Specification of kinetochore-forming chromatin by the histone H3 variant CENP-A

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SUMMARY

The mechanisms that specify precisely where mammalian kinetochores form within arrays of centromeric heterochromatin remain largely unknown. Localization of CENP-A exclusively beneath kinetochore plates suggests that this distinctive histone might direct kinetochore formation by altering the structure of heterochromatin within a sub-region of the centromere. To test this hypothesis, we experimentally mistargeted CENP-A to non-centromeric regions of chromatin and determined whether other centromere-kinetochore components were recruited. CENP-A-containing non-centromeric chromatin assembles a subset of centromere-kinetochore components, including CENP-C, hSMC1, and HZwint-1 by a mechanism that requires the unique CENP-A N-terminal tail. The sequence-specific DNA-binding protein CENP-B and the microtubule-associated proteins CENP-E and HZW10 were not recruited, and neocentromeric activity was not detected. Experimental mistargeting of CENP-A to inactive centromeres or to acentric double-minute chromosomes was also not sufficient to assemble complete kinetochore activity. The recruitment of centromere-kinetochore proteins to chromatin appears to be a unique function of CENP-A, as the mistargeting of other components was not sufficient for assembly of the same complex. Our results indicate at least two distinct steps in kinetochore assembly: (1) precise targeting of CENP-A, which is sufficient to assemble components of a centromere-prekinetochore scaffold; and (2) targeting of kinetochore microtubule-associated proteins by an additional mechanism present only at active centromeres.

Key words: Centromere, Heterochromatin, Histones, Kinetochore, Mitosis

INTRODUCTION

Kinetochores are proteinaceous structures that form transiently on centromeric heterochromatin to direct chromosome movement along spindle microtubules and regulate progression through mitosis (Gorbsky, 1997; Nicklas, 1997; Craig et al., 1999; Choo, 2000). The mammalian kinetochore appears structurally as an outer electron-dense plate, which is associated with a fibrous corona in the absence of microtubule attachment, a narrow clear zone, and an inner electron-dense plate associated with the underlying centromeric chromatin (Brinkley and Stubblefield, 1966). Centromeric heterochromatin in human cells is composed primarily of repetitive, non-transcribing alpha-satellite DNA arrays, ranging in size from 0.5-5 Mb (Tyler-Smith and Willard, 1993). Interestingly, kinetochores form on only a small fraction of the heterochromatin present at centromeres (Zinkowski et al., 1991). It is unclear what differentiates kinetochore-forming heterochromatin from the large blocks that compose the pairing domain of centromeres between sister chromatids and extend in some cases into chromosome arms. It is also unclear how kinetochore plates are attached to the underlying DNA. Sequence-specific DNA-protein interactions do not appear to be essential for kinetochore formation in eukaryotic organisms, with the exception of *S. cerevisiae* (Karpen and Allshire, 1997; Clarke, 1998; Csink and Henikoff, 1998; Wiens and Sorger, 1998; Choo, 2000).

The centromere proteins CENP-A and -C are each localized exclusively within the chromatin immediately beneath kinetochore plates (Saitoh et al., 1992; Warburton et al., 1997), and both are essential for growth and development in mammals (Fukagawa and Brown, 1997; Kalitsis et al., 1998; Howman et al., 2000). Microinjection experiments have suggested that these two proteins function specifically in the assembly of kinetochores. Microinjection of anti-CENP-A or -C antibodies into interphase cells blocked entry into or progression through mitosis, respectively, whereas microinjection of these antibodies after prophase did not inhibit the completion of mitosis (Tomkiel et al., 1994; Figueroa et al., 1998). The presence of CENP-A and -C exclusively at the active

centromere of dicentric chromosomes and at human neocentromeres tightly correlates the presence of these proteins with kinetochore activity (Earnshaw et al., 1989; Warburton et al., 1997; Tyler-Smith et al., 1999; Saffery et al., 2000).

Based in part on similarities with the homologous yeast centromere proteins, Cse4p and Mif2p, CENP-A and -C have been proposed to form a nucleoprotein complex that stabilizes centromeric chromatin fibers and binds them to kinetochores (Meluh and Koshland, 1995; Meluh and Koshland, 1997; Stoler et al., 1995; Meluh et al., 1998; Sullivan, 1998). Genetic and biochemical studies suggest that CENP-A replaces histone H3 in centromere-specific nucleosomes (Palmer et al., 1991; Stoler et al., 1995; Smith et al., 1996; Meluh et al., 1998; Glowczewski et al., 2000; Yoda et al., 2000). CENP-C possesses a non-sequence-specific DNA binding activity and might act as a centromere-specific linker histone that stabilizes the linkage between centromeric DNA and nucleosomes containing CENP-A (Lanini and McKeon, 1995; Yang et al., 1996; Sullivan, 1998). The discovery of centromere-specific histones also in worms, flies and fission yeast suggest that the specialization of nucleosome structure is a common mechanism for kinetochore formation in divergent organisms (Buchwitz et al., 1999; Henikoff et al., 2000; Takahashi et al., 2000).

To determine if CENP-A is sufficient for kinetochore formation, we experimentally induced it to be mistargeted to non-centromeric regions of chromatin in order to determine if its misplacement subsequently caused the redistribution of various associated centromere-kinetochore components. Exogenous CENP-A is faithfully targeted to centromeres when expressed at near physiologic levels (Sullivan et al., 1994; Shelby et al., 1997). However, higher levels of CENP-A expression result in its faulty incorporation into widely dispersed non-centromeric sites throughout interphase nuclei and along the arms of mitotic chromosomes. We have found that a specific subset of constitutive and facultative centromere-kinetochore components becomes mistargeted to non-centromeric sites along with CENP-A. Our results suggest that CENP-A binding to chromatin modifies nucleosomes to favor the deposition of other centromere-kinetochore proteins, thereby orchestrating the initial stages of centromere-kinetochore assembly.

MATERIALS AND METHODS

Cell culture

CHO-AA8 Tet-Off and HeLa Tet-On cells (Clontech Laboratories Inc., Palo Alto, CA) were cultured in Opti-MEM I (Gibco BRL, Gaithersberg, MD), supplemented with 4% Tet system approved fetal bovine serum (Clontech), 0.1 mg/ml G418 (Sigma Chemical Co., St Louis, MO), and 1% penicillin/streptomycin (Sigma). tTA-HeLa-pUHD10.3-CAepi cells (Shelby et al., 1997) were cultured as above with the addition of 0.4 mg/ml G418 and 330 ng/ml puromycin. Cultures were maintained in a humidified 37°C incubator with 5% CO_2. Tetracycline hydrochloride (Sigma) was added to 2 μg/ml to regulate pUHD10.3-CAepi expression (Gossen and Bujard, 1992). COLO 320DM cells (ATCC# CCL-220) were cultured in RPMI 1640 medium containing 10% FBS (Gibco BRL).

Cell cultures were transfected with DNA using lipofectamine PLUS reagent (Gibco). Cells ~70% confluent in 10 cm dishes were transiently transfected with 2 μg of vector DNA in complex with 8 μl of PLUS reagent and 12 μl of lipofectamine reagent in antibiotic-free medium. Transfected cells were split the next day and harvested two days later, except where noted. A stable, inducible CHO cell line expressing an epitope-tagged version of CENP-A was established by co-transfecting CHO-AA8 Tet-Off cells with 40 μg of pUHD10.3-CAepi (Shelby et al., 1997) and 2 μg of pTK-Hyg (Clontech). Stable transformants were selected by growth in 0.4 mg/ml hygromycin over 4 weeks, then maintained with 0.1 mg/ml. Colonies arising from single cells were grown in 24-well trays and assayed for inducible CENP-A-(HA) expression by western blot.

Mitotic cells with unreplicated genomes (MUGs) were generated as originally described (Brinkley et al., 1988). CHO cells were arrested overnight in medium containing 2 mM hydroxyurea (HU; Sigma). Fresh medium containing 2 mM HU and 5 mM caffeine was then added with 250 ng/ml Colcemid (Gibco) to arrest MUGs as they entered mitosis.

For chromosome preparations, cultures were arrested in mitosis by incubation with 250 ng/ml Colcemid (Gibco) for 3-5 hours. Mitotic cells were selectively harvested by shake-off and swollen for 10 minutes at 37°C in 75 mM KCl containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were then cytospun onto glass coverslips using a Cytospin 3 (Shandon Instruments, Inc., Pittsburgh, PA) at 2000 rpm for 2 minutes. and processed for immunofluorescence as described below.

Expression constructs

The full-length CENP-A expression construct used in this study has been described previously (Shelby et al., 1997). This construct, pUHD10.3-CAepi, contains three copies of the hemagglutinin 1 (HA-1) epitope (Niman et al., 1983) at the C-terminus of the human CENP-A cDNA and is based on the tetracycline-repressible construct described previously (Gossen and Bujard, 1992). A deletion mutant of this construct, which we refer to as CENP-A-core, was generated by amplifying the entire pUHD10.3-CAepi plasmid, less the nucleotides encoding the N-terminal amino acids 2-45 of CENP-A. The following oligonucleotide primers were used for PCR, in combination with pUHD10.3-CAepi as the template: 5′-atcgaaccatg-ggttggctaaaggagatccgaaagc; and 3′-atcgaaccatggcacgccgcagag. The product was digested with the restriction enzyme *Nco*I, the sites for which were introduced in the primer sequences, and then ligated to form a circular DNA. Sequencing of the construct confirmed the desired product, and expression was assessed by western analysis and immunofluorescence microscopy following transfection into HeLa Tet-On cells. The amino-acid sequence of the HA-tagged CENP-A protein is as follows, with the excised residues underlined and the epitope-tag in italics: MGPRRRSRKPEAPRRRSPSPTPTPGPSRR-GPSLGASSHQHSRRRQGWLKEIRKLQKSTHLLIRKLPFSRLAR EICVKFTRGVDFNWQAQALLALQEAAEAFLVHLFEDAYLLTL HAGRVTLFPKDVQLARRIRGLEEGLG*GRIFYPYDVPDYAGYPYD VPDYAGSYPYDVPDYAAQCGR*.

