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Visualization of centromere proteins CENP-B and CENP-C on a stable dicentric chromosome in cytological spreads

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Abstract. We have screened for the presence of two centromere autoantigens, CENP-B (80 kDa) and CENP-C (140 kDa) at the inactive centromere of a naturally occurring stable dicentric chromosome using specific antibodies that do not cross-react with any other chromosomal proteins. In order to discriminate between the active and inactive centromeres on this chromosome we have developed a modification of the standard methanol/acetic acid fixation procedure that allows us to obtain high-quality cytological spreads that retain antigenicity with the anti-centromere antibodies. We have noted three differences in the immunostaining patterns with specific anti-CENP-B and CENP-C antibodies. (1) The amount of detectable CENP-B varies from chromosome to chromosome. The amount of CENP-C appears to be more or less the same on all chromosomes. (2) CENP-B is present at both active and inactive centromeres of stable dicentric autosomes. CENP-C is not detectable at the inactive centromeres. (3) While immunofluorescence with anti-CENP-C antibodies typically gives two discrete spots, staining with anti-CENP-B often appears as a single bright bar connecting both sister centromeres. This suggests that while CENP-C may be confined to the outer centromere in the kinetochore region, CENP-B may be distributed throughout the entire centromere. Our data suggest that CENP-C is likely to be a component of some invariant chromosomal substructure, such as the kinetochore. CENP-B may be involved in some other aspect of centromere function, such as chromosome movement or DNA packaging.

Introduction

The kinetochore is a specialized structure at the surface of the centromere that mediates the attachment of the mitotic chromosomes to the spindle microtubules (Rieder 1982) and may contain the mechano-chemical 'motor' responsible for movement of the chromosomes to the spindle poles at mitosis (Kirschner and Mitchison 1986; Gorbsky et al. 1987; Koshland et al. 1987). The identities of the centromere and kinetochore components responsible for interaction of the chromosomes with the spindle are currently being widely studied by cell biologists.

Abbreviations: *CENP* centromere protein

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The analysis of these components was greatly aided by the discovery that certain patients with rheumatic diseases make high-titer autoantibodies that recognize antigens at the centromere (anti-centromere antibodies or ACA; Moroi et al. 1980). Immunoelectron microscope analysis using the immunoperoxidase method further suggested that at least one of the autoantigens recognized by these sera was located in the kinetochore (Brenner et al. 1981).

In an earlier study from this laboratory we identified a family of three centromeric polypeptide antigens recognized by the anticentromere autoantibodies (Earnshaw and Rothfield 1985). These were designated CENP-A (*CEN*tromere *P*rotein A – 17 kDa), CENP-B (80 kDa) and CENP-C (140 kDa). We subsequently demonstrated by immunoblotting that 100% of 38 sera from different patients with ACA recognized CENP-B plus either CENP-C or CENP-A (Earnshaw et al. 1986). Furthermore, 35 of the 38 sera recognized all three CENP antigens (Earnshaw et al. 1986). The cDNA encoding CENP-B was subsequently cloned and specific monoclonal and polyclonal antibodies were prepared against a CENP-B/β-galactosidase fusion protein expressed in *E. coli* (Earnshaw et al. 1987a). In this study we have utilized these reagents together with a newly prepared antibody that is specific for CENP-C to examine the relationship between the presence of CENPs B and C and centromere activity on a stable dicentric chromosome.

Both classic cytogenetic data (McClintock 1939) and recent studies of artificial chromosomes in *Saccharomyces* (Mann and Davis 1983; Oertal and Mayer 1984; Koshland et al. 1987) have shown that most dicentric chromosomes are mitotically unstable. Attachment of two kinetochores on a single chromatid to opposite spindle poles results in the DNA molecule of that chromatid being simultaneously pulled towards both spindle poles (anaphase bridge formation). Apparently the spindle generates sufficient force to ultimately break the chromatid. As a consequence, most dicentric chromosomes are associated with a mosaic karyotype.

Despite this problem, a number of apparently stable dicentric chromosomes have been described in humans (Distèche et al. 1972; Warburton et al. 1973; see also the numerous references in Earnshaw and Migeon 1985) and other mammals (Zinkowski et al. 1986; Cherry and Wang 1988; Vig and Paweletz 1988). The most common of these have been dicentric X chromosomes, although stable dicentric autosomes have also been reported. Stable dicentric

chromosomes all appear to have one *active* centromere, located at the primary constriction, and one *inactive* (or latent; Hsu et al. 1975) centromere, which may or may not be associated with a secondary constriction. Such dicentric chromosomes are thus stable because they are *functionally* monocentric.

The mechanism of centromere inactivation is unknown, but it does not appear to entail the loss of centromere-associated DNA elements. Inactive centromeres are typically C-band-positive (Distèche et al. 1972; Warburton et al. 1973; references in Earnshaw and Migeon 1985) and, where examined, they have been shown to contain centromere-associated alphoid DNA elements (Jabs et al. 1984). Thus centromere inactivation is likely to result either from subtle DNA sequence alterations or from stable changes in centromere chromatin composition or conformation.

Sera from autoimmune patients have been used to test for the presence of the CENP-A, -B and -C antigens at inactive centromeres in three studies, in which a total of 11 different human dicentric chromosomes were examined by indirect immunofluorescence (Merry et al. 1985; Earnshaw and Migeon 1985; Peretti et al. 1986). No single conclusion emerged from these studies. While four of the chromosomes lacked detectable CENP antigens at the inactive centromere (Earnshaw and Migeon 1985; Peretti et al. 1986), the remaining seven stable dicentrics expressed the antigens at both centromeres (Merry et al. 1985; Peretti et al. 1986). These results therefore confirm that certain inactive centromeres retain the DNA sequences needed for binding centromere-specific polypeptides. [A similar complex picture has emerged from studies of multicentric chromosomes in rodents (Zinkowski et al. 1986; Cherry and Wang 1988; Vig and Paweletz 1988).]

Further examination of these published experiments reveals an interesting difference between autosomal dicentrics and those involving the sex chromosomes. The autoantigens were detected at both the active and inactive centromeres of all dicentric autosomes examined (Merry et al. 1985; Peretti et al. 1986). The level of antibody binding at the inactive centromere was, however, typically less intense than that seen at the active centromere.

In contrast with these results, the centromere autoantigens were undetectable at the inactive centromeres of three of the five dicentric X chromosomes and in the one X,Y fusion examined (Earnshaw and Migeon 1985; Peretti et al. 1986). Furthermore, the two X chromosomes that exhibited fluorescence at the inactive centromere were apparently not completely mitotically stable. Both gave rise to 45,X lines, suggesting that centromere inactivation was not complete (Peretti et al. 1986).

The observation that the most dramatic decreases in the level of detectible centromere antigens are found in dicentric X chromosomes suggests that centromere inactivation might involve processes related to X-chromosome inactivation. That is, either the structure of the centromere chromatin or the organization of centromere proteins might be altered at the inactive centromere. This difference might then be enhanced when it occurs on the inactive X chromosome. [The dicentric X has typically been described in trisomic females, and it is invariably the inactivated X (see the references cited in Earnshaw and Migeon 1985).]

