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Atomic force microscopy reveals the local ordering characteristics of nucleosomal chain from cell†

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Atomic force microscopy was used to investigate the local ordering characteristics of hypotonic spread HeLa nucleosomal chains. Nucleosomal chains were diluted in tridistilled water and dropped on freshly cleaved mica, rinsed with ultrapure water and air dried. We have found that nucleosomal chains show varied structures *in vitro*: beads-on- a-string, irregular arrangement, open rings, closed rings. Neither the hydrolysation of trypsin nor the digestion of micrococcal nuclease or nuclease is necessary for observation of 'beads-on-a-string'. The closed rings formed by a nucleosomal chain have a close relevance to the two marked characteristics of the mild method developed in this work for preparing H1-depleted chromatin. The described method may provide a new reliable way to study the behaviour of the extended chromatin fibre at low ionic strength. These observations may provide a useful clue to elucidate how the structure of the extended chromatin fibre affects DNA transcription, replication and repair. Copyright © 2001 John Wiley & Sons, Ltd.

KEYWORDS: AFM; nucleosomal chain; beads-on-a-string

INTRODUCTION

Understanding the structure of the extended chromatin fibre at low ionic strength is of crucial importance, because extended fibres presumably represent the form in which the DNA template is presented to cellular machinery conducting transcription, replication and repair.[1] The DNA in a eukaryotic cell is folded in a series of hierarchical stages. The first step is the formation of a complex of a histone protein octamer with approximately 146 base pairs (bp) of DNA, which is known as a nucleosome, the fundamental repeating unit of the eukaryotic chromatin.[2,3] In the core particle of the nucleosome, 146 bp of DNA is wrapped in ~1.65 left-handed superhelical turns around a histone octamer consisting of two molecules of each of the core histones H2A, H2B, H3 and H4.[4] Structure determination of nucleosomes based on x-ray crystallography yielded a structure 5.5 nm thick with a diameter of 11 nm.[5] Successive core particles are connected by stretches of linker DNA to H1, the so-called lysine-rich or linker histones. Chromatin is made up of variously folded and compacted chains of nucleosomes and linker DNA, modulated by histone modification, non-histone proteins and other future particles of the nuclear environment. How this linear array of nucleosomal particles is organized in the nucleus remains an enigma.[1,3]

Atomic force Microscopy (AFM)[6] has become an important tool for obtaining ultrastructural information on biological samples.[7] It is used to make highly detailed topographical images with a typical resolution for biological samples of a few nanometres, and the three-dimensionality of the sample can be determined directly. Imaging is done under very mild conditions: no vacuum is required, the samples need not be conducting and can be imaged in air or even in an aqueous environment. Imaging 'in air' can be carried out under ambient air conditions with controlled humidity. In this study we use AFM for the structural research of hypotonic spreading nucleosomal chains.

MATERIALS AND METHODS

Preparation of HeLa cell nuclei

HeLa S3 cells were grown as monolayers in RPMI 1640 supplemented with 10% (v/v) newborn calf serum. Logarithmically growing monolayers were washed twice at room temperature with phosphate- buffered saline (PBS: 0.2 gl^{-1} KCl, 0.2 g l^{-1} KH_2PO_4, 8.0 g l^{-1} NaCl, 1.56 g l^{-1} $Na_2HPO_4 \cdot 7H_2O$) and harvested by centrifugation at 600 g for 7 min. The pelleted cells were then gently dispersed in divalent cation-free PBS and stored at −20 °C.

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Nuclei were isolated from $\sim 10^7$ cells by lysis in 40 mM TRIS·HCl (pH 7.5), 2 mM DTT and 5 mM $MgCl_2$, homogenized with 12 strokes in a glass homogenizer and centrifugation through a glycerol cushion (25% glycerol, 10 mM TRIS·HCl (pH 8.0), 1.5 mM $MgCl_2$). Transmission electron microscopy was used to assess the purity of the nuclear preparations and confirmed the absence of organellar contamination. Nuclei were washed gently in a buffer containing 150 mM KCl, 5 mM $MgCl_2$ and 25 mM TRIS·HCl (pH 7.5).[8] Nuclei were washed again and suspended in a buffer containing 50 mM TRIS·HCl (pH 8.0), 0.1 mM DTT and 0.1 mM EDTA and then stored in liquid nitrogen.

