

Changes in Chromosomal Surface Structure by Different Isolation Conditions*

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Summary. The human cell cycle was synchronized and the chromosomes were isolated by a centrifugation method using two representative solutions for chromosome isolation (a polyamine buffer, PAB and citric acid solution, CAS) and fixatives. The centrifugation method yielded sufficient amounts of human metaphase chromosomes. Observation of the isolated chromosomes by scanning electron microscopy (SEM) revealed two types of surface structure which have been repeatedly reported to date: the human chromosomes in the PAB were relatively smooth but covered irregularly with scaly structures, while the surface of the chromosomes in the CAS exhibited a dense fibrous structure with a uniform diameter of 50–70 nm. Comparison of proteins extracted from chromosomes isolated with the PAB and CAS clearly indicated the removal of linker histones, H1, from chromosomes isolated with the CAS. These findings imply that the two different images of human chromosomes frequently observed by SEM are due to the removal of peripheral chromosomal materials including linker histones and/or the depletion of linker histones which prevent the surface chromatin fibers from scattering.

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A eukaryotic chromosome is a super molecule consisting of two strands of DNA molecules, histones and non-histone proteins, and appears when chromatin fibers form chromosomes at mitosis. Although attempts to elucidate the higher order structure of the chromosome have been made since the beginning of the last century, its precise structure has not yet been clarified due to methodological limitations.

One example of this lies in the importance of accurately maintaining the three-dimensional structure of the chromosome in order to analyze it using a scanning electron microscope (SEM). Sample preparation methods including critical point drying have thus been well examined. It is also important to avoid excess coating with metal, which can hide the true surface structure. Using such sophisticated methods for SEM, it has been reported that the chromosomes of animals (SWENEY et al., 1979) and plants (IWANO et al., 1997) showed globular structure on their surfaces. The knobby surface 65–70 nm in diameter is thought to represent the peripheral tips of radial loops of a 30-nm chromatin fiber (MARSDEN and LAEMMLI, 1979; ADOLPH and KREISMAN, 1985). The three-dimensional folding of the scaffold in histone H1-depleted chromosomes by immunofluorescent staining with an antibody specific to topoisomerase II showed predominantly opposite helical handedness of the sister chromatid (BOY DE LA TOUR and LAEMMLI, 1988). From these observations, one of the most probable higher order structure of a chromosome is that a radial looped chromatin fiber enwraps a scaffold, which is helically coiled by the condensation of chromatin fibers (RATTNER and LIN, 1985). On the other hand, in the mitotic barley and rye chromosome, a rather smooth surface resembling tiles on a

roof or braided strands without showing any fibrous structure has also been reported (MARTIN et al., 1996).

In this paper, we describe the effect of chromosome isolation solutions mainly on the surface structure of human chromosomes, as revealed by SEM. We also suggest that the different surface structures of the isolated chromosomes are due to the depletion of linker histones from the chromosomes.

MATERIALS AND METHODS

Cell synchronization and chromosome isolation

The procedure of chromosome isolation is summarized in Figure 1. First, a human lymphoblast-like cell line, K562, subcultured once in 3 days in RPMI1640 (GIBCO BRL) with 10% Fetal bovine serum (FBS),

was treated with 20 ng/ml colcemid (Sigma, Tokyo) for 12 h to arrest the cell cycle at the M phase. Synchronized cells were collected by centrifugation and resuspended into a hypotonic solution of 75 mM KCl (pH 5.7) or Ohnuki's solution (55 mM KCl:55 mM NaNO_3 :55 mM CH_3COONa =4:2:0.8) (OHNUKI, 1968). After treatment of the KCl hypotonic solution for 30 min or Ohnuki's solution for 2 h, the cells were collected by centrifugation and resuspended into either of the following two solutions for chromosome isolation. One was a modified polyamine buffer (PAB; 15 mM Tris-HCl, 2 mM EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM EGTA, 0.2 mM spermine, 0.5 mM spermidine, pH 7.2) containing 1 mg/ml digitonin (Sigma), 14 mM 2-mercaptoethanol and 0.1 mM PMSF, which was developed and used for the ordinary isolation of mammalian chromosomes in various chromosomal studies (KURIKI and HARUO, 1997,

