

# COMMENTARY

## When is a centromere not a kinetochore?

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### Centromere and kinetochore

The terms 'centromere' and 'kinetochore' were originally coined to describe the region(s) of each chromosome with which the spindle microtubules become associated (Rieger *et al.* 1976). In recent years, the term 'centromere' has largely come to be used by morphologists to describe the primary constriction of mitotic chromosomes. The term 'kinetochore' has been used to refer to the circular plaque structure that rests on the surface of the centromeric chromatin and into which microtubules are inserted (Jokelainen, 1967; Brinkley and Stubblefield, 1966). This commentary is concerned with development of a rationale for assignment of newly identified antigens to specific centromere domains and, in particular, to the kinetochore domain.

The distributions of a number of centromere-associated antigens that have been described in recent years are most conveniently classified if the centromere is subdivided into three principle domains, which we have previously designated the *kinetochore*, *central* and *pairing domains* (Earnshaw and Rattner, 1989). Under this nomenclature, the *kinetochore domain* consists of both the kinetochore and the subjacent chromatin upon which it rests (Earnshaw and Rattner, 1989). The kinetochore is a small (0.4–0.6  $\mu\text{m}$  diameter in PtK1 cells; Roos, 1973) button-like structure that rests on the surface of the centromeric chromatin. The most extensive mammalian kinetochores are seen in the fusion chromosomes of the Indian muntjac, where they may be up to 1.45  $\mu\text{m}$  in length (Comings and Okada, 1971).

At metaphase, the kinetochore consists of a dense osmophilic outer plate 0.025–0.040  $\mu\text{m}$  thick that is separated from the surface of the centromeric chromatin by an electron-lucent zone of similar thickness (Roos, 1973; Rieder, 1982). The surface of the subjacent chromatin is often compacted into a second dense plate. A fibrous corona is seen to radiate outward from the outer plate whenever microtubules are absent (Rieder, 1982).

Much recent interest has been focussed on the kinetochore outer plate, since this is where the majority of microtubules are inserted. The detailed structure of this plate is not known, although a variety of evidence suggests that it may contain a special form of chromatin. This evidence includes the presence of a fibrous substructure seen by electron microscopy (Ris and Witt, 1981; Rattner, 1986; Rattner and Bazett-Jones, 1989), the susceptibility of the structure to digestion with DNase (Pepper and Brinkley, 1980) and the presence of phosphate as revealed

by electron-spectroscopic imaging (Rattner and Bazett-Jones, 1989). While this notion has a number of attractive features, not the least of which would be a potential functional homology between mammalian and yeast kinetochores (Pluta *et al.* 1990; Brinkley, 1990; Bloom *et al.* 1989; Clarke, 1990; Murphy and Fitzgerald-Hayes, 1990), it is important to note that the presence of DNA in the outer kinetochore plate has yet to be definitely established. Thus the structure and composition of the kinetochore remain important subjects for future study.

The majority of microtubules that attach the chromosomes to the spindle during metaphase and anaphase terminate in the outer kinetochore plate. As a result, there has been a tendency in recent discussions of the structure and function of this region to focus solely on the kinetochore *per se*. For example, recent experiments demonstrating that the mechanochemical motor for anaphase chromosome movement is located on the chromosome (Gorbsky *et al.* 1987; Nicklas, 1989) have been broadly interpreted as proof that the motor is at the kinetochore. However, this assumption also has yet to be confirmed directly.

Careful observation of the centromere region of chromosomes in thin sections of metaphase cells reveals that a significant number of microtubules enter the chromatin just lateral to the kinetochore plates (Comings and Okada, 1971; Rattner and Bazett-Jones, 1989). In fact, only about half of the microtubules of the bundle connecting the kinetochore to the spindle pole actually terminate in the kinetochore itself (Rieder, 1990). The rest either pass into or through the chromatin at its periphery, terminate in the inner plate, or terminate prior to reaching the chromosome (Comings and Okada, 1971; Rattner and Bazett-Jones, 1989; Rieder, 1990). The chromatin immediately surrounding the kinetochore differs from the bulk of centromeric chromatin in that it is rich in the CENP autoantigens (Cooke *et al.* 1990). This difference lead us to suggest that this chromatin might play some role in chromosome–spindle interactions (Pluta *et al.* 1990).

