 plasmid

A 0.2-kb poly A fragment was inserted into #212 plasmid (Fukagawa) before ß -actin, neomycin-resistance gene. Thiis plasmid was used for construction of Cenp-H-GFP plasmid.

1) #212 plasmid was digested with SalI, dephosphorylated 5'- phosphate residues with SAP, filled protruding 5' termini with the Klenow fragment, and purified.
2) pEGFP-N1 plasmid (Clontech) was digested with NotI and AflII, filled protruding 5' termini with the Klenow fragment, and purified a 0.2-kb fragment.
3) These fragments were connected by ligase reaction, then transformed and purified.

4.2.2 Cells

Table 4.3 CENP-I Cell lines constructed in this study

All cell lines described below was derived from DT40 (CL18) and made by transfection.

Cell line	Clone No.	Selectant for disrupted CENP-I allele(s)			Randomly integrated cDNA and selectant	
Mis6-1st	2	his	wt	wt		
Mis6-2nd	37	his	puro	wt		
Mis6-2nd+cDNA	11	his	puro	wt	Mis6-pUHD10-3 zeo	
Mis6-3rd (M90)	90	his	puro	neo	Mis6-pUHD10-3 zeo	
Mis6-3rd-GFP		his	puro	neo	Mis6-pUHD10-3 zeo	Mis6-GFP hyg
Mis6-3rd-GFP		his	puro	neo	Mis6-pUHD10-3 zeo	Mis6-GFP blast
Mis6-3rd-Tub		his	puro	neo	Mis6-pUHD10-3 zeo	pEGFP-Tub hyg

his: histidinol, puro: puromycin, neo: neomycin (G418, geneticin), zeo: zeocin, hyg: hygromycin, blast: blasticidin

4.2.3 Culture of DT40 cell line

Medium (500 ml): Store at 4°C, use in a month.

50 ml of FBS (EQUITECH-BIO, Lot No. SFB30-1027)

10 ml of CS (Gibco, Lot No. 294884)

50 μl of 1 M ß-mercaptoethanol

5 ml of Penicillin- Streptomycin (10000 units/ml penicillin and 10000 μg/ml streptomycin, Gibco, 04031)

4.3 Isolation of chicken CENP-I

The chicken CENP-I gene was isolated by degenerate RT-PCR of mRNA extracted from DT40 cells. The sequences of the degenerate primers were 5' - TC(ACG) A(AG)(AG) CA(AG) TG(AGT) AT(AG) TT and 5'- TA(CT) AA(AG) TT(CT) TT(CT) GC(ACT) CC. We screened a chicken testis cDNA library (Stratagene) constructed in λZAPII phage with the RT-PCR product as a probe. Several independent positive clones were sequenced and identified as full-length chicken CENP-I cDNAs. The translational initiation site was confirmed by 5'-RACE method. We used the chicken CENP-I cDNA as a probe to isolate genomic clones specific for CENP-I from a DT40 genomic DNA library as described (Fukagawa and Brown, 1997). The genomic structure of the CENP-I clone thus obtained was determined by PCR and partial sequencing.

4.4 Electroporation of chicken DT40 cells

4.4.1 Preparation of linearlized plasmid DNA solution for stable transfection

1) Digest the plasmid DNA with appropriate restriction enzyme described below for overnight.
2) Check the structure of plasmid in 1 μl aliquot of the reaction mixture followed by gel electrophoresis.
3) Extract the reaction mix with phenol: chloroform.
4) Precipitate the plasmid DNA by adding salt and ethanol. After centrifuge, manipulations should be done in a clean bench for cell culture.
5) Rinse the pellet 70% ethanol and store the open tube at room temperature until the ethanol has evaporated. Do not dry up until no fluid is visible in the tube.
6) Add an appropriate volume of PBS described the table below. Store the DNA solution at 4 °C over 1h to dissolve the pellet completely.

Table 4.4 Digestion of plasmid DNAs for stable transfection

Plasmid DNA	*Volume (μg)*	*Restriction enzyme*	*Final PBS (ml)*
Mis6-his	30	Not I	0.2
Mis6-puro	30	Not I	0.2
Mis6-pUHD10-3	100	Xho I	0.1
tTA-Bleo	15 or 20	Sca I	0.1
Mis6-neo	30	Not I	0.2
pEGFP-tub	100	Mlu I	0.1
#257	25	BamH I	0.1
Mis6-GFP	100	Afl II	0.1
#257	25	BamH I	0.1
Mis6-GFP	100	Afl II	0.1
#134	20	Not I	0.1
CenpH-GFP	30	Not I	0.2

4.4.2 Preparation of plasmid DNA solution for transient transfection

1) Precipitate 5.0-7.5 μg plasmid DNA (not linearlized) as described step 4)-5) above.
2) Dissolve the pellet in 200 μl of PBS and store at 4°C over 1h.

4.4.3 Stable transfection

1) To maximize the effect of transfection, feed cells to be the mid- to late-logarithmic phase of growth at a concentration of ~5 x 10^5 cells/ml in 75 cm^2 flask with fresh medium about 24 h prior to the experiment.

2) Transfer cell suspension into a 50 ml tube, centrifuge at 1000rpm for 5min. Resuspend the cell pellet in 1 ml PBS (-).

3) Mix 40 μl aliquots of the cell suspension and 160 μl Trypan-Blue (GIBCO BRL) in a 1.5 ml tube, estimate the number of living cells using a hemocytometer. During cell count, cell suspension in the 50 ml tube should be on ice.

4) Adjust the concentration of the cell suspension at 1.667 x 10^7 cells/ml by PBS (-) or centrifuge and resuspend in smaller volume of PBS (-).

5) Add an aliquot of 0.6 ml single cell suspension (1 x 10^7 cells) and 0.2 ml linearized plasmid DNA solution (described above) to a 0.4 cm Gene Pulsar cuvette (BioRad, 165-2088).

6) Mix by pipetting gently and incubate on ice for 5 min. Do not introduce air bubbles into the suspension during the mixing step.

7) Set the parameters on the BioRad Gene Pulsar II as see the table below.

8) Wipe out water around the cuvette, put in the BioRad Gene Pulsar II, and discharge the device. After the electroporation, incubate the cuvette on ice for 5 min.

9) Dilute the electroporated cells in a fresh medium without selectant (s) as described the table below.

10) Feed 100 μl cell suspension per well and incubate 37°C for 24 h.

11) Add 100 μl fresh mediums with selectant (s) in double concentration.

12) Incubate at 37°C for 1-2 weeks by the time that colonies should be visible by naked eye.

13) Suspend the colony by pipetting and transfer the cell suspension to 24 well plates. Then glow cells in two 25-cm2 flasks.

14) Harvest the cell of one flask in the mid- to late-logarithmic phase of growth by centrifuge at 1000rpm for 5min. Suspend the pellet in 2 ml freezing medium (Cell Banker; NZY Biochemicals, BLC-1) and add to two Cryogenic vial s (Nalgene), 1 ml of cell suspension/ vial.

15) The cell of another flask in the late logarithmic phase of growth is used for purification of genomic DNA and southern blotting analysis.

4.4.4 Transient transfection

1) Same as step 1)-3).

2) Adjust the concentration of the cell suspension at 8.335 x 10^6 cells/ml by PBS (-) or centrifuge and resuspend in smaller volume of PBS (-).

3) Add an aliquot of 0.6 ml of single cell suspension (5 x 10^6 cells) and 0.2 ml of 5.0-7.5 μg circular plasmid DNA solution to a 0.4 cm BioRad Gene Pulsar cuvette.