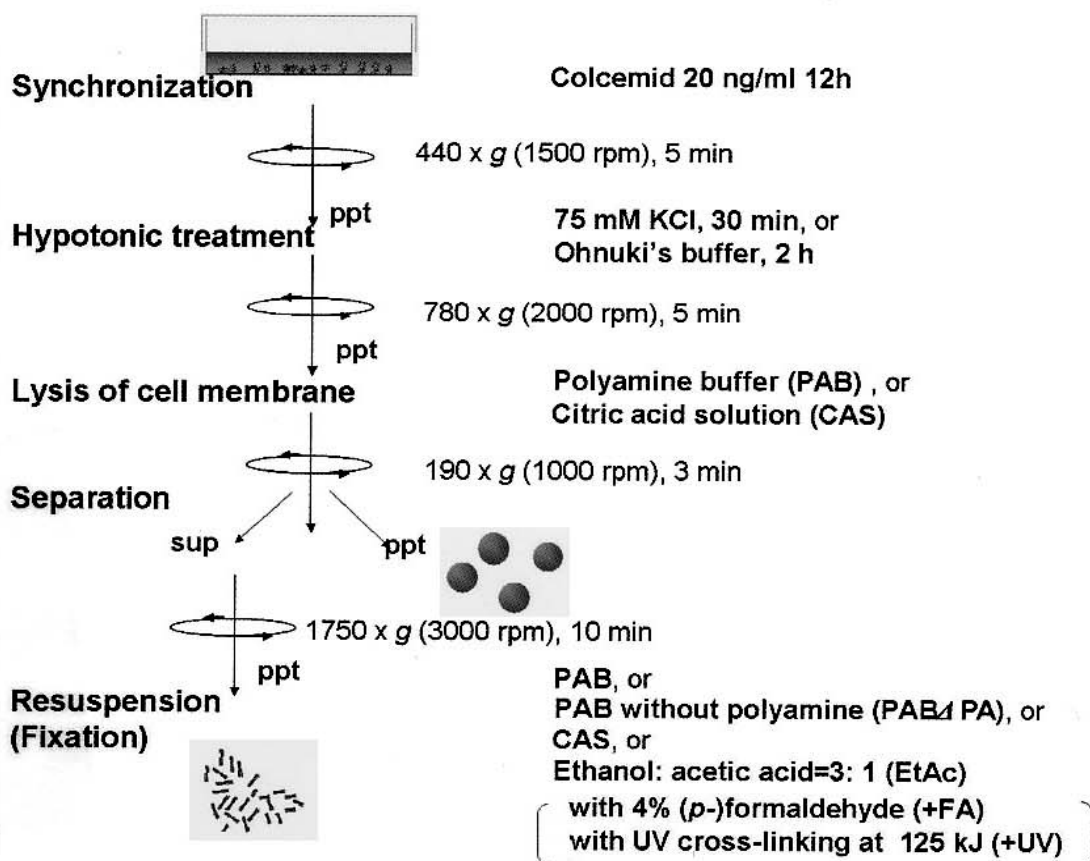


Fig. 1. The procedure for chromosome isolation consists of the five steps of cell synchronization, hypotonic treatment, removal of the cell membrane, separation by centrifugation, and final resuspension with or without fixation.

1997, SPECTOR et al., 1998). The other was a citric acid solution (CAS; 0.1 M citric acid, 0.1 M sucrose, 0.5% Tween20, pH 2.6) that was originally used to fractionate human chromosomes at the border between dextran and polyethylene glycol (PEG) solutions (PINAIEV et al., 1979). After lysis of the cell membrane in either the PAB or CAS, the chromosomal suspension was centrifuged at $190 \times g$ for 3 min at a temperature of 4°C . The chromosome rich fraction (C fraction) was carefully recovered as the supernatant (sup), and the nuclei rich fraction (N fraction) was recovered as the precipitation (ppt). The C fraction was then centrifuged again at $1750 \times g$ for 10 min at 4°C . The precipitated chromosomes were resuspended in a fresh sample of the same chromosome isolation solution. The separation profiles were confirmed by flow cytometry (EPICS Elite, Beckman Coulter) after staining with $35 \mu\text{g}/\text{ml}$ propidium iodide (PI). After separation by centrifugation, several different conditions of fixation were applied as follows: resuspension in the same buffer (PAB or CAS), resuspension in PAB without polyamine (PAB Δ PA), fixation in either of the PAB

or CAS including 4% para-formaldehyde (PAB+FA, CAS+FA), fixation in acetic ethanol (ethanol: acetic acid=3:1, EtAc), or fixation with ultraviolet (UV) light cross-linking at 125 kJ after resuspension in the PAB or CAS (PAB+UV, CAS+UV). For comparison, a conventional method of direct extraction of chromosomes into EtAc after the centrifugation following hypotonic treatment was also employed to examine the yielding efficiency morphological changes by light microscopy.

Chromosome observation by light and scanning electron microscopy

Isolated chromosomes were placed on glass slides, stained with $1 \mu\text{g}/\text{ml}$ 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) and observed with a fluorescence microscope (Axioplan2 Imaging, Zeiss). Then isolated chromosomes were mounted on a plastic sheet (Wako, Tokyo), which was coated with poly-L-lysine (Sigma) and fixed with 4% para-formaldehyde in PAB or CAS. After washing with the same solution, the specimens were dehydrated in an ethanol series and freeze-dried using the t-butanol drying

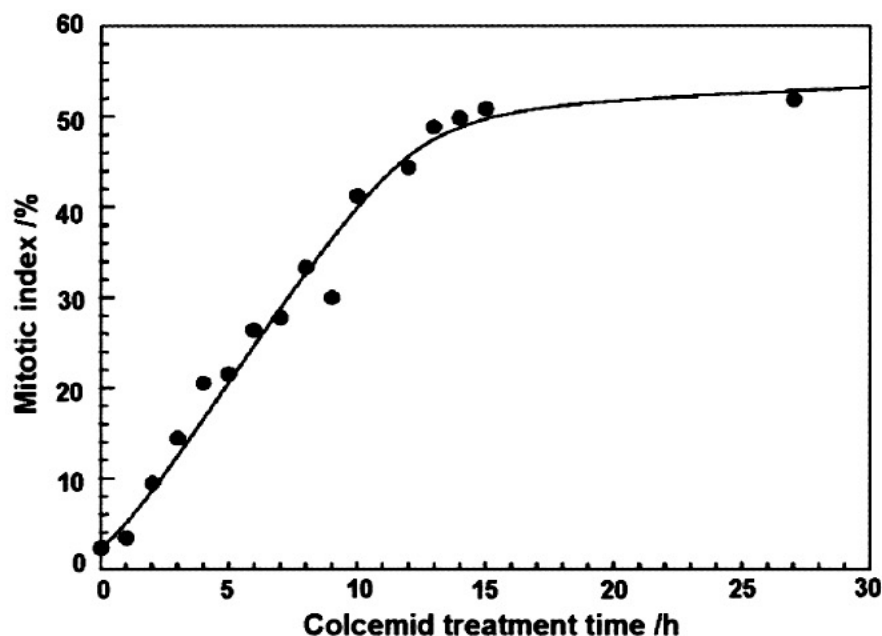


Fig. 2. Cell synchronization with colcemid treatment. More than 50% of the cells were arrested at the M phase after 12-15 h of treatment.