Chromatin preparation

All operations were carried out at 0–4 °C. The preparation of chromatin from purified nuclei was carried out according to the method of Oudet *et al*.[9] and Maurushige *et al*.,[10] modified in order to prevent DNAase action and drastic shearing as much as possible. All homogenizations were done manually. Nuclei from 4 g of HeLa cell were lysed in 250 ml of a buffer containing 10 mM TRIS·HCl (pH 7.9), 80 mM NaCl, 20 mM EDTA and 0.01% Triton X-100 using a Teflon glass homogenizer. After 10 min of centrifugation at 12000 g, the pellet was suspended in 40 ml of a buffer containing 10 mM TRIS·HCl (pH 7.9), 150 mM NaCl, 20 mM EDTA and 0.01% Triton X-100 and homogenized. A 20 ml aliquot of the suspension was then centrifuged for 3 h at 54 000 g through two layers of sucrose, the upper one (10 ml) containing 15 mM TRIS·HCl (pH 7.9), 150 mM NaCl, 20 mM EDTA and 0.01% Triton X-100 and 1.65 M sucrose, whereas the lower one (25 ml) contained 15 mM TRIS·HCl(pH 7.9), 150 mM NaCl, 20 mM EDTA and 0.01%Triton X-100 and 1.7 M sucrose. The pellet was then homogenized in 50 ml of a buffer containing 10 mM TRIS·HCl (pH 7.9), 150 mM NaCl and 20 mM EDTA and then centrifuged for 10 min at 12 000 g. The purified chromatin pellet was finally suspended in 5 ml of 10 mM TRIS·HCl (pH 7.9), 5 mM sodium bisulphite and 0.1 mM dithiothreitol and stored at −20 °C.

Preparation of lysine-rich histone-depleted chromatin for AFM imaging

All operations were carried out at 0–4 °C. The preparation of lysine-rich histone-depleted (H1-depleted) chromatin was carried out according to the method of Oudet *et al*.[9] and modified for AFM observation. Purified chromatin was diluted to 100 μg DNA ml^{-1} in a buffer containing 10 mM TRIS·HCl (pH 8.0), 700 mM NaCl and 5 mM sodium bisulphite (buffer A). Chromatin solubilization, which was accompanied by a marked increase in viscosity, was achieved in 2–3 h at 4 °C. A 1 ml align of the very viscous solution was diluted in 19 ml of tridistilled water, homogenized, kept at 4 °C for 1 h and then centrifuged for 20 min at low speed. The supernatant was taken out for AFM imaging. A drop of the supernatant (~5 μl), was placed on freshly cleaved mica, compressed nitrogen gas was applied to the sample in such a way that the liquid spread out on the mica surface, the sample was incubated at room temperature for 10 s and then the surface was rinsed with ultrapure water (>18 MΩ·cm^{-1}; Milliq-UV, Millipore Co., Bedford, MA) for 1 min and air dried.

Microscopic analysis was carried out by using a commercial atomic force microscope (Digital Instruments, Santa Barbara, CA). All AFM images were obtained in air at room temperature by using contact mode with a spring contact of 0.12 nN nm^{-1}. The cantilever was 200 μm in length.

Analysis of AFM images

The lateral dimensions of biological structures in AFM are overestimated because of the finite dimensions of the tips. In order to minimize the broadening effect of AFM, we measured the diameters of nucleosomes and fibres with full width at half-maximum height.

RESULTS

Imaging by AFM does not require complicated specimen preparation procedures, such as staining and shadowing, yet it does provide molecular resolution. When we applied the AFM technique to the native chromatin, this technique detected a well-known linear bead structure. (Fig. 1). A 'beads-on-a-string' pattern is created by nucleosomal cores connected by linker DNA, which is a characteristic feature of lysine-rich histone-depleted chromatin. This typical appearance of nucleosomal chains was described previously by electron microscopy[11] or AFM investigations.[12–14] The nucleosomes have an average diameter of $\sim 35 \pm 6$ nm ($n = 50$) and a mean height of 4.0 ± 1.3 nm ($n = 50$). The width values are similar to the results obtained in previous contact mode AFM studies of chromatin fibre with the characteristic 'beads-on-a-string' appearance: nucleosomes in native chromatin from *Tetrahymopna thermophila*, 31.2 ± 4.9 nm[15]; nucleosomes in native chicken chromatin, 30 ± 3 nm; nucleosome cores assembled *in vitro* into chromatin fibres, ~30–40 nm, with the smallest cores measuring 27 nm.[16]

When corrected for the broadening of image width in AFM as a function of the radius of curvature of the scanning tip,[17–20] these measurements are consistent with a maximum width of ~11 nm for each nucleosome. These height values

