CENP-A-containing Nucleosomes: Easier Disassembly versus Exclusive Centromeric Localization

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CENP-A is a histone variant that replaces conventional H3 in nucleosomes of functional centromeres. We report here, from reconstitutions of CENP-A- and H3-containing nucleosomes on linear DNA fragments and the comparison of their electrophoretic mobility, that CENP-A induces some positioning of its own and some unwrapping at the entry–exit relative to canonical nucleosomes on both 5S DNA and the α-satellite sequence on which it is normally loaded. This steady-state unwrapping was quantified to 7(±2) bp by nucleosome reconstitutions on a series of DNA minicircles, followed by their relaxation with topoisomerase I. The unwrapping was found to ease nucleosome invasion by exonuclease III, to hinder the binding of a linker histone, and to promote the release of an H2A-H2B dimer by nucleosome assembly protein 1 (NAP-1). The (CENP-A-H4)₂ tetramer was also more readily destabilized with heparin than the (H3-H4)₂ tetramer, suggesting that CENP-A has evolved to confer its nucleosome a specific ability to disassemble. This dual relative instability is proposed to facilitate the progressive clearance of CENP-A nucleosomes that assemble promiscuously in euchromatin, especially as is seen following CENP-A transient over-expression.

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Introduction

Centromeres represent a specific chromosome region in eukaryotic cells that defines the site of kinetochore formation, thereby directing spindle attachment and chromosome segregation during mitosis.¹ Maintenance of centromere identity for millions of years appears to result from a subtle evolutionary interplay between genetic features (the rapidly diverging and highly repetitive hundreds to thousands of kbp-long centromeric DNA;² e.g. the 171 bp repeated α-satellite in human), and epigenetic features.³ The main candidate for the centromeric epigenetic mark is CENP-A, a histone H3 variant that replaces H3 in centromeric nucleosomes⁴,⁵ and has diverged significantly from species to species, in contrast to canonical H3, which has remained nearly invariant. The C-terminal two-thirds of CENP-A (the histone fold) is relatively homologous (62% identical) to H3, but its N-terminal one-third is unique.

An early question raised about CENP-A is how it targets to the centromere. Although CENP-A and the CENP-A orthologs in different organisms (also referred to as CENH3s⁶) are produced throughout the cell cycle, like the main H3 variant enriched in transcriptionally active chromatin, H3.3, but unlike the major H3 (H3.1/2) that is synthesized exclusively during DNA replication,⁷–¹⁰ they incorporate into...
centromeres only during embryonic anaphase in Drosophila,\(^\text{11}\) or early G1 in humans cells.\(^\text{12}\) Replacing CENP-A residues within either the L1 loop or the α2 helix with the corresponding H3 residues results in chimeric histones that fail to target to centromeres,\(^\text{13,14}\) while replacement of both the L1 loop and the α2 helix of H3 with the corresponding features from CENP-A, to give the H3\(^\text{CATD}\) chimera (Figure 1(a)), is sufficient for targeting (CATD stands for CENP-A targeting domain).\(^\text{15}\) In addition to being targeted, H3\(^\text{CATD}\) and its yeast version were found to functionally replace CENP-A in human cells or its counterpart (Cse4p) in budding yeast, with no adverse consequence on kinetochore formation, chromosome segregation or cell viability.\(^\text{16}\) Interestingly, the functionality of the CATD correlates with its property to compact the (CENP-A/H3\(^\text{CATD}\)-H4) tetramers, as observed by gel filtration, and to slow the hydrogen/deuterium exchange rate in portions of CENP-A/H3\(^\text{CATD}\) and H4 within the tetramers, relative to tetramers made with H3.1/2 and H3.3.\(^\text{15}\) Remarkably, this latter property was preserved upon tetramer association with H2A-H2B dimers to form nucleosomes on α-satellite and 5 S DNAs.\(^\text{17}\)

Over-expression of CENP-A or its orthologs results in accumulation of the variant at non-centromeric chromosomal sites and in rare cases leads to the formation of neocentromeres lacking repetitive DNA and significant sequence homology to α-satellite DNA.\(^\text{18-20}\) Proteolysis, mediated, at least in part, by the ubiquitin-proteasome pathway, regulates the level of the transiently over-expressed variant in the soluble pool in *Saccharomyces cerevisiae*,\(^\text{21}\) and clears it progressively from the chromosomal arms in Drosophila,\(^\text{22}\) in contrast to centromere-localized subunits, which were resistant.\(^\text{21,22}\) Perhaps relevant to the targeting problem is the purification from Drosophila cells of a soluble pre-assembly complex containing the CENP-A ortholog (CID), H4 and the generic assembly factor RbAp48 (p48).\(^\text{23}\) The occurrence of this simple complex is consistent with the ability of p48 to bind CID *in vitro* (in addition to H4), but not H3.\(^\text{24-25}\) This finding is in addition to p48 co-existence with several other proteins in various chromatin remodeling and modifying complexes, and in histone chaperones CAF-1 and HIRA (involved in nucleosome assembly during replication and transcription, respectively).\(^\text{8}\) An at least indirect involvement of p48, in conjunc- tion with p46, was demonstrated in human cells by the observation that their simultaneous, but not individual, RNAi knockdown abolished targeting.\(^\text{26}\) A similar simple pre-assembly complex may not exist in other species, however, since p48 was absent from a CENP-A nucleosome-associated complex (NAC) purified from human centromeric chromatin, although it was present along with p46 as a CAF-1 component in the H3.1 equivalent complex.\(^\text{27}\)

Using single nucleosomes assembled on a series of DNA minicircles, followed by their relaxation with topoisomerase I (the so-called "DNA minicircle approach"),\(^\text{28,29}\) three conformational states have been identified between which conventional nucleosomes can thermally fluctuate.\(^\text{30,31}\) The first state, we referred to as the "closed negative" state, introduces a topological deformation in linking number relative to naked DNA between −1.4 and −1.7 (ΔLkn(i) in equation (3) in Materials and Methods), and has DNAs entering and exiting the nucleosome negatively crossed. This form corresponds to the canonical conformation with ~147 bp of wrapped DNA, and usually is energetically favored. The second state, where the entry–exit DNAs are uncrossed (we termed this previously the "open" state (ΔLkn = −0.7 to −1)), concomitantly involves the unwrapping of ~21 bp through breakage of the most distal H3 αN/DNA-binding sites at superhelix locations (SHL)±6.5.\(^\text{32}\) The third, "closed positive", state has ΔLkn = −0.4 to −0.6 with positively crossed entry–exit DNAs, and is energetically the least favored, as expected from the DNA left-handed wrapping around the histones. Modeling has defined the topological and energetic (see above) parameters of these conformational states and their dependence on effectors of nucleosome dynamics, such as the modification status of the histone tails,\(^\text{30}\) or the DNA sequence.\(^\text{31}\) Remarkably, nucleosomes in chromatin fibers showed very similar values for these parameters, as observed in recent single-molecule experiments.\(^\text{33}\)

