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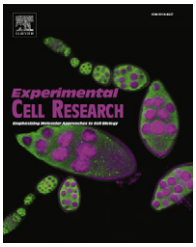

Review Article

Chromatin organization — The 30 nm fiber

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ABSTRACT

Despite over 30 years of work, the fundamental structure of eukaryotic chromatin remains controversial. Here, we review the roots of this controversy in disparities between results derived from studies of chromatin in nuclei, chromatin isolated from nuclei, and chromatin reconstituted from defined components. Thanks to recent advances in imaging, modeling, and other approaches, it is now possible to recognize some unifying principles driving chromatin architecture at the level of the ubiquitous '30 nm' chromatin fiber. These suggest that fiber architecture involves both zigzag and bent linker motifs, and that such heteromorphic structures facilitate the observed high packing ratios. Interactions between neighboring fibers in highly compact chromatin lead to extensive interdigitation of nucleosomes and the inability to resolve individual fibers in compact chromatin *in situ*.

Contents

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Introduction

Historically, determining the structure of an individual cell component and relating it to its function(s) has followed a well established path: the component is often first recognized in light or electron micrographs, then isolated for biochemical and further structural work, perhaps culminating in x-ray diffraction studies. Microtubules and actin filaments are examples of this route that come to mind — for others, somewhat different routes have been taken, but we now have a great deal of structural information about most cell components, providing a solid basis for further experimental work. Surprisingly, despite its central role in the cell, and the availability of x-ray data on nucleosomes, the structure of chromatin as it exists in living cells remains controversial. Here, we discuss the problems that have impeded progress, and consider strategies for moving forward, with emphasis on the 30 nm chromatin fiber.

Examination of the chromatin literature reveals a basic problem: when isolated from the nucleus, chromatin tends to adopt a fiber-like conformation. However, when nuclei are observed *in situ* using transmission electron microscopy (TEM) of sections, chromatin most commonly appears as rather uniform darkly staining masses with no obvious fiber-like properties. There are some exceptions to this rule which are informative in understanding the basis of the apparent discrepancy between the *in vitro* versus *in situ* observations. The frustrations of researchers in trying to come to grips with the problems of elucidating chromatin structures have been evident in such titles as 'Chromatin Higher Order Structure: Chasing a Mirage' [1] and 'Chromatin Structure: does the 30 nm fibre exist *in vivo*?' [2]. Indeed, at times, the situation has been reminiscent of the tale of the blind men assigned to describe an elephant, resulting in wildly differing accounts based on each's interpretation of a different portion of its anatomy. Here, we attempt to summarize the present state of the field, first examining chromatin as seen in nuclei and the heroic attempts to create preparations that are minimally disruptive and maximally informative, and then summarizing the much more extensive work on isolated chromatin. Finally, we consider hypotheses that may reconcile the apparent discrepancies that emerge from these two approaches.

The terminology used in describing chromatin structures has evolved over the years, and can be confusing. For example, the term 'native chromatin' was originally used as a descriptor for the unperturbed *in vivo* state, contrasting it with chromatin exposed to conditions that could lead to structural changes. More recently, the term 'native' has been applied to chromatin observed in or isolated from organisms, contrasting it with chromatin reconstituted from purified components *in vitro*. Here, we use the earlier definition. The term '10-nm chromatin fiber' has also been used to describe different states of chromatin. In some cases, the term has been applied broadly to any unfolded chromatin in the 'beads-on-a-string' conformation. The other, more restricted use, refers to a stack of nucleosomes forming a fiber ~10 nm in diameter as might be expected, for example, to result from a partially uncoiled solenoidal structure. For an open, uncondensed array of nucleosomes, the mean diameter is clearly considerably less than 10 nm, and in this sense, the term best avoided, or at least carefully defined. Finally, as noted previously [3], the term 'order' in 'higher order structure' has the same sense as in 'order of magnitude'. Nevertheless, perhaps because of the expectation that arrays of more-or-less identical units would fold into an 'ordered' array, the phrase has often been used in this more restrictive sense.

