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Human centromeres and neocentromeres show identical distribution patterns of >20 functionally important kinetochore-associated proteins.

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Using combined immunofluorescence and fluorescence *in situ* hybridization (FISH) analysis we have extensively characterized the proteins associating with two different homologue human neocentromeres at interphase and prometaphase/metaphase, and compared these directly with those found with normal human centromeres. Antisera to CENP-A, CENP-B, CENP-C, CENP-E, CENP-F, INCENP, CLIP-170, dynein, dynactin subunits p150^{Glued} and Arp1, MCAK, Tsg24, p55CDC, HZW10, HBUB1, HBUBR1, BUB3, MAD2, ERK1, 3F3/2, topoisomerase II and a murine HP1 homologue, M31, were used in immunofluorescence experiments in conjunction with FISH employing specific DNA probes to clearly identify neocentromeric DNA. We found that except for the total absence of CENP-B binding, neocentromeres are indistinguishable from their alpha satellite-containing counterparts in terms of protein composition and distribution. This suggests that the DNA base of a potential centromeric locus is of minimal importance in determining the overall structure of a functional kinetochore and that, once seeded, the events leading to functional kinetochore formation occur independently of primary DNA sequence.

INTRODUCTION

Proteins that associate with normal mammalian centromeres can be broadly classified into two groups based on their spatial positioning throughout the cell cycle. The first class, constitutively associated with the centromere, comprises CENP-A, -B and -C, which are thought to have structural roles in kinetochore formation (1–3), and the recently identified CENP-G, which has an unknown function (4). The second class, known as passenger proteins, associate with the centromere transiently during the cell cycle, usually between late G2 and early prophase, before dissociating prior to the completion of mitosis (5). This group comprises many diverse proteins which can be classified further according to their apparent role in cell division. Some are involved in spindle capture and chromosome congression [CLIP170 (6), dynein (7,8), dynactin (9,10)], metaphase-to-anaphase transition [BUB1 (11,12), BUBR1 (12,13), BUB3 (14), MAD1 and -2 (15,16), ZW10 (17), ERK (18), 3F3/2 epitope (19), p55CDC (20), Tsg24 (21)], resolution and segregation of separating chromatids, or sister chromatid cohesion, whereas others have apparently multiple roles [INCENP (22,23), CENP-E (24), MCAK (25,26)], or as yet undefined roles in centromere function [CENP-F (27), M31 (28), M33 (29), PcG (30), HP1 (31), topoisomerase II (32,33), SUVAR39H1 (34)]. To date, CENP-A, CENP-C, CENP-E, CENP-F, MCAK, dynein, dynactin subunits, MAD2 and HZW10, have been found to associate only with active centromeres on dicentric chromosomes, suggesting a direct link with kinetochore function (35–38).

A novel class of non-repeat DNA-based centromeres known as neocentromeres has recently been described in humans and *Drosophila* (39,40). These structures form at apparently euchromatic DNA regions devoid of the repeats traditionally associated with centromere function (reviewed in ref. 39). A common feature of human neocentromeres is the absence of associated CENP-B binding, which is directly related to the absence of satellite repeats (39). The best characterized of these structures arose in a marker chromosome at a position corresponding to 10q25 following a complex rearrangement of a normal chromosome 10 (41,42). This neocentromere was shown to be derived from non-rearranged euchromatic DNA, with a core centromere protein-binding domain which is totally unremarkable in its structure and DNA content (43). To date, proteins that are considered markers of active centromere function, CENP-A, -C and -E, have been shown to localize to neocentromeres at metaphase (39,42,44,45), and we have recently shown localization of CENP-F and INCENP to chromosome 20- and chromosome 1-derived neocentromeres (44,45). The status of other centromere-associated proteins on neocentromeres, however, remains unknown. Given the fundamental difference in DNA sequence composition between normal centromeres and neocentromeres, it is important to determine which of the proteins associating with normal centromeres also associate with neocentromeres. Specifically, it would be of interest to know whether any differences exist in the protein-binding profile which may further our under-

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Table 1. Association status of different proteins with normal human centromeres and neocentromeres

Antisera	Antigen	Function	Reference	α-satellite centromere		10q25 neocentromere		20p12 neocentromere	
				Interphase	Metaphase	Interphase	Metaphase	Interphase	Metaphase
Structural proteins									
αCenpa	CENP-A[a]	S	76	DF	+	+	+[b]	ND	+[c]
2D-7	CENP-B	S?[d]	58	DF	+	–	–[b]	ND	–[c]
αCenpc	CENP-C[a]	S	77	DF	+	+	+[b]	ND	+[c]
Motor-related proteins									
HX1	CENP-E	MTC, CC	78	–	+	–	+	–	+[c]
70.1	Dynein	MTC, CC	Sigma	–	+	–	+	–	+
αp150	p150^{Glued}	MTC, CC	80	–	+	–	+[b]	–	+
A27	Arp1	MTC, CC	53	–	+	–	+	–	+
2D6, 4D3	CLIP-170	MTC	80	–	+	–	+[b]	–	+
αMCAK	MCAK	MTC, AP	25	–	+	–	+[b]	–	+
Other passengers									
D10	CENP-F[a]	U	27	–	+	–	+[b]	–	+[c]
RaDB26	INCENP	CS, CK	86	–	+	–	+[b]	–	+[c]
Checkpoint control mechanism									
αBUB1	HBUB1[e]	CPC	79	–	+	–	+	–	+
αBUBR1	HBUBR1[e]	CPC	79	–	+	–	+	–	+
αBUB3	BUB3[e]	CPC	This study	–	+	–	+[b]	–	+
αMAD2	MAD2[e]	CPC	81	–	+	–	+	–	+
αhZW10	hZW10[e]	CPC	17	–	+	–	+[b]	–	+
3F3/2	Unknown[e]	MA	83	–	+	–	+[b]	–	+
ppERK	Erk1[e]	MA	New England Biolabs	–	+	–	+	–	+
αp55CDC	p55cdc[e]	APC	85	–	+	–	+	–	+
αTsg24	Tsg24[e]	APC	84	–	+	–	+[b]	–	+
DNA/chromatin modifiers									
p170 topoII	TopoII	U (CR?)	TopoGEN	DF, BG	+[f]	NDI	+[f]	NDI	+
MAC353	M31	U (CM)	28	DF, BG	+/–[g]	NDI	+/–	NDI	ND

S, structural; MTC, microtubule capture; CC, chromosome congression; U, unknown; CS, chromosome segregation; CK, cytokinesis; AP, anaphase progression; CPC, checkpoint control; MA, metaphase-to-anaphase transition; APC, anaphase-promoting complex; CR, chromatid resolution; CM, chromatin modification; DF, distinct antibody foci visible; BG, high background; NDI, no data obtained due to the high level of interphase staining; ND, no data obtained.