4) Same as step 6)-8).

5) Glow the cell in 75 cm^2 flasks. Incubate at 37°C for 36 h, then harvest cells for immunocytochemistry.

Table 4.5 Parameters for electroporation

Parameter	Stable transfection	Transient transfection
Cells per electroporation (suspend in 0.6 ml PBS)	1 x 10^7 cells	5 x 10^6 cells
Voltage	0.55 kV	0.25 kV
Resistance	25 μF	975 μF
Resulting time constant	0.6-0.68 msec	18.0-20.0 msec

Table 4.6 Dilution of cell suspension after electroporation for stable transfection

Clone	Selectant(s)	Medium	Concentration	96 well plate
M6-1st	his	50 ml + α	x 1	x 5
M6-2nd	his, puro	50 ml + α	x 1	x 5
M6-2nd+cDNA	his, puro, zeo	70 ml + α	x 1	x 7
M6-3rd (M90))	his, puro, zeo, G418	100 ml + α	x 1	x 10
M6-3rd-GFP	his, puro, zeo, G, hyg	50 ml + α	x 1	x 10
M6-3rd-GFP	his, puro, zeo, G, blast	100 ml + α	x 1	x 10
M6-3rd-Tub	his, puro, zeo, G, hyg	100 ml + α	x 1	x 10
CENP-H-GFP	G418	150 ml	x 1	x 3
			x 1/2	x 3
			x 1/4	x 8

his: histidinol, puro: puromycin, G: G418 (geneticin), zeo: zeocin, hyg: hygromycin, blast: blasticidin

Table 4.7 Selectants used in stable transfection

Selectant (supplier)	Concentration of stock solution	Final concentration for DT40 cells	Dissolve in
L-histidinol (Sigma, H-6647)	100 mg/ml	1 mg/ml	PBS (-) *1
puromycin (Clontech, 8052-1)	1 mg/ml	0.5 μg/ml	dd H_2O

zeocin (Invitrogen, 45-0430)	100 mg/ml	1 mg/ml	Purchased in liquid
geneticin (Sigma, G-9516)	100 mg/ml	2 mg/ml	dd H_2O
hygromycin B (Wako, 085-06153)	100 mg/ml	2.5 mg/ml	PBS (-)
blasticidin S hydrochloride (Waken, KK-400)	10 mg/ml	25 μg/ml	dd H_2O
tetracycline (Sigma, T-7660)	1 or 10 mg/ml	2 μg/ml	50% EtOH

*1: Adjust pH to 7 with 1 M NaOH (0.33 volumes).

4.5 Southern blotting and hybridization

4.5.1 Plating bacteriophage

Materials

LB medium

1mM $MgSO_4$ (100 ml): Sterilize by autoclave and store at room temperature.

SM buffer (plus gelatin; 1 litter): Sterilize by autoclave and store at room temperature.

5.8 g of NaCl

2.0 g of magnesium sulfate

50 ml of Tris-Cl (pH 7.5)

0.1 g of gelatin

NZY plates (5-6 of 150-mm plates/1 litter of NZY): Sterilize plates by irradiating UV for 30 min in a bench before pouring autoclaved medium.

5.0 g of NaCl

2.0 g of magnesium sulfate

5.0 g of yeast extract

10.0 g of NZ amine

Adjust pH at 7.5 with NaOH and adjust the volume (NZY medium).

Add 15 g of agar and sterilize autoclave. Cool to 37°C, pour into plates, harden at room temperature and store at 4°C.

NZY Top agarose (200 ml): Add 1.4 g of agarose to 200 ml of NZY medium. Sterilize by autoclave. Melt the top agarose just before use by autoclave and store in a 47°C water bath to prevent the agarose from solidifying until needed.

Method

1) Incubate NZY medium with a single bacterial colony of the *E. coli* XL1-Blue MRA (P2) (Stratagene) strain at 30°C.
2) Centrifuge the cells at 3000 rpm for 10 min. Discard the supernatant, and resuspend the pellet in 5 ml of 10 mM $MgSO_4$.
3) Measure the OD_{600} of the resuspended cells, dilute the cells to a final concentration of 0.5 OD_{600} with 10 mM $MgSO_4$. Chill on ice.
4) Make a dilution (in SM plus gelatin) of the bacteriophage stock. Mix aliquots of the diluted bacteriophage stock with 300 μl of the bacteria suspension. Incubate the infected bacterial cultures for 15 min at 37°C.
5) Add to each aliquot of infected cells 6.5 ml of molten (47°C) top agarose. Pour the contents of each tube onto separate, numbered 150-mm agar plates.

6) Close the plates, allow the top agarose to harden, and incubate at 37°C in an inverted position until plaques appear (10-12 h).

7) Chill the plates for at least 1 hr at 4°C to allow the top agarose to harden.

4.5.2 Transfer DNA from gel-plated phage to nylon membranes

Materials

Whatman 3MM paper (Whatman, 3030 917): Cut a piece of paper square with sides 25 centimeters long.

Hybond-N^+ (Amersham, RPN303B): Cut a piece of membrane square with sides 20 centimeters long.

21-gauge needle

Denaturing solution (1 litter): Store at room temperature.

87.66 g of NaCl

20.0 g of NaOH

Neutralizing solution (pH 7.2; 1 litter): Sterilize by autoclave and store at room temperature.

60.6 g of Tris

87.7 g of NaCl

0.37 g of EDTA

40.4 ml of 14 N HCl

20x SSC (1 litter): Sterilize by autoclave and store at room temperature.

175.2 g of NaCl

88.2 g of trisodium citrate dihydrate

0.4 N NaOH (500 ml): Store at room temperature in a plastic bottle.

5x SSC (1 litter): Sterilize by autoclave and store at room temperature.

Method

1) Number the dry filters with a soft-lead pencil then remove the plates from the cold room or refrigerator.

2) Place a dry, labeled filter neatly on to the surface of the top agarose so that it comes into direct contact with the plaques. Do not attempt to adjust the position of the filter and be careful not to trap air bubbles.

3) Mark the filter in five or more asymmetric, peripheral locations by stabbing through it and into the agar beneath with a 21-gauge needle. Mark the same point at the bottom of plate with a permanent marker, too.

4) After 1-2 min placing the filter on the top agarose, peel the filter from the plate gently.

5) Transfer the filter, plaque side up, to a sheet of Whatman 3MM paper impregnated with denaturing solution in a tray for 7 min.

6) Transfer the filter, plaque side up, to a sheet of Whatman 3MM paper impregnated with neutralizing solution in a tray for 3 min.

7) Transfer the filter, plaque side up, to a sheet of Whatman 3MM paper impregnated with 2x SSC in a tray for 5 min.

8) Transfer the filter, plaque side up, to a sheet of Whatman 3MM paper, and dry at the room temperature for 20 min to 1 h.

9) Transfer the filter, plaque side up, to a sheet of Whatman 3MM paper impregnated with 0.4 N NaOH in a tray for 20 min.

10) Soak the membrane in 5x SSC for 1 min. Dry the filter on a sheet of Whatman 3MM paper.

11) Store the filter in a heat-sealable bag at room temperature.

4.5.3 Digestion of genomic DNA and fractionation by gel electrophoresis

1) Grow cells in logarithmic growth phase in a 25 cm^2 flask.

2) Harvest cells and purify their genomic DNA by QIAGEN DNeasy Tissue kit.

