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Chromosomes without a 30-nm chromatin fiber

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How is a long strand of genomic DNA packaged into a mitotic chromosome or nucleus? The nucleosome fiber (beads-on-a-string), in which DNA is wrapped around core histones, has long been assumed to be folded into a 30-nm chromatin fiber, and a further helically folded larger fiber. However, when frozen hydrated human mitotic cells were observed using cryoelectron microscopy, no higher-order structures that included 30-nm chromatin fibers were found. To investigate the bulk structure of mitotic chromosomes further, we performed small-angle X-ray scattering (SAXS), which can detect periodic structures in noncrystalline materials in solution. The results were striking: no structural feature larger than 11 nm was detected, even at a chromosome-diameter scale (~1 μm). We also found a similar scattering pattern in interphase nuclei of HeLa cells in the range up to ~275 nm. Our findings suggest a common structural feature in interphase and mitotic chromatins: compact and irregular folding of nucleosome fibers occurs without a 30-nm chromatin structure.

Keywords: 30-nm chromatin fiber, chromatin, cryo-EM, fractal nature, interphase nuclei, irregular folding, mitotic chromosomes, X-ray scattering

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Introduction

In current molecular biology textbooks, initial DNA packaging and organization into a chromosome or nucleus is often depicted as shown in **Figure 1** (e.g., ref. 1). The current assumption is that DNA is wrapped around histones, forming "nucleosomes" (beads-on-a-string), followed by nucleosome folding into a 30-nm chromatin fiber (e.g., refs. 2–5). The famous "hierarchical helical folding" model asserts that the 30-nm chromatin fiber is folded progressively into larger fibers (i.e., ~100-nm and then ~200-nm fibers) to form the final mitotic chromosomes.[6-9] A similar hierarchy is thought to exist in interphase nuclei (e.g., ref. 10).

To visualize mitotic chromosomes in a close-to-intact state, we performed cryoelectron microscopy (cryo-EM), in collaboration with Eltsov, Frangakis and Dubochet.[11] For cryo-EM, mitotic HeLa cells were collected and frozen by high-pressure freezing. Thin sections were then prepared at low temperature and observed. Cryo-EM and subsequent computational image processing did not reveal 30-nm chromatin structures in the mitotic chromosomes, but rather a uniform disordered texture, strongly arguing against the current established hypothesis[11](also see refs. 12 and 13). However, cryo-EM observations were limited to examining a portion of a chromosome because the section thickness was only ~70 nm, which may have prevented observation of hierarchical regular structures in the chromosomes.

Small-Angle X-Ray Scattering (SAXS)

To investigate the bulk structure of mitotic chromosomes in solution, we performed small-angle X-ray scattering (SAXS). When X-rays are used to irradiate non-crystalline materials, scattering at small angles generally reveals periodic structures within samples (**Fig. 2A**) (e.g., ref. 14). A typical scattering

