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Irregular Orientation of Nucleosomes in the Well-Defined Chromatin Plates of Metaphase Chromosomes[†]

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ABSTRACT: In previous studies with partially denatured metaphase chromosomes, we detected platelike structures instead of the chromatin fibers currently considered in different structural models for chromosomes. Here we have observed that dilution of compact metaphase chromosomes with hyposmotic solutions can transform whole chromatids into extended plates formed by many layers. Since this treatment is soft and it does not change the ionic conditions, these observations indicate that native chromosomes are formed by stacked plates. This strengthens our hypothesis about the multilayer structure of chromosomes, which was originally based on results obtained using stronger denaturing conditions. We have investigated the structure of plates emanated from chromosomes using electron tomography. Our three-dimensional reconstructions demonstrate conclusively that the surface of the plates is very smooth and do not show repetitive structures supporting any regular organization of nucleosomes; even the nucleosomes in plate edges show irregular orientations. Furthermore, we have used polarizing microscopy for the study of whole chromosomes in metaphase cells in aqueous solution. Our results show that condensed chromosomes are not birefringent under structuring ionic conditions similar to those used with plates. This observation is incompatible with the existence of parallel columns of nucleosomes within chromosomes. In summary, we have not detected any regular orientation of nucleosomes, but at the same time, our results indicate that the bulk of chromatin in native chromosomes is organized forming very well-defined plates, in which the nucleosomes of the successive layers are interdigitated. Presumably, this dense structure is required for safe transfer of DNA to daughter cells.

The packaging of extremely long DNA molecules in chromosomes during mitosis is one of the most fascinating problems of molecular biology. We know that DNA is associated with histone octamers ($H2A_2-H2B_2-H3_2-H4_2$) forming nucleosome core particles (*1*, *2*). Under adequate ionic conditions, the nucleosome filament complemented with histone H1 is folded into a 30 nm chromatin fiber which has been modeled in different laboratories (*3*−*6*). In general, current structural models for the metaphase chromosome suggest that the chromatin fiber is the fundamental structural element for the formation of condensed chromatids (*7*, *8*). However, in our previous transmission electron microscopy (TEM)[1] study (*9*), in which we investigated the structure of human metaphase chromosomes using different conditions, we found that chromatin fibers were observed only in experiments performed at extremely low ionic strength. Similar conditions were used in the early TEM studies that suggested fibrillar models for the metaphase chromosome structure (*10*−*12*). In contrast, under a wide range of ionic conditions, including cation concentrations approaching those found in metaphase cells (*13*), we observed that chromosomes were highly compact without visible fibers. Recently, Eltsov et al. (*14*) have reported that the cryo-electron microscopy analysis of vitreous sections of chromosomes in mitotic cells does not indicate the presence of 30 nm fibers.

Instead of chromatin fibers, we observed multilayered platelike structures surrounding compact human chromosomes partially denatured by incubation at 37 °C (*9*). This was an unexpected finding because these structures had never been described before. In a more recent study (*15*), we showed that plates can also be found in metaphase chromosomes from chicken lymphocytes. We studied plates using atomic force microscopy in aqueous solution at room temperature and cryo-electron microscopy. Plates are very thin but maintain the nucleosome filament tightly bound; we needed incubations under low-ionic strength conditions to produce emanations of chromatin fibers from the plate edges. We proposed that metaphase chromosomes are formed by many stacked plates oriented perpendicular to the chromatid axis. This structure has never been suggested before for typical chromosomes, but in the case of dinoflagellate chromosomes (which do not contain histones), Livolant and Bouligand (*16*) proposed that DNA is packed forming a complex multilayer structure. Our thin-plate model can explain easily the chromosome banding obtained in cytogenetic studies using diverse staining procedures.

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[1]Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TEM, transmission electron microscopy.

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In this work, we have developed gentle procedures for the disassembly of metaphase chromosomes into plates. We have investigated the structure of plates emanated from chromosomes using electron tomography. The resulting tomographic reconstructions have allowed us to study the three-dimensional (3D) structure of plates and the organization of nucleosomes within them. On the other hand, we have used polarizing microscopy for the analysis of whole chromosomes in metaphase cells, in aqueous solution under structuring ionic conditions similar to those used in our study of individual plates emanated from chromosomes. We have not detected any regular orientation of nucleosomes. However, our results show that this apparently irregular structure is compatible with the fact that in metaphase chromosomes the bulk of chromatin is perfectly organized in a structure composed of very well-defined plates that occupy the entire volume of the chromatid.

MATERIALS AND METHODS

Metaphase Chromosomes. Chromosomes from HeLa cells were isolated and purified on sucrose step gradients as described in previous publications (*9*, *15*). These gradients contained four layers of sucrose (30−60%) and 5 mM Pipes (pH 7.2), 1 mM EGTA, 5 mM NaCl, and 5 mM $MgCl_2$. Chromosome suspensions collected from the 40−50 and 50−60% sucrose interphases were mixed and diluted with 1−2 volumes of the same solution, but without sucrose, and spread on carbon-coated grids.

