CENP-C is necessary but not sufficient to induce formation of a functional centromere

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CENP-C is an evolutionarily conserved centromeric protein. We have used the chicken DT40 cell line to test the idea that CENP-C is sufficient as well as necessary for the formation of a functional centromere. We have compared the effects of disrupting the localization of CENP-C with those of inducibly overexpressing the protein. Removing CENP-C from the centromere causes disassembly of the centromere protein complex and blocks cells at the metaphase–anaphase junction. Overexpressed CENP-C is associated with an increase in errors of chromosome segregation and inhibits the completion of mitosis. However, the excess CENP-C does not disrupt the native centromeres detectably and does not associate with another conserved centromere protein, ZW10. The distribution of the excess CENP-C changes during the cell cycle. In metaphase, the excess CENP-C coats the chromosome arms. At the metaphase–anaphase transition, the excess CENP-C clusters, and during interphase it is present in large bodies which form around pre-existing centromeres which are also clustered. These results indicate that CENP-C is necessary but not sufficient for the formation of a functional centromere and suggest that the structure of CENP-C may be regulated during the cell cycle.

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Introduction

At mitosis in eukaryotes, spindle microtubules bind to a specific region of each chromosome termed the centromere (Pluta *et al.*, 1995) and direct chromosome segregation. Centromeres also regulate the activity of the mitotic checkpoint pathway which prevents anaphase initiation until all the centromeres are attached to the spindle (Rudner and Murray, 1996; Allshire, 1997; Gorbsky, 1997; Amon, 1999). Although errors of chromosome segregation are an important cause of genetic diseases including cancer (Lengauer *et al.*, 1998), the problem of how centromeres interact with the microtubules of the spindle at cell division is not understood.

The first method used to study centromeres was to analyse mutations which cause errors of chromosome segregation. This method has been applied most extensively in the yeasts, but also in *Drosophila melanogaster*, and has led to the identification of many genes encoding

centromere components. For example, several encode members of the evolutionarily conserved mitotic checkpoint pathway (Hyman and Sorger, 1995; Chen *et al.*, 1996; Taylor and McKeon, 1997) that are localized to the centromeres before they attach to the spindle. Eight constitutive centromere components have also been identified in *Saccharomyces cerevisiae*, but only those encoded by the *CSE4* (Stoler *et al.*, 1995) and *MIF2* genes (Brown *et al.*, 1993) have homologues that are found at the centromeres of other organisms. The *Drosophila* centromere protein ZW10 has no detectable homologue in yeast, but is conserved in vertebrates and in *Caenorhabditis elegans* (Starr *et al.*, 1997). The second method for analysing the centromere has been to identify the *cis*-acting centromeric sequences (Carbon, 1984) and then to study the proteins which bind to them. This approach has been applied most widely in the budding yeast and has led to the identification of several proteins including a large protein complex termed CBF3 (Lechner and Carbon, 1991), which contains four proteins encoded by genes previously identified in mutational studies (Doheny *et al.*, 1993).

It has been impossible to apply genetic analysis to vertebrate centromeres because of the lack of suitable assays for mutations which affect the accuracy of chromosome segregation. Controversy about the identity of vertebrate centromeric DNA has also restricted the identification of vertebrate centromere-binding proteins by biochemical techniques. Vertebrate centromere proteins have therefore been identified mainly by immunological approaches. Antibodies against centromeric proteins have been isolated either from patients with autoimmune disease (Moroi *et al.*, 1980) or from immunized animals (Compton *et al.*, 1991). This has led to the identification of seven centromeric protein antigens, CENP-A, -B, -C, -D, -E, -F and -G (Pluta *et al.*, 1995; He *et al.*, 1998). Of these, CENP-A and CENP-C seem to be particularly important. CENP-A is a 140 amino acid centromere-specific protein of which the C-terminal 90 amino acids are 60% identical to histone H3 (Shelby *et al.*, 1997). CENP-A is found only at active centromeres (du Sart *et al.*, 1997; Karpen and Allshire, 1997; Warburton *et al.*, 1997). CENP-A copurifies with nucleosomes and, together, these data suggest that it is a component of a special type of nucleosome that is formed only at active centromeres (Vafa and Sullivan, 1997). The *S.cerevisiae* protein CSE4p is a homologue of CENP-A, contains a C-terminal region which is 60% identical to H3 and is a constituent of a specific type of nucleosome that forms at yeast centromeres (Meluh *et al.*, 1998). It therefore seems likely that a centromeric nucleosome containing a specific variant of H3 is a fundamental feature of eukaryotic centromere organization.

CENP-C is the only component other than CENP-A to

show clear homology to a yeast centromere protein (Brown, 1995; Lanini and McKeon, 1995; Meluh and Koshland, 1995). Its yeast homologue, MIF2p, binds the centromeric DNA (Meluh and Koshland, 1997) and is encoded by an essential gene. Mutating or overexpressing MIF2p causes errors of chromosome segregation (Brown *et al.*, 1993). CENP-C is also an essential (Fukagawa and Brown, 1997; Kalitsis *et al.*, 1998) DNA-binding protein (Yang *et al.*, 1996) and, like CENP-A, is found only at active centromeres (Earnshaw *et al.*, 1989; Sullivan and Schwarz, 1995). These properties suggest that MIF2p and CENP-C may perform homologous functions at the centromere in yeast and vertebrates and that both are likely to be associated either directly or indirectly with the centromere-specific nucleosome. Ultrastructural studies have played an important role in influencing ideas about CENP-C function. Conventional electron microscopic preparations of the vertebrate kinetochore indicate that the microtubules bind to a 35–40 nm thick disc that is separated from the underlying heterochromatin by a 15–30 nm electron-lucent zone. In these preparations, CENP-C is located adjacent to the heterochromatin in an inner plate beneath the electron-lucent zone. Injection of antibodies to CENP-C leads to destruction of the kinetochore as detected by electron microscopy and inhibits cells transiting through mitosis (Tomkiel *et al.*, 1994). The long distance between the apparent location of CENP-C and the microtubule-binding region in the kinetochore led to these results being interpreted to suggest the idea that CENP-C is necessary for chromosome stability and that it functions in an architectural role, perhaps assembling a platform upon which other components assemble (Saitoh *et al.*, 1992; Tomkiel *et al.*, 1994).

