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Scanning Electron Microscopy of C-Banding

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ABSTRACT—The mechanism of C-banding was analyzed on the basis of the structural changes of the 30 nm chromatin fibre using scanning electron microscopy (SEM). SEM of non-banded metaphase spreads of L-cells revealed chromosomes consisting of 30 nm chromatin fibre loops along the entire length. No marked difference in both the dimension and appearance of such looped structures was discernible between the centromeric region and the rest of the chromosome. In contrast, C-banded chromosomes exhibited a conspicuous alteration of the fibre conformation in the centromeric region. The looped, fibrous structures were almost completely lost from this region, while the non-centromeric region still exhibited fibrous structures with slightly different appearances compared with those observed in the control chromosomes. On the other hand, results obtained using fluorescence microscopy showed that more DNA retained in the centromeric region than in the non-centromeric region. Since the analytical experiments exhibited that the characteristic collapsed state of the centromeric region occurred only with the alkali treatment but neither with the 2 x SSC nor acid treatments, the centromeric heterochromatin seemed to contain some specific protein which should be sensitive to alkali. The structurally collapsed but subsecuently compact centromeric region may become more, or still, resistant to the DNA extraction due to the 2 x SSC treatment and the centromeric chromatin thus retained may be visualized as the C-band.

INTRODUCTION

It is well known that the technique of C-banding selectively stains the constitutive heterochromatin located mainly in the centromere region of the metaphase chromosome. The technique originally came from the *in situ* hybridization study of mouse heterochromatin in the chromosomes prepared using the air-drying method (Pardue and Gall, 1970) and accomplished by Arrighi and Hsu (1971) as the most reliable method for detecting C-bands. Comings *et al.* (1973) claimed that C-banding was independent upon the differential renaturation of DNA and that C-bands were produced when the more delicate chromatin structures in the arms were disrupted. The use of transmission electron microscopy (TEM) for examining the mechanism of C-banding revealed and supported that the selective loss of non-centromeric chromatin was involved in the production of C-bands (Burkhorder, 1975; Comings *et al.*, 1973). Jack *et al.* (1985) carried out the SEM of C-banded chromosomes and reported that the regions corresponding to the C-bands were highly condensed with tightly packed chromatin fibers, resembling non-banded chromosomes. On the other hand, the present SEM study on the C-bands revealed that the tightly packed state of the chromatin fibres observable in the C-banded chromosomes did not resemble the appearance of the chromatin fibres discernible in the centromeric regions of the non-banded chromosmes and that marked structural changes occurred in the centromeric regions of the C-banded chromosomes. On the basis of the present findings both from the SEM and fluorescence examination, the mechanism of C-banding was discussed.

MATERIALS AND METHODS

Mouse L-cells were grown in Eagle's MEM supplemented with 5% fetal calf serum. After colchicine treatment (0.5 μg/ml medium) for 1 hr, cells were treated with 0.075 M KCl, fixed in methanol/ acetic acid (3/1) and air-dried on clean glass slides at 5°C in a cooling box.

C-banding was carried out on the basis of Sumner's BSG (barium hydroxide/saline/ Giemsa) method (Sumner, 1972). Briefly, chromosome preparations were placed in a freshly prepared 5% aqueous solution of barium hydroxide at 50°C for 15 min, incubated for 1 hr at 60°C in 2 × SSC (0.3 M sodium chloride containing 0.03 M tri-sodium citrate) and stained with 2% Giemsa in PBS for 15 min. C-banded chromosomes were photographed and then processed for SEM. Some preparations treated with the C-banding procedure were directly processed for SEM without Giemsa staining. For analyzing the action of the C-banding procedure, some other preparations were treated with alkali or acid alone and examined on the chromosome DNA *in situ*. The effect of 2 × SSC treatment alone was examined as well. As a simple way for *in situ* detection of DNA, Hoechst 33258 staining combined with fluorescence microscopy was adopted. Preparations were stained with Hoechst 33258 (0.1 μg/ml) for 10 min, and then examined under a fluorescence microscope using the G-stimulated ultraviolet light. For confirming the aging effect on C-banding, preparations immediately after air-drying and ones aged for various times ranging from 1 to 7 days were examined.