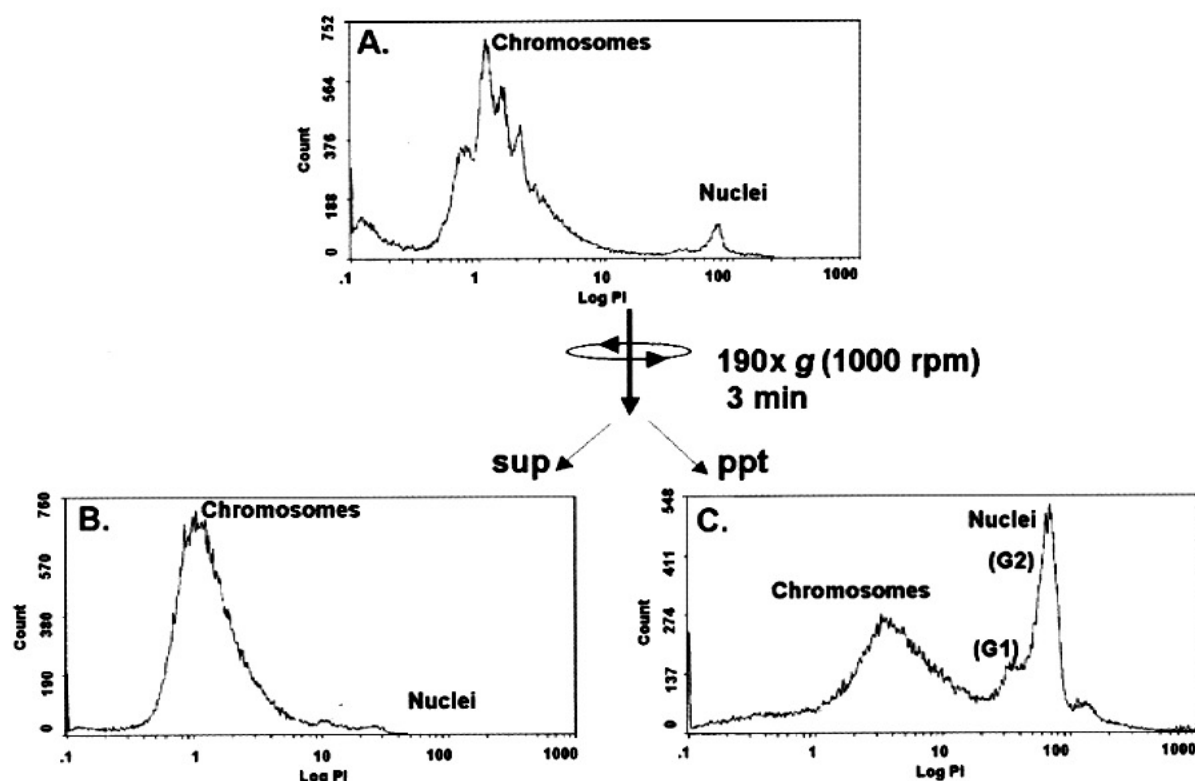


Fig. 3. The separation profiles of the chromosome-rich fraction (C fraction) and the nucleus-rich fraction (N fraction) in a polyamine buffer (PAB), separated by centrifugation at $190\times g$ for 3 min at 4°C . Each fraction and the cell lysate before centrifugation were stained with propidium iodide (PI) and analyzed by flow-cytometry. **A.** Before centrifugation. **B.** Supernatant (sup, C fraction). **C.** Precipitation (ppt, N fraction). Almost no nuclei peak was detected for the C fraction compared with the samples before centrifugation. On the other hand, the peaks of G2 nuclei and G1 nuclei are clearly detected for the N fraction. The results show that most nuclei were precipitated and chromosomes remained in the supernatant.

method (INOUE and OSATAKE, 1988). The specimens were osmium-coated with an osmium plasma coater (OPC40, Nippon Laser) and examined in a scanning electron microscope (S-4700, Hitachi) at 10 or 15 kV.

Isolation and identification of the chromosomal proteins

Isolated chromosomes with PAB or CAS were transferred into a microtube and collected by centrifugation at $1900\times g$ for 10 min at 4°C followed by resuspension into phosphate buffered saline (1x PBS). Proteins were extracted by the acetic acid method (HARDY et al., 1969; IZUTSU et al., 2001) with a minor

modification. Briefly, a 1/10 volume of 1 mM MgCl_2 and 2 volumes of acetic acid were added to the extract and the mixture was stirred for 1 h at 4°C with a microtube stirrer. After centrifugation at $4400\times g$ for 10 min, the supernatant was dialyzed three times against 2% acetic acid with Spectra/P membrane (MWCO=1000, Spectrum Medical Industries Inc.). The solutions of extracted proteins were frozen by liquid nitrogen and lyophilized. The lyophilized proteins were diluted into 1x SDS sample buffer and applied to 12% SDS-PAGE gel. After the electrophoresis, the gel was stained with Coomassie Brilliant Blue (CBB).