Recently, it has become clear that centromeres in metazoans are not fixed structures but can form at a variety of ectopic loci in the genome which share no detectable sequence homology (Karpen and Allshire, 1997). Nothing is known about the mechanism of such 'centromere activation' events, and we would like to understand how they occur. Our experimental system is the chicken DT40 cell line (Buerstedde and Takeda, 1991). This cell line enables the study of cell-autonomous functions by reverse genetic approaches because modification of endogenous genes by homologous recombination is very efficient in DT40 cells. In previous work (Fukagawa and Brown, 1997), we used homologous recombination to engineer a conditional loss-of-function mutation into the CENP-C gene. This mutant demonstrated that functional CENP-C was essential for viability and suggested that CENP-C was necessary for the metaphase to anaphase transition. One idea for the mechanism of centromere activation is that CENP-C is a 'master molecule' which can direct centromere formation (Karpen and Allshire, 1997) and that 'centromere activation' arises as a result of the ectopic localization of CENP-C. We have tested this idea by overexpressing CENP-C and studying the consequences. The logic of our experiment is that if CENP-C were able to seed centromere formation, then overexpressed CENP-C would sequester other centromere components, disrupt the native kinetochore and thus block the metaphase–anaphase transition. Our results suggest that although CENP-C alone cannot cause formation of a functional centromere, it does have a spindle-disrupting

activity. The structural properties of the excess CENP-C may indicate why ectopic centromere formation is rare.

Results

CENP-C is necessary for the assembly of the full centromere protein complex and for the metaphase–anaphase transition

We previously described a conditional loss-of-function mutant generated by tagging a single endogenous CENP-C gene with a mutant mouse oestrogen receptor (Fukagawa and Brown, 1997). These cells (referred to as Δ /CENP-C–ER) proliferated as wild-type in the presence of the steroid receptor agonist 4-hydroxytamoxifen but died when 4-hydroxytamoxifen was removed from the culture. Cytological analyses of the mutant cells suggested that under restrictive conditions the mutant blocked at the metaphase–anaphase boundary. We wanted to examine the phenotype in detail and to test the idea that the presence of CENP-C was necessary for full assembly of the centromere protein complex. Earlier work had shown that the Δ /CENP-C–ER cells took 48 h to accumulate in mitosis following removal of the 4-hydroxytamoxifen (Fukagawa and Brown, 1997). We therefore cultured the Δ /CENP-C–ER cells for 30 h in the absence of 4-hydroxytamoxifen, synchronized the population in mitosis with nocodazole for 6.5 h and then analysed the cells by measuring their DNA content using a fluorescence activated cell sorter (FACS, Figure 1A) and histone H1 kinase activity in p34^{CDC2} immunoprecipitates (Figure 1B) after the cells were released from the nocodazole block. This analysis established that the cells accumulated with a 4N DNA content and high levels of mitotic CDC2 kinase when grown under restrictive conditions and were thus in metaphase, as had been suggested by the cytological appearance of the cells. We raised an antiserum against the N-terminal 303 amino acids of chicken CENP-C and used this to localize the protein under both permissive and restrictive conditions (Figure 1C). This experiment established that under restrictive conditions, CENP-C was lost from the centromere. In order to assess the consequences of CENP-C loss upon centromere assembly, we needed to examine chromosomes isolated from cells grown under restrictive conditions for the presence of another centromere protein. We chose to use the chicken homologue of the conserved metazoan centromere protein ZW10 for these experiments because this protein, like CENP-C, is present only at active centromeres (Faulkner *et al.*, 1998). We therefore cloned the chicken homologue of the ZW10 protein, raised an antiserum against the protein and used this to localize chicken ZW10 in the CENP-C mutant under both permissive and restrictive conditions (Figure 1C). These experiments established that growth of the Δ /CENP-C–ER cells under restrictive conditions also led to loss of the ZW10 protein from the centromere. We therefore conclude that the presence of CENP-C is necessary for the assembly of the full centromere protein complex.

Testing the idea that CENP-C is sufficient for centromere assembly

The requirement for CENP-C for centromere assembly, its localization within the inner plate of the kinetochore

Fig. 1. Cells expressing a conditionally inactive CENP-C-oestrogen receptor fusion protein fail to assemble a full centromere complex under restrictive conditions and arrest at metaphase. **(A)** Δ CENP-C-ER cells were cultured in the presence or absence of 4-hydroxytamoxifen (4-OHT) for 30 h. Cells were then synchronized by nocodazole for 6.5 h. After release from the block, cells were harvested at the indicated times and analysed for DNA content by FACS after staining with propidium iodide. The scale bar corresponds to 10 μ m. **(B)** Cell cycle-dependent histone H1 kinase activity in chicken p34^{CDC2} immunoprecipitates was measured in the cells prepared in **(A)**. The kinase activity was measured using purified histone H1 as a substrate. Total radioactivity of histone H1 was measured by an Instant Imager. **(C)** CENP-C or ZW10 localization on metaphase chromosomes of Δ CENP-C-ER cells grown in the presence or absence (restrictive conditions) of 4-OHT for 48 h. Rabbit antibodies to either chicken CENP-C or ZW10 were applied to metaphase chromosomes and detected with Cy3-conjugated second antibodies (red). DNA was counterstained by DAPI (blue).

and its conserved sequence suggested that the presence of CENP-C might be sufficient to assemble a functioning centromere. To test this idea, we overexpressed CENP-C and analysed the phenotypic consequences. One would predict that if the presence of CENP-C was sufficient to seed the formation of a new centromere, then the excess CENP-C would bind the limited pool of other centromere components which in turn would lead to the disruption of

native centromeres and consequently to a failure of the overexpressing cells to transit from metaphase to anaphase.

In order to engineer a DT40 cell line which overexpressed CENP-C, we first of all constructed a cell line which constitutively expressed a fusion protein (tTA) between the Tet repressor and the activating domain of herpes virus VP16 trans-activating protein (Gossen and Bujard, 1992) and then introduced into these cells a

Fig. 2. Immunoblotting of overexpressed CENP-C. Clone C-27 cells containing the tetracycline-inducible transgene were cultured for 10 h in the presence or absence of tetracycline (TET), nuclei were isolated, solubilized in loading buffer and subjected to SDS-PAGE. The gels were blotted, and the blots were probed with an affinity-purified anti-CENP-C antibody and developed with HRP-labelled anti-rabbit IgG using chemiluminescence (ECL). The signal intensity was measured using a Molecular Dynamics Storm imaging system which demonstrated that CENP-C was expressed ~130-fold more in the absence of tetracycline than in its presence. Relative cell numbers are shown (1 corresponds to 7.5×10^5 cells).

plasmid encoding chicken CENP-C driven by a promoter dependent for activity upon bound tTA fusion protein. These cells therefore overexpressed CENP-C in the absence of tetracycline, and expression was extinguished by the addition of tetracycline. We used the antiserum against CENP-C to monitor expression (Figure 2). Western blotting of the protein extracted from cells grown in the absence of tetracycline demonstrated that the cells overexpressed the native CENP-C ~130-fold. The level of protein in cells grown in the presence of tetracycline (Figure 2, lane 1) was similar to the levels in native DT40 cells, indicating that the background level of CENP-C expression from the tetracycline-repressible promoter was negligible (data not shown).

