CENP-C Is Required for Maintaining Proper Kinetochore Size and for a Timely Transition to Anaphase

John Tomkiel, Carol A. Cooke, Hisato Saitoh, Rebecca L. Bernat, and William C. Earnshaw

Department of Cell Biology and Anatomy, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract. The human autoantigen CENP-C has been demonstrated by immunoelectron microscopy to be a component of the inner kinetochore plate. Here we have used antibodies raised against various portions of CENP-C to probe its function in mitosis. We show that nuclear microinjection of anti-CENP-C antibodies during interphase causes a transient arrest at the following metaphase. Injection of the same antibodies after the initiation of prophase, however, does not disrupt mitosis. Correspondingly, indirect immunofluorescence using affinity-purified human anti-CENP-C antibodies reveals that levels of CENP-C staining are reduced at centromeres in cells that were injected during interphase, but appear unaffected in cells which were injected during mitosis. Thus, we suggest that the injected antibodies cause metaphase arrest by reducing the amount of CENP-C at centromeres. Examination of kinetochores in metaphase-arrested cells by electron microscopy reveals that the number of trilaminar structures is reduced. More surprisingly, the few remaining kinetochores in these cells retain a normal trilaminar morphology but are significantly reduced in diameter. In cells arrested for extended periods, these small kinetochores become disrupted and apparently no longer bind microtubules. These observations are consistent with an involvement of CENP-C in kinetochore assembly, and suggest that CENP-C plays a critical role in both establishing and/or maintaining proper kinetochore size and stabilizing microtubule attachments. These findings also support the idea that proper assembly of kinetochores may be monitored by the cell cycle checkpoint preceding the transition to anaphase.

THE high fidelity transmission of chromosomes to daughter cells at each cell division is dependent on the interaction between the spindle apparatus and the *cis*-acting region of each chromosome known as the centromere. A number of essential mitotic functions either occur at, or are controlled by this highly specialized chromosomal domain. In addition to capture of spindle microtubules, the growth and disassembly of microtubules is somehow regulated at the centromere. The complex series of movements that chromosomes undergo to congress at the metaphase plate, and subsequently travel poleward, is controlled and generated at least in part by force-generating motor proteins that reside at the centromere. Finally, the centromere is responsible for regulating the cohesion and timely release of sister chromatids during mitosis (for a recent review see Bloom, 1993).

In recent years there has been rapid progress towards identifying and understanding the function of centromere components. In the simplest system studied, the budding yeast *Saccharomyces cerevisiae*, a complex has been identified which binds to both the essential *cis*-acting DNA, CDEIII (Lechner and Carbon, 1991), and to microtubules (Kingsbury and Koshland, 1991), and is capable of generating microtubule-based motor activity in vitro (Hyman et al., 1992). Three major components of this complex have been identified by genetical and biochemical means, and their individual roles at the centromere are currently under investigation (Doheny et al., 1993; Goh and Kilmartin, 1993; Jiang et al., 1993). However, while yeast promises to provide a fruitful model system for centromere function, it is unlikely that the centromeres of higher eukaryotes will simply be iterations of an *S. cerevisiae* centromere subunit. Even the centromeres of the fission yeast *Schizosaccharomyces pombe* are much more complex, and involve higher order repeats of centromeric DNA (Clarke and Baum, 1990; Chikashige et al., 1989; Nakaseko et al., 1986).

Electron microscopy studies have revealed that the higher eucaryotic centromere is also more complex in structural organization (Comings and Okada, 1971; Ris and Witt, 1981; Reider, 1982). A physical structure, known as the kinetochore, is found at the surface of the centromeric heterochromatin. The kinetochore mediates attachment of the

Address all correspondence to W. C. Earnshaw, Department of Cell Biology and Anatomy, Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205.

Dr. Saitoh's present address is La Jolla Cancer Research Foundation, 10901 North Torrey Pines Rd., La Jolla, CA 92037.

Dr. Bernat's present address is Department of Genetics, Stanford University School of Medicine, 300 Pasteur Dr., Stanford, CA 94305-5120.

chromatin to the spindle microtubules, and may be required to organize the underlying centromeric DNA into a single functional unit. It consists of three layers with differential staining properties. An electron-dense inner plate lies directly on the surface of the chromosome, and may represent a complex of *cis*-acting centromere DNA and centromere-specific chromatin proteins. This region is separated from an electron-dense outer plate by a electron-translucent middle zone. The compositions of the mid-zone and outer plate are unknown. It is generally believed that the mid-zone is a space, largely devoid of material. Several models have proposed that the outer plate is composed of chromatin loops (Rattner, 1987; Zinkowski et al., 1991; McEwen et al., 1993), although a concerted effort has failed to detect DNA in the outer plate (Cooke et al., 1993).

In mammals the comparatively large size of the centromere and the complexity of the centromeric heterochromatin have made biochemical dissection of the centromere a formidable task. Nonetheless, numerous components have been identified, either due to their fortuitous recognition by human autoantibodies (Moroi et al., 1980; Guldner et al., 1984; Earnshaw and Rothfield, 1985; Kingwell and Rattner, 1987), or by a more directed approach of raising antibodies to purified scaffold-associated proteins (Compton et al., 1991; Yen et al., 1991). Of the mammalian centromere proteins identified to date, only four have been characterized enough to provide a clue of their function at the centromere. These are known as the CENPs (CENtromere proteins)[1]. One of these, CENP-E, which was identified as a chromosome scaffold-associated protein, is a member of the kinesin superfamily (Yen et al., 1992). Inhibition of CENP-E by antibody injection during prometaphase delays the onset of anaphase (Yen et al., 1991), thus CENP-E may be involved in generating chromosome movement towards the poles. The remaining three of these centromere proteins have been identified through their reactivity with human anticentromere autoimmune (ACA) sera. Unlike CENP-E, these appear to be structural rather than motor proteins. CENP-A is believed to be a histone H3 variant because of its co-isolation with mononucleosomes (Palmer et al., 1987) and peptide sequence similarity with H3 (Palmer et al., 1991). It contains the histone-fold domain joined to a large amino-terminal domain that is unrelated to other proteins in the database (K. Sullivan, personal communication). CENP-B is an alpha-satellite DNA-binding protein (Masumoto et al., 1989) located in the heterochromatic central region of the centromere, interior to the kinetochore plates (Cooke et al., 1990). Finally, CENP-C is a component of the trilaminar kinetochore. Its localization is restricted to the inner kinetochore plate, at the interface between the centromeric heterochromatin and the outer kinetochore plate to which microtubules attach (Saitoh et al., 1992). Preliminary in vitro DNA-binding studies suggest that CENP-C may bind DNA (C. H. Yang, H. Saitoh, and W. C. Earnshaw, unpublished results). Thus, CENP-C may interact directly with the *cis*-sequences that specify centromere formation, and may play a role in establishing the kinetochore suprastructure.

1. *Abbreviations used in this paper*: ACA, anticentromere autoimmune; CENP, CENtromere protein; CF-PAC1, cystic fibrosis pancreatic cancer; DAPI, 4',6-diaminidino-2-phenyl-indole; MUG, mitosis with unreplicated genomes; NuMA, nuclear-mitotic apparatus protein; pk, porcine kidney.

Previous studies have demonstrated that injection of IgG purified from human ACA disrupts both kinetochore assembly and progression through mitosis and meiosis (Bernat et al., 1990; Simerly et al., 1990; Bernat et al., 1991). These studies provided the first evidence that the human centromere autoantigens play functionally important roles in cell division. Conclusions from these studies were limited, however, since the injected IgG was purified from autoimmune sera which recognized several centromeric antigens, including CENP-A, CENP-B, and CENP-C. Thus, the role of each individual antigen could not be accurately assessed. We have now raised antibodies specific for CENP-C, which we use here to investigate the role of CENP-C in centromere assembly and function.

Materials and Methods

Cell Culture

HeLa JW cells were cultured in monolayers in RPMI plus 5% fetal calf serum. Cystic fibrosis pancreatic cancer (CF-PAC1) cells were grown in Iscove's modified Dulbecco's medium without L-glutamine (BioWhittaker, Inc., Walkersville, MD) plus 5% fetal calf serum. LLCPK (porcine kidney) cells were grown in DME plus 5% fetal calf serum. Just prior to microinjection, 20 mM Hepes (pH 7.4), 10 μg/ml streptomycin, and 10 μ/ml Penicillin (GIBCO BRL, Gaithersburg, MD) were added to cultures.

Antibodies

β-galactosidase fusion proteins containing various regions of human CENP-C were produced in *E. coli* using the T7 polymerase-based expression system as previously described (Saitoh et al., 1992). These were used as antigens to raise rabbit anti-CENP-C antisera (see Fig. 1). In the case of the fusion protein containing CENP-C residues 296-943, two antisera were raised. One was raised against glutaraldehyde-fixed antigen in hopes of producing better antibodies for immuno-electron microscopy, which requires glutaraldehyde fixation to preserve structure. This antiserum is referred to as G296-943. IgG for injection was purified from all sera on immobilized protein A (Repligen, Cambridge, MA) as previously described (Bernat et al., 1990), dialyzed in Dulbecco's PBS (D-PBS) and concentrated to 5 mg of protein/ml using centricon 30 filters (Amicon Corp., Arlington Heights, IL). Antibodies were affinity purified from bacterially produced full-length CENP-C immobilized in situ in SDS-polyacrylamide gels as per (Madara et al., 1990). Affinity purified antibodies were similarly dialyzed and concentrated to 2.5 mg protein/ml.

Affinity Purification of Human Anti-CENP-C Antibodies

Autoimmune serum GS, which was used for the initial identification of CENP-A, -B, and -C (Earnshaw and Rothfield, 1985), was affinity purified against protein encoded by λ gt11 clone CNPC3 (Saitoh et al., 1992) as described in Earnshaw et al. (1989). This clone produces a partial CENP-C peptide containing amino acid residues 296-943. Briefly, phage were grown on bacterium Y1090 and induced onto nitrocellulose filters presoaked in 10 mM isopropylthiogalactoside. Filters were incubated with serum GS for 4 h, washed with PBS, and then eluted with glycine, pH 2.5. Eluted antibodies were neutralized with 1 M KPO_4, pH 9.0, and used undiluted for immunofluorescence and for immunoblotting.

Immunoblotting

HeLa chromosomes were isolated as previously described (Bernat et al., 1990), separated by SDS-PAGE on a 10% acrylamide gel, and transferred to nitrocellulose. Strips of these blotted chromosomes were probed with either human ACA serum GS (at 1:2,500), purifed IgG from anti-CENP-C rabbit polyclonal antisera (at 1:1,000), or antibodies affinity purified from bacterially expressed CENP-C (at 1:1,000). The CENP-C fusion protein used for affinity purification was the β fragment, which includes full-length CENP-C (Saitoh et al., 1991). Antibodies were detected by autoradiography after incubation of the strips with ^{125}I-protein A.