Ultrastructure of chromatin *in situ*

From the earliest work applying transmission EM to thin sections of cells, it was apparent that the techniques that were so successful with membranous and cytoskeletal components provided little information concerning the structural organization of chromatin and other nuclear components. Moreover, little improvement was seen as advances in specimen preparation were applied. Nevertheless, there was an expectation that chromatin adopted a fiber-like conformation which would be apparent if a suitably gentle technique for preparing thin sections could be developed. As early as 1963, J. G. Gall obtained compelling images of chromatin fibers obtained by spreading nucleated amphibian erythrocytes on a water surface [4]; this was followed by a more comprehensive exploration of techniques and a wider range of chromatin sources that resulted in ~30 nm diameter fibers [5]. One interpretation of these studies was that chromatin that appeared as a more-or-less undifferentiated dense mass in thin section TEM became decondensed when exposed to low ionic strength conditions, revealing the underlying ~30 nm fiber organization. This suggested that the original dense chromatin *in vivo* contained fibers that were so close packed that they could not be discriminated. This property underscores a major difference between chromatin and other fiber-like cellular components such as microtubules which retain their individuality in thin section TEM in the close-packed state.

A nagging possibility for the apparent absence of a fiber organization in TEM sections of native nuclei has been that chromatin is more liable to disruption by the procedures needed to prepare thin sections than other cellular components. Therefore, as each advance in TEM specimen preparation has been developed (cryofixation, freeze substitution, low temperature embedding, freeze-etch etc.), it has been applied to nuclei [6–8]. More advanced processing of electron micrographs, including tomographic 3D reconstruction has also been used in attempts to distinguish substructure in chromatin masses, in some cases revealing very short fiber-like regions with a variety of diameters [7]. Cryo-TEM of thin sections obtained from vitrified cells observed in the frozen hydrated state, perhaps the present 'gold standard' for retaining structural integrity [9] has also been applied to cultured cells in interphase and metaphase [10,11]. None of these techniques has enabled large scale visualization of ~30 nm chromatin fibers *in situ*. There are also concerns regarding potential limitations of cryo-techniques. Does the local heating resulting from the passage of the microtome knife through the vitrified sample perturb the native structure? Is the rate of cooling sufficient to arrest the extremely rapid conformational fluctuations that occur over microsecond timescales [12]? Alternate hypotheses for the non-appearance of chromatin fibers based on the premise that individual chromatin fibers do not exist in native chromatin *in situ* have also been put forward. These are based on the property of chains of nucleosomes to interdigitate via face-to-face interactions between non-adjacent nucleosomes [13] or form a 'molten globule' [11] when packed closely at the very high concentrations that exist *in vivo*. In a very recent study by Nishino and colleagues using both small angle x-ray scattering (SAXS) and cryo-sections [14] of isolated metaphase chromosomes,

no evidence for 30 nm-like higher-order structures was found. However, the SAXS signal did indicate structure at the level of the nucleosome diameter (11 nm) and strong face-to-face interactions between the nucleosome discs (6 nm) providing key evidence supporting a non-fiber arrangement of nucleosome chains, perhaps mediated by interface interactions between distal nucleosomes.