We were intrigued by the observation that whenever fluorescence was detected at the inactive centromere, this was typically less intense that the fluorescence at the active centromere (Merry et al. 1985; Peretti et al. 1986). We have considered three possible reasons for this. First, one or more of the three centromere autoantigens might be missing from the inactive centromere. Second, all three centromere antigens might be present at the inactive centromere, but they might be present in reduced amounts. Third, the composition of the active and inactive centromeres might be identical, but their conformations might differ. That is, the structure of the inactive centromere might be such that the antibodies only gain access to a small percentage of the antigen molecules present (thus resulting in decreased antibody binding).

Both published studies that described the presence of CENP autoantigens at inactive centromeres used whole patient sera (Merry et al. 1985; Peretti et al. 1986). Because such sera recognize a minimum of two different centromere antigens (Earnshaw et al. 1986), it was not possible to distinguish between the above models. The purpose of the present study was therefore to take advantage of our recently prepared mono-specific antibody probes for CENP-B and CENP-C to examine the distribution of these polypeptide antigens at the active and inactive centromeres of a stable dicentric autosome. To do this, we developed a modification of the standard cytological spreading procedure that is compatible with the detection of centromere antigens.

Materials and methods

Cells and antibodies. The chromosome studied was a dicentric inverted duplication of one maternal chromosome 13, abbreviated dic(13). This chromosome is present in a primary amniocyte line established at the Johns Hopkins University School of Medicine Prenatal Diagnostic Center from a fetus diagnosed in utero with holoprosencephaly (Rijhsinghani et al. 1988). This cell line is grown in Eagle's Minimal Essential Medium with 15% fetal calf serum.

Autoimmune serum GS, which was used for the initial identification of CENP-A, -B and -C (Earnshaw and Rothfield 1985), was used previously to obtain a cDNA clone encoding CENP-B (Earnshaw et al. 1987). A β-galactosidase fusion polypeptide encoded by that clone was used to immunize a rabbit, yielding serum ra-ACA 1, which is specific for CENP-B in immunoblots (Earnshaw et al. 1987). Immunoblots of purified chromosomes from HeLa cells with these sera are shown in Figure 1. Lane A is an immunoblot with whole serum GS diluted 1:2500 and lane D is an immunoblot with ra-ACA 1 diluted 1:500. (For a Coomassie blue stain of a similar gel sample see Earnshaw and Rothfield 1985.)

Antibodies specific for CENP-C were affinity-purified from serum GS using as antigen, protein encoded by a cDNA clone from human placental cDNA library (Clonetech) in λgt11 (Young and Davis 1983a, b). Greater than 4×10^6 recombinant bacteriophages were screened with serum GS, and many immunopositive clones were obtained. Most of these clones contained sequences in common with CENP-B (as determined by plaque hybridization); however, six appear to encode an epitope that is present on CENP-C. This was shown using the method of Snyder et al. (1988) as follows. Phage were plated on a lawn of bacterium Y1090 at a density of about 1×10^4/9-cm petri plate at 42° C. When plaques were visible (at approximately 4 h), a 9 cm diameter nitrocellulose filter (which had been presoaked

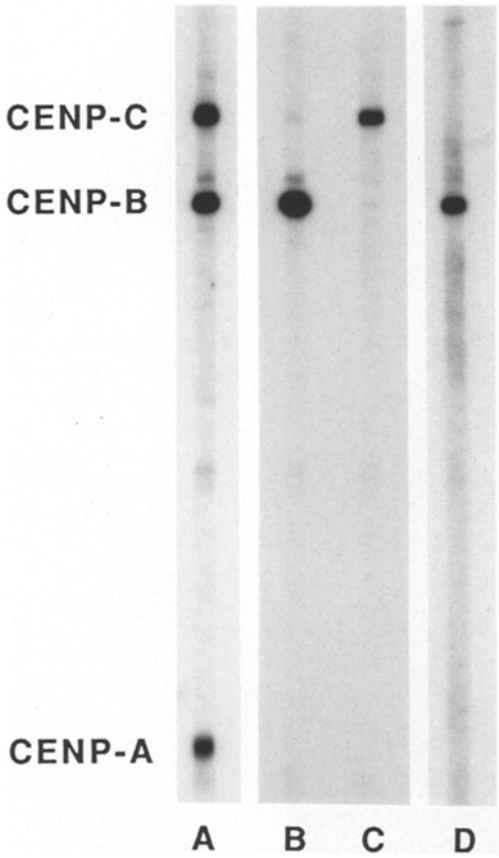

Fig. 1. Immunoblots of isolated human chromosomes with various anti-centromere antibodies. Blot strips were probed with: *lane A* serum GS 1:2500; *lane B* serum GS affinity-purified from a CENP-B clone (undiluted); *lane C* serum GS affinity-purified from a cDNA clone encoding an epitope present on CENP-C (undiluted); and *lane D* ra-ACA 1 (rabbit serum elicited by injection with cloned CENP-B) diluted 1:500

in 10 mM isopropylthiogalactoside) was placed on the bacterial lawn for 4 h at 37° C. This filter was then removed from the plate, washed in PTX buffer (Earnshaw et al. 1984), blocked by incubation with 4% BSA in PTX for 20 min, and incubated with serum GS for 16 h at room temperature. The filter was then washed in PTX buffer (1 × for 5 min), PBS (1 × for 5 min), and bound antibodies were eluted by treatment with 3.5 ml 0.2 M glycine/HCl, pH 2.5 for 2 min at 37° C. The solution of freshly eluted affinity-purified autoantibodies was immediately neutralized by addition of 1.75 ml 1 M KPO_4, pH 9.1.

When these affinity-purified antibodies were used to probe a nitrocellulose blot of human chromosomal proteins, they bound only to CENP-C (Fig. 1, lane C). Thus, these cDNAs encode an epitope that is present on CENP-C. For comparison, lane B shows an identical nitrocellulose blot of chromosomal proteins that was probed with GS antibodies that were affinity-purified in parallel from plaques of a phage encoding a CENP-B fusion protein. The detailed characterization of these clones will be published elsewhere (H. Ratrie and W.C. Earnshaw, unpublished results).

Solvent staining. Fixation of mitotic cells with a mixture of methanol and acetic acid is an important part of the standard cytological spreading procedure for preparation of karyotypes. Unfortunately, when mitotic chromosome spreads prepared by this method were probed with anti-centromere antibodies, no centromere fluorescence was observed (Fig. 2). It was therefore necessary to develop a modification of the standard chromosome spreading procedure that was compatible with the use of immunological probes for centromere antigens.

Amniocytes cultured on glass coverslips were blocked for 2–3 h with 0.125 μg/ml Colcemid. The following procedures were then performed at room temperature. Cells were swollen in a hypotonic solution (0.8% Na citrate) for 25 min. An equal volume of methanol/acetic acid (3:1) was then added to the citrate solution for 2 min. The liquid was removed by aspiration, and 2 ml methanol:acetic acid (3:1) added for 5 min. After removal of most of the fixative, the coverslips were dried by blowing on them with an aquarium pump (Hagen 800 or 800D, using a cut 1-ml plastic pipette as a nozzle; David C. Peakman, personal communication). As soon as the surface was dry, the coverslips were rehydrated by immersion in a 1X solution of PBS-azide (10 mM Na PO_4 pH 7.4, 0.15 M NaCl, 1 mM EGTA, 0.01% NaN_3) for 5–15 min. The chromosomes were then swollen by washing the coverslips three times (2 min each) with TEEN (1.0 mM triethanolamine: HCl pH 8.5, 0.2 mM NaEDTA, 25 mM NaCl)+0.5% Triton X-100+0.1% BSA. The antibody (diluted appropriately in 1X TEEN+0.5% Triton X-100+0.1% BSA) was then added to the coverslips for 30 min at 37° C. Antibody was removed by washing three times with KB^-[10 mM Tris:HCl pH 7.7, 0.15 M NaCl, 0.1% BSA] for 2 min, 5 min, and 3 min at room temperature. Subsequent steps in the immunofluorescence procedure were as previously described (Earnshaw and Migeon 1985) except that KB^- was used for all washes. We will refer to this protocol as the solvent spreading method.

