

## PUBLISHER'S NOTE

# Expression of Concern: CENP-C binds the alpha-satellite DNA in vivo at specific centromere domains

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This Expression of Concern relates to *J. Cell Sci.* (2002) **115**, 2317-2327 (doi:10.1242/jcs.115.11.2317).

A reader contacted Journal of Cell Science with concerns about a potential image duplication in Fig. 2A of this article, which was also highlighted on the PubPeer website. Possible duplication was highlighted for the  $\alpha$ -sat (1, 5, 19) and  $\alpha$ -sat (X) total DNA dilution series dot blots in Fig. 2A.

We contacted the authors of the article to request the original data for this experiment. We were informed by Professor Giovanni Perini, co-first author of the article, that the corresponding author, Professor Giuliano Della Valle, retired in 2010 and passed away in 2016. Consequently, the data and laboratory records relating to Fig. 2A are no longer available.

Professor Perini informed us that he cannot exclude the possibility that the  $\alpha$ -sat (1, 5, 19) and  $\alpha$ -sat (X) total DNA dilution series dot blots in Fig. 2A are duplicated images. However, Professor Perini also highlighted that the legend for Fig. 2A states that the filters were sequentially hybridised with the alpha-satellite probes, raising the possibility that the similarities between these two dot blots, which are shown for semi-quantitative assessment of the corresponding ChIP dot blots, could be the result of rehybridisation of the same filter with the different probes. The authors state that the major conclusions of the article are not affected by this issue.

Because the original data and records for this experiment are not available, we are unable to determine whether Fig. 2A shows duplicated data for the  $\alpha$ -sat (1, 5, 19) and  $\alpha$ -sat (X) total DNA dilution series. The journal is therefore publishing this Expression of Concern to make readers aware of this issue.

# CENP-C binds the alpha-satellite DNA in vivo at specific centromere domains

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## Summary

CENP-C is a fundamental component of the centromere, highly conserved among species and necessary for the proper assembly of the kinetochore structure and for the metaphase-anaphase transition. Although CENP-C can bind DNA in vitro, the identification of the DNA sequences associated with it in vivo and the significance of such an interaction have been, until now, elusive. To address this problem we took advantage of a chromatin-immunoprecipitation procedure and applied this technique to human HeLa cells. Through this approach we could establish that: (1) CENP-C binds the alpha-satellite DNA selectively; (2) the CENP-C region between amino acids 410 and 537, previously supposed to contain a DNA-binding domain, is indeed required to perform such a function in vivo; and (3) the profile of the alpha-satellite DNA associated with CENP-C is essentially identical to that

recognized by CENP-B. However, further biochemical and ultrastructural characterization of CENP-B/DNA and CENP-C/DNA complexes, relative to their DNA components and specific spatial distribution in interphase nuclei, surprisingly reveals that CENP-C and CENP-B associate with the same types of alpha-satellite arrays but in distinct non-overlapping centromere domains. Our results, besides extending previous observations on the role of CENP-C in the formation of active centromeres, show, for the first time, that CENP-C can associate with the centromeric DNA sequences in vivo and, together with CENP-B, defines a highly structured organization of the alpha-satellite DNA within the human centromere.

Key words: Alpha-satellite DNA, CENP-C, CENP-B, Centromere, Chromatin-immunoprecipitation

## Introduction

Centromeres are essential nucleo-proteinaceous structures required for the proper segregation of eukaryotic chromosomes during mitosis and meiosis. The centromere region is structurally and functionally complex being composed of at least three main domains: the kinetochore, the central domain and the pairing domain (Earnshaw and Rattner, 1989). Electron microscopy studies have shown that the kinetochore, the structure responsible for the attachment and movement of chromosomes along the microtubules of the spindle, is constituted by two electron-dense layers (inner and outer plates) separated by an electron translucent layer called the middle plate (Rieder, 1982). Only the inner plate contains both proteins and DNA, while the other two plates seem to be formed largely of proteins (Cooke et al., 1993). The central domain contains highly condensed constitutive heterochromatin and is located just beneath the kinetochore (Pluta et al., 1990), whereas the inner pairing domain is necessary for sister-chromatid cohesion (Vagnarelli and Earnshaw, 2001). At a molecular level, the human centromere mainly consists of alpha-satellite DNA arrays complexed to a number of different proteins whose role in centromere assembly and function is still unclear. The alpha-satellite DNA

is present at all centromeres of human chromosomes and composed of tandemly repeated units of 171 bp that can extend from 100 kb up to several megabases of DNA (Wu and Manuelidis, 1980; Willard, 1985). The organization of alpha-satellite DNA arrays is highly structured and can differ from one chromosome to another (Willard and Wayne, 1987).

Recent observations indicate that the alpha-satellite DNA may play a pivotal role in the organization of the human centromeres. In fact, at least three important centromeric proteins bind the alpha-satellite DNA. CENP-B, a nonessential protein situated in the central domain, recognizes a specific 17 bp sequence (CENP-B box) within the alpha-satellite repeats (Cooke et al., 1990; Muro et al., 1992; Pluta et al., 1992; Yoda et al., 1992) and may facilitate the packaging of the alpha-satellite arrays. Poly(ADP-ribose)polymerase (PARP) binds to a 9 bp sequence (pJ $\alpha$  box) in alpha-satellite DNA (Earle et al., 2000). PARP binds preferentially to active centromeres and to a sequence in cloned human neocentromere DNA, but its role at centromeres is not yet known. CENP-A is an essential histone H3-like protein, which localizes to active centromeres at the inner plate of the kinetochore (Earnshaw and Rothfield, 1985; Warburton et al., 1997). CENP-A is specifically associated with alpha-satellite DNA in vivo although it is not

yet clear whether there are sequences that it preferentially recognizes (Vafa and Sullivan, 1997).

Other proteins have been found in the inner plate of the kinetochore, which participate in centromere organization. For instance, CENP-C is an essential factor involved in the assembly of the kinetochore and in the correct segregation of sister chromatids (Saitoh et al., 1992; Tomkiel et al., 1994; Fukagawa and Brown, 1997; Kalitsis et al., 1998; Fukagawa et al., 1999). Because of its localization, it has been long hypothesized that it might recognize the centromeric DNA (Sugimoto et al., 1999). Several *in vitro* studies have established that it is possible to define a minimal domain of CENP-C that binds DNA (Sugimoto et al., 1994; Yang et al., 1996; Sugimoto et al., 1997). This binding domain seems to overlap with the domain required for the targeting of CENP-C to the centromere *in vivo* (Yang et al., 1996). However, several attempts to identify *in vitro* putative CENP-C-binding sequences have failed. This scenario is complicated by the observation that CENP-C, CENP-A and PARP also associate with active neocentromeres that apparently do not contain repetitive sequences typical of conventional centromeres, suggesting that their localization to centromere may occur through epigenetic mechanisms (Choo, 1997; Depinet et al., 1997; Choo, 2000; Sullivan, 2001).