Contrasting with these results are observations on a special class of nuclei where fibers can be observed *in situ*. These include nucleated erythrocytes and echinoderm sperm and share the following properties: transcription largely or completely absent; presence of specialized, more highly charged H1-type histones; a longer nucleosome repeat length; and a low proportion of non-histone chromatin proteins. These nuclei also give x-ray scattering profiles indicative of a more prominent internal structure than active nuclei [15]. When observed with thin section TEM either in the native state, or after a very mild decondensation, these nuclei are seen to contain clear ~30 nm diameter chromatin fibers, just as would be predicted based on the appearance of fibers in decondensed and spread nuclei [4,5]. Fibers are evident in these specialized nuclei both in frozen hydrated [16], and in more conventional thin section TEM where tomographic reconstruction revealed irregular zigzags of nucleosomes and linker DNA [17]. Here, it is important to emphasize that even in these specialized nuclei, the appearance of fibers may require mild decondensation. For example, a recent cryo-EM tomographic study of chicken erythrocyte chromatin used isolated nuclei exposed briefly to low salt [18]. Nevertheless, the ability to determine the 3D locations of individual nucleosomes in fibers provides valuable insight, allowing comparisons with results using fibers isolated from nuclei or created *in vitro* by reconstitution. The aforementioned EM cryo-tomography study was able to recognize structural motifs within 30 nm fibers from avian erythrocytes [18] that mirrored the zigzag organization seen in isolated fibers. Vertebrate retinal rod photoreceptor cells provide another special case. These nuclei have a central mass of compact heterochromatin surrounded by layers of less compact chromatin. When samples of retina were vitrified after dextran cryoprotection, freeze-substituted, embedded in epoxy at 60 C, and sections subjected to tomographic analysis, the 3D reconstructions revealed concentric layers of chromatin of varying compaction ranging from a central mass in which no fibers were apparent, to less compact regions with ~30 nm fibers. In the outer, most decondensed layers, beads-on-a-string nucleosomal arrays could be identified [19].

There are two common structural features derived from tomographic studies where the 3D locations of nucleosomes have been determined. Firstly, nucleosomes tend to occupy the fiber periphery, with linker DNA more centrally located, favoring a zigzag arrangement, and secondly, the fibers are irregular, and lack a distinct symmetry.

30 nm fiber structure based on studies of chromatin isolated from nuclei

To isolate chromatin for *in vitro* studies, controlled nuclease fragmentation followed by elution from the nuclei with low-salt or physiological solutions is typically employed. The fragments containing from one to several hundred nucleosomes may then be probed by biochemical, biophysical, or imaging techniques.

Due to the size and complexity of soluble chromatin, electron microscopy (EM) has been most effective in analysis of salt-dependent folding of chromatin fibers in solution. A classic example is the study of Thoma et al. [20] showing a progressive salt-dependent folding of chromatin isolated from rat liver nuclei. At low salt concentration, the nucleosome arrays fold into fibers with ca. 30 nm diameter and distinct zigzag path of the nucleosome linkers in the middle of the fiber, leaving the nucleosome cores at the fiber periphery. Upon increasing the ionic strength to about 100 mM, the chromatin fibers contract without much change in diameter suggesting a longitudinal folding along the main axis of the fiber [20]. Later imaging of isolated chromatin fibers with nucleosomal resolution in the partially condensed form using transmission EM [21], atomic force microscopy [22] and cryo-EM [23] revealed an intrinsic heterogeneity of nucleosome packing within the 30 nm fiber attributed to the variability in nucleosome orientation resulting from the helical nature and length variability of linker DNA [21]. This structural heterogeneity persists even when chromatin fibers are strongly compacted by divalent cations as observed by cryo-EM tomography [24]. Electron microscopy also revealed the formation of "stem" motifs by closely juxtaposed linker DNA [23,25], suggesting that the formation of the linker DNA "stem" coupled with the neutralization of the negative charge of linker DNA by linker (H1 type) histone promote the longitudinal compaction of the 30 nm fiber.

Because the 30 nm diameter appeared to be relatively independent of the salt conditions and nucleosome packing heterogeneity *in vitro* and was close to the fiber diameter measured *in situ* (see earlier discussion), the term "30 nm fiber" is now generally applied to describe the almost universal higher-order structure of compact chromatin fibers in solution. However, within the most compact fibers, individual nucleosomes often cannot be revealed by EM and there is still debate whether the nucleosome linkers become bent or remain straight during the transition from the open zigzag to the folded form and whether the conformational heterogeneity seen in the open zigzag is maintained in the compact fiber.