The ratio of immunostaining at the active and inactive centromeres was determined in two ways. First, two different observers estimated the average intensity ratios by direct observation in the microscope. This yielded an average estimate of 1.7:1 (active:inactive). Second, images were taken under linear conditions with a SIT video camera and the fluorescence intensity at the active and inactive centromeres was determined using a Perceptics Biovision image analysis system. In theory, this method should yield an accurate determination of the fluorescence from the two centromeres; however, in practice we found that the ratios determined in this way varied from 2–6:1 (active:inactive). The scatter in these measurements is probably due to two factors. First, the area of the fluorescence was small, so that we were able to use only a small number of pixels for the determination. This greatly increases the uncertainty of the determination. Second, the signal-to-noise ratio was low for the dicentric chromosome. This meant that the subtraction of local background had a large effect on the final values obtained. Because of these uncertainties, we have used these observations only for a crude estimate of the ratio of antibody binding to the active and inactive centromeres.

Aqueous staining. We have not yet been able to get the affinity-purified anti-CENP-C antibodies to stain the centromeres of chromosomes prepared by the above method. The reason for this is unknown, although it could reflect

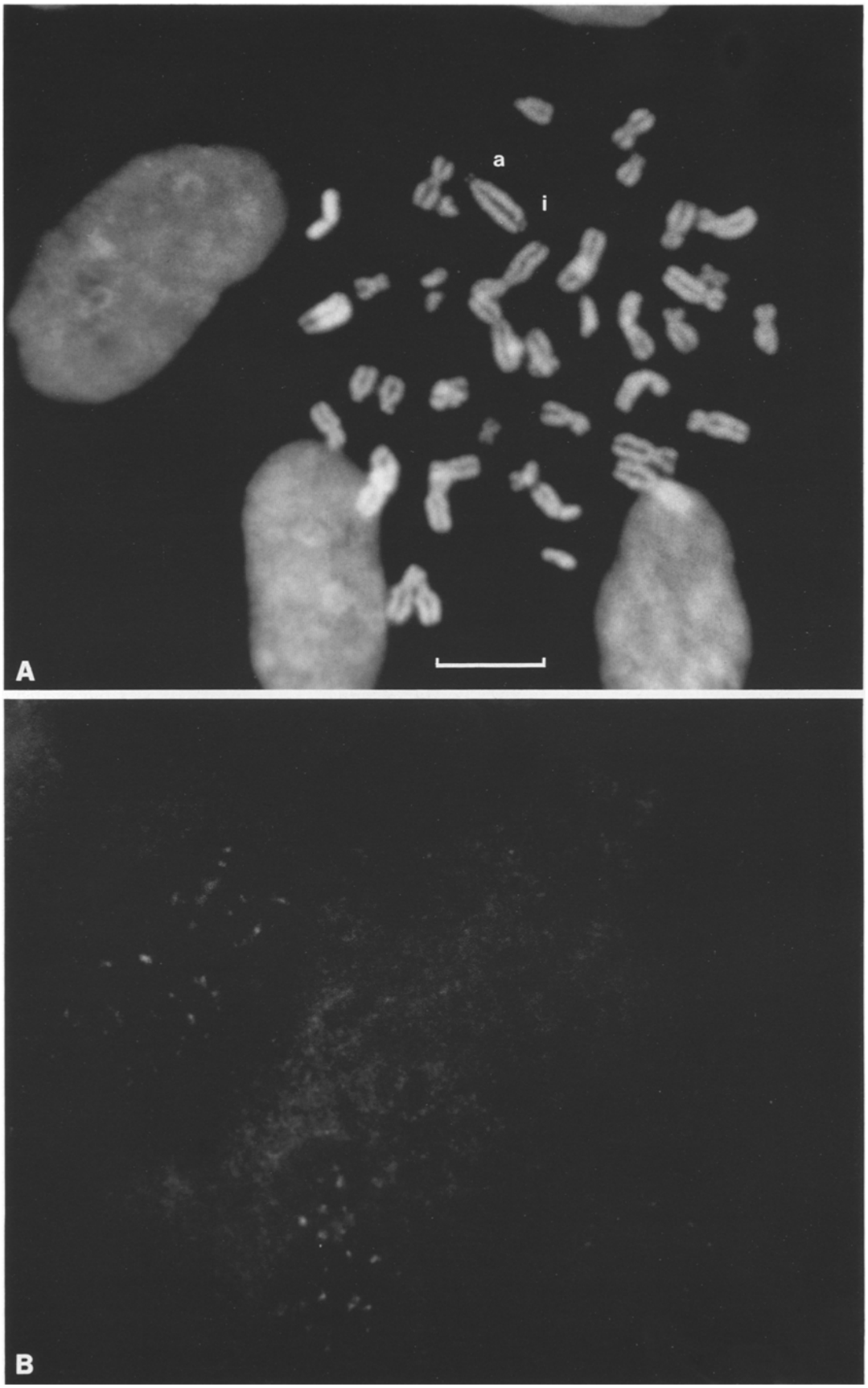

Fig. 2A, B. Failure of autoantiserum to bind to the centromeres of chromosomes spread after treatment with methanol/acetic acid (3:1). In this and all subsequent micrographs the active (*a*) and inactive (*i*) centromeres of the dic(13) chromosome have been indicated. **A** DAPI staining for DNA (Williamson and Fennell 1975). **B** Indirect immunofluorescence using serum GS diluted 1:10000, performed under conventional conditions. No staining of the centromeres of the mitotic chromosomes is seen, but faint labelling of the interphase centromeres of two of the nuclei is detected. In all micrographs bars represent 10 μm