[a]Localizes specifically to kinetochores.

[b]Identical colocalization was seen in the BE2Cl-18–5f somatic cell hybrid line.

[c]Voulliare *et al.* (44).

[d]A specific centromere role for CENP-B has yet to be established.

[e]Experiments carried out on nocadazole-arrested cells.

[f]Variable results obtained in different experiments.

[g]Distinct centromere signals were not always apparent.

standing of the processes of neocentromere formation and function. In this study we have directly compared the localization of >20 centromere-associated proteins with varying functions on normal alpha satellite-based centromeres and neocentromeres.

RESULTS

The 10q25 and 20p12 neocentromeres and strategy for antibody detection

We used two previously described mitotically stable neocentromeres in this study. The first is located on a marker chromosome derived following a complex rearrangement of a normal human chromosome 10, which involved the deletion of the normal centromere, followed by activation of a neocentromere at a position corresponding to 10q25 (41,42). We used two different cell types as sources of the neocentromere. The first cell type was a transformed lymphoblast cell line derived directly from the patient (42), and the second was a somatic cell hybrid line containing the mar del(10) chromosome in a Chinese hamster ovary (CHO-K1) background lacking the normal human chromosome 10 (42,46). The second neocentromere used in this study is located on a marker chromosome which resulted from a breakage of a normal chromosome 20 at 20p11.2 followed by an inverted duplication of the p arm sequences and activation of a neocentromere at one of the 20p12-derived regions within this chromosome (44). We used a transformed lymphoblast cell line derived directly from the patient as a source of this neocentromere (44). For combined fluorescence *in situ* hybridization (FISH)/immunofluorescence studies on the

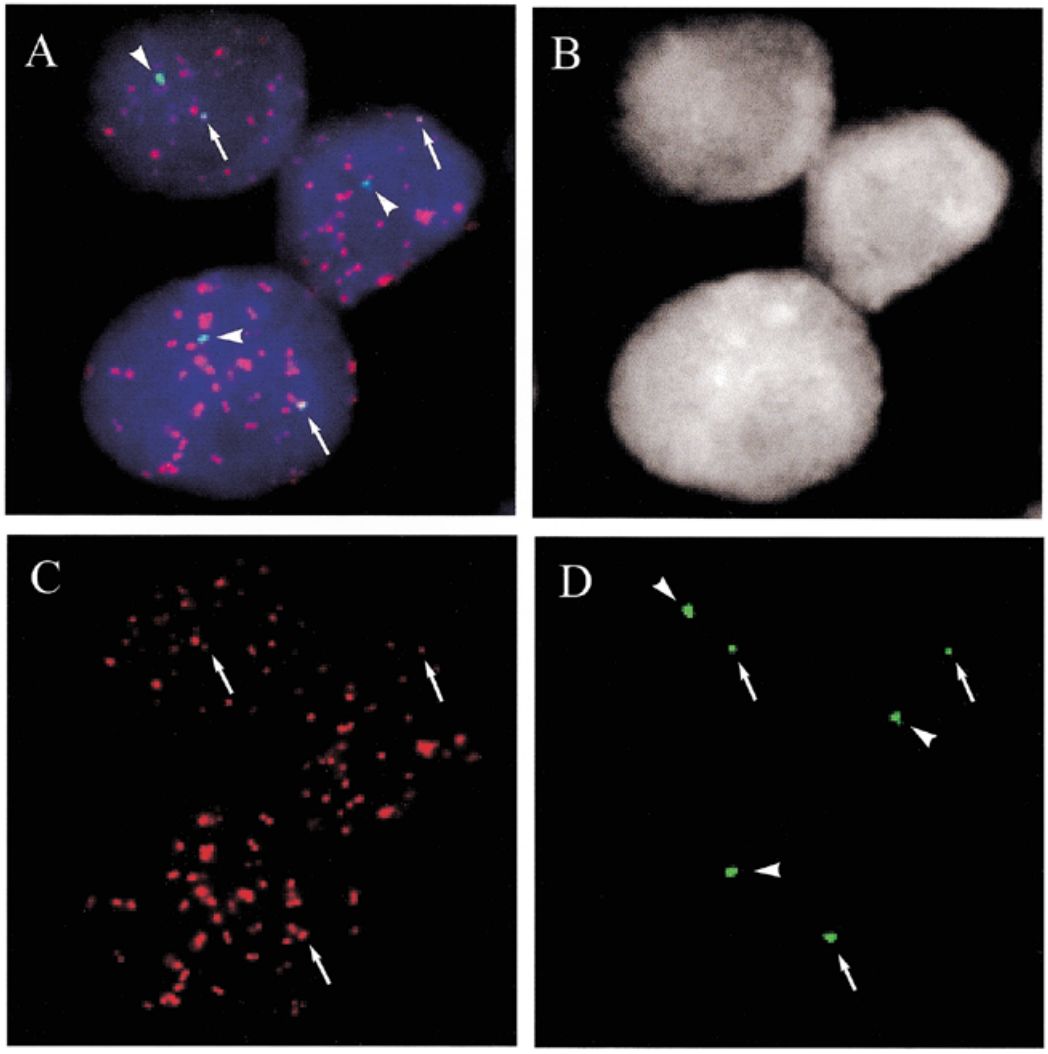

Figure 1. CENP-A remains associated with the 10q25 neocentromere throughout the cell cycle. Combined FISH/immunofluorescence on interphase lymphoblast nuclei using anti-CENP-A antisera (red) and a 10q25 neocentromere-derived BAC DNA probe (E8; green). (**A**) Combined image showing antisera, FISH and DAPI (blue) signals. Blue FISH signals corresponding to the normal 10q25 region are indicated by arrowheads. Overlapping red and green signals are indicated by small arrows. (**B**) Corresponding DAPI image. (**C**) CENP-A antisera signals only. (**D**) E8 FISH signals only. Signals resulting from hybridization of E8 with the 10q25 region of normal chromosome 10 are indicated by arrowheads.