For SEM, air-dried preparations were processed by a modification

(Takayama and Hiramatsu, 1993) of the osmium-thiocarbohydrazide (OTO) technique (Ip and Fishman, 1979). Immediately after air-drying, or after processing for light microscopy including fluorescence microscopy, chromosome preparations were immersed in phosphate buffer, pH 6.8 for 10 min at 37°C, fixed in 3% glutaraldehyde in buffer for 60 min, and washed in buffer. The preparations were then fixed in 0.5% OsO_4 in buffer for 2 min, washed in distilled water, immersed in an aqueous saturated solution of thiocarbohydrazide (TCH) for 2 min and washed in distilled water. The procedure of osmium fixation followed by TCH incubation was repeated more than six times. The preparations were dehydrated in a graded ethanol series and finally dried by a modification (Takayama *et al.*, 1989) of the freeze-drying method including *t*-butyl alcohol substitution (Inoue and Osatake, 1988). Briefly, preparations in ethanol were transferred to *t*-butyl alcohol three times, put on the cooling plate (-20°C) of a drying apparatus (RMC-Eiko ID-2) and the frozen *t*-butyl alcohol covering the specimens was completely sublimated by evacuation for about 90 min. The dried preparations were lightly sputter coated with gold-palladium alloy in an ion-coater (Meiwa SC-500A). Chromosome structures were examined and photographed with a high resolution SEM (JEOL JMS-880). The most SEM micrographs excepting some special ones were taken without tilting specimens.

RESULTS

According to our experience, aged chromosome preparations made by the air-drying method are in general not suitable for obtaining satisfactory SEM images. The use of preparations immediately after the air-drying, however, invariably resulted in poor C-bands that were accompanied with G-band like segments in the arms. Typical C-bands were obtained using 7-day-old preparations (Fig. 1a). As is seen in Fig. 1b, these C-banded chromosomes at lower magnification show a lower intensity of SEM signal in the C-band regions compared with the rest of the chromosomes, which suggests that a distinct structural difference exists between the centromeric and non-centromeric regions. An enlarged SEM image of one of these C-banded chromosomes (Fig. 1c) clearly reveals such a structural difference. The C-banded region appears severely collapsed and no fibrous structures are discernible. In contrast to this, the non-centromeric region still shows some fibrous structures whereas they appear somewhat disruptive. On the other hand, no structural difference was discernible between the centromeric and non-centromeric region of the non-banded chromosomes (Fig. 1d). The examination utilizing Hoechst fluorescence also revealed that fairly even distribution of fluorescence along the chromosomes with slightly accented spots at the centromeres in the non-banded chromosomes (Fig. 1e). The fluorescence pattern in the C-banded chromosomes, however, appeared identical to the Giemsa C-banding pattern (Fig. 1f, g). Since the SEM results obtained seemed contradictory with the fluorescence data, a series of analytical examination on the C-banding procedure was carried out.

For obtaining reliable SEM images, experiments were performed exclusively with the 1-day-old preparations. Firstly, to know the 30 nm chromatin fiber configuration in the control, non-banded chromosomes were examined in detail along the length of them. As is shown in Fig. 2a and c, each of the non-banded chromosomes was seen to consist of uniform fibrous structures along the entire length including the centromeric region. In the images at higher magnification (Fig. 2b, d), the fibrous structures were seen to be entangled 30 nm chromatin fibre loops, and no discernible differences of the looped structures in both dimension and appearance were found between the centromere and the non-centromeric region. A stereo pair of the images at higher magnification (Fig. 2d) clearly reveals that the centromeric region appears as well ridged as the non-centromeric region does.