3) Set up the reaction according to the following pipetting scheme:

purified genomic DNA	75 μl
buffer	20 μl
restriction enzyme (see the table below)	5 μl
Final volume	100 μl

Clone	Restriction enzyme
M6-1st	Sac I
M6-2nd	Sac I
M6-2nd + cDNA	EcoRI I
M6-3rd + cDNA	Sac I

4) Incubate at 37°C for 16-24 h. Concentrate the DNA fragments by ethanol precipitation.

5) Dry the DNA at room temperature to remove ethanol and dissolve the DNA in 10 μl of 10x gel-loading buffer.

6) Separate the fragments of DNA by electrophoresis through a 0.7% agarose gel in 1x TAE under 40 volts.

7) Stain the gel with ethidium bromide and photograph the gel.

4.5.4 Transfer DNA from agarose gels to nylon membranes

Materials

Hybond-N^+ (Amersham): Cut square with sides 12.5 centimeters long.

VacuGene XL vacuum blotting system (Amersham, 82-1266-24)

Whatman 3MM paper (Whatman, 3030 917)

a heat-sealable bag

0.25 N HCl (1 litter): Store at room temperature.

Denaturing solution (1 litter): Described above.

Neutralizing solution (pH 5.0; 1 litter): Sterilize by autoclave and store at room temperature.

121.1 g of Tris

116.9 g of NaCl

84 ml of 14 N HCl

20x SSC (1 litter): Sterilize by autoclave and store at room temperature.

175.2 g of NaCl

88.2 g of trisodium citrate dihydrate

0.4 N NaOH, 5x SSC: Described above.

Method

1) Place a gel supporting screen and a mask, which has window(s) of a slightly smaller than gel size in center of it, to a blotting unit.
2) Soak a piece of membrane in ddH_2O and place in the window of mask. Make sure that there are no air bubbles between the membrane and the screen. Transfer the gel to the membrane, make sure again that there are no air bubbles between the gel and the membrane.
3) Fasten clips and vacuum at 20 mbar. Add 50 ml of 0.25 N HCl on the gel, vacuum at 40 mbar for 5 min. If the vacuum pressure doesn't go up, make sure no gaps between the gel and the mask.
4) Remove the solution as possible with vacuuming. Add 50 ml of denaturing solution on the gel and vacuum for 6 min.
5) Remove the solution as possible with vacuuming. Add 50 ml of neutralizing solution (pH 5.0) on the gel and vacuum for 6 min.
6) Remove the solution as possible with vacuuming. Add calmly 20x SSC until the gel soak in the solution, vacuum for 1 h. During vacuuming, add 20x SSC several times to soak the gel.
7) Remove the solution and write sample name or the date on the membrane in soft-lead pencil.
8) Place the membrane, labeled side up, on a piece of Whatman 3MM paper wet with 0.4 N NaOH in a tray. Fix the DNA for 20 min.

9) Soak the membrane in 5x SSC for 1 min. Dry the membrane on a piece of Whatman 3MM paper.

10) Irradiate UV at 0.12 J/ cm^2 to cross-link the DNA to the membrane (Not essential).

11) Store the membrane in a heat-sealable bag at room temperature.

4.5.5 Hybridization of radiolabeled probes to nucleic acids

Materials

a heat-sealable bag

Megaprime DNA labeling systems (Amersham, RPN1604)

X-ray film (Scientific Imaging Film; Kodak, 165 1512)

Imaging plate (Fuji, BAS III)

Church mix (1 litter): Store at room temperature. Heat at 60°C just before use.

10g of BSA

250 ml of 2 M phosphate buffer (pH 7.0)

700 ml of 10% SDS

Stir the mixture gently on a heating block (~65°C) until dissolved, then adjust volume.

2x SSC/0.5% SDS (2 litter): Prepare the day before use. Heat at 60°C.

200 ml of 20x SSC

100 ml of 10% SDS

1x SSC/0.5% SDS (2 litter): Prepare the day before use. Heat at 60°C.

100 ml of 20x SSC

100 ml of 10% SDS

0.5x SSC/0.5% SDS (2 litter): Prepare the day before use. Heat at 60°C.

50 ml of 20x SSC

100 ml of 10% SDS

Method

1) Filtrate 50 ml of Church mix with 0.8 μm membrane and add in a 50 ml tube.

2) Add 40 ml of filtrated Church mix to a heat-sealable bag containing DNA immobilized membrane(s), squeeze gently as much air as possible from the bag. A bag can contain two membranes that the DNA blotted sides are back to back each other.

3) Seal the open end of the bag with a heat sealer and then make a second seal. Residual Church mix in a 50 ml tube and the bag incubate at 60°C.

4) Add 3 μl of the probe DNA and 27 μl ddH_2O to a 1.5 ml tube with holes on the cap by a needle to avoid boiling over suddenly.

5) Add 5 μl of Primer solution and spin briefly. Denature by heating to 98°C for 5 min in a boiling water bath.

6) Spin briefly, then add 10 μl of Reaction buffer, 5 μl of ^{32}P, and 2 μl of Enzyme. Mix gently by pipetting, spin briefly, and incubate at 37°C for 10 min.

7) If needed, add 0.5-1 μl of ^{32}P-labelled λ/Hind III DNA stored at -20°C.

8) Denature the labeled DNA by heating to 98°C for 5 min in a boiling water bath, then chill on ice. Spin briefly just before hybridization.

9) Add the denatured labeled DNA to residual Church mix in the 50 ml tube. Cut the sealed open end of the bag with a pair of scissors, deliver the solution into the bag. Squeeze gently as much air as possible from the bag, reseal with the heat sealer. Hybridize at c for 16-24 h with gentle agitation. Prepare solutions for wash and heat at 60°C.

10) Pour several hundred milliliters of preheated (60°C) 2x SSC/0.5% SDS to a plastic box, transfer the membrane. Wash briefly, change the solution and wash at 65°C for 5 min with gentle agitation. Repeat the wash one more in fresh solution. Be careful with radioactive contamination.

11) Wash twice in preheated (60°C) 1x SSC/0.5% SDS at 65°C for 8 min with gentle agitation. Then wash once in preheated (60°C) 0.5x SSC/0.5% SDS described above.

12) Place the membrane on a pad of paper towels and remove most of the liquid.

13) Place the damp membrane on a sheet of Saran Wrap. If needed, spot the holes in the membrane with radioactive ink. Cover the membrane with a sheet of Saran Wrap and seal with Scotch tape.

14) Expose the membrane to X-ray film for 16-24 h at -70°C with an intensifying screen, or to Imaging plate for 1-24 hr at room temperature, in an exposure cassette.

15) Develop the X-ray film in a darkroom or image the plate by Storm imaging system (Molecular Dynamics).

4.6 Antibody production

A chicken CENP-I expression construct (amino acids 307 - 525) was created in vector pET28a (Novagen). The histidine-tagged recombinant protein was expressed in *E. coli* BL21 (DE3) cells after 4 h induction with 0.5 mM IPTG and purified by nickel column chromatography. Purified recombinant CENP-I was used to immunize a rabbit. Serum was affinity purified against recombinant CENP-I protein on a CNBr-activated Sepharose 4B column.

4.7 Western blotting

4.7.1 Preparation of protein from DT40 cell

1) Grow cells in logarithmic growth phase in a 25 cm^2 flask.
2) Collect cells by centrifuge at 1000 rpm for 5 min at 4°C.
3) Wash in 10 ml of PBS (-).
4) Suspend the pellet in 1 ml of PBS (-), transfer to a 1.5 ml tube.
5) Centrifuge at 3000 rpm for 5 min at 4°C and remove the supernatant as possible. It can be stored at -80°C.
6) Dissolve the pellet on ice, add 110 μl of cold PBS (-).
7) Pippete an aliquot of 10 μl cell suspension into a 15 ml tube, determine of its protein concentration by using Bio-Rad *DC* Protein Assay (Bio-Rad).