10q25 neocentromere, we used an 80 kb bacterial artificial chromosome (BAC) DNA probe (designated E8), derived directly from the mar del(10) chromosome containing the NC DNA sequence previously described (43,46). For identification of the inv dup(20p) marker chromosome we used a 173 kb BAC (designated 859D4; Genbank accession no. AL035668) which localizes to 20p13 on the normal chromosome 20 and produces two distinct signals on the inv dup(20p) marker following FISH analysis. Using various anti-centromere protein antisera in combination with these BACs we were able to show direct colocalization of centromere-associated proteins with the 10q25- and 20p12-derived neocentromeres. The results are summarized in Table 1 and discussed below.

Constitutive structural proteins associate with the 10q25 neocentromere throughout the cell cycle

Centromere antibody signals could be divided into distinct classes based on the pattern observed in chromosome spreads and interphase nuclei. Antisera to the constitutive structural proteins CENP-A and -C produced clear centromere signals at metaphase in agreement with previous results (42,44). The signals produced on both normal and neocentromeres were characteristic double dot patterns at the site of primary centromeric constriction (data not shown). Both of these proteins demonstrated 10q25 neocentromere association in interphase nuclei, as evidenced by colocalization of antisera immunofluorescence signals and E8 neocentromere FISH signals (CENP-A) (Fig. 1). We were unable to investigate the association of these proteins with the 20p12 neocentromere at interphase due to a lack of a neocentromere-specific DNA probe for this chromosome. CENP-A and -C were the only two proteins in this study that could be detected clearly at the 10q25 neocentromere throughout the cell cycle. In agreement with previous results we were unable to detect CENP-B association with the 10q25 or 20p12 neocentromeres at any stage of the cell cycle (data not shown).

Neocentromere association of motor-related proteins and other passengers

The most commonly observed pattern of antibody staining involved the absence of distinct signals in interphase nuclei, which was indicative of an absence of antigen association with all centromeres, including the 10q25 and 20p12 neocentromeres. Distinct centromere and neocentromere signals were visible in chromosome spreads, however, as indicated by colocalization of antibody and BAC signals on the mar del(10) chromosome and specific antibody signals on the inv dup(20p) chromosome. This result was obtained for passenger proteins with varying functions, including the motor-associated proteins CENP-E, dynein, dynactin subunits $p150^{Glued}$ and Arp1 (data not shown), CLIP-170 (Fig. 2A and B), and MCAK (Fig. 2C and D). Similar results were also obtained for CENP-F and INCENP; however, some chromatid staining was apparent with INCENP antisera, consistent with previous results (44,47, data not shown). No antisera signals were observed for any of these proteins on the corresponding 10q25 region on the normal chromosome 10 or the 20p12 regions of the normal chromosome 20, or the other arm of inv dup(20), and where examined identical results were obtained in the CHO-based somatic cell hybrid line containing the mar del(10) chromosome (data not shown; Table 1).

Proteins controlling metaphase-to-anaphase transition localize to all centromeres, including the 10q25 and 20p12 neocentromeres

We were unable to detect distinct interphase centromeric signals for proteins associated with the mitotic checkpoint control mechanism and the anaphase-promoting complex (HBUB1, HBUBR1, BUB3, MAD2, HZW10, 3F3/2 epitope, ERK1, p55CDC and Tsg24). These proteins showed centromere association during the early stages of mitosis, however, in agreement with previous studies (reviewed in refs 48,49). Consistent centromeric staining patterns were apparent in chromosome spreads for each of these proteins, where association with the 10q25 and 20p12 neocentromeres was also observed (Fig. 3). We also examined the status of several of these proteins at the 10q25 neocentromere in a CHO somatic cell hybrid line, and demonstrated identical patterns of association for MAD2, BUB1, BUB3, Tsg24, ERK1 and 3F3/2 epitope (data not shown; Table 1). No signals could be detected using HBUBR1 or p55CDC in the somatic cell hybrid line containing the mar del(10) chromosome, apparently reflecting human specificity of these antisera. No proteins involved in metaphase-to-anaphase transition were detected on the corresponding 10q25 or 20p12 regions of the normal chromosomes 10 and 20.

DNA and chromatin modifiers accumulate at neocentromeres

Variable results were observed with polyclonal antisera to topoisomerase II on metaphase chromosome spreads. We routinely found variation of signal distribution between cells; however, the most frequently observed pattern was one of punctate staining of interphase nuclei with some discrete foci apparent (Fig. 4A). The diffuse nature of this staining did not permit the association status with neocentromeres to be determined. Generalized chromatid association on metaphase chromosomes was always present, with a distinct accumulation of signal at centromeres. A similar accumulation of topoisomerase II was seen on the 10q25 and 20p12 neocentromeres; however, no accumulation was observed at the corresponding 10q25 and 20p12 regions on the normal chromosomes (Fig. 4A).

Attempts at localization of chromatin-modifying proteins to metaphase chromosomes were made with varying degrees of success. The murine heterochromatic protein 1 (HP1) homolog, M31, produced the most variable results of any antisera tested in this study. Limited amounts of antisera confined our analysis to the 10q25 neocentromere. Interphase nuclei showed generalized background staining with several (five to twenty) large regions of specific staining, consistent with previous results (28). The nature of this signal did not allow the association status with the 10q25 neocentromere to be determined. Chromatid staining was also apparent on some chromosomes with specific centromeric signals visible in only a proportion of chromosome spreads (Fig. 4B). Where detected, antisera signals were seen at most centromeres, including the 10q25 neocentromere; however, the signals were more diffuse than those observed with antisera to the constitutive or passenger proteins described above. No accumulation of M31 antisera signals was observed on the corresponding 10q25 region on the normal chromosome 10.

DISCUSSION

This study represents an extensive characterization of centromere-associated antigens in individual human cell lines. We have confirmed the association of >20 individual mammalian centromere proteins with normal human kinetochores, and have compared the association pattern directly with two human neocentromeres.