The above discussion is intended to suggest that questions of kinetochore structure and function have moved to a level of detail where it now becomes important to make precise assignments of antigens to the fibrous corona, outer and inner kinetochore plates and immediately adjacent chromatin. In order to develop detailed and accurate models for the functional organization of the

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Key words: centromere, kinetochore, spindle.

**Table 1. Antigenes assigned to the kinetochore**

Antigen	$M_r$ $\times 10^{-3}$	Published location	Evidence	Comments	Ref.*
Calmodulin	17	Kt	EM peroxidase	Also stains SP, spindle vesicles	1
CENP antigens	ND	Kt	EM peroxidase	See result for CENP-B below	2
CENP-A	~17	CEN	IF	No monospecific antibodies yet	3
CENP-B	80	CEN (central)	Immuno-gold	Located beneath Kt	4
CENP-C	140	CEN	IF	No monospecific antibodies yet	4
CENP-D	50	CEN	IF	No EM	5
RCC1?	47	Kt	IF	=CENP-D? no EM	6
Tubulin	52	Kt (corona)	Immuno-gold	Only in colcemid-blocked cells	7
Topoisomerase I	100	Kt	IF	Disputed result, others think it is unlikely to be centromere; no EM	8
Cytoplasmic dynein	440	Kt	IF	Also stains k-MTs (Met); S (Ana); no EM	9
	150	Kt	IF	Also stains k-MTs (Met); S (Ana); no EM	9
	70	Kt	IF	Also stains k-MTs (Met); S (Ana); no EM	9
	400	Kt	IF	Also stains k-MTs (Met); SP (Ana)	10
	79	Kt	IF	Also stains k-MTs (Met); SP (Ana); no EM	10
37A5 antigen	140/155	Kt	IF	MB (Ana); no EM	11
Class I	250	CEN/Kt	IF	Kt (Met), MB (Ana); no EM	12
Class II	275	CEN/Kt	IF	Also stains SP	
Class III	205	CEN/Kt	IF	Also stains SP (Met); MB+SP (Ana)	12
Class IV	205	CEN/Kt	IF	Also stains SP	12
Class V	205	CEN/Kt	IF	Only chromosomal in colcemid	12
Class VI	205/215	CEN/Kt	IF	Only chromosomal in colcemid	12
p34 <sup>cdc2</sup>	34	Kt	IF	Also stains SP, k-MTs & MB; no EM	13
Phosphoproteins		Kt	IF	Also stains SP & MB; no EM	14

Abbreviations: ND, not determined; IF, indirect immunofluorescence; EM, immunoelectron microscopy; Kt, kinetochore; CEN, centromere, S, entire spindle; MB, central spindle and midbody; SP, spindle poles, k-MTs, microtubules connecting kinetochores to spindle poles; Met, metaphase; Ana, anaphase (if not specified staining is the same through all stages of mitosis).

\*References: 1 (Dedman *et al.* 1980), 2 (Brenner *et al.* 1981); 3 (Guldner *et al.* 1984; Earnshaw and Rothfield, 1985); 4 (Earnshaw and Rothfield, 1985; Cooke *et al.* 1990); see also: 5 (Kingwell and Rattner, 1987); 6 (Bischoff *et al.* 1990); 7 (Mitchison and Kirschner, 1985); 8 (Maul *et al.* 1986), (see also Haaf *et al.* 1990); 9 (Steuer *et al.* 1990), 10 (Pfarr *et al.* 1990); 11 (Pankov *et al.* 1990), 12 (Compton *et al.* 1991), 13 (Rattner *et al.* 1990); 14 (Vandré *et al.* 1984)

centromere/kinetochore it is essential that components assumed to be located in the kinetochore actually are localized in the trilaminar plate structure, and not in the surrounding chromatin or elsewhere in the centromere.

There is thus need for the development of a generally accepted protocol by which to determine the location of newly identified centromere components precisely.

