Nucleosome spacing periodically modulates nucleosome chain folding and DNA topology in circular nucleosome arrays

The length of linker DNA that separates nucleosomes is highly variable, but its mechanistic role in modulating chromatin structure and functions remains unknown. Here, we established an experimental system using circular arrays of positioned nucleosomes to investigate whether variations in nucleosome linker length could affect nucleosome folding, self-association, and interactions. We conducted EM, DNA topology, native electrophoretic assays, and Mg\(^{2+}\)-dependent self-association assays to study intrinsic folding of linear and circular nucleosome arrays with linker DNA length of 36 bp and 41 bp (3.5 turns and 4 turns of DNA double helix, respectively). These experiments revealed that potential artifacts arising from open DNA ends and full DNA relaxation in the linear arrays do not significantly affect overall chromatin compaction and self-association. We observed that the 0.5 DNA helical turn difference between the two DNA linker lengths significantly affects DNA topology and nucleosome interactions. In particular, the 41-bp linkers promoted interactions between any two nucleosome beads separated by one bead as expected for a zigzag fiber, whereas the 36-bp linkers promoted interactions between two nucleosome beads separated by two other beads and also reduced negative superhelicity. Monte Carlo simulations accurately reproduce periodic modulations of chromatin compaction, DNA topology, and internucleosomal interactions with a 10-bp periodicity. We propose that the nucleosome spacing and associated chromatin structure modulations may play an important role in formation of different chromatin epigenetic states, thus suggesting implications for how chromatin accessibility to DNA-binding factors and the RNA transcription machinery is regulated.

Understanding the mechanisms by which eukaryotic DNA is tightly packed in the interphase chromatin and metaphase chromosomes and at the same time remains accessible to the biological machinery remains one of the most fundamental problems in cell biology. Most textbooks, based on structural studies of biochemically defined linear nucleosome arrays in vitro, describe chromatin folding as helical fibers, either the one-start solenoidal helix with bent DNA or the two-start helical zigzag with relatively straight linker DNA. However, recent evidence from in vivo and in situ studies suggests that regular helical structures are absent from nuclear chromatin and that in most proliferating cells the chromatin is highly irregular in situ without any distinct order above the nucleosome diameter (10 nm) (1, 2) but still showing the two-start zigzag signature nucleosome proximities (3–6). These experimental and imaging studies provide key experimental evidence for earlier modeling suggesting that native nucleosome chains fold into a nonhelical zigzag chain in which linker DNA length and configuration mediate the compaction of the nucleosome chain and proximity of the nearest neighbor nucleosomes (7–9).

Bulk linker DNA length (L\(_L\))\(^{3}\) varies substantially between different organisms and tissues ranging from 10 bp found in fission yeast to ~100 bp in echinoderm sperm (10). Within the same nucleus, the linker DNA length is preferentially quantized with ~10-bp repeat (one DNA double helix turn) suggesting that it reflects steric features of chromatin folding (11). Earlier biochemical experiments showed that the DNA linkers contain noninteger numbers of DNA turns, such as \(L = 5, 15, 25\) bp (12). Here, the type of DNA linkers with integer number of turns is denoted as \([10n]\) and the linker lengths shifted by 5 bp as \([10n+5]\). Subsequent genome-wide mapping of yeast nucleosomes by parallel sequencing has also showed linker DNA distribution with peaks at \([10n+5]\) bp (13). More recent studies using chemical nucleosome mapping method that eliminates MNase digestion artifacts confirmed the preferential \([10n+5]\) linker DNA length in yeast (14, 15). Chemical mapping also revealed the preferential \([10n+5]\) linker DNA length in mouse embryonic stem cells (16). Genome-wide nucleosome posi-
Nucleosome linkers modulate chromatin folding and topology

The highly variable nature of eukaryotic chromatin makes it necessary to create adequate in vitro and in silico models that could account for the complex and dynamic mechanism(s) and factors that mediate chromatin folding in vivo. Biochemically defined nucleosome arrays assembled from histone octamers and synthetic clone 601 nucleosome positioning DNA provide a convenient experimental system for mechanistic studies of nucleosome chain folding in vitro that can be matched by computational modeling of nucleosome arrays with variable nucleosome spacing. Using this system, an increase of linker DNA length from about 20 to 60 bp in ~10-bp increments was shown to considerably alter chromatin fiber folding in vitro (20, 21) consistent with 3D modeling on chromatin fiber folding (22, 23). Sedimentation and imaging analysis of clone 601–based nucleosome arrays with linker DNA lengths varying by small, 2- to 3-bp increments (24) allowed us to observe a periodic modulation of chromatin folding with strongest changes between the compact arrays with \( L = 20 \) (10\( n \)) and unfolded nucleosome chains with \( L = 25 \) bp (10\( n + 5 \)). These experimental findings were consistent with later Monte Carlo simulations showing that 3D structure of the (10\( n + 5 \)) linkers precludes the nucleosomes from coming into close contact by their histone interfaces in contrast to the (10\( n \)) linkers (25). Furthermore, linker DNA sequence–dependent bending was sufficient to significantly modulate nucleosome array folding (26).

Nuclear chromatin substantially differs from the linear arrays models by its ability to form topologically closed loops and thus to maintain different levels of DNA supercoiling. It also does not have free DNA ends and terminal nucleosomes that may promote unnatural end-to-end interactions in vitro. However, nucleosome arrays can be also assembled on circular covalently closed DNA forming circular nucleosome arrays in vitro and minichromosomes in vivo. Recently, using precisely positioned clone 601–based circular nucleosome arrays, we have conducted topological analysis showing that a 5-bp difference leading to rotational change of half turns between nucleosomes could strongly affect DNA topology in the circular arrays (27). This observation, based on analysis of relatively short 20- and 25-bp linkers typical for yeast genome, poses new questions: Whether the effect on DNA topology could be extended to larger DNA linkers such as those found in eukaryotic cells and whether the nucleosomes in topologically constrained circular nucleosome arrays would adopt a folded structure substantially different from those found in linear arrays.