Antibody Injection into HeLa Cells

HeLa cells were grown into microcolonies on etched locator grid coverslips (BellCo Glass, Inc., Vineland, NJ) as previously described (Bernat et al., 1990). Antibodies were microinjected into each cell within a microcolony (into the nuclei in the case of interphase cells) using a Narishige micromanipulator and a Nikon PLI-188 microinjector. Cells were maintained at 37° using a Nikon NP-2 microscope stage incubator at all times during injection and subsequent observation. Injected cells were monitored once per hour for entry into mitosis. All cells included in the analysis were observed for at least 3 h after entry into mitosis, and the time spent in mitosis was noted at the time of fixation. For electron microscopy, colonies were fixed and processed when a suitable number of cells in a small area had accumulated in mitosis.

Immunofluorescence

Cells were rinsed for 1 min in D-PBS and fixed for 5 min in 4% formaldehyde in D-PBS. Cells were then processed as previously described (Bernat et al., 1990). Briefly, cells were rinsed and stained in lysis/wash buffer (150 mM NaCl, 10 mM Tris, pH 7.7, 0.1% Triton-X, 0.1% BSA). To visualize centromeres, cells were stained sequentially with patient serum GS at 1:1,000 (Earnshaw and Rothfield, 1985; Bernat et al., 1990) or undiluted affinity purified anti-CENP-C antibodies from this serum, biotinylated goat anti-human antibody (Vector Labs., Burlingame, CA) at 1:1,000, and streptavidin/Texas red (Bethesda Research Labs, Gaithersburg, MD) at 1:800. Serum GS recognizes CENPs A, B, and C on immunoblots of HeLa chromosomal proteins. Tubulin was stained with mouse anti-α (Accurate Chem. & Sci. Corp., Westbury, NY) and mouse anti-β tubulin antibodies (Amersham Corp.) at 1:50 and fluoresceinated goat anti-mouse antibodies (Dako Corp., Carpenteria, CA) at 1:500. For nuclear-mitotic apparatus protein (NuMA) and CENP-E staining, cells were stained at a 1:5,000 dilution with monoclonal antibody 1F1 (Compton et al., 1991) or 177 (Yen et al., 1991), respectively, followed by fluoresceinated goat anti-mouse antibodies at 1:500. Each incubation period was for 1 h at 37°C and was followed by 3 × 5 min washes with lysis/wash buffer at room temperature. DNA was stained with 4',6-diaminidino-2-phenyl-indole (DAPI) at 1 μg/ml for 10 min. Coverslips were mounted in Mowiol in PBS (Osborn and Weber, 1982). Slides were examined on an Olympus Vanox microscope. Images were obtained using a DAGE SIT camera driven by a Perceptics Hyperscope image processing program. Images were subjected to a fourier sharpening algorithm and photographed on T-max 100 film using GCC print manager software and a Colorfast digital film recorder (GCC Technologies, Bedford, MA).

Electron Microscopy

Cells were rinsed for 1 min with PHEM buffer (60 mM Pipes, pH 6.9, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$), permeabilized for 1 min with PHEM plus 0.1% Triton X-100, and fixed for 1 h in 2% glutaraldehyde in PHEM. Cells were then stained 30 min in 0.05% tannic acid in PBS, rinsed three times in PHEM, post-fixed in 1% osmium tetroxide in 0.1 M cacodylate, pH 7.4, for 20 min, and stained with 3% uranyl acetate in water for 2 h. After dehydration through a graded ethanol series, cells were embedded in Epon overnight at 60°C. Injected cells were identified using the impression of the grid pattern on the Epon block. Cells were serial sectioned and sections were collected on Formvar-coated slot grids. Grids were stained with uranyl acetate and lead citrate and examined with a Zeiss 10A transmission electron microscope at 60 kV.

Kinetochore Measurements

Kinetochores were photographed and printed at a magnification of either 16,000× or 48,000×. Two independent measurements of each kinetochore outer plate were made using a Fowler and NSK electronic digital caliper. The average of these two measurements was taken as the true value. Comparisons of control preimmune kinetochore sizes and anti-CENP-C kinetochore sizes were made using Student's *t*-test after transformation of the raw values by the formula $X = X^2$ to approximate a normal distribution of the data.

Results

Inhibition of CENP-C Causes a Transient Arrest at Metaphase

We used microinjection of anti-CENP-C antibodies to ask whether CENP-C performs an essential role in the chromosomal events of mitosis. Briefly, HeLa cells were allowed to form microcolonies on indexed coverslips. All members of selected microcolonies were microinjected with IgG purified from rabbit polyclonal sera, or antibodies affinity purified from bacterial CENP-C fusion protein which had been immobilized in polyacrylamide gels. Interphase cells as well as cells in various stages of mitosis were injected. The cells were then allowed to progress through the remainder of the cell cycle, and were monitored at least once per hour using phase contrast optics to determine the time of entry into mitosis (in the case of interphase-injected cells) and the time required to complete mitosis. When a suitable number of cells were observed to enter mitosis, the coverslip was fixed and processed for immunofluorescence with anti-tubulin antibodies, human ACA serum and DAPI (to permit visualization of the chromosomes). The fates of all cells that entered mitosis at any time during the observation period were recorded.

The antibodies used for injection were raised by immunization of rabbits with fusion proteins shown in Fig. 1 *A*. Control experiments involved microinjection of IgG purified from preimmune sera from these same rabbits. An immunoblot of HeLa chromosomal proteins probed with the IgG preparations used for these experiments is shown in Fig.1 *B*. All of the anti-CENP-C IgG used in the microinjections clearly react with CENP-C, and only weakly recognize other proteins. Similar results were obtained when antibodies were tested on immunoblots of total cell protein (data not shown). Fig. 1 *B* (lane *1*)is probed with an autoimmune serum that recognizes CENP-A and CENP-B as well as CENP-C, and thus indicates the positions at which these proteins have migrated. It is important to note that we see no cross-reactivity of our rabbit anti-CENP-C IgGs with these other centromere proteins, even when the blot is significantly overexposed. In a previous study we found that some of these anti-CENP-C sera apparently recognized a protein that comigrated with CENP-B (Saitoh et al., 1992). It is possible that such cross-reacting antibodies were lost during the IgG purification; a significant fraction of anti-CENP-B antibodies in some human patients is of the IgA subclass (Hildebrandt et al., 1990). However, to ensure that any phenotypes observed were due to inhibition with CENP-C, we also injected antibodies affinity-purified using a CENP-C fusion protein. These antibodies recognize only CENP-C (Fig. 1 *B*, lane *14*), even on extended exposures.

We found that introduction of anti-CENP-C antibodies into HeLa cells prior to nuclear envelope breakdown disrupted the subsequent mitosis (Table I). The predominant phenotype observed was a prolonged delay at metaphase. We obtained this same result for all of the anti-CENP-C antibodies tested, including the affinity-purified antibodies. Abnormal mitoses were observed at only a low frequency in cells injected with IgG purified from preimmune sera, at a level comparable to that observed in uninjected cells (7%). The effect of the antibodies was not specific to HeLa cells. We observed a similar metaphase delay in two other cell lines tested, a human CF-PAC1 and porcine kidney cell line (LLCPK) (Table I).

Our assessment of the metaphase delay phenotype is supported by the distribution of both NuMA (Lydersen and Pettijohn, 1980; Tousson et al., 1991; Yang and Snyder, 1992; Yang et al., 1992) and CENP-E (Compton et al., 1992; Yen

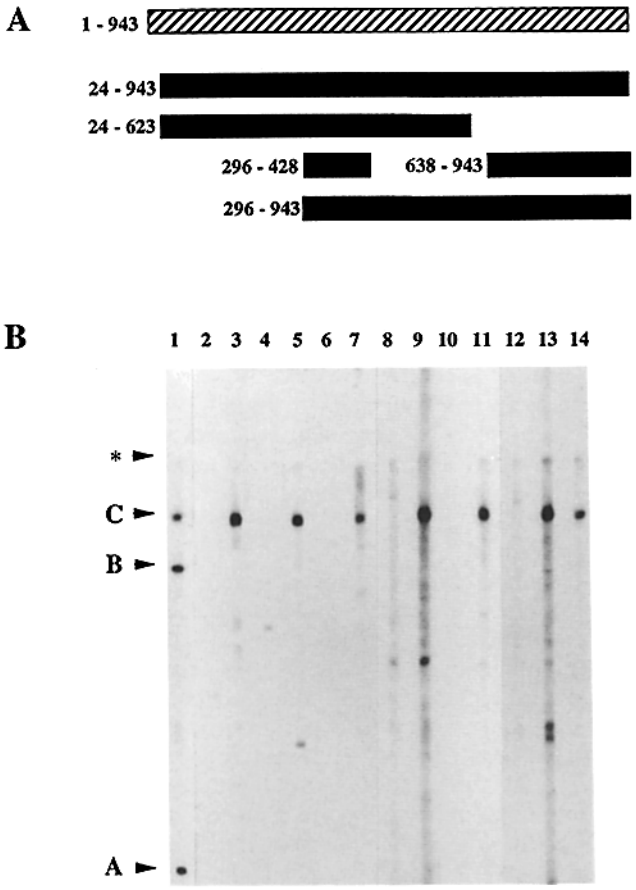


Figure 1. Anti-CENP-C antibodies used for injections. (*A*) The hatched upper bar represents full-length CENP-C. Each solid bar indicates a region of CENP-C expressed in *E. coli* as part of a fusion protein. These fusion proteins were used as immunogens to raise rabbit polyclonal antibodies. The numbers to the left of each bar indicate the amino acid residues of CENP-C contained within each fusion protein. (*B*) An immunoblot of crude HeLa chromosomal proteins probed with GS serum (human ACA serum, lane *1*) and the IgG from preimmune and immune rabbit sera raised to the CENP-C proteins diagrammed in *A* in the following order (preimmune and immune, respectively): anti-24-943 (lanes *2* and *3*), anti-24-623 (lanes *4* and *5*), anti-296-943 (lanes *6* and *7*), anti-296-428 (lanes *8* and *9*) and anti-638-943 (lanes *10* and *11*) anti-glutaraldehyde-fixed 296-943 (G296-943, lanes *12* and *13*). Lane *14* is probed with antibodies from rabbit serum G296-943 which were affinity purified from bacterially produced CENP-C 24-943. GS serum recognizes CENPs A, B, and C, as indicated by the arrows. The asterisk indicates the interface between the resolving and separating gels.

et al., 1991, 1992) in arrested cells. Both antigens showed their characteristic metaphase distributions in microinjected HeLa cells that were fixed after 1–2 h at metaphase. Given the disruption of the kinetochore structure caused by the injection of CENP-C antibodies (see below), the proper localization of CENP-E is surprising. CENP-E has been postulated to reside in the kinetochore, and immunofluorescence staining shows that it is located peripherally both to CENP-B (Yen et al., 1991) and to CENP-C (data not shown). We should note, however, that we cannot eliminate the possibility that the amount of CENP-E at centromeres is decreased in these anti-CENP-C-injected cells.

Although all the anti-CENP-C antibodies from the various rabbit sera produced metaphase delay, the percentage of cells that exhibited this phenotype varied depending on the antibodies injected. This quantitative difference may be due to different titres or binding affinities of anti-CENP-C antibodies in the various sera, or more efficient inhibition of CENP-C function by antibodies directed against certain epitopes. However, since we saw no unique pattern of mitotic disruption for any antibody directed against a particular region of CENP-C, we are unable to draw conclusions about functional domains from our data. Injection of monoclonal antibodies to defined epitopes may be more informative in this respect.

