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Role of the Centromere/Kinetochore in Cell Cycle Control

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OVERVIEW

Sera from autoimmune patients with anticentromere antibodies (ACAs) recognize a family of three structurally related human centromere proteins. Immunoelectron microscopy shows that these proteins are distributed throughout the heterochromatin subjacent to, and surrounding, the kinetochore. IgGs purified from these sera (ACA-IgG) disrupt mitotic events when microinjected into tissue-culture cells. The phenotype observed depends on the cell cycle position at the time of injection. When cells are injected from prophase onward, there is no apparent disruption of the subsequent mitosis. When cells are injected during the G_1 and S phases of the cell cycle, they subsequently become arrested at prometaphase. When ACA-IgGs are introduced into the nucleus during the G_2 phase of the cell cycle, the chromosomes successfully complete prometaphase but then remain blocked in metaphase for several hours. These observations define two execution points for antibody action: the S/G_2 transition and the onset of prophase. The fact that ACA-IgGs must be introduced into the nucleus during interphase in order to disrupt mitosis suggested to us that they might influence one or more aspects of kinetochore assembly. This hypothesis has been confirmed by serial-section electron microscopy. These results demonstrate that one or more antigens found in the heterochromatin surrounding the kinetochore are required for the assembly and/or function of this organelle in mitosis. Furthermore, they provide the first direct evidence that the kinetochore is involved in the timing of the metaphase/anaphase transition.

INTRODUCTION

Biochemical characterization of the proteins of the human centromere began with the discovery that sera of certain patients with scleroderma spectrum disease contain autoantibodies that react with the centromere region of mitotic chromosomes (Moroi et al. 1980). After some initial controversy (Cox et al. 1983; Ayer and Fritzler 1984; Earnshaw et al. 1984; Guldner et al. 1984; Valdivia and Brinkley 1985; van Venrooij et al. 1985; Nyman et al. 1986), these sera were shown to recognize four chromosomal autoantigens, designated CENPs A through D (*cen*tromere *p*roteins) (Earnshaw and Rothfield 1985; Kingwell and Rattner 1987). In the years since these antigens were first described, significant progress has been made in their characterization.

CENP-A is now known to be a centromere-specific histone H3 isotype (Palmer et al. 1991) that is found in the mononucleosome fraction following digestion of cellular chromatin (Palmer and Margolis 1987). A cDNA encoding CENP-B was cloned and shown to encode a multidomain protein with two highly acidic regions (Earnshaw et al. 1987). This protein was initially postulated to bind to DNA on the basis of the variability seen when chromosomes were stained with monoclonal antibodies (Earnshaw et al. 1987). This was confirmed when it was shown that CENP-B binds to a 17-bp sequence in α-satellite DNA in vitro (Masumoto et al. 1989). This sequence was termed the CENP-B Box (Masumoto et al. 1989). Unfortunately, the demonstration that CENP-B binds α-satellite DNA tells us little about CENP-B function. Nothing is known about α-satellite DNA function in vivo since this DNA is silent by all functional criteria thus far applied (Willard 1990).

CENP-C shares at least one structural determinant (presumably a posttranslational modification) with CENP-B (Earnshaw et al. 1987). Furthermore, the presence of CENP-C at a given centromere correlates strongly with the activity of that centromere in mitosis. CENP-C is not detectable at the inactive centromere of a stable dicentric chromosome (Earnshaw et al. 1989). A cDNA encoding CENP-C has recently been cloned and sequenced (H. Saitoh et al., in prep.). A polypeptide comigrating with CENP-D has recently been purified and shown to be the product of the *RCC1* gene, a negative regulator of the entry into mitosis (Nishimoto et al. 1978; Ohtsubo et al. 1987; Bischoff et al. 1990). It was quite surprising that the RCC1 protein would be found specifically at the centromere, and understanding of the significance of this observation will require further study. The purpose of the studies described below was to begin to determine the function of the CENP antigens in vivo.

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RESULTS

The Presence of CENP-B Does Not Correlate with the Ability of the Centromere/Kinetochore to Interact with the Spindle

We and other investigators originally assumed that CENP-B was likely to be involved in the function of the kinetochore. This was shown to be unlikely by two observations that found the protein to be neither sufficient nor necessary for kinetochore function. When we examined the distribution of CENP-B on a stable dicentric chromosome where one of the centromeres is inactive in attachment to the mitotic spindle, we found that the protein was present at both the active and inactive centromeres (Earnshaw et al. 1989). This argued that CENP-B was unlikely to be directly involved in the binding of microtubules to chromosomes, as had been suggested by in vitro cross-linking experiments (Balczon and Brinkley 1987).

Further analysis of the distribution of CENP-B in human chromosomes led to the discovery that the protein is undetectable at the centromere of the Y chromosome (Fig. 1). This now makes sense, given the observation that the CENP-B box sequence is not detected in any α-satellite sequences cloned to date from the Y chromosome (Masumoto et al. 1989). One widely held opinion is that α-satellite DNA is important for establishment of the overall structure of the centromere region. It therefore seems likely that CENP-B may have a structural role in the centromeric heterochromatin. If this is the case, then we predict that the Y chromosome must contain a homologous protein. If so, this second protein must be sufficiently divergent from CENP-B that it is not detected by either the antibody or DNA probes available to us at present. Such divergence within a gene family has previously been seen in the tubulin gene family, where the specialized β6-tubulin of chick erythrocytes is not detected with many antibody and DNA probes directed against other tubulin isotypes (Murphy et al. 1987).