Guided by these questions, we constructed and studied linear and circular arrays containing 12-mer nucleosomes with 41 and 36-bp linker DNA corresponding to 188 and 183 nucleosome repeat lengths (NRLs) previously observed with human HeLa cell chromatin (28, 29). First, by analytical ultracentrifugation, agarose gel mobility, transmission EM, and DNA topology assays we observed that the NRL = 188 bp imposes a more compact chromatin folding of the linear arrays than the NRL = 183 bp arrays, thus showing that linker DNA length affects topology of a nucleosome array for NRLs typical of higher eukaryotes. These changes fall within the periodic structural modulations of nucleosome arrays with 20- and 25-bp linkers.

Despite the substantial difference in DNA topology, the chromatin higher-order structure including salt-dependent chromatin compaction and self-association appeared to be rather similar and thus not affected by DNA topology and by the absence of open DNA ends in circular arrays. Remarkably, by EM-assisted Nucleosome Interaction Capture (EMANIC) we observed that the interactions between any two nucleosomes separated by one nucleosome (\( i \pm 2 \)) is notably stronger with NRL = 188 whereas interactions between any two nucleosomes separated by two other nucleosomes (\( i \pm 3 \)) is stronger with NRL = 183 in linear as well as in circular arrays. Our new computational model that combines multiple nucleosome rotational settings with dynamic opening of linker DNA was able to accurately recapitulate all experimentally observed properties of the positioned nucleosome arrays and extend experimental prediction for wide range of arrays (\( L = 20–60 \) bp) effectively covering nucleosome repeat length in most eukaryotic genomes.

Based on our experiments and modeling, we suggest that the \( \{10n\} \) DNA linker length may play an important role in formation of folded chromatin fiber structures and DNA topology typical of heterochromatin, whereas \( \{10n + 5\} \) linkers support the more flexible and amenable chromatin structures, thus providing new implications for regulation of chromatin accessibility to DNA binding factors and RNA transcription machinery.

Results

Modulation of chromatin folding by nucleosome spacings typical of human cells

To investigate whether the difference in nucleosome spacing between 183- and 186-bp NRLs typical of higher vertebrates, in particular human HeLa cells (28, 29), could affect nucleosome array folding we have reconstituted nucleosome arrays with 12-mer repeats of the 147-bp nucleosome core from the Widom’s clone 601 sequence (19) plus linker DNA sequences of 36 and 41 bp (Fig. S1). Proper histone/DNA ratios in the reconstituted linear arrays were monitored as we described previously (24, 26) by restriction nuclease protection assay with BamH1 sites available in the linker DNA and by EM counting of the number of nucleosomes per array: 11.90 for \( 183 \times 12 \) and 11.88 for \( 188 \times 12 \) (Fig. S2, A and B).

To monitor the extent of salt-dependent chromatin folding in the linear \( 183 \times 12 \) and \( 188 \times 12 \) arrays, we fixed the nucleosome arrays with 0.1% glutaraldehyde in a buffer containing either 5 or 150 mM NaCl. The fixed arrays were then subjected to deoxynucleoprotein (DNP) agarose gel electrophoresis. The agarose gel (Fig. 1A) shows that whereas at 5 mM NaCl the \( 183 \times 12 \) and \( 188 \times 12 \) arrays migrate with the same mobility (lanes 2, 3, 6, 7), at 150 mM NaCl the electrophoretic mobility of \( 188 \times 12 \) is notably higher (Fig. 1B), consistent with its tighter compaction.

Next, by analytical ultracentrifugation (AUC), we observed that at physiological salt concentration (150 mM NaCl) the \( 188 \times 12 \) arrays undergo a more compact folding (\( s_{20w}^0 = 42.0 \)) than the NRL = 183-bp arrays (\( s_{20w}^0 = 39.0 \)) (Fig. 1, C–E). At low salt concentration (5 mM NaCl) no difference between the
Figure 1. Folding of linear nucleosome arrays shows a periodic linker DNA length dependence. A and B, DNP electrophoresis of linear 183 × 12 and 188 × 12 arrays fixed by glutaraldehyde under the specified conditions. C and D, distributions of sedimentation coefficients, c(S), for 183 × 12 and 188 × 12 linear nucleosome arrays in the compact form (150 mM NaCl). E, sedimentation coefficient averages of four independent experiments for 183 × 12 and 188 × 12 arrays at 5 mM NaCl and 150 mM NaCl. F, comparison of main sedimentation coefficient peaks for 12-mer oligonucleosome linear arrays with varying NRL (165, 167, 169, 172, 177, 200, 205, 207, and 209 bp from Ref. 24 and 183 and 188 bp from this work) at 5 mM NaCl, 150 mM NaCl, and 1 mM MgCl2. G, box graphs show numbers of nucleosomes per unit length (11 nm) of 183 × 12 and 188 × 12 linear arrays at 5 mM NaCl and 150 mM NaCl. The graphs show the median (horizontal lines), the mean (X-cross), and data points (diamonds) within the standard deviations (box) as well as minimal and maximal points (whiskers). H, electron micrograph (uranyl acetate staining, dark-field imaging) of 183 × 12 and 188 × 12 arrays fixed at 5 mM NaCl, standard buffer (left panels) and 150 mM NaCl (right panels). Error bars in bar graphs show S.D. p values represent probabilities associated with Student’s t test.
two arrays was observed (Fig. 1E) showing that the observed difference in sedimentation velocity is because of folding and not molecular mass or other difference in DNA or protein composition. The average of four AUC experiments indicated a statistically significant difference in sedimentation velocity between 183 × 12 and 188 × 12 at 150 mM NaCl (t test: p < 0.001; Fig. 1E). A comparison with previous data (24) suggests that the changes in the salt-dependent nucleosome array compaction are periodically modulated in phase with periodic changes in linker DNA length in the NRL 165- to 188-bp range (Fig. 1F).