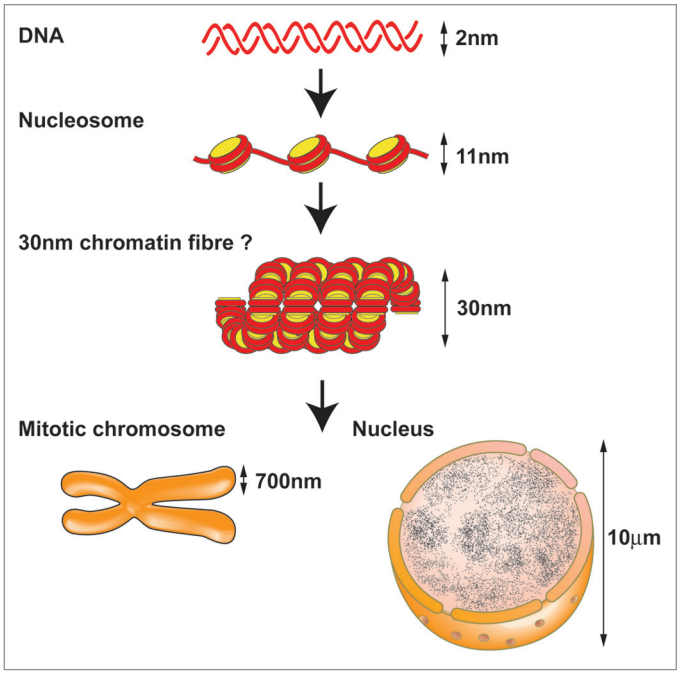

Figure 1. In the textbook model, a long DNA molecule is wrapped around a basic core histone octamer that consists of H2A, H2B, H3 and H4 histone proteins, and forms a nucleosome with a diameter of 11 nm. The nucleosome has long been assumed to be folded into 30-nm chromatin fibers before the higher-order organization of mitotic chromosomes or interphase nuclei occurs. We show a typical one-start helix model between two well-known structural models for 30-nm chromatin fibers: one-start helix (solenoid) and two-start helix (zigzag). The images are reproduced with minor modifications from reference 60 with permission from Elsevier.

pattern of SAXS composed of concentric rings is shown in **Figure 2C**. The signals at smaller angles (closer to the center) reflect larger periodic structures and vice versa.[14]

At the SPring-8 synchrotron radiation facility in Japan, isolated human chromosomes at the bottom of a glass capillary were exposed to a synchrotron X-ray beam and the scattering patterns were recorded (**Fig. 2B**).[15] Note that the chromosomes were not fixed or dehydrated to avoid possible artifacts caused by such treatments.[16,17] The typical scattering pattern of mitotic chromosomes showed three peaks at 6, 11 and 30 nm,[15] consistent with previous experiments by Langmore and Paulson.[18,19] The 6- and 11-nm peaks are believed to come from edge-to-edge and face-to-face positioning of nucleosomes, respectively.[18-20] The 30-nm peak is assumed to represent the side-by-side packing of 30-nm chromatin fibers,[18-20] which has long been regarded as strong evidence for the existence of these fibers in chromosomes. However, based on cryo-EM, these 30-nm structures are not apparent in mitotic chromosomes.[11-13]

Mitotic Chromosome Organization

To examine the nature of the 30-nm peak observed by SAXS, the isolated chromosomes were examined by cryo-EM. As noted above, no 30-nm chromatin structures were observed inside the chromosomes.[15] However, the cryo-EM images unexpectedly showed that the chromosome surface was coated with electron-dense granules the size of ribosomes.[15] Western blotting and immunostaining with specific antibodies confirmed that there were contaminating ribosomes on the chromosome surface.[15] The ribosomes were regularly stacked at ~30-nm spacing.[15]

We removed the ribosomes from the chromosome surface by washing with an isotonic buffer containing polyamine and EDTA (buffer A; see refs. 19, 21 and 22),[15] while maintaining the size and shape of the chromosomes.[15] Strikingly, in the chromosomes, no 30-nm peaks were detected by SAXS,[15] although other peaks coming from an internal structure of nucleosomes remained.

Previous reports have suggested that chicken erythrocyte nuclei, which are almost completely transcriptionally silenced, contain 30-nm chromatin fibers[23,24]; therefore, these were used as positive controls. SAXS and cryo-EM analyses demonstrated 30-nm chromatin fiber structures in chicken erythrocyte nuclei, which lacked ribosomes.[15] Therefore, we should have detected 30-nm fibers in the human mitotic chromosomes if they were present. We concluded from the combined SAXS and cryo-EM data that regularly folded 30-nm chromatin fibers are not present in human mitotic chromosomes.[15]

Next, we investigated an entire region of mitotic chromosomes using a newly developed apparatus for ultrasmall-angle X-ray scattering (USAXS).[25] Again, we found no regular periodic structures between 50 and 1,000 nm.[15] The cryo-EM, SAXS and USAXS data collectively indicate that irregular folding of nucleosome fibers is the bulk structure of human mitotic chromosomes.[15]