Scanning transmission electron microscopy (STEM) as well as neutron scattering allows one to determine the mass of the imaged chromatin array and thus calculate mass per unit length. For chromatin fibers containing repeated nucleosomes of known molecular mass, this parameter is usually represented as number of nucleosomes per 11 nm of fiber (diameter of a single nucleosome). At physiological concentrations of monovalent cations, the mass per unit length may reach 8 nucleosomes/11 nm [26,27]. These STEM studies also confirmed a strong dependence of mass per unit length upon the solution ionic strength, again without much change in diameter, consistent with longitudinal folding of the 30 nm fiber. The diameter and the mass per unit length determined in these early studies remain among the most consistent characteristics of native chromatin measured *in vitro*.

Studies of the 30 nm fiber structure using reconstituted nucleosome arrays

Biochemically defined nucleosome arrays may be assembled from isolated or recombinant histones and DNA (native or recombinant) by dialysis from high to low salt concentration [28]. A significant breakthrough in understanding the internal organization of the chromatin fiber was facilitated by construction of

polynucleosome templates for precise positioning of histone octamers — initially from repeats of 5S ribosomal DNA [29] and later from clone 601 DNA selected from random synthetic DNA sequences [30]. Construction of defined polynucleosome arrays greatly improved the precision and reproducibility of *in vitro* experiments, allowing dissection of the contributions of particular chromatin structural elements such as linker histones, core histone N-terminal domains, posttranslational histone modifications, and histone variants that modify the nucleosome surface, to 30 nm fiber structure (see [31]).

Because of the high uniformity and reproducibility of reconstituted nucleosome arrays, sedimentation velocity analysis is especially powerful in identifying small structural changes dependent on histone composition, DNA length, and solution components. Earlier sedimentation studies (reviewed in [32]) provided reference data for subsequent work on chromatin fiber in solution. Most of the sedimentation studies employed 12-mer nucleosome repeats with 200–208 bp nucleosome repeat lengths. The correspondence between S values, ionic conditions, and chromatin compaction levels as seen by EM under the same conditions (see later) is schematized in Fig. 1.

Since each nucleosome is positioned, it is possible to construct arrays of precise length and calculate their compaction very accurately. EM images of 12-mer nucleosome arrays based on clone 601 are very consistent with the sedimentation values for 12-mer nucleosomes in the open, partially folded, and completely folded states [33,34] thus providing a reliable link between sedimentation analysis (Fig. 1), EM imaging, and computational modeling (Fig. 2).

Since compact 12-mers are too short to measure fiber diameters accurately, a series of polynucleosome DNA templates containing several dozen clone 601 repeats with different nucleosome repeat lengths was constructed by D. Rhodes and colleagues providing fibers where diameters could be measured accurately and mass/unit length calculated. Measurements of chromatin fibers reconstituted with various nucleosome repeat lengths (177–237 bp) and completely folded by linker histone and divalent ions suggested a stepwise increase in chromatin diameter from ~33 nm for chromatin with 177–207 bp repeat to ~44 nm for 217–237 bp repeat. This nucleosome repeat length-dependent increase in fiber diameter was associated with a jump in nucleosome packing from 10–11 to 14–17 nucleosomes per 11 nm [35]. It thus appears that the packing density promoted by linker histone and divalent ions exceeds the compaction seen with native chromatin *in vitro* [24,36] and *in situ* [18].

Models and experimental studies of intrinsic 30 nm fiber architecture

Despite more than 30 years of research, the exact configuration of nucleosome linker DNA and how nucleosomes interact with each other inside the 30 nm chromatin fiber are not completely understood [37]. Based on studies of native chromatin fibers, Finch and Klug proposed the first post-nucleosomal model of the chromatin fiber — a one-start solenoid-type helix with approximately 6 nucleosomes per turn (~11 nm) and bent DNA linkers that continued the helical trajectory established in the nucleosome core and with each nucleosome making close contact with its immediate neighbor in the array [38].

A few years later, Worcel et al. [39] and Woodcock et al. [27] proposed alternative zigzag structures consisting of a two-start helical ribbon with straight DNA linkers. Several other topologies besides the basic zigzag and solenoid models have been proposed: for example the interdigitated solenoid [40] where planes of nucleosomes coming from the adjacent turns of the solenoid crisscross one another.