4.7.2 SDS-PAGE and transfer proteins to a membrane

Materials

5-20% Gradient gel cassette (Real Gel Cassette; Bio Craft, SDG-571)

Hybond-P (Amersham, RPN303F): Cut a piece of membrane rectangle 7 by 9 centimeters.

Whatman 3MM paper (Whatman, 3030 917): Cut a piece of paper rectangle 9 by 11 centimeters, and 15 by 20 centimeters.

Slab gel electrophoresis system (Bio Craft, BE-140)

Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, 165-5052U)

PBS (-)

2x sample buffer (10 ml):

2 ml of 0.5 M Tris-Cl (pH 6.8)

4 ml of 10% SDS

1.2 ml of ß-mercaptoethanol

2 ml of glycerol

0.8 ml of ddH_2O

an appropriate volume of bromophenol blue

10x Running buffer (1 litter): Store at room temperature.

30 g of Tris-base

143 g of glycine

10 g of SDS

Blotting buffer (2 litter): Store at room temperature.

6.1 g of Tris-base

28.8 g of glycine

400 ml of MeOH

Method

1) Add 100 μl of 2x sample buffer to 100 μl of the cell suspension and mix well. Homogenize by sonication for 3 sec (x5) on ice.
2) Add an aliquot of 10 μg of protein to a new 1.5 ml tube. Denature by heating at 95°C for 5 min. Residual samples store at -20°C.
3) Insert the gel cassette into the electrophoresis apparatus. Add electrode buffer to upper and lower electrode chambers. Be sure to remove air bubbles beneath the slab gel and form the sample wells.
4) Wash wells, load the protein samples and marker (BenchMark Prestained Protein Ladder; Amersham).
5) Attach the electrode wires and apply 40 mA at constant current until the dye is 1 cm from the bottom of the gel.
6) During electrophoresis, write the name of sample and antibody on a piece of Hybond-P membrane in soft-lead pencil. Soak the membrane in MeOH, rinse in deionized H_2O and immerse in blotting buffer with a plastic tray.
7) Turn off the current. Remove the gel cassette, pry the plates apart carefully (a spatula is useful) and transfer the gel into blotting buffer.
8) Soak three peaces of Whatman 3MM paper (15 by 20 cm) in blotting buffer and place on the electric plate. In step 8)-11), ensure that no air bubbles are present between the layers.
9) Assemble the materials in blotting buffer: a sheet of Whatman 3MM paper (7 by 9 cm), gel, membrane and a sheet of paper (7 by 9 cm).
10) Place this sandwich on the three peaces of paper (15 by 20 cm) with gel side up (membrane side bottom).
11) Soak new three peaces of Whatman 3MM paper (15 by 20 cm) in blotting buffer and place on the sandwich.
12) Transfer at 25V for 40 min.

4.7.3 Detection by blocking and probing with antibody

Materials

TBS-T (3 litter): Store at room temperature.

14.5 g of Tris-base

48.0 g of NaCl

Adjust pH at 7.6 with HCl

Add 3 ml of Tween 20

0.5% BSA/TBS-T (200 ml): Prepare just before use.

a heat-sealable bag

ECL Plus Western blotting detection reagents (Amersham, RPN2131)

Method

1) Following electrophoretic transfer, soak the blotted membrane in 0.5% BSA/TBS-T for 1 hr at room temperature with agitation or for 16-24 hr at 4°C with no agitation to block nonspecific binding sites.
2) Remove solution and rinse the blot twice with TBS-T (2x 1 min).
3) Dilute primary antibody with TBS-T as described table below. Prepare 2.5-5 ml of diluted solution/ membrane and centrifuge briefly.
4) Add the membrane and diluted primary antibody solution to a heat-sealable bag. Squeeze gently as much air as possible from the bag, seal the bag with a heat sealer and then make a second seal.
5) Incubate at room temperature for 1 h with agitation. Remove solution.
6) Rinse with TBS-T at room temperature. Rinse once rapidly, then wash once for 15 min and twice for 5 min,
7) Dilute secondary antibody with TBS-T as described table below. Prepare 100-200 ml of diluted solution/ membrane in a tray.
8) Incubate at room temperature for 45 min with agitation. Remove solution.
9) Rinse as in step 6), then twice more in TBS-T.
10) Perform detection protocol as described in ECL Plus Western blotting detection reagents.
11) Image the labeled sample by Storm imaging system (Molecular Dynamics).

Table 4.8 Primary antibodies for western blotting

Antibody	Dilution rate	Reference or supplier
Rabbit anti-chicken CENP-I	1: 2500	Nishihashi et al., 2002
Monoclonal anti-α-tubulin (mouse)	1: 1000	Sigma, T-5168

Table 4.9 Secondary antibodies for western blotting

Antibody	Dilution rate	Reference or supplier
Peroxidase-conjugated goat anti-rabbit IgG	1: 10000	Jackson ImmunoResearch Lab., 111-035-003
Peroxidase-conjugated rabbit anti-mouse IgG	1: 10000	Jackson ImmunoResearch Lab., 315-035-003

4.8 Cell cycle analysis

4.8.1 Cell count by hemocytometer for growth curve

1) Grow cells in mid-logarithmic growth phase in a 75 cm^2 flask.
2) Harvest cells by centrifuge and suspend the pellet in an appropriate volume of medium.
3) Mix 40 μl aliquots of the cell suspension and 160 μl Trypan Blue Stain 0.4% (Gibco, 15250-061) in a 1.5 ml tube, estimate the number of living cells using a hemocytometer.
4) Feed the cell in 7-10 of 25 cm^2 flasks at 5x 10^5 cells/ flask.
5) At the time point of 0, 12, 24, 36, 48, 60, 72 and 96 h, harvest cells from one of 75 cm^2 flasks. Determine the number of living cells by counting as described in step 3).

4.8.2 Preparation of DT40 cells with BrdU-labeled DNA for flow cytometry

Materials

10 mM bromodeoxiuridine (BrdU; Roche, 280-879): Store at 4°C in the dark.

PBS (-)

70% EtOH (100 ml): Cold at –20°C.

1% BSA/PBS (-) (200 ml): Prepare just before use.

4 N HCl/0.5% Triton X-100 (20 ml): Prepare just before use.

5.7 ml of 14 N HCl

100 μl of Triton X-100

anti-BrdU primary antibody (Becton Dickinson, 347580)

10 mg/ml propidium iodide (PI; Sigma, P-4170) (5 ml): Store at 4°C in the dark.

Method

1) Grow cells in 25 cm^2 or 75 cm^2 flasks to be mid-logarithmic growth phase at the time point of 0, 12, 24, 36, 48, 60, 72 and 96 h.
2) Add BrdU to a final concentration of 10 μM 20 min before of harvest. Do not expose to the light.
3) Harvest cells and centrifuge at 1000rpm for 5 min.
4) Suspend the pellet in 10 ml of PBS (-) and centrifuge described step 3).
5) Add 10 ml of precold (-20°C) 70% EOH to a 15 ml tube wrapped in aluminum foil. Keep it on ice.
6) Suspend the pellet 50 μl of PBS (-) by gently tapping and drop into the tube. Store at -20°C for 30 min- a month.
7) Mix cell suspension by inverting and transfer an aliquot of 7.5 ml to a new tube. Residual cell suspension store at -20°C again.
8) Collect cells by centrifuge at 1700 rpm for 3 min.

