Three-dimensional Helical Coiling Structures and Band Patterns of Hydrous Metaphase Chromosomes Observed by Low Vacuum Scanning Electron Microscopy

Sumire Inaga¹, Keiichi Tanaka² and Akihiro Inoo¹

Division of Genome Morphology¹, Department of Functional, Morphological and Regulatory Science, Tottori University Faculty of Medicine, Yonago; and Tanaka SEM Institute, Yonago, Japan

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Summary. Helical coiling structures and band patterns of hydrous metaphase chromosomes were documented three-dimensionally by low vacuum scanning electron microscopy (SEM). Fixed or unfixed isolated Chinese hamster metaphase chromosomes were stained with platinum blue (Pt blue) and observed in the backscattered electron mode for low vacuum SEM without any hypotonic treatment or drying processes.

Fibrous structures were shown both in the fixed and unfixed hydrous chromosomes; helical chromatid coils and their subcoils were clarified especially in the fixed chromosomes having contrasting alternative bands of light and darkness, while the translucent perichromosomal matrix and compact fibrous structures were recognized in the unfixed chromosomes. The helical coils were more clearly represented in a loosened chromatin of metaphase chromosomes. Treatment with a tris-HCl buffer solution and Pt blue staining in a hydrous condition successfully produced banding patterns similar to G-bands on metaphase chromosomes. These banded chromosomes observed by low vacuum SEM were also analyzed stereoscopically by field emission SEM after critical point drying.

These findings indicate that: 1) native or unfixed chromosomes maintain the compact arrangement of high-order helical structures covered with the perichromosomal matrix; 2) helical coiling appearances of chromatids frequently observed in previous papers might be caused by loosening of the final level of the high-order structure of the metaphase chromosome; and 3) banding patterns might be produced by the rearrangement or reorganization of chromatin fibers at the 30 nm fiber level after the extraction of some chromosomal components including the peri- or intrachromosomal materials during the banding procedure.

In regard to the three-dimensional structure of the metaphase chromosome, several models have been proposed based on previous investigations by light microscopy (LM), and transmission and scanning electron microscopy (TEM and SEM): these include the randomly folded fiber model (Dupraw, 1965), the successive helical coiling fiber model (Sedat and Manuelidis, 1978), the scaffold and radial loop model (Laemmli, et al., 1978), the helical coiling of radial loops model (Ratner and Lin, 1985) among others. In these models, the 30 nm chromatin fiber consisting of DNA and histones is commonly considered as the elementary structure of the metaphase chromosome, although the higher-order structures are different from each other. Some SEM investigators have insisted that the 30 nm chromatin fiber is folded into a helical chromatid in the metaphase (Ratner, 1992; Yan, 1993; Takayama and Taketani, 1996); we have also elucidated the hierarchical coiling appearance by the combined use of SEM and TEM after a loosening of the compact chromatin organization (Inaga et al., 1994). Recently, the atomic force microscope (AFM) (Binnig et al., 1986), which provides the topographical information at atomic-level resolution, has been further applied to chromosome observation (De Grooth and Putman, 1992; Firtzsche et al., 1994, 1995; McMaster et al., 1996a, b; Ushiki et al., 1996; Tamayo and Miles, 2000). Despite of these studies, the manner by which the 30 nm fiber is organized into the metaphase chromosome during the condensation process of the cell division remains
controversial, probably because the intrinsic structure of chromosome is easily distorted during preparation procedures such as hypotonic treatment, fixation, dehydration, or drying for conventional SEM and TEM preparations.

The correlation between the chromosome structure and the banding pattern has been also studied by various investigators since KATO and YOSHIDA (1972) first reported the intimate relationship between the G-banded and the helical structure from LM findings of mammalian chromosomes. TAKAYAMA (1976) also demonstrated the transitional state between the helical configuration and the banded one by LM of Chinese hamster chromosomes. Thereafter, some studies by SEM have examined the three-dimensional structure of banded chromosomes in mammalian cells (HARRISON et al., 1981, 1983; UTSUMI, 1982; TANIGUCHI and TAKAYAMA, 1986). Concerning the structure of banded chromosomes by AFM (MUSIO et al., 1994, 1997; SHAHN et al., 2000; HOSHI and USHIKI, 2001), previous investigators suggested that the surface topography of the G-banded chromosome varied according to the sample preparation including drying methods, although definite conclusions on both high-order structure and the banding mechanism of metaphase chromosomes have yet to be reached.

