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Combination of fluorescence in situ hybridization and scanning force microscopy for the ultrastructural characterization of defined chromatin regions

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Although the internal arrangement of interphase chromatin is still a matter of conjecture, there exists a large body of evidence for the compartmentalization of chromosomal domains. A study based on combined scanning force and optical microscopy of supramolecular chromatin spreads produced by isotonic lysis of cells suspended in phosphate-buffered saline has been conducted. The ultrastructure of fluorescent labeled chromosomes was resolved with the topographical contrast provided by the scanning force microscope. Fluorescence in situ hybridization was used to label specific DNA sequences. The location of different pericentromeric chromosome regions was determined by fluorescence microscopy and correlated with scanning force microscope topography. Using a single DNA probe, discrimination between labeled chromosome pairs of an aneuploid cell was possible, based on the different intensities of fluorescence signals. The results show that the in situ hybridization technique with fluorescence labeling is compatible with scanning force microscopy. The combination of these methods can be used for the specific identification and lateral localization of DNA sequences in spread chromatin, opening the possibility for the ultrastructural characterization of defined genes in the scanning force microscope. © 1996 American Vacuum Society.

I. INTRODUCTION

The length of human genomic extended DNA is about 1.8 m. The DNA in association with histone and nonhistone proteins constitutes the system of chromatin fibers and is packaged in an interphase cell nucleus ~5 μm in diameter. In the course of the cell cycle, the chromatin undergoes further compaction, culminating in the highly condensed metaphase chromosomes. The fundamental morphological feature of chromatin is the nucleosomal chain, constituted of DNA and histone proteins and establishing the typical “beads-on-a-string” conformation in the electron microscope or scanning force microscope (SFM). This nucleosome chain can be further compacted into a ~30 nm fiber under low ionic strength conditions. Chromatin fibers 100 nm in width have also been shown in G1 interphase nuclei. The mechanism of further compaction of chromatin fibers in interphase nuclei and the transition from the relaxed interphase chromatin to the very compact metaphase chromosomes is poorly understood. There is evidence for compartmentalization of chromosomal domains in interphase, and some information has been derived from statistical analysis of metaphase spreads based on the assumption that the metaphase chromosome distribution should reflect, at least to some extent, the topology of chromosomes inherited from the preceding interphase. Studies of sectioned metaphase cells and metaphase spreads show that larger chromosomes have a more peripheral position.

SFM is a new high-resolution technique with an increasing number of applications in structural biology, including studies of chromatin (for a review see Ref. 16). Investigations of isolated chromatin fibers and spread nuclei have been reported. The use of spreading techniques that preserve, at least to some extent, the spatial relations of
neighboring chromatin sites in the interphase nucleus permits the investigation of chromatin at a spatial resolution otherwise hindered by the tight packaging of the genetic material. One of these techniques, isotonic lysis,\textsuperscript{21} leads to spreading of interphase chromatin without applying detergent or resorting to hypotonic conditions.

The investigation of single chromosomes during interphase is hampered by the decondensation of chromatin such that identification according to structural features is impossible; however, a labeling of specific DNA sequences in chromatin can be achieved by in situ hybridization. There are several ways of visualizing the labeled DNA sequences, one of them being the attachment of fluorescent dyes to the probe, designated as fluorescence in situ hybridization (FISH). By using labeled chromosome specific libraries, FISH allows the detection of chromatin material corresponding to single chromosomes in interphase nuclei.\textsuperscript{22}

In situ hybridization combined with cytochemical techniques has been used for SFM investigations of metaphase chromosomes.\textsuperscript{23,24} An enzyme-catalyzed growth of amorphous material at the hybridization site was used for detection. The resolution was limited by the detection method and the extreme condensation of the chromatin in the metaphase chromosome. The use of fluorescence-based detection applied to spread chromatin should result in a considerable increase in the lateral resolution of the hybridization site. The application of simultaneous fluorescence and SFM investigations of the same specimen of spread chromatin was demonstrated previously.\textsuperscript{20} In this study we demonstrate the compatibility of FISH and SFM, a combined tool ideally suited for ultrastructural investigations of chromatin related to specified DNA sequences.