To accomplish tetracycline-regulated ectopic expression of CENP-C in HeLa Tet-On and CHO Tet-Off cells, a human CENP-C cDNA (Saitoh et al., 1992) was sub-cloned from pBluescript (Stratagene, La Jolla, CA) into pTRE (Clontech) using flanking *Bam*HI and *Hin*dIII sites. The desired product was confirmed by DNA sequence analysis and immunofluorescence microscopy.

Immunofluorescence microscopy

As previously described in detail (Van Hooser and Brinkley, 1999), cells on coverslips were first rinsed with PBS, and then soluble proteins were extracted with 0.5% Triton X-100 (TX-100) in PEM (80 mM K-Pipes, pH 6.8, 5 mM EGTA, pH 7.0, 2 mM $MgCl_2$) for 2 minutes at 4°C prior to fixing with 4% formaldehyde (Polysciences Inc., Warrington, PA) in PEM for 20 minutes at 4°C. Nonspecific binding of antibodies was blocked overnight at 4°C with 5% milk in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20). Primary antisera were diluted in TBST and incubated on coverslips for 1 hour at 37°C. Human auto-antiserum SH-CREST (Valdivia and Brinkley, 1985) was diluted 1:1000 for immunofluorescence. Monoclonal antibody HA.11 (BAbCO, Richmond,

CA) was diluted to 2 μg/ml, rabbit anti-hSMC1 1:100 (Schmiesing et al., 1998), rabbit anti-HZW10 1:250 (Starr et al., 1997), rat anti-HZwint-1 1:250 (Starr et al., 2000), rabbit and murine monoclonal anti-CENP-B 1:250 (Cooke et al., 1990), rabbit anti-CENP-C 1:250 (Saitoh et al., 1992), murine monoclonal anti-CENP-E IgG 1:500 (Yen et al., 1991), rabbit anti-CENP-F (BAbCO) to 2 μg/ml, and rabbit anti-phosphorylated (Ser10) H3 (Upstate Biotechnology, Lake Placid, NY) to 0.2 μg/ml (Hendzel et al., 1997). Goat anti-human, anti-rabbit, anti-rat, and anti-mouse IgG (H+L) secondary antibodies (Jackson Immunoresearch Laboratories Inc., West Grove, PA), conjugated to FITC or TXRD, were diluted in TBST and incubated with samples for 45 minutes at 37°C. DNA was counterstained with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in TBST. Coverslips were mounted on glass slides using Vectashield anti-fade medium (Vector Laboratories Inc., Burlingame, CA). All figures are composite images obtained with a Deltavision, deconvolution-based optical workstation (Applied Precision, Issaquah, WA). Z-series stacks of multiple focal planes were used to render 3D volumes.

Nuclear matrix preparation

Chromatin was eluted from cells fixed in formaldehyde, as described (Nickerson et al., 1997). Cells growing on coverslips were washed in cold PBS, and soluble proteins were extracted for 5 minutes at 4°C in CSK (10 mM K-Pipes, pH 6.8, 0.3 M sucrose, 0.1 M NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 20 mM vanadyl riboside complex (VRC), 1 mM PMSF; 1 μg/ml each of aprotinin, leupeptin, antipain, and pepstatin) containing 0.5% TX-100. Preparations were fixed with 4% formaldehyde in CSK for 20 minutes at 4°C, rinsed in CSK twice, and once in DIG (10 mM K-Pipes, pH 6.8, 0.3 M sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA). Chromatin was removed by digestion with 400 units/ml RNase-free DNase I (Roche Molecular Biochemicals, Indianapolis, IN) in DIG for 50 minutes at 32°C. The preparations were washed briefly with 0.25 M ammonium sulfate in DIG, then in DIG and CSK alone prior to immunostaining.

Immunoblotting

Cells were harvested from culture flasks using 0.03% EDTA in Puck's saline and pelleted at 1000 ***g*** for 6 minutes. Cell pellets were resuspended in 1× SDS-PAGE sample buffer for whole-cell preparations (Laemmli, 1970). For nuclear extracts, cell pellets were resuspended in PEM containing 0.5% TX-100 and multiple protease inhibitors, and then incubated on ice for 20 minutes. The extracted cells were pelleted for 10 minutes at 1000 ***g*** at 4°C and resuspended in 1× SDS-PAGE sample buffer. Protein samples were heated at 95°C for 5 minutes and resolved by electrophoresis on 4-20% gradient gels (BioRad Laboratories, Hercules, CA) for 2.5 hours at 120 V. Proteins were transferred to polyvinylidene fluoride microporous membranes (Millipore Corporation, Bedford, MA) overnight at 50 mA in an electroblotting apparatus (BioRad). Filters were blocked with 5% milk in TBST for 90 minutes at room temperature (RT) and rinsed briefly in TBST. Primary antibodies were diluted in TBST and incubated with filters for 1 hour at 37°C. SH-CREST auto-antiserum was diluted 1:2000 for immunoblotting. Monoclonal antibody HA.11 (BAbCO) was diluted to 1 μg/ml, rabbit anti-hSMC1 1:2000 (Schmiesing et al., 1998), and rabbit anti-CENP-C 1:1000 (Saitoh et al., 1992). Filters were washed in TBST and incubated with alkaline-phosphatase-coupled goat anti-IgG (H+L) secondary antibodies (Pierce, Rockford, IL) diluted 1:2500 in TBST for 1 hour at RT. The filters were rinsed in TBST and AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5), then reacted with BCIP/NBT (Sigma) for 5-60 minutes at RT.

RESULTS

Mistargeting of CENP-A to non-centromeric chromatin

CENP-A is normally localized exclusively at the centromeres of chromosomes (Sullivan et al., 1994). Fig. 1A shows the centromere-specific targeting of epitope-tagged human CENP-A protein, expressed from a cDNA-based construct integrated in a stable HeLa cell line (Shelby et al., 1997). Centromeres are marked by staining with human autoimmune serum from a patient with CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) variant scleroderma (Moroi et al., 1980). This CREST antiserum, designated SH, recognizes CENPs-A, -B, and -C (Valdivia and Brinkley, 1985; Earnshaw and Rothfield, 1985). CENP-A staining is restricted to the kinetochore region of centromeres, whereas CREST additionally stains the pairing domain between sister chromatids due to the dispersal of CENP-B throughout most alpha-satellite DNA (Masumoto et al., 1989; Cooke et al., 1990).

Higher levels of CENP-A expression, achieved by re-transfecting the stable HeLa cell line with the same cDNA expression construct encoding CENP-A, resulted in the mistargeting of CENP-A to non-centromeric regions of the chromosomes (Fig. 1B). Following ~24 hours of expression, CENP-A staining was observed at high levels along the lengths of chromosome arms (Fig. 1B). Interestingly, the exogenous CENP-A was not targeted to the pericentric heterochromatin of certain chromosomes (Fig. 1B, arrows). These pericentric regions did, however, contain phosphorylated histone H3 (Ser10), as detected by an epitope-specific antibody (Hendzel et al., 1997), indicating that they were not refractory to immunofluorescence and had incorporated H3 (Fig. 1B). A similar staining pattern has also been observed for ectopically expressed histones H2B and H3 and is thought to be caused by the depletion of these histones as cells progress through S-phase, resulting in very low levels available for incorporation into late-replicating heterochromatin (Henikoff et al., 2000). In agreement with this conclusion, ectopic CENP-A was found to target the entire lengths of chromosomes, including pericentric heterochromatin, when expressed at high levels in transiently transfected cells for periods longer than 24 hours (Fig. 2; Figs 3-12). CENP-A, therefore, displayed properties similar to other histones when ectopically expressed in cultured cells.

To directly test if CENP-A alone is sufficient for kinetochore assembly, we compared the distributions of several other centromere-kinetochore components in the presence and absence of CENP-A overexpression and mistargeting (Table 1).

CENP-A does not recruit CENP-B to non-centromeric chromatin

CENP-B is a constitutive centromere protein (present throughout the cell cycle) that binds a specific 17-bp DNA sequence motif present within the centromeres of most human chromosomes (Masumoto et al., 1989). Given that CENP-B is a sequence-specific DNA binding protein, it seemed unlikely that it would be recruited to non-centromeric sites with mistargeted CENP-A. Indeed, specific antibodies to CENP-B stained exclusively the centromeres of chromosomes in cells overexpressing CENP-A (Fig. 2).