The effect of the alkali treatment alone which is used as the first step in the C-banding technique was examined without the following treatment of the saline. The chromosomes thus treated and stained with Giemsa exhibited no C-bands (Fig. 3a). Fluorescence examination of them, however, revealed accented fluorescent spots at the centromeres together with a fluorescence pattern resembling the G-bands in the non-centromeric regions, suggesting that selective DNA extraction occurred to some extent (Fig. 3b). SEM images of these chromosomes (Fig. 3c, e) showed markedly altered centromeres which quite resembled those of the typical C-banded chromosomes (Fig. 1b). A highly tilted SEM image (Fig. 3d) and a stereo pair of ones (Fig. 3f) clearly reveal that the centromeric regions are markedly depressed and that the fibrous structures are almost completely lost from these regions, whereas the non-centromeric regions still exhibit neat looped structures of 30 nm chromatin fibres. To confirm the uniqueness of the fiber disrupting effect of alkali, the 2 x SSC treatment was carried out omitting the alkali treatment. Giemsa staining of the chromosomes treated with the 2 x SSC alone resulted in the formation of G-bands (Fig. 4a). Hoechst fluorescence examination of the treated chromosomes showed accented fluorescence at the centromeres together with G-band like, weak fluorescence patterns (Fig. 4b), suggesting the occurrence of some selective DNA loss during this treatment. On the other hand, SEM images of these chromosomes revealed the identical appearances in both the centromeric region and the rest of the chromosomes (Fig. 4c). As is seen in Fig. 4d and e, enlarged images of them revealed that both the centromeric and non-centromeric regions of the chromosomes consisted of the looped structures of 30 nm chromatin fibres which appeared considerably dispersed compared to those in the control chromosomes. The highly tilted image shown in Fig. 4e exhibits that the centromeric region appears still as well ridged as the rest of the chromosome does. These SEM results evidently show that in contrast to the alkali treatment, the 2 x SSC treatment brought about no differential alteration of fibrous structures in both the centromeric and non-centromeric region.

The effect of the treatment with acid instead of alkali on the fibrous structures was examined by treating chromosomes with HCl at a variety of concentration at room temperature for 60 min without the SSC treatment. Chromosomes treated with 0.2 N HCl exhibited no discernible alteration in the fibrous structures along the entire length of the chromosomes (Fig. 5a). After treatment with 0.5 N HCl, however, loss of the fibrous