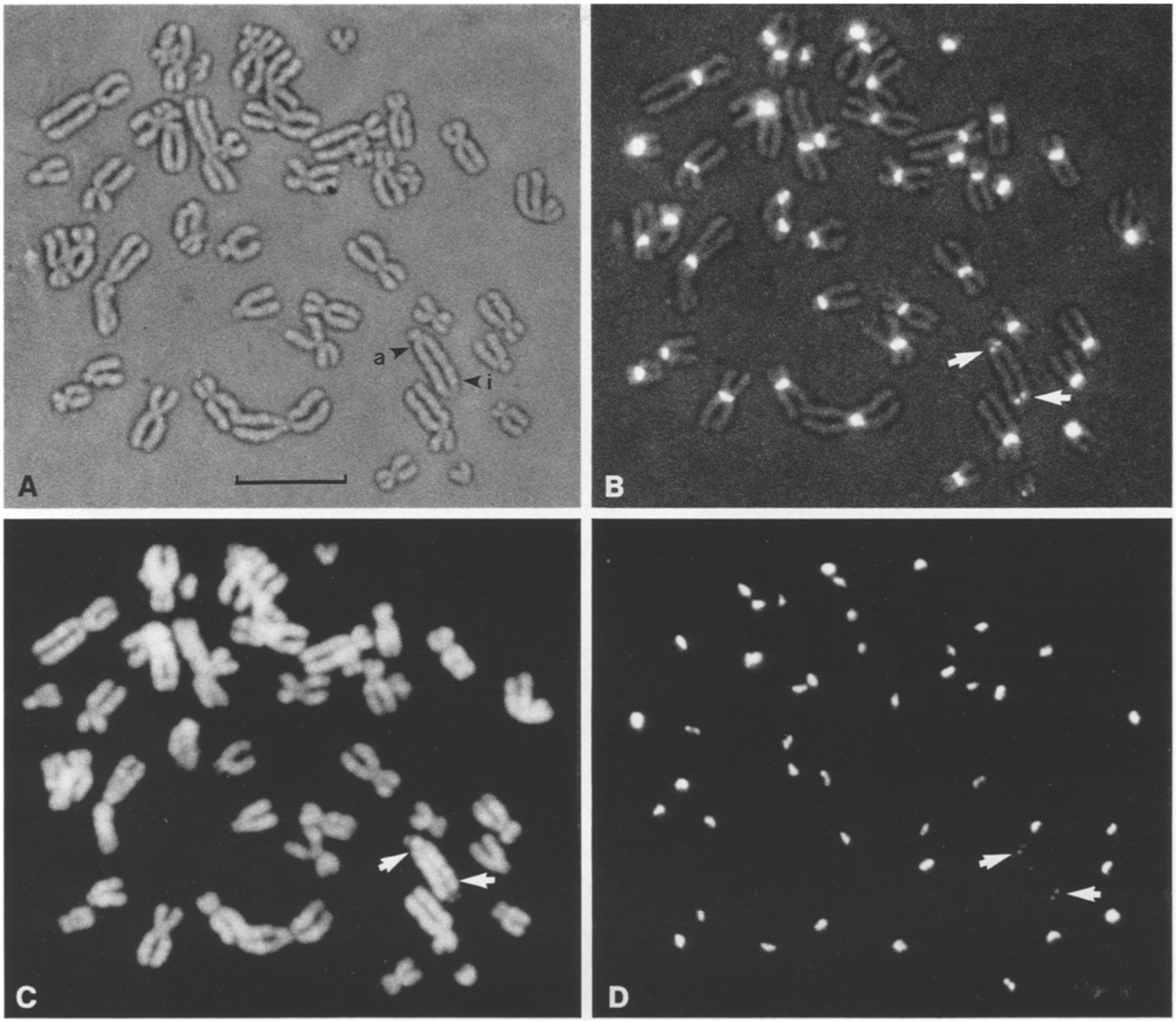

Fig. 3A–D. Binding of autoimmune serum GS to the active and inactive centromeres of a stable human dicentric chromosome in a cytological spread when the antibody is added under conditions when the chromatin structure has been disrupted. **A** Phase contrast image. **B** Simultaneous double exposure of the phase contrast image and indirect immunofluorescence using serum GS diluted 1:10000. The *arrows* indicate sites of antibody binding to the active and inactive centromeres. **C** DAPI staining for DNA. Note the presence of structural satellites at either end of the dicentric chromosome. **D** Indirect immunofluorescence with patient serum GS. The *arrows* indicate the locations of the active and inactive centromeres

either destruction of the CENP-C epitope by the organic solvents or instability of the affinity-purified antibodies in the low ionic strength buffers used for the solvent spreading method.

As a result, an alternative aqueous, protocol for obtaining mitotic chromosome spreads was developed in order to determine the distribution of CENP-C in dicentric chromosomes. Blocked cells on coverslips were swollen with Na citrate as described above. After 25 min, excess citrate was removed, leaving 5–10 µl per coverslip. The coverslips were then placed on a filter paper in a plastic petri dish and centrifuged up to a speed of 2400 rpm in a Beckman TJ6 table top centrifuge. After centrifugation, coverslips were briefly immersed in PBS-azide, fixed for 5 min in 3% formaldehyde in PBS-azide, permeabilized by washing three times (2 min each) with KB (identical to KB^- but containing 0.1% Triton X-100), and the first antibody added in KB for 30 min at 37° C. Subsequent processing was as described above for the solvent spreading method.

Results

Solvent spreading method

Our initial immunochemical studies of specific chromosome abnormalities were hampered by the inability to identify marker chromosomes in metaphase spreads since recognition of many marker chromosomes requires fixation and spreading procedures used by cytogeneticists for chromosome identification and banding analysis. This posed a