CENP-A recruits CENP-C to non-centromeric chromatin

CENP-C is a constitutive centromere protein that is essential for the faithful transmission of chromosomes to daughter cells during mitosis (Tomkiel et al., 1994; Fukagawa and Brown,

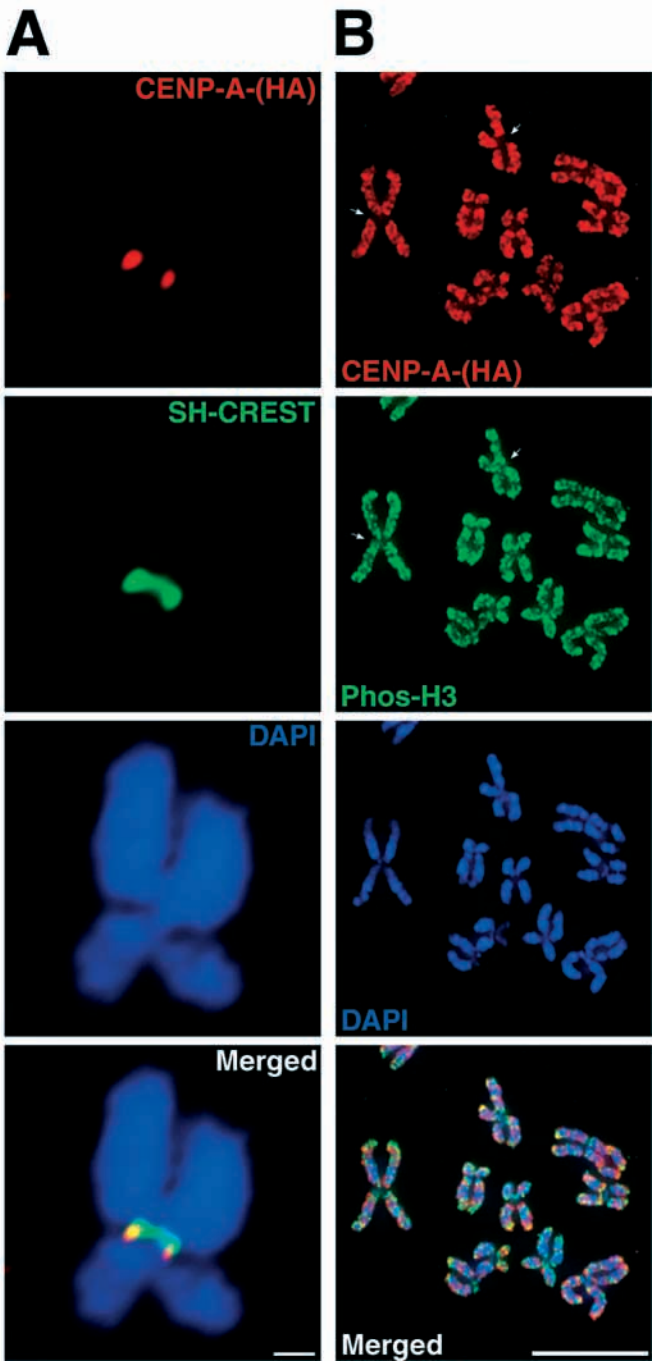

Fig. 1. CENP-A is targeted to the kinetochore region of centromeres and, at higher levels of expression, along chromosome arms. (A) HA-tagged CENP-A was immunolocalized with anti-HA antibody (red) in a stable HeLa cell line that expresses the gene in *trans* from a CMV-based tetracycline-regulated promoter (Shelby et al., 1997). Cells were arrested in mitosis with Colcemid and harvested by mitotic shake-off. Centromeres were detected using SH-CREST auto-antiserum (green), which recognizes antigens in both the pairing and kinetochore domains of centromeres. DNA was counterstained with DAPI (blue). Bar, 1 μm. (B) HA-epitope-tagged human CENP-A cDNA was re-transfected transiently into the stable HeLa cell line. Under these conditions, HA-tagged CENP-A was found to target the lengths of chromosomes (red). Following ~24 hours of expression, CENP-A-(HA) was predominately found on the euchromatic arms of chromosomes, with the pericentric heterochromatin of certain chromosomes containing reduced levels of staining (arrows). Phosphorylated histone H3 was detected using a specific antibody (green) and was found to be interspersed with the mistargeted CENP-A along the arms and within the pericentric heterochromatin of all chromosomes. Bar, 10 μm.

1997; Kalitsis et al., 1998). CENP-C is normally localized with CENP-A exclusively within the centromeric heterochromatin directly beneath kinetochores (Fig. 3) (Saitoh et al., 1992). However, when CENP-A was overexpressed in cells, we found that CENP-C colocalized with the mistargeted CENP-A at ectopic regions along the lengths of mitotic chromosomes. Untransfected cells on the same coverslips demonstrated the characteristic centromere-specific staining of anti-CENP-C antibody (Fig. 3). Interphase chromatin also showed non-centromeric staining of CENP-C when CENP-A was overexpressed and mistargeted in the cells (see below).

The mistargeting of CENP-C that was induced by CENP-A overexpression was confirmed by two lines of experimentation, both using human autoimmune sera. First, we took advantage of the previous finding that two of the three centromere proteins recognized by SH-CREST antiserum are nuclear matrix antigens: CENP-B and CENP-C (Ouspenski and Brinkley, 1993; He and Brinkley, 1996). CENP-A is also recognized by the serum, but can be eluted from cell preparations with other histones by DNase and high salt treatments (Ouspenski and Brinkley, 1993; He and Brinkley, 1996). We found that CENP-A could be eluted from the chromatin of overexpressing cells, yet SH-CREST staining remained at non-centromeric and centromeric sites, indicating the presence of CENP-B and/or -C (Fig. 4). Given that the localization of CENP-B was found to be unchanged by CENP-A overexpression using specific sera,

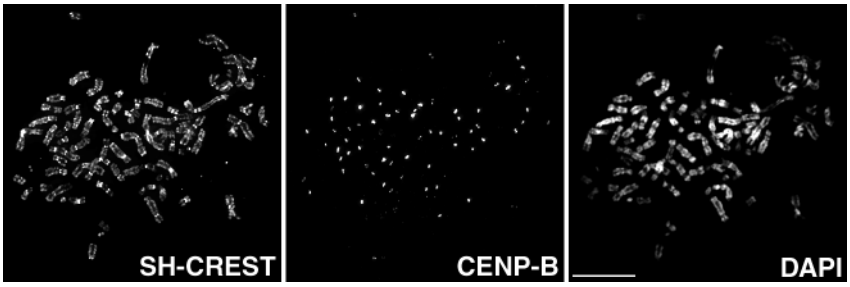

Fig. 2. CENP-B is not recruited to non-centromeric regions of chromatin containing mistargeted CENP-A. HA-tagged human CENP-A cDNA was transiently transfected and overexpressed in HeLa cells. Cells were arrested in mitosis with Colcemid for ~3 hours following ~48 hours of CENP-A-(HA) expression and were harvested by mitotic shake-off. SH-CREST antiserum, which recognizes CENP-A, was localized throughout the mitotic chromosomes of transfected cells. CENP-B was immunolocalized using a specific antiserum. Anti-CENP-B antibody stained exclusively the centromeres of chromosomes in HeLa cells overexpressing CENP-A. DNA was counterstained with DAPI. Bar, 10 μm.

Table 1. Effects of CENP-A mistargeting on centromere-kinetochore proteins

Native Protein	Centromere-kinetochore association	Effect of CENP-A mistargeting (mitosis)	Effect of CENP-A mistargeting (interphase)	Effect of CENP-A (core domain only) mistargeting	Effect of CENP-C mistargeting
CENP-A	Constitutive	–	–	–	Stays at centromeres
CENP-B	Constitutive	Stays at centromeres	Stays at centromeres	Stays at centromeres	Stays at centromeres
CENP-C	Constitutive	Mistargets with CENP-A	Mistargets with CENP-A	Stays at centromeres	–
CENP-E	Prometaphase-anaphase	Stays at kinetochores	Unchanged	Stays at kinetochores	Stays at kinetochores
HZW10	Prometaphase-anaphase	Stays at kinetochores	Unchanged	Stays at kinetochores	Stays at kinetochores
HZWint-1	Prophase-late anaphase	Mistargets with CENP-A	Mistargets with CENP-A	Stays at kinetochores	Stays at kinetochores
hSMC1	Prophase-late anaphase	Mistargets with CENP-A	Unchanged	Stays at kinetochores	Stays at kinetochores

the above result confirms that CENP-C is recruited to non-centromeric regions of chromatin containing mistargeted CENP-A.

A second experimental approach used a CREST autoimmune serum, designated EK (Mole-Bajer et al., 1990), which recognizes CENP-B and CENP-C, but not CENP-A, by western. Although EK-CREST staining is normally restricted to centromeres in HeLa and CHO cells, non-centromeric staining was observed when CENP-A was overexpressed (data not shown).

CENP-C does not recruit CENP-A to non-centromeric chromatin

To determine if the opposite reaction is also possible, we induced CENP-C to be mistargeted through its overexpression and examined the subsequent localization of CENP-A. It has previously been shown (Fukagawa et al., 1999) that excess CENP-C associates with chromosomes during early mitosis when overexpressed in the chicken DT40 cell line, is redistributed into perichromosomal foci during late mitosis, and then forms nuclear inclusions during interphase. In HeLa cells, we found that overexpressed CENP-C showed a pattern of mistargeting similar to that of CENP-A, characterized by high levels at centromeres and lower levels throughout the interphase euchromatin (Fig. 5). Despite the mistargeting of CENP-C during interphase, CENP-A was faithfully targeted to centromeres (Fig. 5). HeLa cell growth was potently inhibited by CENP-C overexpression, and mitotic cells were not observed.

Mechanism of CENP-A-induced mistargeting

In cells overexpressing CENP-A, the mistargeting of CENP-C could be due to the recruitment of CENP-C to non-centromeric chromatin by CENP-A, or possibly due to an increase in CENP-C expression induced by excess CENP-A. To investigate this point, the amount of CENP-C protein was compared by western analysis between CHO cells induced to express exogenous CENP-A over 50+ passages and the same cell line uninduced over the same number of passages. The total level of CENP-C protein in cells appeared equal in