Excess CENP-C delays the completion of mitosis

We wanted to compare the effect of excess CENP-C upon DT40 cells with the consequences of inactivating CENP-C using the conditional loss-of-function mutant. We first of all examined the consequences of overexpressing CENP-C upon cell cycle progression. Figure 3 presents the results of experiments designed to investigate the effect of excess CENP-C upon one cell cycle. DT40 cells which potentially overexpress CENP-C were synchronized in mitosis by culturing in nocodazole for 6.5 h, released from the nocodazole block and then cultured either in the presence of tetracycline (control) or in the absence of tetracycline for 12 h to induce the overexpression of CENP-C. Cells were analysed at 2 h intervals for DNA content using a FACS (Figure 3A), for histone H1 kinase activity in p34^{CDC2} immunoprecipitates (Figure 3B) and for the stage of mitosis at 2 h intervals (Figure 3C and D) by cytological techniques. Twelve hours after releasing these cells from the nocodazole block, 78% of the experimental cells had accumulated with a 4N DNA content while 65% of the control cells had returned to a 2N content (Figure 3A). The cells had inactivated their mitotic CDC2 kinase (see the 12 h time point in Figure 3B), indicating that they had traversed the metaphase–anaphase boundary. Cytological analysis (Figure 3C and D) demonstrated that the cells accumulated in anaphase (Figure 3D) without loss of viability (Figure 3C and D).

In order to help us to understand how CENP-C might be having this effect, we used the antibody to localize

CENP-C in the overexpressing cells (Figure 4). At metaphase, the overexpressed CENP-C covered the chromosomes, both over the centromere and over the arms (Figure 4B). At anaphase, however, most of the CENP-C was lost from the chromosome arms and was detected in small aggregates (Figure 4D), most of which were not bound by the chromosomes but were distributed around them.

We wanted to define where the cells were being delayed in the cell cycle. In so far as the mitosis is clearly aberrant, it was by no means certain that cells such as those illustrated in Figure 4D were actually in anaphase. In order to resolve this point, we needed to be able to see a specific chromosome clearly in the karyotype and to establish whether segregation of the sister chromatids had occurred. We therefore made use of a DT40 hybrid cell line containing a mini-chromosome derived from the human Y chromosome. The mini-chromosome that we used, called mini-2.5, was derived from the intact human Y chromosome by telomere-directed chromosome breakage of the proximal long arm (Shen *et al.*, 1997). Mini-2.5 therefore contains the short arm, the centromere and the proximal long arm of the human Y chromosome. Mini-2.5 was moved into DT40 cells by micro-cell fusion to create the cell line DT2.5 and was stably maintained in the hybrids for hundreds of generations in the absence of any applied selection (not shown). We established a variant of DT2.5 which overexpressed CENP-C and which we called DT2.5-5 (CENP-C). We combined fluorescence *in situ* hybridization (FISH) with a human alphoid DNA probe and immunofluorescence to co-localize the mini-2.5 and CENP-C in these cells after overexpression of the CENP-C (Figure 4E–H). These experiments confirmed what we had seen with the DT40 cells which overexpressed CENP-C but contained no mini-chromosome, both with respect to the changing distribution of CENP-C and the interpretation that the excess CENP-C either slowed poleward movement during anaphase itself or delayed the anaphase–telophase transition. It is important to note that although excess CENP-C did disrupt mitosis, there was nothing in the phenotype to suggest that the excess CENP-C was inducing new centromeres to form or was disrupting the native centromere. The bouquet of sister chromatids at anaphase had a distribution that was similar to that of the wild-type (Figure 4D and H).

Excess CENP-C inhibits cytokinesis but does not delay progress through interphase

We wanted to know whether overexpressing CENP-C had any effects during interphase, and so we analysed a second cell cycle. We therefore synchronized the cells with nocodazole as before and analysed the cells by FACS at 4 h intervals for 28 h to measure the amount of nuclear DNA, and by cytological techniques to estimate the cell cycle stage (Figure 5). Cytological analysis showed that 4N cells accumulated in the second cycle. In the early part of the cycle, this was due to the accumulation of anaphase cells suggested by the previous results, but binucleate cells also accumulated in the culture (Figure 5B–D) after the completion of the prolonged anaphase. The DNA and cytological analyses showed that despite the excess CENP-C, the cells traversed the second cycle with no apparent disruption of interphase. Furthermore, these

Fig. 3. Effect of excess CENP-C on cell cycle progression. (A) DT40 cells potentially capable of overexpressing CENP-C were blocked at metaphase by nocodazole and released into fresh medium in the presence or absence of tetracycline (TET) for 12 h. At the indicated times, cells were harvested and analysed for DNA content by FACS after staining with propidium iodide. The positions of the cell populations with 2N and 4N DNA contents are indicated. (B) DT40 cells potentially capable of overexpressing CENP-C were blocked at metaphase by nocodazole and released into fresh medium in the presence or absence of tetracycline (TET) for 12 h. At the times indicated, cells were harvested and the CDC2 kinase activity was measured using purified histone H1 as a substrate. Total radioactivity of histone H1 was measured by an Instant Imager. (C and D) indicated DT40 cells potentially capable of overexpressing CENP-C were blocked at metaphase by nocodazole and released into fresh medium in the presence (C) or absence (D) of tetracycline (TET) for 12 h. Cells were harvested at the indicated time after release of the nocodazole block and cyto-centrifuged onto a standard microscope slide to assess the stage in the cell cycle. After fixation and staining with DAPI, the proportion of the indicated cell type was counted under a fluorescent microscope. At least 1000 cells were scored at each time point. Viability was determined by Trypan blue exclusion.

cells went into the second round of mitosis on schedule (at 20 h) and, by 28 h, 20% of the cells had accumulated with an 8N DNA content (Figure 5A). Chromosomes which were elongated and kinked were present in the 8N mitotic cells at 28 h (Figure 5E). We noticed similarly deformed chromosomes in the cells at the end of the first cycle and wondered whether they were sister chromosomes isolated from anaphase cells or whether they were deformed metaphase chromosomes. In order to resolve this point, we counted the number of large chromosomes in such figures and observed that even though the chromosomes were thinner than usual in appearance, which suggested that they might in fact be sister chromatids, the chromosome number was ~12. DT40 are karyotypically very similar to normal chicken cells, are nearly diploid

and contain 12 large chromosomes, and so we conclude that the abnormally shaped chromosomes that we see in these spreads are metaphase chromosomes and not anaphase sister chromatids.