To study the role of CENP-C in centromere formation, we took advantage of a chromatin-immunoprecipitation assay (ChIP) that allows the analysis of specific DNA-protein complexes *in vivo* (Orlando et al., 1997). This technique has been successfully used to study the DNA-binding activity of more than 40 proteins, including general transcription factors, trans-activators, repressors and structural components of chromatin (Solomon et al., 1988; Dedon et al., 1991; Strutt and Paro, 1998; Chen et al., 1999; Hsu et al., 1999; Parekh and Maniatis, 1999; Tanaka et al., 1999; Orlando, 2000; Partridge et al., 2000; He et al., 2001). Moreover, a thorough study by Toth and Biggin indicates that formaldehyde crosslinking occurs mainly at the protein-DNA interface and reflects the *in vivo* binding of a specific factor to its cognate DNA sites (Toth and Biggin, 2000). On the basis of previous results showing that CENP-C can both bind chromosomal DNA *in vitro* and co-localize with centromeric DNA in interphase nuclei, we decided to use the ChIP assay to examine the dynamics of how CENP-C associates to the centromeric DNA sequences *in vivo*.

## Materials and Methods

### Chromatin-immunoprecipitation assay

#### Formaldehyde crosslinking

HeLa cells, grown in DMEM medium supplemented with 10% (v/v) FCS (Life Technologies) and 2 mM L-glutamine, were processed for a crosslinking and immunoprecipitation experiment.  $2 \times 10^8$  cells were treated *in situ* for crosslinking by adding formaldehyde to a 1% final concentration directly to the culture medium and incubated at room temperature for 15 minutes. The crosslinking reaction was stopped by adding glycine to a final concentration of 0.125 M. After 5 minutes, cells were washed twice with  $1 \times$  PBS and collected in 5 ml of TES buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 150 mM NaCl and 1 mM PMSF). After centrifugation, the cell pellet obtained from 10 plates was resuspended in 2 ml of FA lysis buffer (50 mM Hepes, 200 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 1 mM PMSF and Roche Protease inhibitors mix). Cells were sonicated eight times for 8 seconds at medium sonication

intensity. Afterwards the lysate was transferred to eppendorf tubes and centrifuged for 10 minutes at 10,000 *g* at 4°C. The supernatant was recovered and 200  $\mu$ l of lysate were subjected to immunoprecipitation with 5  $\mu$ l of rabbit pre-immune serum (PI serum) or 2  $\mu$ g of polyclonal antiserum against CENP-B, CENP-C, ScII and the HA tag (Santa Cruz). A 200  $\mu$ l aliquot of lysate was set apart and further used as a control of the total chromatin input in the immunoprecipitation experiments. After 5 hours of incubation, 50  $\mu$ l of a 50% slurry trisacryl-proteinA beads solution (Pierce Chemical Co., Rockford, IL) were added to each lysate sample and incubated for 1 hour at 4°C at a constant rotation. The antibody-chromatin complex was then recovered by centrifugation and washed twice with 1 ml of FA lysis buffer, once with 1 ml of buffer FW3 (10 mM Tris-HCl pH 8.0, 0.25 M LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA, 1 mM PMSF and protease inhibitors) and once with 1 ml of TE buffer. To reverse the crosslinking, the beads were resuspended in 200  $\mu$ l of PK buffer with 150  $\mu$ g/ml of proteinase K (Roche) and incubated at 65°C for at least 6 hours on a thermomixer (Eppendorf). DNA was then phenol-chloroform treated and ethanol precipitated with the addition of glycogen as a carrier. The control chromatin input has been similarly treated.

### UV crosslinking

$2 \times 10^8$  HeLa cells were grown for 24 hours in DMEM supplemented with 2 mM L-glutamine, 10% FCS and 20  $\mu$ M 5-bromo-2-deoxyuridine. Cells were trypsinized, collected in a 50 ml falcon tube, washed three times with cold  $1 \times$  PBS and resuspended in 8 ml of  $1 \times$  PBS. They were transferred into a 100 mm petri dish, kept on ice and directly exposed for 15 minutes to a source of 366 nm UV light positioned at a distance of about 3 cm from the dish. Cells were finally collected, washed once with  $1 \times$  PBS, resuspended in 2 ml of FA lysis buffer and from here on treated by following the same steps of the formaldehyde crosslinking procedure.

### Dot blot and Southern blot analysis of immunoprecipitated DNA

Total input DNA and immunoprecipitated DNA samples were digested with *Eco*RI, resolved on 1.5% agarose gel and transferred to a Hybond-N<sup>+</sup> filter (Amersham Pharmacia Biotech). The filter was pre-hybridized and hybridized with a solution containing 7% SDS and 0.5 M Na<sub>3</sub>PO<sub>4</sub>, pH 7.0. Probes were [ $\alpha$ -<sup>32</sup>P]-labeled with the Megaprime kit (Amersham) and added to the hybridization solution at a specific activity of  $2 \times 10^9$  cpm/ $\mu$ g. The alpha-satellite DNA probes (Archidiacono et al., 1995) used for the hybridization were: (1) pZ7.6B (680 bp) detecting chromosome 7; (2) pZ21.A (850 bp) detecting chromosomes 13 and 21; (3) pZ17-1.6A (1.02 kb) detecting chromosome 17; (4) pZ5.1 (680 bp) detecting chromosomes 1, 5 and 19; (5) pZ8.4 (1.2 kb) detecting chromosome 8; (6) pDMX1 (2 kb) detecting chromosome X. Other probes recognizing non-centromeric sequences were: BLUR-8 (300 bp) detecting Alu repeats (Jelinek et al., 1980); and p(291)LSau (291 bp) detecting beta-satellite (Agresti et al., 1987). Dot blot analysis was performed by transferring total input DNA and the immunoprecipitated DNA samples onto a Hybond-N<sup>+</sup> filter (Amersham) with a Bio-Dot Apparatus (Bio-Rad). The filter was pre-hybridized and hybridized using the same conditions as for the Southern blots.

### Semiquantitative PCR

The region containing part of the human renin gene promoter was amplified by using primer REF: 5'-CAGCTGTTGCTTTTCCTGCC-3' and RER: 5'-AAACAGCACTGTACAGGGCTA-3'. Amplification conditions were as follows: 94°C for 30 seconds, 58°C for 15 seconds and 72°C for 30 seconds. 10  $\mu$ l aliquots of the PCR products were

taken after 30, 35 and 40 cycles respectively, run on 2% agarose gel and visualized by ethidium bromide.