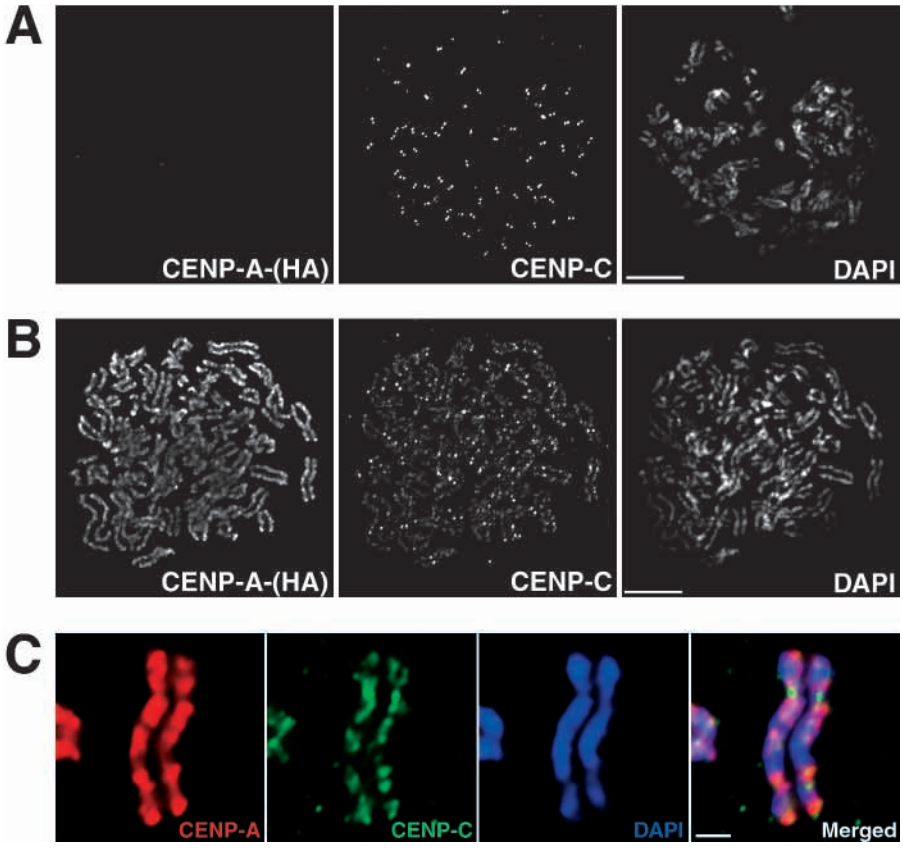

Fig. 3. CENP-C is mistargeted with CENP-A during mitosis. To determine if CENP-C is recruited to non-centromeric regions of chromatin to which CENP-A has been mistargeted, HA-tagged human CENP-A cDNA was transiently transfected into HeLa cells, and CENP-C was immunolocalized using a specific antiserum. Cells were arrested in mitosis with Colcemid for ~3 hours following ~48 hours of CENP-A-(HA) ectopic expression and harvested by mitotic shake-off. (A) Control cells, in which CENP-A was not overexpressed, demonstrated anti-CENP-C antibody staining exclusively at centromeres. A low level of anti-HA antibody background staining was observed. DNA was counterstained with DAPI. Bar, 10 μm. (B) In transfected cells, anti-CENP-C antibody was found to colocalize along the lengths of chromosome arms with ectopic CENP-A, detected using anti-HA antibody. Bar, 10 μm. (C) Higher magnification view of a chromosome from panel B. Bar, 1 μm.

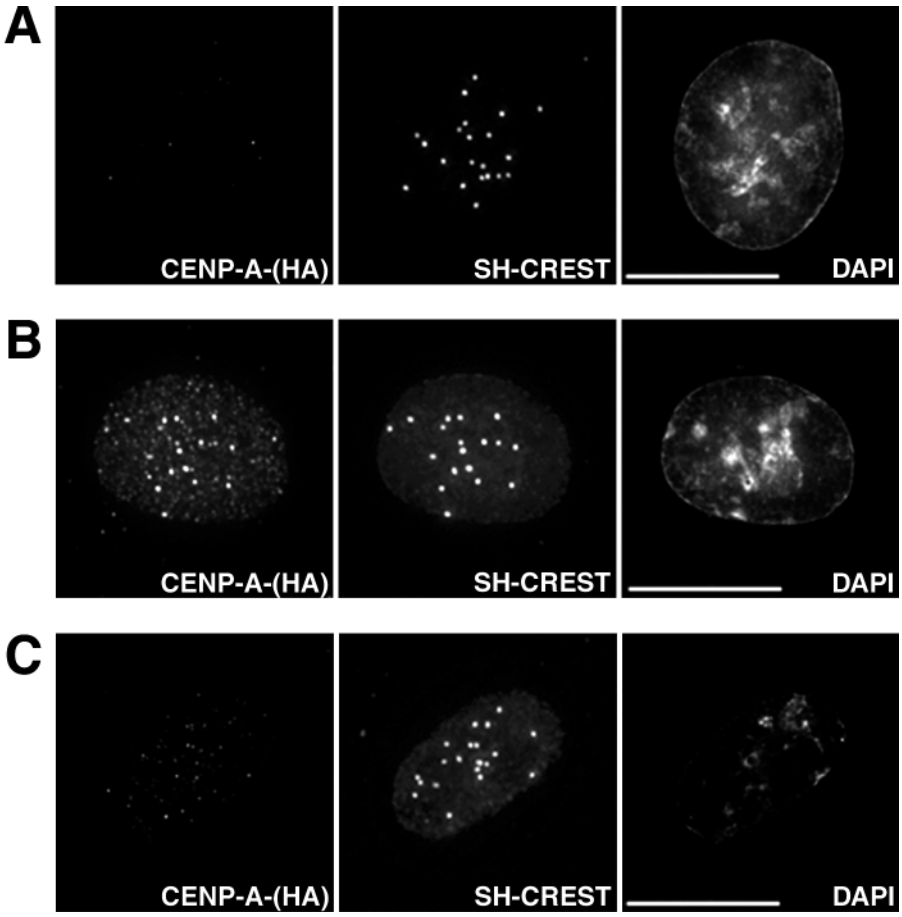

Fig. 4. CENP-C is mistargeted with CENP-A during interphase. To determine if CENP-C is recruited to non-centromeric regions of chromatin to which CENP-A has been mistargeted, CENP-A was overexpressed and co-localized with other CENPs recognized by human autoimmune sera. A stable CHO cell line was used that expresses an HA-tagged human CENP-A cDNA from a CMV-based tetracycline-regulated promoter. (A) SH-CREST antiserum, which primarily recognizes CENPs-A, -B, and -C, stains exclusively centromeres in uninduced control interphase CHO cells. A low level of anti-HA antibody background staining is observed. DNA was counterstained with DAPI. (B) SH-CREST was found to localize throughout the interphase chromatin of CHO cells that were induced to express the HA-tagged CENP-A transgene. CENP-A-(HA) was detected using anti-HA antibody. (C) To determine if the non-centromeric staining of CREST observed following CENP-A overexpression was in part due to the mistargeting of factors other than CENP-A recognized by the serum, CENP-A was overexpressed in the CHO cell line, and then eluted from fixed cell preparations by salt and DNase treatment prior to immunostaining. Exogenous centromeric and non-centromeric CENP-A was significantly reduced in extracted preparations when compared with unextracted (B), as detected by immunofluorescence using anti-HA antibody. Speckles of CENP-A remained at non-centromeric sites in the extracted preparations, perhaps corresponding to matrix-associated regions of the genome. Importantly, centromeric and non-centromeric SH-CREST staining remained following the elution of CENP-A, indicating the presence of CENP-B and/or -C at the ectopic sites. Bars, 10 μm.

the presence and absence of CENP-A overexpression (Fig. 6). The amount of CENP-B in cells was also observed to be equivalent between the two samples, as was expected given the lack of CENP-B association with non-centromeric sites of CENP-A targeting (Fig. 6). The amount of CENP-C protein associated with the chromatin also appeared to be equal in the presence and absence of CENP-A overexpression (Fig. 6). These results suggest that endogenous CENP-C is recruited away from centromeres to regions of the chromosome containing mistargeted CENP-A.

Recruitment of CENP-C requires the CENP-A tail

The homology between CENP-A and H3 resides exclusively in the C-terminal globular core domain of the two histones, whereas the N-terminal tails of the two histones, exposed at the surface of nucleosomes, share little homology (Sullivan et al., 1994). We therefore sought to determine if the unique tail of CENP-A was required for recruitment of CENP-C to ectopic regions of chromatin. The expression vector used in the above experiments was modified to remove the sequence encoding amino acids 2-45 of CENP-A N-terminus (see Materials and Methods). This resulted in an identical complement of

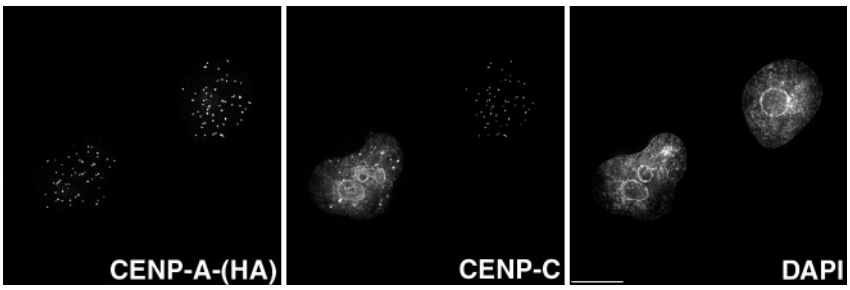

Fig. 5. Mistargeted CENP-C does not recruit CENP-A to ectopic loci. Similar to CENP-A, CENP-C can be mistargeted to non-centromeric regions of chromatin by overexpression. To determine if mistargeted CENP-C is capable of recruiting CENP-A to non-centromeric chromatin, a tetracycline-regulated CENP-C expression vector was constructed and transfected into a stable HeLa cell line in which HA-tagged CENP-A is expressed at near-physiological levels and faithfully targeted to centromeres (Fig. 1A). CENP-C and CENP-A were coexpressed for 48 hours in the cells. Cells were grown and harvested on coverslips. Overexpressed CENP-C was observed to mistarget throughout interphase chromatin (cell on left), visualized using an anti-CENP-C antibody that stains exclusively centromeres in untransfected cells (cell on right). CENP-A was immunolocalized with anti-HA antibody and was found to be faithfully targeted to centromeres despite the overexpression and mistargeting of CENP-C. DNA was counterstained with DAPI. Bar, 10 μm.