Excess CENP-C is associated with errors of chromosome segregation

In yeast, excess MIF2p is associated with an increase in the frequency of chromosome mis-segregation (Brown *et al.*, 1993). We wanted to determine whether overexpression of CENP-C had similar consequences. Chromosome analysis is difficult in chicken cells because chicken chromosomes are poorly characterized. We therefore made use of the DT40 hybrid cell line containing the mini-chromosome derived from the human Y chromosome

Fig. 4. Excess CENP-C is chromosome associated at metaphase and dispersed at anaphase. (A–D) DT40 cells containing the tetracycline-repressible CENP-C gene were grown either in the presence (A and C; control) or the absence of tetracycline (B and D; CENP-C overexpression) and the CENP-C localization examined using an affinity-purified anti-CENP-C antibody. CENP-C signals were detected by Cy3-conjugated second antibody (red). DNA was counterstained by DAPI (blue). (E–H) DT40 cells containing the tetracycline-repressible CENP-C gene and the human Y-derived mini-chromosome 2.5 were grown either in the presence (E and G; control) or the absence of tetracycline (F and H; CENP-C overexpression). CENP-C (green) was localized using an affinity-purified anti-CENP-C antibody and the mini-chromosome (red) localized by fluorescent *in situ* hybridization. DNA was counterstained by DAPI (blue). The scale bars correspond to 10 μ m.

which overexpresses CENP-C (DT2.5-5). DT40 cells are potentially useful for analysing many different aspects of vertebrate chromosome biology, and the use of a hybrid cell line containing a well-characterized human chromosome to assay chromosome segregation accuracy may have general applicability, and so we present some of the raw data in Figure 6. Measuring errors of chromosome segregation by this method indicated that the Y-derived mini-chromosome failed to segregate accurately in ~20% of the cell divisions when CENP-C is present in excess (Table I). We used separate telomere (Cooke *et al.*, 1985) and centromere probes (Tyler-Smith and Brown, 1987) in the cytogenetic analysis and so the experiment also enabled us to determine whether excess CENP-C was associated with chromosome breakage. The number of nuclei containing unequal numbers of centromeres and telomeres is increased in the presence of excess CENP-C, but the increase is very small, and thus we conclude that the level of chromosome breakage, although detectable, is low in these overexpressing cells. We also analysed conventional chromosome spreads for evidence of chromosome breakage and confirmed the small but detectable increase in the frequency of chromosome breakage (data not shown).

Excess CENP-C disrupts the mitotic spindle

CENP-C is a potential component of the kinetochore microtubule-binding complex, and so one cause of the anaphase prolongation might be spindle disruption. Immunocytochemical analysis of cells overexpressing CENP-C confirmed that this was the case. Light microscope analysis of control cells grown in the presence of

tetracycline and immunocytochemically stained with anti-tubulin antibody demonstrated a well ordered spindle at metaphase (Figure 7A) and anaphase (Figure 7C). However, the cells which overexpressed CENP-C showed extensive spindle disruption (Figure 7B, D and E). We carried out the analysis of the spindle disruption in the DT2.5-5 cells which contained the human mini-chromosome in order to help to define the ploidy and cell cycle stage of the cells with the disrupted spindle. The results indicated that although there was massive spindle disruption in these cells which was sometimes associated with the formation of apparently multi-polar spindles, there was no evidence in the distribution of the abnormal mitotic spindle to suggest that the CENP-C was binding the microtubules (Figure 7E). In metaphase cells, the microtubules did not coat the chromosomes as one would predict on the basis of the distribution of the CENP-C.

Excess CENP-C does not disrupt assembly of the native centromere and CENP-C is not sufficient for full centromere formation

There are several possible explanations for how excess CENP-C might disrupt mitosis. According to one explanation, the excess CENP-C could be acting by disrupting the spindle without disrupting the function of the centromere; according to a second explanation, the excess CENP-C could be titrating one or more centromere proteins which in turn leads to defective centromere function. We needed to be able to detect centromere disruption if we were to decide between these explanations. We therefore used the presence or absence of the ZW10 antigen as a marker for

Fig. 5. Cytokinesis is inhibited in the presence of excess CENP-C. (A) DT40 cells potentially capable of overexpressing CENP-C were blocked at metaphase by nocodazole and then cultured in fresh medium in the presence (control) or absence of tetracycline (TET) for 28 h. At 4 h intervals, cells were harvested and analysed for DNA content by FACS after staining with propidium iodide. The positions of cell populations with 2N, 4N or 8N DNA content are illustrated. (B) DT40 cells potentially capable of overexpressing CENP-C were cultured in the absence of tetracycline for 28 h to induce CENP-C overexpression. Cells were then fixed in 70% ethanol, and the DNA was stained with DAPI and imaged using fluorescence and differential interference contrast. A merged image illustrating two binucleate cells is shown. (C and D) DT40 cells potentially capable of overexpressing CENP-C were blocked at metaphase by nocodazole and released into fresh medium in the presence (C; control) or absence of tetracycline (D) for 12 h. Cells were harvested at the indicated times after release of the nocodazole block and cyto-centrifuged onto a standard microscope slide. After fixation and staining with DAPI, the proportion of the indicated cell type was counted under a fluorescent microscope. At least 1000 cells were scored at each time point. Viability was determined simultaneously by trypan blue exclusion. (E) Cells containing the tetracycline-repressible transgene were cultured in the absence of tetracycline for 30 h to induce CENP-C overexpression. Cells were then swollen in hypotonic buffer, fixed in methanol:acetic acid, chromosomes spread on a slide and stained with DAPI. The chromosomes are pseudo-coloured red. The scale bar in (E) corresponds to 10 μm.

an intact centromere and detected the ZW10 using the antiserum discussed above. We considered ZW10 to be a good marker for these experiments because it is present at active human centromeres (Faulkner *et al.*, 1998) as well as at *Drosophila* neo-centromeres (Williams *et al.*, 1998). We therefore analysed the effect of overexpressing CENP-C on the structure of the native centromere using the ZW10 antibody. In both control cells and the overexpressing cells, the centromere was intact as judged by the presence and amount of detectable ZW10 antigen (Figure 8). One limitation of this experiment is that it is not demonstrated directly that the chromosomes illustrated in Figure 8D have accumulated excess CENP-C. In order to resolve this point, we established a cell line overexpressing CENP-C tagged with the green fluorescent protein (GFP). These cells had a mitotic phenotype that was similar to that of the cells which overexpressed CENP-C, and we used the GFP tag to co-localize the CENP-C and the ZW10 in the overexpressing cells. The

results confirmed the single labelling experiments and demonstrated that excess CENP-C does not disrupt the distribution of the ZW10 (Figure 8F). We therefore concluded that the effects of overexpressing CENP-C are unlikely to arise as a consequence of the excess CENP-C titrating some key centromere component and thereby disrupting centromere structure and function. We also observe that overexpressing CENP-C leads to no detectable ectopic binding of the ZW10 protein to the chromosome arms, and so we also conclude that CENP-C itself is not sufficient for centromere assembly.