#### Construction and expression of CENP-C mutants

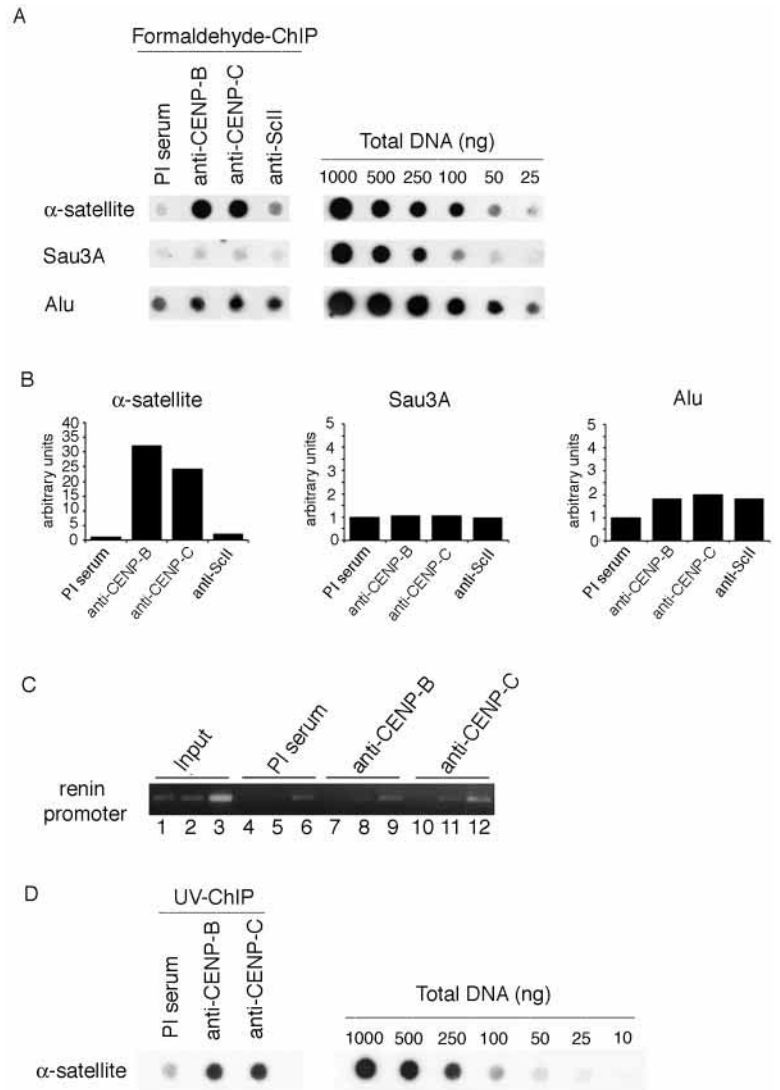
Expression constructs encoding the HA-tagged truncations of CENP-C were generated by PCR as follows. HA::23/943: primer 23F, 5'-AAAGGGGGATCCGCACGTGACATTAA-CACAGAG-3' and primer 943R, 5'-AAAGGGGAATTCTCATCTTTTATCTGAGTAAAAAG-3'; HA::192/537: primer 192F, 5'-AAAGGGGGATCCATGCTGCCTTCAA-GTACAGAGG-3' and primer 537R, 5'-AAAGGGGAATTCTCACTCCTCTGATTTTACCACCC-3'; HA::23/410: primer 23F and primer 410R, 5'-AAAGGGGAATTCTCA-TGGTTTTCTGCATTCTTGG-3'.

PCR products were digested with *Bam*HI and *Eco*RI and directionally cloned into the pCDNA3.1 vector, in frame with a HA-tag located at the N-terminal of the proteins. For each mutant, 20 µg of expression construct were used to transfect HEK293T cells by means of the lipofectamin method (Roche). HEK293T cells were grown in DMEM medium supplemented with 10% FCS, 2 mM L-glutamine. Expression levels of the truncated proteins were tested by western blot. Transfected cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% N-dodecylsulfate, 0.1% SDS) and sonicated. 20 µg of total cell extract were separated on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The filter was blocked with TBS (150 mM NaCl, 25 mM Tris-HCl pH 8.0) and 8% nonfat dry milk. After incubation with the specific antibodies, the filter was washed three times with TBST (TBS plus 0.1% Tween-20) and incubated with anti-rabbit peroxidase-conjugated secondary antibodies (Amersham). Proteins were revealed by ECL (Amersham).

#### Ultrastructural immunocytochemistry

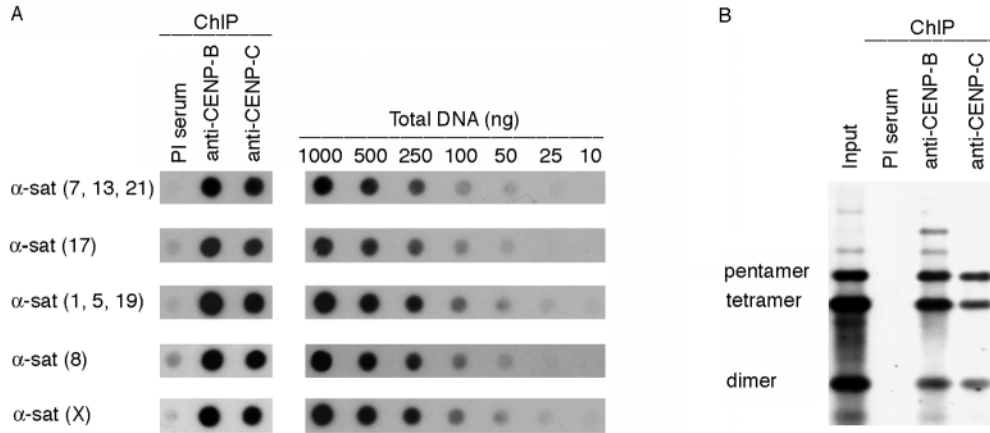
Subconfluent HeLa cells, grown as previously described, were fixed overnight in 4% paraformaldehyde in 0.2 M PIPES, pH 7.0 at 4°C. After rinsing in cold PBS, the cells were scraped and pelleted at low speed. Pelleted cells were infused with a mixture of sucrose and polyvinylpyrrolidone before freezing, as described (Tokuyasu, 1989). Thin cryosections were cut with a Drukker diamond knife (Drukker Co., Almere, The Netherlands) on a Reichert Ultracut S ultramicrotome (Leica) equipped with cryoattachment.

Grids with sections were first incubated in 0.2% BSA-c (Aurion, Wageningen, The Netherlands) to reduce unspecific labeling. For the CENP-B and CENP-C mapping, the respective sera were diluted 1:100 in PBS containing 0.1% BSA-c. The bound antibodies were revealed by 6 or 10 nm protein A-gold complexes (Aurion). In the co-localization experiments, consecutive immunolabeling of the two CENPs was performed as described (Griffiths, 1993). The results of parallel labelings (i.e. single labeling, as well as double labeling with interchanged sizes of the gold complexes for the two CENPs), gave consistent results. In the negative control experiments, the primary antibodies were omitted; the positive control involved an unrelated rabbit p80-coilin antibody kindly provided by E. K. Chan, The Scripps Research Institute, La Jolla, CA (Andrade et al., 1993). Antibodies and protein A-gold complexes were incubated for 60 minutes. After the labeling was completed, sections were fixed with 1% glutaraldehyde in PBS for 20 minutes and treated for 15 minutes in 2% OsO<sub>4</sub> in PBS. Sections were extensively washed in water and postembedded in 0.3% uranyl acetate in



**Fig. 1.** CENP-C binds the alpha-satellite DNA in vivo. (A) HeLa cells were formaldehyde treated and chromatin was immunoprecipitated (Formaldehyde-ChIP) with the different sera. After reversal of crosslinking, the immunoprecipitated DNA samples were analyzed by dot blot. The level of enrichment of different human highly repetitive sequences is shown (left). Decreasing amounts of total DNA are included to provide an internal quantitation of the hybridization signals (right). The filters were sequentially hybridized with the following [ $\alpha$ -<sup>32</sup>P]-labeled DNA probes: pZ7.6B (680 bp) and pZ21.A (850 bp) alpha-satellite DNAs, specific for chromosomes 7, 13 and 21; Sau3A repeat (291 bp) and Alu repeat (300 bp). PI serum (rabbit pre-immune serum), anti-CENP-B, anti-CENP-C and anti-ScII indicate the DNA recovered with the corresponding antibodies. (B) Densitometric quantitation of the hybridization signals. The intensity of the hybridization signal in the pre-immune samples was set to 1 arbitrary unit. (C) Level of enrichment of a single copy sequence (renin promoter region ~350 bp) tested by semiquantitative PCR in the different immunoprecipitated samples. For each sample, three conditions of PCR (30-35-40 cycles) were used. Lanes 1-3, input (1 ng); lanes 4-6, PI serum; lanes 7-9, rabbit anti-CENP-B serum; lanes 10-12, rabbit anti-CENP-C serum. (D) HeLa cells were exposed to UV light for 15 minutes, lysed and chromatin was immunoprecipitated with the different sera. Immunoprecipitated DNA samples were hybridized with a cocktail of the pZ76.B and pZ21.A probes.

polyvinyl alcohol (Tokuyasu, 1989). Grids were observed by EM Zeiss 109.