RESULTS

Synchronization of cell cycles of human K562 cell line

By the 20 ng/ml colcemid treatment the proportion of K562 cells arrested at mitosis (mitotic index, MI) increased gradually to reach a general plateau at 50% up to 12–15 h after the treatment (Fig. 2). The colcemid treatment was then carried out for 12 h in further experiment.

Chromosome isolation and the separation profiles

After resuspension of the hypotonic treated cells in the PAB or CAS, the cells were burst and the individual chromosomes were suspended in the solution, thus enabling the collection of the chromosomes with centrifugation. The separation profiles for PAB were examined by flow cytometry (Fig. 3). Before centri-

fugation, four chromosomal peaks and a single nuclear peak were clearly observed by flow-karyotyping (Fig. 3A). After centrifugation, one merged peak was detected for the chromosome fraction (C fraction) (Fig. 3B), and the peaks of G2 and G1 nuclei became prominent in the nuclear fraction (N fraction) together with a peak of the reduced amount of chromosomes (Fig. 3C). These data show that nuclei were precipitated and most of the isolated chromosomes remained in the supernatant.

Two different isolation solutions were combined with several fixation methods in order to examine their effects on the chromosome isolation according to the following three criteria: yielding efficiency, maintenance of morphology, and purity of isolated chromosomes. The results are summarized in Table 1. Figure 4 also depicts DAPI-stained chromosomes under four representative isolation and fixation conditions (D, E, H, J in Table 1) observed by light microscopy. Chromosomes isolated with the PAB

Table 1. Chromosome preparation under various conditions

Conventional method		Yielding	Morphology	Purity
O	Ethanol : Acetic acid = 3 : 1	++	++	ND
O'	Ohnuki's buffer → EtAc (55 mM KCl : 55 mM NaNO ₃ : 55 mM CH ₃ COONa = 4 : 2 : 0.8)	++	++	ND
Centrifugation method		Yielding	Morphology	Purity
A	Polyamine buffer (PAB) (15 mM Tris-HCl, 2 mM EDTA 80 mM KCl, 20 mM NaCl, 0.5 mM EGTA, 0.2 mM spermine, 0.5 mM spermidine, pH 7.2, with 1 mg/ml digitonin, 14 mM 2-mercaptoethanol, 0.1 mM PMSF)	+++	+	+
B	PAB → EtAc	++	++	+
C	PAB + 4%(p)-formaldehyde (PAB + FA)	++	+	-
D	PAB + UV cross-linking (UV)	++	+	+
E	PAB without polyamine (PABΔPA)	++	-	+
F	PABΔPA + FA	++	+	-
G	Citric acid solution (CAS) (100 mM citric acid, 100 mM sucrose, 0.1% Tween 20, pH 2.7)	+++	+	+
G'	Ohnuki's buffer → CAS	++	++	+
H	CAS → EtAc	++	+++	++
H'	Ohnuki's buffer → CAS → EtAc	++	++	++
I	CAS + FA	++	++	-
J	CAS + UV	++	+	++

(In the methods with a prime, Ohnuki's buffer was used as a hypotonic solution. In the other methods without a prime, 75 mM KCl was used. Arrows indicate an exchange of suspending solution.) +++: very high or well preserved, ++: high or preserved, +: moderate or moderately damaged, -: low or damaged, ND: not determined.