In this work, we investigated the intrinsic properties of CENP-A nucleosomes, with respect to their structure, dynamics and stability, in comparison with those of conventional (H3) nucleosomes, in the hope that they could explain some of the peculiarities of centromeric chromatin mentioned above. When reconstituted on linear DNA fragments and subjected to electrophoresis, CENP-A nucleosomes were retarded relative to H3 nucleosomes, which suggested some unwrapping at their entry–exit. Consistently, the "DNA minicircle approach" revealed that CENP-A nucleosomes prefer the open, uncrossed conformation, in contrast to H3 nucleosomes, which prefer the closed negative conformation (see above). The resulting 7±2 bp steady-state unwrapping was sufficient to compromise the binding of a linker histone and to promote dissociation of a H2A-H2B dimer by nucleosome assembly protein 1 (NAP-1). Once dimers are removed, the (CENP-A-H4)\(_2\) tetramer is also more easily released with heparin than the (H3-H4)\(_2\) tetramer. We conclude that, at least in the absence of the above-mentioned additional CENP-A\(^\text{NAC}\) components that are recruited by CENP-A nucleosomes to functional centromeres, CENP-A nucleosomes are easier to disassemble than canonical nucleosomes. The potential physiological relevance of this result is discussed.

**Results**

**CENP-A-dependent DNA unwrapping and phasing of nucleosomes**

The sequences in the domains of interest of the histone H3 family used in the present work are compared in Figure 1(a). Here, we focus on the SHL ± 6.5
and SHL±2.5 (incidentally ±1.5) which are shown, for the positive ones, in the upper gyre of the crystallized nucleosome core particle. These histones were mixed with stoichiometric amounts of H4, H2A and H2B to form the octamers that are displayed in Figure 1(b).

**Figure 1.** Histones and nucleosome crystal structure. (a) Upper panel: H3 is the chicken sequence. H3tl is derived from a Xenopus H3 by deletion of the first 26 residues of the N-terminal tail (xH3 has a single conservative G-A substitution at residue 102 relative to cH3 which is not expected to make any difference in the assays reported here). CENP-A is the human version, and CENP-Atl was derived from it by deletion of the first 25 residues of its N-terminal tail. H3(RK) and H3tl(RK) are identical with H3 and H3tl, respectively, except for an R49K substitution in $\alpha_N$. H3CATD is a chimera of human H3 (black; the part of hH3 present in H3CATD is identical with cH3) and hCENP-A (blue). Gaps in the alignment are indicated by dashes. Residue numbers are those of H3. Arrowheads indicate H3 arginine residues whose lateral chain enters the small groove at SHL±6.5,±1.5 and±2.5 of the double helix, or residues at equivalent positions of other histones (bold red symbols). Lower panel: The SHL are indicated in the upper gyre of the crystallized core particle (adapted from Figure 1(d) of Luger et al.32). (b) Electrophoretic patterns of reconstituted octamers (see Materials and Methods), compared to the native octamer from chicken erythrocyte chromatin, in an SDS-containing 18% (w/v) polyacrylamide gel (acrylamide to bisacrylamide, 29:1, w/w), stained with Coomassie brilliant blue. Note that tailless H3 (H3tl) comigrates with H4, and that H3CATD migrates between CENP-A and H3. CENP-Atl and H3tl(RK): tailless CENP-A and H3(RK).
Octamers were used to reconstitute mononucleosomes on linear ~350 bp 5 S and α-satellite DNA fragments (Figure 2). We first note, from the amounts of residual unreconstituted naked DNAs in the patterns, that similar histone/DNA ratios led to similar reconstitution levels on either 5 S or α-satellite DNA. This argues for the absence of a significant preference of the CENP-A octamer to bind its homologous α-satellite DNA (compare H3 and CENP-A patterns in Figure 2(a), (b) and (c), respectively).

A resemblance between H3 and CENP-A patterns is observed on both DNAs, despite a general decrease in mobility and some differences in positioning of CENP-A-nucleosomes relative to H3 nucleosomes. N3, a strong position of canonical nucleosomes on 5 S DNA,35 is hardly occupied by CENP-A nucleosomes, and N4 is reduced (Figure 2(a)). N2 was missing from the α-satellite pattern and was replaced by two new bands (Figure 2(b); stars). These results are at variance with previous work that failed to detect CENP-A-specific positions on the same α-satellite sequence.36 The discrepancy may simply reflect the shorter sizes of the fragments used (186 bp and 192 bp, against 350 bp here).

Partially specific CENP-A phasing is not conferred by the CATD (see Introduction and Figure 1(a)), because H3CATD-containing nucleosomes yield a migration pattern identical with that observed with canonical H3 (Figure 2(c)). The removal of CENP-A unique N-terminal tail (CENP-Atl; Figure 1(a)) does not alter the positioning (starred positions in Figure 2(c)). Removal of H3 N-terminal tails, to give H3tl (Figure 1(a)), does not modify the conventional nucleosome pattern either, except for some decrease in band mobility (compare middle bands of H3 and H3tl in Figure 2(c)).

Retardations of H3(RK) and H3tl nucleosomes relative to H3 nucleosomes are expected to signal DNA unwrapping at their entry–exit. The reason is that the R49K substitution, in weakening entry–exit histone/DNA-binding sites, should be similar in its consequences to removal (or acetylation) of the tails whose net effect is to increase the repulsion between entry–exit DNAs and favor the open conformation on DNA minicircles.30

These data, along with the identical sedimentation rates of CENP-A and H3 nucleosomes in sucrose gradients (not shown), and with the histone composition analysis and the DNase I and micrococcal nuclease footprints of others,5,36 provide ample evidence that CENP-A can replace H3 efficiently in assembling bona fide nucleosomes with their full histone complement. This conclusion holds even if CENP-A nucleosomes may be somewhat unwrapped at their entry–exit, which appears to result, in part, from CENP-A substitution of arginine 49 by lysine. The partially specific CENP-A phasing is not related to its unique N-terminal tail, nor is it linked to its peculiar feature that targets it to the centromere, the CATD.

Nucleosomes were also made with an H3 mutant termed H3(RK), where arginine 49 was replaced with lysine (lysine is found at the corresponding position in CENP-A; Figure 1(a)). This substitution is expected to weaken the αN/DNA-binding sites at nucleosome entry–exit (SHL±6.5), since this arginine in histone H3 intercalates into the small groove and stabilizes histone/DNA-binding at these positions,32 which a lysine presumably will not do. H3(RK) does not affect nucleosome positioning relative to H3, but otherwise mimics CENP-A in generating some nucleosome retardation (compare H3 and H3(RK) in Figure 2(a)–(c)).