The most likely reason for the long-standing inability to solve the 30 nm fiber structure was the high degree of intrinsic irregularity of the native chromatin due to linker length variations, various histone modifications, histone variants, and variable linker histone level. Therefore, more recent experiments addressing the internal structure of the 30 nm fiber have combined highly regular repeats with strong nucleosome positioning sequences such as Widom's clone 601-based arrays, with highly uniform recombinant histones.

Based on their observations of the role of histone H4 N-tail in compacting the 30 nm fiber [41], Richmond and colleagues designed an elegant experiment where core histones H4 and H2A were modified to facilitate bifunctional chemical crosslinking between the histone H4 N-tail and the H2A/H2B acidic patch that interact at the interface of crystallized nucleosomes [42]. Crosslinking was initiated under conditions that induce the most compact state of the 30 nm with high Mg^{2+} concentrations, after which the fibers were unfolded by exposure to low salt and imaged by transmission EM. The results provided compelling evidence for a zigzag structure with two-start nucleosome arrangement [43].

Zigzag folding was also evident in the subsequent crystallographic study of a tetranucleosome [44] with a short, 167 bp, repeat and without linker histones. X-ray analysis showed that under the high Mg^{2+} crystallization conditions, nucleosome core

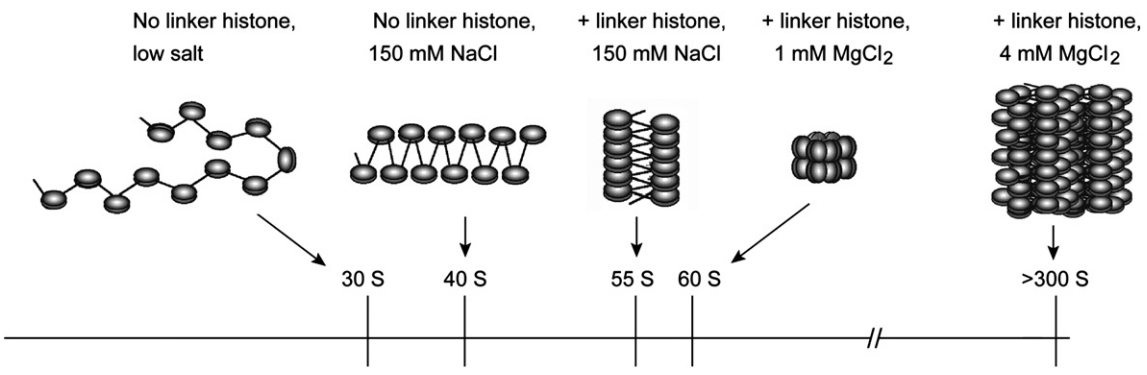

Fig. 1 – Scheme showing correspondence between proposed *in vitro* compaction states and sedimentation coefficients of 207×12 nucleosomal arrays.