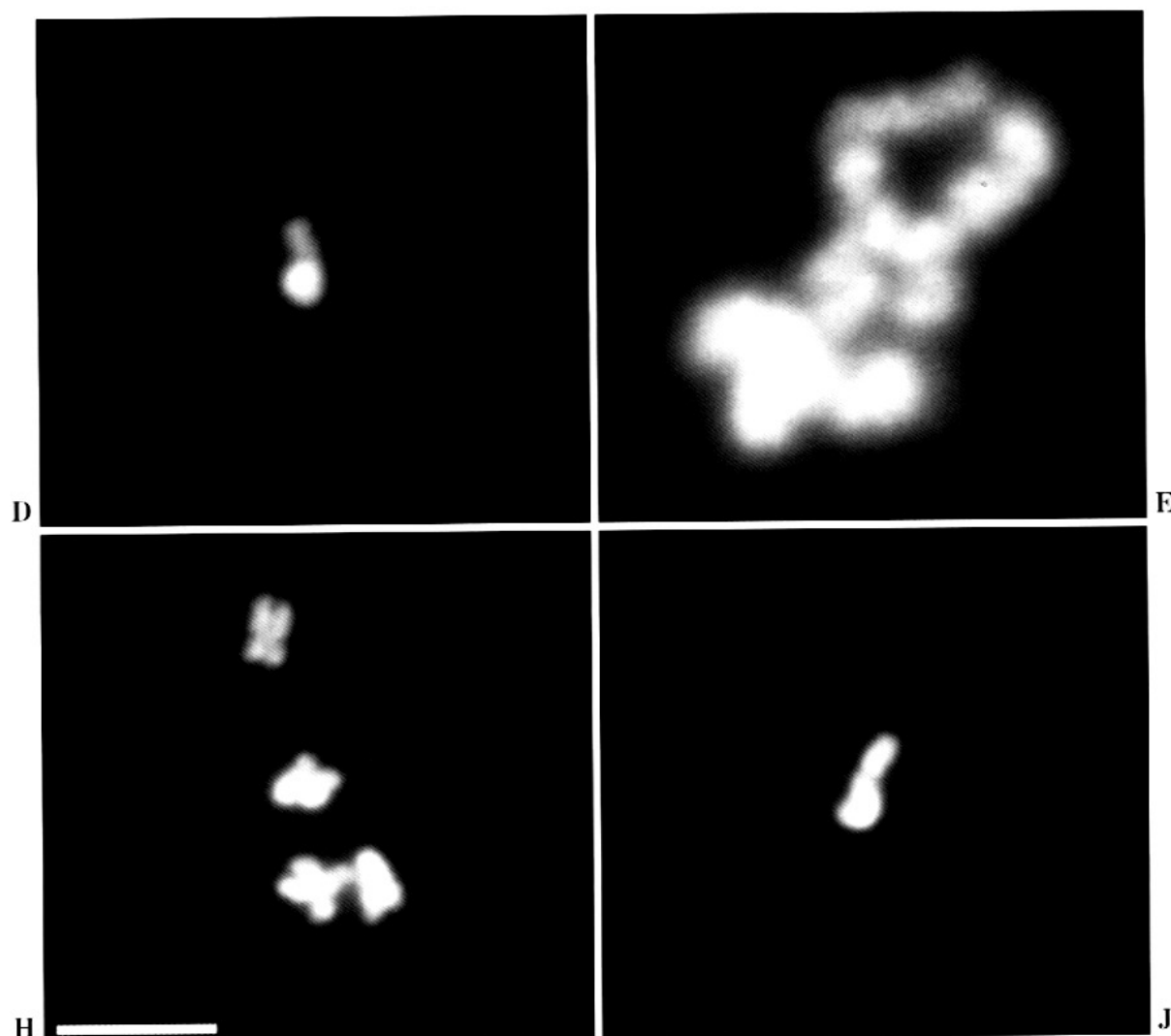


Fig. 4. Fluorescent images of DAPI-stained chromosomes isolated by different conditions. The alphabetical indications correspond to those in Table 1. **D.** Resuspension into the modified polyamine buffer (PAB) followed by UV cross-linking (PAB+UV). **E.** Resuspension into the PAB without polyamine (PAB Δ PA). Chromosomes are extensively swollen. **H.** Resuspension into the citric acid solution (CAS) followed by the exchange of the solution to ethanol: acetic acid=3:1 (EtAc). Sister chromatids of each chromosome are clearly observed in this treatment. **J.** Resuspension in the CAS followed by UV cross-linking (CAS+UV).

followed by UV cross-linking (PAB+UV) showed a compact morphology (Fig. 4D). The chromosomes isolated with PAB and resuspended into the PAB without polyamine (PAB Δ PA) were extremely swollen and damaged but still preserved their morphological characteristics such as centromeric constriction and sister chromatid cohesions (Fig. 4E). The morphology of the isolated chromosomes with the CAS was better preserved than those with the

PAB, especially that followed by fixation with EtAc. They also showed a well-preserved morphology with centromeric constriction and sister chromatids cohesion (Fig. 4H). Chromosomes isolated with the CAS followed by UV cross-linking (CAS+UV) showed a compact morphology (Fig. 4J) similar to those isolated with PAB+UV. According to the results obtained, chromosomes were best harvested when the PAB and CAS were used for chromosome isolation,