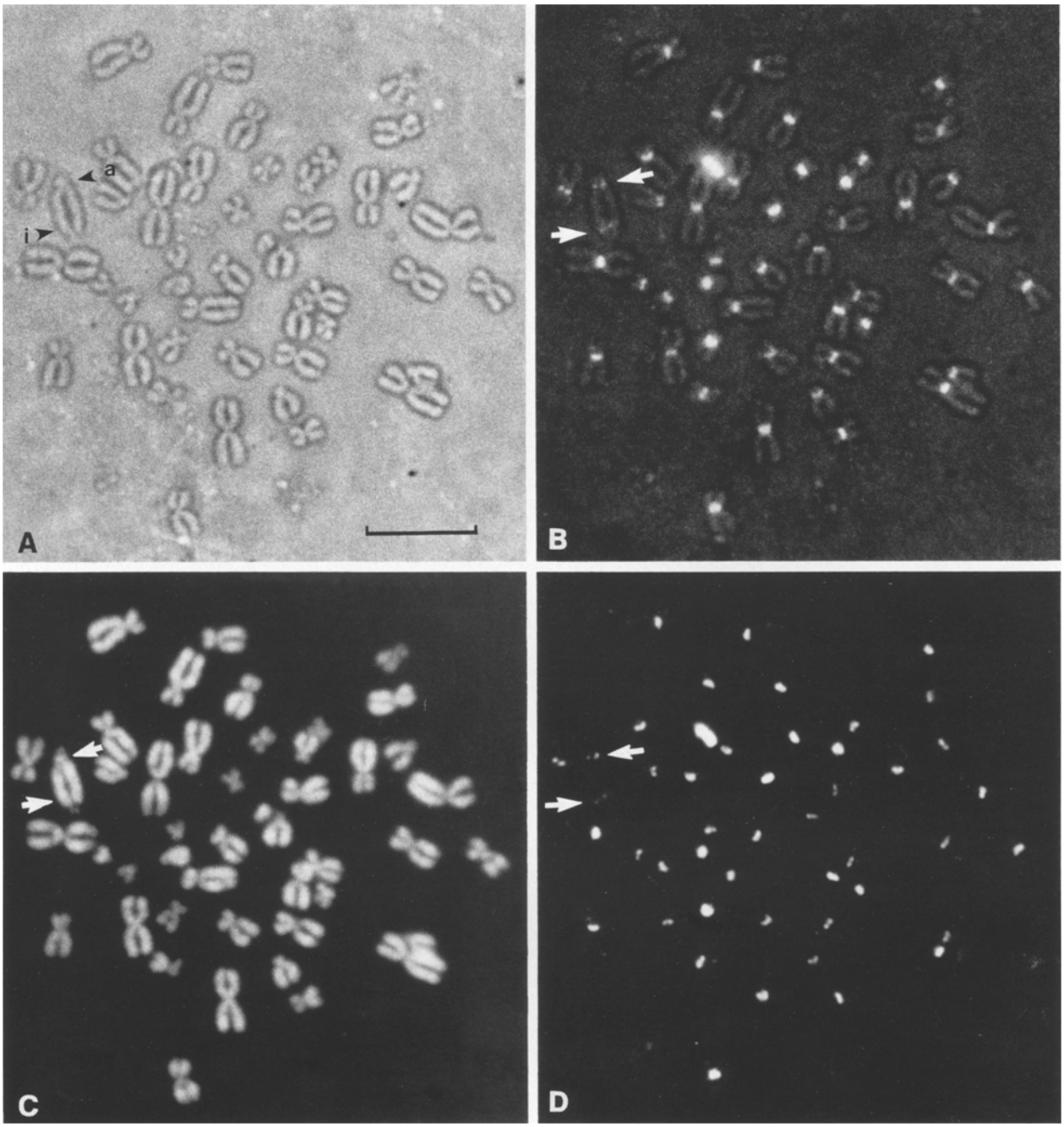

Fig. 4A–D. Binding of rabbit anti-CENP-B to both active and inactive centromeres of the stable dicentric chromosome. **A** Phase contrast image. **B** Simultaneous double exposure of the phase-contrast image and indirect immunofluorescence using rabbit serum ra-ACA 1 diluted 1:2000. **C** DAPI staining for DNA. **D** Indirect immunofluorescence with rabbit serum ra-ACA 1

problem, since exposure of mitotic cells to methanol/acetic acid fixative prior to preparing spreads virtually abolishes the immunoreactivity with the anti-centromere antibodies. This is shown in Figure 2, where chromosomes spread following methanol/acetic acid pretreatment of the cells were stained with antiserum GS. Panel A shows the spread chromosomes stained with DAPI (a non-intercalating DNA-binding dye; Williamson and Fennell 1975). Panel B shows the corresponding indirect immunofluorescence image. No staining is seen over the mitotic chromosomes, although some fluorescent spots seen over an adjacent interphase nucleus appear to result from (albeit greatly decreased) staining of interphase centromeres.

The immunoreactivity of the spread mitotic chromosomes could have been abolished for two reasons. The antigens could have been extracted or destroyed by exposure to the organic solvents. Alternatively, exposure to methanol/acetic acid could have affected the packaging of the centromeric chromatin, so that the antigens were no longer accessible to the antibodies.

The observation that interphase nuclei (in which the chromatin is less condensed than it is in mitotic chromosomes) show a low level of specific antibody staining led us to test the latter possibility. To do this we added antibody to spread chromosomes whose chromatin packaging was deliberately disrupted. This was accomplished with

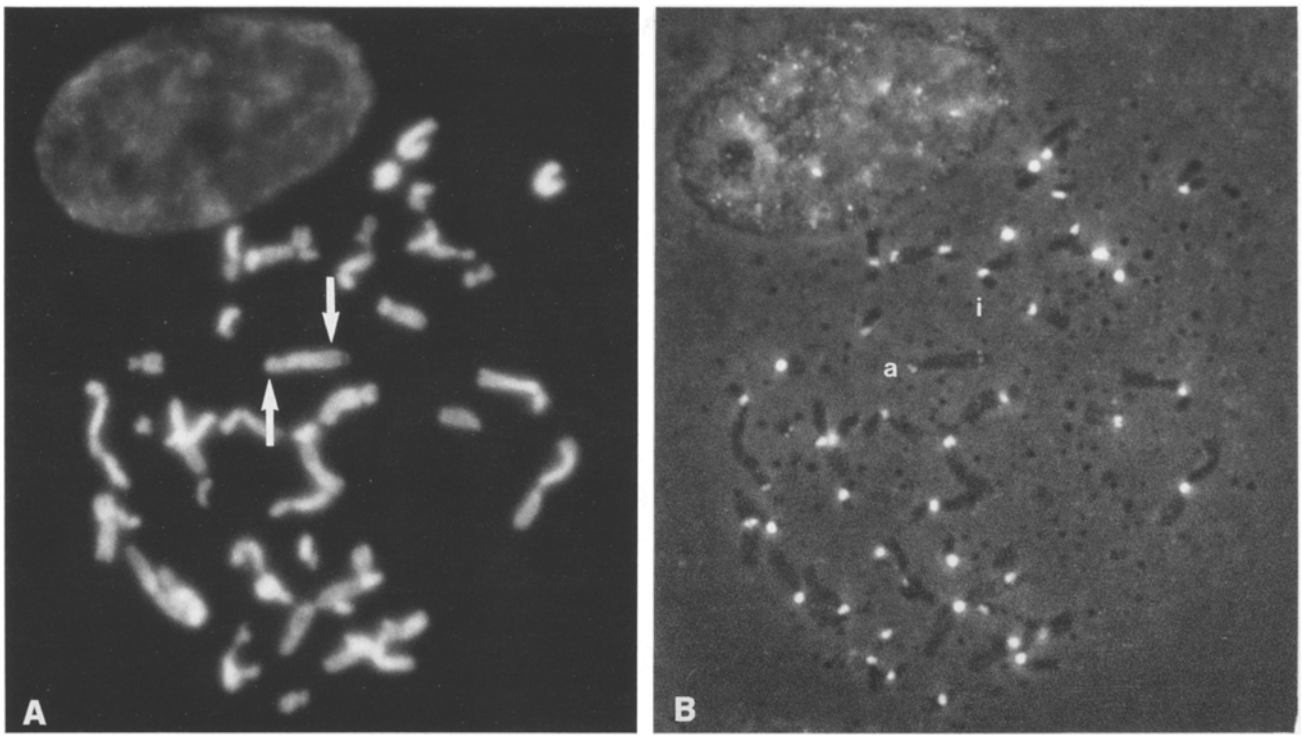

Fig. 5A, B. Staining of CENP-B on the dicentric chromosome in an aqueous spread. **A** DAPI staining for DNA. **B** Simultaneous double exposure of the phase contrast image and indirect immunofluorescence using rabbit serum ra-ACA 1 diluted 1:2000

a low ionic strength EDTA-containing buffer that causes the 30 nm chromatin fiber (which represents the higher order packing of nucleosomes) to unravel into the more extended "beads-on-a-string" conformation (Thoma et al. 1979; Labhart and Koller 1981; Earnshaw and Laemmli 1983). In the presence of this buffer the radial chromatin loops spread outward, and the chromosomes expand by about 6- to 10-fold (Earnshaw and Laemmli 1983). Anti-centromere antibody was then applied in this buffer. Under these conditions it is doubtful that significant conformational constraints on antibody binding would occur. The chromosomes were returned to a buffer solution at physiological ionic strength for all subsequent treatments during the immunofluorescence protocol. This reverses the effect of the EDTA swelling and causes the extended chromatin loops to collapse back onto the chromatid axes (Earnshaw and Laemmli 1983). Chromosomes can survive multiple rounds of such swelling and shrinking treatments without noticeable distortions (Cole 1967).