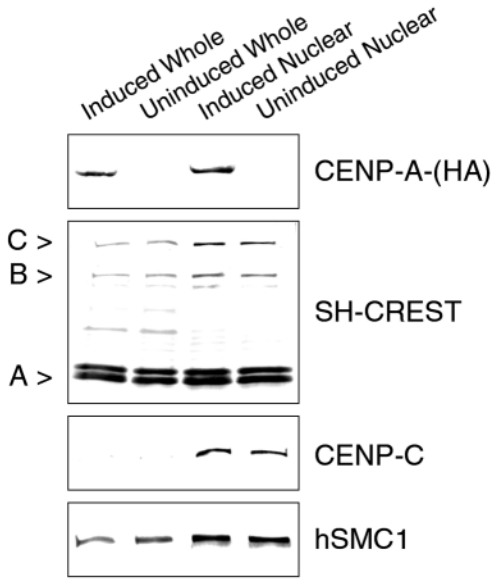

Fig. 6. Ectopic expression of CENP-A does not alter the level of CENP-B, CENP-C or hSMC1 that is soluble in the cell or associated with chromatin. The levels of various centromere-kinetochore proteins were compared between a stable CHO cell line induced to express HA-tagged CENP-A over 50 passages and the same cell line uninduced over the same number of passages. Protein samples from whole cells (50,000 cell-equivalents per lane) and detergent-extracted nuclear enrichments (100,000 cell-equivalents per lane) were compared by western analysis. Expression of CENP-A-(HA) was determined using anti-HA antibody and SH-CREST antiserum. SH-CREST also recognizes a non-centromeric 20-25 kDa antigen of unknown identity that displays certain biochemical properties similar to CENP-A (Earnshaw and Rothfield, 1985; Valdivia and Brinkley, 1985; Ouspenski and Brinkley, 1993; He and Brinkley, 1996). CENP-C was detected using a specific antibody and with SH-CREST antiserum. hSMC1 was detected using a specific antiserum. CENP-B was detected using SH-CREST. The subcellular localization of CENP-B was unaltered by CENP-A overexpression and serves as a control for the relative amount of protein loaded in each lane.

transcriptional and translational control elements, but expressing a tailless CENP-A peptide. We found that the N-terminal 44 amino acids of CENP-A are not required for its targeting to centromeres (Fig. 7), similar to results obtained previously (Sullivan et al., 1994). Lower levels of non-centromeric targeting upon overexpression were also observed, identical to the full-length CENP-A molecule (Fig. 7). However, we found that CENP-C was not recruited to non-centromeric regions when the CENP-A tail was missing (Fig. 7) (Table 1).

CENP-A assembles kinetochore components on chromatin

It was recently discovered that the human structural maintenance of chromosomes protein 1 (hSMC1) is localized to centromeres-kinetochores from early prophase to late anaphase (H.C.G. and K.Y., unpublished). The heterodimeric hSMC1/hSMC3 complex appears to be involved in chromosome alignment and metaphase progression in human cells (Schmiesing et al., 1998). We found that when CENP-A was overexpressed in HeLa and CHO cells, anti-hSMC1 antibody predominately stained kinetochores during mitosis, but some staining was also colocalized with mistargeted CENP-A along the lengths of chromosomes (Fig. 8). In untransfected cells within the same preparations, anti-hSMC1 stained exclusively the kinetochores (Fig. 8A). Mistargeting of hSMC1 to chromosome arms was observed between early prophase and late anaphase, displaying similar dynamics to the faithfully targeted hSMC1 protein at kinetochores (Fig. 8B,C). During interphase, hSMC1 is targeted to nuclear matrix foci that do not correspond to prekinetochores. This pattern appeared

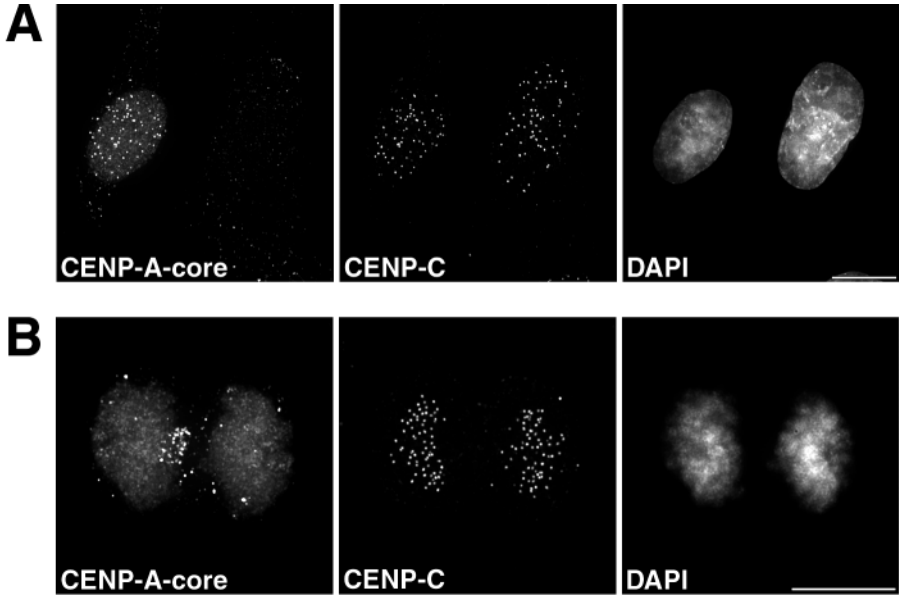

Fig. 7. The CENP-A N-terminal tail is required for the recruitment of CENP-C to ectopic sites. A DNA construct encoding the C-terminal core domain of CENP-A with an HA-epitope tag (referred to as CENP-A-core) was transiently transfected into HeLa cells and detected by immunofluorescence using anti-HA antibody. Cells were grown and processed on coverslips. CENP-C was co-stained with a specific antibody. (A) Anti-CENP-C staining is centromere-specific in untransfected control cells (cell on right), in which CENP-A-core expression was undetectable. Similarly, CENP-C localization remained centromere-specific in transfected cells (cell on left), which demonstrated the localization of CENP-A-core throughout the nucleus and most predominately at centromeres. DNA was counterstained with DAPI. (B) Cells expressing the CENP-A-core peptide appeared to progress through mitosis normally. As during interphase, CENP-C was found exclusively at centromeres. The core domain of CENP-A appears to be targeted to chromatin less efficiently than the full-length protein and some accumulations were observed in the cytoplasm (Sullivan et al., 1994). Bars, 10 μm.

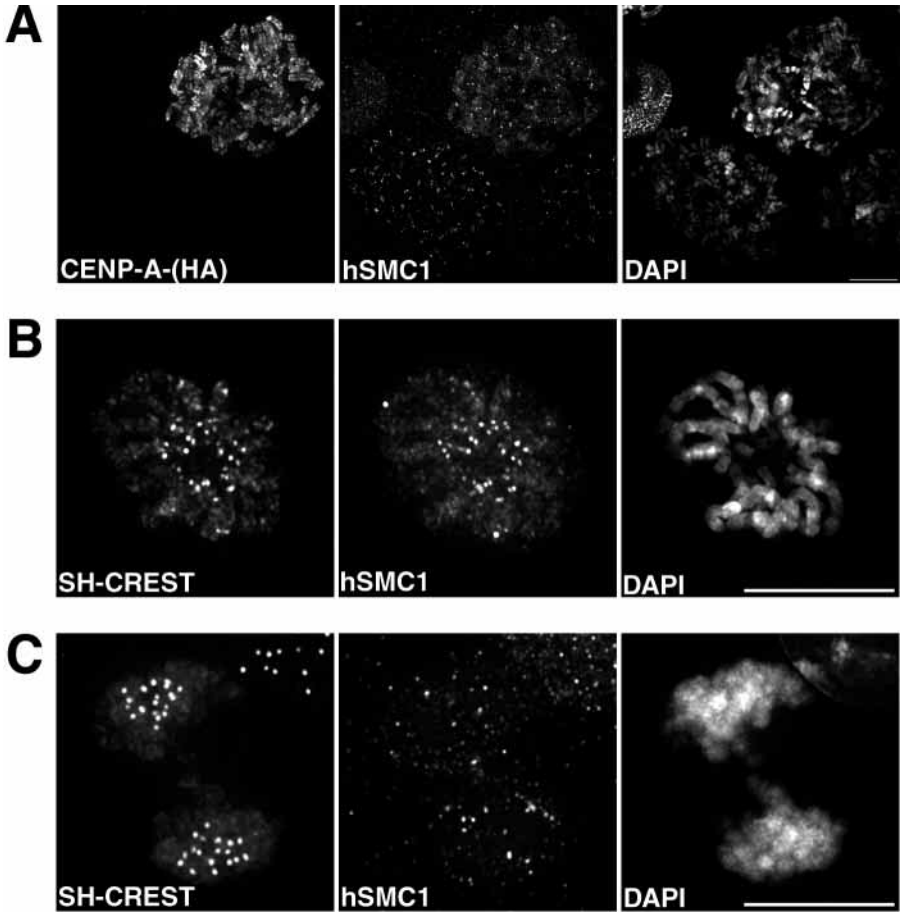

Fig. 8. hSMC1 is mislocalized along chromosome arms with CENP-A specifically during mitosis. (A) hSMC1 was immunolocalized in HeLa cells that were transiently transfected with HA-tagged CENP-A ~48 hours prior to arrest in mitosis. Cells overexpressing CENP-A-(HA), detected with anti-HA antibody, demonstrated anti-hSMC1 mislocalized along the arms of chromosomes (cell on right). Control untransfected cells within the same preparation (cell on left) demonstrated the characteristic centromere-kinetochore-exclusive staining of anti-hSMC1 during mitosis. DNA was counterstained with DAPI. (B) CHO cells were grown and processed on coverslips to examine ectopic and centromeric hSMC1 at all stages of mitosis. hSMC1 was observed along the lengths of chromosomes as early as prometaphase. SH-CREST was used to detect the mistargeted CENP-A. (C) Centromeric and non-centromeric anti-hSMC1 staining was redistributed to perichromosomal foci during late anaphase. Centromeric and non-centromeric SH-CREST staining, however, was observed at all stages. Bars, 10 μm.

unchanged by the overexpression and mistargeting of CENP-A (data not shown). The total level of hSMC1 protein in cells and associated with chromatin appeared the same in the presence and absence of CENP-A overexpression (Fig. 6). hSMC1 was not recruited to non-centromeric sites when the CENP-A core domain alone was mistargeted (data not shown), indicating that the CENP-A tail is required for recruiting hSMC1 to ectopic sites, identical to the results found for CENP-C recruitment (Fig. 7). The overexpression and mistargeting of CENP-C was not sufficient to recruit hSMC1 to non-centromeric regions (data not shown; Table 1).