Recently, the techniques for observing hydrous materials by low vacuum SEM, which was originally designed by ROBINSON (1975), have been improved by using platinum blue (Pt blue) staining method in the backscattered electron (BSE) mode (TANAKA and INAGAKI, 1993; TANAKA et al., 1997). Using this technique, the fine structure of fixed hydrous Chinese hamster chromosomes was observed in relation to the configuration of chromatin fibers in the metaphase chromosome (TANAKA et al., 1998). The fixed hydrous chromosomes consisted of basic fibers (about 70 nm thick); these fibers coiled into a 200–400 nm thicker thread which produced a chromatin of 1 μm in diameter by winding up to the final coil. These findings indicate that the chromatin fibers keep their intrinsic arrangement in the hydrous chromosomes, — in contrast with in dried ones and that low vacuum SEM is suitable for observing the structure of hydrous chromosomes.

In the present study, a comparative observation of fixed and unfixed hydrous chromosomes was performed by low vacuum SEM to investigate more clearly the three-dimensional intrinsic arrangement of high-order chromatin fibers in metaphase chromosomes with fewer artifacts. Moreover, banding treatments were attempted under the hydrous condition to elucidate the structure of banded chromosomes by low vacuum SEM.

MATERIALS AND METHODS

Hydrous chromosomes

Metaphase chromosomes were isolated from cultured bone marrow cells or lung fibroblast cells of the Chinese hamster according to the method of WRAY and STUBLEFIELD (1970). The isolated chromosomes in an isolation buffer solution (1.0 M hexylene glycol, 0.5 mM CaCl₂, 0.1 mM PIPES; pH 6.5) were mounted on the glass slide (10 × 5 mm) which had previously treated with 0.1% poly-L-lysine. After keeping them for 10 min in a sealed Petri dish to maintain the moist condition, some were fixed with 1% paraformaldehyde in the isolation buffer, while others were left in an unfixed condition.

For G-banding, treatment of the 0.02 M tris-HCl buffer solution (pH 7.0, 37°C, 20 min) introduced by KATO and MORIWAKI (1972) was performed in the unfixed chromosomes without any hypotonic treatment or drying process. All specimens were then prepared according to the procedure described previously for low vacuum SEM (TANAKA et al., 1997, 1998). Briefly, they were rinsed in the isolation buffer and stained with a 3% Pt blue aqueous solution (TANAKA and INAGAKI, 1993) for 15 min at pH 9.0. They were immersed in 20% dimethyl sulfoxide solution (DMSO) for 20 min to avoid ice crystal damage during the low vacuum SEM observation below 0°C. Each specimen attached onto a specimen stub with a starch glue (Yamato Paste, YAMATO Ltd., Tokyo) and adjusted on the cooling stage of a Hitachi S-2400N low vacuum SEM. Observations were performed in the BSE mode of SEM at 20 to 10°C with a vacuum grade from 90 to 270 Pa and at an accelerating voltage of 25 kV.

Some of G-banded chromosomes prepared in the hydrous state were also critical point-dried after low vacuum SEM observation. They were coated with platinum-palladium and observed with a Hitachi S-4500 field emission SEM (FESEM) at 5 kV in the secondary electron (SE) mode. Stereo pairs of micrographs were taken at a 0° and 10° tilt.

Air-dried chromosomes

Air-dried G-banded chromosomes were prepared according to the standard banding method; they were hypotonic treated with a 0.075 M KCl solution, fixed with a mixture of methanol and acetic acid (3:1), spread onto a glass slide and air dried. They were then treated with a 0.02 M Tris-HCl buffer solution (pH 7.0, 37°C, 20 min) and stained with a Giemsa solution. After LM observation, they were examined in a low vacuum SEM in a dried condition at 90 Pa.
They were then stained with Pt blue and observed again in a low vacuum SEM.

RESULTS

**Helical coiling structures of hydrous chromosomes**

Fibrous components of metaphase chromosomes were observed both in the fixed and unfixed samples in the hydrous state of the low vacuum SEM (Fig. 1). In unfixed chromosomes, the arrangement of chromatin fibers was slightly obscure and the outline of these chromosomes was smooth. This is probably due to the presence of the perichromosomal matrix, appearing like a thin translucent layer surrounding the surface of the unfixed chromosome.