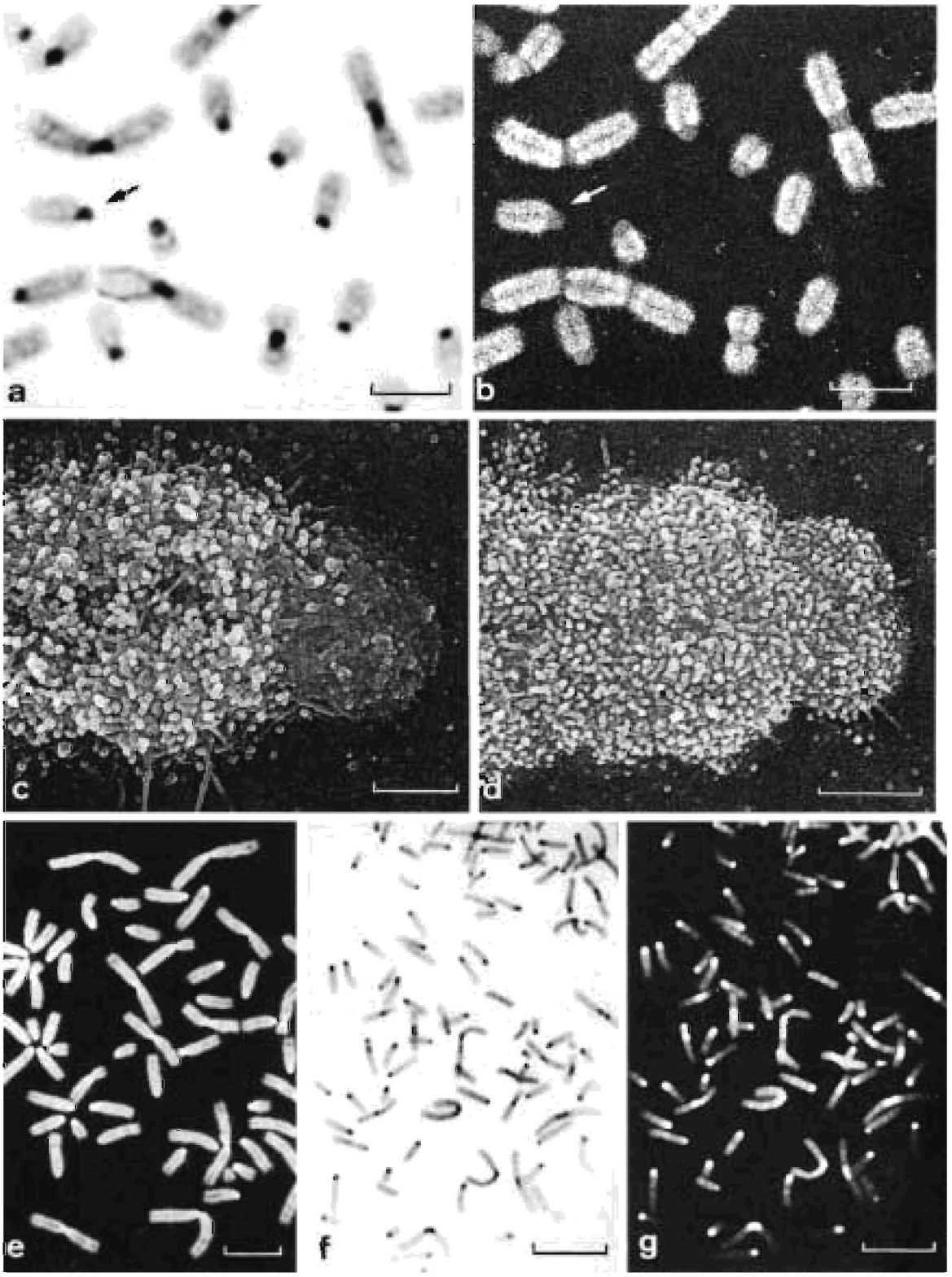

Fig. 1. C-banded and non-banded metaphase chromosomes from 7-day-old preparations of L-cells. Bars represent 5 μm for a, b, e, 500 nm for c, d and 10 μm for f and g, respectively. (a) Metaphase chromosomes with typical C-bands. (b) SEM images of the same chromosomes as shown in a. (c) Enlargement of the same chromosome as pointed with an arrow in a and b. (d) SEM image of a non-banded chromosome. (e) Fluorescence micrograph of non-banded chromosomes after staining with Hoechst 33258. (f) C-banded metaphase spread. (g) The same metaphase as shown in f, using fluorescence microscopy.