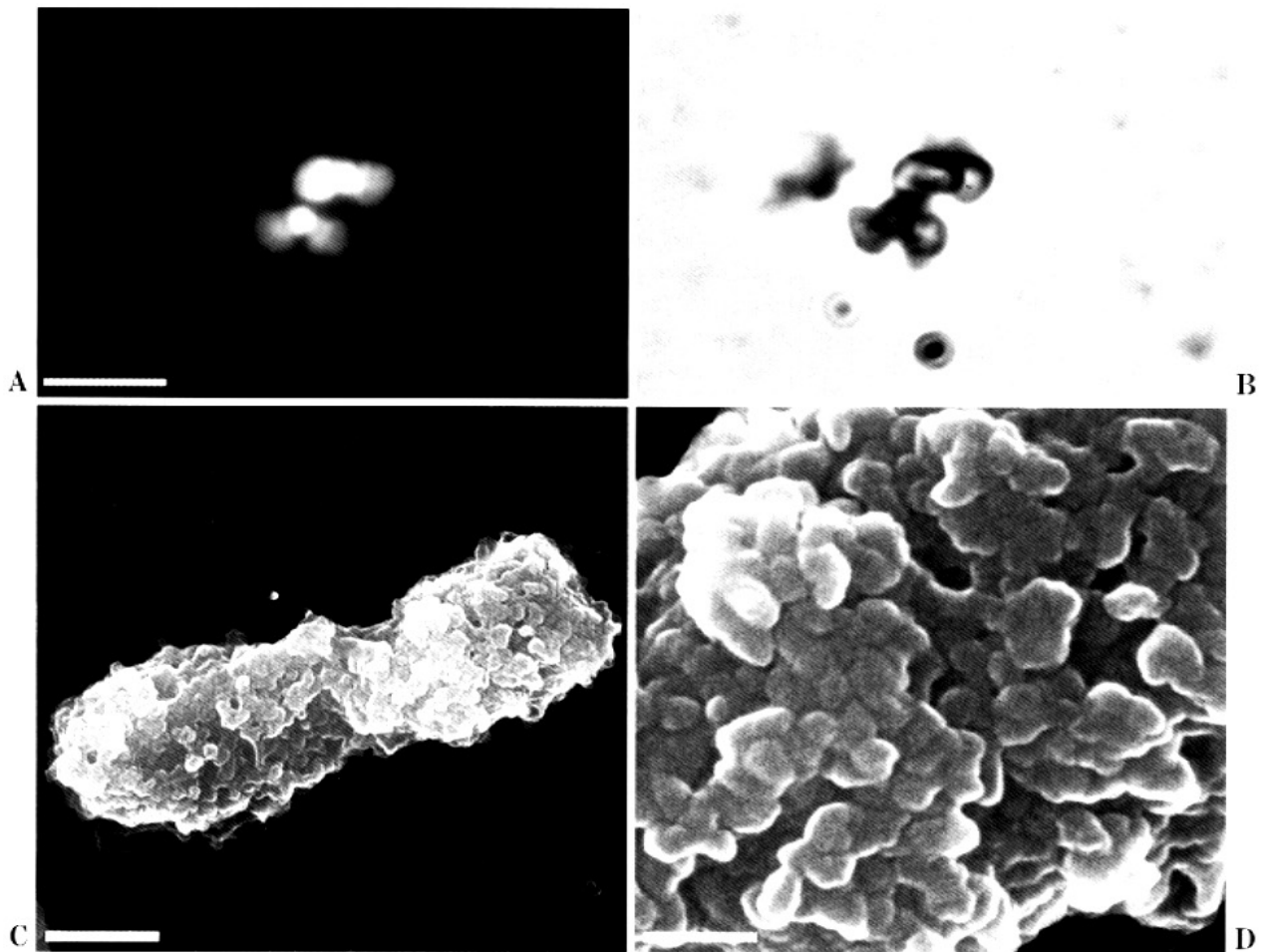


Fig. 5. The overall morphology and the surface structure of chromosomes isolated with the PAB. **A.** Fluorescent images of DAPI-stained chromosomes. **B.** Phase contrast image of the same portion of **A.** Scale bars (**A**, **B**) = 5 μ m. **C.** SEM image of a chromosome, which appear rather smooth in surface. Bar indicates 500 nm. **D.** Closer view of a part of **C.** The scaly structures are present in the surface of the chromosome. Bar: 125 nm

regardless of the fixation conditions.

Surface structure of isolated chromosomes

The overall morphology and surface structures of isolated chromosomes with the PAB and CAS were observed before fixation by light microscopy and SEM (Figs. 5, 6).

The chromosomes isolated with the PAB and CAS were stained with DAPI and observed both by fluorescence microscopy (Figs. 5A, 6A), and by phase contrast microscopy (Figs. 5B, 6B). Although the overall morphology of the chromosomes isolated with the

PAB was not clear (Fig. 5A, B), those chromosomes isolated with the CAS clearly showed centromeric constriction and sister chromatids in both DAPI-staining and phase contrast images (Fig. 6A, B). The width of the chromosomes isolated with the CAS was larger than that of the chromosomes isolated with the PAB and the structure of two sister chromatids in the former chromosomes were similar to those isolated with conventional methods with EtAc (O or O' in Table I).

When the chromosomes were observed by SEM, a conspicuous difference was observed between their surface structures. The chromosomes isolated with