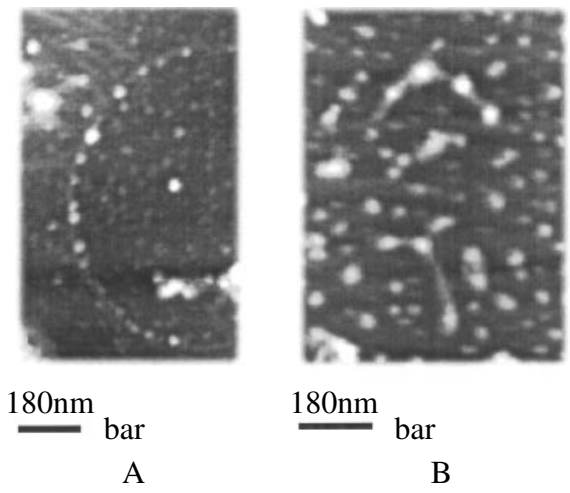

Figure 1. Contact mode AFM images showing a typical beads-on-a-string structure. Height scale is 0–15 nm.

are also similar to those for nucleosomes assembled *in vitro* (2.5–6.0 nm)[17] and to the height established for the short axis of a nucleosome by x-ray diffraction (5.7 nm)[21] and neutron scattering (5.5–6.0 nm).[22,23] These data suggest a possible face-on deposition of the nucleosomes that may result from a strong nucleosome–surface interaction; the observed height depends upon the orientation of the adsorbed nucleosomes. The flat orientation should be clearly preferred during adsorption because of energetic constraints, and the flexibility of the extended nucleosomal chain due to the linker DNA gives the nucleosomes enough freedom of movement to reach this favourable conformation. Moreover, the surface tension occurring during air drying would probably flatten other orientations (e.g. standing on the edges).[14] This reduced height value suggests sample compression by the AFM tip and sample collapse due to surface tension effects during deposition. The drying process of the sample may cause a certain reduction of height. In Fig. 1 the nucleosomal chains show an arc pattern.

Figure 2 shows the morphology of extended polynucleosome filaments on mica. Nucleosomal chains show an irregular arrangement, with nucleosome particles of various sizes attached to the filamentous elements. Arrows point to chromatin regions where nucleosomes are in close contact and the nucleosome centre-to-centre distance is much shorter than that of spreading regions. From Figs 1 and 2 we can see that nucleosomal chains are prone to bending.

The circles of nucleosomal chain reveal a characteristic 'beads-on-a-string' appearance (Figs 3 and 4). The diameters of the globular particles, which are in close contact or are separated by a variable distance, range from 36 to 43 nm. The rings can be divided into two classes: open rings (Fig. 3) and closed rings (Fig. 4). Most of the rings are asymmetrically closed and the others are open rings. Usually, the open

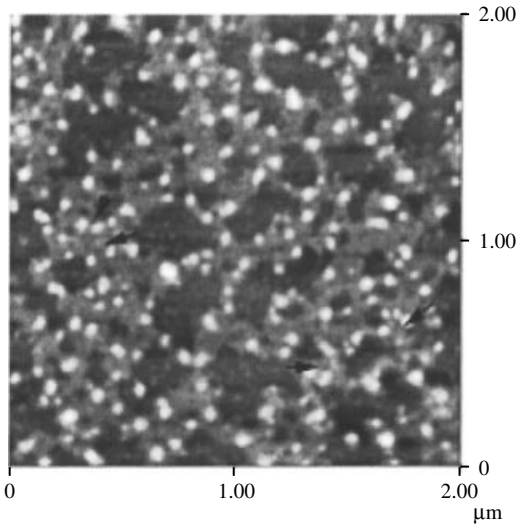

Figure 2. Atomic force microscopy image showing the morphology of extended polynucleosomal filaments. Arrows point to the regions where nucleosomes are in close contact. Height scale is 0–15 nm.