![Figure 2](image-url)
Figure 3. Exonuclease III as a probe of unwrapping at CENP-A nucleosome entry-exit. (a) Core particles (NUC) were reconstituted with H3 and CENP-A octamers on 32P-end-labelled ~147 bp DNA fragments extracted from native core particles prepared from a micrococcal nuclease digest of chromatin in chicken erythrocyte nuclei. They were subsequently electrophoresed before (lanes C) or after digestion at 37 °C with 1000 units/ml of exonuclease III (Fermentas) for 4 min (lanes 1 and 4), 9 min (lanes 2 and 5) and 20 min (lanes 3 and 6) in 10 mM Tris–HCl, (pH 7.5), 50 mM NaCl, 1 mM β-mercaptoethanol, 4 mM MgCl2. (b) Gel slices (brackets) were cut out of the gel shown in (a), and aliquots of the eluted DNA products were electrophoresed in a 7 M urea/10% polyacrylamide gel (acylamide/bisacrylamide = 29:1, w/w) in 90 mM Tris–borate–EDTA (pH 8.3). Lanes were numbered according to their origin in (a). CDNA, starting ~147 bp DNA; M, 32P-end-labeled pUC 18 HpaII digest. (c) A scheme showing how the digestions of labeled nucleosomes in (a) (probes) were checked through the prior addition of a carrier chromatin made of unlabeled H3 core particles on the same DNA. (d) Same as (b), but after DNA end-labeling with 32P using DNA kinase. Note the similar digestions of carrier chromatin in lanes 1 and 4, 2 and 5, and 3 and 6. The SHL corresponding to the bands are indicated. Autoradiograms are shown.
Exonuclease III as a probe for unwrapping of CENP-A nucleosomes

Exonuclease (exo) III is known to pause every ten nt or 11 nt when invading the nucleosome from the 3' ends, which reflects the DNA periodical binding onto the histone surface. If, as could be expected, the pauses are modulated by the binding strength, then exo III should pause for a shorter time upon entering CENP-A nucleosomes. To test that prediction, CENP-A- and H3-containing core particles were reconstituted on chicken ∼147 bp core DNA fragments, digested with exo III and subjected to electrophoresis (Figure 3(a)). Unreacted naked DNA disappears

Figure 4. The minicircle approach and its application to nucleosomes on α-satellite 356 bp ΔLk −3 topoisomer. (a) A diagram of the approach. (b) Mononucleosomes (NUC) were reconstituted on that topoisomer (C_{DNA}) with the indicated octamers (Figure 1(b)) (lanes 1, 4, 7, and 10), incubated in buffer R without (lanes 2, 5, 8, and 11) or with topoisomerase I for 30 min. at 37 °C (lanes 3, 6, 9, and 12), and electrophoresed as nucleoproteins at room temperature. (c) Gel slices corresponding to relaxed nucleosomes (brackets in (b)) were cut out, the DNA products eluted and electrophoresed in a chloroquin-containing polyacrylamide gel, along with marker topoisomers (M1 and M2). Autoradiograms are shown. (d) Radioactivity profiles of the lanes in (c).

Figure 5. Relaxation data of nucleosomes on α-satellite and pBR DNAs. (a) Topoisomer relative amounts in Figure 4(d) (red) and in similar relaxations of the same nucleosomes on the DNA minicircles of the two series were plotted as functions of their ΔLk. Data points were usually the mean of two or three independent experiments for each histone. Error bars are the standard deviations of four to eight measurements for H3 and H3^{CATD} (Native in Figure 1(b), H3, H3l and H3^{CATD} behave similarly in relaxation assays and were combined for the error calculation) and two to four measurements for H3(RK) (H3(RK) and H3l(RK) combined) and CENP-A (CENP-A and CENP-Atl combined). Smooth curves were calculated for each DNA from fitting of the three-state model to the H3 plus H3^{CATD} data points combined, as described in Materials and Methods. The pBR curve virtually superimposes on that previously obtained with the same DNA and the native octamer in Figure 1(b). (b) H3 curves are those in (a), and other curves were similarly obtained from fitting to the appropriate data points in (a). Nucleosome conformations corresponding to the three peaks of the pBR H3 profile are shown.
Figure 5 (legend on previous page)
rapidly upon digestion, while nucleosomes resist. DNAs were extracted (brackets) and subjected to electrophoresis in a denaturing gel (Figure 3(b)). The 147 nt band resists digestion in H3-containing nucleosomes (lanes 1–3), while it is rapidly digested away in CENP-A nucleosomes (lanes 4–6). As a control, we checked that carrier H3 core particles (schematics in Figure 3(c)) were digested identically in equivalent lanes of the two nucleosomes (compare lanes 1 and 4, 2 and 5, and 3 and 6 in Figure 3(d)). These results support the notion of weaker histone/DNA-binding sites, i.e. of an unwrapping, at the entry–exit of CENP-A nucleosomes.

**Topological measurement of the unwrapping in CENP-A nucleosomes**

The minicircle approach involves three steps (Figure 4(a)): (i) reconstitution of mononucleosomes on a series of negatively supercoiled DNA minicircles; (ii) their relaxation with topoisomerase I, followed by their gel electrophoresis as nucleoproteins; and (iii) the purification of the relaxed DNA products, and their analysis. The example of H3, H3(RK), H3CATD and CENP-A nucleosomes assembled on topoisomer (topo) −3 (ΔLk ≈ −3; see equation (1) in Materials and Methods) of the α-satellite 356 bp DNA minicircle is shown in Figure 4(b). Relaxed nucleosomes (+ topoisomerase: circled lane numbers) were excised (brackets) and the purified DNAs were subjected to electrophoresis (Figure 4(c)). Three adjacent topoisomers were obtained, whose relative amounts vary depending on the octamer used. H3 nucleosomes showed two major peaks, with slightly more topo −1 than topo −2 (Figure 4(d), upper left). Upon replacement of H3 with CENP-A, the amount of topo −2 greatly decreases relative to topo −1 (Figure 4(d), lower right), indicating a lower proportion of nucleosomes in the closed negative conformation to the benefit of nucleosomes in the open conformation (see Introduction). The R49K mutation in H3 decreases the amount of topo −2 to an intermediate extent (Figure 4(d), upper right), suggesting the substitution at this position in CENP-A is responsible, in part, for the altered topoisomer equilibrium distribution. In contrast, the CATD substitution into H3 has no influence on the distribution (Figure 4(d), lower left), i.e. on nucleosome wrapping.