Transmission EM (TEM) imaging was used to confirm results of DNP electrophoresis and AUC experiments. The 183 × 12 and 188 × 12 nucleosome arrays were fixed at 5 mM NaCl and 150 mM NaCl, attached to carbon-coated EM grids and imaged (Fig. 1H). At 5 mM NaCl, both 183 × 12 and 188 × 12 showed an open “beads on a string” conformation. At 150 mM NaCl, both arrays underwent a significant compaction with only a few nucleosomes protruding from the compacted chromatin fibers. To quantify the linear compaction of the nucleosome arrays, we measured the number of nucleosomes per unit length for each construct (Fig. 1G). At low salt, the two constructs do not show significant differences in compaction. The increasing concentration of NaCl led to an ~3-fold linear compaction and a notable difference between the 183 × 12 and 188 × 12 arrays. At 150 mM NaCl, the number of nucleosomes per 11 nm was significantly higher in 188 × 12 than in 183 × 12 arrays (t test: p < 0.01). Thus, the TEM measurements confirm the 188 × 12 arrays undergo stronger longitudinal compaction at physiological ion conditions.

**DNA topology in circular nucleosome arrays depends on nucleosome spacing**

Covalently closed circular DNA is characterized by the linking number (Lk) defined as the number of turns of one strand of the DNA double helix around the other (30). Negative DNA supercoiling around histone octamer introduces Lk difference (∆Lk) between –1 and –1.5 depending on the linker DNA length (27). To investigate whether the longer nucleosome linkers typical of higher vertebrates (~40 bp) could affect DNA linking number, we reconstituted circular nucleosome arrays using two different methods described by us previously (27). In the first method, we used plasmid-based templates containing the same 183 × 12 and 188 × 12 nucleosome templates inserted into pUC19 vector. In these 4.7-kb–long circles, about half of DNA belongs to pUC19 vector, which is not a nucleosome positioning sequence. To ensure that the total plasmid DNA does not affect the resulting measurements of ∆Lk, we inserted an additional 60-bp linker after the 183 × 12 repeat so that the net length of the 183 × 12 and 188 × 12 DNA are equal. The isolated plasmid DNA extracted from *Escherichia coli* has superhelical density σ = −0.06 (30), which facilitates formation of nucleosomes.

We reconstituted these circular plasmids with histone octamers by the standard salt dialysis method. Examination of the resulting circular plasmid nucleosome arrays by restriction nuclease protection assay and EM analysis both show practically complete incorporation of DNA into the nucleosomes at the histone octamer/nucleosome ratio of ~1.0 (Fig. S2, C and D). After reconstitution with histones, the nucleosome arrays were relaxed with topoisomerase I, deproteinized, and the supercoiled DNA analyzed on agarose gels containing intercalator chloroquine (CQ) that is needed to increase positive DNA supercoiling and resolve the fast-migrating DNA topoisomers (31).

Comparison of the electrophoretic mobility of single copy DNA obtained with variable histone loading shows the increase in the superhelical density of DNA that accompanies formation of nucleosomes (Fig. 2A). Without core histones, both DNA circles, p-183 × 12 and p-188 × 12 are equally relaxed (∆Lk = 0) and run near the bottom of the gel (lanes 1 and 2). Gradual increase in the core histone loading increases the superhelical density and accordingly changes the electrophoretic mobility, which first increases and then decreases so that for DNA with 80–100% histone loading we achieve the optimal resolution of DNA topoisomers on the gel in the presence of 1.5 μg/ml CQ (Fig. 2A).

Using densitometry scanning of the agarose gels we can accurately determine the difference in the DNA linking number, ∆(Lk), induced by formation of nucleosomes. Evaluation of the linking number difference between the peak values in p-183 × 12 and p-188 × 12 nucleosome arrays gives a change of ∆(Lk) = 4 supercoils (sc) (Fig. 2B). Provided that the nucleosome arrays have the same number of “601” nucleosomes and the vector-associated nucleosomes are not supposed to change between the two samples, our data show a substantial difference in ∆(Lk) = 0.33 (per “601” nucleosome) between the p-183 × 12 and p-188 × 12 constructs.

In a complementary approach, needed to ensure that the Lk difference between the 182 × 12 and 188 × 12 arrays is not affected by vector DNA, we reconstituted minicircles containing only the repeats of 183 × 12 and 188 × 12 nucleosomes. The supercoiled DNA minicircles were prepared by ligation and relaxation by topoisomerase I in the presence of 4.0 μg/ml of EtBr yielding formation of 11 (±1) nucleosomes per minicircle (27). Reconstitution of nucleosomes with histone octamers leading to full saturation of the nucleosome templates as assayed by BamHI digestion (Fig. S3) provided a result qualitatively similar to that in the case of the plasmid-based circles, namely, DNA in 188 × 12 arrays is more negatively supercoiled than in 183 × 12 arrays (Fig. 2, C and D), with the topological difference ∆(Lk) = 3.5 sc.

To evaluate the absolute ∆Lk values in these minicircles, we used the same approach we employed recently analyzing the 167 × 12 and 172 × 12 minicircle arrays (27). As a reference we used the supercoiled 188 × 12 minicircles (denoted sc188 in Fig. 2, C and D), having on average 12 negative supercoils determined as described (27). Comparison with sc188 gives us ∆Lk = −11.5 sc for 183 × 12 arrays and −15 sc for 188 × 12 arrays (Fig. 2, C and D). The ∆Lk values recalculated per nucleosome (Table 1) demonstrate a very good agreement between our topological gel assay (Fig. 2) and Monte Carlo simulations (32). Thus, with two different approaches, we were able to show that the linker DNA length difference in the nucleosome repeats typical of higher vertebrates (NRL = 183–188 bp) could significantly affect DNA topology consistent with computational modeling (25, 32).
Circular nucleosome arrays show reduced longitudinal folding and similar degree of self-association compared with linear arrays

To monitor salt-dependent compaction of the circular nucleosome arrays, we conducted TEM imaging with subsequent linear compaction measurements. Because the circular nucleosome arrays provide opportunity to observe the effect of the unconstrained supercoiling (DNA supercoiling not absorbed by the nucleosomes) on chromatin compaction, here we also analyzed the p-183 \times 12 and p-188 \times 12 plasmid arrays in their supercoiled and topoisomerase I–relaxed forms. The relaxed and supercoiled circular nucleosome arrays were fixed by glutaraldehyde either at 5 mM NaCl or at 150 mM NaCl, applied on carbon-coated EM grids and subjected to TEM. At 5 mM NaCl, the topoisomerase-relaxed nucleosome arrays produced mostly open circles in a “beads on a string” conformation whereas at 150 mM NaCl we observed a strong compaction (Fig. 3A). The supercoiled p-183 \times 12 and p-188 \times 12 arrays showed a very similar degree of compaction under EM (Fig. S4).