TEM and Electron Tomography. For the spreading, the chromosome suspension (100 μL) above the grid (placed in the cap of an inverted tube) was centrifuged at 4 °C for 10 min at 1500*g* (or 900*g* in some experiments) on a swinging bucket rotor. The extensions were fixed with 2.5% glutaraldehyde (reaction for 10 min at room temperature), washed twice by floating the grid on deionized water (1 mL) for 5 min, rinsed in ethanol for 2−3 s, and allowed to dry on a filter paper. Finally, the grids were rotary-shadowed with platinum using the MED 020 high-vacuum coating system (BAL-TEC, Balzers, Liechtenstein); the thickness of the platinum coating in our samples was approximately 1 nm, as measured with the QSB 060 film thickness monitor (BAL-TEC). Imaging and tomography were performed using the JEM-1400 microscope (Jeol, Tokyo, Japan), equipped with a computer-controlled tilt specimen holder. For tomography, images were recorded at tilt intervals of 1°, from −60° to 60°, using a cooled CCD camera (MultiScan TM600w, Gatan, Pleasanton, CA). We used the Gatan-DigitalMicrograph software package for the acquisition of fully automated tilt series, and IMOD (version 3.11) (*17*) for the detection of the specimen tilt axis, image alignment, and tomographic reconstructions. In some experiments, before the sample had been spread, 5 nm colloidal gold particles were deposited on the carbon-coated grids to facilitate further micrograph alignments, but in many cases, the high contrast of the images allowed marker-free alignment.

Polarizing Microscopy of Metaphase Cells. HeLa cells were accumulated in metaphase using colcemid as described elsewhere (*9*), swelled by incubation with 75 mM KCl at 37 °C for 10 min, centrifuged at 275*g* for 5 min at 4 °C, and finally resuspended in diverse aqueous solutions: (a) 15 mM triethanolamine-HCl (pH 7.4), 2 mM EDTA, 0.5 mM EGTA, 20 mM NaCl, 80 mM KCl, 0.2 mM spermine, and 0.5 mM spermidine, (b) 5 mM Pipes (pH 7.2), 1 mM EGTA, 5 mM NaCl, and 5 mM $MgCl_2$, and (c) buffer b with 20 mM $MgCl_2$. The birefringent properties of the resulting samples were analyzed with an Axiotron polarizing microscope (Zeiss, Oberkochen, Germany) at room temperature. Images were obtained with a DC200 CCD camera (Leica, Wetzlar, Germany); we used long exposure times (4−9 s) for the acquisition of images with crossed polars. Squid (*Loligo vulgaris*) sperm heads and small fragments of finely ground calcite were used as internal references. Swelled cells under different ionic conditions were also observed using phase contrast and fluorescence microscopy; in the latter case, chromosomes were stained with DAPI.

RESULTS

Dilution of Metaphase Chromosomes with Hyposmotic Solutions Favors the Emanation of Multilayer Plates. Condensed chromosomes purified by centrifugation on sucrose step gradients containing 5 mM Mg^{2+} show the typical compact structure observed in metaphase cells (see the inset of Figure 1). In previous studies (*9*, *15*), we found that incubation at 37 °C and diverse mechanical treatments cause the emanation of multilayer plates from uncrosslinked chromosomes. In this work, we have found that softer treatments are able to transform chromosomes into extended structures containing a large amount of multilayer plates (see examples in Figure 1 and Figures S1−S3 of the Supporting Information). In these experiments, we simply diluted the chromosome suspension with an aqueous solution with the same ionic composition as the step gradient but without sucrose. The dilution with this hyposmotic solution probably causes a rapid influx of the external water solution into the chromatids; the whole chromosome becomes inflated, and this favors disassembly.

Chromosomes treated with hyposmotic solutions exhibit different degrees of disassembly. We have observed almost intact chromosomes and chromosomes having extrusions of planar structures only in the telomere region (Figure S1A,B of the Supporting Information). We have also found chromosomes completely unstructured. In some cases (Figure 1B), the remains of the initial chromosome (i.e., the intense white regions in our reverse contrast micrographs) appear also as a multilayered structure, suggesting that plates are apparently occupying the space corresponding to whole chromatids. In other images, it is not possible to recognize the location of the original chromatids (Figures S2 and S3 of the Supporting Information); the plates can be seen as isolated structures either because the chromatids have been entirely converted into extended plates or because they have been split off from unstructured chromosomes.