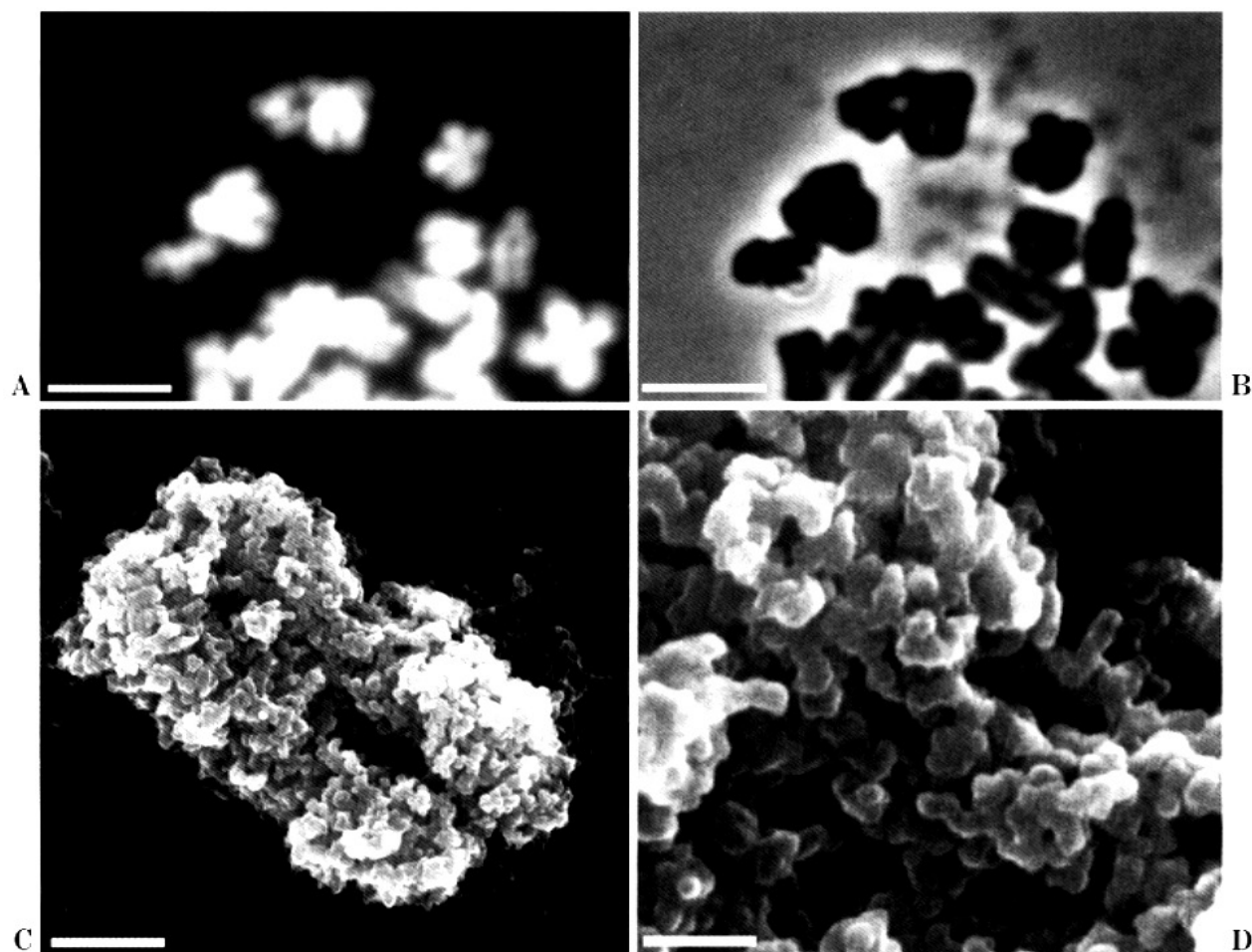


Fig. 6. The overall morphology and the surface structure of chromosomes isolated with the CAS. **A.** Fluorescent image of DAPI stained chromosomes. **B.** Phase contrast image of the same portion of **A.** Scale bars (**A**, **B**) = 5 μ m. **C.** SEM image of a chromosome, whose surface is covered with fibrous structures. Bar = 500 nm. **D.** Closer view of a part of **C.** Bar = 125 nm

the PAB had a rather smooth surface (Fig. 5C) and the surface of the chromosome appeared to be covered with flat, scaly structures with differences in size and shape that became obvious at higher magnification (Fig. 5D). This structure seems to occur by densely covering the inner structure with a certain matrix, which has been known as the chromosome peripheral matrix on the chromosome sheath. On the other hand, these chromosomes isolated with the CAS were observed as dense fibrous structures of rather uniform diameter (50–70 nm) (Fig. 6C, D). Moreover, the chromosomes isolated with the CAS

showed a more relaxed morphology with emergence of two sister chromatids, compared with those isolated with the PAB as already shown by light microscopy. Although the chromosomes isolated with the CAS showed a swollen morphology, the overall chromosomal characteristics, such as sister chromatid cohesion and centromeric constriction, were well maintained.

Protein content of isolated chromosomes

The morphological differences between the two iso-

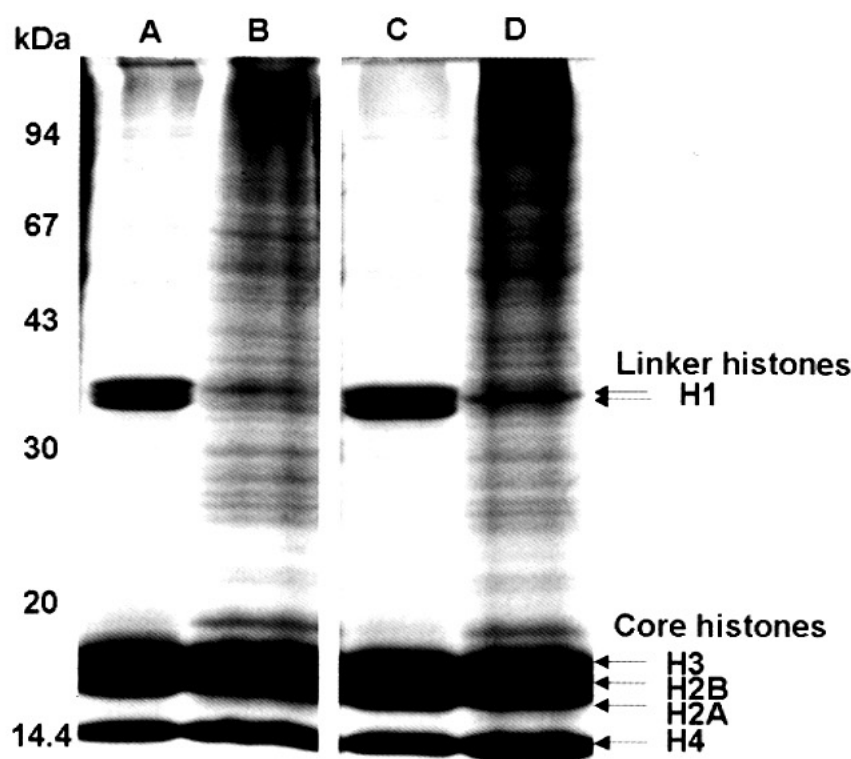


Fig. 7. The chromosomal and nucleic proteins were extracted by the acetic acid method and applied to 12% SDS-PAGE. **A.** Chromosomal fraction in the PAB. **B.** Chromosomal fraction in CAS. **C.** Nuclear fraction in the PAB. **D.** Nuclear fraction in the CAS. For both the chromosomal and nuclear proteins, the core histones were unaffected by differences in isolation solutions, but the linker histones were almost eliminated for the chromosomes and nuclei isolated with the CAS.