When the solvent spreading method was applied to the cell line containing the dic(13) chromosome using autoantiserum GS (which recognizes the three CENP antigens), the centromeres of all chromosomes were stained with the antibody (Fig. 3). Thus the effect of methanol/acetic acid fixation on chromosomes is apparently not to extract or destroy the centromere antigens, but to alter the conformation of either the centromere or the proteins within it so that the antigenic sites are no longer accessible to anti-centromere antibodies.

The dic(13) chromosome is readily identified in these spreads (Fig. 3A). This chromosome has a characteristic appearance with structural satellites at both ends (Fig. 3C). Although previous cytogenetic analysis indicated that both centromeres are derived from a single maternal homologue (Rijhsinghani et al. 1988), the two look distinctly different (Fig. 3). One (labelled "a") resembles the centromeres of the other chromosomes, in that the sister chromatids are fused (Fig. 3A, C). At the second centromere (labelled "i"), the two sister chromatids, while closely associated, do not appear to fuse. We assume that the former is the active centromere and the latter its inactive counterpart.

Even though the sister chromatids do not fuse at the inactive centromere, they do tend to stay associated (see Figs. 2, 3, 4, 6, 7). It is possible that the arms are briefly fused at this centromere, but separate early as has been proposed for other inactive centromeres (Zinkowski et al. 1986). Regardless of the mechanism, at least one component involved in holding the chromatids together at the centromere is apparently missing from the inactive centromere.

When probed with autoimmune serum GS, both the active and inactive centromeres were stained (Fig. 3B, D). As in the previously published studies of other autosomal dicentrics, the active centromere was typically stained roughly two to five times as intensely than its inactive counterpart (determined as described in Materials and methods). This ratio varied from spread to spread, and in the spread shown in Figure 3 the two centromeres bound approximately equal amounts of antibody (but cf. Fig. 5).

Detection of CENP-B at the inactive centromere

Because serum GS recognizes all three of the CENP antigens (Fig. 1), the above result could indicate that the inactive centromere expresses either a reduced amount of all three antigens or normal amounts of one and reduced amounts of the others. We have therefore used our monospecific probes for CENP-B and -C to compare the levels of these antigens at the active and inactive centromeres.

To ensure selective detection of CENP-B, we prepared solvent spreads of mitotic cells as described above and

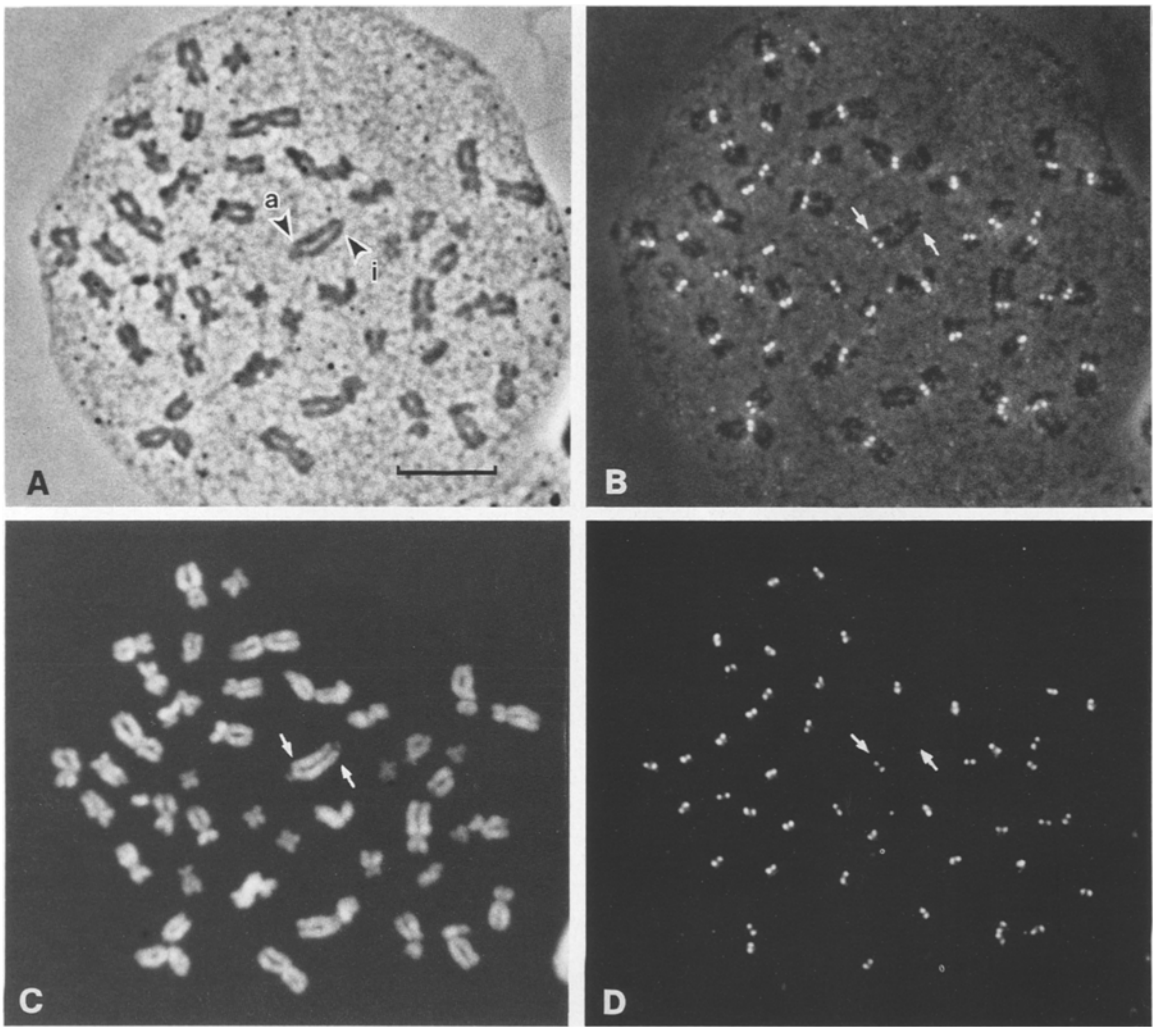

Fig. 6A–D. Selective binding of affinity-purified anti-CENP-C to the active centromere of the stable dicentric chromosome (aqueous staining protocol). **A** Phase contrast image. **B** Simultaneous double exposure of the phase contrast image and indirect immunofluorescence using affinity-purified anti-CENP-C diluted 1:2. **C** DAPI staining for DNA. **D** Indirect immunofluorescence with affinity-purified anti-CENP-C diluted 1:2

probed them by indirect immunofluorescence using a polyclonal antibody prepared against CENP-B fusion protein (Earnshaw et al. 1987). This antibody recognizes only CENP-B in immunoblots of chromosomal proteins (Fig. 1). Immunoreactive CENP-B was detected at both the active and inactive centromeres of the dic(13) chromosome (Fig. 4B, D). The active centromere appears to express roughly two to five times as much CENP-B antigen as does its inactive counterpart.