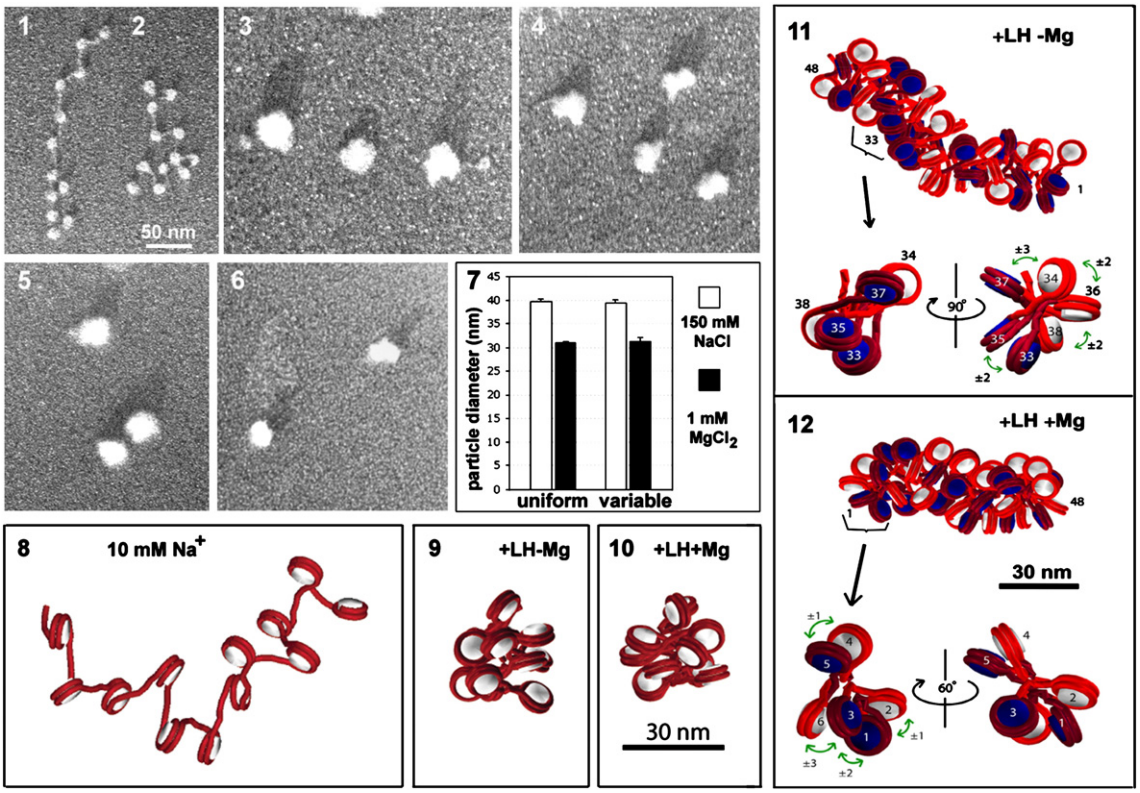

Fig. 2 – Electron microscopy and modeling of unfolded nucleosome arrays and compact chromatin fibers.1,2: Electron micrographs (platinum shadowing) of regular 207×12 core arrays (no linker histone) in 5 mM NaCl (1,2). 3–6: linker histone-containing regular 207×12 arrays condensed in 150 mM NaCl (3) and 1 mM $MgCl_2$ (4) and variable (207±2)×12 arrays condensed in 150 mM NaCl (5) or 1 mM $MgCl_2$ (6). 7: Average diameter of uniform and variable linker histone-containing arrays condensed in 150 mM NaCl (open columns) and 1 mM $MgCl_2$ (black columns). Error bars represent standard errors of mean.8–10: Models based on Monte Carlo (MC) simulation of 12×207 oligonucleosomes at 0.01 M monovalent salt without linker histone (8), and 0.15 M monovalent salt with linker histone (9), and with linker histone and Mg^{2+} (10) highlight compaction effects of linker histones and Mg^{2+}.11, 12: Space-filling models based on MC simulation of 48-unit oligonucleosome chains compacted at 0.15 M monovalent salt with linker histone (11), and with linker histone and Mg^{2+} (12). 11: compaction at 150 mM NaCl leads to a two-start zigzag chromatin fiber with predominantly i±2 interactions between the most proximal nucleosomes 33 and 35, 34 and 36, 36 and 38) shown by green arrows. 12: in the presence of 1 mM $MgCl_2$ several nucleosomes have bent linkers resulting in i±1 interactions (nucleosomes 1 and 2, 4 and 5), interspersed with i±2 (1 and 3) and i±3 (6 and 3) interactions. Odd-numbered nucleosomes are indicated by burgundy for the DNA and blue for the cores. Even-numbered nucleosomes are indicated by red for the DNA and white for the cores. Modified from Ref. [33].

arrays can spontaneously fold into a regular structure [44] consistent with earlier two-start zigzag models [15,27]. Because of the relatively low resolution achieved with tetranucleosome crystals [44], the forces and interactions stabilizing the compact structure were not revealed.