On the other hand, the arrangement of chromatin fibers in fixed chromosomes was distinct. These fixed chromosomes exhibited the segmental or coiling structure of chromatids with peripheral looping fibers about 70 nm thick. Stereoscopic observations of the fixed chromosomes further showed two levels of the helical coiling structures: the coils of chromatid itself and the subcoils of chromatid fibers 70–100 nm thick (Fig. 2). Gaps or clefts in the chromosomes were produced by the spiralization of chromatids. The direction of the sister chromatid coils was symmetrically opposite helical handedness. The diameter of the chromatid was 600–800 nm and that of fibers forming the chromatid was 300–400 nm. The chromatid fibers were further composed of thinner fibers of 70–100 nm in diameter. These chromatids showed the alternative patterns of bright and dark contrast, similar to the pattern of G-bands observed by LM. In some chromosomes, one side of a paired sister chromatids appeared to be accidentally elongated due to an uncoiling or stretching of the final chromatid coils (Fig. 3).
Fig. 2. Two sets of stereo pairs of fixed hydrous chromosomes observed by low vacuum SEM (cross-view images). Helical coiled sister chromatids (a) and subcoils (arrows of a and b) in the chromatids are observed three-dimensionally. The direction of a paired sister chromatid coils is partially symmetrical opposite handedness. Alternative bright and dark regions are recognized in each chromatid. Scale bars = 1 μm
Fig. 3. An uncoiled chromatid shown in a fixed hydrous chromosome by low vacuum SEM. a. The elongate chromatid is found only in one side (thick arrow) of sister chromatids. b. A closer view of a. Coils of the chromatid and their subcoils are evident. The arrangement is shown schematically in the inset of this figure. Scale bars = 1 μm

Fig. 4. LM (a) and low vacuum SEM (b, c) images of air-dried chromosomes treated with tris-HCl for banding. Chromosomes are very flat due to the effect of the high surface tension of water during air-drying. Giemsa-positive bands (arrows) are not distinguished by low vacuum SEM in the specimens treated only with Giemsa-staining (b). After Pt blue staining (c), signals are enhanced obviously at the G-positive regions, which are comparable with G-bands of LM images (a). Scale bar = 5 μm

**Banding patterns of hydrous chromosomes**

Air-dried chromosomes treated with a tris-HCl buffer solution were observed by LM and low vacuum SEM (Fig. 4). Giemsa-positive bands were not distinguished under the low vacuum SEM in the BSE mode when the specimens were only stained with Giemsa. Pt blue staining of the chromosomes obviously increased BSE signals at the G-positive regions, which were identified by comparing the images with LM images. Observation of chromosomes treated with a tris-HCl buffer solution followed by Pt blue staining without drying also successfully produced the banding patterns on metaphase chromosomes (Fig. 5). The banding patterns almost coincide with the G-bands. In addition, fibrous components were further observable...
in the chromosomes, although the arrangement of the chromatin fibers could not be sufficiently traced, probably because the resolution was not very high in the present low vacuum SEM (possible magnification at most ×25,000). To overcome this problem, the banded chromosomes observed by low vacuum SEM were critical point dried, metal-coated and stereoscopically observed with a FESEM at higher magnification (Fig. 6). These images revealed that the chromosomes were composed of 30 nm fibers. The compact and sparse regions of 30 nm fibers were formed alternatively in the chromosomes, which corresponded to the positive bands and interbands of G-band staining, respectively. In the compact regions, the strong coiling of 30 nm chromatin fibers into the thicker ones was evident, while the parallel arrangement of the independent fibers was remarkable in the sparse regions.

DISCUSSION

Chromosomes exhibit different profiles depending on the preparation methods and observation techniques. In the present study, we have investigated the intrinsic high-order structure of metaphase chromosomes under the hydrous state by low vacuum SEM to avoid preparation artifacts, and clarified the correlation between the high-order structure and banding structure of metachromase chromosomes.

We have succeeded here in observing fixed and unfixed hydrous chromosomes by low vacuum SEM of Pt blue stained specimens. As shown in the previous paper (TANAKA et al., 1998), chromatin fibers were intensely stained with Pt blue. The mechanism of Pt blue staining may be explained by the fact that Pt blue has a good affinity for DNA, which results in staining of the chromatin fibers. It is true that not only the chromatin fibers but also cell organelles such as mitochondria cristae could be clearly observed in the hydrous state after Pt blue staining by low vacuum SEM (TANAKA et al., 1997). Furthermore, the perichromosomal matrix appeared to be also stained slightly in unfixed chromosomes. Further studies are needed to clarify these points.