We then considered how the nucleosome fiber is organized into a mitotic chromosome. Because condensin[26] and topoisomerase IIα,[27] which are essential for chromosome condensation, form an axis in the chromosome[22,28-32] (**Fig. 2D**), we assumed that they globally secure the nucleosome fibers around the chromosomal center. Locally, a nucleosome fiber is folded in an irregular manner toward the chromosome center (**Fig. 2D**). In addition, a genomic site is rather randomly incorporated into a wide, but not reproducibly specific, region in the chromosome during the condensation process. This view is consistent with the observation by the Belmont group that three-dimensional positions of GFP-labeled genomic loci showed intrinsic variability between sister chromatids in the chromosome.[33]

Interphase Chromatin Organization

To examine the bulk periodic structure of interphase chromatin, we next focused on interphase nuclei of HeLa cells. Langmore and Paulson reported SAXS peaks at ~30, ~11 and ~6 nm in the nuclei of HeLa cells or mouse lymphocytes.[18,19] These authors suggested that the 30-nm peak represents

the side-by-side packing of 30-nm chromatin fibers,[18,19] and this has long been considered strong evidence for the existence of these fibers in interphase chromatin. Consistent with their data, we also detected similar peaks at ~30, ~11 and ~6 nm in the HeLa nuclei, which were isolated using their procedure (**Fig. 3A**). Because we detected ribosome components in the nuclei sample by western blotting (**Fig. 3B**), in analogy with the case of the mitotic chromosomes, we again removed them by washing the nuclei with buffer A.[19,21,22] This treatment removed most of the ribosome aggregates from the nuclear fractions (**Fig. 3B**). As expected, the 30-nm peak in the SAXS pattern disappeared almost completely (**Fig. 3C**), again suggesting the absence of the 30-nm structure. Note that the remaining peaks at larger angles, which came mainly from the internal structures of the nucleosomes, were unchanged (**Fig. 3A and C**). Similar to the case for mitotic chromosomes, the scattering profile of interphase chromatin also showed that the ~6-nm peak (face-to-face positioning) predominated over the ~11-nm peak (edge-to-edge positioning) (**Fig. 3C**). As formation of the 30-nm chromatin fiber requires similar frequencies of face-to-face (~6-nm peak) and edge-to-edge (~11-nm peak) positioning,[15] the scattering profile in **Figure 3C** also supports the near absence of regular 30-nm chromatin fibers in interphase chromatins.

Consistent with our finding, interphase nuclei in most higher eukaryote cell types examined by cryo-EM reportedly contain no regular 30-nm chromatin fiber (e.g., refs. 16, 34 and 35). Using a combination of a chromosome conformation capture (3C) technique and polymer modeling, Dekker also found that the chromatin in a transcriptionally active domain in yeast did not form a compact 30-nm fiber, but instead was extended with a rather loose arrangement of nucleosomes.[36] More recently, the Bazett-Jones group made a similar observation by electron-spectroscopic imaging (ESI), providing phosphorus and nitrogen mapping with sufficient contrast and resolution to visualize 10-nm nucleosome fibers.[37,38] Although the cryo-EM and ESI, which are both EM-based methods, examine only a limited portion of nuclei, our SAXS study corroborates the notion that 30-nm chromatin fibers are absent in interphase chromatin.