The observed plates are of very different sizes, and their edges show very different shapes. This heterogeneity is probably due to a random splitting of plates produced by mechanical stress during chromatid disassembly. Nevertheless, some plates observed in our micrographs are very large. There are plates with a surface area larger than the cross section of a chromatid (~0.3 μm^2 considering a chromatid diameter of ~0.6 μm). For instance, the big plates shown in Figure S3 of the Supporting Information have a surface area of ~1 μm^2. Since plates are very thin and easily split when they are extruded from the chromatids, the observation of large plates in some of our preparations suggests the possibility that they could form a continuous structure within native chromosomes (see Discussion).

Electron Tomography Reconstruction of the 3D Structure of Chromosome Plates. TEM images suggest that plates emanated from chromosomes are flat and smooth. The contrast produced by rotary-shadowing with platinum facilitates the

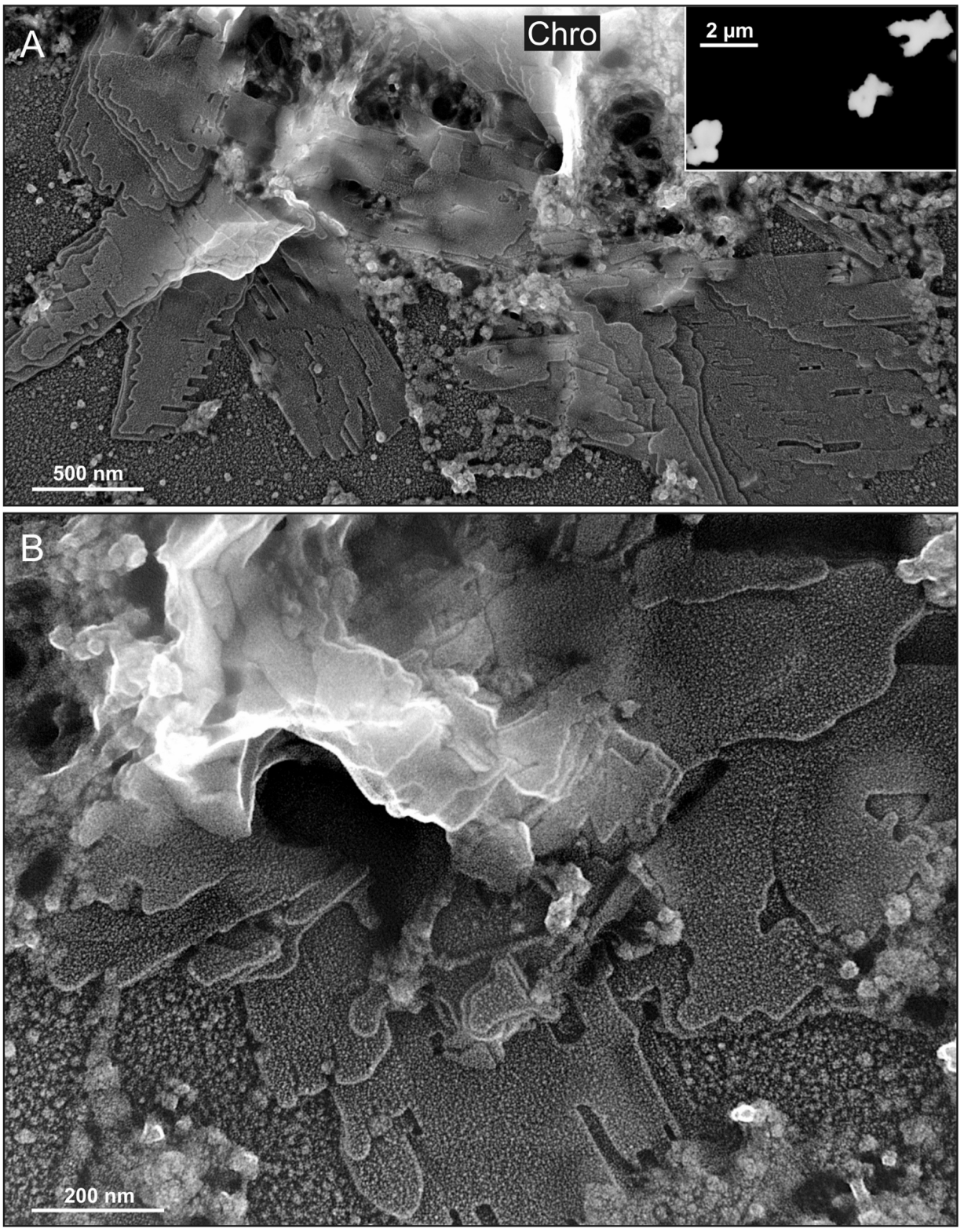

FIGURE 1: Metaphase chromosomes in an aqueous solution containing ~50% sucrose and 5 mM Mg^{2+} were diluted with 2 volumes of the same solution without sucrose, spread by centrifugation on carbon-coated grids, rotary-shadowed, and visualized by TEM (A and B). Chro indicates the remains of a chromatid. The inset shows spread chromosomes not subjected to the dilution step. Additional images showing diverse degrees of chromosome disassembly are presented in Figures S1−S3 of the Supporting Information.

visualization of the plate edges. At a high magnification, there are many bars visible in the edges (see Figure 7 of ref *15*), and we interpreted that these bars correspond to the turns of DNA of the nucleosomes located in the plate edges. Unfortunately, TEM can give us only two-dimensional (2D) images lacking depth sensitivity. To obtain reliable information about the 3D structure of the plates, we have conducted an extensive electron tomography study.