Overall, more than two-thirds of the anti-CENP-C-injected cells remained at metaphase for more than 2 h, and some remained in mitosis for up to 10 h (i.e., until fixation and immunostaining). In cells arrested for such extended periods, we usually could no longer detect a metaphase plate after 4 h of arrest using phase contrast microscopy. Upon examination of arrested cells more closely by immunofluorescence, we found that chromosomes appeared to fall off the metaphase plate with time. In cells arrested for 1 h at metaphase, the majority of the chromosomes were aligned at the plate, and the spindles appeared normal (Fig. 2, *A–C*). However, in cells arrested for longer periods, the chromosomes assumed a more scattered configuration and the spindles were correspondingly disrupted (Fig. 2, *D–I*). The morphology of the spindles appeared to be largely determined by the position of the chromosomes, which suggested that at least some of the chromosomes that had left the metaphase plate were still attached to spindle microtubules.

Using immunofluorescence microscopy, it was difficult to ascertain if these cells had entered anaphase, since the chromatin was often clumped and individual chromosomes could only rarely be seen. However, examination of similar cells by electron microscopy revealed that sister chromatids eventually separated, although they failed to segregate to the poles (Fig. 3).

Approximately 10% of the cells injected with anti-CENP-C antibodies either died or detached from the coverslip after an extended mitotic arrest. A similar result was obtained when cells injected with IgG purified from anti-centromere autoantibodies were observed by time lapse video microscopy (Bernat et al., 1990). It is important to note that these cells die a number of hours after injection of the antibody, and only after remaining blocked at metaphase for an extended period. We therefore attribute the cell death to the mitotic block rather than physical damage incurred during injection.

The Effects of Anti-CENP-C Antibodies Are Cell Cycle Dependent

In contrast to our results with interphase-injected cells, we found that injection of these same anti-CENP-C antibodies during mitosis caused only a slight lengthening of M phase (up to 1 h), but did not disrupt cell division. A similar pausing was observed for cells injected with IgG purified from preimmune sera, and probably reflects the physical trauma of the injection itself. We have observed this same phenomenom in previous microinjection studies (Bernat et al., 1990).

Of the HeLa cells which were injected with anti-CENP-C

Table I. Microinjection of Anti-CENP-C IgG into Interphase Cells Causes Cells to Delay at Metaphase

IgG injected	Number injected	Number mitotics scored	Phenotypes observed: Metaphase delay*	Reverted to interphase	Divided micronucleated abnormalities‡	Other abnormal
	%					
G296-943§	1,826	365	115	16	79	12
affinity purified	501	68	23	4	16	10
preimmune	357	139	2	0	4	5
296-943	680	93	64	1	23	0
preimmune	111	15	0	0	0	0
296-428	176	73	44	0	8	6
preimmune	132	44	1	0	0	1
638-943	156	54	30	5	8	1
preimmune	119	25	1	0	0	0
24-623	244	39	20	7	1	0
preimmune	79	10	0	0	0	1
24-943	371	18	11	1	3	1
preimmune	301	14	1	0	0	1
HeLa Totals						
anti-CENP-C	3,954	710	307 (42.5%)	34 (4.9%)	138 (19.6%)	30 (4.2%)
preimmune	1,099	247	6 (2.4%)	0	4 (1.6%)	7 (2.9%)
LLCPK						
296-643	205	34	12	0	6	1
preimmune	43	23	0	0	1	0
CF-PAC1						
296-643	532	72	39	3	20	0
preimmune	90	10	0	0	1	0

* These cells were observed at metaphase for at least 2 h. Approximately 10% of these died and/or detached from the cover slip prior to fixation. The rest remained in mitosis until the time of fixation (from 2–10 h after a metaphase plate was first observed).
‡ These include anaphase and telophase bridges, tripolar metaphases, failures in cytokinesis, and unequal divisions in which daughter cells produced were grossly unequal in size.
§ Antiserum raised against glutaraldehyde-fixed antigen. See Materials and Methods for details.

antibodies during mitosis, 93% (67/72) divided within 1 h to produce two phenotypically normal daughter cells, (10 of these were injected in prophase, 13 in prometaphase, 37 in metaphase, 9 in anaphase, and 3 in telophase). The remaining 7% (5 cells) exhibited various abnormalities, including production of micronuclei, incomplete cytokinesis resulting in a binucleate daughter cell, and tripolar metaphases. These abnormal phenotypes were also observed in about 5% of uninjected cells, which likely represents the baseline frequency of abnormal divisions in this cell line. Thus we conclude that anti-CENP-C antibodies have no detectable effect when introduced subsequent to the initiation of prophase.

In interphase-injected cells, we noticed a more subtle cell cycle-dependent effect of the antibodies. Since we knew the time each cell entered mitosis after injection, we could estimate the time spent at each stage in the cell cycle using previously generated FACS data (FACS is a registered trademark of Beckton Dickinson & Co. Mountainview, CA) (Bernat et al., 1990). The proportion of cells that entered mitosis during observation was approximately equal between cells injected with anti-CENP-C IgG and control cells injected with preimmune IgG. This suggests that the anti-CENP-C antibodies did not significantly delay progression through interphase. Thus, we could roughly classify cells by their cell cycle stage at the time of injection. By this criterion, antibodies were most effective at disrupting mitosis if injected prior to S phase. More than 80% of the cells injected in G1 were affected, while slightly less than 50% of cells injected in S and G2 were affected. This same trend was observed for all the antibodies tested, and the combined data are shown in Fig. 4.

Antibody-induced Arrest Correlates with a Decrease in CENP-C Staining at Centromeres

In previous work we were able to show that IgG from human anticentromere sera bound centromeres in vivo (Bernat et al., 1990). However, here we could not detect the microinjected anti-CENP-C antibodies at centromeres in most cells, even when cells were permeabilized prior to fixation to remove unbound antibody. Our difficulty in detecting CENP-C might reflect that it is not as readily accessible to antibodies as other centromere antigens in vivo, perhaps due to structural changes which occur during kinetochore assembly. However, since we observed a mitotic phenotype in anti-CENP-C injected cells, a tenable alternative is that microinjected anti-CENP-C antibodies disrupt the proper localization of CENP-C.

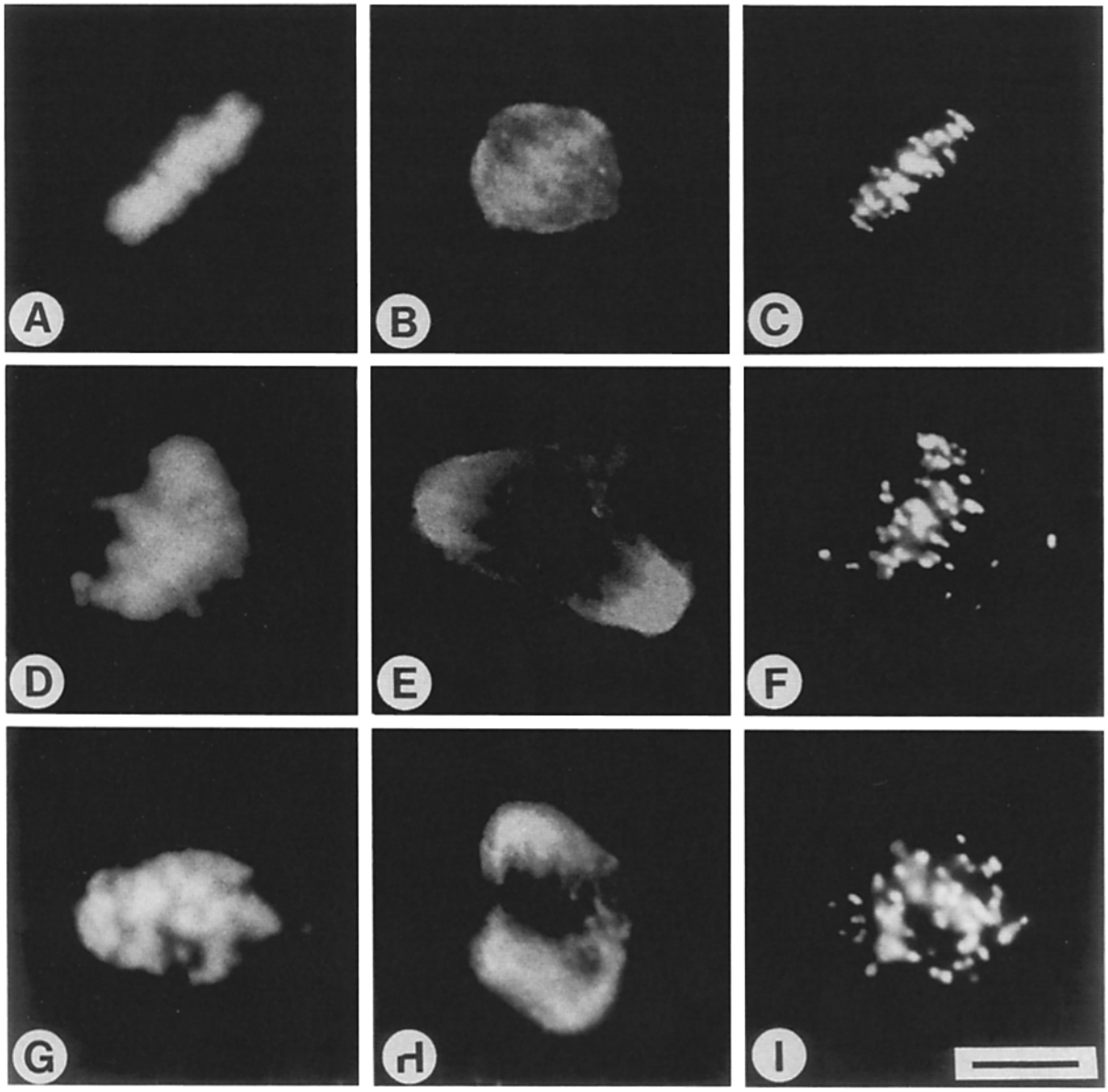


Figure 2. Chromosome and spindle morphology in HeLa cells arrested at metaphase at time of fixation. Cells shown here were injected with anti-CENP-C 296-943 IgG during the preceding interphase. *A*, *D*, and *G* show DAPI staining of DNA. *B*, *E*, and *H* show indirect immunofluorescence staining for tubulin. *C*, *F*, and *I* show indirect immunofluorescence staining for centromeres using human ACA serum GS. The cells shown had been in mitosis for the following times before fixation: (*A–C*) 1 h; (*D–F*) 3 h; (*E–I*) 7 h. Bar, 10 μm.

To examine this possibility more carefully, we used a second reagent to detect CENP-C in microinjected cells. We affinity purified human anticentromere antibodies against λgt11 plaques producing a peptide containing the carboxy half of CENP-C (amino acids 296-943). We then used these affinity-purified antibodies to detect CENP-C in cells that had been injected with rabbit antibodies directed against the amino half of CENP-C (amino acids 24-623). We chose this antibody for injection to minimize the possible overlap in epitopes recognized by the human antibodies, so that the binding of the injected antibodies would not block subsequent binding of the antibodies used for detection. We tested this in fixed cells by staining first with the IgG to be used for injection, and subsequently with the affinity-purified antibodies. The rabbit and human antibodies colocalized to centromeres in both mitotic and interphase cells (Fig. 5, *A* and *B*).