As discussed earlier, using DNA templates containing several dozen clone 601 repeats with different nucleosome repeat lengths, D. Rhodes and colleagues were able to demonstrate a very tight compaction of reconstituted chromatin fibers especially for repeats above 207 bp [35]. In terms of internucleosomal interactions, these results were interpreted as evidence for the interdigitated solenoid model [40], as the observed high nucleosome packing ratio was not compatible with other zigzag or solenoidal models [35,45]. However, these compact structures are also consistent with the polymorphic multi-start chromatin fiber model with extended nucleosome linkers [46] showing that bent linker DNA conformation does not uniquely fit into the compact 30 nm fiber.

For very short nucleosome repeat lengths typical of yeast and neuronal cells (~167 bp) a distinctly smaller fiber diameter of about 21 nm with apparent 2-start organization was observed by EM [47], consistent with the nucleosome stacking observed in tetranucleosome crystals [44] and sedimentation [34]. Thus the 167 bp repeat fiber is the first chromatin higher-order structure for which there is a good agreement between biophysical characteristics, EM imaging, and X-ray crystallography.

The internal organization of the 30 nm fiber can also be probed by single-molecule force spectroscopy (SMFS) in which reconstituted or native chromatin fibers are conjugated with synthetic beads that may then be captured by optical or magnetic traps. Applying a pulling force on these beads and analysis of the resulting fiber extension provides a way to examine the internal forces between nucleosomes and relate them to different chromatin models. Results of earlier SMFS experiments conducted at physiological concentration of monovalent cations were consistent with

a zigzag fiber architecture [48]. In contrast, a more recent SMFS study of nucleosome arrays folded by divalent cations suggested a fundamental one-start solenoid organization for the chromatin fiber, and a stabilizing rather than essential contribution of the linker histone to fiber compaction [49]. This interpretation argues for the classic rather than the interdigitated solenoid and thus does not explain the higher packing ratio achieved in the presence of divalent cations by free 30 nm fibers [35,45]. Alternative interpretations include the likelihood that the fiber structure was heterogeneous due to the partial unfolding of the outer DNA segments in the nucleosome core [50], or that under tension, nucleosome rearrangement to form irregular "superbeads" was occurring [51].

Another approach that gives a more holistic view of chromatin organization is provided by contemporary *in silico* modeling that aims to recreate the 30 nm fiber starting with arrays of nucleosome cores on DNA and incorporating known chemical and physical interactions, factors and associated charges (counterions, linker histone, histone N-tails, and their modifications). The nucleosome models are then subjected to multiple simulated random conformational variations by the Monte Carlo method to determine the most energetically favorable conformations (for reviews see [31,52]. While precise atomic modeling is not yet feasible because many important interactions inside the chromatin fibers are not fully understood, the computations typically result in the prediction of a limited number of energetically favorable structures that can be tested empirically. In one recent example, a "mesoscopic" computational model of the 30 nm fiber was derived and coupled with an EM-assisted nucleosome interaction capture (EMANIC) technique. Both approaches revealed an internal structural heterogeneity of 30 nm fibers compacted with linker histone and Mg^{2+} [34]. Although the fibers showed a predominantly two-start organization, the structures were interspersed with partially bent linker DNA where interactions between consecutive nucleosomes typical of one-start solenoids occurred. These experimental data were in excellent agreement with results of Monte Carlo simulations (Fig. 2) which revealed that linker DNA crossing in the center of the fiber hinders longitudinal zigzag compaction. Remarkably, the spontaneous bending of only one linker per 5–6 nucleosomes was sufficient to achieve an energetically favorable longitudinal folding in the presence of divalent cations.

The combined results of EMANIC and computational modeling reconcile a two-start zigzag topology with the type of linker DNA bending that defines solenoid models and suggests a single heteromorphic chromatin fiber structure. The heteromorphic model (Fig. 2) is fully consistent with the internal heterogeneity of native chromatin fibers observed by EM and Cryo-EM [21,24] as well as with the fiber diameter and mass-per-unit length of chromatin isolated from nuclei [36].The ability of the chromatin fiber to reach full compaction despite internal heterogeneity apparently reflects the unique property of the 30 nm fiber to incorporate multiple conformations resulting from dynamic interactions of the sort that are likely to occur *in vivo*.