HZwint-1 (human ZW10 interacting protein 1) localizes to kinetochores from prophase to late anaphase (Starr et al., 2000). We found that HZwint-1 was mislocalized along the lengths of mitotic chromosomes together with mistargeted CENP-A (Fig. 9A). Interestingly, HZwint-1 colocalized with CENP-A throughout interphase chromatin as well, but was not as predominant at centromeres-prekinetochores (Fig. 9B). HZwint-1 is normally cytoplasmic during interphase (Starr et al., 2000), as was observed in untransfected cells within the same preparation (Fig. 9B). Cells in which CENP-C was overexpressed, rather than CENP-A, did not show the same redistribution of HZwint-1 to non-centromeric regions of interphase chromatin (Fig. 9C). HZwint-1 was not recruited to non-centromeric sites when the CENP-A core domain alone was mistargeted (data not shown), indicating that the CENP-A tail is required for recruiting HZwint-1 to ectopic sites.

Microtubule-associated proteins are not assembled by CENP-A

The kinetochore component ZW10 is necessary to recruit the microtubule-associated motor dynein to kinetochores and is essential for proper chromosome segregation in *Drosophila* (Williams et al., 1992; Starr et al., 1998). ZW10 is localized to kinetochores from prometaphase through anaphase and is distributed along kinetochore microtubules during metaphase (Williams et al., 1992). We found that the human homologue, HZW10 (Starr et al., 1997), remained at kinetochores when CENP-A was mistargeted to chromosome arms (Fig. 10A).

CENP-E is a kinesin-related mitotic motor protein, localized to kinetochores from prometaphase through anaphase (Yen et al., 1991; Yen et al., 1992; Cooke et al., 1997). CENP-E was also not recruited to the arms of chromosomes along with mistargeted CENP-A (Fig. 10B).

CENP-A-directed chromatin assembly is not sufficient for neocentric activity

The stable CHO and HeLa cell lines used in this study display similar growth rates over multiple passages whether expression of ectopic CENP-A is induced or uninduced (data not shown). The apparent lack of mitotic defects in these cells suggests that mistargeting of CENP-A does not significantly alter the normal pattern of chromosome segregation. Electron microscopy demonstrated the presence of trilaminar kinetochore plates and spindle microtubules at the primary constriction only and not elsewhere along the chromosomes in these cells (data not shown).

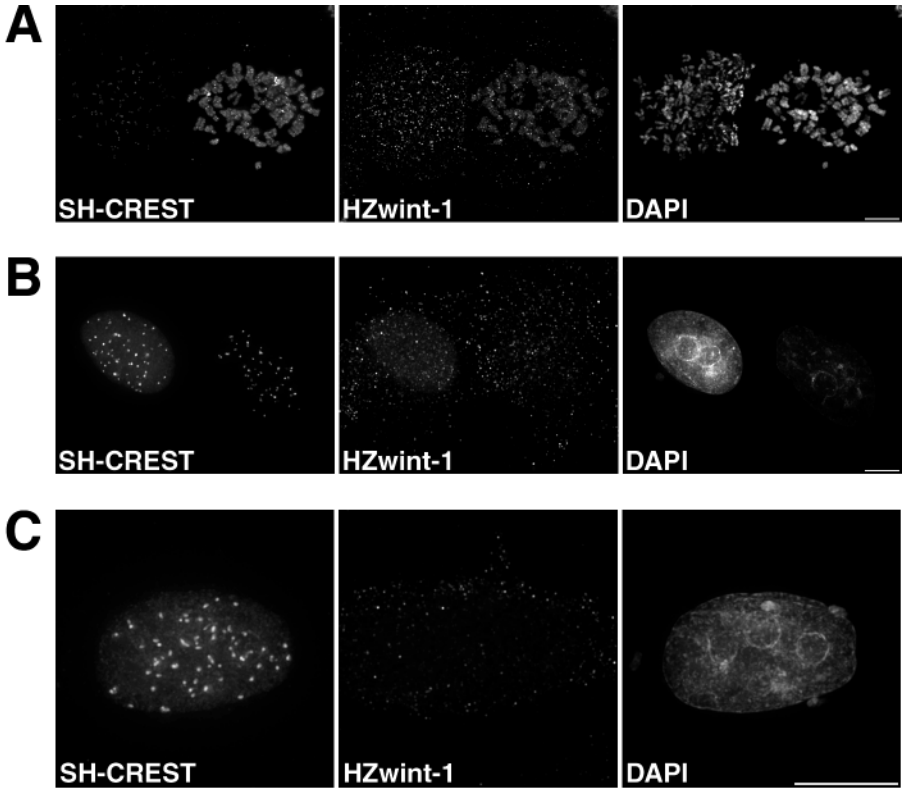

Fig. 9. HZwint-1 is mislocalized with CENP-A, but not with CENP-C, during mitosis and interphase. (A) HZwint-1 was immunolocalized in HeLa cultures transiently transfected with HA-tagged CENP-A. Cells were arrested in mitosis with Colcemid for ~3 hours following ~48 hours of CENP-A-(HA) expression and harvested by mitotic shake-off. Mitotic cells overexpressing CENP-A, detected with SH-CREST antiserum, demonstrated mislocalization of anti-HZwint-1 along chromosome arms (cell on right). Untransfected cells within the same preparation (cell on left) demonstrated the characteristic staining of anti-HZwint-1 exclusively at kinetochores. DNA was counterstained with DAPI. (B) Cells were grown and stained on coverslips to examine all phases of the cell cycle. Anti-HZwint-1 staining was observed throughout interphase chromatin in cells overexpressing CENP-A (cell on left). Anti-HZwint-1 staining was exclusively cytoplasmic in untransfected cells (cell on right). (C) Overexpression of CENP-C, detected using SH-CREST antiserum, did not result in the mistargeting of HZwint-1 during interphase. Untransfected mitotic cells on the same coverslips were used as a positive control for HZwint-1 staining (data not shown). Bars, 10 μm.

To further determine if CENP-A is sufficient for kinetochore assembly and function, we tested the capacity of individual non-centromeric chromosome fragments containing CENP-A for mitotic movement in mitotic cells with unreplicated genomes (MUGs). With the MUG procedure, caffeine is used to induce premature entry into mitosis and chromosome fragmentation in cells arrested at the G_1/S-phase boundary (Brinkley et al., 1988; Zinkowski et al., 1991; Ouspenski and Brinkley, 1993). Under these conditions, fragments of the centromere-kinetochore disassociate from chromosome arms and align on the metaphase plate, whereas non-centromeric chromosome fragments are displaced from the spindle and segregate randomly. We found that mistargeted CENP-A did not support the movement of arm-derived chromosome fragments in this system, as would have been expected if mistargeted CENP-A had conferred

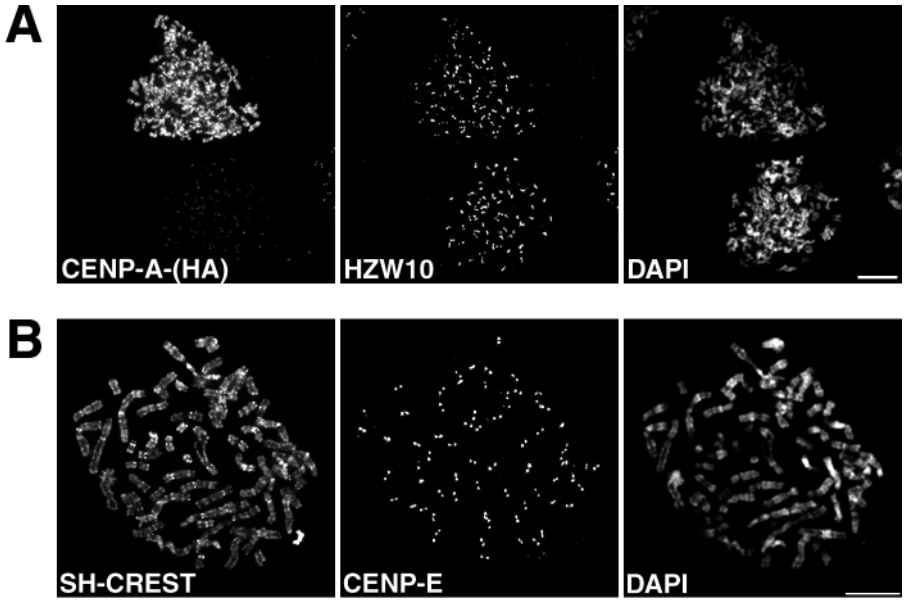

Fig. 10. Kinetochore components HZW10 and CENP-E are not mistargeted to ectopic regions with CENP-A. To determine if kinetochore microtubule-associated proteins are recruited to non-centromeric regions of chromatin to which CENP-A has been mistargeted, HA-tagged CENP-A was overexpressed in HeLa cells, and human ZW10 and CENP-E were immunolocalized using a specific antisera. Cells were arrested in mitosis with Colcemid for ~3 hours following ~48 hours of CENP-A-(HA) expression and harvested by mitotic shake-off. (A) Anti-HZW10 antibody was localized exclusively to kinetochores in mitotic cells overexpressing CENP-A (top cell), as was found in untransfected controls within the same preparations (bottom cell). CENP-A-(HA) was detected using anti-HA antibody. DNA was counterstained with DAPI. (B) CENP-E was similarly localized exclusively to kinetochores in cells overexpressing CENP-A. Ectopic CENP-A and centromeres were detected using SH-CREST antiserum. Bars, 10 μm.