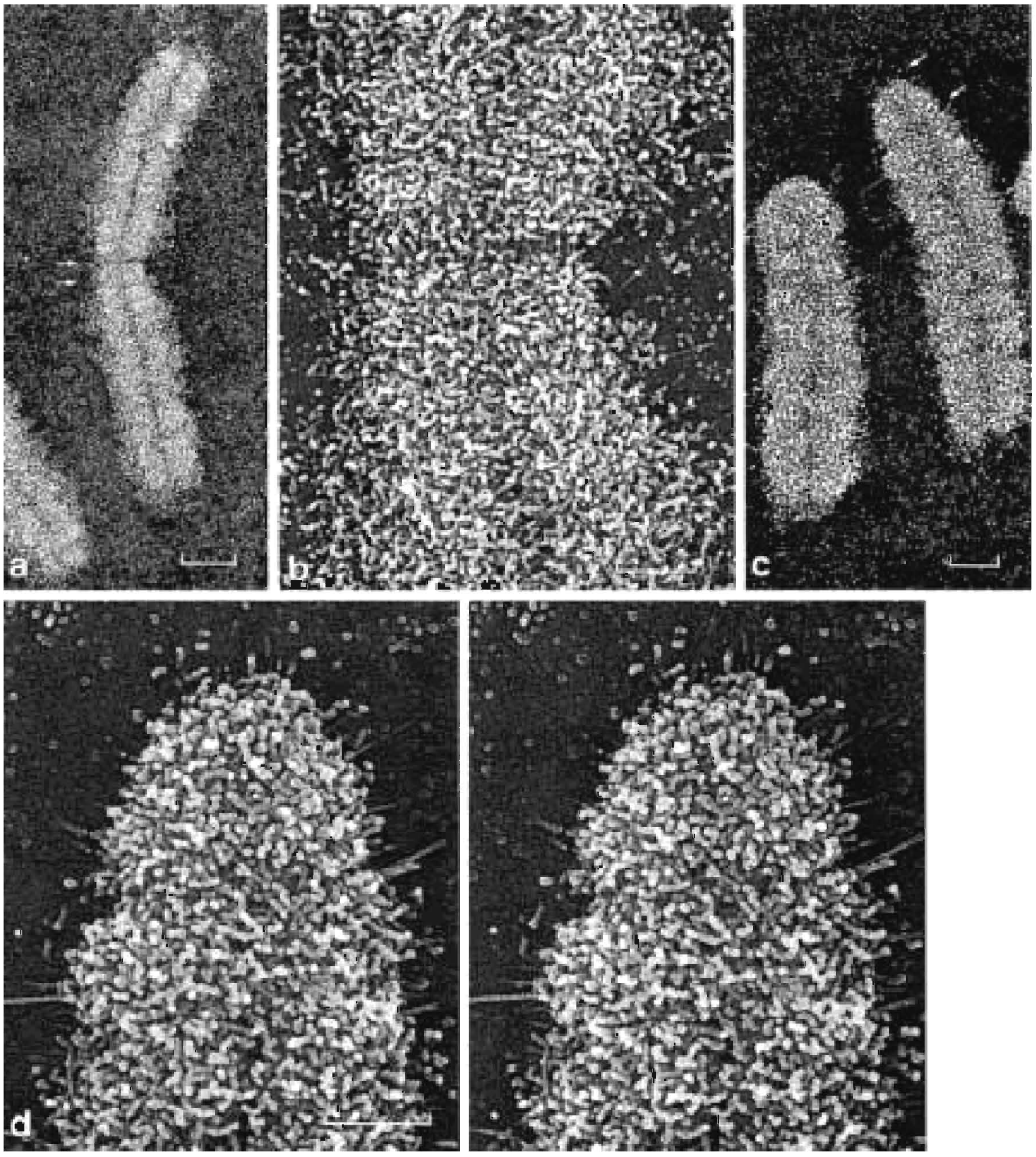

Fig. 2. Non-banded chromosomes from 1-day-old preparations. Bars represent 1 μm for a, c, and 500 nm for b, d, respectively. (a) Metacentric chromosome. The centromere is shown with arrows. (b) Enlargement of the centromere and pericentromeric region of the same chromosome as shown in a. (c) Acrocentric chromosomes. (d) Stereo pair of the enlargement of the centromere and pericentromeric region of the chromosome pointed with arrows in c, photographed at 0° and 8° of tilting.

structures occurred in both the centromeric and non-centromeric region (Fig. 5b). As the concentration of HCl was increased, damages of fibrous structures increased without showing any structural differentiation between the centromeric and non-centromeric region.

DISCUSSION

Jack *et al.* (1985) stated that C-banded regions, which stained positivbely in LM, were highly condensed with tightly packed chromatin fiber, resembling non-banded chromosomes. They, however, did not present any SEM photographs showing such a highly condensed state of the non-banded chromosomes in their paper. On the other hand, our present findings clearly demonstrated that the SEM image of the tightly packed stated of the centromeric region of the C-banded chromosome did represent the drastic alteration from the not packed state of the non-banded chromosome. This is quite evident when the SEM image of C-banded chromosome shown in Fig. 1c is compared with that of non-banded