As in our previous experiments with this and other antibodies specific for CENP-B (Earnshaw and Rothfield 1985; Earnshaw et al. 1987), the intensity of immunostaining was found to vary from chromosome to chromosome. Surprisingly, however, we also observed a consistent difference between the level of staining of the normal and dic(13) centromeres. We estimate that the centromere of the normal chromosome 13 binds roughly three times as much anti-CENP-B as does the active centromere of the dic(13) [which was among those that stain least well (Fig. 4)]. Little is known as yet about the extent to which antigen levels for a given chromosome vary between individuals, and the reason for this observed difference is not known.

In any case, these experiments demonstrate that the presence of CENP-B is not sufficient for the tight apposition of sister chromatids that is characteristic of the primary constriction, since the two sister chromatids are separated at the inactive centromere.

Failure to detect CENP-C at the inactive centromere

We next sought to test for the presence of CENP-C at the inactive centromere using the affinity-purified antibodies presented in Figure 1. Unfortunately, the solvent spreading procedure appeared to abolish the immunoreactivity with these antibodies. There are several possible explanations for this. First, the solvent treatment might either extract or destroy CENP-C. Second, the EDTA swelling procedure might not be sufficient to reverse the conformational effects of solvent treatment in masking the CENP-C antigen. Finally, the affinity-purified antibodies might be unstable during dilution or dialysis into EDTA swelling buffer.

In order to visualize CENP-C on the dicentric chromosome we have developed a modification of the spreading

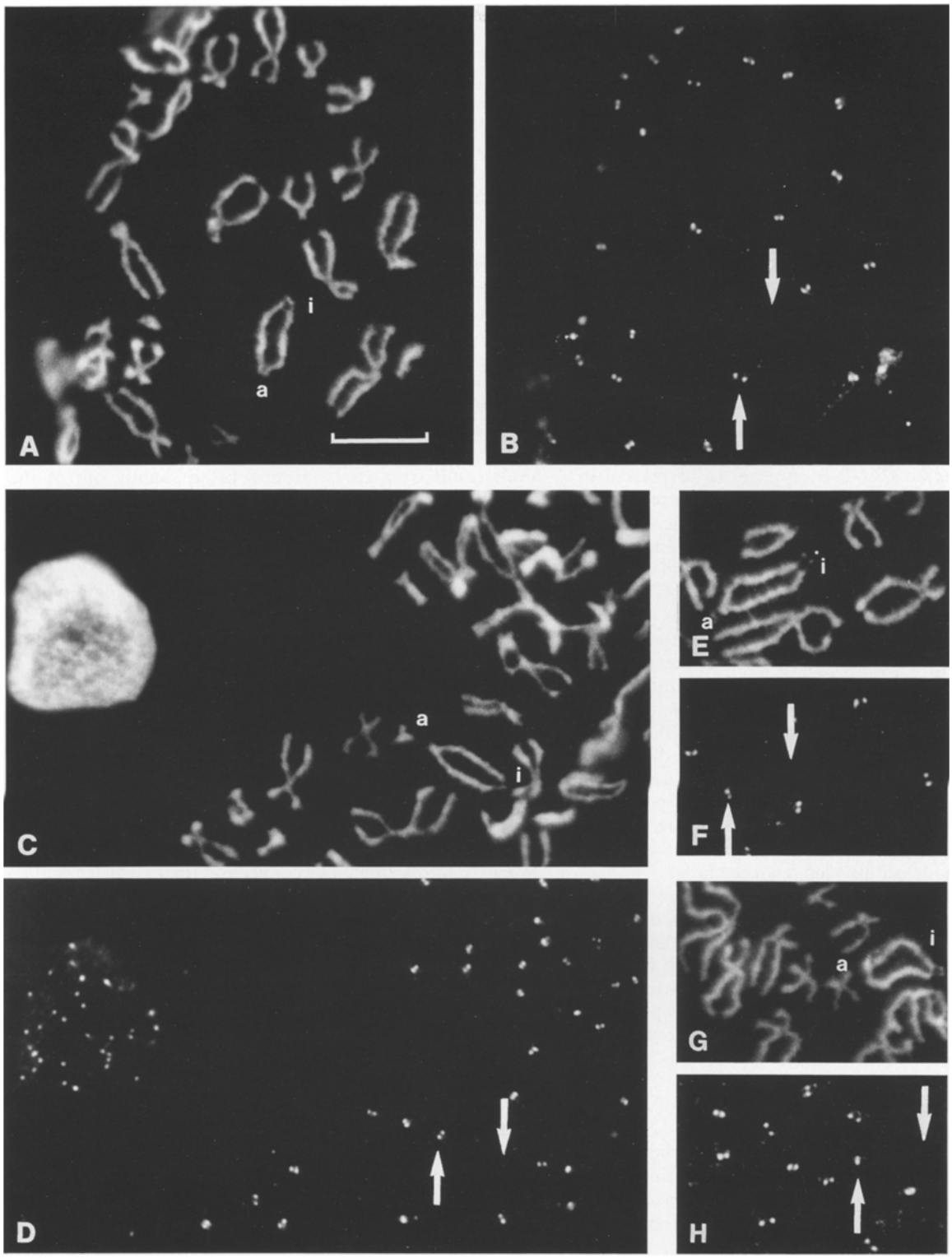

Fig. 7A–H. Detection of CENP-C on the dic(13) chromosome in aqueous spreads with the antibody added under conditions where the chromatin higher-order structure was disrupted. **A**, **C**, **E**, **G** DAPI staining for DNA. **B**, **D**, **F**, **H** Binding of affinity-purified anti-CENP-C (prepared in TEEN chromosome swelling buffer and used undiluted). Only the active centromere is labelled

method described above in which the dicentric chromosome could be identified without exposure of the cells to organic solvents. In this procedure, which we will refer to as *aqueous spreading*, cells grown on coverslips were swollen with Na citrate as described in Materials and methods, and spread by centrifugation as described previously (Earnshaw and Migeon 1985). This procedure permits routine identification of the dic(13), but the morphology of the other chromosomes is not typically sufficiently well preserved to allow their unambiguous identification.

We first confirmed that the aqueous and solvent spreading procedures yield similar results with the autoantibody and with the polyclonal anti-CENP-B. When mitotic cells were spread by the aqueous method the pattern of staining of the dicentric chromosome with either autoantibody GS or the CENP-B-specific polyclonal antibody resembled that seen with the solvent spreading method. Both the active and inactive centromeres were stained, and as before, the inactive centromere bound less antibody than did its active counterpart. A typical example of staining for CENP-B is shown in Figure 5. Thus both fixation and spreading procedures demonstrate clearly that CENP-B is located at the inactive centromere of this dicentric chromosome.

Use of the aqueous spreading procedure enabled us to use the affinity-purified antibodies specific for CENP-C to localize this antigen in mitotic spreads containing the dicentric chromosome (Fig. 6). Three significant differences were obtained from the staining pattern observed with antibodies specific for CENP-B. First, all chromosomes appear to bind an approximately equivalent amount of antibody. Thus CENP-C, unlike CENP-B, appears to be present in roughly equal amounts at all centromeres. Second, only the active centromere of the dic(13) chromosome was seen to bind antibody. Third, while the signal obtained with the CENP-B-specific antibody often gave a single merged spot for the two sister centromeres, the CENP-C staining pattern typically showed two well-resolved spots.