9) Add 10 ml of 1% BSA/PBS (-) to the pellet and centrifuge described step 8).

10) Add 2 ml of 2-4 N HCl/0.5% Triton X-100, incubate at room temperature for 30 min.

11) Collect cells by centrifuge at 1700 rpm for 3 min.

12) Wash twice in 5 ml of 1% BSA/PBS (-). Remove the supernatant as possible and vortex the pellet gently.

13) Add 30 μl of anti-BrdU primary antibody (Becton Dickinson) and incubate at room temperature for 1 h. During incubation, mix a few times cell suspension by gently tapping.

14) Add 5 ml of 1% BSA/PBS (-) and centrifuge at 1700 rpm for 3 min. Repeat the wash one more.

15) Mix 1 μl of FITC-conjugated anti-mouse IgG and 19 μl of 1% BSA/PBS (-), centrifuge briefly.

16) Add the diluted secondary antibody solution to the pellet. Mix well and incubate at room temperature for 30 min in the dark place. During incubation, mix cell suspension once by gently tapping.

17) Add 5 ml of 1% BSA/PBS (-) and centrifuge at 1700 rpm for 3 min.

18) Prepare 5 μg/ml PI/1% BSA/PBS (-).

19) Remove the supernatant as possible, suspend the pellet in 500 μl of 5 μg/ml PI/1% BSA/PBS (-). Incubate at room temperature for 1 h or store at 4°C for overnight in the dark.

20) Subsequent flow-cytometry is performed with an Epics Altra cytometer (Beckman-Coulter). Fluorescence data are displayed as dot plots using Altra analysis software (Beckman-Coulter).

4.8.3 Preparation of DT40 cells with synchronized culture for flow cytometry

Materials

PBS (-)

500 μg/ml nocodazole (Sigma, M-1404): Sterilize by filtration and store at -20°C.

0.8 M mimosine (Sigma, M-0253): Sterilize by filtration and store at -20°C.

Dissolve mimosine in small amount of 12 N HCl and then adjust volume.

70% EtOH (200 ml): Cold at –20°C.

Buffer A (200 ml): Store at room temperature.

8 ml of 1M Tris-Cl (pH 7.4)

16 ml of 10% NaCl

4.2 ml of 1 M $MgCl_2$

Sterilize by autoclave. Cool down at room temperature.

Add 100 μl of Nonidet P-40 (Nacalai tesque, 252-23).

10 mg/ml propidium iodide (PI; Sigma) (5 ml): Store at 4°C in the dark.

Staining buffer (3 ml): Prepare just before use.

30 μl of 100 mg/ml RNase A (QIAGEN, 19101)

15 μl of 10 mg/ml PI

2.95 ml of Buffer A

Method

1) Grow cells in mid-logarithmic growth phase in a 75 cm^2 flask.
2) Add 3-5 ml cell suspension to a 225 cm^2 flask with fresh medium (0 h in Fig. 2.6A).

   Synchronization at G1/M phase transition

   After 11 hr incubation, add nocodazole to a final concentration of 500 ng/ml to synchronize M phase gently (11 h). Incubate for 4 h, collect cells by centrifuge at 1000 rpm for 5min, wash cells with PBS (-) three times and suspend in fresh medium containing 0.8 mM mimosine (15 h). Incubate for 15 h.

   Synchronization at M phase

   After 22 h incubation, add nocodazole to a final concentration of 500 ng/ml (22 h) and incubate for 8 h.
3) Collect cells by centrifuge at 1000 rpm for 5min, wash cells with PBS (-) three times. Incubate the cells in 7-8 of 75 cm^2 flasks with fresh medium (30 h).
4) At the time point of 30, 32, 34, 36, 38, 40 and 42 h, harvest cells from one of 75 cm^2 flasks. Wash cells in 10 ml PBS (-), suspend the pellet in 1 ml PBS (-). Add 10 ml of precold (–20°C) 70% EtOH to a new 15 ml tube, drop cell suspension in the 70% EtOH. Store at –20°C for 1 hr to a month.
5) Mix cell suspension by inverting and transfer an aliquot of 5 ml to a new tube. Residual cell suspension store at -20°C again.
6) Centrifuge at 2000 rpm for 15 min at 4°C. Suspend the pellet in 10 ml PBS (-) and centrifuge 800 rpm for 5min at 4°C. Repeat the wash one more.
7) Prepare staining buffer described above.
8) Suspend the pellet in 0.5 ml staining buffer and incubate for over 30 min in the dark.
9) Subsequent flow-cytometry is performed with an Epics Altra cytometer (Beckman-Coulter). Fluorescence data are displayed as dot plots using Altra analysis software (Beckman-Coulter).

Table 4.10 Reagents used in cell cycle analysis

Reagent (supplier)	Concentration of stock solution	Final concentration for DT40 cells
Brd U (Roche, 280 879)	10 mM	20 μM

PI (Sigma, P-1470)	10 mg/ml	10 μg/ml
Colcemid (Gibco, 15212-012)	10 μg/ml	100 ng/ml
L-mimosine (Sigma, M-0253)	0.8 M	0.8 mM
Nocodazole (Sigma, M-1404)	0.5 mg/ml	0.5 μg/ml

4.9 Immunocytochemistry

4.9.1 Methanol fixation

Materials

glass slides: Draw a circle or lines by scratching the surface to mark the region for dropping cell suspension. Write sample name in margin with pencil.

plastic coverslips and coverglasses

Coplin jars: Wrap in aluminum foil.

humidified dark box: Soak Whatman filter paper in PBS (-). Warm at 37°C.

PBS (-)

Methanol: Cold at –20°C.

0.56% hypotonic (pH 7.4; 1 litter): Store at room temperature.
Warm at 37°C in a water bath just before use.

16 ml of 2.5 M KCl

1 ml of 500 mM EDTA (pH 8.0)

20 ml of 1 M HEPES (pH 7.4)

KCM (500 ml): Store at room temperature.

4.47 g of KCl

0.584 g of NaCl

5 ml of 1 M Tris-Cl (pH 8.0)

500 μl of 0.5 M EDTA (pH 8.0)

500 μl of Triton X-100

TEEN (50 ml): Prepare just before use.

500 μl of 0.1 M Triethanolamine hydrochloride (pH 8.5)

20 μl of 0.5 M EDTA (pH 8.0)

250 μl of 5M NaCl

50 μl of Triton X-100

0.05 g of BSA

KB (-) (1 litter): Prepare just before use.

10 ml of 1M Tris-Cl (pH 7.7)

30 ml of 5 M NaCl

1 g of BSA

3% PFA/250 mM HEPES (500 ml): Described below.

0.1 μg/ml DAPI (1 ml): Store at 4°C in the dark.

Dissolve 10 mg of DAPI (Roche, 236 276) in 1 ml of VECTASHIELD (Vector Laboratories, H-100).

Prepare 10 μg/ml DAPI by adding 1 μl of 10 mg/ml DAPI in 999 μl of distilled deionized H_2O.

Dilute 10 μg/ml DAPI to 0.1 μg/ml with VECTASHIELD.