Excess CENP-C leads to centromere clustering during interphase

We wanted to understand why overexpressed CENP-C had no detectable effect upon the rate of progress through interphase and so we immunocytochemically analysed the distribution of CENP-C in overexpressing interphase cells. This analysis demonstrated that in the overexpressing

Fig. 6. Chromosome mis-segregation and breakage in a DT40 hybrid cell line which contains a human mini-chromosome and overexpresses CENP-C. (A–E) Examples of double-colour FISH analysis signals on interphase nuclei of DT40 cells which contain a human mini-chromosome and which overexpress CENP-C (2.5-5 cells) after growth in the absence of tetracycline for 20 h. Probes specific for the Y chromosome centromere (alphoid DNA, labelled green and abbreviated G) or the pseudo-autosomal telomere (labelled red and abbreviated R) were used to identify either errors of segregation (R:G, 2:2 or R:G, 0:0) or chromosome breakage events (R:G, 1:2, 2:1, 0:1 and 1:0). The nuclei containing the relevant figures are indicated by arrows. FISH analysis was performed on cells grown in the presence or absence of tetracycline. Signal distribution was counted in ~1000 nuclei of cells from both cultures. The results are summarized in Table I. The scale bar corresponds to 10 μ m.

Table I. Aberrant segregation of a mini-chromosome in cells which overexpress CENP-C

R:G	1:1	2:2	0:0	1:2	1:0	2:1	0:1	Others
Single nucleus								
–TET	741 (64.5%)	117 (10.2%)	61 (7.9%)	10 (0.9%)	6 (0.5%)	7 (0.6%)	4 (0.4%)	3 (0.3%)
+TET	932 (96.5%)	14 (1.4%)	12 (1.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (%)
R:G	1:1 + 1:1	2:2 + 0:0	1:2 + 1:0	2:1 + 0:1	Others			
Binuclei								
–TET	123 (10.7%)	39 (3.4%)	3 (0.3%)	2 (0.2%)	3 (0.3%)			
+TET	8 (0.8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)			

We used fluorescent *in situ* hybridization to measure the accuracy of the segregation of a mini-chromosome derived from the human Y chromosome in DT40 cells which overexpress CENP-C or in the same cells grown in the presence of tetracycline and which do not express CENP-C. Probes specific for the Y chromosome centromere (alphoid DNA, labelled green) or the pseudo-autosomal telomere (labelled red) were used to identify either errors of segregation (R:G, 2:2 or R:G, 0:0) or chromosome breakage events (R:G, 1:2, 2:1, 0:1 and 1:0). Signal distribution was counted in ~1000 nuclei of cells from both cultures. The table indicates the number of each type of figure in either mononucleate or binucleate cells.

cells, the CENP-C was distributed in ~30 large spherical structures in the nucleus which replaced the 80 or so CENP-C-containing kinetochores found in control cells (Figure 9A and B). The decrease in the number of CENP-C-containing structures occurred despite the increase in the amount of CENP-C and suggested that the excess CENP-C had assembled at pre-existing centromeres which had then clustered. If so, the centromeric but not the telomeric DNA should be associated with these large CENP-C-containing structures in the overexpressing cells. In order to investigate this point, we made use of the overexpressing cells containing the human Y-derived mini-chromosome [the DT2.5-5 (CENP-C) cells] (Figure 9C). We analysed these cells by combined FISH and immunocytochemistry with antibodies to CENP-C and probes to either the Y chromosome centromere, the Y chromosome

short arm telomere or the chicken *hprt* gene which is found very close to the telomere at the short arm of chicken chromosome 4 (Fukagawa *et al.*, 1999). This analysis established that the CENP-C-containing structures included the centromeric sequences but not the telomeres. We therefore conclude that in interphase cells, the excess CENP-C preferentially targets to and assembles at pre-existing centromeres.

Discussion

In order to investigate the possible functions of CENP-C, we have compared the effects of overexpressing CENP-C with the consequences of disrupting the localization of CENP-C to the centromere. When CENP-C was removed from the centromere, the mutant cells blocked at the

Fig. 7. Excess CENP-C disrupts the mitotic spindle. DT40 cells containing the tetracycline-repressible CENP-C gene and the mini-chromosome 2.5 were grown either in the presence (A and C; control) or in the absence of tetracycline (B, D and E; CENP-C overexpression) and the tubulin localization was examined using the YL1/2 rat monoclonal anti-yeast tubulin antibody and FITC-conjugated anti-rat IgG (green). The mini-chromosome (red and indicated with an arrowhead) was localized subsequently by FISH. DNA was counterstained by DAPI (pseudo-coloured red). The scale bar corresponds to 10 μ m.

metaphase–anaphase boundary and another member of the centromere protein complex, ZW10, was lost from the centromere. During interphase, overexpressed CENP-C accumulates within the nucleus around pre-existing centromeres and causes them to cluster. During metaphase, the excess CENP-C coats the chromosome arms. Neither interphase, the metaphase–anaphase transition nor the native centromeres are disrupted detectably by the excess CENP-C, but the microtubule spindle and the accuracy of chromosome segregation itself are massively disturbed. Cells overexpressing CENP-C are delayed in mitosis with low H1 kinase levels, demonstrating that they are in anaphase, and cytokinesis itself is inhibited. Here we discuss the significance of these observations for our understanding of the centromere and its role in mitosis.

CENP-C and the mitotic spindle

Although the observation that the overexpression of a centromere protein inhibits mitosis might be considered obvious, this is not so; overexpression of CENP-A, for example, is associated with no strong phenotype (Vafa and Sullivan, 1997; our unpublished observations). The

Fig. 8. Overexpressing CENP-C does not disrupt the native centromere. (A–D) CENP-C or ZW10 localization on metaphase chromosomes of DT40 cells potentially capable of overexpressing CENP-C grown in the presence (control) or absence of tetracycline (CENP-C overexpressing) for 24 h. Rabbit antibodies to either chicken CENP-C or to ZW10 were applied onto a metaphase chromosome slide and bound antibodies were detected with Cy3-conjugated second antibodies (red). DNA was counterstained by DAPI (blue). The scale bar corresponds to 10 μ m. (E and F) ZW10 localization on metaphase chromosomes of DT40 cells potentially capable of overexpressing a CENP-C–GFP fusion protein grown in the presence (control) or absence of tetracycline (CENP-C–GFP overexpressing). Rabbit antibodies to ZW10 were applied onto a metaphase chromosome slide and bound antibodies were detected with Cy3-conjugated second antibodies (red). DNA was counterstained by DAPI (blue). CENP-C–GFP is pseudo-coloured green. The scale bars correspond to 10 μ m.