Results for the whole series of α-satellite and pBR\textsuperscript{30,31} DNA minicircles are shown in plots of the topoisomerase relative amounts in the equilibria versus their ΔLk (Figure 5(a)). CENP-A nucleosomes on both DNAs show a shift of their profiles toward less negative ΔLk values relative to canonical nucleosomes. A smaller shift is observed with H3(RK)-containing nucleosomes. The theoretical profiles\textsuperscript{31} in Figure 5(b) obtained by fitting with a thermodynamic-three-state model (see Materials and Methods) yield topological (ΔLk\textsubscript{α}) and energetic parameters (ΔG\textsubscript{α}) of the three conformations of each nucleosome (Table 1). As observed with the 5 S DNA (see the legend to Table 1), ΔLk\textsubscript{α} of α-satellite nucleosomes are less negative than their pBR counterparts. Thus, both of the sequences that provide nucleosome assembly sites in vivo (α-satellite and 5 S DNAs), but not the prokaryotic pBR DNA, show a net overwinding relative to naked DNA upon wrapping around the histone surface.\textsuperscript{30,31}

Relative to the open state, the free energies of the two crossed-entry-exit states (negative and positive) increase equally from H3 to H3(RK) nucleosomes and from H3 to CENP-A nucleosomes: 0.7–0.8 kT and 1.6–1.7 kT, respectively, on both DNAs (Table 1). These results provide the first demonstration that the αN/DNA-binding sites at nucleosome entry–exit stabilize both negative and positive states equally. The data show also that the destabilization of the entry–exit binding sites resulting from the arginine-lysine substitution in H3(RK) is ~45 (0.75/1.63) % of that achieved by CENP-A. The probability of occupancy of each state for a nucleosome with a nicked loop free to rotate under thermal fluctuations can be calculated subsequently. Taking into account the wrappings in the different states, the extra steady-state unwrappings in H3(RK) and in CENP-A nucleosomes relative to H3 nucleosomes, averaged on the two DNAs, is respectively 3(±2) bp and 7(±2) bp (Table 2).

**Hindered linker histone binding onto CENP-A nucleosomes**

The linker histone seals the two turns of the DNA superhelix\textsuperscript{30,40,41} and may have difficulty in binding an unwrapped nucleosome. H5 (a H1 variant in nucleated erythrocytes) was unable to bind CENP-A

### Table 1. Conformational state parameters for nucleosomes on α-satellite and pBR DNAs

<table>
<thead>
<tr>
<th>State</th>
<th>ΔLk\textsubscript{α} (kT)</th>
<th>ΔG\textsubscript{α} (kT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-sat</td>
<td>pBR</td>
</tr>
<tr>
<td></td>
<td>H3</td>
<td>H3(RK)</td>
</tr>
<tr>
<td>Negative</td>
<td>−1.55</td>
<td>−1.5</td>
</tr>
<tr>
<td>Open</td>
<td>−0.79</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>−0.47</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Fitting (see Materials and Methods) was done simultaneously for the three relative amounts versus ΔLk curves in Figure 5(b) using nine parameters: three ΔLk\textsubscript{α} which were assumed to be the same for all three nucleosomes on each DNA, and six ΔG\textsubscript{α} energies. Independent fitting of each curve with five parameters (three ΔLk and two ΔG\textsubscript{α}) led to similar values (not shown). Error bars are between ±0.02 and ±0.04 for ΔLk\textsubscript{α} and between ±0.1 and ±0.3 for ΔG\textsubscript{α}. Former ΔLk\textsubscript{α} values (negative, open and positive states) were −1.69, −1.04 and −0.56 for pBR and −1.40, −0.72 and −0.41 for 5 S.\textsuperscript{31}
nucleosomes on α-satellite 356 bp topo −1 and topo −2 under conditions where binding to H3 nucleosomes was robust (Figure 6(a)). Some binding is observed with topo −3, presumably due to the large topological constraint in the loop of that nucleosome ($\Delta L_k \sim -1.3$; see equation (3) in Materials and Methods), which strongly stabilizes the two superhelical turns. We conclude that CENP-A-containing nucleosomes are easier to disassemble than H3-containing nucleosomes. 

### Table 2. Probability of state occupancy and mean wrapping

<table>
<thead>
<tr>
<th>State</th>
<th>Wrapping (bp)</th>
<th>α-sat</th>
<th>CENP-A</th>
<th>pBR</th>
<th>CENP-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>147</td>
<td>0.75</td>
<td>0.63</td>
<td>0.51</td>
<td>0.65</td>
</tr>
<tr>
<td>Open</td>
<td>126</td>
<td>0.17</td>
<td>0.31</td>
<td>0.46</td>
<td>0.27</td>
</tr>
<tr>
<td>Positive</td>
<td>147</td>
<td>0.08</td>
<td>0.06</td>
<td>0.03</td>
<td>0.08</td>
</tr>
<tr>
<td>&lt;Wrapping&gt; (±maximal deviation)</td>
<td>143.4(±0.6)</td>
<td>140.5(±0.6)</td>
<td>137.4(±0.7)</td>
<td>141.5(±1.4)</td>
<td>138.1(±1.5)</td>
</tr>
<tr>
<td>Unwrapping (bp) relative to H3</td>
<td>2.9(±1.2)</td>
<td>6.0(±1.3)</td>
<td>3.4(±3)</td>
<td>8.7(±3)</td>
<td></td>
</tr>
</tbody>
</table>

The equilibrium probability of state $i$ ($i=1–3$), $f(i)$, was calculated for each histone from the state free energy in Table 1, $\Delta G_n(i)$, using the equation $f(i)=\exp[-\Delta G_n(i)]/\sum_i \exp[-\Delta G_n(i)]$. Mean wrappings were calculated by weighting the wrapping in each state by its probability. Errors were calculated from the errors on $\Delta G_n$.

### Figure 6. H5 binding potentials of H3/CENP-A and H3/H3(RK) nucleosomes

(a) Mononucleosomes (NUC) were reconstituted on α-satellite 356 bp topoisomers −1, −2 and −3 with H3 and CENP-A octamers, and with H5 at the H5:octamer molar ratios indicated (see Materials and Methods), and electrophoresed. NUC: H5-bound nucleosome. (b) Same as (a) for nucleosomes reconstituted with H3 and H3(RK) octamers. Nucleosome conformations on the different topoisomers before H5 binding are shown. Autoradiograms are shown.
virtually unable to bind H5, unless an unrealistically large unconstrained topological stress is applied. H1, which is less positively charged than H5 and hence a poorer binder, should similarly fail to bind.

It was interesting to check H5 binding potential of H3(RK)-containing nucleosomes. These nucleosomes bind H5 on all three topoisomers, although the extent of binding is reduced substantially on H3.