The original TEM images corresponding to two multilayer plates are presented in Figure 2A,B. We have obtained 121 images at different tilt angles for each plate, and after aligning all these images, we have reconstructed the 3D structure (Figure 2C,D).

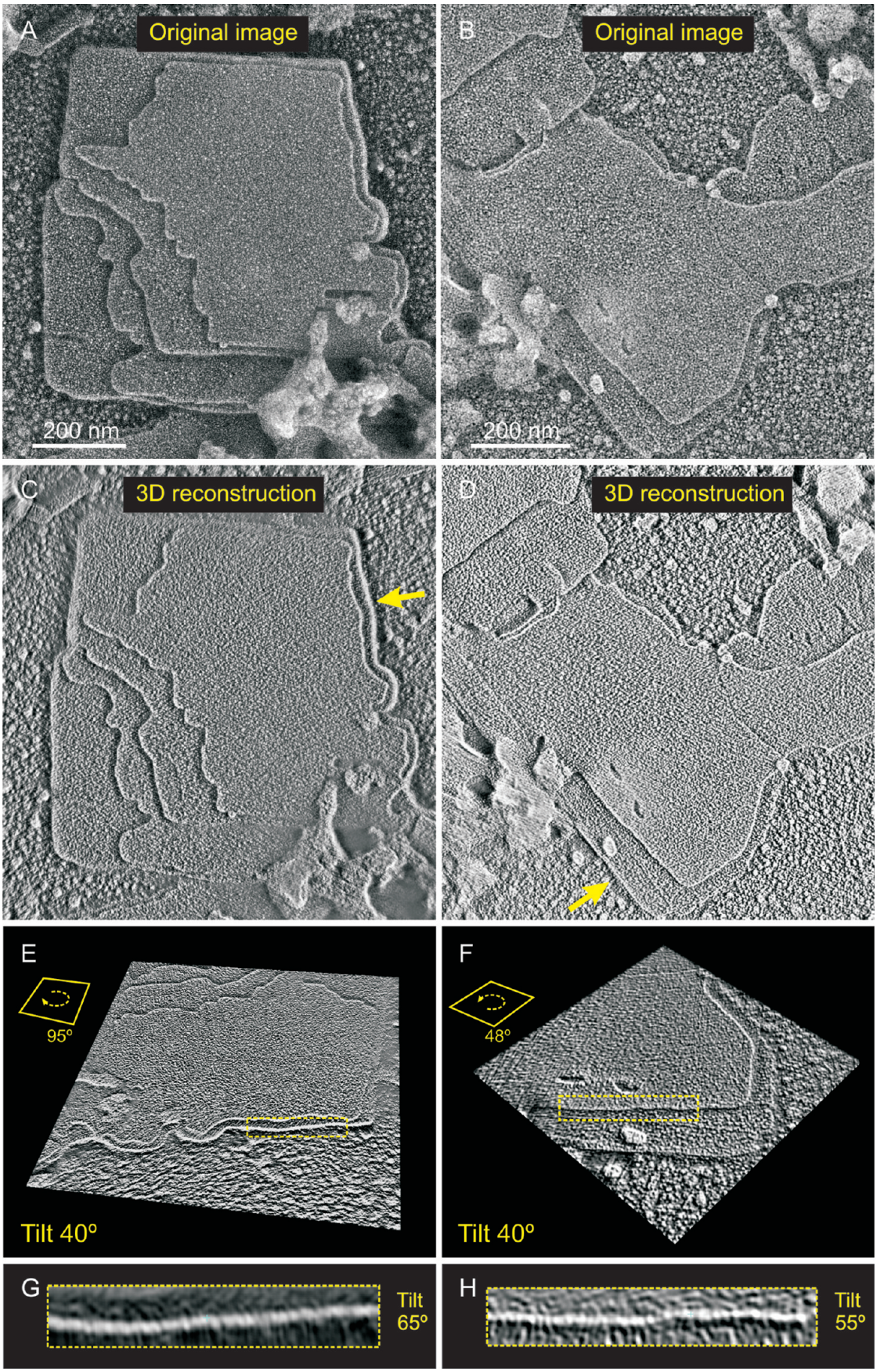

FIGURE 2: Examples of electron tomography analysis of chromatin plates that emanated from metaphase chromosomes treated with hyposmotic solutions. Normal TEM images (A and B) and the corresponding 3D reconstructions (C and D). The yellow arrows indicate the views selected for further analysis in panels E and F; the plate edges marked with a yellow rectangle were magnified and are visualized with a higher tilt angle in panels G and H.