All functionally important kinetochore-associated proteins bind identically to normal centromeres and neocentromeres

Given that neocentromeres appear functionally equivalent to their alpha satellite-based counterparts, it is not surprising that they share some common protein components. A fundamental difference between the two centromere types, however, is the DNA on which they are based. Whereas normal human centromeres contain large blocks of relatively uniform alpha satellite repeats, neocentromeric DNA appears to be totally devoid of such elements (42,43). Until now, it has been unclear what effect, if any, this has on the development and overall structure of a functional neocentromere kinetochore and its associated proteins.

Centromere-associated proteins can be broadly classified based on their presumptive function during kinetochore formation and cell division. Structural proteins CENP-A and -C, although apparently DNA binding (1,3,50,51), associate only with active centromeres (35,36), and have previously been shown to associate with the 10q25 and 20p12 neocentromeres at metaphase (42,44). We have confirmed that these proteins remain associated with the 10q25 neocentromere throughout the cell cycle in a similar way to normal alpha satellite-containing centromeres, suggesting maintenance of a common

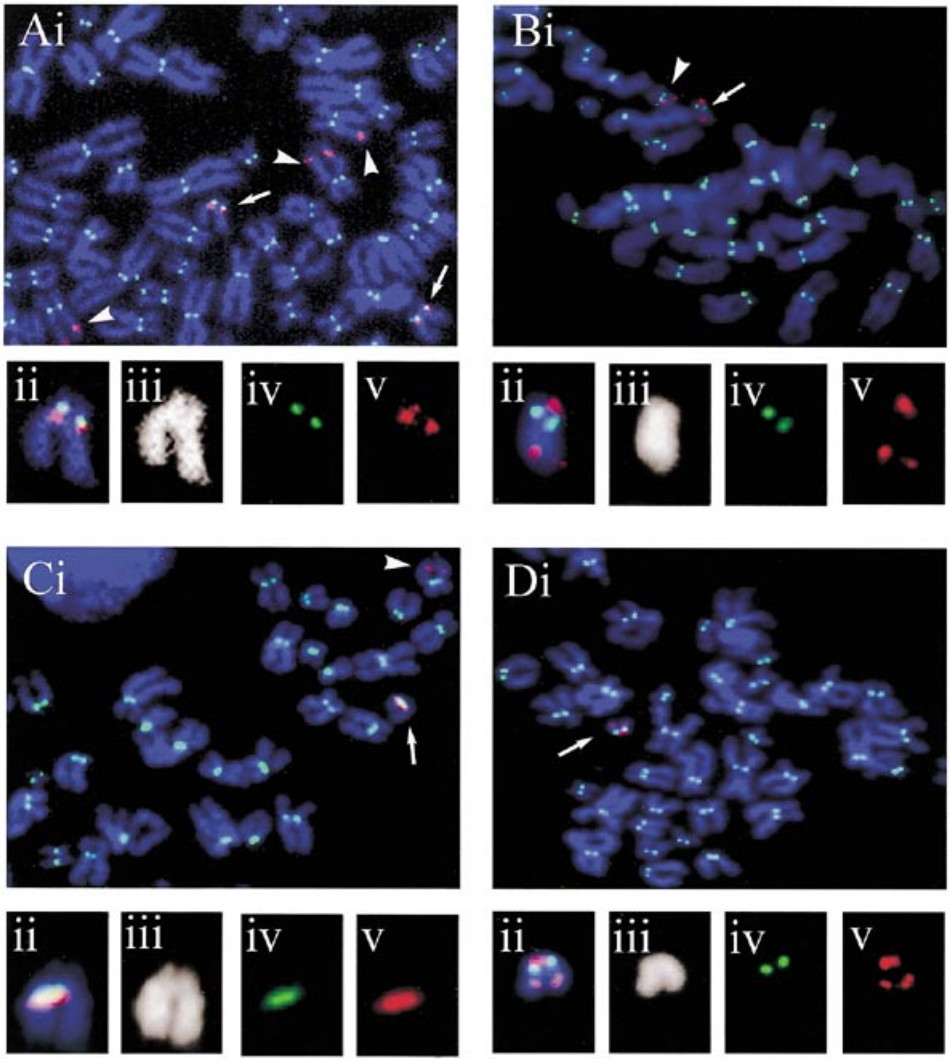

Figure 2. Examples of localization of motor-related proteins to neocentromeres. Combined FISH/immunofluorescence on human chromo- somes using antisera to motor-related proteins (green) and either a 10q25 neocentromere or 20p13 BAC DNA probe (red). (**A**) (i) CLIP-170 antisera/E8 FISH signals on a polyploid cell containing mar del(10). Three copies of normal chromosome 10 (arrowheads) show no overlap of E8 and anti-CLIP-170 signals, whereas two copies of the mar del(10) chromosome (small arrows) show overlap of signals. (ii) Combined E8/CLIP-170 signals; (iii) corresponding DAPI signal; (iv) CLIP-170 signal alone; and (v) E8 signal alone, on the mar del(10) chromosome. (**B**) Normal chromosome 20 (arrowhead) shows an anti-CLIP-170 signal (green) distinct from the more terminal 859D4 BAC signal at 20p13 (red). In contrast the inv dup(20p) (small arrow) shows two distinct 859D4 signals one of which is closely positioned to the 20p12 neocentromere. Note the absence of antibody signal on the duplicated 20p12 region on the same chromosome. (ii) Combined 859D4/CLIP-170 signals; (iii) corresponding DAPI signal; (iv) CLIP-170 signal alone; and (v) 859D4 signal alone, on the inv dup(20p) chromosome. (**C**) (i) Anti-MCAK antisera signals (green) are absent from interphase nuclei but present on all metaphase centromeres. Normal chromosome 10 (arrowhead) shows no overlap between E8 (red) and anti-MCAK (green) signals, whereas mar del(10) (small arrow), shows overlap of these signals at the neocentromere. (ii) Combined E8/MCAK signals; (iii) corresponding DAPI signal; (iv) MCAK signal alone; and (v) E8 signal alone, on the mar del(10) chromosome. (**D**) (i) Anti-MCAK results on a cell containing the inv dup(20p) marker chromosome. The inv dup(20p) (small arrow) shows two distinct 859D4 BAC signals (red) one of which is closely positioned to the anti-MCAK antibody signals (green). Note the absence of antibody signal on the duplicated 20p12 region on the same chromosome. (ii) Combined 859D4 BAC/MCAK signals; (iii) corresponding DAPI signal; (iv) MCAK signal alone; and (v) 859D4 BAC signal alone, on the inv dup(20p) chromosome.

protein–DNA structural base for normal centromeres and neocentromeres throughout the cell cycle, independent of primary DNA sequence.