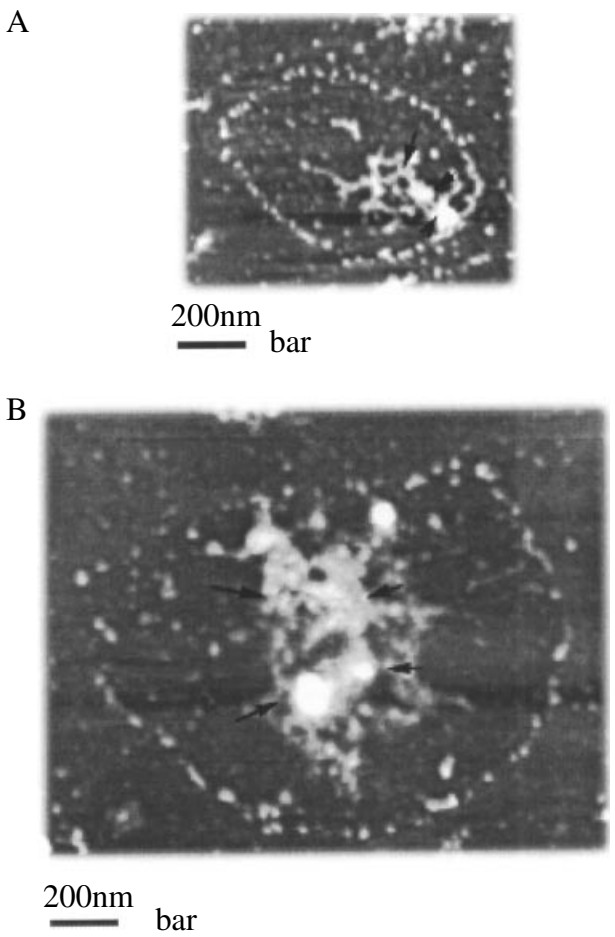

Figure 3. The open rings formed by the nucleosomal chain surrounding compacted chromatin (which the arrows point to). Height scale is 0–15 nm.

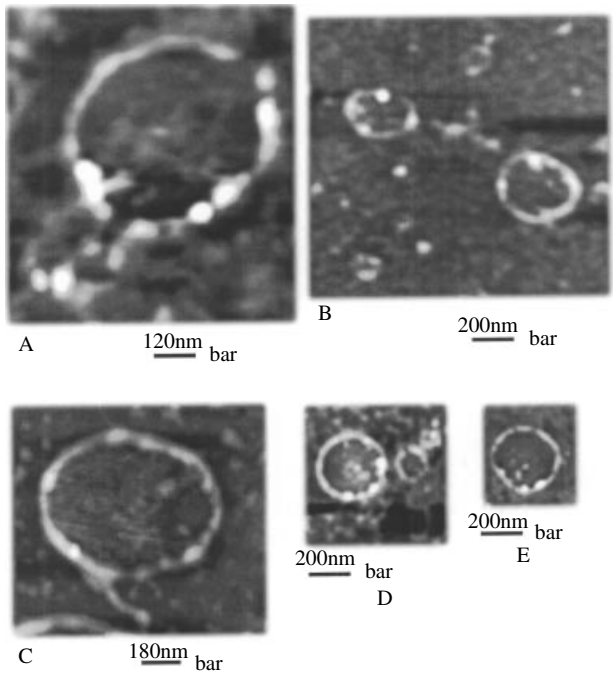

Figure 4. The circles formed by the nucleosomal chain, revealing a characteristic 'beads-on-a-string' appearance. Height scale is 0–15 nm.

rings are larger in diameter than the closed rings. We have measured the contour lengths of the rings. The open rings are very variable in size, ranging between ~2.0 and ~4.0 μm in circumference. For the closed rings, the smallest measured ring had a fibre length of ~1.0 μm and the largest of ~2.5 μm; the average length of the rings is ~1.5 μm ($n = 40$). Furthermore, open rings and asymmetric rings not shown in Fig. 4 can be detected. Therefore, ring formation cannot be explained by a single strand cut in the DNA followed by reannealing of its ends.

The formation of rings of nucleosomal chain depends on two chief requirements: a substantial degree of stiffness in the nucleosomal chains, and the force for its bending. In Fig. 3—the ring of nucleosomal chain surrounding compacted chromatin (arrows point to it)—nucleosome-on-a-string can be found in the unravelling part of the compacted chromatin. It is possible that the mild method for nucleosome preparation cannot completely unfold the closely folded native chromatin, and it is also probable that the extended chromatin recondensed *in vitro*. But it is impossible that open rings were formed by condensation of the nucleosome chain. Possibly the surface tension and a substantial degree of stiffness of the chain facilitated the formation of an open ring during folded chromatin spreading.

DISCUSSION

Chromatin has been imaged by AFM in air and in liquid.[15,24–26] Imaging a chromatin sample in air is more reproducible than imaging in liquid. The spatial resolution of the AFM images of chromatin fibres obtained in liquid is still significantly lower than that achieved in images of the same samples in air. Therefore, AFM in air is generally used.