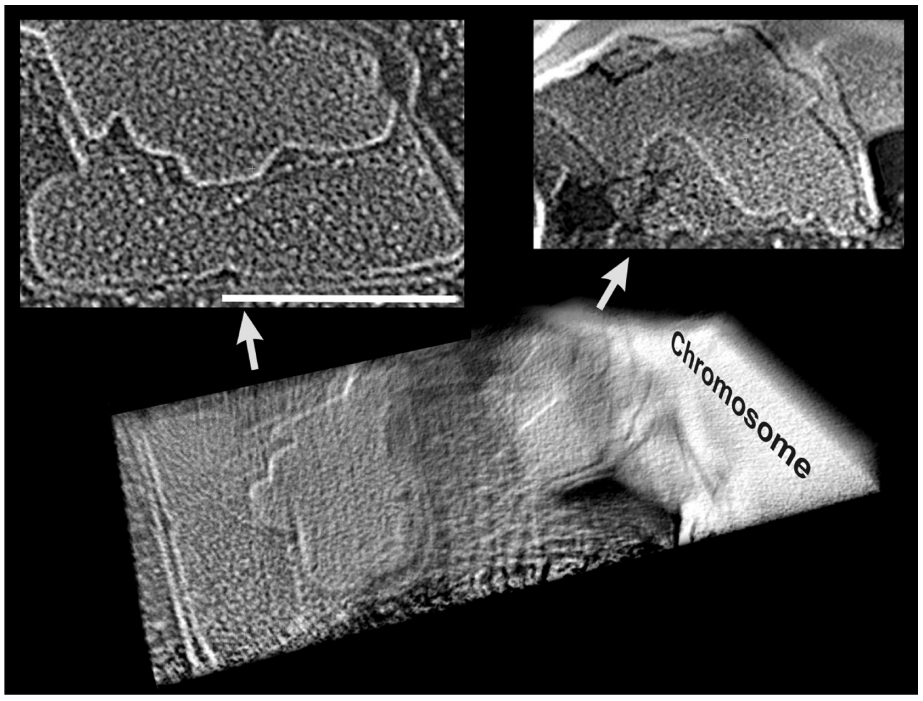

FIGURE 3: 3D tomographic reconstruction of a plate that emanated from a chromosome. This plate was probably split into two pieces during the spreading, with one part attached to the chromosome (right; it is highly tilted with respect to the carbon plane) and the other in contact with the carbon surface (left); the two parts are shown with different orientations at a higher magnification in the top part of this figure. The bar is 200 nm.

The analysis of the tomograms using different view angles (see examples in Figure 2E,F) confirms that plates can be really very flat. The plates that are not in contact with carbon have diverse orientations and may be curved (Figure 3, right) but show the same structural characteristics as the plates in contact with the carbon surface (Figure 3, left). These results and the observation of many typical plates that are far from the carbon surface (for instance, the plates on top of the thick region in Figure 1B and the plates in the central region of Figure S2 of the Supporting Information) indicate that plate structure is independent of the substrate.

Furthermore, using high tilt angles, it is possible to measure directly the thickness of the plates. The histogram in Figure 4B corresponds to the measurements of the height of a step in a multilayer plate (6.1 ± 0.5 nm; $n = 18$). The mean value obtained for layers in contact with the carbon film is higher [7.8 ± 0.5 nm; $n = 16$ (Figure 4A)]; nevertheless, the actual height of the bottom layers is probably even higher than this value because there is unorganized chromatin in the carbon surface around the plates (see, for instance, Figure 2C,D) which may reduce the measured height. We obtained similar values in plate height measurements performed using unidirectional shadowing and atomic force microscopy in aqueous solution (*15*).

The surface of the 3D reconstructed structures is very smooth (see Figures 2C−F and 3). There is no sign of any regular organization of the bars that presumably corresponds to nucleosomal DNA. Equivalent observations have been reported by Eltsov et al. (*14*) in their analysis of cryosections of mitotic chromosomes in situ. Bars are more easily seen in the plate edges of tilted tomograms. The visualization of the edges using progressively increasing tilt angles (see examples corresponding to high tilt angles in Figure 2G,H) shows that bars are continuous structures that span the entire thickness of the layer that contains them. This excludes the possibility that the bars are due to imaging artifacts. Furthermore, the irregular orientation of the