To compare folding of the circular nucleosome arrays with linear ones, we measured the number of nucleosomes per unit length (Fig. 3B). In the unfolded form at 5 mM NaCl, the relaxed p-183 \times 12 arrays were slightly less compact than other arrays whereas the total compaction (about 1 nucleosome per 11 nm) was very similar to that of linear arrays (cf. Fig. 1G). When condensed at 150 mM NaCl, the supercoiled p-188 \times 12 showed...
the highest rate of compaction (about 1.8 nucleosomes per 11 nm). The agarose gel (Fig. 3C) also shows that the supercoiled p-188 × 12 arrays migrate with higher mobility at 150 mM NaCl than other circular arrays. Still, the compaction of p-188 × 12 arrays was significantly lower than 2.9 nucleosomes per 11 nm observed for the linear 188 × 12 arrays (cf. Fig. 1G).

Mg²⁺-dependent precipitation assay has been widely used to test propensity of chromatin arrays for self-associating or forming tertiary structures in vitro (33). However, the previously reported self-association studies were all employing linear arrays where the unnatural nucleosome end-to-end interactions might have considerably affected efficiency of self-association. Because circular arrays are structurally heterogeneous as they contain both the superhelical and relaxed or nicked forms of DNA, here we assayed self-association using agarose DNA electrophoresis that can discriminate between the superhelical and relaxed forms of DNA. The self-association of linear and circular (relaxed and supercoiled) arrays was measured by scanning the agarose gels resolving the precipitated and soluble DNA and recording the concentration of MgCl₂ at which 50% of the reconstituted chromatin precipitated (Fig. 4, A and B). Efficiency of self-association of circular and linear nucleosome arrays with NRL = 188 bp appeared to be very similar and independent of the relaxed or stressed topological states (Fig. 4, C and D). With NRL = 183 bp circular nucleosome arrays we observed a notable difference between self-association at 2.1 mM Mg²⁺ in the supercoiled form and at 3.2 mM Mg²⁺ in the relaxed form (Fig. 4D). Thus, the combined experiments suggest that the presence of unconstrained supercoiling may promote linear folding (secondary chromatin structure) for {10n} arrays and self-association (tertiary chromatin structure) for {10n+5} arrays. However, the circular nucleosome arrays do not fold as compactly as the linear arrays and thus may present a more flexible form of chromatin whose higher-order structure is amenable for regulation by DNA supercoiling and external factors mediating chromatin folding.

### Linear and circular nucleosome arrays with (10n+5) linkers show internucleosomal interactions typical of mixed two-start and three-start zigzag

To resolve nucleosome interactions within condensed chromatin, we have previously developed EMANIC (34). Here, we for the first time applied EMANIC to circular nucleosome arrays in parallel with linear arrays. We used partial formaldehyde cross-linking to fix a limited number of internucleosomal contacts (10–20%) in 150 mM NaCl-condensed state as described before (34). The chromatin was then decondensed at low salt, and EM conducted for quantitative assessment of internucleosomal interactions (Fig. 5A) compared with non-cross-linked controls. EM images of individual control and cross-linked linear and circular arrays overlaid with scoring masks for EMANIC are included in supporting EM datasets (see also Table S1).

First, we conducted EMANIC with linear 183 × 12 arrays and compared it with EMANIC data for 188 × 12 arrays (26). For both arrays, we observed significant increase in total looping interactions upon cross-linking (Fig. S5, A and B). In agreement with previous models (24, 25), with 188 × 12 arrays we observed a significant increase in i ± 2 interactions indicating folding according to the two-start zigzag. However, with 183 × 12, we observed weaker i ± 2 but stronger and more significant i ± 3 interactions than with 188 × 12 arrays (Fig. 5, cf. B and C and D).

EMANIC analysis of the topoisomerase-relaxed circular nucleosome arrays cross-linked at 150 mM NaCl also showed significant increase in total looping interactions upon cross-linking (Fig. S5, C and D). Analysis of individual loops (Fig. 5, D and E) showed an even higher increase of i ± 2 interactions in p-188 × 12 compared with the control and cross-linked p-183 × 12. Plotting of differential interactions between EMANIC and control datasets (Fig. 5, F and G) showed a remarkable similarity between the linear and circular arrays in considerably stronger gains of interactions at i ± 2 in p-188 × 12 and i ± 3 in p-183 × 12. i ± 3 appears to be the only significant type of interactions (p <0.05) in both circular and linear arrays (Fig. 5I). Thus, EMANIC shows that the {10n} linkers promote a stronger two-start zigzag folding consistent with previous modeling and structural studies suggesting close contacts and stacking interactions in the {10n} arrays. The {10n+5} linkers support a relaxed zigzag where the nucleosomes may interact with their i ± 2 and i ± 3 neighbors.

### 3D computational modeling reveals periodic modulation of nucleosome array folding with amplitude reducing upon increase in linker DNA length

Previously, we applied MC simulation to analyze nucleosome array folding for different sequence-dependent linker DNA conformations (26). Comparison of the experimentally measured and computed values of sedimentation velocity showed that our predictions were very precise for the “rigid” linkers with inherent DNA curvature induced by A-tracts. However, modeling of 12-mer nucleosomes with mixed sequence linkers (identical to that used for 188 arrays here) predicted the nucleosome arrays to be more compact than was actually observed by the experiments (26). Therefore, we modified our simulations by introducing dynamic opening of linker DNA at the nucleosome exit/entry sites, thereby increasing flexibility of nucleosome arrays (32) (see “Experimental procedures”).