Toward a unifying concept of chromatin organization

Despite the disparities discussed above both between and within *in situ* and *in vitro* approaches, we now seem to be converging on a few unifying themes and it is possible to propose a hypothesis based on two properties of arrays of nucleosomes. First, under appropriate 'physiological' conditions, arrays of nucleosomes in solution adopt a 30 nm fiber conformation which is broadly based on a zigzag arrangement of nucleosomes and linker DNA. These fibers are not highly ordered, but even when constructed from identical components, show many irregularities that are likely related to the energetic costs of retaining a strict zigzag during compaction. In chromatin obtained from nuclei, additional irregularities arise from variations in linker DNA length, occupancy of linker histone, post-synthetic modifications of core and linker histones, and the presence of histone variants. As chromatin concentration increases, both *in vitro* and *in vivo*, the forces maintaining intra-fiber integrity compete with inter-fiber nucleosome-nucleosome interactions, allowing their interdigitation. At this point, EM images fail to convey the underlying fiber organization, rather, suggesting a 'molten globule' sea of close-packed nucleosomes. However, if it were possible to follow a chain of nucleosomes in this 'sea', an irregular pleated fiber-like trajectory would be traced out. A strong signal corresponding to face-to-face nucleosome interactions (6 nm) detected by small-angle X-ray scattering in condensed metaphase chromosomes [14] suggests that this type of interaction may constrain the pleated fibers in a manner similar to the hydrogen bonds that stabilize folded β-strands in a protein. Thus, the conformation might be considered a 'constrained pleated fiber' resembling protein β-sheets rather than helices. Conversely, as compaction-inducing conditions are relaxed, intra-fiber interactions begin to dominate, and fibers become observable by EM as separate entities with the helical structural features discussed earlier.

Future prospects

Technical advances in the past decade have been instrumental in providing critical new information, and it is likely that this trend will continue. As computing power grows, *in silico* chromatin modeling is likely to incorporate more factors, and perhaps advance from the mesoscale level of 'resolution'. Similarly, the increasing power and availability of EMs designed specifically for cryo-tomography of low contrast specimens is likely to propel that aspect of chromatin structure examination.

One of the limitations of most current EM imaging is its inability to discriminate based on composition. However, that limitation may be ending. A recent study of chromatin organization using EM sections was based on the incorporation of large segments of foreign DNA into cultured cells coupled with antibodies specific to the foreign chromatin. Immunogold labeling revealed that the foreign chromatin adopted a distinct fiber-like conformation which was not visible in unlabeled nuclei [53]. However, the fibers were in the order of 120 nm in diameter, and their relationship with 30 nm fibers remains unclear.

X-ray microscopy and tomography of vitrified cells provides a novel high resolution imaging approach that is becoming applied to cell structures, including nuclei [54]. The high coherence and penetrating power of synchrotron x-rays may provide the resolution and contrast to complement data obtained with TEM.

Perhaps especially informative in the near future will be the application of 'super resolution' light microscopy. The resolution 'gap' between light and electron microscopy has long been

recognized as limiting structural analysis of nuclei and chromosomes, and the ability to bridge it with techniques such as photo-activated localization microscopy (PALM) is already yielding important new information [55]. These authors used sophisticated image processing techniques to examine PALM images of Drosophila embryo chromosomes carrying an EGFP-labeled H2A variant. Filamentous structures ~70 nm in diameter were consistently seen. Again, the relationships between these structures and 30 nm fibers remain to be determined. With predicted resolutions as small as 20 nm, these advanced imaging techniques have the potential to open up the field of nuclear structure, to include multiple levels of organization. We are still largely ignorant of chromatin organization above the 30 nm fiber, and there is little evidence for the strict hierarchy of folding levels once predicted. A full discussion of this topic is beyond the scope of this review, but some aspects are covered in [3,56]. Indeed, the emergence of other techniques, such as those based on 'Chromatin Conformation Capture' that interrogate the 3D structure of the nucleus augurs a bright and exciting future.

Conflict of interest

The authors declare no conflicting interests.

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