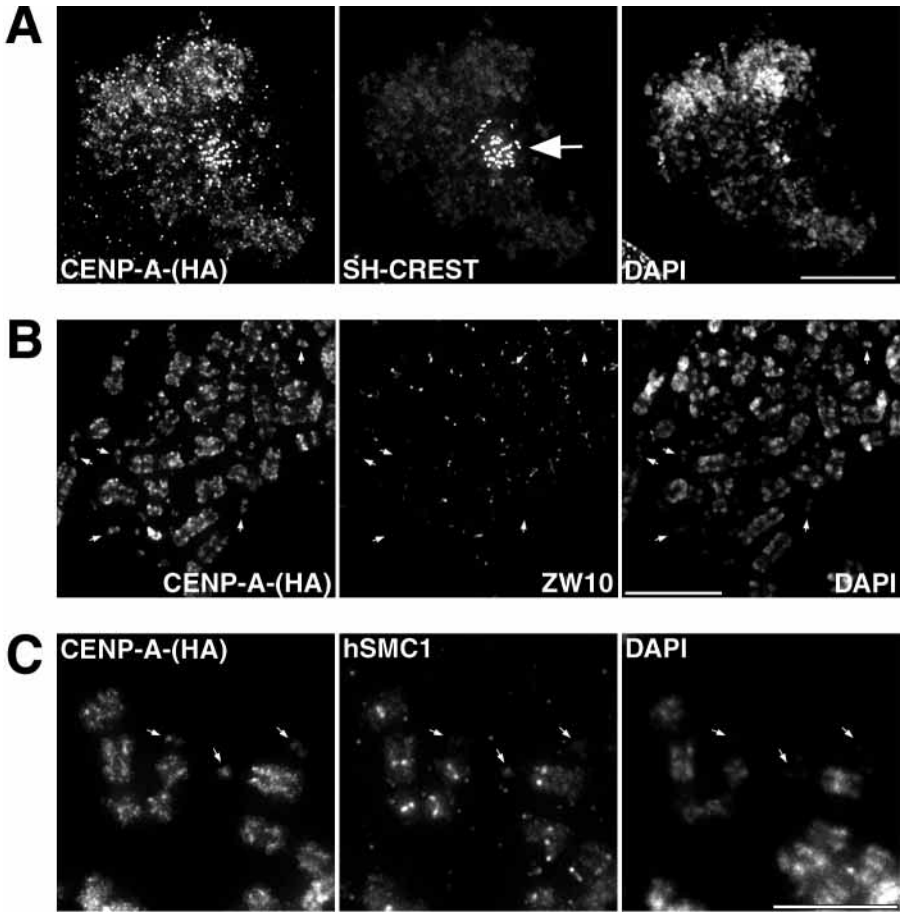

Fig. 11. Mislocalized CENP-A does not support the mitotic movement of non-centromeric chromosome fragments. (A) CHO cells expressing HA-tagged CENP-A were arrested at the G_1/S-phase boundary with hydroxyurea, then induced to prematurely enter mitosis and fragment their genome by caffeine treatment. Centromere-kinetochore subunits, which stain intensely with SH-CREST antiserum, were observed to congress at the metaphase plate (arrow). Whereas non-centromeric genome fragments containing mistargeted CENP-A, detected using anti-HA antibody, stained less intensely with SH-CREST antiserum, were immotile and lay in clusters around the cell periphery. DNA was counterstained with DAPI. (B) CENP-A was transiently transfected and overexpressed in COLO 320DM cells, which contain numerous acentric double-minute (DM) chromosomes (arrows). Targeting of CENP-A to the DMs was not sufficient to assemble kinetochores, as demonstrated by an apparent lack of anti-ZW10 antibody reaction. (C) hSMC1, detected with a specific antibody, was observed to be recruited to the DMs (arrows) following the mistargeting of CENP-A, detected with anti-HA antibody, to these acentric chromosomes. Bars, 10 μm.

neocentromeric activity to chromosome arms (Fig. 11A). The only fragments that appeared capable of aligning on the metaphase plate were those that stained very intensely with SH-CREST antibody and thus corresponded to true centromere-kinetochore fragments, as shown previously by EM (Fig. 11A) (Zinkowski et al., 1991).

We hypothesized that the native centromere on chromosomes might be suppressing the formation of kinetochores at distal sites where CENP-A was experimentally mistargeted. Evidence that such a mechanism exists comes mostly from the study of dicentric chromosomes, in which one centromere is, in most cases, inactivated, thereby stabilizing the chromosome during cell division (Karpen and Allshire, 1997; Wiens and Sorger, 1998). Similarly, neocentromeres can form on fly and human chromosomes within regions of DNA that previously did not display centromere activity (Karpen and Allshire, 1997; Choo, 2000). Thus, mechanisms must exist to suppress this neocentromeric activity when an endogenous centromere is functioning at a different position on the same chromosome (Karpen and Allshire, 1997).

To determine if CENP-A has the capacity to form complete kinetochores at ectopic sites in the absence of any suppressive effect caused by the endogenous centromere on the same chromosome, we mistargeted CENP-A to double-minute (DM) chromosomes, which lack centromeres in most cases. For these experiments, a human colorectal carcinoma cell line, COLO 320DM, was used that contains DMs in >24% of the metaphases. We found that the mistargeting of CENP-A to DMs resulted in a protein complex similar to that formed along the lengths of normal chromosomes. As discussed above, this complex contained CENP-A, CENP-C, hSMC1 and HZwint-1, but did not contain the microtubule-associated kinetochore proteins ZW10 and CENP-E (Fig. 11; and data not shown). These results indicate that factor(s) unique to the centromere locus are required in addition to CENP-A for the assembly of kinetochore microtubule-associated factors. These results also suggest that centromere-kinetochore factors are not spread along chromosomes from endogenous centromeres when CENP-A is mistargeted, but appear to be recruited de novo to non-centromeric regions.

CENP-A function on dicentric chromosomes

Active centromeres on dicentric chromosomes are defined as the locus at which kinetochores are formed and as the final point of sister chromatid cohesion prior to anaphase onset (Vig and Rattner, 1989). Inactive centromeres do not form kinetochores and separate earlier in mitosis with the chromosome arms. Both active and inactive centromeres can be detected by immunostaining with CREST antiserum, due to the presence of CENP-B at both sites (Merry et al., 1985; Earnshaw et al., 1989), although all other known centromere and kinetochore proteins are limited to the active centromere only (Sullivan and Schwartz, 1995; Warburton et al., 1997; Faulkner et al., 1998; Starr et al., 2000). We found that when exogenous CENP-A was overexpressed in the COLO 320DM cell line, which contains several dicentric chromosomes, active

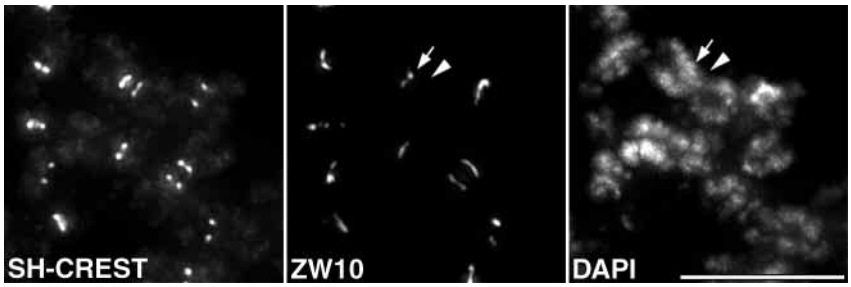

Fig. 12. Mistargeting of CENP-A to the inactive centromere of dicentric chromosomes is not sufficient for kinetochore formation. CENP-A was overexpressed for ~48 hours in a cell line (COLO 320DM) carrying multiple dicentric chromosomes and was immunolocalized using SH-CREST autoimmune serum. Cells were arrested in mitosis with Colcemid and harvested by mitotic shake-off. In untransfected control cells, CENP-A is only observed at active and not inactive centromeres (not shown). In cells overexpressing the protein, CENP-A was observed along the lengths of chromosome arms and at inactive centromeres. Despite the mistargeting of CENP-A, anti-ZW10 antibody localized only at the active centromere (arrow) and not at the inactive centromere (arrowhead) of dicentric chromosomes. DNA was counterstained with DAPI. Bar, 10 μm.

centromeres were the preferential site of CENP-A targeting (Fig. 12A). Similar results have been reported for the distribution of endogenous CENP-A on a dicentric chromosome (Warburton et al., 1997). We have confirmed that CENP-C, HZwint-1 and hSMC1 are all targeted exclusively to the active centromere of dicentric chromosomes along with CENP-A (data not shown; Earnshaw et al., 1989; Starr et al., 2000) (H.C.G. and K.Y., unpublished). This data is consistent with a central role for CENP-A in assembling these factors to form kinetochore-specific chromatin.

Potentially, the occlusion of CENP-A from chromatin could be a mechanism for inactivating extra centromeres when fusion events produce a chromosome with more than one centromere. To experimentally test this hypothesis, we examined the distribution of kinetochore components on dicentric chromosomes following the mistargeting of CENP-A to inactive centromeres. At high levels of ectopic expression, CENP-A was observed to target the lengths dicentric chromosomes, including inactive centromeres (Fig. 12A; and data not shown). CENP-A appeared to target these regions with a reduced efficiency relative to the active centromeres. We found that the partial centromere-prekinetochore complex recruited by CENP-A to inactive centromeres resembled that formed along the chromosome arms: containing CENP-A, CENP-C, hSMC1 and HZwint-1, as discussed above. The complex formed at inactive centromeres lacked HZW10 and CENP-E, resembling the chromosome arms rather than the active centromere (Fig. 12B). Thus, CENP-A is not sufficient to restore activity to inactive centromeres, and the control of CENP-A targeting is not the only element regulating centromere activity on multicentric chromosomes.