Next we examined CENP-C localization in cells injected either during mitotis or interphase. In 85% (23/27) of cells microinjected during mitosis and subsequently fixed, we could clearly detect CENP-C staining at centromeres using the affinity-purified antibodies (Table II, Fig. 5 *C*). These cells were all fixed within 1 h of injection so that most were still in mitosis at time of fixation. The four exceptions which failed to stain were in telophase at the time of injection, and had entered G1 prior to fixation.

In contrast, we failed to detect CENP-C at centromeres in 77% (111/144) of the cells injected during interphase. Our inability to detect CENP-C at the centromere appeared to correlate with the amount of time that the antibody was pres-

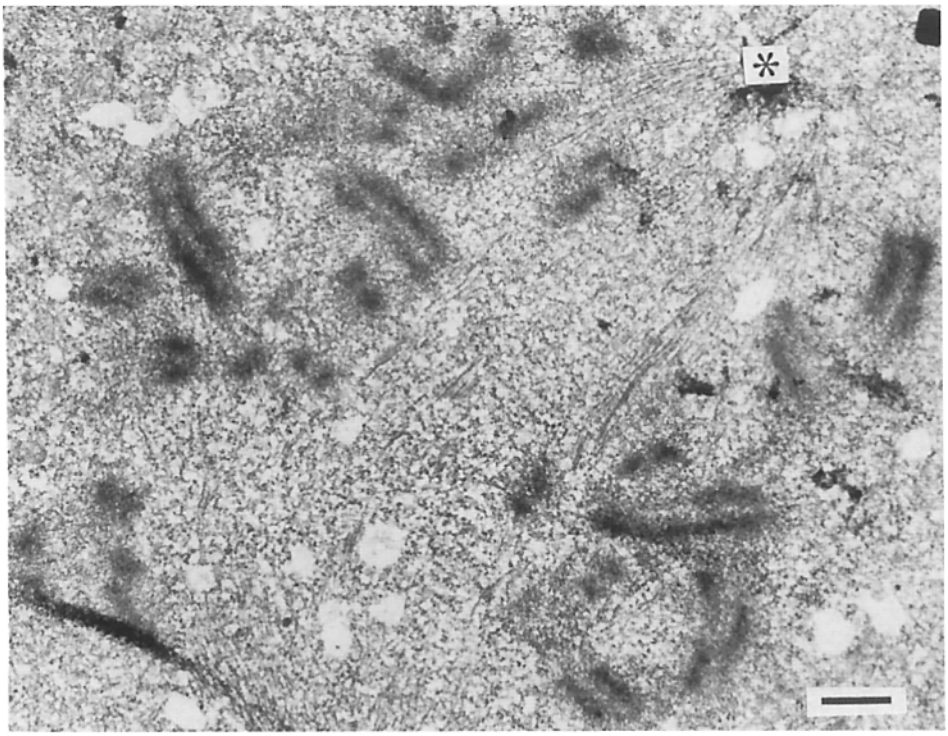


Figure 3. Sister chromatids separate but fail to segregate in anti-CENP-C-injected cells. The figure shows an electron micrograph of a cell that had been arrested in mitosis for 6 h before fixation. This cell was injected with anti-CENP-C G296-943 IgG during the preceding interphase, 10 h before entering mitosis. A metaphase plate had been observed in this by phase microscopy during the first 2 h of mitosis, but was no longer visible at time of fixation. The asterisk indicates the position of one spindle pole. Chromosomes are no longer congressed at the metaphase plate, but are scattered throughout the cell. Sister chromatids have separated, but have failed to segregate, as indicated by their close parallel associations. Bar, 1 μm.

ent before fixation. We observed CENP-C staining at centromeres in half of the cells fixed within 3 h of injection, although the staining was often markedly reduced when compared to staining in preimmune-injected or non-injected cells (Fig. 5, *E* and *F*). However, when antibody was introduced into interphase cells more than 3 h before fixation, we could detect centromeres in fewer than 30% of injected cells. Of note are three cells which were injected during interphase but arrested at metaphase prior to fixation. We failed to detect centromeres in any of these cells (Fig. 5 *D*). This suggests that the difference in staining of interphase and mitotic cells is a property of the cell cycle time of injection, and is not dependent on the cell cycle stage at fixation.

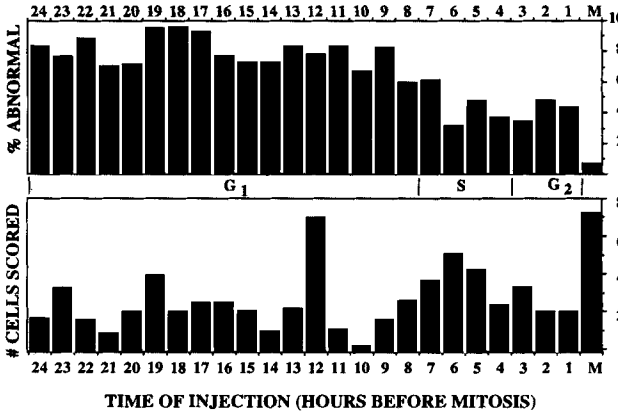


Figure 4. Injection of anti-CENP-C antibodies during G1 causes a more severe effect than injection later in the cell cycle. The upper panel shows a plot comparing the time at which cells were injected with anti-CENP-C antibodies (relative to M) versus the percentage of those cells which exhibited abnormal mitosis. The lower panel indicates the total number of injected cells scored at each time point. The approximate cell cycle phase is indicated as previously determined for this line of HeLa cells by FACS analysis (Bernat et al., 1990). This plot represents the combined data from the various anti-CENP-C antibody injections.

We considered that the elimination of CENP-C staining at centromeres may merely depend on the amount of time that the injected antibody is present, rather than the cell cycle time of injection. To examine this, we added colcemid to cells immediately after microinjecting the anti-CENP-C antibodies. Thus, cells that were in mitosis at the time of injection remained in mitosis for the duration of the experiment. Of 25 mitotic-injected cells, 23 still had strong CENP-C staining at centromeres after being held at mitosis for 4–5 h (Fig. 5 *G*), whereas centromere staining was undetectable in 16/20 interphase-injected cells in the same experiment (Fig. 5, *H* and *I*). Two of these latter cells had entered mitosis prior to fixation, yet still failed to show centromere staining (Fig. 5 *H*).

Based on these results, we suggest that introduction of anti-CENP-C antibodies during interphase directly interferes with localization of CENP-C at centromeres. This may ultimately result in disruption of the following mitosis. The simplest explanations for the lack of effect of antibodies microinjected during mitosis are based on the inability of the injected antibody to disrupt localization. CENP-C may become resistant to the antibody inhibition during prophase kinetochore assembly due to conformational changes or interactions with other centromere proteins, or its binding at centromeres may be stabilized such that it is less susceptible to displacement by antibodies.

Anti-CENP-C-injected Cells Can Eventually Progress through Mitosis

About one third of the cells that were delayed at metaphase

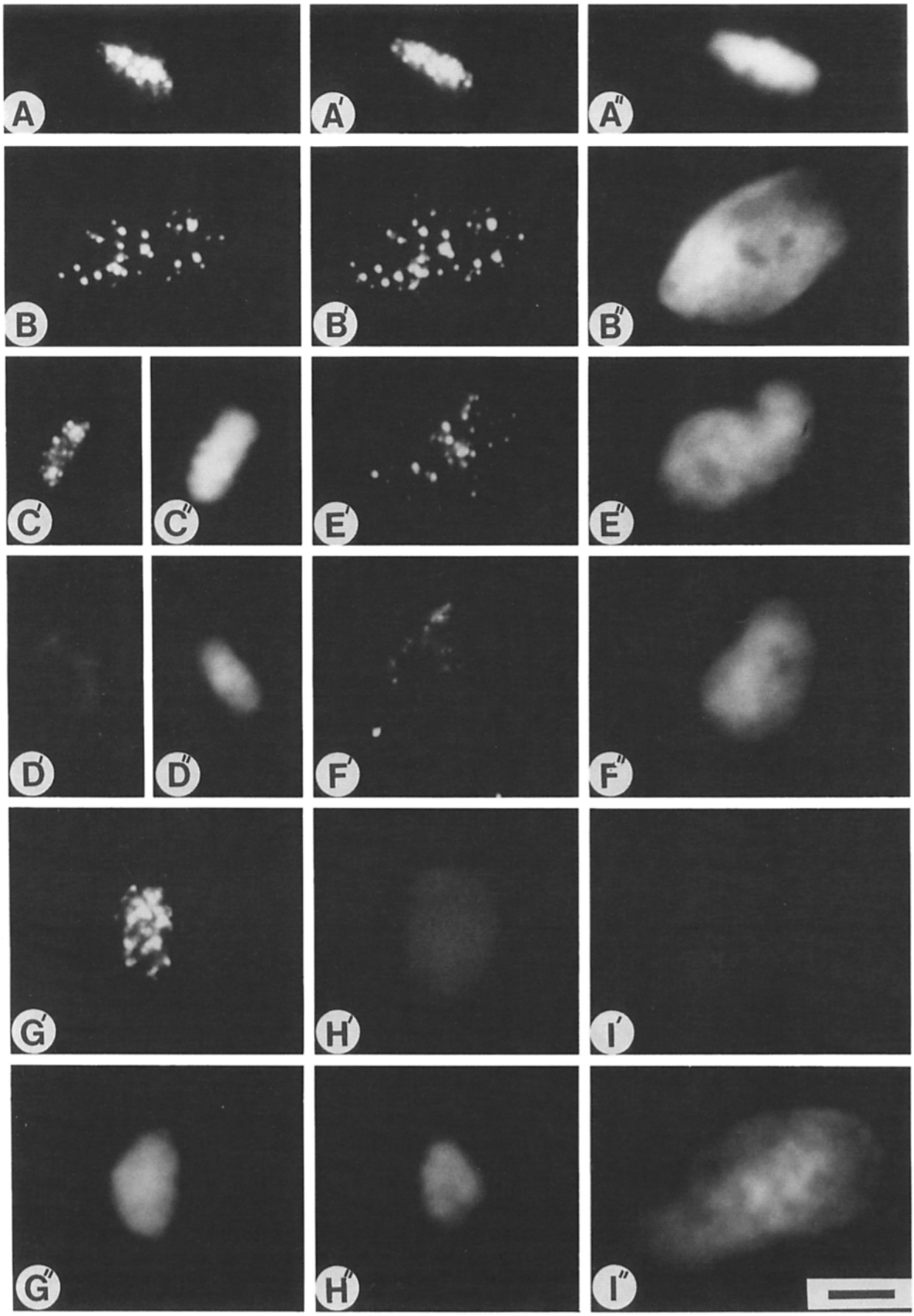

A
A'
A''
B
B'
B''
C'
C''
E'
E''
D'
D''
F'
F''
G'
H'
I'
G''
H''
I''

Table II. Detection of CENP-C at Centromeres in HeLa Cells Microinjected with Anti-CENP-C IgG

Antibody with injected	Cell cycle stage at injection	Number of cells injected	Time of fixation, hours after injection	Percent centromere staining
Anti-CENP-C 24-623	Mitosis			
	prophase	2	0–1	100
	prometaphase	4	0–1	100
	metaphase	17	0–1	100
	telophase	4	0–1	0
	interphase	8	2–3	50
		15	3–4	27
		30	5–7	23
		91*	16–18	21
preimmune	mitosis	8	0–1	100
	interphase	56	2–18	96
Colcemid treated Anti-CENP-C 24-623	mitosis	25	4–5	92
	interphase	20‡	4–5	20

Antibodies were injected into nuclei of HeLa cells, which were then incubated for the indicated time before fixation. In one experiment, cells were arrested in mitosis for 4–5 h by the addition of colcemid to the media immediately after injection. CENP-C at centromeres was detected using human autoantibodies that had been affinity purified against lambda gt11 plaques expressing amino acid residues 296-943 of CENP-C.