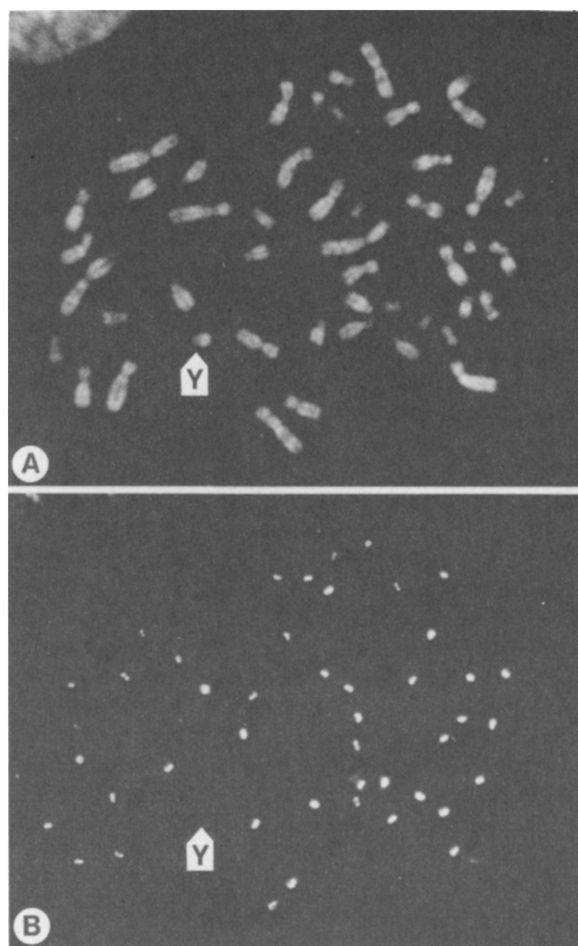

Figure 1. CENP-B is not detectable at the centromere of the human Y chromosome. (*A*) DAPI staining; (*B*) immunolocalization of CENP-B using a monospecific rabbit antibody, ra-ACA1 (Earnshaw et al. 1987). A cytogenetic spread of a human male cell line was prepared and stained as described previously (Earnshaw et al. 1989).

CENP-B and the Other Antigens Are Located in the Heterochromatin Beneath and Around the Kinetochore

The cytogenetic evidence cited above suggested that CENP-B is not an essential component of the kinetochore. To formulate models for the function of this protein in vivo more precisely, we decided to locate CENP-B precisely within the centromere, using immunoelectron microscopy with colloidal gold probes.

It is necessary at this point to define clearly the terms that we will use to describe the structures at the centromere region. We refer to the centromere as the entire region of the primary constriction, where sister chromatids are held in closest apposition. We will adopt a morphological definition of the kinetochore as the trilaminar plate structure at the surface of the centromere (for an extensive discussion, see Rieder 1982). Although the kinetochore cannot be distinguished during interphase, the centromere region remains highly condensed, as shown by immunoelectron microscopy with ACAs (Brenner et al. 1981; Moroi et al. 1981; Cooke et al. 1990). Detailed serial-section electron microscopy analysis has shown that the kinetochore undergoes a morphological maturation during mitosis. It first becomes visible as a fuzzy ball during prophase and develops into a trilaminar disk during early prometaphase (Rieder 1982).

When immunoelectron microscopy with 1 nm colloidal gold probes was used to map the location of CENP-B (Cooke et al. 1990), it was found that the protein is distributed throughout the heterochromatin beneath the kinetochore (Fig. 2). When a statistical analysis was performed, we were able to localize less than 1% of the grains to the kinetochore plate itself. Twenty-eight grains were found over the outer plate, whereas the remainder (6219) was distributed over the heterochromatin beneath it. When this experiment was repeated with patient serum recognizing CENPs A through C, a similar result was obtained (3% of 1992

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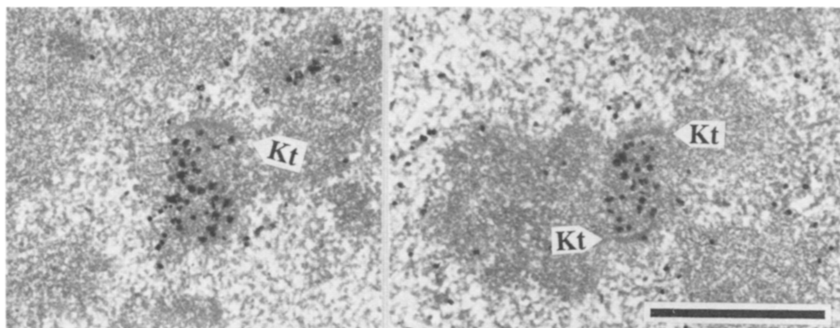

Figure 2. CENP-B is found in the heterochromatin beneath the kinetochore (Kt) plate. HeLa chromosomes were exposed to monospecific rabbit anti-CENP-B, which was subsequently localized with 1 nm colloidal gold as described recently (Cooke et al. 1990). Bar, 1μm.

grains in the outer plate), although subtle differences led us to suggest that at least one of the antigens appears to encircle the kinetochore closely. Recent experiments confirm that the antigen closely encircling the kinetochore is likely to be CENP-C (H. Saitoh et al., unpubl.).

Our results opened a controversy, since a similar experiment had previously suggested that the proteins recognized by patient serum were localized to the kinetochore itself, at least in rat kangaroo and Chinese hamster (Brenner et al. 1981). We now believe that the differences between the two studies are likely to arise both from interspecies differences in antigen distribution and from experimental methods (use of peroxidase detection versus the use of colloidal gold).

We conclude from our experiments that the CENP antigens are not predominantly part of the kinetochore but are instead heterochromatin proteins. Until now, there has been no evidence that the heterochromatin has any role in kinetochore structure or function. However, as described below, the results of microinjection experiments suggest strongly that the region of heterochromatin containing CENP antigens is essential for the assembly and function of the kinetochore in mitosis.