**Figure 7.** NAP-1 depletion of core particles. (a) Core particles (NUC; diagram) were reconstituted with H3- and CENP-A-containing octamers on the \( ^{32}\)P-labeled \(~147\) bp DNA, and were subsequently supplemented with a ninefold excess (DNA:DNA; w/w) of a carrier chromatin made of pUC18 plasmid DNA reconstituted with H3-containing octamers. Mixtures were incubated without (lanes 2) or with yeast NAP-1 at molar ratios of 7 and 20 dimers per histone octamer (lanes 3 and 4) in TE buffer supplemented with 100 mM NaCl and 100 \( \mu \)g/ml of BSA for 1 h at 37 °C, and were electrophoresed as nucleoproteins (residual unreconstituted naked DNA not shown). 12% and 20% of the H3 nucleosomes, and 21% and 36% of the CENP-A nucleosomes, respectively, were converted into hexasomes (HEX) by treatment with NAP-1 in lanes 3 and 4 (after subtraction of hexasome initial amounts in lanes 2). CNUC, starting nucleosomes in TE buffer. CTET, control tetrasomes (TET) reconstituted with the corresponding tetramers. Strikingly, no CENP-A tetrasomes could be reconstituted on the \(~147\) bp DNA at any histone to DNA ratio, but only nucleosome-like particles presumably made of two tetramers stacked on top of each other (lane 5). (The absence of tetrasomes was confirmed by sedimentsations in sucrose gradients; not shown.) (b) Electrophoresis and sedimentation profiles in linear sucrose gradients of H3 core particles before (CNUC) or after incubation for 4 h with yNAP-1 at a molar ratio of 20 dimers per histone octamer (CNAP), and of H3 tetrasomes (CTET). Aliquots of fractions from the gradients were electrophoresed. Hexasomes sediment between nucleosomes and DNA, and tetrasomes on the faster side of the DNA peak, as expected from their respective histone contents. Note that control nucleosomes contain minor amounts of hexasomes that are well visible in fractions 16–18 of the gradient.

Easier Disassembly of CENP-A-containing Nucleosomes
(RK) nucleosomes compared to H3 nucleosomes at lower H5/octamer ratios (Figure 6(b)). These results show that H5 is a sensitive probe of nucleosome unwrapping.

**CENP-A nucleosomes are easier to disassemble**

**NAP-1 assays**

Core particles were reconstituted with H3 and CENP-A octamers onto the ~147 bp core DNA and they were subsequently incubated with increasing molar ratios of yeast NAP-1 (Figure 7(a)). This resulted in the progressive appearance of faster-migrating particles, which were identified previously in similar assays as hexasomes (one (H3-H4)$_2$ tetramer plus one H2A-H2B dimer). Centrifugation in sucrose gradients (Figure 7(b)) showed these particles sedimenting halfway between control tetrasomes (one tetramer; TET) and core particles (NUC), as expected for hexasomes. Quantification of the radioactivity in the bands showed the formation of almost twofold more hexasomes with CENP-A than with H3 at either of the two concentrations of NAP-1 used (see the legend to Figure 7(a)).

Facilitated removal of the first dimer in CENP-A compared to H3 core particles may be the consequence of the steady-state unwrapping described above. In this case, the first dimer should be even less stable in open-state nucleosomes (~21 bp unwrapped), but more stable in negative-state, fully wrapped, nucleosomes. In agreement with this prediction, a large depletion is observed in nucleosomes reconstituted on topo −1, but none in nucleosomes on topo −2 (Figure 8). Hexasomes are again formed, plus some tetrasomes with CENP-A. Interestingly, the net depletions are essentially the same with H3 and CENP-A (see the legend to Figure 8). Note that depletions on topo −1 are substantially larger than those observed on the ~147 bp DNA at the same NAP-1 molar ratio (compare depletions in the legends to Figures 7(a) and 8), showing that core particles lie somewhere in between nucleosomes on topos −1 and −2 with respect to their stability.

**Heparin assays**

Like NAP-1, heparin (an acidic polyelectrolyte) mostly removes a single dimer. Nucleosomes on topo −1 or −2 were converted into hexasomes, which were eventually destroyed without release of tetrasomes. This conclusion is obvious from the band mobilities on topo −1 (Figure 9(a)), and was checked

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**Figure 8.** NAP-1 depletion of nucleosomes on topos −1 and −2. (a) H3 nucleosomes on α-satellite 356 bp ΔLk = −1 and −2 topos were supplemented with pUC18 chromatin and incubated as indicated in Figure 7(a) without (lanes 2) or with yNAP-1 at molar ratios of 2.5, 7 and 20 dimers/histone octamer (lanes 3–5). 6%, 32% and 50% of H3 nucleosomes, respectively, were converted into hexasomes by treatment with NAP-1 on topo −1 (taking into account their initial amounts in lanes 2), but none on topo −2. (b) Same as (a) with CENP-A nucleosomes. 10%, 37%, and 48% of the nucleosomes on topo −1 were converted into hexasomes/tetrasomes in lanes 3–5, and again none on topo −2. Starting nucleosome conformations are shown. Autoradiograms are shown.
for topo −2 by sedimentation in sucrose gradients similar to those displayed in Figure 7 (b) (not shown). When the H3/CENP-A N-terminal tails are removed, however, the second dimer is released earlier on topo −1 (but not on topo −2) and a hexasome–tetradsome transition is observed at intermediate concentrations of heparin (see H3tl and CENP-A in Figure 9(b)).

The concentration of heparin required to destroy half of the initial nucleosomes is $C_{\text{NUC}}^{50}$, while the concentration that leads to half of the total increase in the amount of naked DNA is $C_{\text{DNA}}^{50}$ (Figure 10(a)). $C_{\text{NUC}}^{50}$ and $C_{\text{DNA}}^{50}$ are somehow the measures of the apparent stabilities of the first dimer and of the hexamer/tetramer, respectively.

Histograms of $C_{\text{NUC}}^{50}$ in Figure 10(b) (left panel) show: (1) an equally low stability of the first dimer in CENP-A and H3 nucleosomes on topo −1 (CENP-A/ H3 $C_{\text{NUC}}^{50}$ ratio ~1). As with NAP-1, this is expected to reflect the complete breaking of entry–exit binding sites on topo −1, which obliterates the difference between the two nucleosomes; (2) a stabilization of the first dimer on topo −2, as observed with NAP-1 in Figure 8. Interestingly, the extent of this stabilization is much lower in H3 than in CENP-A nucleosomes (topo −2/topo −1 $C_{\text{NUC}}^{50}$ ratio ~3, against ~12, respectively). As a result, the first dimer is strongly destabilized in CENP-A relative to H3 nucleosomes on topo −2 (CENP-A/ H3 $C_{\text{NUC}}^{50}$ ratio ~1/4). This suggests that $\alpha$N/ DNA-binding sites are too weak to stabilize the negative state in CENP-A nucleosomes on topo −2, a result consistent with the difficulty of these nucleosomes binding to H5 (Figure 6(a)); and (3) a stabilization of the first dimer upon tail removal. The stabilization increases from nucleosomes on topo −1 (H3tl/H3 and CENP-A/H3 $C_{\text{NUC}}^{50}$ ratios ~2.5 and 1.5, respectively) and CENP-A nucleosomes on topo −2 (CENP-A/H3 $C_{\text{NUC}}^{50}$ ratio ~2), to H3 nucleosomes on topo −2 (H3tl/H3 $C_{\text{NUC}}^{50}$ ratio ~5). We conclude that tail removal largely increases the deficit in stability of the first