**Fig. 2.** CENP-B and CENP-C bind the alpha-satellite DNA of different human chromosomes and recognize the same subfamilies of DNA sequences. (A) Filters were prepared as described in Fig. 1A and sequentially hybridized with alpha satellite probes recognizing, at high stringency, only a subset of human centromeric sequences. The specific chromosomes recognized by each probe are indicated. PI serum (pre-immune serum), anti-CENP-B and anti-CENP-C indicate the DNA recovered with the corresponding antibodies. (B) HeLa cells were formaldehyde-treated

and chromatin was immunoprecipitated with the different sera. After reversal of crosslinking, DNA was digested with *EcoRI*, run on a 1.5% agarose gel and then blotted onto nylon membrane. Alpha-satellite DNA was revealed by hybridizing the filter with probes specific for alpha-satellite of chromosomes 7, 13 and 21. Dimers, tetramers and pentamers are multimers of the 171 bp alpha-satellite monomeric unit.

To estimate the mutual distribution of CENP-C and CENP-B, the most external CENP-B (or CENP-C) particles were joined by straight lines and the CENP-C (or CENP-B) particles inside and outside the formed polygon were counted. Data from 65 centromeres were evaluated using the Wilcoxon rank test.

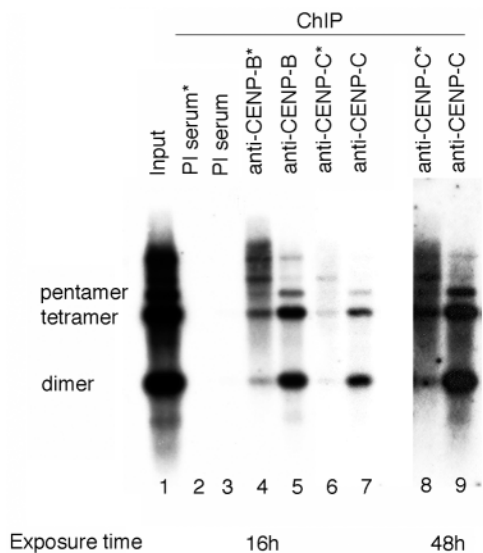
## Results

### CENP-C binds the alpha-satellite DNA in vivo

To analyze the centromeric DNA associated with CENP-C in vivo we have performed a chromatin-immunoprecipitation assay (ChIP) on HeLa human cells. Cells were treated with formaldehyde to allow the formation of crosslinks between DNA and its associated proteins. Chromatin was sonicated to reduce the DNA size to an average of 0.5-5 kb and then immunoprecipitated with a rabbit polyclonal anti-CENP-C serum. As a positive control, DNA was also immunoprecipitated with a rabbit polyclonal anti-CENP-B serum, since CENP-B is known to bind in vitro a 17 base-pair sequence within the alpha-satellite DNA. To validate the specificity of the co-immunoprecipitation we also performed a ChIP assay using an anti-ScII serum. ScII (SMC2) is a chromosome scaffold protein that binds along the chromosome arms, including the centromere, without displaying any DNA sequence specificity (Saitoh et al., 1994). Thus the immunoprecipitation with anti-ScII serum should not show any preferential enrichment for specific DNA sequences. After reversal of crosslinking, all immunoprecipitated DNAs and decreasing amounts of total input DNA (from 1  $\mu$ g to 25 ng), used as a calibrated standard of the hybridization, were transferred onto a nylon filter by dot blot. The filter was sequentially hybridized with DNA probes detecting alpha-satellite DNA of chromosomes 7, 13 and 21 (pZ7.6B and pZ21.A), satellite Sau3A and Alu sequences, the latter representative of non-centromeric repetitive elements (Jelinek et al., 1980; Agresti et al., 1987; Wayne et al., 1987; Wevrick and Willard, 1991; Wevrick et al., 1992; Archidiacono et al., 1995; Harrington et al., 1997; Ikeno et al., 1998; Masumoto et al., 1998). The results of Fig. 1A show that anti-CENP-C serum, among different types of repetitive DNA sequences, can immunoprecipitate the alpha-satellite DNA specifically.

Densitometric analysis of the alpha-satellite DNA recovered after co-immunoprecipitation shows that the enrichment is around 32 and 24-fold, respectively, for anti-CENP-B and anti-CENP-C samples (Fig. 1B), whereas the enrichment for the Sau3A and Alu repetitive sequences was not significantly different from that of the controls (PI serum and anti-ScII). Furthermore, to exclude the possible enrichment of other types of sequences such as single copy genes, we used semi-quantitative PCR to amplify the promoter region of the human renin gene, known to map to chromosome 1q32 (Cohen-Haguenaer et al., 1989). Fig. 1C shows that the renin promoter region is not enriched in the anti-CENP-B and anti-CENP-C samples (compare lanes 6, 9 and 12). These results indicate that CENP-B and CENP-C can bind DNA in vivo and provide strong evidence that CENP-C associates with the centromeric alpha-satellite DNA.

Although the formaldehyde concentration used in this work has been widely employed to study protein-DNA interactions in vivo we cannot exclude the possibility that it may induce covalent links between proteins and thus may have the potential to connect proteins to DNAs that they do not directly contact (Strahl-Bolsinger et al., 1997). To demonstrate that the results obtained with the formaldehyde crosslinking reflect a direct interaction of CENP-C to the alpha-satellite DNA, we used an alternative procedure based on an in vivo UV crosslinking assay (Rzepecki et al., 1998). It is well established that UV light can generate covalent links either between dimers of pyrimidines or between DNA and proteins directly associated to it (Rzepecki et al., 1998; Toth and Biggin, 2000). HeLa cells were grown for 24 hours in the presence of 5-bromo-2-deoxyuridine before being exposed for 15 minutes to a 366 nm UV light. 5-bromo-2-deoxyuridine can replace thymidine during DNA replication and is more efficient than thymidine at forming covalent links when exposed to UV light. Since the alpha-satellite DNA possesses a high A+T content, the use of 5-bromo-2-deoxyuridine should increase the chances of crosslinking CENP-C to the alpha-satellite DNA. Cells were lysed and chromatin was then immunoprecipitated and processed by following the same steps described for the formaldehyde crosslinking. Immunoprecipitated DNAs, transferred onto a nylon filter by dot blot, were hybridized with



**Fig. 3.** CENP-C recognizes DNA within the alpha-satellite arrays. Immunoprecipitated DNA samples were digested with *EcoRI* either before (lanes marked with an asterisk) or after the reversal of crosslinking. Alpha-satellite DNA was detected as previously described. For the immunoprecipitation with anti-CENP-C antibodies, two exposure times of the filter are presented: 16 hours and 48 hours. The alpha-satellite immunoprecipitated with anti-CENP-C and anti-CENP-B antibodies show a very similar band profile when digested with *EcoRI* either before or after the reversal of crosslinking.

a cocktail of the [ $\alpha$ - $^{32}$ P]-labeled pZ7.6B and pZ21.A probes. Fig. 1D shows that both anti-CENP-B and anti-CENP-C antibodies can specifically immunoprecipitate the alpha-satellite DNA. The results of the UV crosslinking indicate that CENP-C, as well as CENP-B directly contacts the alpha-satellite DNA *in vivo*.