CENP Antigens Participate in Interphase Events That Are Required for Mitotic Progression

Microinjection of ACA-IgG into HeLa cells has profound effects on mitotic events, demonstrating that one or more of the CENP antigens are essential for mitotic progression. We do not yet have definitive proof of the identity of the antigen responsible for the inhibitory effects; however, we have strong suggestive evidence that CENP-B is involved. IgGs purified from 9 of 11 sera of patients with ACA inhibited mitotic events when injected into HeLa cells (Bernat et al. 1990). As shown in Figure 3, these sera recognize different combinations of the major CENP antigens. Particularly notable is the IgG from serum GS (lane 1), which strongly inhibited mitosis and had detectable antibodies only against CENP-B.

IgGs from two ACA-positive sera (Fig. 3, lanes 6 and 7) did not inhibit mitotic events. We have calculated that with our most concentrated preparations of IgG, we have only been able to inject approximately 50,000 molecules of CENP-B per cell (Bernat et al. 1990). This is roughly equimolar with the level of

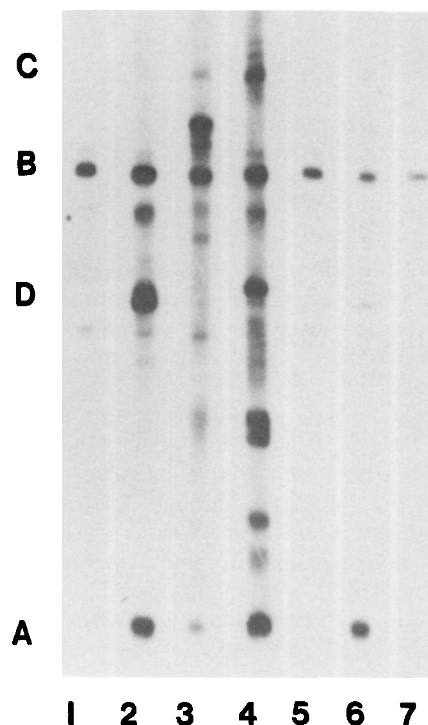

Figure 3. Different ACA-IgGs used for microinjection recognize different combinations of the CENP antigens in immunoblots of HeLa chromosomal proteins. (*1–5*) ACA-IgGs disrupted mitosis following microinjection; (*6, 7*) no disruption. The sera from which these IgGs were isolated are: (*1*) GS (low anti-CENP-A and anti-CENP-C); (*2*) AP (low anti-CENP-C); (*3*) F-2248; (*4*) J-3051; (*5*) H-345 (low anti-CENP-A and anti-CENP-C); (*6*) G-2016 (low anti-CENP-C); (*7*) G-1942 (low levels of all three antibodies). The positions of CENPs A through D are indicated at the left. Note that only serum AP consistently recognized CENP-D. (Reprinted from Bernat et al. 1990, *The Journal of Cell Biology*, vol. 111, p. 1529, in modified form, by copyright permission of the Rockefeller University Press).

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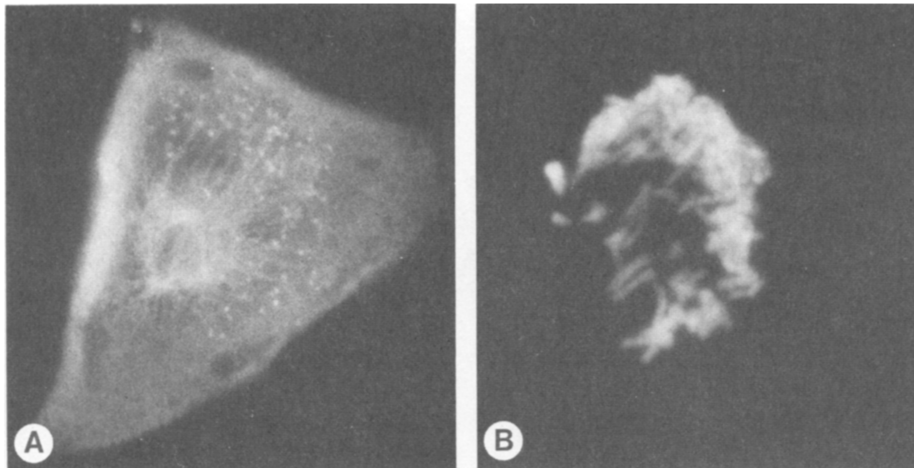

Figure 4. HeLa cell arrested in pseudoprometaphase at time of fixation. (*A*) Superimposed indirect immunofluorescence staining for tubulin (streaks), and the microinjected antibody (dots). (*B*) DAPI stain of the DNA. Conditions of microinjection and immunolocalization were carried out as described by Bernat et al. (1990).

CENP-B determined by quantitative immunoblotting (20,000–50,000 copies) (R.L. Bernat, unpubl.). The immunoblots with the two inactive IgG preparations (Fig. 3, lanes 6 and 7) are noticeably weaker than those in the active lanes, suggesting that these preparations were less concentrated and may have been injected in substoichiometric amounts. It should be noted that IgGs purified from nine control sera had no effect on mitotic progression (Bernat et al. 1990). These control sera included four with antitopoisomerase I, one with antitopoisomerase II (Hoffmann et al. 1989), one with antibodies to two high-molecular-weight chromosome scaffold proteins, and three from normal individuals.

The protocol used for the microinjection experiments enabled us to determine roughly the cell cycle phase at which antibodies were injected, and this turned out to be crucial for the interpretation of the results. Cells were plated sparsely and then grown into isolated microcolonies of 20–50 members on coverslips that had been shadowed through electron microscope finder grids to create an index pattern. All members of a microcolony were injected and observed periodically thereafter to monitor their entry into mitosis. Although the cells in the microcolony were asynchronous, the approximate cell cycle phase at the time of injection could be back-calculated from the time elapsed between injection and entry into mitosis. Thus, cells that entered mitosis 1 hour after injection must have been injected in G_2, and so on. The approximate lengths of the cell cycle phases under these growth conditions were determined by flow cytometry of HeLa cultures grown in parallel.