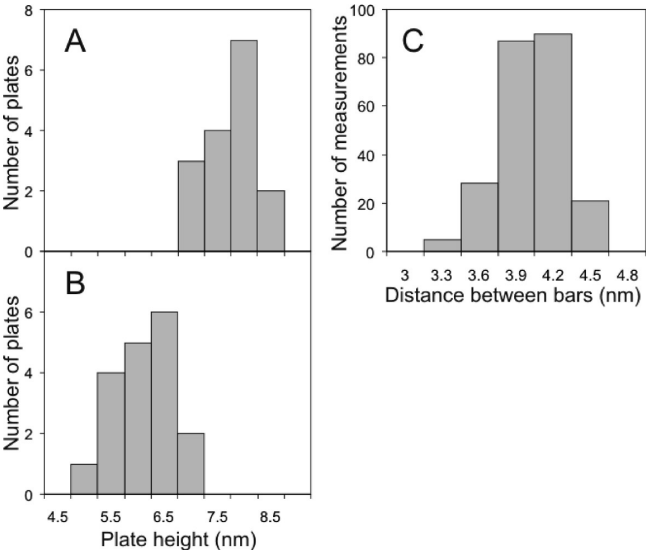

FIGURE 4: Histograms corresponding to measurements performed using 3D tomographic reconstructions. (A) Heights of plates closest to the carbon surface. (B) Heights of a step in multilayer plates. (C) Distances between the dense bars observed on the edges of chromatin plates.

bars in the edges indicates that nucleosomes are not organized forming regular piles with a repetitive spacing. Our 3D reconstructions have allowed us to obtain the mean distance between bars in the plate edges [calculated by dividing the length of a particular region in an edge by the number of bars observed in the same region (see the histogram in Figure 4C)]. The mean value obtained is 4.0 ± 0.3 nm ($n = 259$). This distance is significantly larger than the distance between the turns of DNA in columns of nucleosomes stacked regularly [~2.8 nm, as determined by TEM (*18*), X-ray crystallography (*19*), and cryo-electron microscopy (*20*)].

Polarizing Microscopy Results Also Suggest an Irregular Orientation of Nucleosomes in Metaphase Chromosomes in Aqueous Solution. The results described above suggest an irregular orientation of nucleosomes in the plates emanated from chromosomes. In contrast, early analysis of vitrified cryosections of metaphase cells (*21*) suggested that nucleosomes within chromosomes may be stacked forming columnar structures. To clarify this apparent contradiction, we have investigated the organization of nucleosomes within native chromosomes.

Leforestier et al. (*20*) showed that isolated nucleosome core particles in the presence of polyamines form ordered aggregates containing parallel columns of stacked core particles. Using polarizing microscopy, these authors observed that these regular columnar structures are birefringent; when the nucleosome columns are parallel to the slide and the coverslip surfaces, they were illuminated between crossed polars. This is due to the fact that the optical properties of the core particles are dependent on the orientation of the birefringent DNA molecule with respect to the polarized light. Taking into account all these observations, we have used polarizing microscopy to analyze the structure of condensed chromosomes in cells arrested in metaphase.

Cells were suspended in aqueous solutions containing polyamines. As one can see in Figure 5B, when observed between crossed polars, chromosomes are not illuminated. The same results were obtained when the cells were treated with aqueous solutions containing 5 and 20 mM Mg^{2+} (Figure 5D,F). In contrast, birefringent squid sperm cells (or calcite fragments) in adequate orientations are highly illuminated.

In these experiments, cells were unfixed and highly swelled during the observation. Furthermore, microtubules were depolymerized by colcemid. Thus, in principle, chromosomes were free to adopt any orientation. In fact, we have seen that chromosomes are randomly oriented in the polarizing microscope with parallel polars (Figure 5A,C,E) and in phase contrast and fluorescence microscopy observations (Figure S4 of the Supporting Information). Thus, since chromosomes appeared as dark objects in all orientations between crossed polars, we can conclude that in aqueous solution, under different ionic conditions, condensed chromosomes are optically isotropic. According to all the considerations mentioned above, this result excludes the possibility that nucleosomes are oriented regularly, forming parallel columns inside metaphase chromosomes.