#### The localization problem

Until recently, relatively few candidates for centromere and/or kinetochore proteins were known. However, this chromosomal region has recently begun to attract an increased level of interest, and new centromeric antigens are being described at an ever increasing rate. The most commonly used method for establishing the chromosomal location of such antigens involves the use of specific antibodies for indirect immunofluorescence microscopy. Chromosomes from colcemid-blocked cells are typically used as substrates for this analysis. At present, as shown in Table 1, a number of the newly identified antigens have been assigned to the kinetochore on the basis of the results of immunofluorescence analysis.

There are two difficulties with this approach. First, immunofluorescence analysis alone cannot be used as evidence that a component is located in the kinetochore plates, since the spacing between the outer kinetochore plate and the subjacent chromatin (0.025–0.04  $\mu$ m; Rieder, 1982) is well below the limit of resolution of the light microscope, even if computer image processing is used to enhance image contrast. This is true even in the chromosomes of the Indian muntjac, where the kinetochore is much larger in lateral extent, but is still closely apposed to the surface of the centromeric chromatin. Second, the protein composition of the centromere region of chromosomes from cells incubated with microtubule-

blocking agents is apparently different from that of mitotic chromosomes in unperturbed cells.

Because it is impossible to distinguish the kinetochore from the underlying chromatin by light microscopy, we developed the term *kinetochore domain*, which was to be understood as comprising both the kinetochore and the subjacent chromatin on which it rests (Earnshaw and Rattner, 1989). We then proposed that all antigens localized to the surface of the centromeric chromatin by light microscopy be designated as components of the *kinetochore domain* (Earnshaw and Rattner, 1989). It is essential to note that assignment of an antigen to the kinetochore domain does not mean that the antigen is a component of the kinetochore plates themselves.

We then went on to suggest that the further designation of specific components as being localized to the kinetochore proper should only be made under conditions where the kinetochore can be visualized directly as a laminar plate structure (Earnshaw and Rattner, 1989). This requires antigen localization by electron microscopy, preferably using nondiffusible probes. While this continues to be a difficult set of experiments to perform, recent advances in immunolocalization technology, particularly the use of 1 nm colloidal gold probes, have simplified somewhat the mapping of chromosomal antigens (Cooke *et al.* 1990).

One demonstration of the need for precise immunoelectron microscope localization of centromere components comes from the study of the centromeric autoantigens recognized by ACA. While they were first described as being located in centromeres (Moroi *et al.* 1980), these antigens have lately come to be thought of as components of the kinetochore. This shift in thinking was largely based on a study in which the immunoperoxidase method was used to localize the ACA antigens to the kinetochores of Chinese hamster and rat kangaroo chromosomes (Brenner *et al.* 1981), although a contempor-

ary study found that the antibodies stained the entire centromere region of human chromosomes (Moroi *et al.* 1981).

When we used the immunogold procedure to localize the centromeric autoantigens recognized by the autoimmune patient sera, we were surprised to find the gold distributed in the chromatin beneath and around the kinetochore plates, but not over the plates themselves (Cooke *et al.* 1990). In one set of experiments using antibodies specific for CENP-B (the  $80 \times 10^3 M_r$  autoantigen recognized by the great majority of ACA from autoimmune patients; Earnshaw *et al.* 1986), we scored 28 gold grains over the kinetochore plates and 6219 grains in the subjacent chromatin (Cooke *et al.* 1990). Similar results were obtained with the whole autoimmune serum (Cooke *et al.* 1990).

Differences between the results of immunoperoxidase and immunogold localization have been observed in other systems, and ascribed to localized diffusion of the peroxidase reaction product. For example, such diffusion has been proposed to explain the different results obtained for the localization of protein C23 in the nucleolus when using immunoperoxidase and immunogold methods to map the distribution of this antigen (Spector *et al.* 1984; Biggiogera *et al.* 1989). In the kinetochore, artifactual labeling of the outer plate could potentially arise from the nature of structure itself, where a dense outer plate is separated from the highly concentrated centromeric chromatin by a less dense space only  $0.025 \mu\text{m}$  in width (Rieder, 1982), across which the peroxidase reaction product might diffuse. This might also explain our unpublished observations that antibodies recognizing the INCENP antigens, which are present in centromeres but clearly not in the kinetochore domain (Cooke *et al.* 1987), also occasionally gave labeling of the outer kinetochore plate when the immunoperoxidase method was used.