The motor-associated proteins CENP-E, cytoplasmic dynein intermediate chain, components of the dynactin complex, CLIP-170, MCAK and CENP-F [which has recently been shown to interact with CENP-E (52)] associate only with active centromeres in multicentric chromosomes (37). Cytoplasmic dynein, dynactin and CLIP-170 have also recently been shown to colocalize at microtubule distal ends (53). We have shown that each of these proteins associate in an identical manner with neocentromeres and normal human centromeres at metaphase, whereas no centromeric foci, and hence no neocentromere association, was apparent in interphase nuclei. Recently, HZW10 has been shown to recruit dynein and dynactin to the centromere in *Drosophila* (54) and we have shown that this protein also associates with neocentromeres. Similar results were obtained for INCENP, which has recently been implicated to have a role in correct chromosome segregation and cytokinesis (22,23). These results would suggest that

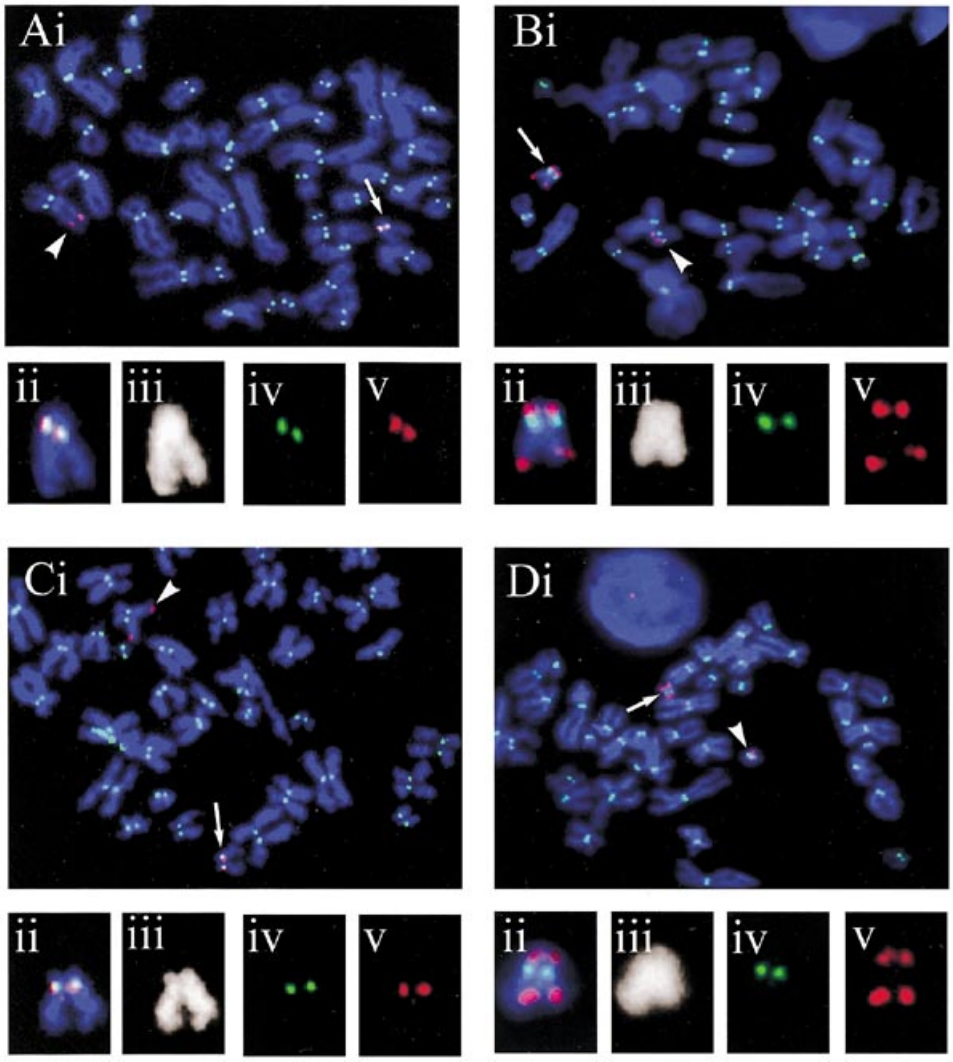

Figure 3. Examples of localization of proteins involved in metaphase-to-anaphase transition to the neocentromeres. Combined FISH/immunofluorescence on chromosomes using antisera to mitotic checkpoint-associated proteins (green) and neocentromere-derived BAC DNA probes (red). (**A**) (i) hZW10 antisera and E8 FISH signals show no overlap on the normal chromosome 10 (arrowhead) and no hZW10 signals are present on interphase nuclei (data not shown). Mar del(10) (small arrow) shows colocalization of anti-hZW10 (green) and E8 signals (red) at the neocentromere. (ii) Combined E8/hZW10 signals; (iii) corresponding DAPI signal; (iv) hZW10 signal alone; and (v) E8 signal alone, on the mar del(10) chromosome. (**B**) The normal chromsome 20 (arrowhead) shows a distinct anti-ZW10 signal (green) distinct from the more terminal 859D4 BAC signal at 20p13 (red). In contrast the inv dup(20p) (small arrow) shows two distinct 859D4 BAC signals one of which is closely positioned to the 20p12 neocentromere. Note the absence of antibody signal on the duplicated 20p12 region on the same chromosome. (ii) Combined 859D4 BAC/ZW10 signals; (iii) corresponding DAPI signal; (iv) ZW10 signal alone; and (v) 859D4 BAC signal alone, on the inv dup(20p) chromosome. (**C**) (i) Anti-MAD2 (green) and E8 (red) signals show no overlap on the normal chromosome 10 (arrowhead) whereas mar del(10) (small arrow) shows colocalization of anti-MAD2 (green) and E8 signals (red) at the 10q25 neocentromere. (ii) Combined E8/MAD2 signals; (iii) corresponding DAPI signal; (iv) MAD2 signal alone; and (v) E8 signal alone, on the mar del(10) chromosome. (**D**) The normal chromsome 20 (arrowhead) shows a distinct anti-MAD2 signal (green) and a single 859D4 BAC signal at 20p13 (red). In contrast the inv dup(20p) (small arrow) shows two distinct 859D4 BAC signals one of which is closely positioned to the 20p12 neocentromere. Note the absence of antibody signal on the duplicated 20p12 region on the same chromosome. (ii) Combined 859D4 BAC/MAD2 signals; (iii) corresponding DAPI signal; (iv) MAD signal alone; and (v) 859D4 BAC signal alone, on the inv dup(20p) chromosome.