Monte Carlo simulations reveal a substantially more compact equilibrium structure for the 188 × 12 arrays (Fig. 6; see stereo images on Fig. S6) with smaller diameter, reduced length,
and higher sedimentation velocity than for 183 × 12. The MC simulations are also consistent with the difference in DNA topology between the 188 × 12 and 183 × 12 arrays observed by topological assays (Table 1). The representative conformations indicate that the 188 × 12 mainly folds into a two-start zigzag configuration with frequent contacts between i and i + 2 nucleosomes (black arrows on Fig. 6B) in contrast to 183 × 12 in which i ± 3 interactions (red arrows on Fig. 6A) are observed even more frequently than i ± 2. However, the overall probability of occurrence of the internucleosome contacts is much smaller for the 183 × 12 (Fig. 6C), which is one of the main signatures of an open and flexible fiber array. Earlier, we described this seemingly counterintuitive effect (i.e. the array with the shorter linker being more flexible than the array with the longer linker) in the context of topological polymorphism of nucleosome arrays (25).

To compare modeling predictions with EMANIC we calculated the frequency of internucleosome interactions (FINI) in our MC ensembles (32). Because formaldehyde cross-links DNA and proteins at a very short distance, we only consider nucleosomes with the center-to-center distance not exceeding 110 Å (two nucleosome radii). Setting the nucleosome proximity threshold at 110 Å in FINI, we found the probability of cross-link formation in the MC ensembles for all i ± n interactions (Fig. 6C), as well as the detailed distribution of distances for the i ± 2 and i ± 3 interactions (Fig. S7). The predicted ratios of i ± 2 or i ± 3 interactions in 188 × 12 and 183 × 12 arrays (Fig. 6C) shows an excellent agreement with EMANIC data for linear and circular arrays (Fig. 5, F and G).

The overall probability of occurrence of the internucleosomal contacts, which is one of the main signatures of an open and flexible fiber array, is much smaller for the 183 × 12 than
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for 188 × 12 arrays as shown by FINI (Fig. 6C), also consistent with EMANIC (cf. “loops” Fig. S5, A and B). Earlier, we described this seemingly counterintuitive effect (i.e. the array with the shorter linkers being more flexible than the array with the longer linkers) in the context of topological polymorphism of nucleosome fibers (25).

Finally, we have calculated sedimentation coefficient ($s_{20, w}$) for MC ensembles of 12-mer arrays with NRL varying between 162 and 212 bp, and compared them with experimental data on nucleosome chain folding (Fig. 6D). The MC simulations were made using internucleosomal stacking energy $E = -5$ kT (32). Remarkably, our MC simulations are in an excellent agreement with the experimental data for nucleosome arrays with variable NRLs (Fig. 6D).

To address sensitivity of our system to the possible internucleosomal stacking energies variations, we compared sedimentation velocity and FINI calculations obtained using three different energies ($E_1 = -2.7$, $E_2 = -5$, and $E_3 = -8$ kT). With all three energies, we observed that despite the expected increase in the overall frequency of contacts and sedimentation velocity upon increase in stacking interaction, the relative FINI intensities and sedimentation peak periodicity remain unchanged (Fig. S8). Thus, our modeling and experiments reveal a prominent oscillating periodicity in overall folding of the nucleosome arrays determined by rotational internucleosomal settings, with the oscillation amplitude reducing upon the increase in linker DNA length (translational nucleosome settings).
Recent chromosome conformation capture studies have revealed chromatin looping anchored by SMC proteins (condensin and cohesin) and self-association between similar epigenetically marked chromatin states as the two universal principles of structural and functional organization of eukaryotic genome (35). Remarkably, loops anchored by the SMC proteins could be strong enough to maintain a locally altered DNA supercoiling within a closed loop (36). Chromatin loops play an important functional role in mediating interactions between transcription-activating DNA elements such as enhancers and promoters (37, 38). However, the molecular mechanisms of nucleosome folding and self-association confined within the topologically closed loops remain unclear. In this regard, we analyzed whether forming a covalently closed nucleosome chain, modeling a chromatin loop with anchored ends, would dramatically change the higher-order folding of the nucleosome array. Despite the topoisomerase I relaxation of the reconstituted nucleosome arrays creating the predicted topological difference between the 183- and 188-bp NRLs (Fig. 2), we observed no significant effect of nucleosome array circularization on either folding in cis (the secondary level of chromatin higher-order structure) or self-association in trans (the tertiary level of chromatin higher-order structure) of the nucleosome arrays. An important consequence of our experiments is that multiple previous biochemical and modeling studies of chro-

**Figure 5. EMANIC analysis of internucleosomal interactions within the linear and circular nucleosome arrays.** A, representative TEM images of the folded and unfolded formaldehyde–cross-linked p-183 × 12 nucleosome arrays and scheme of EMANIC scoring showing some frequently observed internucleosomal interactions. B–E, box graphs show percentage of individual loops (i = 1 to i = 7), and combined larger loops (i = 8 to i = 11) within linear and circular nucleosome arrays scored either without cross-linking (control) or after formaldehyde cross-linking in the presence of 150 mM NaCl. Elements of the graph are as described in the legend for Fig. 1G. F and G, bar graphs show difference in percentage of cross-linked interactions minus non–cross-linked controls for individual loops (i = 1 to i = 7), and combined larger loops (i = 8 to i = 11). H and I, bar graphs show Student’s t test p values for the difference in percent of cross-linked interactions minus non–cross-linked controls.
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Crystalline and cryo-EM structures of nucleosome arrays were employing linker DNA lengths close to \[10n\] values, with \(NRL = 157, 167, 177,\) and \(187\) bp (42–44). These studies revealed nucleosome linkers forming zigzag crossing inside the fiber, with nucleosomes forming two stacks of the fiber perpendicular where each nucleosome number \(i\) interacts not with its nearest neighbor \((i \pm 1)\) but with the nucleosome preceding or succeeding the nearest neighbor \((i \pm 2)\). Our previous EM and modeling studies showed that the higher-order folding imposed by \([10n + 5]\) linker (25 bp) is notably less ordered and compact compared with the \([10n]\) linker (20 bp) although both structures were predicted to have the two-start zigzag organization (24, 25).