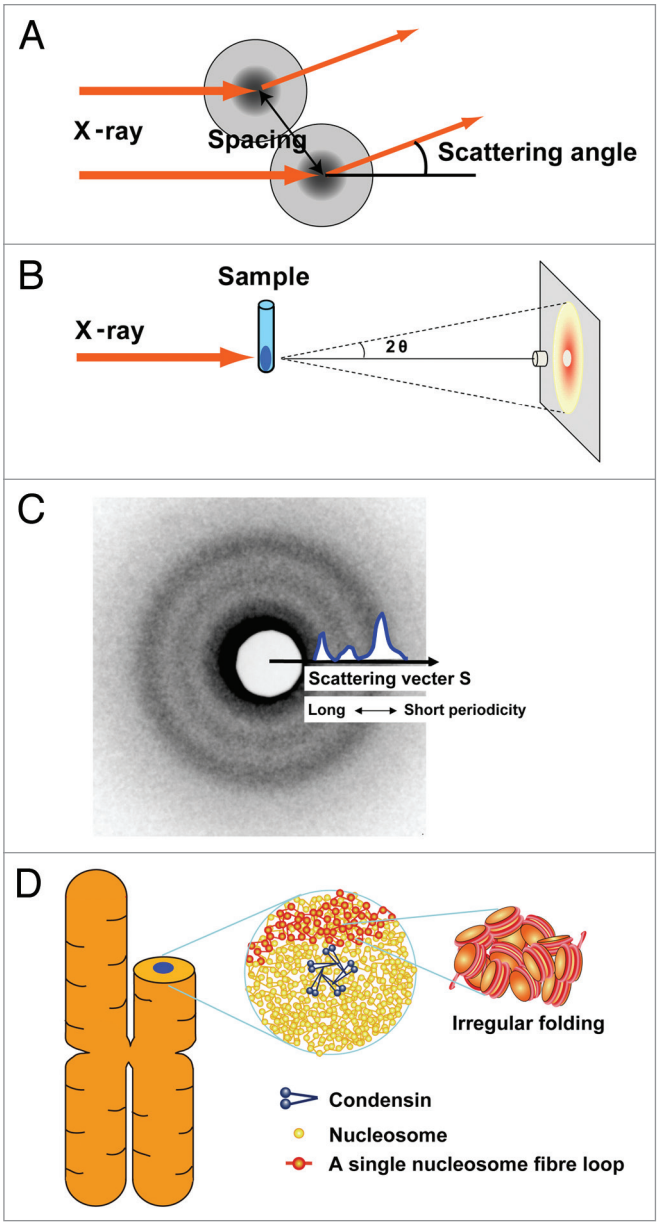

Figure 2. (A) When non-crystal materials are irradiated with X-rays, small-angle scattering generally reflects the size and spacing of internal structures. (B) Experimental setting: a chromosome pellet in a quartz capillary tube was exposed to a synchrotron X-ray beam and the scattering patterns were recorded with a CCD camera or imaging plate. The details of the setting are described in Nishino et al.[15] (C) A typical scattering pattern is composed of concentric rings. The signals at smaller scattering angles [smaller size of the scattering vector (*S*) closer to the center] reflect larger periodic structures and vice versa. In **Figure 3**, the singles on the concentric rings are averaged and shown in a one-dimensional plot. The size of the scattering vector is defined by $S = 2\sin(\theta)/\lambda$, where λ is the wavelength and 2θ is the scattering angle. A periodic length is given by the inverse of *S*. Thus, "30-nm peak" refers to a scattering peak at $S = 0.033$ nm^{-1}. (D) Chromosomes consist of irregularly folded nucleosome (beads on a string) fibers. Condensins (blue) hold the nucleosome fibers (red) globally around the chromosome center. Locally, the nucleosome fiber is folded in an irregular or disordered manner, forming loop structures that are collapsed toward the chromosome center (blue). The collapsed fiber (red) forms a domain that could be compatible with the large module observed by the Belmont group.[8]

Furthermore, using the USAXS apparatus, we observed no periodic structures in the interphase chromatins in the range