DISCUSSION

Our data suggests that CENP-A acts as a primary determinant in establishing where kinetochores are initially formed on chromosomes. Experimentally elevating the level of CENP-A expression throughout the cell cycle results in the mistargeting of this centromere-specific histone along the lengths of mitotic chromosomes and throughout interphase nuclei, interspersed with histone H3. We have found that the constitutive centromere protein CENP-C is recruited to sites where CENP-A is mistargeted, suggesting a role for CENP-A in organizing the fundamental structure of centromeric heterochromatin beneath kinetochores. CENP-B was not recruited away from centromeres by mistargeted CENP-A, which was to be expected, given that CENP-B is a sequence-specific DNA binding protein (Masumoto et al., 1989). CENP-C possesses a non-sequence-specific DNA binding activity and is targeted to the centromere by a domain that is distinct from, and partially overlapping with, the DNA-binding domain (Lanini and McKeon, 1995; Yang et al., 1996). CENP-C has been proposed to act analogously to a linker histone that stabilizes nucleosomes containing CENP-A (Sullivan, 1998). Our results extend the previously reported finding that CENP-A is required for recruitment of CENP-C to mouse centromeres (Howman et al., 2000) and demonstrate that CENP-A, specifically, is sufficient for this recruitment.

CENP-A-containing chromatin further assembled the kinetochore components hSMC1 and HZwint-1 at non-centromeric sites. In addition, another facultative kinetochore protein CENP-F was observed in some cases, at very low levels, to mistarget to non-centromeric sites with CENP-A and its associated factors (data not shown). The kinetochore proteins that were recruited by CENP-A share commonalties in their timing of localization to kinetochores and in their protein structure that make them distinct from other kinetochore components. First, these factors arrive at prekinetochores before microtubule-associated factors and remain longer. Specifically, hSMC1, HZwint-1 and CENP-F localize to prekinetochores between G_2 and early prophase and remain until telophase (Rattner et al., 1993; Liao et al., 1995; Starr et al., 2000) (H.C.G. and K.Y., unpublished). The microtubule-associated factors CENP-E and ZW10, which were not recruited to ectopic regions with CENP-A, are targeted to kinetochores later, during prometaphase, and appear in some cases to redistribute down spindle microtubules at metaphase (Yen et al., 1991; Williams et al., 1992). A second feature shared by hSMC1, and HZwint-1, and CENP-F is the presence of extensive coiled coil forming domains in their protein structure (Rattner et al., 1993; Liao et al., 1995; Odgren et al., 1996; Schmiesing et al., 1998; Starr et al., 2000). Interactions between coiled coil-containing proteins are thought perhaps to play a scaffolding role during kinetochore assembly by allowing the formation of fibrous structures or protein-protein binding sites that target other kinetochore components (Liao et al., 1995; Starr et al., 2000). It therefore appears that CENP-A is sufficient to assemble, at least in part, a centromere and a ‘prekinetochore scaffold’.

The ability of CENP-A to assemble into chromatin and recruit other centromere-prekinetochore components appears to be a unique property of this histone. Overexpression of other centromere-kinetochore proteins discussed in this study

resulted in their aberrant accumulation in the cytoplasm or nucleus and/or was toxic to cells (data not shown). CENP-C alone displayed a similar association with interphase chromatin as CENP-A did when overexpressed, although CENP-C did not assemble the same centromere-prekinetochore complex. Our results are consistent with those of Fukagawa et al., who demonstrated that CENP-C alone, mislocalized by overexpression, did not recruit the dynein-associated kinetochore protein ZW10 to non-centromeric sites (Fukagawa et al., 1999). Similarly, we have shown that CENP-C in complex with other factors recruited by CENP-A is also not sufficient for assembling ZW10 or CENP-E to non-centromeric regions.

Centromeric association of the yeast cohesin complex, which is essential for sister chromatid cohesion, has been shown to require Cse4p and Mif2p, homologues of CENP-A and -C, respectively (Guacci et al., 1997; Michaelis et al., 1997; Tanaka et al., 1999). Our results demonstrate that in mammalian cells, CENP-A is sufficient for targeting the cohesin subunit hSMC1 to chromosomes during mitosis, but not during interphase. As CENP-C was mistargeted along with CENP-A, these factors could have recruited hSMC1 together. Mistargeting of CENP-C alone was not sufficient to recruit hSMC1 (data not shown). Although the function of hSMC1 at mammalian centromeres-kinetochores remains elusive, it is interesting to note that the targeting of this cohesin to chromosome arms did not detectably interfere with sister chromatid separation.

The centromere-prekinetochore scaffold assembled by CENP-A is not sufficient for complete centromere activity, indicating the presence of one or more additional steps in the kinetochore assembly pathway. Kinetochore plates were not observed by electron microscopy at non-centromeric sites of CENP-A localization, and neocentromeric activity was not detected by the MUG assay. CENP-A was also not sufficient for the assembly of complete kinetochores on acentric DM chromosomes. This experiment indicates that the lack of complete kinetochore assembly by CENP-A at non-centromeric regions was not due to *cis*-acting suppression by the endogenous centromere on the same chromosome. Rather, the endogenous centromere appears to contain information in addition to CENP-A that is specifically required for the targeting of microtubule-associated factors and assembly of kinetochore plates on chromosomes.

CENP-A was similarly not sufficient for the complete assembly of kinetochores when experimentally targeted to the inactive centromeres of dicentric chromosomes. These inactive centromeres, like active centromeres on the same chromosome, contain alpha-satellite DNA, low levels of histone acetylation, and are late replicating (A.A.V.H., I.I.O. and B.R.B., unpublished). Thus, the determinants unique to active centromeres that are required in addition to CENP-A for kinetochore formation must be distinct from these factors. A key step in defining the kinetochore locus might be the post-translational marking of the centromere proteins themselves and the transmittance of these mark(s) at the active centromere locus between cell generations (Everett et al., 1999). If centromere proteins are indeed marked, it would be of interest with regard to the above data to determine if this mark is retained when centromere proteins are experimentally mistargeted to non-centromeric regions.

CENP-A potentially could target other centromere-kinetochore components to chromatin through protein-protein interactions, or perhaps by modulating chromatin structure. Previous studies have suggested that the functional specialization of chromatin by histone variants is associated with changes in the protein-protein interactions that occur at the surface of nucleosomes between histone tails and non-histone proteins (Wolffe and Pruss, 1996). Biochemical and genetic data have shown an interaction between Cse4p and the Ctf19p-Mcm21p-Okp1p complex, which is potentially involved in kinetochore assembly and kinetochore-microtubule interactions (Ortiz et al., 1999; Chen et al., 2000). This interaction requires a specific, essential domain within the N-terminal tail of the histone (Keith et al., 1999; Chen, et al., 2000). We have found that the distinctive N-terminal tail of CENP-A is required for the recruitment of CENP-C and components of the 'prekinetochore scaffold' to ectopic regions. As our experiments with the truncation mutant were conducted in a normal background containing endogenous CENP-A, they do not address whether the CENP-A tail is required for targeting these factors to their native centromere-kinetochore location.

A yeast two-hybrid screen using the CENP-A tail as bait has identified a number of proteins that putatively interact, but this set does not include the proteins discussed in the present study (I.I.O, A.A.V.H. and B.R.B., unpublished). Similarly, immunoprecipitation of detergent-soluble CENP-A from CHO cells did not reveal any interaction between this fraction of CENP-A and CENP-B, -C, or hSMC1 (data not shown). These data suggest that CENP-A is not in a complex with the other factors prior to their deposition at centromeres. Thus, we favor a model in which CENP-A recruits centromere-prekinetochore scaffold components to chromatin. A more detailed study including the isolation of CENP-A from chromatin without disruption of kinetochore structure would likely be required to establish a direct interaction, if any.

Alternative to a model in which this recruitment occurs by protein-protein interactions, a particular structural conformation may be given to the chromatin in which CENP-A is incorporated that favors the assembly of a centromere-prekinetochore scaffold. The amino acid sequence of CENP-A diverges from that of histone H3 at a number of N-terminal tail residues that are acetylated in transcriptionally active chromatin and phosphorylated during chromosome condensation (Sullivan et al., 1994; Van Hooser et al., 1999). Acetylation and phosphorylation of the H3 tail is thought to weaken its association with DNA and 'open' chromatin, either directly by altering nucleosome structure, or indirectly by targeting specific nonhistone proteins (Van Hooser et al., 1999). CENP-A might confer a specific structure to nucleosomes within a subregion of the centromere and allow a higher-order state of chromatin to develop as a necessary precursor to kinetochore assembly (Meluh and Koshland, 1997; Van Hooser et al., 1999).

Based on the results reported here, we extend previously proposed models for mammalian kinetochore assembly. CENP-A targeting appears as an initial step in forming a kinetochore. The constitutive centromere protein CENP-C is recruited to nucleosomes containing CENP-A during interphase to form a nucleoprotein complex that structurally defines kinetochore-specific chromatin within extended

regions of centromeric heterochromatin. Kinetochore assembly is insulated from the dynamics of interphase chromatin remodeling due to the presence of CENP-A and the subsequent reduction of nucleosome modifications associated with H3 in transcriptionally active chromatin (Meluh and Koshland, 1997; Van Hooser et al., 1999). As cells enter mitosis, subunits of centromeric heterochromatin containing the CENP-A/CENP-C nucleosome complex assemble to form a 'prekinetochore scaffold,' composed of HZwint-1, hSMC1 and CENP-F, as a necessary precursor for kinetochore plate formation (Zinkowski et al., 1991). Targeting of microtubule motor-associated proteins to the prekinetochore scaffold is governed by additional factor(s) present only at the active centromere site, distinct from alpha-satellite DNA, patterns of histone hypoacetylation, and late DNA replication timing. CENP-A and/or CENP-C potentially carry unique patterns of post-translational modifications that (1) mark where the active locus is transmitted on chromosomes between cell generations, and (2) are required for kinetochore plate assembly and microtubule attachment (Everett et al., 1999; Choo, 2000). The principal role of the CENP-A nucleoprotein complex during mitosis may be to maintain the conformation of chromatin beneath kinetochores as mechanical stress is applied to centromeres (Sullivan, 1998).

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