the mechanisms underlying alignment and resolution of chromosomes containing a neocentromere are similar to those of their alpha satellite-based counterparts.

Proteins associated with the anaphase-promoting complex (Tsg24, p55CDC), the checkpoint protein complex (BUB1, BUBR1, BUB3, MAD2) and other proteins involved in controlling metaphase-to-anaphase transition (ERK1, 3F3/2 epitope, HZW10) associate with centromeres during the early stages of mitosis prior to commencement of anaphase, where they function to ensure correct alignment of all chromosomes prior to anaphase onset (reviewed in refs 48,49). The status of these proteins on active versus inactive centromeres of dicentric chromosomes is generally unknown; however, MAD2 and HZW10 appear to localize specifically to active centromeres on dicentric chromosomes (37). In agreement with previous reports, we found association of all of these proteins with normal centromeres in nocadazole-arrested cells. Signals for these proteins were generally observed on the outer regions of the centromeric constriction consistent with previous reports in various mammalian systems (48,49). The localization of

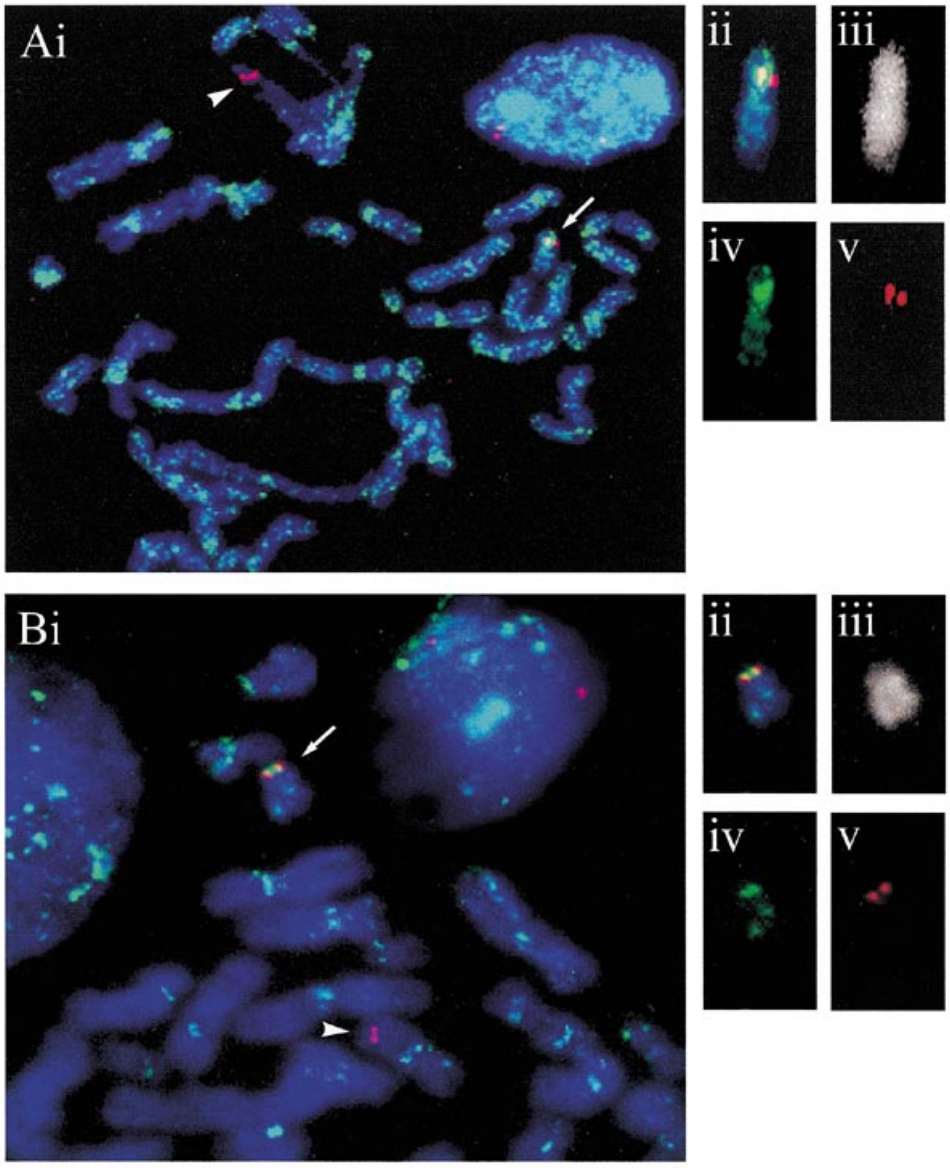

Figure 4. (**A**) Localization of topoisomerase II to the 10q25 neocentromere. (i) Interphase nuclei show a punctate staining pattern of anti-topoisomerase II signals (green) whereas metaphase chromosomes show some generalized chromatid staining with accumulation of signal at centromeres. Distorted chromosome 10q25 region of normal chromosome 10 (arrowhead) shows no overlap of anti-topoisomerase II and E8 signals (red) whereas mar del(10) shows an accumulation of topoisomerase II signal at the neocentromere as evidenced by an overlap of green and red signals. (ii) Combined E8/topoisomerase II signals; (iii) corresponding DAPI signal; (iv) topoisomerase II signal alone; and (v) E8 signal alone, on the mar del(10) chromosome. (**B**) Localization of M31 (murine HP1 homolog) to the 10q25 neocentromere. (i) Interphase nuclei show distinct regions of staining with anti-M31 (green). Metaphase chromosomes show variable signals with some chromatid staining and accumulation of signal at centromeres. Normal chromosome 10 (arrowhead) shows no overlap of anti-M31 and E8 signals (red) whereas mar del(10) shows an accumulation of M31 signal at the neocentromere as evidenced by an overlap of green and red signals (small arrow). (ii) Combined E8/M31 signals; (iii) corresponding DAPI signal; (iv) M31 signal alone; and (v) E8 signal alone, on the mar del(10) chromosome.