The organization of alpha-satellite DNA arrays is highly structured and can differ from one chromosome to another. Moreover, each chromosome carries particular arrays of alpha-satellite DNA sequences that can be revealed by using specific DNA probes. To demonstrate the general nature of the CENP-C binding to the alpha-satellite DNA of human chromosomes, a set of filters carrying the immunoprecipitated DNA samples, along with internal standards, were hybridized with chromosome-specific centromeric DNA probes. The results of Fig. 2A show that CENP-C binds the alpha-satellite DNA of all tested chromosomes.

The different localization of CENP-B and CENP-C in the centromere of metaphase chromosomes (Cooke et al., 1990; Saitoh et al., 1992; Sugimoto et al., 1999) suggests that the two proteins may recognize different subtypes of alpha-satellite DNAs. Conversely, our results indicate that both CENP-B and CENP-C seem to bind the same alpha-satellite DNA higher order repeats. However, the dot blot analysis does not allow a precise discrimination between different subfamilies of alpha-satellite DNA of a given chromosome. To address this issue we have compared the profile of the alpha-satellite DNA immunoprecipitated by both anti-CENP-B and anti-CENP-C antibodies after digestion with *EcoRI*, known to display the long-range periodicity of the alpha-satellite DNA of

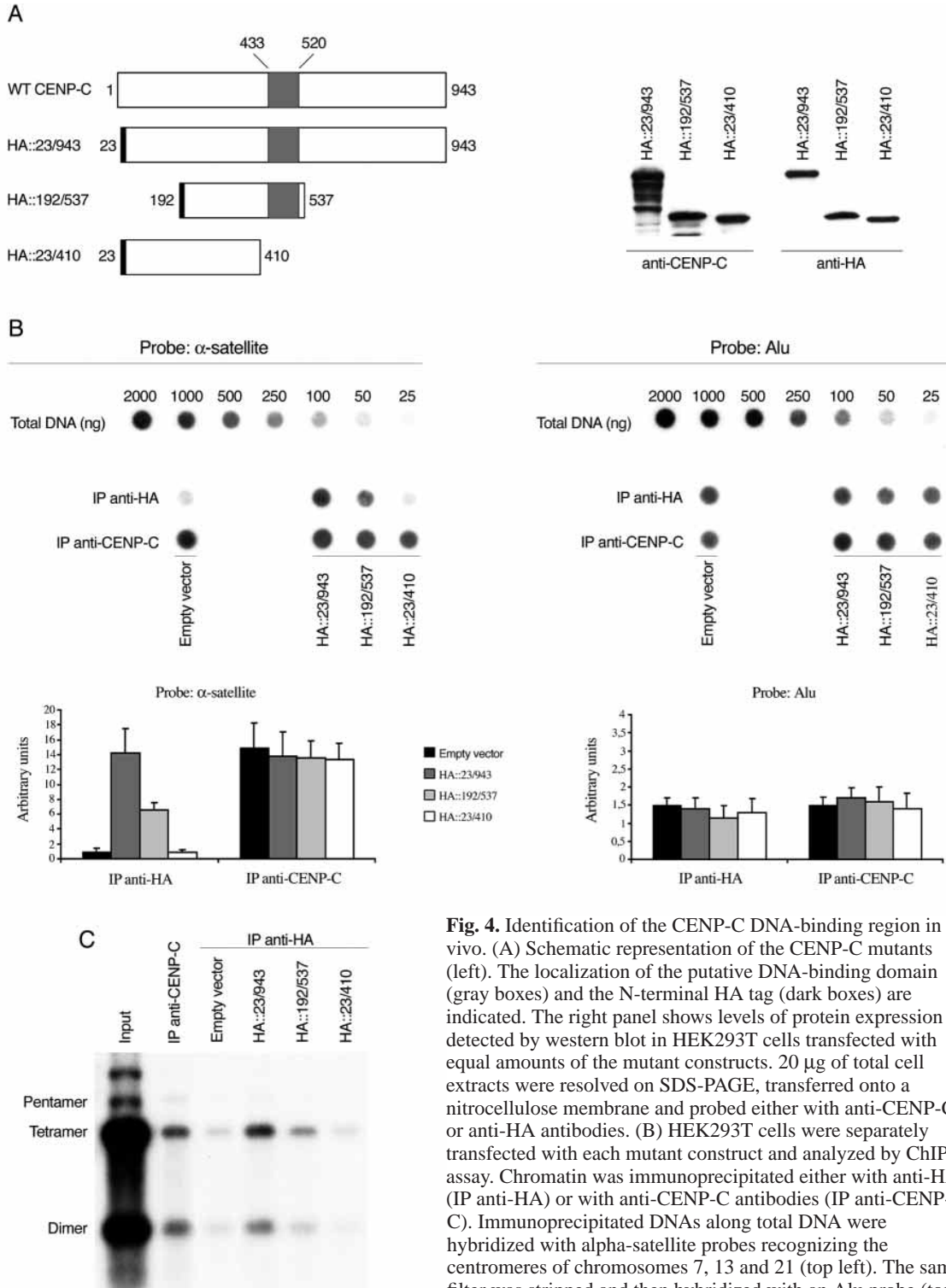
chromosomes 7, 13 and 21. The DNA, run on a 1.5% agarose gel and blotted onto a nylon membrane, was hybridized with a cocktail of the pZ7.6B and pZ21A DNA probes. Fig. 2B shows that both CENP-B and CENP-C recognize the same dimeric, tetrameric and pentameric subtypes of alpha-satellite DNA.

The DNA region associated with CENP-C are situated within the alpha-satellite arrays

Although anti-CENP-C antibodies specifically immunoprecipitate chromatin fragments containing the alpha-satellite DNA, we cannot exclude the possibility that such an interaction may occur in different DNA regions within these fragments that flank or are interspersed in the alpha-satellite arrays. To address this question we have modified the ChIP assay as follows. Before reversal of the crosslinking, DNA was digested with *EcoRI* when the Ig-CENP-C-DNA complexes were still immobilized on the trisacryl-proteinA beads (see Materials and Methods). Furthermore, the complexes were thoroughly washed with FA buffer to remove the DNA fragments released by *EcoRI*. The digestion of the immunoprecipitated DNA would be expected to generate two alternative and mutually exclusive patterns depending on where CENP-C binds the DNA. If CENP-C binds the DNA within the alpha-satellite, then the alpha-satellite arrays would remain part of the complexes retained on the beads and would be further recovered and purified. On the contrary, if CENP-C binds sequences adjacent to or interspersed in the alpha-satellite arrays, then the alpha-satellite DNA would be released from the beads upon *EcoRI* digestion, lost during the extensive washings and thus undetected by Southern blot hybridization. Although the digestion was not complete, probably because chromatin proteins crosslinked to DNA partially prevent the access of the endonuclease to its cognate restriction sites, Fig. 3 shows that the alpha-satellite-CENP-C complexes were always retained on the beads in spite of the fact that the *EcoRI* digestion was performed either before or after the reversal of crosslinking (Fig. 3, compare lanes 6 and 7, and, at a longer exposure, lanes 8 and 9). As a control, we analyzed CENP-B that binds DNA inside the alpha-satellite. As expected, the profile of the alpha-satellite DNA recovered by anti-CENP-B antibodies is unchanged in both conditions (lanes 4,5). Moreover, the DNA profiles of the alpha-satellite DNA bound by CENP-B and CENP-C were practically identical (lanes 4,8). Taken together these results suggest that CENP-C binds DNA within the alpha-satellite DNA arrays.