No mitotic phenotype was seen when antibodies were injected into mitotic cells from prophase onward (Bernat et al. 1990). One trivial explanation for such a result could have been that the antibodies did not have sufficient time to bind to their antigen prior to its action in mitosis. This explanation was ruled out by injecting early prophase cells and then holding them in prometaphase for 2 hours in the presence of nocodazole. These cells traversed mitosis normally following removal of the drug. This result defines the onset of prophase as an *execution point* (Pringle and Hartwell 1981) for the action of the antibodies. After this time, the antigens have completed all functions essential for the subsequent mitosis that are sensitive to antibody inhibition.

In contrast to the above results, injection of ACA-IgG into cells during interphase had a profound effect on subsequent mitotic events. If the antibodies were introduced into nuclei prior to 2–3 hours before mitosis, the cells became arrested at prometaphase; i.e., the chromosomes condensed, the nuclear envelope

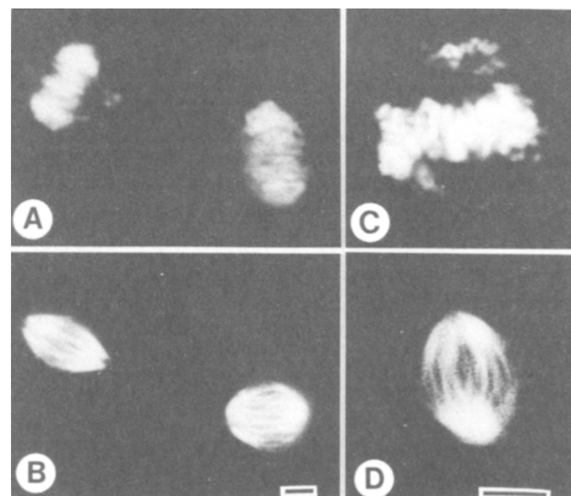

Figure 5. Cells arrested at metaphase following injection of ACA-IgG. (*A*,*C*) DAPI stain for DNA; (*B*,*D*) indirect immunofluorescence staining for tubulin. The cells had been in mitosis for the following times prior to fixation: (*A*,*B*) left, 5 hr; (*A*,*B*) right, 4 hr; (*C*,*D*) 4 hr. Bars: (*B*,*D*) 10 μm. (Reprinted from Bernat et al. 1990, *The Journal of Cell Biology*, vol. 111, p. 1524, in modified form, by copyright permission of the Rockefeller University Press.)

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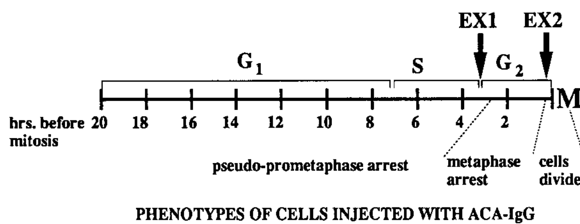

Figure 6. Diagram showing the mitotic phenotypes of cells following nuclear injection with ACA-IgG. The positions of the two execution points are indicated. (Reprinted from Bernat et al. 1990, *The Journal of Cell Biology*, vol. 111, p. 1530, in modified form, by copyright permission of the Rockefeller University Press.)

broke down, the spindle began to form, but the chromosomes never moved significantly from their position at nuclear envelope breakdown. In favorable instances, it appeared that the chromosomes were able to bind microtubules, but were apparently unable to move along them to the spindle equator (Fig. 4). Different results were obtained when cells were injected with antibody during the G_2 phase (within 2–3 hr of the onset of mitosis). Chromosomes in these cells were able to complete prometaphase movements and congress to the metaphase plate. However, the cells subsequently become arrested at metaphase (Fig. 5).

The interphase microinjection results permitted us to define a second execution point for antibody interference with mitotic events (Fig. 6). This point occurs about 2–3 hours prior to mitosis, a time that roughly corresponds to the S/G_2 transition in these cells. After this point, some function dependent on the CENP antigens that is required for prometaphase chromosome movements is no longer sensitive to inactivation by the antibodies. The fact that the antibodies must contact their antigen during interphase suggests that they interfere with some aspect of centromere or kinetochore assembly, rather than directly blocking function of their target antigen(s) during mitosis.

DISCUSSION

Concerted studies of mitosis by many workers during the last century have focused on five major problems: (1) the physiological control of the onset of mitosis; (2) the structural transformations in the chromosomes, nucleus, and cytoskeleton that occur at this time; (3) the mechanism of chromosome movement; (4) the mechanisms regulating the onset of anaphase; and (5) the mechanism by which the cleavage furrow is positioned. The studies described above have provided the first link between the structures involved in chromosome movement and mechanisms determining the onset of anaphase. Our understanding of the mechanisms of chromosome movement has grown significantly since the development of in vitro and in vivo systems for the analysis of chromosome:microtubule interactions and movements. Specifically, a significant amount of attention has become focused on the kinetochore as both the site of chromosomal attachment to the spindle and the probable location of the mechanochemical motors that power these movements (Mitchison and Kirschner 1985; Gorbsky et al. 1987; Brinkley et al. 1988; Nicklas 1989).

In the present studies, we have shown that proteins that lie outside the morphologically distinct kinetochore play an essential role in the function of this structure in mitosis; i.e., the centromeric heterochromatin performs an essential role in the organization of the kinetochore. Our experiments suggest that these essential functions of the centromeric heterochromatin are carried out, not during mitosis itself, but during the preceding interphase. If cells are injected with antibody at any time following the onset of prophase, they appear to traverse mitosis normally. This suggests that the antibody is unlikely to bind and inactivate a centromeric component directly and is more consistent with models where the antibody interferes with some aspect of centromere assembly.