The present study is the first to offer SEM images of unfixed hydrous chromosomes in comparison with fixed chromosomes. We have clearly shown that the translucent thin perichromosomal matrix and chromatin fibers were present on the unfixed hydrous chromosomes. It is also interesting that the chromatin fibers in the unfixed chromosomes presented very compact appearances through the covering perichromosomal matrix in comparison with the fixed one. This finding implies that the perichromosomal matrix might play an important role to maintaining its high-order structure in a compact chromosome organization. While the component of this matrix is still unknown, TAMAYO and MILES (2000) stated in their AFM studies that individual chromatin fibers can be observed after mild enzymatic treatment to digest the covering substances which are mainly composed of proteins and RNA.

In the stereoscopical observation of fixed chromosomes, we have demonstrated two levels of the helical structure — the coils of the chromatid and the coiling fibers in the chromatid — without any special treatment for spiralization. These findings were essentially the same as those obtained in the previous study (TANAKA et al., 1998). The present study has further clarified the chromatid coils in a loosened state. These studies and our previous ones by ultrahigh-resolution SEM and TEM (INAGA et al.,
Fig. 6. A stereo pair of a tris-HCl treated human chromosome observed by FESEM after critical point drying and metal-coating (cross view images). Fibrous structures, about 30 nm thick, are clearly observed. The compact regions (⋆) and sparse regions (arrows) of the chromosome correspond to positive bands and interbands, respectively. In the compact regions, coiling of 30 nm chromatin fibers is apparent, while the parallel arrangement of the independent 30 nm chromatin fibers is remarkable in the sparse regions. Scale bar = 500 nm.

1994) suggest that the high-order structure of the metaphase chromosome is composed of hierarchical arrangement of coiling by so-called 30 nm chromatin fibers.

As for the direction of a paired sister chromatid coils, the opposite helical handedness by mirror symmetry has been reported based on a three-dimensional structural analysis of mammalian chromosomes by LM (BOY DE LA TOUR and LAEMMLI, 1988). Similar findings were also observed in the present study. The dynamic changes in the total number of spirals and in the size of chromatid at different mitotic stages has been suggested based on LM (OHNUKI, 1968; INO, 1971), TEM (STUBBLEFIELD, 1973), and SEM (YAN, 1993) studies. In the present study of hydrous chromosomes, variations in the width of chromatids and diameter of chromatid fibers were observed. This is probably due to the degree of contraction in each chromatid.

Another noteworthy finding is that the alternative bright and dark contrast similar to the pattern of G-banded chromosomes was recognized in SEM images of helical chromosomes. In order to clarify the correlation between the intrinsic high-order structure and banding structure of the metaphase chromosome, we then observed chromosomes with banding treatment under the hydrous condition without any drying processes. For this purpose, the tris-HCl buffer solution was used for producing bands (KATO and MÖRIWAKI, 1972). Treatment of this buffer solution is simple and effective as compared with proteolytic enzymes, such as trypsin. Thus, we succeeded in producing the banding patterns of chromosomes in the hydrous condition by low vacuum SEM.

Several interpretations have been made on the banding mechanism. TAKAYAMA (1974, 1976) has suggested that the G-band is due to disruption of the architectural integrity of the chromosome, where the delicate and balanced organization of the chromosomal fibers collapses and the fibers clump together with neighboring ones. BURKHOLDER (1975) referred to TEM observations of trypsinized chromosomes to
state that the band portions were relatively more resistant to dispersion than the interband portions. Some other authors considered that certain negative regions of G-banded chromosomes were produced due to the denaturation of non-histone proteins (Comings and Avelino, 1973). Holmquist et al. (1982), on the other hand, considered that G-negative bands (containing GC-rich DNA) correspond to the early clusters of DNA synthesized and G-positive bands (containing AT-rich DNA) to the late clusters. Based on their AFM studies, Tamayo and Miles (2000) reported that the thicker and thinner regions in the chromosome corresponded to G and R bands, but concluded that no significant structural differences were found between them. The present study, however, support the idea that the banding structures are produced by the rearrangement or reorganization due to the effect of chemical treatment.

In conclusion, the present study has demonstrated the possibility that: 1) the native or unfixed chromosome maintain their high-order structure with the perichromosomal matrix; 2) helical coiling appearances of the chromatids can be found by loosening the chromatid coils; 3) banding patterns is produced by the rearrangement or reorganization of 30 nm thick chromatin fibers after extraction of some structural elements including the peri- or intra- chromosomal materials during the banding treatment. Further studies on the composition or intra-chromosomal materials will be required in relation to the chromosomal structure at each level of high-order structure.

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REFERENCES