Mica is a layered mineral with a negative surface charge. Nucleosomal chain consists of DNA and histones. Each histone has many N-terminals, which are positively charge groups. The coulombian force between the mica surface and the N-terminal is presumably strong, so nucleosomal chain can be stably adsorbed on the mica surface. Chromatin preparation for AFM imaging includes air drying of the samples. Drying can induce structural alterations by flattening. The apparent height of samples using AFM is usually less than the value for the native structure, probably due to flattening during air drying.

We isolated nucleosomal chains from nuclei using a mild method. There are two marked characteristics in our preparation of specimens for AFM observation. Firstly, we did not use trypsin or micrococcal nuclease in the whole process of preparing H1-depleted chromatin. Usually, the light hydrolysation of trypsin[25,27,28] or the reduction of micrococcal nuclease[24,26,29,30] or nuclease is employed for imaging the 'beads-on-a-string' structure. Secondly, we took out the supernatant for AFM after the diluted chromatin was centrifuged for 20 min at low speed (Materials and Methods). This step avoided the effect of compacted chromatin or other heavy component on AFM observation. Our results show a clear 'beats-on-a-string' appearance, and there is no obvious difference with the previous observations.[24–30] But the closed rings formed by nucleosomal chain were not reported in the previous observations. This indicates that the appearance of closed rings formed by the nucleosomal chain has a close relevance to our mild method. Maybe there is a cause and effect relationship between the ring appearance and our method. Closed rings formed by the nucleosomal chain may be a distinctive result of the mild method. In addition, our results demonstrate that neither the hydrolysation of trypsin nor the digestion of micrococcal nuclease or nuclease is necessary for observation of 'beads-on-a-string'. Furthermore, the mild method avoided the influence of extraneous macromolecules, such as trypsin. The formation of the closed rings was bound to have something to do with eliminating the influence of extraneous macromolecules.

Chromatin is made up of variously folded and compacted chains of nucleosomes and linker DNA, modulated by histone H1, non-histone proteins and other future particles of the nuclear environment. We removed non-histone and histone H1 attached to chromatin by the mild method to isolate nucleosomal chains from nuclei. Thus non-histone, histone H1 and other components from nuclei (such as RNA polymerase, DNA topoisomerase, RNA) co-exist with nucleosomal chain in buffer A. In this environment, it is very possible that the nucleosomal chain reconstituted to closed rings by the participation or help of non-histone or histone H1 or RNA or RNA polymerase or DNA topoisomerase, etc. Histone H1 has a multidomain structure consisting of a conserved ~80-amino acid globular domain[31,32] together with more variable, highly basic N- and C-terminals that may have extended the conformation.[33] The globular domain is very easy to associate with DNA. Furthermore, H1 has a high positive charge density, which can neutralize the negative charges of DNA and the electrostatic interaction between H1 and DNA is strong enough to make nucleosomal chains bend. Large doughnut-shaped complexes were formed when H1 was mixed with DNA in amounts that extensively neutralized it; the doughnut shape is the most prevalent form observed by electron microscopy for linear double-stranded DNA.[34] It is probable that H1 caused certain long nucleosomal chains to reconstitute into rings with the help of DNA topoisomerase, etc.in the condition of nucleosome core particles neutralizing most charges of DNA. However, the mechanism of nucleosomal chains reconstituting to rings is very complicated and deeper investigations should be carried out.

From the results, we can infer that the nucleosomal chain has a higher stiffness compared with naked DNA. Chromatin spreading and condensing are reversible to a certain degree, so it is feasible to infer the condensing and unfolding of chromatin in nuclei by studying that *in vitro*. The method developed in this work eliminates the influence of extraneous macromolecules, so it may provide a new, reliable way to study the behaviour of the extended chromatin fibre at low ionic strength. These observations may provide a useful clue to elucidate how the structure of the extended chromatin fibre affects DNA transcription, replication and repair.

CONCLUSION

Our results present the likelihood of structural diversity of the nucleosomal chain. Neither the hydrolysation of trypsin nor the digestion of micrococcal nuclease or nuclease is necessary for observation of 'beads-on-a-string'. The Nucleosomal chain has a substantial degree of stiffness. The closed rings formed by the nucleosomal chain have a close relevance to the two marked characteristics of the described method developed in this work to prepare H1-depleted chromatin. The described method may provide a new, reliable way to study the behaviour of the extended chromatin fibre at low ionic strength.

Acknowledgements

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