Here we for the first time experimentally addressed the internal organization of nucleosomes with \([10n + 5]\) linkers to observe that this linker DNA mediates a mixed type of nucleosome interactions with simultaneous presence of \(i \pm 2\) and \(i \pm 3\) contacts in contrast to arrays with \([10n]\) linkers containing predominantly \(i \pm 2\) interactions. 3D computational modeling and Monte Carlo simulations suggest a likely mechanism of this internal heterogeneity: in arrays with \([10n + 5]\) linkers, the linker DNA configuration promotes \(i \pm 3\) contacts and the dynamic unwrapping and internucleosomal stacking both facilitate \(i \pm 2\) contacts. Indeed, without unwrapping and minimal stacking, the simulated interactions in \([10n + 5]\) arrays become almost entirely of the \(i \pm 3\) type (data not shown). Remarkably, our model correctly predicts other structural and topological features associated with linker DNA variability (Table 1). It shows periodic changes in the nucleosome array compaction with amplitude decreasing upon increasing linker DNA length (Fig. 6D) consistent with sedimentation measurements for \(NRL = 183\) and 188 shown here as well as our previous sedimentation analysis of nucleosome arrays with different NRLs (24). The fact that our experiments are fully consistent with the new computational model incorporating dynamic local unfolding of nucleosomes in addition to electrostatic compaction of the nucleosome array (32) suggests that the spatial configuration of the nucleosome chain trajectory rather than histone tail structure and interactions is the main factor that determines the nucleosome chain compaction.

Interestingly, within the long-range interactions \((i \pm 7)\) in the circular arrays, we observed a notably higher rate of nucleosome interactions with the \([10n]\) than with \([10n + 5]\) linkers. This finding was rather unexpected for us as we have previously shown that stiffening of the \([10n]\) arrays by histone H1 inhibits long-range interactions (64) but is consistent with a recent study of enhancer-promoter communications showing that \([10n]\) spacing \((NRL = 177\) bp) results in a stronger transcriptional activation than \([10n + 5]\) spacing with \(NRL = 172\) bp (45). It thus appears that some intermediate stiffness brought about by \([10n]\) spacing without linker histone is optimal for the enhancer-promoter communication.

Recent genome-wide study of nucleosome interactions in human cells (5) showed the most pronounced \(i \pm 2\) pattern in chromatin domains marked by histone H3 (K9) trimethylation associated with condensed heterochromatin. This pattern was interpreted as an evidence of two-start helical zigzag organization in the heterochromatin state. In contrast, another chromatin higher-order structure employing linear nucleosome arrays (for reviews see Refs. 9, 39–41) appear to be adequate for modeling the naturally looped chromatin.

Despite the genomic studies showing pre-eminence of the \([10n + 5]\) linkers in eukaryotic genomes, all published X-ray
tin epigenetic state marked by histone H3 (K27) trimethylation and associated with polycomb-mediated gene repression showed a mixed pattern of nucleosome interactions (5). Intriguingly, a most recent study in Drosophila showed that the heterochromatin tends to have a linker DNA length of ~30 bp i.e. $[10n]$, whereas the polycomb-repressed chromatin state displays 26-bp linkers (18), i.e. closer to $[10n+5]$. Furthermore, recent observation of a multivalent engagement of nucleosome linker DNA and dimethylated histone H3 K9 in regulating UHRF1 ubiquitin:histone ligase activity (46) indicates a functional significance of different linker lengths and, hence, nucleosome interactions in epigenetic regulation.

Following these genome-wide observations, our studies suggest that the $[10n]$ spacing may facilitate compact zigzag stacking in constitutive heterochromatin whereas the $[10n+5]$ spacing could be associated with the more flexible and less structured reversibly repressed chromatin. Thus, our in vitro experiments and in silico modeling generate specific predictions, based on nucleosome folding mechanism, that could be tested by nucleosome interaction capture in vivo. At present, genome-wide nucleosome interaction studies are limited by sequence-read numbers and do not resolve interactions beyond the nearest-neighbor $i \pm 1$ and $i \pm 2$ nucleosomes (3, 5). However, EMANIC in combination with immunofractionation of chromatin states marked by specific genome modifications could be a powerful technique for revealing nucleosome interactions at a wider spectrum of nucleosome spacing and folding patterns associated with different epigenetic states.

**Experimental procedures**

**Nucleosome positioning templates and arrays**

A 147-bp DNA template for the positioned nucleosome core was derived from clone 601 DNA (19), to which 41-bp and 183-bp linker DNA sequences were added (Fig. S1). A monomer DNA with 41-bp linker (24) was used as the template, and oligonucleotide primers for 36-bp linkers were incorporated by PCR. The monomeric templates were expanded into 12-mer arrays using an XbaI restriction site at the 5’-end and SpeI and SphI sites at the 3’-end as described (24). The 12-mer DNA repeats were ligated into pUC19 vector using XbaI and SpeI, transformed into *E. coli* Stbl2 competent cells (Invitrogen, catalog no. 10268-019), and grown in the presence of 50 μg/ml ampicillin. For circular DNA preparation, the plasmids were grown in 2 liters of Miller’s LB media (Invitrogen catalog no. 12795-084) and isolated using four QIAfilter Plasmid Maxi Kits (Qiagen catalog no. 12262) on four columns as described in Qiagen manual. The Qiagen-isolated plasmids were additionally treated with equal volumes of phenol/chloroform, precipitated by ethanol, and redissolved in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Typical yield was 1 mg of plasmid DNA.

Circular plasmids (p-183 × 12 and p-188 × 12) were reconstituted with chicken erythrocyte histone octamers by salt dialysis as described above for linear DNA but without carrier DNA and, hence, without ultracentrifugation. Plasmid reconstitutes were analyzed by digestion with restriction enzymes BamHI and HindIII to ensure proper histone loading and transmission EM to determine the number of nucleosomes per array (Fig. S2, C and D).

DNA minicircles containing only 12-mer 601 nucleosomes with repeat lengths of either 183 or 188 bp inserted into pUC19 vector as above but with additional 60 bp added to 183 × 12 to make it the same DNA length as the 188 × 12 plasmid. These plasmids were transformed into *E. coli* Stbl2 competent cells (Invitrogen, catalog no. 10268-019), and grown in the presence of 50 μg/ml ampicillin. For circular DNA preparation, the plasmids were grown in 2 liters of Miller’s LB media (Invitrogen catalog no. 12795-084) and isolated using four QIAfilter Plasmid Maxi Kits (Qiagen catalog no. 12262) on four columns as described in Qiagen manual. The Qiagen-isolated plasmids were additionally treated with equal volumes of phenol/chloroform, precipitated by ethanol, and redissolved in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Typical yield was 1 mg of plasmid DNA.