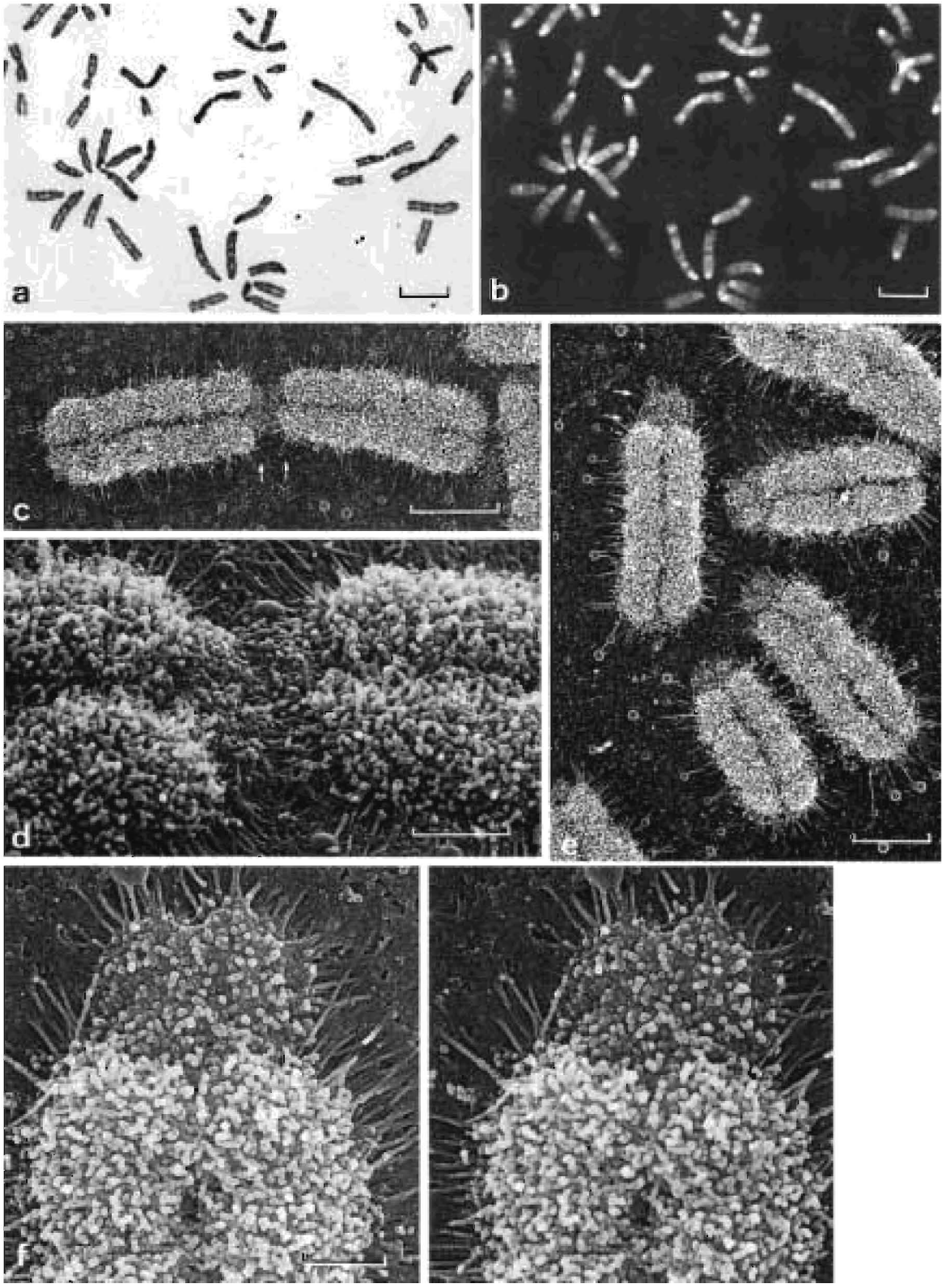

Fig. 3. Chromosomes treated with $Ba(OH)_2$ alone for 10 min but not followed with 2 x SSC treatment. (a) Giemsa staining. Bar represents 5 μm. (b) The same chromosome spread as a, stained with Hoechst dye and photographed under a fluorescence microscope. (c) Metacentric chromosome with a collapsed centromere (arrows). Bar represents 2 μm. (d) Enlargement of the same chromosome as c, at 45° of tilting. Bar represents 500 nm. (e) Four acrocentric chromosomes with collapsed centromeres. Bar represents 2 μm. (f) Stereo pair of the enlargement of the same chromosome as pointed with arrows in e. Bar represents 500 nm.