#### Definition of the CENP-C domain needed to bind the alpha-satellite DNA *in vivo*

To define the CENP-C domain required to bind the alpha-satellite DNA, we analyzed the binding activity of specific CENP-C mutants *in vivo*. We have used these mutants essentially as controls to confirm that the DNA-binding domain of CENP-C defined on the basis of *in vitro* results was also involved in the *in vivo* binding of CENP-C to DNA. The CENP-C mutants were chosen and designed based on *in vitro* results showing that the CENP-C DNA-binding activity resides in the central region of the protein (Sugimoto et al., 1994; Yang et al., 1996; Sugimoto et al., 1997), approximately between amino



**Fig. 4.** Identification of the CENP-C DNA-binding region in vivo. (A) Schematic representation of the CENP-C mutants (left). The localization of the putative DNA-binding domain (gray boxes) and the N-terminal HA tag (dark boxes) are indicated. The right panel shows levels of protein expression detected by western blot in HEK293T cells transfected with equal amounts of the mutant constructs. 20  $\mu$ g of total cell extracts were resolved on SDS-PAGE, transferred onto a nitrocellulose membrane and probed either with anti-CENP-C or anti-HA antibodies. (B) HEK293T cells were separately transfected with each mutant construct and analyzed by ChIP assay. Chromatin was immunoprecipitated either with anti-HA (IP anti-HA) or with anti-CENP-C antibodies (IP anti-CENP-C). Immunoprecipitated DNAs along total DNA were hybridized with alpha-satellite probes recognizing the centromeres of chromosomes 7, 13 and 21 (top left). The same filter was stripped and then hybridized with an Alu probe (top right). A histogram representation of the relative enrichment of each mutants is shown. For each specific condition of immunoprecipitation (IP anti-HA or IP anti-CENP-C), the enrichment (expressed in arbitrary units) is defined as the ratio of the hybridization signal obtained for each sample versus the hybridization signal obtained from cells transfected with the empty vector and subjected to IP with the anti-HA antibody (IP background). The standard error was calculated on the results of three independent experiments. (C) Wild-type and truncated forms of the CENP-C protein are shown to bind the same alpha-satellite subfamilies. Chromatin of HEK293T cells transfected with the empty vector was immunoprecipitated either with anti-CENP-C or with anti-HA antibodies. The mutant proteins were immunoprecipitated with anti-HA antibodies. The analysis of the DNA profile was performed as described in Fig. 2.

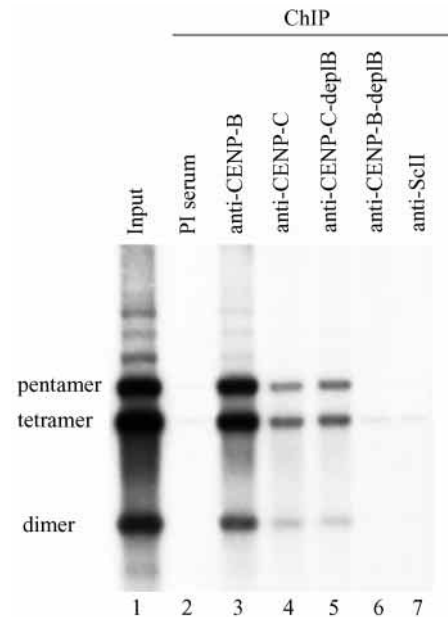
alpha-satellite (bottom left) and Alu sequences (bottom right) obtained for each mutants is shown. For each specific condition of immunoprecipitation (IP anti-HA or IP anti-CENP-C), the enrichment (expressed in arbitrary units) is defined as the ratio of the hybridization signal obtained for each sample versus the hybridization signal obtained from cells transfected with the empty vector and subjected to IP with the anti-HA antibody (IP background). The standard error was calculated on the results of three independent experiments. (C) Wild-type and truncated forms of the CENP-C protein are shown to bind the same alpha-satellite subfamilies. Chromatin of HEK293T cells transfected with the empty vector was immunoprecipitated either with anti-CENP-C or with anti-HA antibodies. The mutant proteins were immunoprecipitated with anti-HA antibodies. The analysis of the DNA profile was performed as described in Fig. 2.

acid 433 and 520 (Yang et al., 1996). Specifically, we have generated three HA-tagged CENP-C mutants either carrying (HA::23-943; HA::192-537) or missing (HA::23-410) the putative DNA-binding region. All truncated CENP-C mutant proteins (Fig. 4A, left) were expressed at a comparable level in transfected HEK293T cells and could be recognized by both anti-CENP-C and anti-HA antibodies in western blot (Fig. 4A, right). According to previous studies (Yang et al., 1996), all three mutants accumulate in the nucleus and specific staining of the centromeres could be clearly detected only in the cells expressing the mutants carrying the putative DNA-binding region (HA::23-943; HA::192-537) (data not shown). To investigate the binding activity of the truncated proteins to the alpha-satellite DNA we have applied the ChIP assay to HEK293T cells transfected with each of the described constructs. DNA, immunoprecipitated with anti-HA antibodies, was analyzed with a mixture of the pZ7.6.B and pZ21.A alpha-satellite DNA probes. Decreased amounts of total input DNA were also included in the filter. As shown in Fig. 4B (left), anti-HA antibodies recover the alpha-satellite DNA from cells transfected with the HA::23-943 and HA::192-537 constructs but not from cells transfected with the HA::23-410 one. To exclude that the overexpression of the different mutant proteins may affect the interaction of CENP-C with the alpha-satellite DNA, transfected cells were also subjected to immunoprecipitation with anti-CENP-C antibodies. The anti-CENP-C antibodies cannot discriminate between endogenous and truncated CENP-C proteins and thus, if transfection did not alter chromatin organization, the amount of recovered alpha-satellite DNA should be roughly identical in all cell samples. The results of Fig. 4B (left) show that this was the case. Furthermore, the background level, determined by the Alu hybridization, is similar in all DNA samples either immunoprecipitated with anti-HA or anti-CENP-C antibodies (Fig. 4B, right). The relative enrichment of both alpha-satellite DNA and Alu sequences is illustrated in the two graphs in Fig. 4B (bottom). Finally, we have verified whether the overexpression of CENP-C mutants might in some way alter the typical *EcoRI* profile observed with the pZ7.6.B and pZ21.A probes. The results of Fig. 4C indicate that the wild-type and CENP-C mutants, HA::23-943 and HA::192-537, bind the same subfamilies of alpha-satellite DNA, whereas the HA::23-410 mutant cannot. In addition, this excludes the possibility that the simple overexpression of the CENP-C proteins can interfere with their correct loading onto the centromeric DNA.