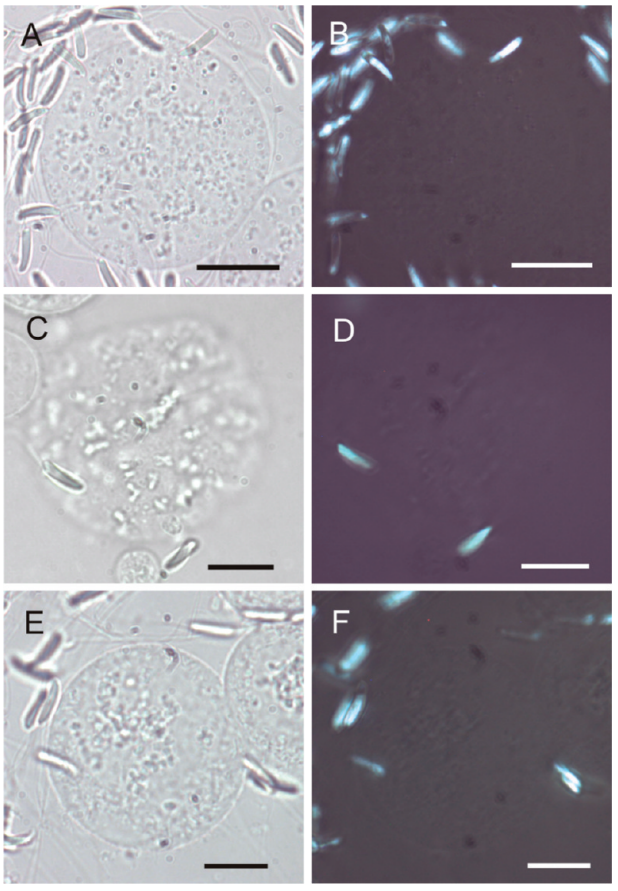

FIGURE 5: Imaging of metaphase chromosomes with polarizing microscopy. HeLa cells arrested in metaphase by colcemid were incubated at room temperature with aqueous solutions containing 0.7 mM polyamines (A and B), 5 mM Mg^{2+} (C and D), and 20 mM Mg^{2+} (E and F). Images A, C, and E were obtained with parallel polars; images B, D, and F were obtained with crossed polars. Birefringent squid sperm heads (elongated cylinders seen around cells) were used as internal controls in these images. The bars are 15 μm.

DISCUSSION

Our results show that the treatment with hyposmotic solutions transforms condensed chromosomes into extended planar structures formed by many layers. In some TEM images, it is clear that both the remains of the chromatids and the extended material around them have the same platelike structure. Since this treatment is soft and maintains the structuring ionic conditions, we can assume that there is no change in the bulk structure of chromatin. These observations strongly suggest that the entire native chromosome is formed by plates. Furthermore, these observations strengthen our original thin-plate model for chromosome structure, which was based on results obtained using stronger denaturing conditions (*15*).

Our electron tomography results have allowed us to study the actual 3D structure of the plates. Tomographic reconstructions demonstrate conclusively that in the 3D space plates are constituted by well-defined layers having a thickness of approximately 6 nm. Our 3D reconstructions also demonstrate conclusively that plates are very smooth; they do not have long striations or repetitive circular structures (with a periodicity of ~11 nm) corresponding to the regular organization of nucleosomes considered in Figure 6B. Our results indicating that even the nucleosomes in the plate edges show irregular orientations are compatible with the structure schematized in Figure 6C. Since the layer thickness is smaller than the nucleosome diameter (Figure 6A), the successive layers are probably interdigitated. The interdigitation of nucleosome-containing structures has been proposed previously by different laboratories to explain the internal compaction of chromatin fibers (*22*−*25*) and lateral association between fibers (*26*−*28*).

Diverse research evidence (*2*, *7*, *20*, *29*−*32*) has indicated that face-to-face interaction of nucleosomes may be involved in chromatin compaction. The irregular orientation of nucleosomes in Figure 6C does not exclude the possibility of face-to-face association between nucleosomes in adjacent layers. Our polarizing microscopy results showing that condensed chromosomes are not birefringent are incompatible with the existence of parallel columns of nucleosomes within chromosomes. Nevertheless, irregularly oriented nucleosomes may associate, forming piles with diverse relative orientations (not shown in the schematic drawing in Figure 6C). Such a structure has many diverse orientations of the nucleosomal DNA, and consequently, in