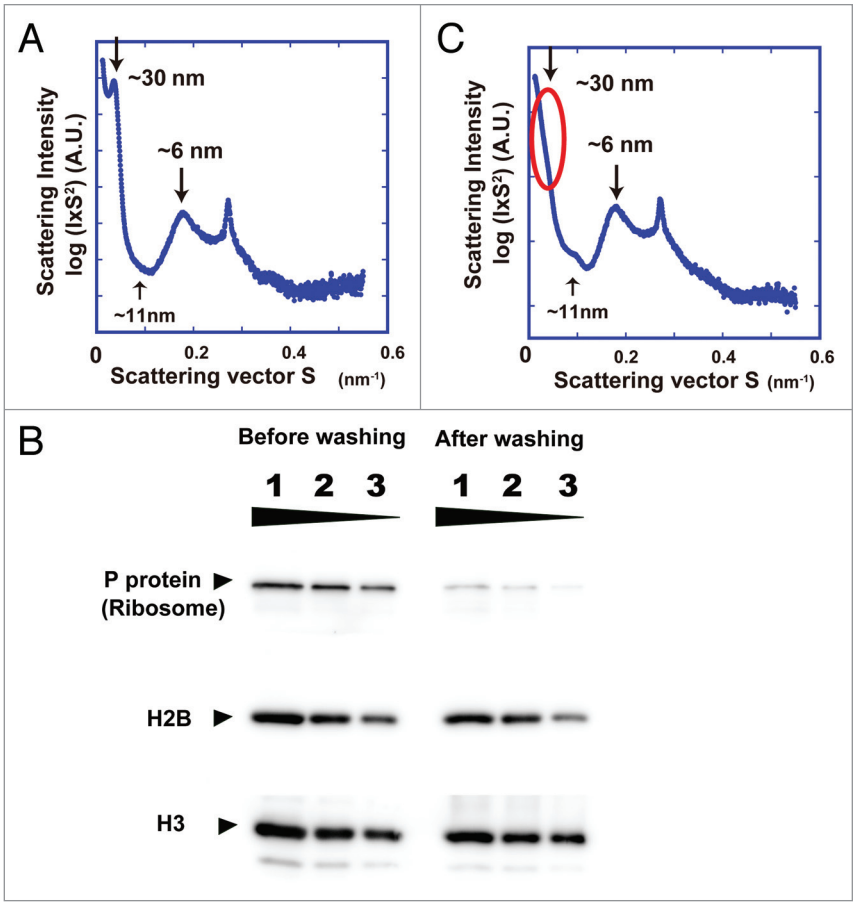

Figure 3. (A) SAXS profile of HeLa interphase nuclei, which were isolated using the Langmore and Paulson procedure.[18,19] Three peaks at ~6, ~11 (faint) and ~30 nm were detected (arrows). In the plot of log(I x S^2) vs. S, I is the measured average intensity and S is the size of the scattering vector, the inverse of the structure size or spacing (for details, see ref. 15). The 6- and 11-nm peaks are thought to come from edge-to-edge and face-to-face positioning of the nucleosomes, respectively. The 30-nm peak was assumed to represent the side-by-side packing of 30-nm chromatin fibers. (B) The presence and removal of ribosomes in the HeLa interphase nuclei were verified by western blotting. Nuclear lysates of ~2 x 10^4, 1 x 10^4, and 5 x 10^3 cells were loaded into lanes 1–3, respectively. Proteins were separated by SDS-PAGE and then transferred to membranes. Ribosomal P-protein[64] and histone H2B (control) and histone H3 (control) were detected using specific antibodies (H2B, Upstate 07–371; H3 Abcom ab1791). P-protein was detected in the original nuclei, but much less in the nuclei after washing, whereas histones H2B and H3 were similarly observed in both nuclei. (C) Only the 30-nm peak disappeared after removal of ribosome aggregates, whereas the other peaks remained.

between ~50 and ~1,000 nm (**Fig. 4A**). Taken together with the SAXS data, we suggest that the bulk interphase chromatin also consists of irregularly folded nucleosome fibers (**Fig. 4D**).

Similarity of Organization between Interphase and Mitotic Chromatins

Notably, a comparison of the USAXS scattering profiles of mitotic and interphase chromatins shows similarities in a range up to ~275 nm, suggesting a similar organization in this range (**Fig. 4A and B**). Beyond this range, the slope in interphase chromatin decreases in magnitude (**Fig. 4A**), and the curve becomes flat.

We found that both scattering patterns follow the power-law relationship between scattering intensity and scattering vector S over a wide range (**Fig. 4C**; ref. 15). This scattering property suggests a fractal nature,[39] although we cannot rule out the possibility that other forms of organization might lead to a similar scattering pattern. The power-law relationship continues up to ~1,000 nm in mitotic chromosomes and ~275 nm in interphase chromatin (Fig. 4C and E in ref. 15). This suggests that mitotic and interphase chromatin have common structural features that are similar at many magnifications, at least in a range up to ~275 nm. Consistent with this finding, evidence for a fractal structure of human interphase chromatin was recently obtained[40-43] (also see ref. 44).