Method

1) Grow cells in mid-logarithmic growth phase in a 25 cm^2 or 75 cm^2 flask.
2) If needed, add colcemid (KaryoMax; GIBCO BRL) with 30 ng/ml in the cell suspension (e.g. 100 μl of 10 μg/ml colcemid in 30 ml of the culture), incubate 1.5-2 hr. Following steps do not require sterile conditions.
3) Collect cells by centrifuge at 1000 rpm for 5min.
4) Suspend the pellet in 10 ml PBS (-) by gently tapping with a 15 ml tube and centrifuge as described step 3).
5) Discard the supernatant, resuspend the pellet in residual PBS (-) by gently tapping.
6) Add 10 ml of 0.56% hypotonic solution and invert the tube gently.
7) Incubate at 37°C for 10 min in a water bath. Invert the tube gently once during the incubation.
8) Collect cells as described step 3). Discard the supernatant and resuspend the pellet in residual hypotonic solution by gently tapping.
9) Add 10 ml of precold (-20°C) methanol and invert the tube gently. Store at -20°C for 25 min.
10) Collect cells as described step 3). Add 0.2-0.5 ml of precold (-20°C) methanol and mix the cell suspension by gently tapping.
11) Drop 30 μl of cell suspension on the previously marked region of slide glasses from above. Dry up slightly and transfer slides immediately into a Coplin jar with KCM. Incubate at room temperature for 15 min.
12) Dilute primary antibody with TEEN as described table below. Prepare 100 μl of diluted solution/ slide and centrifuge briefly.
13) Pipette 100 μl of diluted primary antibody solution onto the previously marked region of slide. Cover with a plastic coverslip and incubate 37°C for 1h in a humidified dark box with PBS (-).
14) Transfer the slides with coverslip into a Coplin jars with KB (-). Take coverslips in the jar and wash at room temperature for 5min with gently shaking. Repeat the wash two more times with fresh KB (-).
15) Dilute secondary antibody with KB (-) as described table below. Prepare 100 μl of diluted solution/ slide and centrifuge briefly.
16) Pipette 100 μl of the secondary antibody solution onto the previously marked region of slide. Cover with a plastic coverslip and incubate 37°C for 45 min in a humidified box with PBS (-).
17) Take the coverslip and wash described as step 14).

18) Wash in a Coplin jar with PBS (-) at room temperature for 5min with gently shaking.

19) Re-fix cells with 3% PFA solution in a Coplin jar at room temperature for 15 min.

20) Wash described as step 18).

21) Wipe residual PBS (-) from around the marked region of slide, mount the slide with 0.1 μg/μl of DAPI solution and seal under coverglass with paper bond. Store at 4°C in the dark.

4.9.2 Cytocentrifuge and PFA fixation

Materials

plastic coverslips, coverglasses, Coplin jars, humidified dark box, PBS (-), 0.1 μg/ml DAPI (1 ml): Described above.

glass slides: Draw lines by scratching the surface to mark the region collected cells by cytocentrifugion. Write sample name in margin with pencil.

Cytoclip slide clip, Cytofunnel sample chamber, Filter cards and Cytospin (ThermoShandon): Write sample name in Filter card with pencil. With Cytoclip in open position, fit glass slide, Filter, and then Sample chamber against the clip. Bring spring clip up and secure under the two retaining hooks. Set Cytoclip assembly in rotator for Cytospin.

3% PFA/250 mM HEPES (500 ml): Store at 4°C in the dark.

Add 15 g of paraformaldehyde (do not breath the dust) in 300 ml of distilled deionized H_2O.

Stir the mixture gently on a heating block (~65°C) in a chemical fume food and add 2.5- 3 ml of 1 N NaOH until the paraformaldehyde is dissolved.

Cool the mixture to room temperature.

Then add 125 ml of 1 M HEPES and adjust the final volume to 1 litter with distilled deionized H_2O.

Check the pH in range from 7.0 to 8.0 by testing paper.

0.5% NP40/PBS (-) (250 ml): Prepare just before use.

1.25 ml of NP40 in 250 ml of PBS (-)

0.5% BSA/PBS (-) (1 litter): Prepare just before use.

5 g of BSA in 1 litter of PBS (-)

Method

1) Same as step 1)~5) in methanol fixation.

2) Add 1-3 ml of PBS (-) and mix by gently tapping.

3) Transfer 300 μl aliquots of cell suspension into a Cytofunnel assembly setting in rotator. Cytocentrifuge at 800rpm for 5min by Cytospin.

4) After spin, immediately transfer the slide into a Coplin jar with 3% PFA solution, fix cells at room temperature for 15 min.

5) Wash in a Coplin jar with PBS (-) at room temperature for 5min.

6) Permealize cells with 0.5% NP40/PBS (-) in a jar for 15 min.

7) Wash in a Coplin jar with 0.5% BSA/PBS (-) at room temperature for 5min. Repeat the wash two more times with fresh 0.5% BSA/PBS (-).

8) Dilute primary antibody with 0.5% BSA/PBS (-) as described table below. Prepare 100 μl of diluted solution/ slide and centrifuge briefly.

9) Pipette diluted primary antibody solution to the slide and incubate described step 13) in methanol fixation.

10) Transfer the slides with a plastic coverslip into a Coplin jar with 0.5% BSA/PBS (-). Take coverslips in the jar and wash at room temperature for 5min with gently shaking. Repeat the wash two more times with fresh 0.5% BSA/PBS (-).

11) Dilute secondary antibody with 0.5% BSA/PBS (-) as described table below. Prepare 120 μl of diluted solution/ slide and centrifuge briefly.

12) Pipette diluted secondary antibody solution to the slide and incubate described step 16) in methanol fixation.

13) Take the coverslip and wash described as step 10.

14) Wash in a Coplin jar with PBS at room temperature for 5min with gently shaking.

15) Wipe residual PBS (-) from around the marked region of slide, mount and store the slide described step 21) in methanol fixation.

Table 4.11 Primary antibodies for immunocytochemistry

Antibody	Dilution rate	Reference or supplier
Monoclonal anti-α-tubulin –FITC conjugate	1: 50	Sigma (F-2168)
Rabbit anti-chicken CENP-I	1: 2000	Nishihashi et al., 2002
Rabbit anti-chicken CENP-A	1: 50	Fukagawa et al., 2001
Rabbit anti-chicken CENP-C	1: 1000	Fukagawa et al., 1999
Rabbit anti-chicken CENP-H	1: 2000	Fukagawa et al., 2001
Rabbit anti-chicken ZW10	1: 200	Fukagawa et al., 1999
Rabbit anti-chicken BubR1	1: 500	Dodson and Earnshaw, unpublished
Anti-HA	1: 50	Sigma (H-6908)

Table 4.12 Secondary antibodies for immunocytochemistry

Antibody	Dilution rate	Reference or supplier
Cy3-conjugated goat anti-rabbit IgG	1: 1000	Amersham (PA43004)
FITC-conjugated rabbit anti-mouse IgG	1: 200	Jackson ImmunoResearch Laboratories (315 095 003)

4.10 FISH

4.10.1 Preparation of painting prove

Preparation of chromosomes and staining for sorting of DT40 cells

Materials

500 μg/ml nocodazole: Sterilize by filtration and store at -20°C.

PBS (-)

0.2% NaCl/0.2% KCl (500 ml): Sterilize by autoclave and store at room temperature.

1 g of NaCl

1 g of KCl

PA buffer (500 ml): Sterilize by filtration and store at room temperature.

7.5 ml of 1 M Tris-Cl (pH 7.4)

2 ml of 0.5 M EDTA (pH 8.0)

20 ml of 2 M KCl

2 ml of 5 M NaCl

500 μl of 200 mM spermine (Sigma, S-2876)

500 μl of 500 mM spermidine (Sigma, S-2626)

500 μl of ß-mercaptoethanol

DIG-PA buffer: Sterilize by filtration and store at 4°C. Dissolve completely at 37°C.