The microinjection experiments permitted us to define two execution points for the antibody interference with mitotic events (see Fig. 6). If ACAs are injected into human cells 2–3 hours before mitosis (a time that roughly corresponds to the S/G_2 transition), the cells become arrested at prometaphase. In contrast, if cells are injected with antibody during the G_2 phase, they are able to complete prometaphase movements and congress to the metaphase plate, but they subsequently become arrested at metaphase. We discuss the implications of these results separately below.

Antibody-induced Prometaphase Arrest

A recent series of studies examining early prometaphase events in vivo has confirmed that chromosomes become attached to the spindle by capturing microtubules whose assembly is initiated at the spindle poles (Mitchison and Kirschner 1985; Mitchison et al. 1986). Surprisingly, two studies have shown that the earliest chromosome movements are apparently associated with lateral attachment of the chromosomes to the microtubules (Rieder et al. 1989; Merdes and De Mey 1990), and one of these studies has shown that these lateral attachments are mediated by the corona region of the kinetochore (Rieder et al. 1989). Upon attachment, these chromosomes move poleward rapidly, at speeds of up to 55 μm/min (Rieder et al. 1989). This suggests that the movements might involve the sliding of chromosomes along the microtubule surface under the influence of cytoplasmic dynein.

This suggestion has recently garnered support from the observation that antibodies to cytoplasmic dynein decorate the centromere regions of chromosomes from cells arrested in mitosis with antimicrotubule agents (Pfarr et al. 1990; Steuer et al. 1990). Although the percentage of cytoplasmic dynein associated with centromeres is significantly lower in cells with intact spindles, some immunoreactivity appears to be associated with the chromosomes under these conditions. The

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initial reports of cytoplasmic dynein localization predicted that within the centromere, the dynein was localized to the kinetochore (Pfarr et al. 1990; Steuer et al. 1990). The antigen has since been localized to the kinetochore corona by immuno-gold electron microscopy (Wordeman et al. 1991). Thus, a consistent picture has emerged in which it seems likely that some, if not all, early prometaphase movements occur as a result of interactions between microtubules and cytoplasmic dynein in the kinetochore corona.

Our observation that injection of ACA into cells prior to the S/G_2 transition blocks prometaphase movements serves to complicate this picture. We have shown that the pattern of antigen recognition by the injected ACA strongly implicates CENP-B and possibly others of the CENP autoantigens in the prometaphase arrest. However, none of these polypeptides are detectable in the kinetochore plate or corona by immunoelectron microscopy (see Fig. 2) (see also Cooke et al. 1990). Furthermore, serial-section electron microscopy analysis of cells arrested in prometaphase following antibody injection reveals that the microtubule binding and motility functions of the kinetochore have become uncoupled. Chromosomes in these cells are able to bind microtubules but are apparently unable to move along them (Bernat et al. 1991).

Our electron microscopy studies suggest that under these injection conditions, ACAs interfere with the assembly of the outer kinetochore plate while leaving the corona apparently intact (Bernat et al. 1991). The corona is a rather ill-defined structure at best, however, and subtle changes in morphology would not have been detected. Nevertheless, these studies suggest that assembly of some component into either the outer kinetochore plate or the corona region is dependent on one of the antigens recognized by ACA and is essential for prometaphase movements. This assembly is presumably completed by early S/G_2 phase (Fig. 6).

CENP-B is an obvious candidate for the target of this inhibition for two reasons. First, it is the only major chromosomal antigen to be recognized by all of the inhibitory sera (see Fig. 1). Second, the cell cycle timing of the inhibitory effect makes sense, given what we know about the biology of this protein. CENP-B has been shown to bind to α-satellite DNA in vitro (Masumoto et al. 1989), and its binding to chromosomal subfragments correlates with the levels of α-satellite in vivo as well. The replication of α-satellite DNA in human cells was the subject of two recent studies, which showed that this DNA replicates late in S phase. We therefore suggest that the ACA-sensitive event that is identified by the execution point at the S/G_2 transition may be the assembly of CENP-B onto newly replicated α-satellite DNA. That CENP-B would be involved in assembly of a motor component into the kinetochore corona is unexpected, given that the protein is detected solely beneath the kinetochore plate in human cells (Cooke et al. 1990).

CENP-B may play some role in the overall organization of the centromeric heterochromatin. Such a role is consistent with the model of the kinetochore as consisting of DNA regions that organize the microtubule-binding components being interspersed between α-satellite loops that make up the compacted heterochromatin beneath the kinetochore (Zinkowski et al. 1991). Even so, it is not obvious why interference with the assembly of CENP-B onto α-satellite DNA should permit microtubule binding but inhibit chromosome movement. Two possibilities suggest themselves. (1) Some component necessary for prometaphase chromosome movement may be unable to bind properly to the kinetochore unless the CENP-B-containing heterochromatin structure is normal. We are currently in the process of determining the distribution of cytoplasmic dynein and a series of other putative kinetochore components in prometaphase-arrested cells. (2) The assembly of the kinetochore plate may be required in order to impart a directionality to the chromosomal movements. If kinetochores contain both dyneins and kinesins, the overall architecture of the plate and corona may segregate the two components so that they do not work against one another.

Regardless of the detailed mechanism, these experiments have shown that one or more of the CENP antigens perform functions prior to the S/G_2 transition that are essential for chromosome movements later on in prometaphase. In addition, these experiments also show that microtubule binding by the kinetochore can be uncoupled from movement. This suggests that unless the ACA-blocked prometaphase is in a "rigor" state, the binding and motility functions of the kinetochore may involve distinct components.

Involvement of the CENP Antigens in the Metaphase/Anaphase Transition

One of the most dramatic aspects of mitosis is the sudden transition from metaphase to anaphase. At metaphase, the chromosomes form a tight mass at the spindle midzone. Although some oscillations of individual chromosomes along the spindle axis occur at this time, this portion of the mitotic cycle is typically characterized as a period of waiting. With the onset of anaphase, sister chromatids abruptly undergo a coordinate separation and begin their movement to the spindle poles.