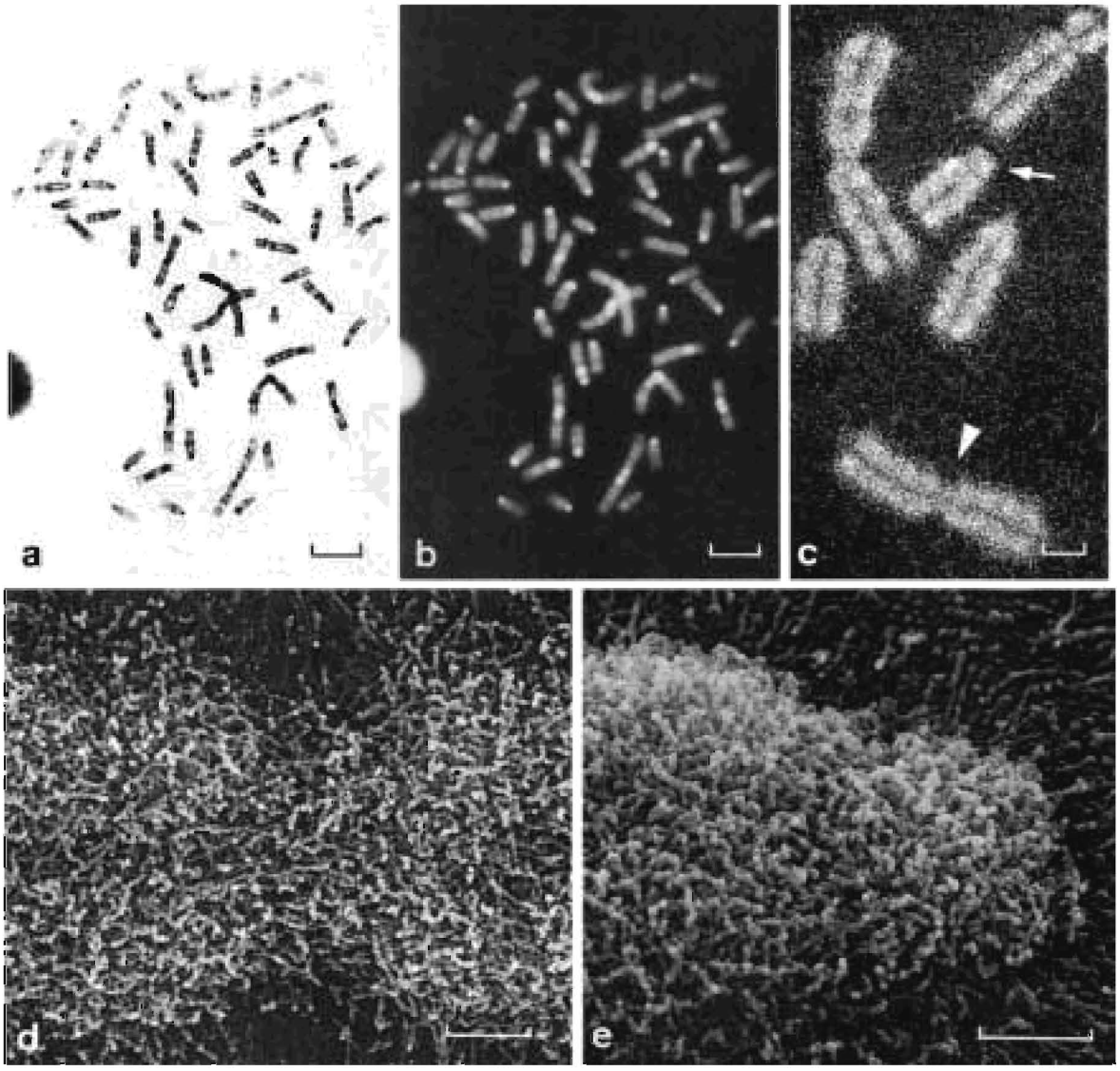

Fig. 4. Chromosomes treated with 2 x SSC alone without Ba(OH)2 pretreatment. (a) Giemsa staining. Bar represents 5 μm. (b) The same chromosome spread as a, stained with Hoechst dye and photographed under a fluorescence microscope. (c) SEM images of treated chromosomes. Bar represents 2 μm. (d) Enlargement of the same chromosome as pointed with an arrowhead in c. Bar represents 500 nm. (e) Enlargement of the same chromosome as pointed with an arrow in c, at 45° of tilting. Bar represents 500 nm.

chromosome (Fig. 1d). The results from our analytical experiments offer further convincing evidence, i.e., the alkali treatment alone resulted in the drastic change of the fibrous structures in the centromeric region together with the retention of these in the non-centromeric region (Fig. 3d, f). It is, therefore, evident that Jack *et al.*'s interpretation and the results of the present study are in contradiction.

On the other hand, the results of fluorescence examinations using the Hoechst dye following both the typical C-banding procedure and the single treatment using alkali or 2 x SSC invariably suggested the selective extraction of DNA from non-centromeric regions with a greater proportion being retained in the centromeric regions. These results seem consistent with the mechanisms so far proposed by several authors (Burkhorder, 1975; Burkhorder and Duczek, 1982; Comings *et al.*, 1973; Holmquist, 1979), whereas these seem incompatible with the conclusion from the present SEM study. One of possible explanations in this problem is as follows: the compaction of the centromeric chromatin substance as a result of the collapse of the fibrous structures induced by the alkali treatment makes the centromeric region more, or still, resistant than the non-centromeric region to the action of DNA extraction by the 2 x SSC treatment and DNA thus retained in the centromeric region is stained with Giemsa as the C-band. Since no drastic changes of the 30 nm chromatin fibre structures are seen in G-banded chromosomes as shown after the 2 x SSC treatment alone, the mechanism of C-banding seems considerably different from that of G-banding. In the latter the interaction between the chromatin and the Giemsa dye is probably much more complicated than in the former.