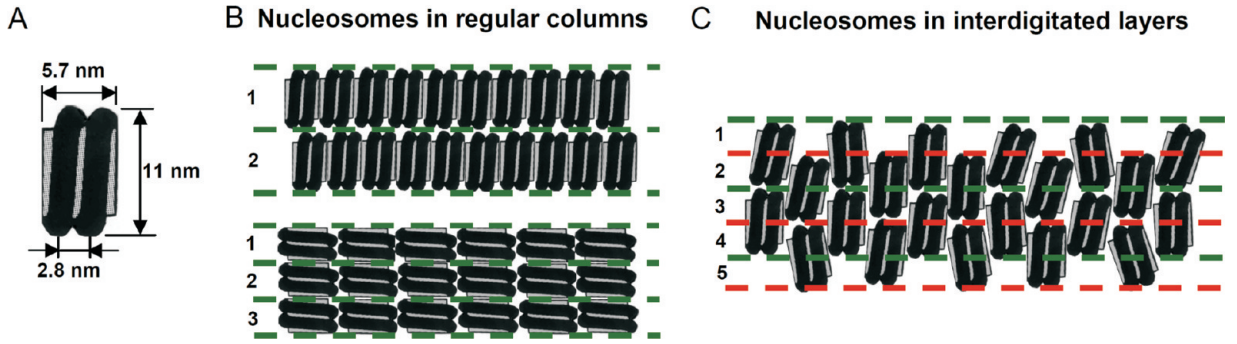

FIGURE 6: Side-view drawings of different possibilities for nucleosome organization in chromatin plates. Our results are not compatible with regular models having parallel columns of nucleosomes (B) and favor an irregular structure with interdigitated layers (C); the successive layers are indicated by numbers at the left. The approximate dimensions of the nucleosome are shown in panel A. According to the 3D reconstructions (Figure 4B) and our previous TEM and atomic force microscopy measurements (*15*), the thickness of a layer is ~6 nm. Thus, in the structure presented in panel C, the successive layers are interdigitated to justify the small thickness of each individual layer in comparison to the nucleosome diameter (~11 nm). Note that in the structures formed with regular columns of nucleosomes (B, top) the average distance between DNA turns is ~2.8 nm. However, in the 3D reconstructions of plate edges, the dense bars that presumably correspond to the turns of nucleosomal DNA are not regularly oriented and the observed average distance between them is greater [~4 nm (Figure 4C)]; similar distances were found in our previous TEM and cryo-electron microscopy studies [3.7 and 3.9 nm, respectively (*15*)]. Therefore, in the structure presented in panel C, nucleosomes are irregularly oriented. The model shown in panel C is merely schematic for the purpose of illustrating the main structural elements considered above; the observed plates are more compact and have a smoother surface than that represented in this simplified drawing.

agreement with our experimental results, it must not exhibit birefringence. It is known that face-to-face interactions involve an acidic surface formed by histones H2A and H2B and basic residues of the N-terminal tail of histone H4 (*2*, *29*−*32*); the other basic tails of core histones and histone H1 could be involved in the intralayer interactions. Furthermore, considering that diverse lines of evidence have shown that nucleosomes have a remarkable structural plasticity (*33*), the possibility that nucleosomes could have an altered structure inside the plates should also be considered in future studies.

Our images showing many large plates emanated from disassembled chromosomes, and in particular the observation of plates larger than the cross section of a chromatid, together with early results indicating that metaphase chromosomes may adopt a global helical folding (*34*−*36*), allow us to speculate that each chromatid could be formed by a single plane folded as a single helicoid. In this hypothetical structure, condensed chromatids should have the adjacent turns in close contact, giving rise to a helicoid with a small pitch (~6 nm). Note that the successive turns in this structure are equivalent to the stacked layers (orthogonal to the chromatid axis and having a thickness of ~6 nm) considered in our original thin-plate model (*15*); the interdigitation of nucleosomes proposed in the structure schematized in Figure 6C explains the observed small thickness of the layers. Furthermore, a continuous helicoidal structure with a very small pitch is also compatible with the easy formation of cytogenetic bands perpendicular to the chromatid axis and with the observed splitting of these bands in stretched chromosomes (*37*), and could be related to the micromechanical properties of mitotic chromosomes (*38*, *39*).

According to our results, in native condensed chromatids, we expect an interdigitation of the successive chromatin layers (or of the helicoidal turns considered in the preceding paragraph), forming a compact structure with a DNA density compatible with the high concentration observed experimentally in metaphase chromosomes of different species (*40*). The high degree of compaction in all directions of space and the global irregular orientation of nucleosomes observed in the 3D reconstructions and in our polarizing microscopy study are compatible with the results of Eltsov et al. (*14*) showing that in the vitreous sections of intact chromosomes the bulk of the chromatin is very compact and apparently completely disordered. In the native chromosome, the stacked plates are completely hidden and the DNA is oriented in many different directions both in each layer and in the entire volume of the chromatids, producing an optically isotropic structure. These properties make difficult the detection of plates in native chromosomes, and even in their cryosections. Fortunately, the relative sliding between layers observed in disassembled chromosomes facilitates the visualization of the surface and edges of many plates. This fact has allowed us to discover and investigate this basic structural pattern of chromatin folding in chromosomes.

We know that after mitosis chromosomes are decondensed, but the organization of chromatin fibers in the interphase nuclei is controversial (*4*, *5*, *41*, *42*). When considering plate−fiber transformations, one must take into account the fact that in fibers the dominant interactions are produced between close neighbor nucleosomes on the chromatin filament, but for the formation of thin plates, the chromatin filament has to be flexible enough to allow interactions between more distant nucleosomes. The results of Zhou et al. (*31*) showing that compact fiber formation inhibits the oligomerization of individual nucleosome arrays are in agreement with these considerations.

We think that the main function of the densely packed chromatin plates is the safe storage of the single DNA molecule contained in each chromatid during mitosis. We expect that in the future it will be possible to define the local modifications of this dense structure that allow the specific placement of topoisomerase II and condensin in the chromatid axis (*43*, *44*) and the formation of the centromere constriction. The results obtained in this work and our previous findings introduce a new geometry based on planar rather than on linear chromatin that may transform completely our current understanding of chromosome structure and function.

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SUPPORTING INFORMATION AVAILABLE

Different degrees of chromosome disassembly produced by treatment with hyposmotic solutions (Figures S1−S3) and random orientation of chromosomes in HeLa cells arrested in metaphase with colcemid (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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