The scattering similarity is also in good agreement with our notion that interphase and mitotic chromatin are locally indistinguishable[17](also see refs. 45–46). Even in the interphase nuclei, numerous chromatin domains like "chromatin liquid drops" are already formed (**Fig. 4D**). For chromosome assembly, such chromatin domains are folded together, presumably by condensins and/or other protein factors, to create a rod-like chromosomal shape.[17] The chromatin domains in interphase were originally identified as replication foci containing ~1 Mbp of the genome region by using pulse labeling.[47-49] The domains have been further analyzed by super-resolution microscopy[50] and Hi-C assay[51] (a method to study the three-dimensional architecture of genomes). Recently it was reported that the domains are correlated with fractal globule structures.[52] The fractal globule, which was identified by Lieberman-Aiden et al. using Hi-C assay,[41] is an interesting structure, in which chromatin fibers are little entangled.[41,42]

Consistently, typical heterochromatin regions in plant or mammalian nuclei that have been visualized by cryo-EM have been reported to look very similar to mitotic chromosomes.[34,35] Taken together, interphase chromatin and mitotic chromosomes have similar local organizations (e.g., chromatin domains, chromatin liquid drops, or fractal globules), showing compact irregular folding of nucleosome fibers without a 30-nm chromatin fiber (**Figs. 2D and 4D**), although with different morphology at the micrometre scale.

30-nm Chromatin Fibers In Vitro and in Some Specific Cells

Although we suggested that 30-nm chromatin fiber is nearly absent in mitotic and interphase cells, there might be short stretches of 30-nm fibers or small amounts

of other regularly folded hierarchies in the cells.[15,53] In additon, clear "30-nm chromatin fibers" have been observed under the microscope (e.g., refs. 24 and 54–59). Why? A possible explanation is as follows: The formation of a 30-nm fiber requires the selective binding of nucleosomes, which are close neighbors on the DNA strand (intra-fiber nucleosomal association). A simple way to construct an intra-fiber nucleosomal association is "isolation of nucleosome fibers." Such isolation could occur under dilute conditions, as in in vitro systems, in which interactions between nucleosome fibers are negligible. In particular, under low-salt conditions with 1–2 mM $MgCl_2$, nucleosome fibers can gently repulse each other and easily form a 30-nm chromatin fiber (e.g., refs. 55 and 58). In conventional EM observations, such formation might be further stabilized through chemical cross-linking (e.g., glutaraldehyde fixation) and shrinkage resulting from alcohol dehydration during sample preparation.[60] Isolation of nucleosome fibers might also occur when nucleosome fibers are looped out from the chromatin domain or chromosome territory for transcription (**Fig. 4D**, top).

In addition, specific cell types have nuclei containing apparent 30-nm chromatin fibers, including starfish sperm,[24,61] chicken erythrocytes[23,24,62] and mouse photoreceptor cells.[63] Although the formation of a 30-nm fiber requires intra-fiber nucleosomal association, inter-fiber nucleosome associations are considered dominant in cells (**Figs. 2D and 4D**).[11,60] Nucleosome fibers are highly interdigitated, such that they are prevented from forming 30-nm chromatin fibers, leading to irregular folding of the nucleosome fibers (polymer-melt-like structures) (**Figs. 2D and 4D**).[11,15,60] In some specific cells that contain the regular 30-nm chromatin fibers, a unique mechanism may be present to increase intra-fiber nucleosome association, presumably via specific histone modifications or the binding of specific proteins.