lated chromosomes are most likely caused by the removal of certain components from the surface and inner part of the chromosomes. The result of the SDS-PAGE clearly showed differences in the protein content between the chromosomes isolated with the PAB and CAS (Fig. 7, lanes A, B). A similar tendency was also observed in the protein content between the nuclear fractions isolated with the PAB and CAS (Fig. 7, lanes C, D). Large quantities of linker histones (histone H1) were detected both in the nuclear and chromosomal fractions isolated with the PAB (Fig. 7, lanes A, C). This fact indicated that the linker histones within the nuclei and chromosomes were well

preserved after the isolation procedures using the PAB. On the other hand, nuclei and chromosomes isolated with the CAS contained only a few quantities of linker histones (Fig. 7, lanes B, D). This indicated that the linker histones were removed from the chromosomes and nuclei in the course of their isolation. On the other hand, no remarkable differences were observed in the quantity of core histones of H2A, H2B, H3 and H4 between PAB- and CAS-treated chromosomes and nuclei, although some amount of core histones could be also depleted from the chromosomes and nuclei.

DISCUSSION

As the surface structures of human and plant chromosomes have been frequently studied by numbers of researchers, the patterns of surface structure have been categorized into two representative patterns: a fibrous and knobby surface (SWENEY et al., 1979; ADOLPH and KREISMAN, 1985; IWANO et al., 1997) and a rather flat scale-covered surface (RIZZOLI et al., 1994; MARTIN et al., 1996). Our present observations of the surface structure of chromosomes isolated with the PAB (Fig. 5C, D) and with the CAS (Fig. 6C, D) also fall into these two categories. From our results, the most plausible explanation for the difference between the two is a dissociation of certain surface complex substances on the chromosome by the effect of the CAS.

The presence of a surface complex of chromosomes has been reported by several researchers, and the peripheral chromosomal material has long been known as a "chromosome sheath". The main component of the peripheral chromosomal material has been considered to be several nuclear and nucleolar proteins as well as ribonucleoproteins (RNPs), including snRNAs (HERNANDEZ-VERDUN and GAUTIER, 1994). Components of nuclear lamin and many nucleolar proteins such as fibrillarin are also associated with the chromosome surface (GLASS and GERACE, 1990; YASUDA and MAUL, 1990). For a detailed analysis of the surface structure by SEM or scanning force microscopy, the peripheral chromosomal materials could be an obstacle; they were removed by enzymatic treatment, which sometimes causes the loss of a three-dimensional volume of the chromosomes (TAMAYO and MILES, 2000). Our results indicated that the CAS treatment also effectively removes the surface complex covering the detailed structure of chromosomes. The finding that the chromosomes isolated with the CAS clearly showed the shape of sister chromatids could also be explained by the removal of the peripheral chromosomal material between two chromatids (GAUTIER, et al., 1992). From this viewpoint, our results of SDS-PAGE indicate that one of the main components of the surface complex is the linker histones, H1 (Fig. 7), although there has been no report showing them to be components of the surface complex. It is, however, also probable that some other proteins such as nuclear lamin, which is located on the surface of chromosomes during mitosis and is carried into the sister nuclei, were eliminated together with linker histones in the course of chromosomal isolation with the CAS. Further proteomic analysis to identify constitutive proteins

contributing to the higher order structure of chromosomes is indispensable in this regard.

It is interesting that many researchers reported that the folding of chromatin fibers is destabilized when linker histones are removed from chromatin fibers in conditions of low ion strength (USACHENKO et al., 1996; CARRUTHERS et al., 1998). It has also been well established that the linker histones in a low ionic strength conditions, are selectively depleted from the chromosomes (BRODY, 1974). Another explanation for the changes in surface structure can be theorized from the role of linker histones to link the looped chromatin fibers in order to prevent the surface chromatin loops from scattering. This explanation is consistent with the radial-loop model of chromosome structure (RATTNER and LIN, 1985). ADOLPH et al. (1985) has successfully controlled the surface structure of chromosomes by adjusting the concentration of divalent cations (Mg^{2+}) which affects the content of linker histones in chromatins and the states of their compaction (CARRUTHERS et al., 1998). Their findings are consistent with our findings that the chromosomes treated with the CAS (which does not contain any divalent cations like Mg^{2+}) were depleted of linker histones (Fig. 7) while overall morphological characteristics, such as sister chromatid cohesion and centromeric constriction, were maintained (Fig. 6C, D). As polyamines can take the place of divalent cations, the chromosomes isolated with the PAB kept a smoother surface (Fig. 5C, D) than the chromosomes isolated with the CAS.

In conclusion, the present study showed that the chromosomal structure could easily be altered by isolation conditions such as the isolation solutions of chromosomes. Therefore, chromosomes should be carefully prepared depending on the objective of the research.

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