* Three cells were arrested at metaphase for at least 2 h before fixation. Centromere staining was not detected in these three cells.

‡ Two cells entered mitosis prior to fixation. Centromere staining was not deteced in these two cells.

eventually attempted cell division during the observation period. Chromosome segregation appeared to fail in these cells, leading to a wide variety of phenotypes (Fig. 6 and 7). In some cells cytokinesis appeared overtly normal, but produced two daughter cells containing several micronuclei. In others, chromosomes that failed to segregate became trapped in the cleavage furrow. The outcome of division in these latter cells seemed to depend on the amount of DNA trapped. Some completed cleavage, with DNA remaining in the midbody. In others, where the majority of the chromosomes remained centrally located, the cleavage furrow formed but subsequently regressed, giving rise to a single daughter cell. Examples of each of these types of abnormal division are shown in time lapse images in Fig. 6.

All of the daughter cells produced from these abnormal divisions contained multiple micronuclei. Centromere staining of these micronucleated cells revealed paired but separated centromeres (Fig. 7 *F*). In some cases there appeared to be a single pair of centromeres per micronucleus. These observations suggest that anaphase sister chromatid separation had occurred in these cells but that sisters had not segregated prior to cell division. This interpretation is consistent with electron microscopy examination of arrested cells described above (Fig. 3).

Kinetochores Are Reduced in Both Size and Number in Anti-CENP-C-injected Cells

We used serial sectioning and electron microscopy to examine kinetochores in mitotic cells that had been injected with anti-CENP-C antibodies. These experiments used total IgG purified from anti-CENP-C G296-943, or antibodies from this same serum that had been affinity purified from bacterially produced full-length CENP-C. Both antibodies gave similar results, which were combined for this analysis. For this experiment, cells were injected in interphase and returned to the incubator for 6–12 h. Injected colonies were monitored until a reasonable number of cells had collected in mitosis, at which point they were fixed and subsequently embedded for EM. Under this protocol, all cells examined are presumed to have been injected in G1. Controls consisted of cells from neighboring colonies that had been injected with preimmune IgG. Anti-CENP-C-injected cells had been in mitosis from 1–6 h at time of fixation, while control cells had entered mitosis less than 30 min before fixation. (The shorter time period reflects the fact that control cells showed no appreciable delay in mitosis.) We examined 80-nm serial sections of each cell, and scored all sections which contained microtubules and/or chromatin.

The number of kinetochores in anti-CENP-C-injected cells was significantly reduced compared to control preimmune-injected cells. In total, we observed 45 different kinetochores in 67 sections of 4 control cells (11.3 kinetochores/cell) and 38 kinetochores in 132 sections of 14 anti-CENP-C-injected cells (2.7 kinetochores/cell). We could divide the anti-CENP-C-injected cells into two groups, based on morphology and length of arrest. Five of the anti-CENP-C-injected cells we examined had been arrested less than 2 h, and the majority of the chromosomes in these cells were at the metaphase plate at time of fixation. These cells accounted for 35 of the 38 kinetochores observed. The remaining nine cells had been arrested in mitosis for 2–6 h, and the chromosomes in these cells were scattered. Thus, the disruption of kinetochores appeared to be correlated with the length of mitotic arrest and/or the ability of chromosomes to remain at the metaphase plate.

We were surprised to find that not only were there fewer

Figure 5. Detection of CENP-C at centromeres in HeLa cells microinjected with anti-CENP-C antibodies. Indirect immunofluorescence images of HeLa cells stained with rabbit anti-CENP-C 24-623 IgG diluted 1:100 (no mark), human affinity-purified anti-CENP-C autoantibodies (′) and DAPI (″). Uninjected cells at metaphase (*A*) and interphase (*B*). Cells microinjected with rabbit anti-CENP-C 24-623 IgG (*C–I*). CENP-C is localized to centromeres in a cell that was microinjected at metaphase and fixed 15 min later (*C*). However, in a cell fixed at metaphase that was microinjected 2 h previously during interphase, CENP-C is not detected at centromeres. This cell was fixed after ~10 min at metaphase (*D*). At 0.5 h after microinjection, CENP-C staining is strongly detected at centromeres in an interphase cell microinjected with preimmune IgG (*E*), but is noticeably reduced in a sister cell that was microinjected with anti-CENP-C IgG (*F*). To determine if a longer incubation would abolish CENP-C staining in metaphase-injected cells, 60 μg/ml colcemid was added to the media at time of injection to arrest cells in mitosis (*G–I*). CENP-C staining at centromeres in a metaphase-injected cell fixed after 4 h in mitosis (*G*). Lack of centromere staining in an interphase cell which entered mitosis ~3 h postinjection (*H*). Lack of centromere staining in an interphase-injected cell which remained in interphase until fixation 4 h postinjection (*I*).

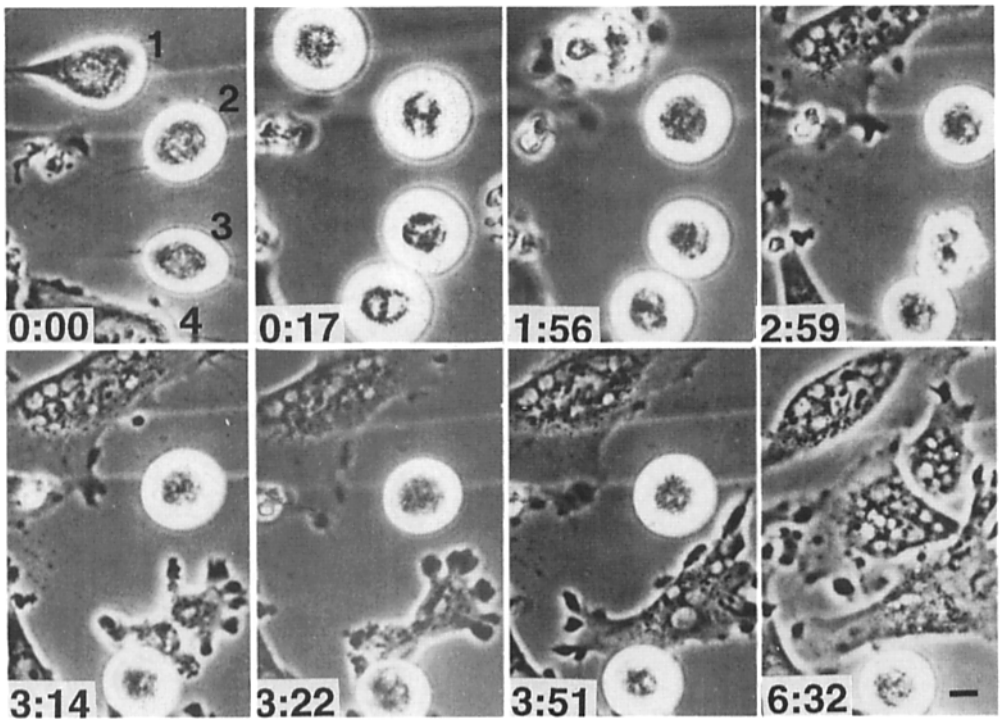


Figure 6. Real time images of abnormal mitoses in anti-CENP-C-injected cells. Phase contrasts images showing typical examples of the abnormal mitoses observed in cells injected with anti-CENP-C G296-943 IgG. The time at which the image was captured is indicated in the lower left corner of each frame, with time zero arbitrarily chosen as the time at which the image in the top left corner was captured. In this image cells 1, 2, and 3 are in late prophase/early prometaphase, while cell 4 is still in interphase. By 0:17, all four cells have achieved metaphase. Note the metaphase plates visible in cells 2 and 4. At 1:56 cell number 1 has progressed through mitosis and is attempting cytokinesis. Cytokinesis in this cell fails, producing a single daughter cell with multiple micronuclei. Cell 3 undergoes a similar fate at 2:59. The formation and regression of the cleavage furrow in this cell is evident in subsequent panels. Cell 2 eventually divides after a delay of 6 h in mitosis, producing two micronucleated daughter cells, while cell four still remains in mitosis at 6:32. Bar, 10 μm.

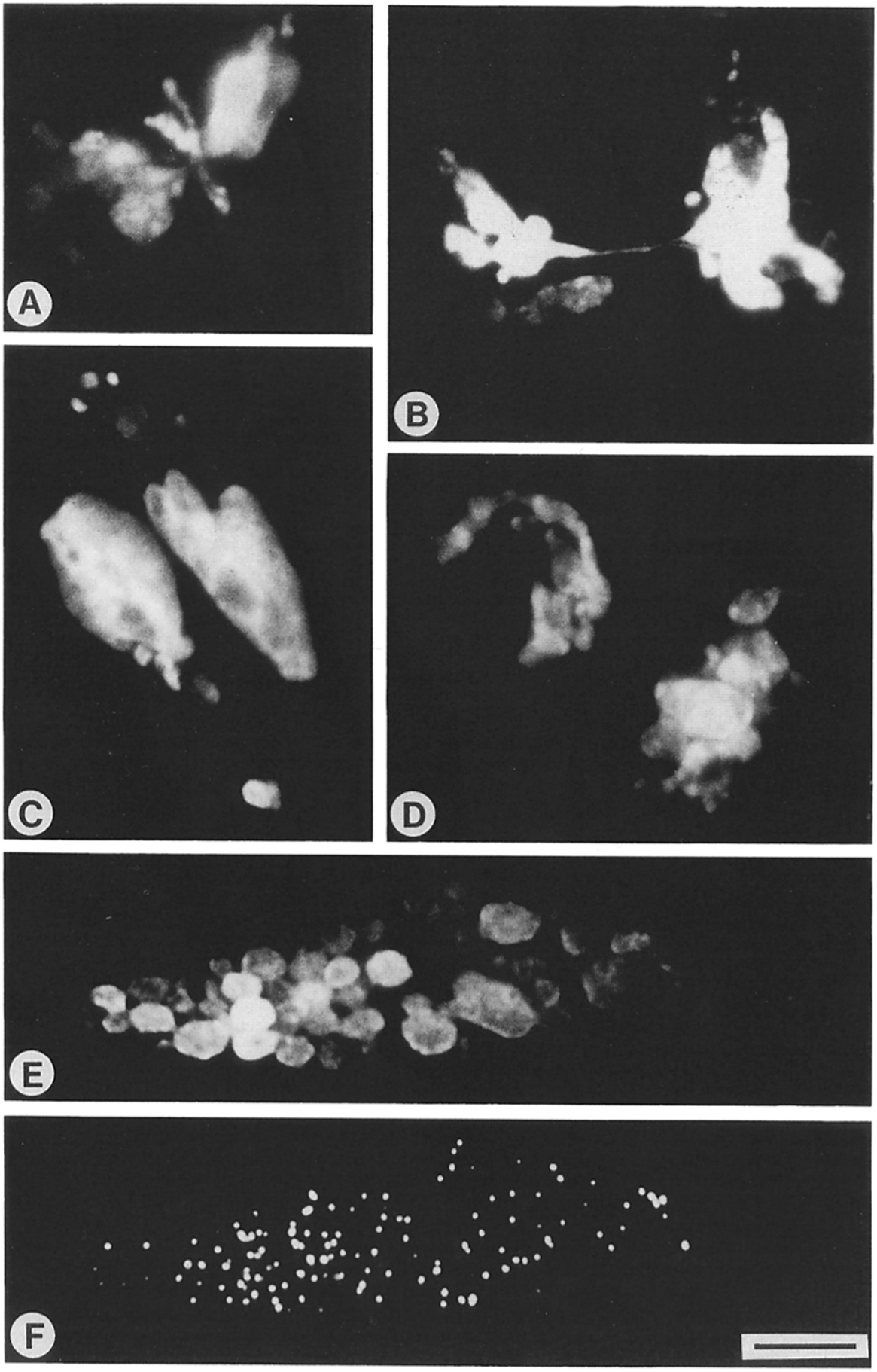
kinetochores in anti-CENP-C-injected cells, but the kinetochores that remained were noticeably smaller than control kinetochores. We measured the diameters of the kinetochore outer plates in both control and anti-CENP-C-injected cells to quantify this size difference. Each kinetochore was measured in all serial sections in which it could be resolved. For the measurements discussed below, we chose the section in which each kinetochore appeared largest. The average size of kinetochores in control cells was 245 $\pm$ 10.6 (SEM) nm, while in anti-CENP-C-injected cells it was a significantly smaller 178 $\pm$ 9.2 nm ($P < 0.001$). This value for our control cells is very similar to the average kinetochore size in rat embryonic cells (0.2 μm; Jokelainen, 1967). Representative examples of kinetochores from both control and anti-CENP-C-injected cells are shown in Fig. 8.