Prepare 1% digitonin (Sigma, D-5628) /PA buffer (10x stock).

Dilute to 1x with PA buffer.

0.5 mg/ml (about 1mM) Hoechst 33258: Sterilize by filtration and store at 4°C.

Chromomycine A3 (Sigma, C-2659): Sterilize by filtration and store at 4°C.

- Dissolve in small amount of EtOH and then adjust volume.

0.6 mM/20 mM sodium citrate (pH 7.0)/2.5 mM $MgCl_2$.

1 M $MgCl_2$ (100 ml): Sterilize by autoclave and store at room temperature.

0.5 M sodium citrate (pH 7.0)

1% Triton X-100

Method

1) Grow cells in mid-logarithmic growth phase in a 75 cm^2 or 225 cm^2 flask.

2) Add 500 μg/ml nocodazole to a final concentration of 500 ng/ml, and incubate for 8 hr.

3) Collect cells by centrifuge at 1000 rpm for 5min.

4) Suspend the pellet in 10 ml PBS (-) by gently tapping with a 15 ml tube and centrifuge as described step 3.

5) Suspend the cells in 10 ml of 0.2% NaCl/0.2% KCl. Incubate on ice for 20 min.

6) Centrifuge at 700 rpm for 10 min at 4°C. Suspend cells in 5 ml of PA buffer.

7) Centrifuge 1300 rpm for 5 min at 4°C.

8) Suspend cells at 3-4x 10^7 cells/ml in DIG-PA buffer (e.g. 1.25 ml DIG-PA buffer for 100 ml of cell culture).

9) Mix by gently voltexing for 1-2 min.

10) Centrifuge 500 rpm for 6 min at 4°C. Save the supernatant fraction carefully. Chromosome samples can be stored at 4°C for 2-3 months.

11) Add 400-200 μl of DIG-PA buffer, 12 μl of 1 M $MgCl_2$, 12 μl of 0.5 mg/ml Hoechst 33258, and 150 μl of 0.6 mM Chromomycin A3 to 600-800 μl of cell suspension. Final concentration is 1.5x 10^7 cells/ml, 10 mM $MgCl_2$, 75 μM Chromomycin A3 and 75 μM Hoechst 33258.

12) Keep at 4°C for 2-3 hr in the dark.

13) Add 120 μl of 0.5 M sodium citrate (pH 7.0) and 40 μl of 1% Triton X-100. Mix by voltexing for 5-10 sec.

14) Collect 5000-10000 of each chromosome by sorting. Fluorescence data is displayed as dot plots showed in Fig. 4.11.

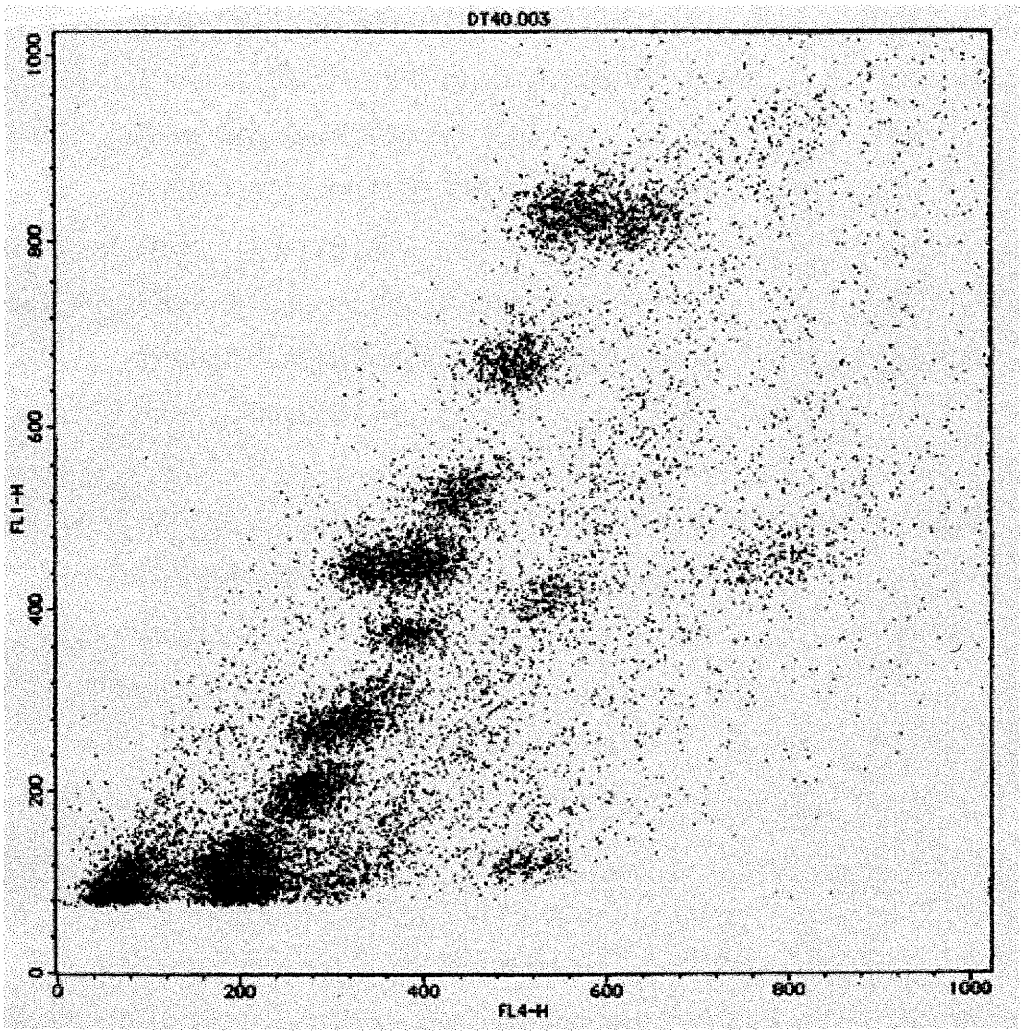


Fig. 4.11 Sorted chromosomes from DT40 cells

4.10.2 Degenerate oligonucleotide primer (DOP) PCR

1) Set up the reaction according to the following pipetting scheme:

probe DNA (sorted chromosome)	1 μl
Thermophillic DNA poly. 10x buffer (Promega)	10 μl
25 mM $MgCl_2$ (Promega)	8 μl
2 mM dNTPs mixture (ABI)	8 μl
DOP primer (20 μM)	5 μl
Taq DNA polymerase (5 units/μl)	1 μl
distilled deionized H_2O	67 μl
Final volume 100 μl	

DOP primer sequence: 5'-CCGACTCGAGNNNNNNATGTGG-3'

2) Carry out amplification using the following parameters:

first cycle	94°C for 9 min
8 cycles	94°C for 15 sec
	30°C for 1.5 min
	72°C for 3 min
25 cycles	94°C for 15 sec
	62°C for 1 min
	72°C for 3 min

After the last cycle, maintain the reaction for 8 min at 72°C and then cool down to 4°C.

3) Set up the reaction according to the following pipetting scheme:

primary PCR product	1 μl
Thermophillic DNA poly. 10x buffer (Promega)	10 μl
25 mM $MgCl_2$ (Promega)	8 μl
2 mM each dATP, dCTP, dGTP mixture	10 μl
1 mM dTTP	16 μl
2 mM biotin-16-dUTP	4 μl
DOP primer (20 μM)	10 μl
Taq DNA polymerase (5 units/ μl)	1 μl
distilled deionized H_2O	40 μl
Final volume 100 μl	

4) Carry out the following amplification protocol.

first cycle	94°C for 3 min

25 cycles 94°C for 1 min

62°C for 1 min

72°C for 1.5 min

After the last cycle, maintain the reaction for 8 min at 72°C and then cool down to 4°C.