**Linear nucleosome array reconstitution**

Core histone octamers were isolated from chicken erythrocyte nuclei using ultracentrifugation under increasing ionic strength and purified using FPLC and a HiLoad™ 16/60 Superdex™ 75 prep grade column as described (26). Reconstitution of chicken erythrocyte histone octamers with the linear 12-mer templates was performed in the presence of carrier DNA generated by digestion of pUC19 vector with DraI and used at a ratio of 2:1 with nucleosome template DNA as described (24). After reconstitution, the carrier DNA was removed by ultracentrifugation on a 5–25% sucrose gradient in buffer containing 10 mM Tris, 1 mM EDTA, 0.5 mM PMSF, at 35,000 rpm at 4°C for 8 h in the Beckman SW41Ti swinging bucket rotor. 1-ml fractions were collected and analyzed on 0.8% Type IV agarose gel run at 80 V/cm in HE buffer for ~1 h (26). Fractions containing 183 × 12 and 188 × 12 arrays without carrier DNA were dialyzed against HNE buffer (10 mM HEPES, 5 mM NaCl, 0.1 mM EDTA, pH 7.5) for ~36 h to remove sucrose.

**Circular nucleosome array reconstitution**

The plasmid-based DNA templates contained 12-mer repeats of clone 601 nucleosome with repeat length of either 183 or 188 bp inserted into pUC19 vector as above but with additional 60 bp added to 183 × 12 to make it the same DNA length as the 188 × 12 plasmid. These plasmids were transformed into *E. coli* Stbl2 competent cells (Invitrogen, catalog no. 10268-019), and grown in the presence of 50 μg/ml ampicillin. For circular DNA preparation, the plasmids were grown in 2 liters of Miller’s LB media (Invitrogen catalog no. 12795-084) and isolated using four QIAfilter Plasmid Maxi Kits (Qiagen catalog no. 12262) on four columns as described in Qiagen manual. The Qiagen-isolated plasmids were additionally treated with equal volumes of phenol/chloroform, precipitated by ethanol, and redissolved in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Typical yield was 1 mg of plasmid DNA.

Circular plasmids (p-183 × 12 and p-188 × 12) were reconstituted with chicken erythrocyte histone octamers by salt dialysis as described above for linear DNA but without carrier DNA and, hence, without ultracentrifugation. Plasmid reconstitutes were analyzed by digestion with restriction enzymes BamHI to ensure proper histone loading and transmission EM to determine the number of nucleosomes per array (Fig. S2, C and D).

DNA minicircles containing only 12-mer 601 nucleosomes with repeat lengths of 183 and 188 bp were prepared by ligations of linear DNA fragments. The (183 × 12) + 60 and 188 × 12 linear DNA fragments were excised by XbaI and SpeI restriction enzyme digestion of the pUC19 plasmids with corresponding inserts. The 183 × 12 + 60 and 188 × 12 DNA templates were separated on 1% agarose gels, extracted using Promega Wizard SV Gel and PCR Clean-Up System (catalog no. A9281), and ligated at low DNA concentration (1 ng/μl) to ensure circle formation rather than ligation of multiple linear fragments as described (27). Ligation mixtures were concentrated and washed in 10 mM Tris-HCl (pH 7.5) using Amicon Ultra-4 Centrifugal Filter Units with a molecular mass cutoff of 100 kDa.

To prepare supercoiled DNA and facilitate assembly of 12 nucleosomes per minicircle (27), 3 μg of DNA samples were treated with 4.0 μg/ml of EtBr in the presence of 10 units of human Topo I (catalog no. TG2005H, TopoGEN) in Topo I buffer (10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, and 5% glycerol) for 2 h at 37°C. The DNA samples were treated with...
equal volume of phenol/chloroform, precipitated by ethanol and redissolved in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Minicircle arrays 183 × 12 and 188 × 12 were reconstituted by mixing minicircle DNA with stoichiometric molecular ratios of recombinant *Xenopus laevis* core histone octamers freshly obtained from The Histone Source, Colorado State University. The reconstitution was performed by salt dialysis as described above with plasmid-based DNA. Minicircles were reconstituted by digestion with restriction enzymes BamHI to ensure proper histone loading (Fig. S3).

**Topoisomerase treatment and DNA topoisomer analysis**

For topoisomerase I treatment, either plasmid or minicircle nucleosome arrays containing 1 μg of nucleosome arrays were mixed with 10 units of human Topo I in Topo I buffer in 50-μl reaction volume and incubated for 40 min at 37 °C. For EM, these samples were dialyzed for 4 h against HNE buffer containing either 5 or 150 mM NaCl. For DNA electrophoresis, proteins were removed by treatment with 1% SDS, 0.2 mg/ml proteinase K for 1 h at 55 °C, and DNA was isolated with Promega Wizard SV Gel and PCR Clean-Up System (catalog no. A9281), eluted in 80 μl H2O, and mixed with 20 μl gel-loading buffer (New England Biolabs, catalog no. B7025S0). DNA samples were run on 1% agarose gels (Sigma, Type 1-A, catalog no. AO169) in Tris/acetate/EDTA (TAE) buffer (Bio-Rad, catalog no. 161-0734) typically at 2.3 V/cm for 15 h with constant buffer recirculation. The gels and the buffer contained different concentrations (1.5–16 μg/ml) of chloroquine (Sigma, catalog no. C-6628) as indicated in the figure legends.