II. EXPERIMENTAL PROCEDURES

JY human B lymphoblastoid cells\textsuperscript{25} were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) in a humidified CO\textsubscript{2} incubator at 37 °C. The cells were spread according to Schlammadinger.\textsuperscript{21} The cell suspension was washed twice in PBS and resuspended to 10\textsuperscript{6} cells/ml. Droplets of 60 μl were deposited on Parafilm. After incubation for 20 min, each droplet was touched with a thoroughly cleaned glass coverslip (Fig. 1). The spreads adhering to the coverslips were fixed for 2 h in 2% paraformaldehyde (PFA) in phosphate buffered saline (PBS), washed in PBS, washed in distilled water, and air dried.

FISH of air-dried samples was performed according to Speleman et al. (Fig. 2).\textsuperscript{26} We used biotinylated DNA probes pUC1.77 and L1.26, a kind gift from Dr. F. Speleman (Dept. of Medical Genetics, University Hospital of Ghent, Belgium). The probe pUC1.77 binds to a 10\textsuperscript{5} base pair long repetitive sequence in the pericentromeric heterochromatin of chromosome 1q,\textsuperscript{27} while the L1.26 recognizes alphoid repetitive sequences in the pericentromeric heterochromatin of chromosomes 13 and 21.\textsuperscript{28} The rehydrated samples were digested with RNase A for 60 min, then with pepsin for 30 min at 37 °C. After 5 min postfixation at room temperature in 4% PFA and 0.5% MgCl\textsubscript{2}, the samples were dehydrated in ethanol series and air dried. The chromatin samples and the probe DNA were denatured together in 60% formamide/2×SSC at 90 °C for 5 min, and hybridized overnight at 37 °C. Probe detection was with a first step of fluorescein-isothiocyanate (FITC) labeled Neutralite-Avidin followed by an amplification step using biotinylated goat anti-avidin and Neutralite-Avidin-FITC. The DNA was labeled using 4',6-diamidino-2’-phenylindole (DAPI) as described earlier.\textsuperscript{20}

Scanning force microscopy was performed with a NanoScope III contact SFM (Digital Instruments, Santa Barbara, CA), operating in the topographic (isoforce) mode, and using a J-tube scanner with a 130° maximum scan range. The calibration is discussed in detail elsewhere.\textsuperscript{29} Si tips with a radius of ~5 nm and a cantilever force con-
were used. Images of 512 \times 512 pixels were obtained at line scan rates of 5–7 Hz. Image processing and analysis were carried out using the NanoScope software. Estimation of the lateral dimensions of topographic features was done at half maximum height.3

Fluorescence imaging was with a Zeiss Axioplan epifluorescence microscope equipped with a Photometrics Series 200 cooled slow scan charge-coupled-device (CCD) camera system incorporating a Kodak KAF 1400 sensor (Photometrics, Tucson, AZ), and interfaced to a Macintosh Quadra 800 computer (Apple Computer, Cupertino, CA). For orienting the samples, images of DAPI-stained dry spreads were taken at low magnification with a Plan-NeoPlan 20X N.A. 0.5 objective. After SFM investigation of selected regions the samples were rehydrated, washed, restained with DAPI, and mounted in Mowiol (Hoechst). Images of DAPI and FITC fluorescence were taken using the appropriate Zeiss filter sets and a Plan-NeoPlan 100X N.A. 1.3 objective. Digitized images were processed and aligned to their SFM counterpart using the program NIH-Image.

III. RESULTS AND DISCUSSION

During isotonic lysis the chromatin of the cells suspended in PBS droplets spreads on the surface of the buffer. The spread chromatin can be transferred to a glass coverslip by touching it to the surface of the droplets (Fig. 1). According to our results the chromatin spreads prepared in this way are
stable enough to allow subsequent in situ hybridization (Fig. 2) and scanning force microscopic investigations of the same specimen.

Figure 3 shows fluorescent and scanning force micrographs of spread chromatin samples. The L1.26 probe was used for the in situ hybridization and visualized thereafter with FITC fluorescence. The DNA content of the chromatin material was detected with DAPI. The two images were overlaid in order to demonstrate the localization of the FISH signals relative to the DNA content. Fluorescent images like those of Fig. 3(a) showed that the chromatin material from single cell spreads formed distinct plaques with some chromatin fibers radiating outward. The signals of the hybridization probe were surrounded by the chromatin, demonstrating the conserved integrity of the spread nuclear content. Neither disruption nor partition of the chromatin of intact nuclei was observed.