delay or inhibition of mitosis that is seen in the presence of excess CENP-C is a reflection of a prolonged anaphase, and is accompanied by the inhibition of cytokinesis and an increase in the frequency of errors of chromosome segregation. Excess CENP-C is associated with spindle disruption and it seems likely that this causes the defective mitosis. Why should excess CENP-C disrupt the mitotic

Fig. 9. Excess CENP-C leads to centromere clustering during interphase. (A) Immunolocalization of CENP-C in interphase nuclei (red) from cells grown in the presence (control) or absence (CENP-C overexpression) of tetracycline (TET) for 20 h. DNA was counterstained by DAPI (blue). The scale bar corresponds to 10 μ m. (B) The number of CENP-C-containing structures in nuclei from cells grown in the presence or absence of tetracycline (TET) for 20 h. In the presence of tetracycline, we observed an average of 75 dots (75 ± 8) closely corresponding to the number of chicken chromosomes ($2N = 78$). In cells grown in the absence of tetracycline and which overexpress CENP-C, the average number of CENP-C-containing structures was 26 ± 11 . (C) Simultaneous detection by FISH and immunofluorescence in interphase of CENP-C and either centromeric or telomeric sequences. DT40 cells containing the human mini-chromosome and expressing CENP-C under the control of the tetracycline-repressible promoter were grown for 20 h in the absence of tetracycline. CENP-C was first localized immunocytochemically (green) and then either centromeric (Y alphoid DNA) or telomeric (pseudo-autosomal telomere and chicken *hprt*) sequences were localized by FISH. FISH signals were detected by Cy3-conjugated avidin (red). DNA was counterstained by DAPI (blue). The scale bar corresponds to 10 μ m.

spindle? We initiated this study in order to test the idea that CENP-C is sufficient to induce centromeres to form. Two observations demonstrate that CENP-C is necessary but not sufficient for centromere formation; excess CENP-C causes no change in the distribution of the centromeric protein ZW10, and the excess CENP-C has

no effect upon the metaphase–anaphase transition. We are therefore forced to conclude that spindle disruption is not caused by ectopic centromere formation. Neither the position of CENP-C in the wild-type centromere (Saitoh *et al.*, 1992) nor any of our observations are consistent with the CENP-C itself binding to microtubules. We

therefore conclude that CENP-C disrupts the spindle by binding to and ectopically localizing one or more factors necessary for accurate spindle formation. The identity of the hypothetical factor or factors remains to be established. It is striking that although the excess CENP-C causes a significant change in the structure of the mitotic spindle, the cells make the transition to anaphase with no delay. How do we explain this? One possibility is that the mitotic checkpoint is disrupted. We tested this idea by examining the consequences of adding colcemid to the cells overexpressing CENP-C. The results (not shown) were clear. The cells blocked in metaphase, and so we conclude that the checkpoint is intact and not disrupted by the excess CENP-C.

Cells which overexpress CENP-C often fail to undertake cytokinesis. This behaviour is similar in some respects to what was seen when anti-centromere antibodies were injected into human fibroblasts (Bernat *et al.*, 1990). In these experiments, the antibody-injected cells were delayed at metaphase with a disrupted spindle but, after transiting to anaphase, often failed to execute cytokinesis. Although cytokinesis is complex, the fact that two quite different approaches provoke the same effect could be caused by the fact that both the antibody injection and the overexpression lead to a dispersal of CENP-C from the centromere.

CENP-C and ectopic centromere formation

An important aspect of our study is the conclusion that the native centromeres appear not to be disrupted in the presence of excess CENP-C. This conclusion is an important one because it underpins the idea that CENP-C, although necessary, is not sufficient to seed centromere formation. However, how strong is this conclusion? One piece of evidence is the observation that the presence of ZW10 at the native centromere is not disrupted by the excess CENP-C. However, ZW10 is just one component of the centromere multi-protein centromeric complex, and it is possible that there are others which are eluted from the centromere and this results in the centromere functioning aberrantly. Although this type of objection can only be answered by a complete characterization of the centromere, the fact that the cells make the metaphase–anaphase transition without any delay in the presence of excess CENP-C strongly suggests that the centromere is intact and fully functional at least until the initiation of anaphase.

We argued above that the spindle disruption characteristic of the cells with excess CENP-C was caused by CENP-C binding to and ectopically localizing a factor necessary for accurate spindle formation. The argument that excess CENP-C does not disrupt the native centromere suggests that this hypothetical factor is not centromeric. Accurate spindle assembly is likely to be sensitive to the levels and locations of many factors. Binding of any of these factors by CENP-C could therefore be responsible for the phenotype. Further characterization of the factors which bind CENP-C during anaphase may explain this aspect of the overexpression phenotype.

CENP-C structural re-organization during the cell cycle

The conclusion that CENP-C is insufficient to cause a centromere to form has the value of excluding one

line of investigation as to the mechanism of centromere activation. A second aspect of our study, although not fully understood, may repay further study with regard to both our understanding of the centromere itself and centromere activation. We have shown that during interphase, the excess CENP-C assembles around pre-existing centromeres in large structures. These structures disperse at the beginning of mitosis, when CENP-C coats the chromosome arms, and appear to start to re-assemble after the initiation of anaphase. Although it is unclear as to whether these large structures have any physiological significance, their regular appearance and the fact that they form at centromeres suggests that they may have. More detailed characterization of the structure of these complexes may suggest new ideas about how CENP-C functions at the centromere. If changes in the distribution of the excess CENP-C are a reflection of changes in the structure and organization of the native centromere, then it will be of interest to identify the modifications of CENP-C which trigger the structural re-organization. Although one possibility is CDC2-catalysed phosphorylation, recent results suggest that CENP-C may also be a target for modification by the small ubiquitin-like protein SUMO-1 (Everett *et al.*, 1999). If CENP-C is modified by SUMO-1, then it will be of interest to determine whether the pattern of SUMO-1 modification changes during the cell cycle.

The fact that excess CENP-C accumulates at pre-existing centromeres during interphase and the observation that CENP-C is necessary for centromere formation provide a simple explanation of why centromeres do not form readily at ectopic sites. According to this explanation, CENP-C normally assembles at pre-existing centromeres and is not available to function at other non-centromeric loci. We initiated our experiments in order to test the idea that CENP-C was sufficient to form a centromere. In one set of experiments not described above, we expressed a fusion between CENP-C and the bacterial DNA-binding protein *lexA* in a cell line which contained a concatamer of 200 *lexA*-binding sites targeted to one allele of the *hprt* gene (Fukagawa *et al.*, 1999). In these cells, we were unable to detect the binding of the *lexA*–CENP-C fusion to the *hprt* locus when we inducibly expressed the *lexA*–CENP-C fusion. In the interphase cells, the inducibly expressed *lexA*–CENP-C was present at centromeres, and in the mitotic cells it had a distribution that was qualitatively similar to that seen with the overexpressed CENP-C described in this report. The affinity of the *lexA* protein for its binding site is in the nanomolar range, suggesting that the affinity of native CENP-C for the centromere is higher than this.