Figure 9. Heparin depletion of nucleosomes on topos −1 and −2. (a) and (b) Nucleosomes (NUC), as reconstituted on $\alpha$-satellite 356 bp $\Delta Lk$−1 and −2 topos with the indicated octamers (see Figure 1(b)), were supplemented with a ninefold excess (DNA:DNA; w/w) of naked form I pUC18 plasmid DNA. Mixtures were subsequently incubated at a constant concentration of DNA for 10 min at 37 °C in TE buffer plus 50 mM NaCl with various concentrations of heparin (sodium salt, grade I-A, from Sigma), and electrophoresed, along with control nucleosomes (CNUC) and/or tetradsomes (CTET). Depletions were found to depend solely on the concentrations of heparin when 20–50% of the initial topoisomer was reconstituted. Higher levels of reconstitution were not tested, while reconstitutions >20% led to larger depletions (not shown). Lanes 0, mock incubation without heparin. A one unit increment in the lane number indicates a heparin concentration multiplied by 4, or by 2 with the half-increment. With a concentration of heparin in lane 1 of $1.35 \times 10^{-3}$ μg/ml, the concentration, $C(n)$, in lane number $n$ is: $C(n)=1.35 \times 10^{-3} \times 4^{n-1}$ μg/ml.
dimer in CENP-A relative to H3 nucleosomes on topo −2 (CENP-Atl/H3tl C\textsubscript{NUC}\textsuperscript{50} ratio ∼1/9, as compared to the above 1/4 value before tail removal).

\( C\text{_{DNA}} \)\textsuperscript{50} values (Figure 10(b), right histogram) indicate: (1) a lower stability of the CENP-A relative to the H3 hexamer/tetramer on both topos −1 and −2 (CENP-A/H3 C\text{_{DNA}}\textsuperscript{50} ratio ∼1/2.5 and 1/2, respectively); (2) a lack of a significant dependence of the stability of the hexamer/tetramer on DNA supercoiling; and (3) a large stabilization of the H3
hexamer/tetramer upon tail removal (H3tl/H3 C_{DNA}^{50} ratios ~ 4 and 8 on topos –1 and –2, respectively), but not of the CENP-A hexamer/tetramer (CENP-Atl/CENP-A C_{DNA}^{50} ratios ~ 1.5 and 0.5, respectively). Thus, tail removal greatly increases the deficit in stability of the CENP-A relative to the H3 hexamer/tetramer (CENP-Atl/H3tl C_{DNA}^{50} ratios ~ 1/7 and 1/35 on topos –1 and –2, respectively, as compared to the above ~1/2.5 and ~1/2 values before tail removal). We conclude that the hexamer/tetramer on topos –1 and –2 responds to tail removal as the first dimer does on topo –2.

We assume that the lower stability of the first dimer in CENP-A nucleosomes on ~147 bp DNA and on topo –2 is due, in part, to the R49K substitution that weakens their entry–exit histone/DNA-binding sites (see above). Similarly, the lower stability of the CENP-A hexamer/tetramer may result from the R83N substitution, since that arginine at the equivalent position of H3 interacts at SHL ± 2.5 (Figure 1(a)) and stabilizes tetrasome entry–exit. Interestingly, H3^{CATD} nucleosomes resemble H3 nucleosomes at the level of the first dimer, but respond like CENP-A nucleosomes with respect to the hexamer/tetramer (Figures 9(b) and 10(b)). This composite behavior is consistent with H3^{CATD} hybrid nature, i.e. with its arginine substitution at tetrasome entry–exit (SHL ± 2.5 at position 83; Figure 1(a)) but not at nucleosome entry–exit (SHL ± 6.5 at position 49).

Discussion

CENP-A versus the α-satellite sequence

It is plausible that an interplay may occur during evolution of CENP-A and the corresponding centromeric DNA sequence (see Introduction). Along this line, a direct influence of the DNA sequence on centromeric chromatin is shown by the recent observation that the length of the α-satellite arrays and their density in CENP-B boxes had a strong impact on nucleation, spreading and maintenance of functional centromeres in human artificial chromosomes.44 We then asked whether our in vitro reconstitution experiments using the minimal components would show any sign of adaptation of CENP-A to its α-satellite sequence?

The answer is no. This is first shown by the observation that the CENP-A octamer has no preference for binding its homologous α-satellite DNA over a heterologous 5 S DNA. Second, a similar 20–30% CENP-A-specific subset of all positions occurs on both DNAs (see Results and Figure 2). CENP-A-specific positions are necessarily tetramer-directed, while positions common to the two nucleosomes give a larger share to the binding energy contribution of H2A-H2B dimers. Removal of the unique N-terminal tail of CENP-A has no effect in our assays, pointing to the histone fold domain being responsible for the new positions. However, the L1 loop and the α2 helix of CENP-A are not responsible alone, since their substitution into H3 leads to H3^{CATD} nucleosomes that position identically with nucleosomes assembled with canonical H3 (Figure 2). The R49K substitution in CENP-A may have no effect either, as shown by the identical positioning of H3 and H3(RK) nucleosomes (Figure 2).

Easier disassembly of CENP-A nucleosomes

The first dimer

Easier removal of the first dimer in CENP-A nucleosomes relative to H3 nucleosomes by NAP-1 or heparin is a direct consequence of the CENP-A-induced 7(±2) bpsteady-state unwrapping at their entry-exit (Table 2). This unwrapping is confirmed by, and at the same time explains, the facilitated invasion of CENP-A nucleosomes by exonuclease III (Figure 3) and micrococcal nuclease.5,36 and the virtual failure of a linker histone to bind (Figure 6(a)). Identification of a hexasome as NAP-1 or heparin disassembly intermediate (Figures 7–9) implies that removal of one dimer stabilizes the other. This behavior has been observed by others upon disassembly by NAP-1 or RNA,43 and during transcription elongation by RNA polymerase II.45 Hexasomes are also the main intermediate in nucleosome assembly with NAP-1.46 Intriguingly, however, the opposite is observed upon octamer assembly from the tetramer and the dimers in high salt: binding of the first dimer enhances binding of the second.47 This result supports the notion that the free tetramer in solution does not have the proper conformation for binding dimers,48 a possible reflection of its chiral flexibility,49,50 and that it acquires it only after binding of the first dimer.