It has long been known that cells possess a mechanism for sensing the completion of the alignment of chromosomes at the midzone of the mitotic spindle. If only a single misaligned chromosome remains in a newt lung cell, the cell will delay entry into anaphase for a significant period (although not indefinitely) (Zirkle 1970b; Rieder and Alexander 1989). These observations have led to the proposal that there is a cell cycle "checkpoint" (Hartwell and Weinert 1989) that senses proper chromosome alignment and spindle assembly at metaphase. This checkpoint presumably operates via an inhibitory circuit that prevents anaphase onset until congression is complete. Our results provide an unexpected opportunity to learn more about the nature of

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the inhibitory circuit, about which nothing is currently known.

As described above, introduction of ACA-IgG into the nucleus within 2 hours of the onset of mitosis results in a distinct mitotic phenotype, in which cells become arrested in metaphase. Prometaphase congression is apparently normal in these cells. We have considered two possible explanations for this antibody-induced metaphase delay. First, the CENP antigens may be essential for assembly of a structurally normal centromere that is able to undergo disjunction normally. Second, the CENP antigens may be involved in some aspect of the signaling pathway responsible for starting the proteolysis cascade that triggers the onset of anaphase (Murray and Kirschner 1989). Our results tend to favor the later explanation.

We have noted that the antibody-induced metaphase delay is typically not permanent. After being arrested in metaphase for up to 8 hours, most arrested cells eventually enter anaphase (Bernat et al. 1990). At this time, the sister chromatids at the metaphase plate disjoin synchronously and move coordinately to the poles (Bernat et al. 1990). Both anaphase A and B movements appear to be normal. This suggests that disjunction per se is not defective and that the anaphase motor is potentially functional during ACA-induced metaphase arrest. Were the sister chromatids "stuck together" as a result of a structural defect in *cis*, we would have expected separation of some of the chromatids to be aberrant.

Electron microscopy of ACA-injected cells arrested in metaphase indicates that the kinetochores in these cells are apparently fragile (Bernat et al. 1991). The basis for this conclusion was the observation that the kinetochores of cells injected during late G_2 appear nearly normal when cells enter mitosis in the presence of colcemid, but they lose their trilaminar morphology and become stretched away from the surface of the chromosome when microtubules are present and exert force on them (Bernat et al. 1991).

Observation of the colcemid-treated cells offers one explanation for the apparent fragility of the kinetochore. The chromatin immediately beneath the kinetochore plate appears to be less compacted than normal in about half of the kinetochores in these cells (Bernat et al. 1991). This may mean that the heterochromatin "foundation" for the kinetochore is weakened in these cells. As a result, when the spindle begins to exert tension, the whole structure apparently pulls away from the surface of the chromosome. That ACA injection would affect the structure of the heterochromatin beneath the kinetochore is consistent with the location of the CENP antigens as determined by our immuno-gold electron microscopy studies, where we showed that the antigens are distributed throughout this region in human chromosomes (Cooke et al. 1990).

These experiments show that injection of ACA into the nucleus during late G_2 causes a metaphase arrest and is associated with abnormal structure of the kinetochore. Thus, whatever the mechanism for sensing the completion of proper metaphase alignment, the centromeric heterochromatin and the kinetochore apparently are essential components of the signaling pathway.

Control of the Metaphase/Anaphase Transition: Spindle-based Models

The mechanism of operation of the metaphase checkpoint remains one of the least-understood aspects of mitotic regulation. Although a variety of published and anecdotal information have implicated both the centromeres and spindle, no clear mechanistic picture has emerged.

Several experiments suggest that the spindle microtubules are somehow involved in the timing of the metaphase/anaphase transition. Delay of spindle assembly by 5 minutes in the highly reproducible early cleavages in sea urchin embryos results in a delay of anaphase onset by 5 minutes (Sluder 1979, 1988). Furthermore, if the size of the spindle is diminished, the onset of anaphase is also delayed (Sluder 1979, 1988).

Our observations of HeLa cells in culture are consistent with a requirement for microtubules in triggering the transition. It has long been known that different cell types respond differently to long-term arrest in the presence of colcemid (Sluder 1979; Kung et al. 1990). For example, HeLa cells will remain blocked in mitosis for at least 24–48 hours in the presence of colcemid (C.A. Cooke and W.C. Earnshaw, unpubl.). We were therefore surprised to note that many HeLa cells blocked in mitosis following ACA injection eventually go on to complete mitosis, even if the cells never achieved a metaphase alignment of the chromosomes (Bernat et al. 1990). This suggests that if microtubules are present, some default mechanism eventually triggers anaphase onset, even when the metaphase checkpoint has not been completed.

If the spindle is involved in triggering the metaphase/anaphase transition, then it might be expected that the structure would exhibit some morphological change at or before the metaphase/anaphase transition. Three such changes have been observed. First, the spindle progressively shortens and becomes more robust during metaphase, adopting a stocky fusiform shape just prior to the transition (Taylor 1959; Rieder 1990). Second, Mazia (1961) referred to a "tightening up" of the spindle during metaphase, indicating that the spindle underwent changes in both its optical properties and its sensitivity to certain chemical agents. Third, the bundles of kinetochore-to-pole microtubules become more birefringent (more ordered) just before anaphase onset, at least in some cases (Inoué 1964).