Other studies of CENP-C disruption

Several groups have studied CENP-C function by either disrupting the function of the molecule with antibodies or using genetic techniques. Originally, Earnshaw and colleagues studied the effects of microinjecting anti-CENP-C antibodies into human fibroblasts (Tomkiel *et al.*, 1994). Subsequently, we used DT40 to generate a conditional loss of CENP-C function mutant, and Choo and colleagues generated and studied a knockout of CENP-C in the mouse (Kalitsis *et al.*, 1998). The main conclusions from each of these experiments are similar, and all

demonstrate that CENP-C is necessary for centromere function and required for the metaphase–anaphase transition. Our results differ from those of Choo and colleagues in one respect that may be significant. When grown under restrictive conditions, the Δ CENP-C–ER accumulate at metaphase with de-condensed chromosomes, while in the mouse knockout the chromosomes accumulate in a hypercondensed state. The basis of this difference is not obvious. The hypercondensed state seen by Choo is typical of what one expects from a cell that has been delayed in metaphase. Why do we see de-condensed chromosomes in our experiments? One possibility is that the ectopically localized CENP-C–ER is binding factors which regulate chromosome condensation. The possibility that CENP-C may be interacting with proteins involved in chromosome condensation is also suggested by the observation that chromosomes with abnormal morphology are isolated from cells grown in the presence of excess CENP-C (Figure 5E). This possibility is in some ways an attractive idea because the centromere is the site of a primary constriction. Further studies of the molecules which associate with CENP-C may cast light on this aspect of both the overexpression and the loss-of-function phenotypes.

Materials and methods

Molecular biology

All plasmids were constructed by standard methods. The transactivator tTA was isolated from pUHD15-1 (Gossen and Bujard, 1992) and cloned into the *Eco*RI site of pPyCAGIRESzeocinP (Niwa *et al.*, 1991), which contain human cytomegalovirus enhancer, chicken β -actin promoter, an internal ribosomal entry site and a zeocin-resistant gene, to create tTA/IRES/Zeo. Full-length CENP-C cDNA was cloned into the *Eco*RI site of pUHD10-3 (Gossen and Bujard, 1992) in order to construct the tetracycline-sensitive expression plasmids referred to as pUHD-CENP-C. Chicken ZW10 was isolated by the degenerate RT–PCR of mRNA extracted from DT40 cells. The sequences of the degenerate primers were 5'-CA(CT) CAC AA(CT) AA(CT) TG(CT) ATG and CTG (AG)A ACA A(ATG) GC (CAT) C (TG) (GA) ATTA. We then screened a chicken macrophage library (gift from John Young, Institute of Animal Health, Compton) using the RT–PCR product as a probe. Two independent positive clones were sequenced and identified as full-length chicken ZW10 cDNAs. Chicken ZW10 is 753 amino acids long and showed 70% identity to the human homologue.

Antibody production and immunoblotting

A chicken CENP-C expression construct (amino acids 1–303) was created in the vector pET14b (Novagen) and expressed in *Escherichia coli* BL21 (DE3) after 4 h induction with 0.5 mM isopropyl- β -D-galactopyranoside (IPTG). The purified recombinant CENP-C was used to immunize two rabbits. Serum was affinity purified against the total bacterial protein and recombinant CENP-C protein on a CNBr-activated Sepharose 4B column (Pharmacia). A fragment of chicken ZW10 cDNA was cloned by RT–PCR and used to isolate two full-length cDNAs. One of these was sequenced and shown to be 70% similar to human ZW10, confirming its identity. Details of the ZW10 cloning and biology will be published elsewhere. A chicken ZW10 expression construct (amino acids 488–753) was created and expressed by a similar method as for the CENP-C protein and was used for rabbit immunization.

We isolated nuclei for immunoblotting analysis. Cells were collected, washed with phosphate-buffered saline (PBS) and resuspended in lysis buffer [15 mM Tris–HCl pH 7.5, 5 mM MgCl₂, 25 mM KCl, 5 mM EDTA, 0.25 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT)]. Cells were lysed by using a teflon Dounce homogenizer 20 times and were then centrifuged at 200 g. The nuclei were washed twice with lysis buffer. Total nuclear protein extracts were prepared by sonication of the nuclei in gel sample buffer. The protein samples were resolved by SDS–PAGE and electrophoretically transferred to Hybond-P membranes (Amersham). The blots were blocked by 3% bovine serum

albumin (BSA) in TBST (15 mM Tris–HCl pH 7.5, 200 mM NaCl, 0.1% Tween-20) for 30 min at room temperature and then incubated overnight at room temperature with affinity-purified anti-CENP-C antibody diluted 1:40 000 in blocking solution. The horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Inc.), diluted 1:20 000 in the blocking solution, were used to detect bound first antibody. The blots were developed using the ECL-plus kit (Amersham) and CENP-C protein was visualized by STORM (Molecular Dynamics). The immunoblotting analysis demonstrated that the native CENP-C had an apparent mol. wt of 130 kDa as judged by SDS–PAGE. The mass of the native protein predicted on the basis of amino acid sequence was 95 kDa. A similar difference between the predicted molecular mass and the mass observed by SDS–PAGE has been noted for human CENP-C (Saitoh *et al.*, 1992) and suggests that CENP-C in both species is either modified extensively post-translationally or that CENP-C has an extended conformation.

Cell culture and transfection

DT40 cells were cultured and transfected as described before (Buerstedde and Takeda, 1991; Fukagawa and Brown, 1997). Δ CENP-C–ER DT40 cells were cultured in the presence of 4-hydroxytamoxifen at 100 nM final concentration. Tetracycline was used at a final concentration of 2 μ g/ml. Histidinol was used at a final concentration of 1 mg/ml and zeocin at a final concentration of 1 mg/ml to select for stable transfectants. In order to create the cell lines expressing CENP-C inducibly, we firstly made tTA-DT40 and tTA-2.5, which were transfected with tTA/IRES/Zeo. Then tTA-DT40 cells and tTA-2.5 cells were co-transfected with *Pvu*I-digested pUHD-CENP-C and the β -actin-his plasmid digested with *Bam*HI, at a ratio of 10:1. After transfection, we picked up colonies and screened for the CENP-C plasmid by Southern hybridization. Expression was confirmed by RT–PCR, immunofluorescence and Western blotting.