It is interesting to note that the drastic structural change of the centromere was induced exclusively with the alkali

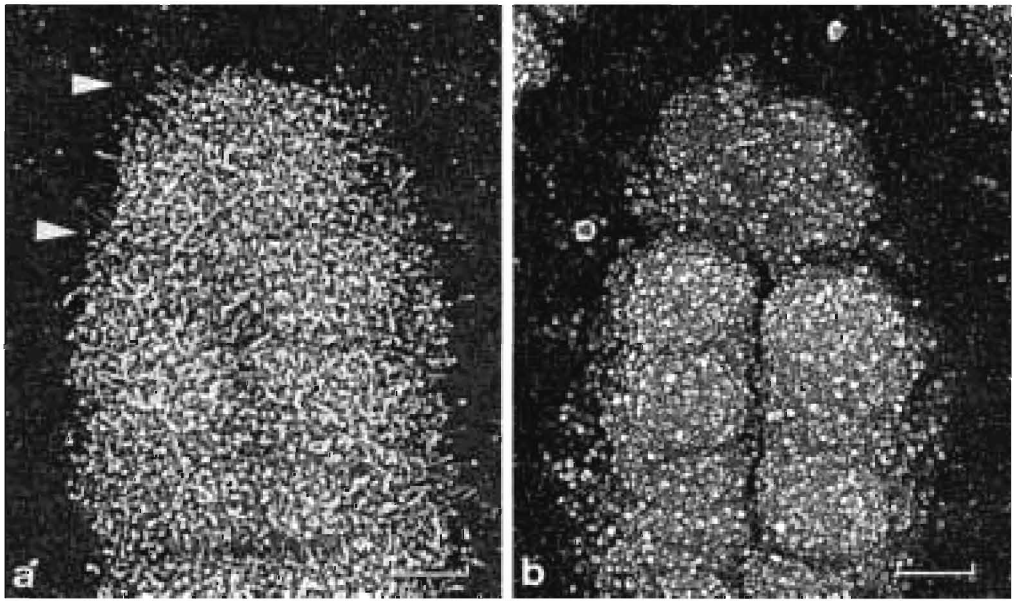

Fig. 5. Chromosomes treated with HCl at room temperature for 60 min instead of $Ba(OH)_2$ without 2 x SSC treatment. Bars represent 500 nm, respectively. (a) Chromosome treated with 0.2 N HCl. Arrowheads show the centromere. (b) Chromosome treated with 0.5 N HCl.

treatment. The result seems suggestive of the existence of some special protein in the centromeric chromatin which should be specifically sensitive to alkali. The fact that the integration of morphology of the 30 nm chromatin fiber in the non-centromeric region is not necessarily affected by the removing of DNA from the fibre seems also significant in characterizing the different sorts of chromatin fibres. At present, molecular mechanisms involved in the higher order chromosome structure remain limited. Topoisomerase II has been identified as a major component of the chromosome scaffold (Earnshaw *et al.*, 1985; Gasser *et al.*, 1986) and proposed to act as a loop fastener by directly interacting with specific DNA sequences (SARs) (Gasser and Laemmli, 1987). Since in spite of the severe collapse of the centromeric chromatin fibres, the fibrous structures in the non-centromeric region were retained, the alkali sensitive protein in question seems to be some non-histone protein other than topoisomerase II, being probably involved in the integration of the 30 nm chromatin fiber of the centromeric heterochromatin. It has been well known that the centromeric heterochromatin of mammalian chromosomes contains highly repetitive DNA sequence. As such a type of DNA, the α-satellite (alphoid) DNA has been known to be located in all the centromeres of the human chromosome complement (Manuelidis, 1978; Waye and Willard, 1987). Earnshaw and Rothfield (1985) identified three chromosomal antigens recognizable with the antibody species obtained from a patient with scleroderma CREST and designated them as CENP (CENtromre Protein) -A, -B, and -C. As Earnshaw *et al.* (1989) had suggested, direct binding by CENP-B of the CENP-B box sequence in the alphoid DNA was proved by Muro *et al.* (1992). CENP-B might be one of the possible candidates for the protein component of the alkali sensitive centromeric heterochromatin.

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