In conclusion, interphase chromatin and mitotic chromosomes have similar local organizations (e.g., chromatin domains, chromatin liquid drops, or fractal globules). The organizations show compact irregular folding of nucleosome fibers without a 30-nm chromatin fiber. Although the term "irregular" or "disordered" might provide an impression that the organizations are likely functionally irrelevant, the irregular folding implies less physical constraint and high dynamism, leading to a high degree of DNA accessibility.[15] The irregular organization may thus have several advantages in template-directed biological processes in interphase nuclei, including RNA transcription and DNA replication, repair and recombination.[15]

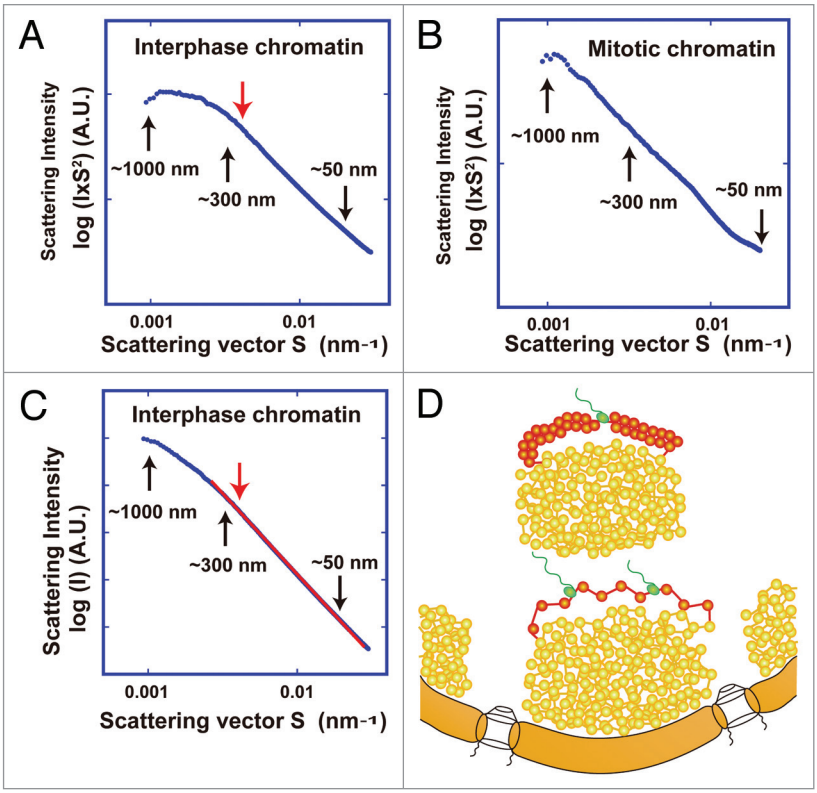

Figure 4. (A) By ultrasmall-angle X-ray scattering (USAXS), no notable structures ~100–150 or 200–250 nm were detected in HeLa interphase nuclei. Beyond the ~275 nm range (red arrow), the slope in interphase chromatin decreased in magnitude. (B) For comparison, a USAXS profile of mitotic chromosomes is reproduced from Nishino et al.[15] (C) The scattering intensity obeys the power law with respect to structure size or spacing. A plot of log (*I*) vs. log (*S*) on a straight line (red line) covers a wide range, extending over nearly three orders of magnitude. Least-squares fitting shows that *I* is proportional to *S* to the power of –3.36 (R = 0.999),[39] suggesting that chromosomes do not possess notable regular structures over a very wide scale and exhibit a fractal nature of genome organization (see also ref. 15). (D) In the interphase nucleus, there are numerous compact chromatin domains like "chromatin liquid drops" (yellow balls).[60] Red, transcribed nucleosome; Green, RNA polymerase and RNA. Formation of a 30-nm chromatin fiber might occur when nucleosome fibers are looped out from the chromatin domain or chromosome territory for transcription (top, see text). In our opinion, the transcriptional silencing can be established through dynamic capturing of transcriptional regions inside the compact chromatin domains. These domains can be considered as drops of viscous liquid, which could be formed by nucleosome-nucleosome interaction and a macromolecular crowding effect.[60] Notably, this view is in line with predictions of the chromosome territory-interchromatin compartment (CT-IC) model[65,66] and previous evidence for an interchromatin compartment as well as the perichromatin region (see ref. 35).

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