Our results show that the differences in the amount of alpha-satellite DNA recovered by the CENP-C mutants seem to reflect their intrinsic ability to bind the alpha-satellite DNA and confirm that the central region of the CENP-C is indeed required to perform this function in vivo.

#### CENP-B and CENP-C localize to alpha-satellite arrays of different centromere domains

The fact that both CENP-B and CENP-C can be crosslinked to the same alpha-satellite subfamilies suggests that CENP-C and CENP-B may co-localize within the alpha-satellite arrays. This, however, appeared to be in contrast with what has been previously observed by immunoelectron microscopy (Cooke et al., 1990) and immunofluorescence (Sugimoto et al., 1999), where CENP-B and CENP-C seem to occupy distinct structural



**Fig. 5.** CENP-B and CENP-C define different alpha-satellite domains. Cell extracts from crosslinked cells were treated for immunoprecipitation with pre-immune serum (lane 2), anti-CENP-B (lane 3), anti-CENP-C (lane 4) or anti-ScII (lane 7) antibodies, or first immunodepleted for the DNA–CENP-B complexes and then subjected to immunoprecipitation with anti-CENP-C (lane 5). As a control, a sample immunodepleted with anti-CENP-B antibodies was subjected to a second round of immunoprecipitation with the same antibody to verify the complete depletion of the DNA–CENP-B complex (lane 6). Alpha-satellite DNA, digested with *EcoRI*, was detected as described in Fig. 2.

domains in the kinetochore/centromere complex of mitotic chromosomes. To investigate this apparent discrepancy we have used a modified version of the ChIP assay. First, crosslinked HeLa cell extracts were immunodepleted, by using anti-CENP-B antibodies, for the DNA–CENP-B complexes. One round of immunoprecipitation with anti-CENP-B antibodies was sufficient to abate the crosslinked DNA–CENP-B complexes to background levels (Fig. 5, compare lane 6 with lane 2). Following the depletion of the DNA–CENP-B complexes, the extract was subjected to immunoprecipitation with anti-CENP-C antibodies. The amounts of DNA–CENP-C complexes recovered before and after the immunodepletion of the CENP-B–DNA complexes were compared. Fig. 5 shows that the amount and the profile of alpha-satellite DNA immunoprecipitated by anti-CENP-C antibodies are not affected by immunodepletion of the DNA–CENP-B complexes (Fig. 5, compare lanes 4 and 5). A similar outcome was obtained for the DNA–CENP-B complexes in the reverse experiment (data not shown). These results strongly suggest that CENP-B and CENP-C localize to distinct non-overlapping centromere domains composed of the same subfamilies of higher order alpha-satellite repeats.

Previous immunoelectron analysis performed on mitotic chromosomes has shown that CENP-B and CENP-C seem to occupy distinct structural domains in the kinetochore (Cooke et al., 1990). However, the relative distribution of the two proteins at the mitotic kinetochore-centromere complex might be masked by the folding of the chromatin fiber in the complex.



**Fig. 6.** CENP-B and CENP-C localize to different centromere domains in interphase chromosomes. (A-F) Immunoelectron microscopy showing simultaneous colocalization of CENP-B (10 nm gold particles) and CENP-C (6 nm gold particles). Each micrograph shows the centromere of an unidentified chromosome from a single cell nucleus. (A'-F') The maximum perimeter occupied by the CENP-B gold particles is marked for illustrative purposes. CENP-C particles within this perimeter are marked in red, while those outside the perimeter are marked in green. The bar in panel F' represents 100 nm.

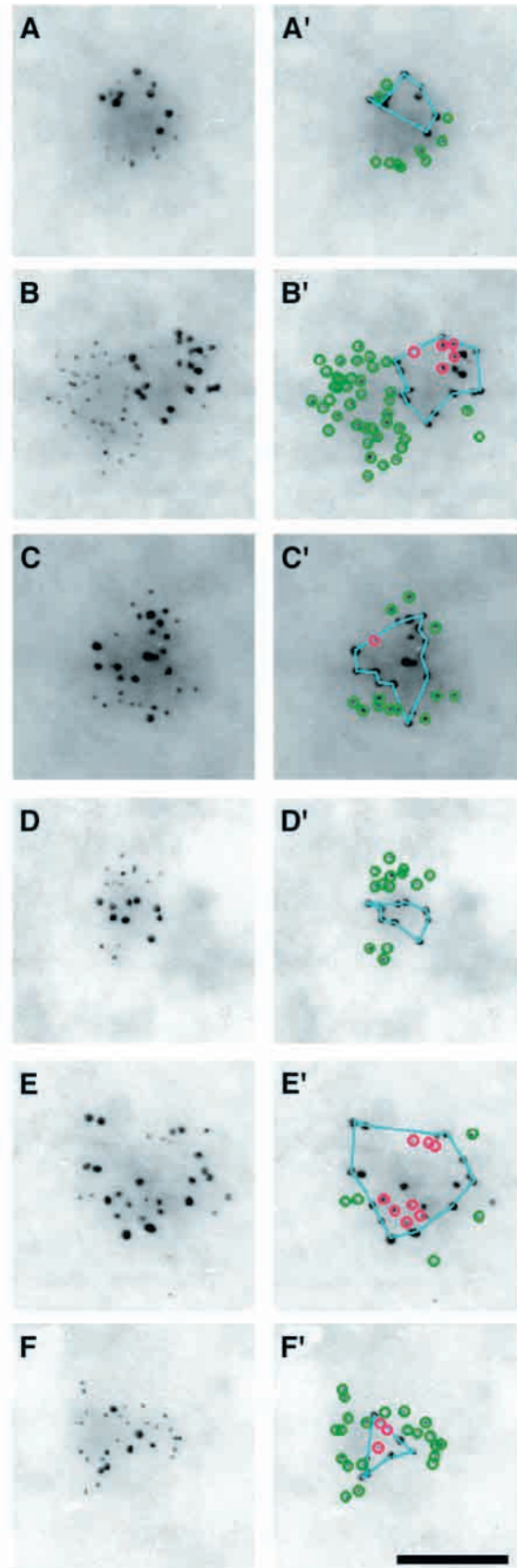
To overcome this problem, we analyzed the relative distribution of the two proteins by double-label immunoelectron microscopy on ultrathin cryosections of interphase HeLa cells.

In this analysis only one section per given cell was considered and this section was more than two orders of magnitude thinner than the cell nucleus. Each micrograph of Fig. 6 shows the centromere of an indiscriminate chromosome from a single cell nucleus. Double labeling of CENP-B (10 nm) and CENP-C (6 nm) proteins with colloidal gold particles resulted in their co-localization in clusters in which the two labels were not randomly interspersed (Fig. 6A-F). A morphometric analysis (see Materials and Methods) showed that CENP-B was concentrated in the interior of the clusters, while CENP-C localized predominantly to the periphery. Specifically, an average of  $7.17 \pm 0.62$  (mean  $\pm$  s.e.) of CENP-B-associated gold particles were located in the interior, while  $2.54 \pm 0.35$  were found in the exterior of these domains. Vice versa,  $1.48 \pm 0.23$  of CENP-C associated particles were located in the interior, while  $11.47 \pm 0.97$  CENP-C were in the exterior. According to the Wilcoxon rank test, the difference between CENP-B and CENP-C localization in the centromeric domain analyzed is significant, with  $P < 0.05$ . Similar results were obtained if the colloidal gold probes were switched (data not shown). These data reinforce the notion that CENP-B and CENP-C occupy distinct domains of the interphase centromere.