These observations have led to two proposed mechanisms to explain the possible role of microtubules in timing of the metaphase/anaphase transition. First, the metaphase/anaphase transition may occur when the spindle contracts below some critical length (C. Reider, pers. comm.). How the cell would sense spindle length is unclear, but one possibility is that each aster emits a

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gradient of inducer. The metaphase/anaphase transition might be triggered when the spindle shortens sufficiently that the concentration of inducer surpasses some threshold level in the vicinity of the chromosomes at the metaphase plate. Although our observations do not address this model directly, we have observed that under some conditions, spindles of metaphase-arrested cells shorten into the fusiform shape characteristic of metaphase/anaphase in newt lung cells (Rieder 1990) while the cells remain blocked in metaphase. In addition, we observed anaphase onset in prometaphase-arrested cells where the chromosomes never congressed to the spindle equator at all. Together, these observations suggest that spindle shortening alone may not be sufficient to induce anaphase onset in HeLa cells.

An alternative model suggests that the cell somehow senses the balance of tension in the spindle and triggers entry into anaphase when this tension reaches a certain level (Snyder et al. 1985). This tension is suggested to arise from the opposition of the compressive forces exerted by the kinetochores pulling the spindle poles inward and the rigidity of the microtubules, which flex as the spindle contracts. Tension levels throughout the spindle may well be different in cells arrested in metaphase as a consequence of ACA injection. In these cells, forces at the point of microtubule attachment to the chromosome result in an extensive stretching of the chromatin away from the surface of the chromosomes. One possible consequence of this stretching is that the tension exerted by the kinetochore microtubule fiber might be abnormal. In these cells, the microtubules are attached to the chromosome via a "weak spring," rather than a robust kinetochore plate. How the cell might monitor the level of tension in the spindle is unknown.

Evidence against a role of spindle tension in initiation of anaphase onset comes from our observation that cells arrested in pseudoprometaphase following injection of ACA-IgG will eventually enter anaphase despite the fact that a metaphase plate is never assembled (Bernat et al. 1990). Likewise, it has long been known that a number of cell types will enter chromosomal anaphase (separation of sister chromatids) even in the absence of spindle microtubules (Sluder 1979). At present, it is not known whether these observations reflect a lack of requirement of microtubules in the inhibitory pathway or whether they reveal the existence of a second, microtubule-independent, default pathway for inducing anaphase onset.

Control of the Metaphase/Anaphase Transition: Centromere/Kinetochore-based Models

UV microbeam ablation experiments reported in abstract form by Zirkle (1970a) suggested that the centromere might be involved in regulation of the metaphase/anaphase transition. First, he observed that a single incorrectly oriented chromosome is sufficient to cause a newt lung cell to delay for a considerable time before entering anaphase (Zirkle 1970b). This observation has been repeated recently, and it has been shown that the delay, although lengthy, is not indefinite (Rieder and Alexander 1989). Zirkle (1970a) then noted that if the centromere region of the detached chromosome was irradiated with a UV microbeam, rendering it incapable of attachment to the spindle, the chromosome now became "invisible" to the regulatory network, and mitosis was initiated normally. If interpreted correctly, this result suggests that an incorrectly oriented centromere "emits a signal" that acts to inhibit anaphase onset.

The difficulty with confirming this result lies in the intrinsic variability of the prometaphase/metaphase period in vivo. Observation of newt lung cells in mitosis leads to the conclusion that the period of time spent in metaphase after proper alignment of the chromosomes at the metaphase plate is extremely variable, making interpretation of timing experiments like those of Zirkle very complex (C. Rieder, pers. comm.). To these results, we now add our observation that microinjection of ACA during late G_2, which appears to have little effect on spindle structures in mitosis but does cause abnormalities of kinetochore structure, results in a delay in metaphase.

We have considered two models in which the injection of ACA might perturb the regulation of anaphase onset. First, some cellular component that normally binds to the centromere/kinetochore only after it has achieved a bipolar orientation at the metaphase plate might be prevented from binding as a consequence of the antibody treatment. This component would be postulated to act as an inhibitor of anaphase onset when in the nonbound state. Although the experiment of Zirkle is clearly consistent with the existence of such an inhibitor, this model remains purely hypothetical at present. In a second model, alignment of the chromosomes at the spindle equator would be required in order to permit subsequent structural changes in the centromere/kinetochore. When completed, these changes would lead to onset of the metaphase/anaphase transition.

Until recently, there has been no evidence for the existence of such changes in the chromosome during metaphase. However, it was known that profound functional changes occur in the kinetochore at the time of anaphase onset. It is possible, albeit with difficulty, to detach a chromosome from the spindle with a microneedle during the approximately 5 minutes prior to anaphase onset (Nicklas and Staehly 1967). If a similar procedure is attempted just after anaphase onset, one finds that the chromosome cannot be detached without destroying the entire spindle (Nicklas and Staehly 1967). The nature of the changes revealed by this observation are unknown at present. A recent study from our laboratory has revealed that profound changes in centromere structure do occur during metaphase. This result came from studies of two proteins that are unrelated to the antigens recognized by ACA.

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A number of years ago, we made monoclonal antibodies to the entire crude chromosome scaffold fraction. One antibody identified a novel pair of chromosomal proteins, which we termed INCENPs (*in*ner *cen*tromere *p*roteins) (Cooke et al. 1987). The unusual fact about these proteins (a doublet of M_r 135/150 kD) was that they are tightly chromosome-associated during interphase and through prometaphase but were seen to completely leave the chromosomes at the metaphase/anaphase transition. They ultimately wind up in the intercellular bridge and are eventually discarded following mitosis (Cooke et al. 1987).

A recent analysis of the distribution of the INCENPs in dividing cells by confocal microscopy yielded an unexpected result when metaphase cells were examined in detail. We were able to observe four structural arrangements of these proteins in metaphase cells and to show that these arrangements correspond to a sequence of stages of metaphase (Earnshaw and Cooke 1991). These stages are illustrated in Figure 7. In "early" metaphase, the INCENPs are present throughout the centromere region as well as on the chromosomal arms, particularly near the telomeres (Fig. 7A,B). In "early-mid" metaphase, the INCENPs are found only at the centromeric region (Fig. 7C,D). In "mid-late" metaphase, a complex pattern of INCENP staining is observed, with some antigen still at centromeres and some antigen seemingly not on the chromosomes at all (Fig. 7E,F). In "late" metaphase cells, the INCENPs appear to have completely separated from the chromosomes (Fig. 7G,H). They now appear as streaks that traverse the metaphase plate in close association with microtubules (Earnshaw and Cooke 1991).