Curiously, these smaller kinetochores always appeared intact and single. That is, we never observed instances of side-by-side kinetochores on a single chromatid, which would occur if one kinetochore split along the axis perpendicular to the chromosome. We might have expected such fragmentation based on observations of mitosis with unreplicated genomes (MUGs) in which kinetochores can apparently be subdivided in this manner (Brinkley et al., 1988). Although we did observe several examples of one or two microtubules attached to the chromatin surrounding small kinetochores (Fig. 8, *D* and *F*), this was also observed in controls, and has been previously described in untreated cells (Comings and Okada, 1971; Witt et al., 1980; Reider, 1982).

There are two points worth noting about the distributions of kinetochore sizes in the control and anti-CENP-C-injected populations (Fig. 9). First, we observed a broad distribution of control kinetochore sizes. Some of this variation in size was undoubtedly due to the different angles at which kinetochores were viewed in cross-section. However, it is our impression that most of this variation is due to actual size differences between kinetochores of individual chromosomes, since we never noted large size differences between sister kinetochores of a single chromosome.

Second, when we compared the control and anti-CENP-C distributions, we noted that the anti-CENP-C distribution did not fit a normal curve, but rather was skewed to the left. We suggest that the skewness of this distribution may reflect a minimum size limit below which the trilaminar kinetochore structure is no longer stable. Since we observed only

Figure 7. Terminal phenotypes in anti-CENP-C-injected cells subsequent to their passage through mitosis. The cells were injected with the following anti-CENP-C IgG during the preceding interphase: (*A*) 296-943; (*B*) 638-943; (*C*) 24-943; (*D*) 296-428; and (*E*) G296-943. DAPI staining (*A–E*) and indirect immunofluorescence of staining by ACA sera (*G*) of anti-CENP-C-injected cells which divided after delays in mitosis ranging from 2–10 h. (*A*) DNA trapped in the cleavage furrow at cytokinesis. (*B*) Micronucleated daughter cells connected by a DNA bridge. (*C* and *D*) Daughter cells showing various degrees of micronucleation. (*E*) A single micronucleated daughter cell resulting from failed cytokinesis. (*F*) Centromeres of the cell in *E*. Note that centromeres appear in loosely associated pairs in each micronucleus. Bar, 10 μm.


A
B
C
D
E
F

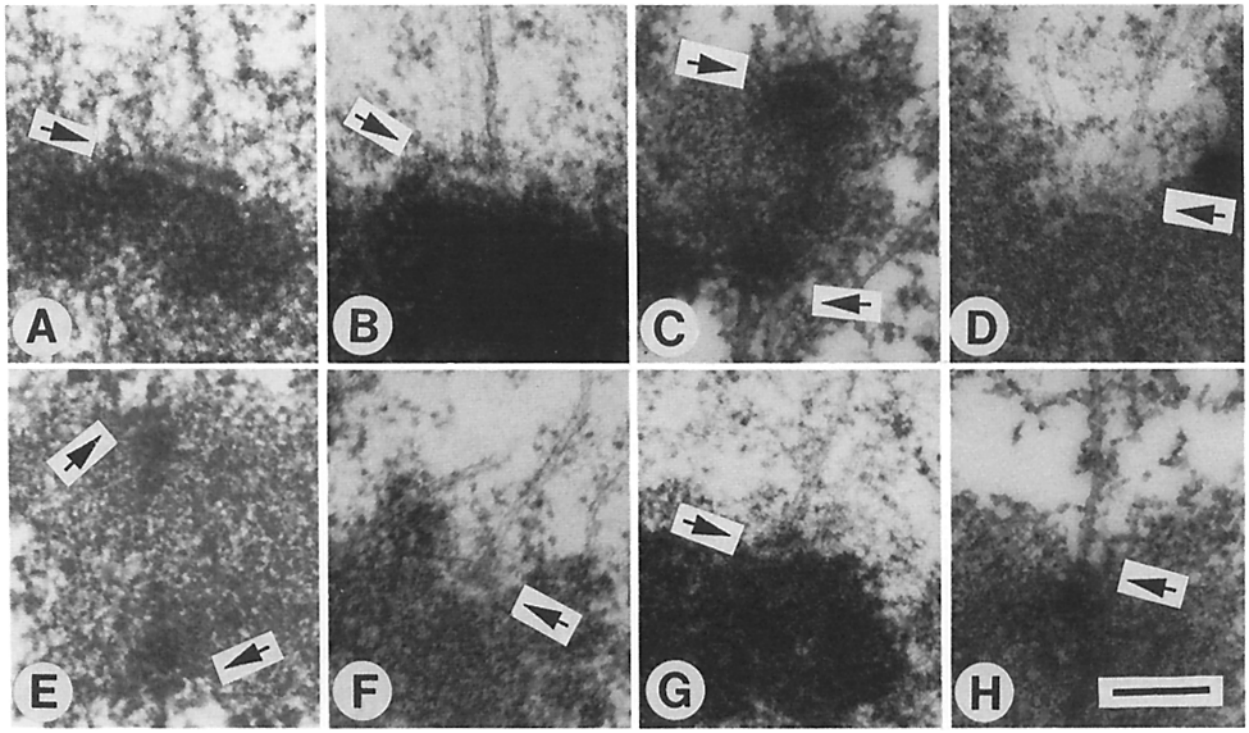


Figure 8. Kinetochore Morphology in anti-CENP-C-injected cells. (*A*) Kinetochore of average size (250 nm) in a cell which was injected with IgG purified from preimmune serum G296-943. (*B–F*) Examples of reduced kinetochores observed in anti-CENP-C-injected cells delayed at metaphase. These examples were all taken from cells injected with affinity-purified anti-CENP-C antibodies from serum G296-943. Kinetochores of both sister chromatids are visible in *C* and *E*. *H* shows the smallest trilaminar structure observed in these cells. Note the microtubule attachment to the flanking chromatin in *D* and *F*. Arrows indicate the positions of the outer kinetochore plates. Bar, 0.2 μm.

a single kinetochore plate less than 100 nm in length, this may be a reasonable estimate of the size cut-off.

In addition to decreasing kinetochore size, our observations suggest that inhibition of CENP-C may also have destabilized microtubule attachments at the kinetochore. In cells which had been arrested in mitosis for longer than 2 h, the majority of chromosomes lacked detectable kinetochores and were no longer attached to microtubules. This is an unexpected finding, since CENP-C is localized to the inner kinetochore plate, and the majority of microtubules appear to end in the outer plate (Comings and Okada, 1971; Witt et al., 1980; Reider, 1982). Furthermore, we know from previous work involving injection of ACA that an intact trilaminar kinetochore structure is not required to maintain microtubule attachments. In ACA-injected cells, it was observed that the majority of chromosomes bound microtubules even though their kinetochores were disrupted (Bernat et al., 1991). However, in cells injected with anti-CENP-C antibodies, we observed only three instances in which microtubules attached directly to chromatin in the absence of a trilaminar structure. The difference between the two results may be attributable to low titres of anti-CENP-C antibodies in the ACA used in the previous study. One interpretation of these results is that stable attachment of microtubules may require a CENP-C-dependent aspect of kinetochore structure.

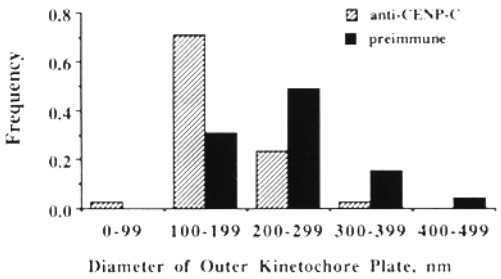


Figure 9. Distribution of kinetochore sizes in cells injected with antibodies purified from either preimmune or anti-CENP-C G296-943 antiserum. Measurements of diameters of 45 kinetochores from cells injected with preimmune IgG, and 38 kinetochores from cells injected with anti-CENP-C IgG or affinity-purified anti-CENP-C antibodies.

Discussion

CENP-C Is Required for Kinetochore Function during Mitosis

Prior to the experiments reported here, a number of lines of correlative evidence suggested a role for the human autoantigen CENP-C in centromere function. First, we showed that CENP-C was present at the active but not the inactive centromere of a mitotically stable dicentric human chromosome (Earnshaw et al., 1989). Second, we localized CENP-C by immuno-electron microscopy to the inner kinetochore plate (Saitoh et al., 1992). Finally, by antibody injection experiments similar to the ones presented here, we and others demonstrated that one or more of the antigens recognized by

anticentromere antibodies from autoimmune patients (ACA) perform essential functions in meiosis and mitosis (Simerly et al., 1990; Bernat et al., 1990). Here we present direct evidence that CENP-C is required for mitotic events.

Introduction of antibodies specific for CENP-C into interphase HeLa nuclei resulted in a prolonged delay at metaphase. From an analysis of the effects of antibody injection at different times during the cell cycle, we conclude that the injected antibodies must interfere with events that occur during interphase in order to disrupt the subsequent mitosis. The lack of detectable CENP-C at centromeres in interphase-injected cells suggests that the antibodies interfere with the localization CENP-C at centromeres, and thus effectively reduce the amount of this protein available during kinetochore assembly. Together with our EM observations of abnormal kinetochores in these cells, these results suggest that CENP-C is required for the interphase assembly of stable, functional kinetochores of normal size.