Cell cycle analysis

Cells were synchronized at metaphase by the addition of 500 ng/ml nocodazole [Sigma, added from a 1000 $\times$ stock in dimethylsulfoxide (DMSO)] for 6.5 h and then released from the nocodazole block by washing three times in medium and re-culturing in fresh medium. At various time points after release, cells were harvested and analysed. For flow cytometric (FACS) analysis, cells were washed twice in ice-cold PBS and fixed in ice-cold 70% ethanol in PBS for 15 min on ice. After fixation, cells were washed and treated with RNase A (100 μ g/ml) in PBS for 30 min at room temperature. The cells were then stained with propidium iodide (10 μ g/ml) in PBS. Samples of 10 000 cells were then analysed on a Becton Dickinson FACScan. CellQuest (Becton Dickinson) software was used for acquisition and manipulation of the data. For cytological analysis, cells were harvested after release of the nocodazole block and cyto-centrifuged onto a standard microscope slide. After fixation and staining with 4',6-diamidino-2-phenylindole (DAPI), the proportion of the indicated cell type was counted under a fluorescent microscope. At least 1000 cells were scored at each time point. There is a quantitative inconsistency between the results of the FACS and cytological analyses of the cell cycle in that the FACS suggest that after 12 h growth in the absence of tetracycline 78% of cells have 4N DNA, while the cytological analyses indicates that only 20% of the cells are in mitosis (Figure 3). We think that the reason for the discrepancy is that mitotic cells are under-represented in cyto-centrifuged preparations either because they are lost during sedimentation or because they cannot be identified after centrifugation onto the slide. We favour the second explanation because we notice that there are many aberrantly shaped cells on the slides which we cannot stage in the cell cycle.

Histone H1 kinase assay

Histone H1 kinase activity in p34^{CDK2} immunoprecipitates was measured as described by Itzhaki *et al.* (1997). Approximately 1 $\times$ 10⁶ cells were washed with PBS twice and suspended in 120 μ l of lysis buffer [20 mM Tris–HCl pH 7.5, 10 mM EDTA, 100 mM NaCl, 1% Triton X-100, 1 mM NaF, 1 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM EGTA and a cocktail of proteinase inhibitors ('Complete', Boehringer Mannheim)] and, after 20 min at 0°C, debris was pelleted by centrifugation. A 100 μ l aliquot of lysate and 2 μ l of an anti-p34^{CDK2} antibody (Krek and Nigg, 1991) were mixed and incubated on ice for 1 h. A 20 μ l aliquot of a protein A–Sepharose beads suspension was then added. After gentle agitation at 4°C for 30 min, beads were washed four times in lysis buffer, the second wash containing 1 M NaCl, and twice in kinase buffer (25 mM MOPS pH 7.2, 15 mM MgCl₂, 5 mM EDTA, 1 mM DTT, 60 mM β -glycerophosphate, 15 mM *p*-nitrophenyl phosphate, 0.1 mM sodium orthovanadate and the complete mix of

protease inhibitors). Pelleted beads were mixed with an equal volume of a reaction mix which consisted of kinase buffer containing Triton X-100 (1%), [γ - 32 P]ATP and histone H1 (1 mg/ml) and incubated at 30°C for 15 min. The reaction was then chilled and stopped by the addition of a one-third volume of 3× gel sample buffer. The samples were loaded on an SDS–polyacrylamide gel. Labelled H1 in dried gels was visualized and quantitated using an Instant Imager (Packard).

Immunocytochemistry

Immunofluorescent staining for whole cells was performed as described previously (Fukagawa and Brown, 1997). Cells were collected onto slides by a cyto-centrifuge and fixed in 3% paraformaldehyde in PBS for 15 min at room temperature, permeabilized in 0.5% NP-40 in PBS for 15 min at room temperature, rinsed three times in 0.5% BSA and stained for 1 h at 37°C in either neat supernatant of the YL1/2 anti-tubulin antibody (Kilmartin *et al.*, 1982) or in anti-CENP-C antibody diluted 1:1000 in PBS/0.5% BSA; these antibodies were then detected using either fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat antibody at 1:66 dilution (Vector) or Cy3-conjugated goat anti-rabbit IgG at 1:1000 (Amersham) dilution in PBS/0.5% BSA.

Metaphase chromosome spreads for immunofluorescence staining were prepared by a method modified from Earnshaw *et al.* (1989). Cells were collected by centrifugation and swollen in a hypotonic solution (40 mM KCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.4) for 10 min at 37°C. After centrifugation, cells were fixed in methanol/acetic acid (3:1) for 25 min at –20°C. Fixed cells were put onto a slide and dried quickly. The slides were treated with KCM (120 mM KCl, 20 mM NaCl, 10 mM Tris–HCl pH 8.0, 0.5 mM EDTA, 0.1% Triton X-100, 0.1% BSA) for 15 min at room temperature. Chromosome spreads were then stained for 30 min at 37°C in either anti-CENP-C antibody diluted 1:1000 in TEEN (1 mM triethanolamine–HCl pH 8.5, 0.2 mM EDTA, 25 mM NaCl, 0.5% Triton X-100, 0.1% BSA) or in anti-ZW10 antibody diluted 1:200 in TEEN; after washing with KB (10 mM Tris–HCl pH 7.7, 0.15 M NaCl, 0.1% BSA), these antibodies were detected using Cy3-conjugated goat anti-rabbit IgG diluted 1:1000 (Amersham) in KB. Antibody-stained cells were counterstained with DAPI at 0.2 µg/ml or propidium iodide at 0.2 µg/ml in Vectorshield antifade. Images were collected using a Digital Pixel (KAF1400) 12-bit slow scan, cooled CCD camera mounted on a Zeiss Axioscop microscope with a Plan Neofluar 100 X/NA1.3 objective together with a filter wheel, and then manipulated in IPLab.

Fluorescence in situ hybridization (FISH) analysis

Metaphase chromosomes were prepared by addition of a hypotonic solution and fixed in methanol/acetic acid (3:1). FISH was carried out according to the method described by Griffin *et al.*, submitted. Biotin-labelled probe was detected with Cy3-conjugated avidin (Amersham, 1:500 dilution with 4× SSC, 0.05% Tween-20, 1% BSA) and digoxigenin-labelled probe was detected with mouse anti-dig antibody (1:666 dilution with 4× SSC, 0.05% Tween-20, 1% BSA) following by staining with FITC-conjugated rabbit anti-mouse IgG (1:1000 dilution with 4× SSC, 0.05% Tween-20, 1% BSA). Chromosomes and nuclei were counterstained with DAPI at 0.2 µg/ml.

Simultaneous detection of FISH and immunofluorescence

For simultaneous detection of FISH and immunofluorescence, we firstly carried out immunofluorescence staining as described above. After a final washing, slides were re-fixed in methanol/acetic acid (3:1) for 30 min at –20°C and dehydrated in 70, 90 and 100% ethanol for 2 min each at room temperature. For subsequent FISH, slides were air-dried overnight and denatured with 0.2 M NaOH in 70% ethanol for 5 min. FISH was carried out as described in the preceding section.

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