The density of the plaques was \( \sim 100/\text{mm}^2 \). The shapes were mostly round, with diameters of \( \sim 10–20 \, \mu\text{m} \). The fluorescent hybridization signals were located in distinct regions of the spread-chromatin plaques (arrows). The SFM image [Fig. 3(b)] revealed the same pattern as seen in the fluorescent microscope. This suggests that the topographic contrast is built up by DNA-containing material, i.e., chromatin. Plaque heights of up to 500 nm were measured. A height difference of 150–200 nm between the signal-containing regions and the surrounding chromatin material was observed. The site to which the L1.26 probe hybridized comprises alphoid repetitive sequences located in close proximity of the nucleolus organizing region on the acrocentric
chromosomes 13 and 21. Thus, the FISH probe is likely to colocalize with the nucleoli, which appeared in the SFM as distinct globular domains of heights well above the remainder of the spread chromatin. Assuming a well-stretched B-DNA, the length of the labeled region of 2 Mbp would be ~700 μm. The observed signals were mostly round with diameters of ~1–6 μm. This is indicative of a well-condensed state of the labeled region.

At higher resolutions fibrous material radiating out from the plaques was more easily detected [Figs. 3(c) and 3(d)]. Filaments with different dimensions could be seen, the thinnest ones having widths of 40–65 nm at half-height and heights of 7–8 nm. These dimensions are within the range of values usually measured for the 30 nm chromatin fiber by SFM. It is reasonable to suppose that drying the samples containing DNA wound about proteins would result in distortion of an otherwise symmetric topology, and cause flattening and widening of the structures. Nevertheless, it cannot be excluded that some of the fibers observed here were built up from several 30 nm filaments that stuck together during the several drying steps required for the in situ hybridization. Furthermore, some may have represented large nonhistone protein complexes attached to the 30 nm filaments.

Hybridization with the pUC1.77 probe yielded similar results. In Fig. 4 fluorescent images of spread chromatin hybridized with the pUC1.77 probe are shown. The fluorescent FISH signals [Fig. 4(b)] were located within the DAPI stained chromatin plaques [Fig. 4(a)]. Since large spreads of a major fraction of the nuclear material such as the one shown in Fig. 3 or that marked by the arrow in Fig. 4(a) were rare events, the probability of finding a spread region that was also labeled with FISH was small. However, such a case was detected; the arrow points at a signal spread over a length of ~5 μm.

Usually six or eight probe-specific signals were observed in one area of DNA-specific (DAPI) fluorescence. They showed a variation in intensity; usually three or four pairs of signals with distinct intensities and sizes could be detected in one plaque. A gallery of such images is collected in Fig. 4(c). Probably the largest pair of signals represents the pericentric regions of intact chromosomes 1, whereas the smaller signals could have resulted from translocations of pericentric chromosome 1 material to other chromosomes.

A pattern similar to that detected with DAPI staining was observed with the scanning force microscope (Fig. 5). The specificity of the DNA labeling allowed the identification of debris revealed by SFM, such as that appearing in Fig. 5(b) on the pole of the plaque opposite to the arrow.

The extended chromatin material containing the probe was also distinguishable with the SFM (arrows). An interesting finding is that hybridization signals of the pUC1.77 probe were somewhat less prominent than those produced by the L1.26 probe. Height differences of 70–150 nm between the signals and the surrounding chromatin were measured. The most likely explanation for this observation is that the function and physical location of the DNA sequences recognized by the probes was different. While L1.26 signals colocalized with nucleoli, the probe pUC1.77 bound to pericentromeric repeats of chromosome 1q, regions not involved in nucleolar organization.

IV. CONCLUSION

We have demonstrated the applicability of the in situ hybridization technique for the labeling of specific DNA sequences of spread chromatin, carried out simultaneously with SFM investigations of the same specimen. The localization of DNA-containing regions with distinct topographic features in the SFM image was made possible by the DNA-specific dye, DAPI. The hybridization process allowed for the identification of specific DNA sequences, while at the same time it did not hinder the spatial resolution of the SFM;
structures as small as 7–8 nm in height and 40–65 nm in width, possibly corresponding to the 30 nm chromatin filaments, were detected. A height difference between the signal-containing and noncontaining spread chromatin material was observed, possibly owing to the deposition of large protein complexes onto condensed target DNA during the hybridization process. Our results show that FISH coupled with SFM can be used for the specific identification and lateral localization of DNA sequences in spread chromatin, enabling the ultrastructural characterization of defined genes in the SFM.

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