While the observation of CENP-B at the inactive centromere yields a straightforward interpretation, the apparent absence of CENP-C must be interpreted with caution. Examples of antigen masking under different conditions of fixation and immunostaining are not unknown (Bravo and Macdonald-Bravo 1987), and we were concerned that the apparent absence of CENP-C from the inactive centromere could reflect such masking. To control for this possibility we have therefore combined aspects of both the aqueous and solvent spreading procedures, taking advantage of the chromosome decondensation step from the latter.

Cells were spread as described above for the aqueous procedure. The resultant coverslips were then placed in EDTA swelling buffer and processed as for the solvent swelling method. Resultant spreads are shown in Figure 7. Chromosomes treated in this way are much more extended, and often remain more swollen than do those processed by the solvent spreading method. Despite these overall distortions, the characteristic morphology of the dicentric chromosome permits its easy identification.

This procedure had no effect on the staining of chromosomes with either anti-CENP-B or anti-CENP-C. As seen in Figure 7, CENP-C remained undetectable at the inactive centromere even when the antibody was applied under conditions where chromatin structure is largely unraveled. We conclude that it is unlikely that CENP-C is present at the inactive centromere.

Discussion

These studies have revealed three differences between the distribution of CENPs B and C in normal and dicentric chromosomes.

1. CENP-B, but not CENP-C, is detected by our antibodies at both the active and inactive centromeres of the dic(13) chromosome. Thus, the presence of CENP-B is not a sufficient condition for kinetochore activity. On the other hand, CENP-C might be essential for microtubule attachment. Although our data argue strongly that CENP-C is absent from the inactive centromere, we cannot absolutely exclude the possibility that CENP-C may be present in a form inaccessible to our immunological probes. However, if CENP-C were present but inaccessible to antibodies at the inactive centromere, it might also be inaccessible to spindle microtubules. Such masking of CENP-C could provide an alternative mechanism for the inactivation of the centromere.

2. The amount of detectable CENP-B antigen varies between different chromosomes in a reproducible way in cytological spreads prepared from a given individual. [That is, some chromosomes, such as chromosome 21, always appear to express large amounts of CENP-B antigen, while others, such as the Y or the dic(13), always express little CENP-B antigen.] In contrast, the level of detectable CENP-C appears to be relatively constant from chromosome to chromosome. It is our impression from microscopic examination that most apparent fluctuations in CENP-C levels result from chromosomes being slightly out of the plane of focus, since the aqueous spreading method often does not result in complete flattening of the mitotic cell.

3. The morphology of the staining patterns observed with anti-CENP-B and anti-CENP-C differs in detail. When the antibodies give rise to double dot staining patterns, the median spacing between the fluorescent dots at the active centromere of the dic(13) is similar for CENP-B (0.68 ± 0.08 μM) and CENP-C (0.66 ± 0.11 μM), suggesting that they occur in similar domains within the centromere. Likewise, the maximum diameter of the fluorescent region is similar for the two (1.15 ± 0.12 μM for CENP-B; 1.05 ± 0.16 μM for CENP-C). The slightly larger maximum diameter for CENP-B staining is probably a consequence of the spread of the CENP-B fluorescence, which is significantly stronger than the CENP-C labelling. In contrast to these similarities, the CENP-B staining is often observed to merge into a single bar connecting the two sister centromeres, rather than two separate dots. This is unlikely to reflect overlap of the sister centromeres caused by the chromosome hitting the coverslip at a skew angle. If such overlap were to occur, the overall diameter of the staining should be decreased by an amount proportional to the extent of overlap. Our measurements indicate that, on the contrary, the widths of double dot and bar centromeres are the same (double dot, 1.15 ± 0.12 μM; bar, 1.16 ± 0.12 μM).

These observations suggest to us that if CENP-B and -C are both components of the kinetochore domain [as suggested by the results of Brenner et al. (1981)], then CENP-B is also abundant in the subjacent centromeric heterochromatin. This suggestion is confirmed by the results of a detailed analysis of the distribution of CENP-B in the chromosome by immunoelectron microscopy of ultrathin sections of mitotic cells. In this study, CENP-B was found to be broadly distributed throughout the compact heterochromatin of the centromere region (C.A. Cooke and W.C. Earnshaw, in preparation).

Possible roles of CENP-B and -C

These results are consistent with a number of possible roles for CENP-B and -C in centromere structure and function. For example, CENP-C appears to be present in nearly equal

amounts at all centromeres, implying that it is a component of an invariant structure that is present on all chromosomes. One obvious candidate is the trilaminar kinetochore plaque structure (1). In contrast, CENP-B is present in variable amounts at the different centromeres. Since the most obvious structural component that varies characteristically between chromosomes is the DNA sequence, this suggests that the binding of CENP-B may be dictated at some level by DNA sequence recognition. CENP-B may be a sequence-specific DNA-binding protein, or it might bind to such a protein.

In one previous in vitro binding study, it was suggested that CENP-B binds to tubulin and thus anchors the microtubules to the kinetochore (Balczon and Brinkley 1987). Our finding that CENP-B is abundant in the subjacent centromeric heterochromatin suggests that CENP-B is unlikely to function solely as a microtubule anchor. Furthermore, the presence of CENP-B at the inactive centromere suggests that CENP-B alone is unable to mediate the chromosome-spindle attachment unless the structure of the inactive centromere is aberrant. (For example, the microtubule binding site might be blocked by some component that exhibits an abnormal binding to CENP-B. It is unlikely that CENP-B itself is altered in this cell line, since the protein appears to be capable of functioning normally at the active centromere.) Our results are consistent with the direct involvement of CENP-C in the attachment of the chromosomes to the spindle, however any interaction of CENP-C with tubulin has yet to be demonstrated (Balczon and Brinkley 1987; Pluta and Earnshaw unpublished work).

Models consistent with our data include the following. (1) The microtubule binding site might be formed partly by CENP-B and partly by a second kinetochore protein (possibly CENP-C). In the inactive centromere this second component might be either missing or assembled incorrectly. (2) CENP-B may act as a binding site for another kinetochore protein(s) (possibly CENP-C) that then interact(s) with the spindle microtubules. This second component might be missing or nonfunctional at the inactive kinetochore, as we have demonstrated for CENP-C. (3) Neither autoantigen may play a direct role in microtubule attachment. CENP-B and/or CENP-C may help establish the proper folding of the kinetochore chromatin fibers or be otherwise involved in assembly of the overall kinetochore structure. Alternatively, CENPs B and C may be involved in some downstream aspect of centromere function. For example, one or both could be involved in the movement of the chromosomes to the poles (Gorbsky et al. 1987; Koshland et al. 1987).

Implications of the immunostaining method

Our ability to "resuscitate" the immunostaining of methanol/acetic acid-fixed chromosomes with the swelling procedure has implications for the interpretation of other immunological studies of chromosomes. The mitotic chromosome is a highly condensed structure that may not be readily penetrated by antibodies under all ionic conditions. Therefore any *apparently* limited distribution of antigen within chromosomes may reflect the accessibility of the antigen to antibody rather than its presence or absence (particularly when antibodies are of the IgM subclass). This is most worrisome for antigens that appear to be restricted to the periphery of mitotic chromosomes [the "perichromin" staining pattern (McKeon et al. 1984; Wataya-Kaneda et al. 1987)]. Our results suggest that such peripheral staining patterns may need to be reexamined using staining protocols similar to that described here.

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