proteins involved in checkpoint control on the 10q25 and 20p12 neocentromeres followed an identical pattern to that seen on normal centromeres, confirming the involvement of neocentromeres in the anaphase-promoting complex and the mitotic checkpoint control mechanism.

DNA-modifying enzymes are an emerging element of centromere function of which relatively little is known. Previous studies have shown that topoisomerase II, a chromosomal matrix-associated enzyme, is required for the resolution of DNA interlockings occurring between sister chromatids during mitosis (32,55). This protein accumulates at centromeres at metaphase before relocating at the completion of cell division (33). We found a similar accumulation of topoisomerase II at the 10q25 and 20p12 neocentromeres, strongly suggesting a direct role of topoisomerase II in centromere function independent of primary DNA sequence. This is supported by other studies showing that inhibitors of topoisomerase II activity disrupt normal centromere function (33,55,56).

Another emerging group of mammalian centromere-associated proteins with as yet unknown importance in centromere function, is the chromatin modifiers [M31 (28), M33

(29), polycomb group (PcG) (30), HP1 (31), SUV39H1 (34)]. A generalized association of a murine heterochromatic protein 1 (M31) with all normal human and mouse centromeres has been reported previously (28) with the amount of M31 signal on human chromosomes varying considerably with varying levels of heterochromatin (28). In this study we demonstrated centromere association of M31 with human centromeres including the 10q25 neocentromere; however, this association was not apparent on all metaphase chromosomes analysed, suggesting either a transient centromere association or disruption of association by the detection method used. When generalized centromere association was observed, the corresponding 10q25 neocentromere association was also present. Mouse M31 and human HP1 show identical amino acid sequences confirming M31 as a murine HP1 homologue (28). Our localization of HP1 to neocentromeres suggests that the chromatin modification associated with centromere function can occur independently of the satellite repeats associated with normal human centromeres. M31 has recently been shown to associate with mouse and human SUVAR39H1 (34), suggesting that other chromatin modifiers may also localize to neocentromeres. Future studies should help to determine exactly how important this chromatin modification is for correct centromere function.

In summary, all of the proteins tested in this study have identical localization patterns on normal and neocentromere kinetochores. The exception is CENP-B, which has been previously reported to be absent on human neocentromeres (39). The lack of CENP-B association with centromeres does not affect centromere function as evidenced by its absence on mammalian Y chromosomes (57) and the absence of mitotic or meiotic defects in CENP-B null mice (58–60). To date, this is the only centromere-associated protein identified that associates both with inactive and active centromeres on dicentric chromosomes (35,61), suggesting a possible role as a specific satellite-associated protein rather than a functional centromere protein.

Implications for functional centromere assembly

In this study we have shown that aspects of chromatin and DNA modification, kinetochore structure, microtubule function and checkpoint control (all involved in active centromere function) occur independently of primary DNA composition. The sequence-independent association pattern of centromere proteins with neocentromeres was demonstrated both in human and CHO cells, suggesting a common mode of centromere assembly in mammalian systems. As a number of the proteins analysed have been specifically localized to defined regions within the kinetochore, their presence indicates that neocentromeres assemble a trilaminar kinetochore in an identical manner to that of normal centromeres. The confirmation that kinetochore function is largely, if not totally, independent of DNA sequence raises several interesting points concerning kinetochore structure and function, and raises the question as to what role the satellite repeats associated with mammalian centromeres perform.

Neocentromere activation can be thought of as a two-step process. Firstly, a rearrangement resulting in a tolerable karyotypic abnormality occurs which separates a potential neocentromere site from the presumptive inhibitory effects of an endogenous centromere and, secondly, an epigenetic modification of the resulting marker chromosome occurs, resulting in the seeding of a fully functional kinetochore. In the case of normal centromeres, this signal is reversible, as evidenced by centromere inactivation in dicentric chromosomes. However, the mechanisms for activation of neocentromeres and inactivation of normal centromeres remain a mystery, so it is unclear whether they involve elements of the same process. Likewise, the exact nature of the presumptive lateral inhibitory signal that prevents multiple sites of centromere activation on a single chromosome has yet to be determined.

Although many neocentromeres are similar to the 10q25 and 20p12 neocentromeres described here in that they are apparently 100% mitotically stable (44,62,63), and some are meiotically competent (64), others are less stable, being represented in only a proportion of cells within a population (45,62,65,66). The reason for this reduced stability is unknown and could reflect either a problem with unbalanced chromosome complement or a problem with kinetochore function during mitosis. It will be interesting to examine the centromere-associated protein localization properties of these neocentromeres in the future.

The confirmation that neocentromere kinetochore structure is essentially identical to normal human centromeres suggests a common primary epigenetic marker for centromere seeding and a common cascade of protein interactions which ultimately result in a fully functional kinetochore regardless of primary DNA sequence. A process of kinetochore formation such as this explains the lack of conservation of centromeric DNA throughout evolution (67), but does not explain the apparent preference for centromere seeding at sites containing large blocks of tandemly repeated DNA. The exact nature of the primary epigenetic marker responsible for kinetochore seeding is unknown; however, various mechanisms such as protein modification through histone acetylation (68,69), DNA modifications by methylation (70) or centromeric chromatin modification through the incorporation of CENP-A or -C (71,72) have been implicated. Whatever the nature of this primary event, it clearly can seed the formation of functional centromeres at multiple genomic locations of varying DNA sequence content, with alpha satellite repeat DNA the preferred sites on human chromosomes. The introduction of large arrays of alpha satellite repeats into human cells is in fact sufficient to seed the *de novo* formation of functional human centromeres (73–75). We speculate that, rather than being involved directly in centromere function, alpha satellite repeats (and possibly other repeat sequences) present the cell with the 'most favourable' environment for centromere seeding and kinetochore formation. In the absence of an inhibition from alpha satellite-based centromeres, other genomic loci with the potential for centromere formation may become activated, forming neocentromeres. Obviously, a prerequisite for the propagation of such centromeres is the maintenance of a viable chromosome complement, which in many instances would select against the propagation of neocentromeres. Future neocentromere studies should allow the processes leading to centromere activation and function to be analyzed without the potential complications associated with satellite-based normal centromeres.