The increase in the apparent stability of the first dimer, i.e. the increase in C_{VNC}^{50}, after removal of H3/CENP-A N-terminal tail (Figure 10(b), left histogram) suggests that heparin uses the tails as “levers”. This is consistent with the tails being heparin primary targets, due to their high positive charge and availability at the outside of the nucleosomes. Interestingly, the lever appears to operate through the proximal αN/DNA-binding sites at nucleosome entry–exit (symbolized by blue dots in the schemes in Figure 10(c)). When the sites are “off” on topo –1, or when they are weakened with CENP-A relative to H3 on topo –2, the lever effect is substantially reduced, i.e. the apparent stabilities do not depend significantly on the presence or the absence of the tails (Figure 10(b)).

The second dimer

The second dimer is usually released together with the tetramer, so that little or no tetrasomes are observed in the depletion patterns (Figures 7–9). On topo –1, however, tail removal strongly destabilizes the second dimer, leading to the early appearance of tetrasomes (see H3tl and CENP-Atl in Figure 9(b)). Such a destabilization of the second dimer upon tail removal is in contrast with the correlative stabilization of the first dimer on the same topoisomer (Figure 10(b)). Such a discrepancy between the responses of
the first and second dimers to tail removal suggests that the tails have a dual role: an intrinsic role in nucleosome stabilization, as anticipated, and an instrumental role in providing heparin a lever for destabilization through the entry−exit histone/DNA-binding sites. The lever is effective to release the first dimer, while the intrinsic stabilization role of the tails dictates the behavior of the second dimer.

Does the second dimer influence tetramer stability? In other words, is the hexamer released at the same concentration of heparin as the tetramer alone? The answer to the second question is yes, at least for H3 on topo −1. The CTET−1 value of H3 tetrasomes on that topoisomer is the same, within the error bar, as the above C_DNA values of the corresponding nucleosomes (not shown). This reinforces our assumption that C_DNA values in the histogram of Figure 10(b) apply indiscriminately to the hexamer or the tetramer.

The hexamer/tetramer

The CENP-A hexamer/tetramer is less stable than its H3 counterpart, and the tails again facilitate heparin release of the second but not of the first (Figure 10(b), right-hand histogram). Thus, except for the destabilization on topo −1 relative to topo −2, which is not observed, the hexamer/tetramer behaves very much like the first dimer (see above). This suggests that the tails again act as levers. The lever can no longer work on the αN/DNA-binding sites at SHL ±6.5, which are broken in single-turn tetrasomes or hexasomes (Figure 10(c), and see the legend). But its effect is then free to propagate down to the L1 loop/DNA-binding sites at tetrasome entry−exit (SHL ±2.5 at residue 83) via the SHL ±1.5 (Figure 1(a)). The fact that binding sites at SHL ±2.5 (and SHL ±1.5) are “on” with both tops −1 and −2 (blue stars in TET-1 and TET-2 in Figure 10(c)) in turn explains the similar apparent stability of the hexamer/tetramer on both topoisomers.

The absence of a lever effect in destabilizing the CENP-A hexamer/tetramer, i.e. its tail-independent apparent stability (Figure 10(b)), may then result from the R83N substitution, which weakens tetrasome entry−exit binding sites at SHL ±2.5 (Figure 1(a)). Relevant to the lever operating via the SHL ±1.5 binding sites, is the observation that in budding yeast CENH3 (Cse4p) arginine 83 is preserved, but arginine 63 at SHL ±1.5 is substituted.

We observed above that the R49K substitution alone (in H3(RK)) achieves only ~45% of the destabilization of nucleosome entry−exit sites provided by CENP-A. The same situation may prevail for CENP-A R83N substitution, which may not entirely account for the lower stability of the CENP-A hexamer/tetramer. This possibility is supported by our observation that tetrasomes containing an H3 R83N mutant were less stable than H3 tetrasomes, but more stable than CENP-A tetrasomes (N. C. e S., unpublished results). This deficit in stability of CENP-A tetrasomes may originate from the higher compaction of the (CENP-A-H4) tetramer relative to the canonical tetramer (see Introduction). This complication is indeed likely to reflect a smaller lateral opening between the two constitutive CENP-A-H4 dimers. (Tetramer lateral opening is known to vary with the DNA sequence and in the course of the tetrasome chiral transition.) In this case, wrapping with a smaller radius of curvature would increase the DNA bending energy and thus decrease tetrasome stability. The failure to reconstitute CENP-A tetrasomes on ~147 bp DNA (Figure 7(a) and see the legend) is a vivid tribute to their low stability.

The use of the H3 tail as a lever for destabilization of the αN helix and/or of the L1 loop may be relevant to in vitro mechanisms. This is suggested by the prevalence of the arginine 49 substitution in CENP-A orthologs from mammals to yeast, with the exception of Drosophila CID where arginine 49 is preserved (but see below). Another general feature pertaining to the facilitated removal of the hexamer/tetramer is the prevalence of the arginine 83 substitution. CID is again an exception, as well as budding yeast Cse4p, with preserved arginine residues at that position. However, arginine 63 (at SHL ±1.5; see Figure 1(a)) is substituted in both organisms (by proline and by serine, respectively), suggesting some kind of compensation for the lack of substitutions at positions 49 and/or 83.

If the normal CENP-A expression level is disturbed by transfection-mediated transient overexpression, then it will mis-localize onto the chromosome arms and easier nucleosome disassembly may promote its subsequent elimination (see Introduction). Histones, and especially the inner ones, are resistant to proteolysis when included in nucleosomes, and for this reason CENP-A elimination presumably requires nucleosome disassembly. This process should be further facilitated by the virtual failure of CENP-A nucleosomes to bind the linker histone, since the absence of H1 is expected to locally break and decondense the chromatin superstructure and favor access of assembly/disassembly factors. In contrast to its lability when mis-localized in euchromatin, CENP-A relatives resist proteolysis when properly incorporated in centromeres, even when tagged with a degradation signal. The existence of a specific block against proteolysis in centromeres is suggested by its relief upon infection of human cells with the HSV-1 virus, in which case CENP-C, a centromere-specific non-histone protein, is correlatively proteolysed.
It was mentioned that in *Drosophila* embryos CID incorporates into centromeres at anaphase (see Introduction). Remarkably, fluorescence recovery after photobleaching (FRAP) showed that this incorporation is accompanied by a high exchange rate of CID, compared to H3 at the same stage of mitosis. 

This discrepancy between H3 and its variant may also reflect the easier CID nucleosome disassembly.