## Discussion

Our study shows for the first time that CENP-C functions as a DNA-binding protein *in vivo*, and preferentially associates with the centromeric alpha-satellite DNA. Interestingly, the use of different centromeric probes indicates that CENP-C binds the alpha-satellite DNA of all chromosomes tested, thus providing a general significance to the role of CENP-C in the human centromere organization. Moreover, the results of the mutational analysis of the protein not only support the idea that CENP-C can bind the alpha-satellite *in vivo* but also reveal that the domain proposed to bind DNA *in vitro* (Yang et al., 1996; Sugimoto et al., 1997) is necessary for the recognition of centromeric DNA *in vivo*. This is in agreement with previous results suggesting that both domains are located in the central region of the protein (Yang et al., 1996).

These results are quite striking since CENP-C does not bind specific DNA sequences *in vitro*. A simple explanation for this discrepancy may reside in the fact that CENP-C-binding activity might be fully manifested as a result of interactions with other centromere proteins *in situ*. This hypothesis is corroborated by recent findings from gene targeting



experiments in mice and conditional loss-of-function analysis in the chicken DT40 cell line, which have shown that CENP-

A and CENP-H are necessary to correctly position CENP-C at active centromeres (Howman et al., 2000; Fukagawa et al., 2001). These data support the model that CENP-C localizes to the centromere through contacts with auxiliary proteins rather than recognizing the kinetochore DNA directly. However, a direct interaction of CENP-C, with either CENP-A or CENP-H, has not been demonstrated and, so far, experiments to prove this point have been unsuccessful. Obviously, it is possible that other centromere proteins concur to target CENP-C to the centromere through this mechanism. However, the situation might be more complex than predicted. In fact, we cannot exclude the possibility that these proteins may contribute to the generation of a particular DNA structure or impose modifications upon specific DNA sequences that would allow CENP-C to be recruited and assembled into the centromere chromatin. Observations by several groups suggest that specific centromeric DNA sequences may play a crucial role in the constitution of a functional human centromere. For instance, Masumoto and colleagues have demonstrated that alphoid DNA from the alpha21-I locus could give rise to artificial chromosomes in HT1080 cells, whereas alphoid DNA from the 21-II locus could not (Masumoto et al., 1998). This strongly suggests that specific blocks of alpha satellite DNA are endowed with peculiar features that allow them to seed centromere formation *in vivo*. The fact that specific blocks of DNA may satisfy the structural and/or sequence requirements for the assembly of an active centromere-kinetochore complex is further supported by recent finding showing that a 330 kb CENP-A-binding domain of the neocentromere found on the human marker chromosome mardel (10) possesses an A+T content (>60%) similar to that of alpha-satellite DNA (Lo et al., 2001).

Although we cannot at present clarify the exact mechanism of recruitment of CENP-C to the centromere, our approach has allowed the analysis of the CENP-C DNA-binding activity as an intrinsic component of the kinetochore and shows that CENP-C contacts the alpha-satellite DNA *in vivo*. This result is important for two reasons: first, it provides a methodological approach to investigate the dynamic of how this protein takes part in the assembly of a functional centromere *in vivo*; and second, it further supports the idea that the alpha-satellite is the kinetochore DNA in normal chromosome.

Another important observation of this work arises from comparison of the DNA sequences recognized by CENP-B and CENP-C. Although CENP-C and CENP-B seem to recognize the same sets of alpha-satellite (although it is possible that CENP-C might recognize other types of alpha-satellite sequences in addition to those also recognized by CENP-B), they appear to be organized topologically in distinct centromere subdomains containing alpha-satellite DNA. This is supported by two results: first, immunodepletion with an anti-CENP-B antibody of the DNA-CENP-B complexes from the crosslinked cell extract does not affect the recovery of the DNA-CENP-C complexes, indicating that the two proteins are not associated to the same alpha-satellite DNA fragments; and second, immunolocalization of CENP-B and CENP-C on interphase chromosomes shows, at the high resolution offered by double-label immunoelectron microscopy, that the tendency of the two antigens to occupy distinct domains is statistically significant. We believe that the best interpretation of these data is that the ChIP assay shows that CENP-B and CENP-C occupy separate alpha-satellite domains, and this is supported by the

immunoelectron microscopy analysis. Our results suggest a subtle and composite organization of the centromere proteins relative to alpha-satellite DNA at the centromere. In fact, both CENP-B and CENP-C bind to the same family of repeated DNA, but only CENP-C binds to the DNA that ends up at the surface of the chromosome associated with the active kinetochore. The mechanism of segregation of CENP-B and CENP-C to different regions of alpha-satellite is not known, but it appears that, in addition to DNA sequence, other factors are likely to be involved. In this context, as previously mentioned, the localization of CENP-C to the centromeres requires at least CENP-A and CENP-H. The knockout of the CENP-A gene in mice (Howman et al., 2000) or the inhibition of its homologue in *C. elegans* by RNA interference (Moore et al., 2001; Oegema et al., 2001), abolishes the ability of CENP-C to target the kinetochore. By comparison, disrupting CENP-C in *C. elegans* has no effect on CENP-A (Oegema et al., 2001). Therefore, CENP-A appears to act upstream of the assembly pathway that drives CENP-C to the kinetochore. Given that the presence of CENP-A with the centromeric DNA is a pre-requisite for the CENP-C localization to the centromere, then the absence of CENP-C from the inactive centromere of stable dicentric chromosomes (Earnshaw et al., 1989; Page et al., 1995; Sullivan and Schwartz, 1995; Fisher et al., 1997; Page and Shaffer, 1998; Sullivan and Willard, 1998) may be causally related to the lack of this preliminary step of the kinetochore assembly. The mechanisms that precisely specify where functional mammalian centromeres form within arrays of centromeric heterochromatin, and how the centromere identity might be propagated at a specific chromosomal site, remain largely unknown. Recent observations indicate that the centromere identity might be established by epigenetic mechanisms (Karpen and Allshire, 1997; Wiens and Sorger, 1998). A centromeric epigenetic mark could be specified by exclusive protein binding, histone modifications, spatial or temporal organization of chromosomal processes, or through the activity of specific centromere-identity loading factors (Sullivan et al., 2001; Sullivan, 2001). In this context, it has been suggested that CENP-A might modify chromatin organization to favor the deposition of other centromere-kinetochore proteins, including CENP-C (Van Hooser et al., 2001). However, mistargeting of CENP-A to DNA outside the conventional centromere regions is not sufficient per se to induce the formation of functional neocentromeres, although it seems to re-direct CENP-C to these sites. This suggests that other factors that include particular DNA sequences or structures may be required for this process.

It is possible that there are different subclasses of alpha-satellite sequences, some of which can contain CENP-C DNA recognition sites. The epigenetic determination of centromere identity and propagation in fact does not rule out the possibility that sequence composition, such as enrichment for repeated sequences or an A+T sequence bias, also has a role in these processes. Although it is clear that the organization of a functional centromere requires the contribution of several factors assembled in a hierarchical fashion, our results strongly indicate that the binding of CENP-C to the alpha-satellite DNA *in vivo* may represent an important step in this process and provide a more comprehensive view of how the architecture of centromeres at human chromosomes is achieved.

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