**Analytical ultracentrifugation**

Sedimentation velocity experiments were performed on an analytical ultracentrifuge (Beckman Optima XL-A) as described (26, 48). Linear nucleosome arrays were dialyzed for 4 h against HNE buffer containing either 5 or 150 mM NaCl and run at DNA concentration 50 μg/ml on the ultracentrifuge rotor An-60 Ti at 20,000 rpm for ~3 h at 20 °C. Scan profiles were collected at a wavelength of 260 nm using ProteomeLab XL-A software. Sedimentation velocity analysis was conducted using the continuous c(S) distribution model of SEDFIT software (49) (http://www.analyticalultracentrifugation.com).4

**EM and EMANIC**

For transmission EM, the linear and circular (Topo I–treated and intact) arrays with DNA concentration ~50 μg/ml were dialyzed for 4 h against HNE buffer containing either 5 or 150 mM NaCl in 10,000 MW/CO membrane dialysis cups at 4 °C and fixed by adding 0.1% glutaraldehyde to the dialysis buffer and dialyzing at 4 °C for 14 h, followed by 5-h dialysis against HNE buffer without glutaraldehyde.

For EMANIC (34), the linear and circular (Topo I–treated and intact) arrays dialyzed against HNE buffer containing either 5 or 150 mM NaCl were brought to room temperature. 0.08% formaldehyde (ACS reagent, Thermo Fisher) was added to the samples and incubated at room temperature for 5 min. The reactions were stopped by adding 100 mM glycine, pH 7.8, to 40 mM and cooling on ice. The samples were dialyzed for 5 h against 10 mM sodium borate, pH 9.0, 0.1 mM EDTA, and then fixed by adding 0.1% glutaraldehyde to the dialysis buffer and dialyzing at 4 °C for 14 h, followed by 5-h dialysis against HNE buffer without glutaraldehyde.

For both TEM and EMANIC, the dialyzed fixed samples were diluted to 1 μg/ml final concentration with 50 mM NaCl, attached to carbon-coated and glow-discharged EM grids (T1000-Cu, Electron Microscopy Sciences), and stained with 0.04% uranyl acetate. Dark-field EM imaging was conducted as described (26) at 120 kV using JEM-1400 electron microscope (JEOL, Peabody, MA) equipped with SC1000 ORIUS 11 megapixel CCD camera (Gatan Inc., Warrendale, PA). EM images were collected at 20,000–25,000 nominal magnification. For EMANIC, linear nucleosome arrays containing 12 ± 1 and circular nucleosome arrays containing 24 ± 2 distinguishable nucleosome cores were selected to score internucleosomal interactions. Minimum 1800 (average 2338) nucleosomes were scored for each sample (Table S1) as previously described (26). Images of all control and cross-linked linear and circular arrays overlaid with scoring masks for EMANIC are shown in supporting EM pdf files (filenames are listed in Table S1). Percentage of individual loops (i ± 1 to i ± 7), and combined larger loops (i ± 8 to i ± 11) and standard deviations were analyzed and was plotted as box graphs using OriginLab data analysis and imaging software. The box graphs show the median, the mean, and data points within the standard deviations as well as minimal and maximal points. Standard deviations were obtained from at least three EM experiments; p values represent probability associated with a Student’s two-sample t test with a two-tailed distribution.

**Mg2+-dependent chromatin self-association assays**

The extent of chromatin self-association was analyzed using selective precipitation in magnesium similar to Ref. 33. Linear and circular nucleosome arrays dissolved in 10 μg/ml HNE buffer at DNA concentration 25 μg/ml were mixed with increasing concentrations of MgCl2 (Sigma-Aldrich, catalog no. M1028 1 ml) and incubated for 20 min on ice. The samples were then centrifuged at 12,000 rpm, 4 °C, for 10 min. Supernatants were collected and mixed with 5% glycerol, 10 mM EDTA, 1% SDS; the pellets were resuspended in 5% glycerol, 10 mM EDTA, 1% SDS. DNA from supernatants and pellets were analyzed on 1% agarose (Lonza, catalog no. M0130 1 ml) and stained with EtBr. Linear DNA was resolved into the superhelical (bottom) band and relaxed (top) band. The percentage of DNA in the supernatant and pellet was determined by DNA band quantification using ImageJ software. The concentration of MgCl2 at which 50% of the reconstituted chromatin was precipitated was recorded.

**3D computational modeling**

The MC ensembles of 188 × 12 and 183 × 12 nucleosome arrays were constructed as described earlier (32). We used the crystal structure of a nucleosome core particle with the Widom “601” DNA sequence resolved by X-ray crystallography (PDB 601...
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ID: 3MVD) (50). The nucleosome cores were fixed during simulation and only went through rigid body motions except for the flexible DNA ends unwrapped from the histone octamer. (The length of DNA unwrapped at each nucleosome core entry/exit site did not exceed 10 bp.) The linker DNA was modeled at the level of dimeric steps, and its trajectory was described by 6 bp step parameters (51). The geometry of the linker DNA fluctuates around the regular B-DNA (25, 32). Therefore, a system of linker length $L$ and average unwrapping $U$ has $6 \times (L + 2U + 1)$ degrees of freedom per nucleosome.

We used the following energy terms: elastic, electrostatic, histone H4 tail-acidic patch interactions, steric hindrance, and the position-dependent adhesion energy of unwrapped DNA (25, 32). The elastic energy was calculated using a knowledge-based quadratic potential function (51). Electrostatic and van der Waals interactions were calculated as described (25). The DNA-histones adhesion energy was obtained by translating the experimental DNA unzipping dwell times (52) into an energy profile (32). Nucleosome stacking mediated by the histidine H4 N-terminal histone H2A/H2B acidic patch interactions was modeled empirically, taking the previously estimated range of the internucleosome interaction between $-8.0$ kT (25) and $-2.7$ kT (53) and an intermediate value $-5.0$ kT.

Every construct was simulated for 150 million MC steps. The final 100 million MC steps were used for statistical analysis of various parameters of the system (e.g. probability distribution of distances).

For any selected conformation of oligonucleosomal array, the sedimentation coefficient $S_{20,w}$ was calculated as described (54). To calculate average sedimentation coefficient values and their respective standard deviations, 100,000 conformations were chosen randomly from the MC ensembles.

We also calculated the FINI for the MC ensembles. This value represents nearest-neighbor interactions along the chromatin fiber. To calculate FINI, two nucleosomes ($i$) and ($i \pm n$) are considered to be in contact if the center-to-center distance is less than 110 Å. Total contacts are normalized to all possible neighbors with distance $n$ along the fiber.

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