In light of these results, it is informative to re-examine the phenotypes observed in previous experiments in which cells were injected with IgG purified from human ACA. Injection of ACA IgG resulted in two discrete phenotypes, depending on the time of the cell cycle at which the antibodies gained entry into the nucleus. If this occurred within 2–3 h of mitosis (G2), cells were delayed at metaphase. Introduction of antibodies into the nucleus more than 3 h before mitosis (G1/S) resulted in a pseudoprometaphase arrest, in which condensed metaphase-like chromosomes were distributed as in prometaphase (Bernat et al., 1990). Both metaphase and pseudoprometaphase arrested cells eventually divided, and produced an array of terminal phenotypes virtually identical to those observed here in anti-CENP-C-injected cells.

Kinetochores were examined by electron microscopy in both the metaphase and pseudoprometaphase arrested cells (Bernat et al., 1991). In both types of arrested cells, kinetochores were disrupted and microtubules were attached directly to the chromatin. It is worth noting, however, that in one metaphase arrested cell a single trilaminar structure was observed which resembled the small kinetochores that we saw in anti-CENP-C-injected cells (see Fig. 4 *E* in Bernat et al., 1991).

Further investigation revealed that kinetochores were assembled in these metaphase-arrested cells, but they appeared to be structurally weakened, similar to kinetochores in cells injected with anti-CENP-C antibodies. When cells were injected with ACA during G2 (under conditions that would produce metaphase arrest) and were treated with colcemid to disrupt microtubules, the number and morphology of kinetochores was nearly normal. It was noted, however, that these kinetochores were often less condensed in the region of the inner plate, exactly where CENP-C is localized. On the other hand, in colcemid-treated cells injected during G1 or S (under conditions that would lead to pseudoprometaphase arrest), a very different phenotype was observed. Very few kinetochores were seen, and those that were observed were abnormal. These observations suggested that ACA prevented the assembly of kinetochores if present during G1/S. Kinetochores could still be assembled in cells injected with ACA during G2, but these became disrupted when microtubules bound, presumably due to microtubule-associated forces involved in chromosome movement.

Thus, the ACA-induced metaphase arrest appears similar to that described here for anti-CENP-C-arrested cells, and it is a reasonable conclusion that inhibition of CENP-C may have contributed significantly to this phenotype. We had previously argued that the mitotic inhibition could be due primarily to antibodies recognizing CENP-B (Bernat et al., 1991), since several of the IgG preparations injected lacked detectable antibodies to either CENP-C or CENP-A, or both, in immunoblots of human mitotic chromosomes. However, we have now shown that anti-CENP-C antibodies are detectable in at least one of these sera, provided that a CENP-C fusion protein is used as a substrate for the immunoblotting experiment (data not shown). We attribute our previous failure to detect CENP-C in part to the low levels of this antigen in chromosomes.

One difference between the ACA-induced metaphase arrest and the anti-CENP-C-induced arrest is that attachment of kinetochore microtubules appeared to be de-stabilized by the rabbit anti-CENP-C antibodies but not by the human autoantibodies. This difference may reflect either a higher titre of CENP-C-specific antibodies in the rabbit antisera or the recognition of different epitopes. The immunolocalization of CENP-C to the inner kinetochore plate and the lack of any known microtubule-binding motif in CENP-C leads us to believe that this putative effect on the stability of microtubule attachment may be indirect. For example, CENP-C could contribute to some structural organization of the kinetochore necessary for stable binding of other proteins which interact directly with microtubules. Even though kinetochores initially appear more normal in anti-CENP-C-injected cells than in ACA-injected cells, attachment or assembly of these proteins at the kinetochore may be more greatly affected by anti-CENP-C antibodies than by ACA.

We never observed a pseudoprometaphase arrest among anti-CENP-C-injected cells, which implies that inhibition of CENP-C alone is not sufficient to generate this phenotype. We also failed to generate this phenotype by injection of either rabbit anti-CENP-B antibodies, or a mixture of rabbit anti-CENP-B and anti-CENP-C antibodies (Bernat et al., 1991; Pluta, A., and J. Tomkiel, unpublished results). Thus, interference with the kinetochore functions necessary for congression at the metaphase plate may require inhibition of other centromere components (perhaps CENP-A). Alternatively, autoantibodies to the CENP antigens may recognize different epitopes than do the rabbit antibodies, which renders their effects qualitatively different. It has been argued that some ACA sera recognize posttranslational modifications present on centromere proteins (Earnshaw and Rothfield, 1985), which presumably would be absent from the bacterial antigens used to raise rabbit antisera.

Disruption of CENP-C Alters the Size of the Kinetochore

It has long been recognized that the centromere of higher eukaryotes could be divided into multiple, functional subunits (McClintock, 1938; Sears, 1952). This idea of the subunit nature of the centromere has more recently been expanded to incorporate more sophisticated observations on the behavioral and physical nature of the kinetochore (Zinkowski et al., 1991). Although the exact substructure of the trilaminar kinetochore is unknown, it is generally accepted that this structure is a collection of multiple microtubule- and motor-binding sites coalesced into a single cohesive functional unit.

CENP-C has been localized to the base, or inner plate, of the kinetochore (Saitoh et al., 1992), and thus resides exactly where one might expect to find a protein involved in organizing or uniting such subunits. Preliminary evidence from our lab indicates that CENP-C binds DNA (Yang, C. H., H. Saitoh, and W. C. Earnshaw, unpublished results), and thus could conceivably act alone or in concert with other proteins to organize the DNA which resides directly beneath the kinetochore.

Our observations are consistent with a model in which CENP-C has a role in coalescing kinetochore components into the trilaminar unit, or stabilizing the lateral associations in that structure once formed. Kinetochores assembled in the absence of CENP-C may be structurally weakened in such a way that they initially dissociate at the edges, maintaining a single central trilaminar structure. This model suggests that kinetochores may be assembled around a central core or nucleation site which is either more resistant to or buffered from disruptive forces associated with microtubule binding. Our observation of only a single kinetochore with a diameter of less than 100 nm suggests that below this size the trilaminar structure is no longer stable. In anti–CENP-C–injected cells, small kinetochores may disappear rapidly as they are reduced below the minimum stable size, while larger kinetochores may initially be only reduced in size.

Alternatively, CENP-C may be a limiting factor during kinetochore assembly, such that kinetochore size is in part dictated by the amount of available CENP-C. We note that this would neither exclude nor require a role of CENP-C in stabilizing or unifying the kinetochore as postulated above. By this view, these small kinetochores may represent a partial assembly rather than a partial disassembly. Even if properly assembled, small kinetochores may not be strong enough to withstand microtubule-associated forces at metaphase. Although observations on MUGs suggest that kinetochore fragments (i.e., small kinetochores) can remain intact in mitosis and apparently segregate normally (Brinkley et al., 1988), it is important to realize that MUGs are not attached to the same large chromosomal cargo as the kinetochores here, and thus may not be subjected to the same counterforces.

Currently, we know nothing about what determines the size of mammalian kinetochores. Our observation here of a wide distribution of kinetochore sizes in control cells suggests that size is determined in part by properties inherent to each chromosome. One candidate could be the copy number of CEN DNA sequences. However, due to the lack of a functional assay for mammalian centromeres, kinetochore-specific sequences have not yet been identified. If CENP-C does indeed bind DNA, as our current data suggest, the sequences which it binds will be prime candidates for such sequences.

Alternatively, kinetochore assembly may require a particular physical conformation of the centromeric chromatin rather than sequence specificity per se. Thus, the size of the kinetochore may depend on the nature of the chromatin condensation at the centromere. In humans, the bulk of the centromeric heterochromatin consists of alpha satellite DNA (for review see Willard and Waye, 1987). There is some suggestion that alpha satellite DNA may be sufficient for some aspects of centromere assembly and function. In some cell lines transformed with alpha satellite repeats, in which these repeats had become amplified on one or more chromosome arms, mitotic phenotypes were observed which were consistent with the presence of functionally dicentric chromosomes (Haaf et al., 1992). By this model, CENP-C could be involved either in establishing or recognizing a unique chromatin structure involving alpha satellite. Binding of CENP-C to this structure could be envisioned to establish the boundaries of kinetochore assembly.

CENP-C and the Metaphase Checkpoint

Observations of mitoses in UV-irradiated cells have suggested that the presence of even a single maloriented chromosome in a mitotic cell may cause a delay in the onset of anaphase (Zirkle, 1970; Rieder and Alexander, 1989). This suggestion has more recently led to the hypothesis that there is a metaphase checkpoint, or feedback system that monitors the state of spindle assembly. Such a system would not normally allow the metaphase:anaphase transition to occur until all chromosomes have achieved a stable bipolar orientation. Mutations have now been identified in *S. cerevisiae* which support this hypothesis (Li and Murray, 1991; Hoyt et al., 1991). Such mutations allow progression through mitosis in the absence of microtubule formation. Interestingly, these mutations all interfere with chromosome segregation. Thus, the wild-type gene products may be responsible for monitoring the formation of stable attachments of microtubules to centromeres.

The results of ACA microinjection experiments suggest that kinetochore assembly is monitored by the metaphase checkpoint (Bernat et al., 1990, 1991). Our present results now indicate that specific disruption of CENP-C function (and thus disruption of the function of the inner kinetochore plate) inhibits the metaphase:anaphase transition.

How might interference with the function of the inner kinetochore plate affect the metaphase checkpoint? Most observations published to date are consistent with a model where the unattached kinetochores of a maloriented chromosome somehow transmit a signal that delays the metaphase:anaphase transition. Proper bipolar attachment of a chromosome to the spindle suppresses the inhibitory signal. Our work suggests that interference with CENP-C function might result in an overall destabilization of the kinetochore, possibly altering the nature of the attachment of microtubules to the outer plate. Kinetochores making such improper or unstable microtubule attachments may continue to transmit the inhibitory signal even after the chromosomes have achieved a bipolar orientation and congressed to the spindle equator. This would cause the affected cell to pause in metaphase. It is interesting to note that in *S. cerevisiae* certain *cis*-acting mutations in CDEIII sequences cause a G_2 delay (which is formally difficult to distinguish from inhibition of the metaphase:anaphase transition in these cells) (Spencer and Hieter, 1992).

Recently, a phosphorylated epitope has been described which is differentially expressed at kinetochores in mitotic cells (Gorbsky and Ricketts, 1993). At metaphase, misaligned chromosomes strongly express this epitope, while chromosomes which are properly congressed at the plate lack expression. Thus, expression of this epitope is a candidate for the inhibitory signal to delay anaphase. We are presently investigating if kinetochores which are disrupted by anti–CENP-C antibodies continue to express this phosphoepitope.

We are grateful to Dr. Duane Compton for generously providing NuMA and CENP-E monoclonal antibodies. We thank Dr. Yuri Labzebnik, Dr. Alastair Mackay, Dr. Ann Pluta, Dr. Charles Yang, and Noriko Saitoh for their critical comments on the manuscript.