It thus appears that immunoelectron microscope mapping of the kinetochore domain requires the use of non-diffusible probes such as colloidal gold.

## Kinetochore proteins

If the rigorous criterion of mapping antigens by immunogold microscopy is applied, only one antigen, tubulin, has conclusively been shown to be located to the kinetochore. Tubulin is associated with the fibrous corona of kinetochores in cells that have been blocked with colcemid (Mitchison and Kirschner, 1985). Most other claims that various antigens are localized to the kinetochore are based solely on indirect immunofluorescence (Table 1). These structural assignments should be interpreted cautiously until the results of electron microscopy are available.

## Effects of microtubule depolymerization on centromere structure

Colcemid and other microtubule-blocking agents have been widely used to free the chromosomes from the cytoskeletal network *in vivo*. Furthermore, kinetochores are often seen best in cells that have been treated with these agents.

Colcemid has widely been assumed to have little effect on the protein composition of the chromosomes, but recent experiments indicate that this is not the case. Colcemid clearly alters the pattern of binding of several recently

identified antigens to the chromosomes. In addition, kinetochores appear slightly different after long exposure to colcemid: the structure is seen to consist of a single electron-dense outer plate with a prominent fibrous corona on its outer surface (Roos, 1973; Rieder, 1982).

This effect of colcemid treatment on the protein composition of the centromere is shown most clearly for the antigens identified in a recent monoclonal antibody analysis (Compton *et al.* 1991). Five of the six components that were located in the kinetochore domain of chromosomes from colcemid-blocked cells were found to bind partly or exclusively to non-chromosomal components in unblocked cells (Table 1). Furthermore, four of the six proved not to be associated with chromosomes at all during anaphase, which is the time at which the kinetochore is presumed to carry out one of its principal motor functions. A similar situation was revealed for cytoplasmic dynein, which appears to be present at the centromeres of prometaphase chromosomes and of chromosomes from both colcemid and vinblastine-blocked cells, but which is predominantly a spindle component during metaphase and anaphase (Pfarr *et al.* 1990; Steuer *et al.* 1990).

These results suggest that centromeres and/or kinetochores from cells lacking microtubules have a significantly different protein composition from their counterparts in normal cells, particularly during anaphase. This may be explained in the case of colcemid to result from the binding of tubulin to the fibrous corona. This tubulin might subsequently provide a binding site for other spindle components. However, such an explanation is ruled out with chromosomes from vinblastine-treated cells, where the kinetochores lack associated tubulin (Mitchison and Kirschner, 1985). In this case two explanations seem plausible. First, the protein composition of the centromere and/or kinetochore may be significantly different between prometaphase and later mitotic stages. If true, this might provide some basis for the differing directions of chromosome movement during prometaphase congression and anaphase. Second, the centromere and/or kinetochore may contain specific binding sites for spindle proteins other than tubulin that are revealed when the cellular microtubules are disassembled. Whatever the explanation, it is clear that analysis of cells blocked in mitosis is not sufficient for the determination of the intracellular distribution of novel centromeric components. Cells cycling normally through mitosis must also be examined.

## Conclusion

Recent experiments have revealed that the centromere and its constituent kinetochore are likely to have an important role both in mediating chromosome attachment to the spindle and in chromosomal movements at anaphase. It is thus essential that the distribution of newly identified centromere proteins be determined precisely in order to permit the development of specific models for how this region interacts with microtubules to cause chromosome movement. Light microscopy of cells arrested in mitosis cannot be used to distinguish kinetochore components from those that bind to the chromatin surrounding the kinetochore plates.

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### Note added in proof:

It has recently been shown by immunogold electron microscopy that dynein is located in the fibrous corona region of the kinetochore (Wordeman, L., Steuer, E. R., Sheetz, M. P. and Mitchison, T. (1991). Chemical subdomains within the kinetochore domain of isolated CHO mitotic chromosomes. *J. Cell Biol.* (in press)).