These observations are consistent with the second model for the regulation of the metaphase/anaphase transition suggested above. In this model, we postulate that as long as the INCENPs are associated with the centromeres, they help to maintain association of sister chromatids and inhibit the onset of anaphase. The transition of INCENP staining during metaphase from centromeric to streaks appears to involve a transfer of the proteins from the centromeres to the bundles of overlapping polar microtubules that penetrate the plate (Earnshaw and Cooke 1991). If the INCENPs require

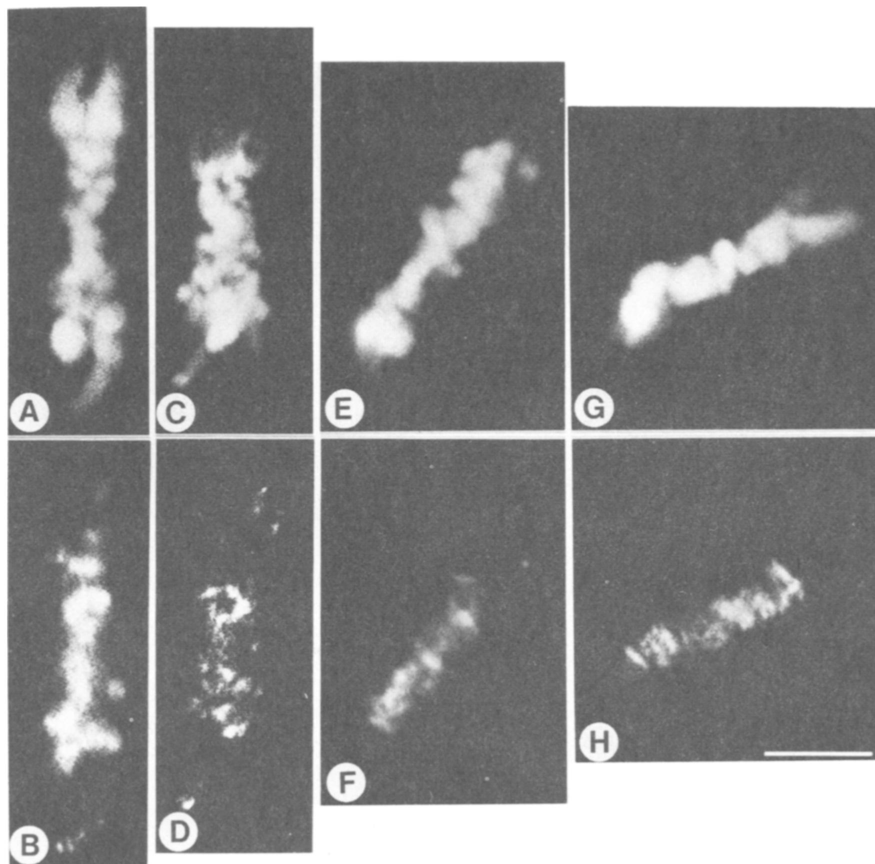

Figure 7. Changes in chromosome structure during metaphase: longitudinal optical sections showing the distribution of INCENPs in cells at various stages of metaphase. (See Fig. 4 [Earnshaw and Cooke 1991] for evidence that these correspond to a temporal sequence of changes throughout metaphase.) (*A*,*B*) Early metaphase (INCENPs on centromeres and protruding chromosome arms); (*C*,*D*) early/mid metaphase (INCENPs concentrated on centromeres); (*E*,*F*) mid/late metaphase (complex distribution of INCENPs with both centromere labeling and streaks between chromosomes); (*G*,*H*) late metaphase (INCENPs in streaks crossing the metaphase plate); (*A*,*C*,*E*,*G*) propidium iodide staining of the DNA; (*B*,*D*,*F*,*H*) INCENP staining with monoclonal antibody 3D3. All images were obtained using a laser scanning confocal microscope as described recently (Earnshaw and Cooke 1991). Bar, 5 μm.

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the presence of antiparallel microtubules in close proximity to the centromeres in order to leave the chromosomes, then this exit may only be possible when the chromosomes are congressed to the spindle midzone. Evidence that the INCENPs do require microtubules in order to exit from the chromosomes comes from the observation that in the presence of colcemid, the antigens become hyperconcentrated in the inner centromere, between sister chromatids (Earnshaw and Cooke 1991). This model predicts that changes in the structure of the centromeric heterochromatin induced by injection of ACA during late G_2 might interfere with the normal pattern of movements of the INCENPs (or related proteins), thereby resulting in the metaphase arrest.

CONCLUSIONS

It is clear from the above discussion that the mechanism by which the inhibitory pathway prevents cells from entering into anaphase in the presence of maloriented chromosomes remains entirely unknown. We have included the extensive discussion of possible models in order to summarize the present state of thinking about the problem and hopefully to stimulate further experimentation. Some of the above models (spindle tension, diffusible inhibitors of anaphase onset) are likely to prove difficult to test, whereas others (the requirement for spindle shortening, interference by ACA with the movements of the INCENPs) may be more amenable to study.

Whatever the mechanism ultimately proves to be, our studies have shown that the centromere (and probably the kinetochore) is an essential component of the control circuit regulating anaphase onset. Furthermore, we have shown that proteins that are not themselves components of the kinetochore, but rather are found in the centromeric heterochromatin surrounding the structure, have profound influences on the function of the kinetochore during mitosis.

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