MATERIALS AND METHODS

Cell culture

Transformed lymphoblasts were cultured in RPMI-1640 medium (Trace Biosciences, Castle Hill, Australia) and 20% fetal calf serum (FCS), whereas the somatic cell hybrid line BE2Cl-18-5f was cultured in KAO-modified HAMs F12 medium (Trace Biosciences) and 12% FCS as previously described (42). Medium was supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco BRL, Life Technologies, Rockville, MD) and both lines were maintained at subconfluence. Microtubule depolymerizing agents colcemid (Gibco BRL) or nocadazole (Sigma, St Louis, MO) were added to medium at concentrations of 10 μM and 0.1 μg/ml, respectively, for 1–2 h prior to cell harvesting.

FISH/immunofluorescence

Combined FISH/immunofluorescence was carried out using a modified procedure described previously (69). Basically, cells were harvested, washed once with phosphate-buffered saline and resuspended at a concentration of 2×10^5 cells/ml in 0.075 M KCl for 15 min at room temperature. Cells (200 μl) were then cytospun onto slides (Shandon Cytospin 3; Life Sciences International, Runcorn, UK) at 1000 r.p.m. (112.9 *g*) for 5 min. Slides were then incubated in prewarmed KCM [120 mM KCl, 20 mM NaCl, 10 mM Tris–HCl, 0.5 mM NaEDTA, 0.1% (v/v) Triton X-100] for 15 min and blotted dry. Primary antibody (50 μl) was then added and the slides incubated at 37°C for 1 h followed by three 5 min washes in KB^- (10 mM Tris–HCl, 150 mM NaCl, 1% bovine serum albumin). The second antibody was then added and the slides were incubated for a further hour at 37°C. A further two KB^- washes were then carried out before fixation in 10% formalin in KCM for 15 min. Three washes in dH_2O were carried out before further fixation in methanol:acetic acid (3:1) for 15 min.

For FISH, slides were dehydrated by sequential emersion in 75, 95 and 100% ethanol (5 min each) followed by complete drying at room temperature. Slides were then immersed in 70% formamide in SSC (83°C) for 4 min followed by ice-cold 75% (2 min), 95% (2 min) and 100% (2 min) ethanol. Slides were then dried completely again at room temperature. Probe/hybridization solution (50% formamide, 2× SSC, 10% dextran sulphate), containing nick-translated DNA labelled with digoxigenin-11-dUTP (DIG; Boehringer Mannheim, Castle Hill, Australia), was denatured at 95°C for 5 min and then 15 μl was added per slide followed by incubation overnight at 37°C. Three post-hybridization washes were carried out in each of 2× SSC at room temperature for 5 min and 60°C for 5 min. Slides were then placed in 2× SSC for 2 min at room temperature. TNB (10% blocking agent, 150 mM NaCl, 100 mM Tris pH 7.5) (80 μl) was added and slides were incubated at 37°C for 30 min. Mouse anti-DIG (50 μl) was added followed by further incubation at 37°C (30 min), three washes in 4× SSC/0.5% Tween, and another incubation (37°C, 30 min) with 50 μl of either anti-mouse FITC or anti-mouse Texas Red. Three final washes in 4× SSC/0.5% Tween were then carried out. Slides were mounted in 30 μl antifade (Oncor, Gaithersburg, MD) containing DAPI and viewed under a microscope.

Immunological reagents

Antisera used in this study are listed in Table 1. Polyclonal anti-CENP-A (76), monoclonal anti-CENP-B (58), polyclonal anti-CENP-C (77) and polyclonal anti-MCAK (25) antibodies have been previously described. Polyclonal anti-CENP-E (78), anti-CENP-F (27), anti-hBUB1 and anti-hBUBR1 (79) were provided by T.J. Yen (Fox Chase Cancer Center, Philadelphia, PA). Anti-dynein (Sigma) and anti-Arp1 (53) were provided by D. Meyer and I.B. Clark (Department of Biological Chemistry, UCLA, CA). Polyclonal anti-p150^{Glued} (80) was provided by K. Vaughan (University of Notre Dame, Notre Dame, IN) and R. Vallee (University of Massachusetts Medical School, Boston, MA). Polyclonal anti-hZW10 (17) was provided by B. Williams and M.L. Goldberg (Cornell University, Cornell, NY), polyclonal anti-MAD2 (81) was provided by E.D. Salmon and B. Howell (University of North Carolina, Chapel Hill, NC), polyclonal anti-ERK (New England Biolabs, Beverly, MA) was provided by Dr N.G. Ahn (University of Colorado, Boulder, CO), monoclonal anti CLIP-170 (82) was provided by Dr F. Perez (University of Geneva, Geneva, Switzerland), monoclonal 3F3/2 (83) was provided by Dr G.J. Gorbsky (University of Virginia, Charlottesville, VA), polyclonal anti-Tsg24 (84) was provided by Dr C. Hoog (Karolinska Institute, Karolinska, Sweden). Polyclonal anti-topoisomerase II was purchased from TopoGEN (Columbus, OH). Polyclonal P55CDC (85) was provided by J. Weinstein (Amgen, Thousand Oaks, CA). Monoclonal anti-M31 (28) was provided by P. Singh (Roslin Institute, Edinburgh, UK). Polyclonal anti-INCENP (86) was provided by W.C. Earnshaw (University of Edinburgh, Edinburgh, UK). Rabbit polyclonal anti-BUB3 was raised against a bacterially expressed mouse Bub3 peptide (residues 166–320) using standard techniques.

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