5) Remove an aliquot of 5 μl of secondary PCR products, check the size of resulting fragments by 1% agarose gel electrophoresis. The probe DNA will be visible as a smear. The majority of the fragments should be between 100 and 500 bp.
6) Incubate residual PCR products at 65°C for 15 min to inactivate enzymes.

4.10.3 Preparation of metaphase chromosome spreads

Materials

PBS (-)

glass slides: Draw lines by scratching the surface to mark the region for dropping cell suspension. Write sample name in margin with pencil.

silica gel containing slide box

MeOH: acetic acid [3:1 v/v] (120 ml): Prepare just before use. Cold to -20°C.

90 ml of MeOH

30 ml of acetic acid

45% acetic acid (10 ml): Prepare just before use.

Method

1) Same as step 1)~8) in methanol fixation.
2) Add pre-cold (-20°C) MeOH: acetic acid [3:1 v/v], initially dropwise while repeatedly tapping the tube to avoid cell clumping. Then fill the volume up to 14 ml with fixative and store overnight at -20°C.
3) Centrifuge at 1000 rpm for 5 min, 4°C. Resuspend pellet in residual solution and add 1-3 ml of precold (-20°C) MeOH: acetic acid [3:1 v/v] with dropwise.
4) Drop 30 μl of 45% acetic acid on the marked region of slide from above. Do not drop on over 5 slides in the same time to avoid evaporating acetic acid.
5) Immediately drop 30 μl of cell suspension on the slide coating acetic acid.
6) Dry the slide, mount a slide with 30 μl of DAPI solution and seal under a coverglass with bond. Check under microscope for good spreading and sufficient mitotic index.
7) Store slides in silica gel containing slide box that is sealed with electric insulation tape at -20°C to keep spreads dry. Spreads can be stored for a year.

4.10.4 Fluorescent in situ hybridization (FISH) of chromosomes

Day 1 (Dehydrogenation)

1) Let the slide box reach room temperature before opening it to prevent condensation on slides when thawing them.
2) Transfer slide in a Coplin jar with 70% EtOH for 2 min, 90% EtOH for 2 min, then 100% EtOH for 2min.
3) Dry at room temperature and keep in a dark box for overnight.

Day 2 (Hybridization)

Materials

Humidified dark box: Soak Whatman filter paper in 2xSSC. Warm up at 37°C.

Hybridization mix (5 ml): Store at 4°C.

2.5 ml of formamide
1 ml of 50% dextran sulfate
500 μl of 20xSSC
100 μl of 2M phosphate buffer (pH 7.0)
50 μl of 100x Denhalt's solution
12.5 μl of 0.2 M EDTA (pH8.0)
6 μl of 100 mg/ml homogenized by sonication sermon sperm DNA
831.5 μl of distilled deionized H_2O

70% formamide/2xSSC (50 ml): Add in a Coplin jar, heat at 70°C in a water bath.

35 ml of formamide
5 ml of 20xSSC
10 ml of distilled deionized H_2O

70% EtOH (50 ml): Add in a Coplin jar and store at -20°C.

70% EtOH, 90% EtOH, 100% EtOH (50 ml): Add in a Coplin jar.

Method

1) Mix 1 μl of painting probe and 14 μl of Hybridization mix.
2) Incubate at 70°C for 10 min in a water bath.
3) Centrifuge briefly and incubate at 37°C for 15 min – 3h.
4) Denature target DNA on the slide at 70°C for 1.5 min in the heated 70% formamide/2x SSC and immediately immerse the slide into pre-cold (-20°C) 70% EtOH for 5 min.
5) Transfer the slide in 70% EtOH for 2 min, 90% EtOH for 2 min, then 100% EtOH for 2min. Dry at room temperature until EtOH on the slide evaporate.

6) Pipette 15 μl of the denatured prove onto the slide in the previously marked region and seal under coverglass with paper bond.

7) Hybridize at 37°C for overnight in a dark box humidified with 2x SSC.

Day 3 (Wash and detection)

Materials

Humidified dark box: Soak Whatman filter paper in 2xSSC. Warm at 37°C.

2x SSC (50 ml): Add in a Coplin jar and warm at 42°C in a water bath.

50% formamide/2x SSC (100 ml): Add in two Coplin jars, warm at 42°C.

50 ml of formamide

10 ml of 20x SSC

40 ml of distilled deionized H_2O.

0.1x SSC (100 ml): Add in two Coplin jars and warm up at 42°C.

4x T solution (1 litter): Store at room temperature.

200 ml of 20x SSC

500 μl of Tween 20

800 ml of distilled deionized H_2O

3% BSA/4x T (10 ml): Prepare just before use.

0.3 g of BSA/10 ml of 4x T solution

1% BSA/ 4x T (10 ml): Prepare just before use.

0.1 g of BSA/10 ml of 4x T solution

2 mg/ml Cy3 labeled avidin (Amersham, PA 43001)

PBS (-)

0.1 μg/ml DAPI/2x SSC (1 ml): Store at 4°C in the dark.

Dilute 10 μg/ml DAPI (described above; methanol fixation) to 0.1 μg/ml with 2x SSC.

Method

1) Transfer the slide in 2x SSC at 42°C, peel off the sealed coverglass and wash for 5 min.

2) Wash twice in 50% formamide/2x SSC at 42°C for 5 min.

3) Wash twice in 0.1x SSC at 42°C for 5 min.

4) Wash in 4x T solution for 5min at room temperature. Repeat the wash two more with fresh solution.

5) Pipette 150 μl of 3% BSA/4x T onto the previously marked region of slide. Cover with a plastic coverslip and incubate 37°C for 20 min in a humidified dark box with 2x SSC.

6) Dilute 2 mg/ml Cy3 labeled avidin to 2 μg/ml with 1% BSA/4x T. Prepare 150 μl/ slide and centrifuge briefly.

7) Transfer the slide with coverslip in a Coplin jar with 4x T solution and take the coverslip.

8) Wipe residual 4x T from around the marked region of slide, pipette 150 μl of diluted Cy3 labeled avidin solution and cover with a coverslip described step 5). Incubate 37°C for 30 min in a humidified dark box with 2x SSC.

9) Take the coverslip in 4x T solution, wash three times described step 4).

10) Wash in a Coplin jar with PBS (-) for 5 min.

11) Wipe residual PBS (-) from around the marked region of slide, mount the slide with 0.1 μg/μl of DAPI/2x SSC and seal under coverglass with paper bond. Store at 4°C in the dark.

4.11 Fluorescence microscopy in living cells

For fluorescence staining of chromosomes, Hoechst 33342 was added to the culture medium to a final concentration of 100 ng/ml. Cells were stained with Hoechst 33342 for 10-15 min and washed three times with culture medium. For microscopic observation, HEPES buffer (pH 7.4) was added to a final concentration of 20 mM to avoid the need for CO_2 gas. Fluorescently stained living cells were observed with an Olympus inverted microscope IX70 with an oil immersion objective lens (PlanApo 60X, NA=1.40). The DeltaVision microscope system used in this study was purchased from Applied Precision, Inc. For temperature control during microscopic observations, the system was assembled in a custom-made, temperature-controlled room. Time-lapse images were recorded at 2 min intervals with an exposure time of 0.2-0.3 s.

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