This work was supported by National Institutes of Health grant GM35212, and an American Cancer Society postdoctoral grant to J. Tomkiel.

Received for publication 10 December 1993 and in revised form 14 February 1994.

References

Bernat, R. L., G. G. Borisy, N. F. Rothfield, and W. C. Earnshaw. 1990. Injection of anticentromere antibodies in interphase disrupts events required for chromosome movement in mitosis. *J. Cell Biol.* 111:1519–1533.

Bernat, R. L., M. R. Delannoy, N. F. Rothfield, and W. C. Earnshaw. 1991. Disruption of centromere assembly during interphase inhibits kinetochore morphogenesis and function in mitosis. *Cell.* 66:1229–1238.

Bloom, K. 1993. The centromere frontier: kinetochore components, microtubule–based motility, and the CEN-value paradox. *Cell.* 73:621–624.

Brinkley, B. R., R. P. Zinkowski, W. L. Mollon, F. M. Davis, M. A. Pisegna, M. Pershouse, and P. N. Rao. 1988. Movement and segregation of kinetochores experimentally detached from mammalian chromosomes. *Nature (Lond.).* 336:251–254.

Chikashige, Y., N. Kinoshito, Y. Nakaseko, T. Matsumoto, S. Murakami, O. Niwa, and M. Yanagida. 1989. Composite motifs and repeat symmetry in S. pombe centromeres: direct analysis by integration of NotI restiction sites. *Cell.* 57:739–751.

Clarke, L., and M. P. Baum. 1990. Functional analysis of a centromere from fission yeast: a role for centromere-specific repeated DNA sequences. *Mol. Cell. Biol.* 10:1863–1872.

Comings, D. E., and T. A. Okada. 1971. Fine structure of kinetochore in Indian Muntjac. *Exp. Cell Res.* 67:97–110.

Compton, D. A., T. J. Yen, and D. W. Cleveland. 1991. Identification of novel centromere/kinetochore-associated proteins using monoclonal antibodies generated against human mitotic chromosome scaffolds. *J. Cell Biol.* 112: 1083–1097.

Compton, D. A., I. Szilak, and D. W. Cleveland. 1992. Primary structure of NuMA, an intranuclear protein that defines a novel pathway for segregation of proteins at mitosis. *J. Cell Biol.* 116:1395–1408.

Cooke, C. A., R. L. Bernat, and W. C. Earnshaw. 1990. CENP-B: a major human centromere protein located beneath the kinetochore. *J. Cell Biol.* 110:1475–1488.

Cooke, C. A., D. P. Bazett-Jones, W. C. Earnshaw, and J. B. Rattner. 1993. Mapping DNA within the mammalian kinetochore. *J. Cell Biol.* 120: 1083–1091.

Doheny, K. F., P. K. Sorger, A. A. Hyman, S. Tugendreich, F. Spencer, and P. Heiter. 1993. Identification of essential components of the *S. cerevisiae* kinetochore. *Cell.* 73:761–774.

Earnshaw, W. C., and N. F. Rothfield. 1985. Identification of a family of three related centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma (Berl.).* 91:313–321.

Earnshaw, W. C., H. Ratrie, and G. Stetten. 1989. Visualization of centromere proteins CENP-B and CENP-C on a stable dicentric chromosome in cytological spreads. *Chromosoma (Berl.).* 98:1–12.

Goh, P., and J. V. Kilmartin. 1993. NCD10: a gene involved in chromosome segregation in *Saccharomyces cerevisiae*. *J. Cell Biol.* 121:503–512.

Gorbsky, G. J., and W. A. Ricketts. 1993. Differential expression of a phosphoepitope at the kinetochore of moving chromosomes. *J. Cell Biol.* 122:1311–1321.

Guldner, H. H., H.-J. Lakomek, and F. A. Bautz. 1984. Human anticentromere sera recognise a 19.5 kD non-histone chromosomal protein from HeLa cells. *Clin. Exp. Immunol.* 58:13–20.

Haaf, T., P. E. Wharburton, and H. F. Willard. 1992. Integration of human α-satellite DNA into simian chromosomes: centromere protein binding and disruption of normal chromosome segregation. *Cell.* 70:681–696.

Hildebrandt, S., E. Weiner, J. Senécal, S. Noell, L. Daniels, W. C. Earnshaw, and N. F. Rothfield. 1990. The IgG, IgM, and IgA isotypes of antitopoisomerase I and anticentromere autoantibodies. *Arth. Rheum.* 33: 724–727.

Hoyt, M. A., L. Totis, and B. T. Roberts. 1991. *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. *Cell.* 66:507–517.

Hyman, A. A., K. Middleton, M. Centola, T. J. Mitchison, and J. Carbon. 1992. Microtubule-motor activity of a yeast centromere-binding protein complex. *Nature (Lond.).* 359:533–535.

Jiang, W., L. Lechner, and J. Carbon. 1993. Isolation and characterization of a gene (CBF2) specifying a protein component of the budding yeast kinetochore. *J. Cell Biol.* 121:513–520.

Jokelainen, P. T. 1967. The ultrastructure and spatial organization of the metaphase kinetochore in mitotic rat cells. *J. Ultrastruct. Res.* 19:19–44.

Kingsbury, J., and D. Koshland. 1991. Centromere-dependent binding of yeast minichromosomes to microtubules *in vitro*. *Cell.* 66:483–495.

Kingwell, B., and J. B. Rattner. 1987. Mammalian kinetochore/centromere composition: a 50 kDa antigen is present in the mammalian kinetochore/centromere. *Chromosoma (Berl.).* 95:403–407.

Lechner, J., and J. Carbon. 1991. A 240 kd multisubunit protein complex, CBF3, is a major component of the budding yeast centromere. *Cell.* 64:717–725.

Li, R., and A. W. Murray. 1991. Feedback control of mitosis in budding yeast. *Cell.* 66:519–531.

Lydersen, B. K., and D. E. Pettijohn. 1980. Human specific nuclear protein that associates with the polar region of the mitotic apparatus: distribution in a human/hamster hybrid cell. *Cell.* 22:489–499.

Madara, P. J., L. R. Banghart, L. J. W. Jack, L. M. Neira, and I. H. Mather. 1990. Affinity purification of polyclonal antibodies from antigen immobilized in situ in sodium dodecyl sulfate-polyacrylamide gels. *Anal. Biochem.* 187:246–250.

Masumoto, H., H. Masukata, Y. Muro, N. Nozaki, and T. Okazaki. 1989. A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. *J. Cell Biol.* 109: 1963–1973.

McClintock, B. 1938. The production of homozygous deficient tissues with mutant characteristics by means of the aberrant mitotic behavior of ring-shaped chromosomes. *Genetics.* 23:315–376.

McEwen, B. F., J. T. Arena, J. Frank, and C. Reider. 1993. Structure of the colcemid-treated PtK_1 kinetochore outer plate as determined by high voltage electron microscopic tomography. *J. Cell Biol.* 120:301–312.

Moroi, Y., C. Peebles, M. J. Fitzler, J. Steigerwald, and E. M. Tan. 1980. Autoantibody to centromere (kinetochore) in scleroderma sera. *Proc. Natl. Acad. Sci. USA.* 77:1627–1631.

Nakaseko, Y., Y. Adachi, S. Funahashi, O. Niwa, and M. Yanagida. 1986. Chromosome walking shows a highly repetitive sequence present in all centromere regions of fission yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1011–1021.

Palmer, D. K., K. O'Day, M. H. Wener, B. S. Andrews, and R. L. Margolis. 1987. A 17 kd centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. *J. Cell Biol.* 104:808–815.

Palmer, D. K., K. O'Day, H. L. Trong, H. Charbonneau, and R. L. Margolis. 1991. Purification of the centromere–specific protein CENP-A and demonstration that it is a distinctive histone. *Proc. Natl. Acad. Sci. USA.* 88:3734–3738.

Rattner, J. B. 1987. The organization of the mammalian kinetochore: scanning electron microscope study. *Chromosoma (Berl.).* 95:175–181.

Reider, C. L. 1982. The formation, structure and composition of the mammalian kinetochore and kinetochore fiber. *Int. Rev. Cytol.* 79:1–58.

Rieder, C. L., and S. P. Alexander. 1989. The attachment of chromosomes to the mitotic spindle and the production of aneuploidy in newt lung cells. *In* Mechanisms of Chromosome Distribution and Aneuploidy. M. A. Resnick and B. K. Vig, editors. Alan R. Liss, New York. 185–194.

Ris, H., and P. L. Witt. 1981. Structure of the mammalian kinetochore. *Chromosoma (Berl.).* 82:153–170.

Saitoh, H., J. Tomkiel, C. A. Cooke, H. Ratrie, M. Maurer, N. F. Rothfield, and W. C. Earnshaw. 1992. CENP–C, an autoantigen in scleroderma, is a component of the human inner kinetochore plate. *Cell.* 70:115–125.

Sears, E. R. 1952. Misdivision of univalents in common wheat. *Chromosoma (Berl.).* 4:535–550.

Simerly, C., R. Balczon, B. R. Brinkley, and G. Schatten. 1990. Microinjected kinetochore antibodies interfere with chromosome movement in meiotic and mitotic mouse embryos. *J. Cell Biol.* 111:1491–1504.

Spencer, F., and P. Hieter. 1992. Centromere DNA mutations induce a mitotic delay in *S. cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 89:8908–8910.

Tousson, A., C. Zeng, B. R. Brinkley, and M. M. Valdivia. 1991. Centrophilin: a novel mitotic spindle protein involved in microtubule nucleation. *J. Cell Biol.* 112:427–440.

Willard, H. F., and J. S. Waye. 1987. Hierarchical order in chromosome-specific human alpha satellite DNA. *Trends Genet.* 3:192–198.

Witt, P. L., H. Ris, and G. G. Borisy. 1980. Origin of kinetochore microtubules in Chinese hamster cells. *Chromosoma.* 81:483–505.

Yang, C. H., and M. Snyder. 1992. The Nuclear–Mitotic Apparatus Protein is important in the establishment of the bipolar mitotic spindle apparatus. *Mol. Biol. Cell.* 3:1259–1267.

Yang, C. H., E. J. Lambie, and M. Snyder. 1992. NuMA: an unusually long coiled–coil related protein in the mammalian nucleus. *J. Cell Biol.* 116: 1303–1317.

Yen, T. J., D. A. Compton, W. C. Earnshaw, and D. W. Cleveland. 1991. CENP–E, a human centromere associated protein released from chromosomes at the onset of anaphase. *EMBO (Eur. Mol. Biol. Organ.) J.* 10: 1245–1254.

Yen, T. J., G. Li, B. T. Schaar, I. Szilak, and D. W. Cleveland. 1992. CENP–E is a putative kinetochore motor that accumulates just before mitosis. *Nature (Lond.).* 359:536–539.

Zinkowski, R. P., J. Meyne, and B. R. Brinkley. 1991. The centromere–kinetochore complex: a repeat subunit model. *J. Cell Biol.* 113: 1091–1110.

Zirkle, R. E. 1970. UV–microbeam irradiation of newt–cell cytoplasm: Spindle destruction, false anaphase, and delay of true anaphase. *Rad. Res.* 41: 516–537.