The above proteolytic pathway may be only an emergency mechanism activated by CENP-A over-expression, which would overwhelm a putative active targeting process. Promiscuous incorporation of normally expressed CENP-A as well would be in keeping with the simple nature of the above-mentioned soluble pre-assembly complex, which indeed points to a passive assembly mechanism. No evidence exists, however, for the occurrence of a similar complex in organisms other than Drosophila (see Introduction). Also intriguing is the capacity of H3CATD to functionally replace CENP-A (see Introduction). It indeed indicates that αN-mediated unwrapping properties of CENP-A (dimers instability) are dispensable, while showing at the same time that the weakening of the interactions between DNA and its L1 histone-fold is essential for function (see the equally low stabilities of H3CATD and CENP-A hexamer/tetramer in Figure 10(b), right histogram). It would be interesting to know, in this respect, whether transiently over-expressed H3CATD, if it also mis-incorporates, would be cleared off the chromosome arms as efficiently as CENP-A.

In conclusion, we have provided evidence that CENP-A nucleosomes are easier to disassemble in vitro than conventional nucleosomes. Remarkably, this relative instability bears on the two layers (dimers and tetramer) of the CENP-A nucleosome. If instability of the first layer appears to be dispensable (see H3CATD data above), instability of the second layer may be an absolute requirement for function. Such a dual instability may explain why and how, following transient over-expression, ectopically localized CENP-As could become specifically susceptible to proteolysis in vitro, ensuring their clearing from all regions of the chromosomes but the centromeres. Whether this scheme applies to normally expressed CENP-As as well, and so contributes to their exclusive centromeric localization, remains to be seen. In any case, the dual relative instability of the centromeric nucleosome uncovered here will likely exert constraints on future models of its function.

**Materials and Methods**

**DNAs**

DNA fragments of the pBR series, 351 bp, 353 bp, 354 bp, 356 bp, 358 bp, 360 bp and 363 bp long originate from plasmid pBR322, and the 357 bp fragment of the S series originates from *Lytechinus variegatus* S rDNA. The α-satellite DNA fragments, 346 bp, 348 bp, 350 bp, 352 bp, 354 bp, 356 bp, and 358 bp long were obtained from human DNA by PCR and cloning in plasmid pUC18, to give recombinant plasmids pNCSα(346-358).

**Histones**

H5 and native octamers (Figure 1(b)) originated from chicken erythrocyte nuclei. Histone H3-containing octamers were reconstituted from native H2A-H2B dimers purified from the native octamers by chromatography using a CM52 (Whatman) column eluted with a linear gradient of NaCl, and (H3-H4)2 tetramers made from the individual chicken erythrocyte histones as follows. H3 and H4 were purified from native octamers by reverse phase HPLC in a Vydac C4 column using an acetonitrile gradient, further processed and mixed in stoichiometric amounts to reform the tetramer as described. To form the octamers, dimers and tetramers, ~1.5 μg/μl each in 2 M NaCl and 1 mM dithiothreitol in TE buffer (10 mM Tris–HCl (pH 7.5), 1 mM EDTA), were mixed together in stoichiometric proportions, without further treatment. Such reconstituted octamers (kept frozen until use) behaved exactly like native octamers in all positioning (Figure 2) and relaxation experiments (Figures 4 and 5) (not shown). Octamers containing tailless H3 (H3tl; see the legend to Figure 1(a)), H3(RK) and H3tl(RK) were made as described above by replacing H3 with its relatives. H3(RK) and H3tl(RK) were derived from H3 and H3tl, respectively, by substitution of arginine 49 by lysine (Figure 1a) and legend) through site-directed mutagenesis (Stratagene QuikChange). Octamers containing CENP-A, tailless CENP-A (CENP-Atl) and H3(RK) and H3tl(RK) were made by mixing the above H2A-H2B dimers with (CENP-A-H4)2, (CENP-Atl-H4)2 and (H3(RK)-H4)-tetramers. Human CENP-A, CENP-Atl and H3(RK) were co-expressed with human H4 in BL21 (DE3), and tetramers were purified as described. The concentrations of component histones required for octamer reconstitutions were measured spectrometrically using ε230max = 2.75 for 1 mg/ml solutions, and final histone stoichiometries were checked by SDS-PAGE and staining with Coomassie brilliant blue (Figure 1(b)).

**Nucleosome reconstitution and relaxation**

Mononucleosomes were reconstituted with octamers on the above 32P-end-labeled DNAs, either before or after circularization into topoisomers, with plasmid DNA as a carrier, using the "salt-jump" method. The method involves a rapid dilution of the histone/DNA mixtures from 2 M to 0.5 M NaCl, at which step H5 was added when appropriate, followed by dialysis against TE buffer. Circular nucleosomes were relaxed with wheatgerm topoisomerase I (Promega Biotech) in buffer R (50 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 50 mM KCl, 5 mM MgCl₂, 0.5 mM dithiothreitol) for 30 min at 37 °C.

**Topology**

Topoisomerases were characterized by their linking number difference, ΔLk, as given by:

\[
\Delta Lk = Lk - Lk_0
\]

in which:

\[
Lk_0 = Tw_0 = N/h_0
\]

and

\[
Tw_0 = \text{DNA most probable twist,}
\]

in buffer R at 37 °C, N (bp) is the minicircle size and h₀ is the helical periodicity.
For a nucleosome in state $i$ ($i=1$–3) on topoisomer $\Delta L_k$, one has:

$$\Delta L_k = \Delta L_{k_0}(i) + \Delta L_{k_l}(i) \quad (3)$$

where $\Delta L_{k_0}(i)$ and $\Delta L_{k_l}(i)$ are the linking number differences associated with the nucleosome and the loop, respectively. Equation (3) gives $\Delta L_{k_0}(i) = \Delta L_k$ when $\Delta L_{k_l}(i) = 0$, which means that $\Delta L_{k_0}(i)$ is the total $\Delta L_k$ of the topoisomer when the nucleosome in state $i$ has a relaxed loop.

Topological and energetic parameters for each state (Table 1) were calculated from the topoisomer relative amounts versus $\Delta L_k$ curves in Figure 5(b), by fitting a thermodynamical three-state model.6,29,31

Briefly, nucleosome free energy (DNA plus protein) in state $i$ on topoisomer $L_k$ is:

$$G(i, N, L_k) = G_{sc}(i) + G_{nt}(i) \quad (4)$$

with:

$$G_{sc}(i) = \left(\frac{K_{sc}(i)}{N_i}\right)^2 \Delta L_{k_l}(i) \quad (5)$$

$G_{sc}(i)$ is the loop free energy of supercoiling, $N_i$ its size, and $K_{sc}(i)/N_i = 12$ its reduced supercoiling force constant previously calculated using the "explicit solutions" theory derived from the elastic rod model for DNA.31,65 $G_{nt}(i)$ represents the bending energy of the torsionally relaxed loop plus all the other contributions to the free energy (originating in particular from the protein). Experimentally, $G_{nt}(i)$ was measured by reference to the open state, and denoted $\Delta G_{nt}(i)$ in Table 1. Fitted parameters were used in reverse to draw the smooth curves in Figure 5.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2007.04.064

References

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