Chromatin Stability at Low Concentration Depends on Histone Octamer Saturation Levels

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ABSTRACT Studies on the stability of nucleosome core particles as a function of concentration have indicated a lower limit of ~5 ng/μL, below which the complexes start to spontaneously destabilize. Until recently little information was available on the effect of low concentration on chromatin. Using the well-characterized array of tandemly repeated 5S rDNA reconstituted into chromatin, we have investigated the effect of dilution. In this study, we demonstrate that the stability of saturated nucleosomal arrays and that of nucleosome core particles are within the same order of magnitude, and no significant loss of histones is monitored down to a concentration of 2.5 ng/μL. We observed that levels of subsaturation of the nucleosomal arrays were directly correlated with an increased sensitivity to histone loss, suggesting a shielding effect. The loss of histones from our linear nucleosomal arrays was shown not to be random, with a significant likelihood to occur at the end of the template than toward the center. This observation indicates that centrally located nucleosomes are more stable than those close to the end of the DNA templates. Itis important to take this information into account for the proper design of experiments pertaining to histone composition and the folding ability of chromatin samples.

INTRODUCTION

Over the last 10 years, the structure and composition of chromatin have emerged as key elements in regulation of gene expression. These chromatin-associated effects can be monitored at the nucleosomal level by investigating histone post-translational modifications, histone variant exchange (1), or the level of higher-order folding and compaction. The vast majority of studies on the effect of chromatin as a regulator of DNA-related nuclear functions have focused on the structure and epigenetic modifications of the primary unit: the nucleosome particle. Recently, a series of publications have demonstrated the importance of potential modulations of chromatin higher-order structure in regulating gene expression and/or DNA repair (see reviews (2–4)). The stability of nucleosomes plays a critical role in histone exchange and DNA accessibility to transcription factors or other regulatory DNA binding proteins. Early studies have associated these regulatory mechanisms with nucleosome dissociation by chromatin remodeling factors (see review by Choudhary and Varga-Weisz (5)) and nucleosome stability as a function of concentration (6–8). The stability of nucleosomes was linked with the potential recruitment of transcription factors or other regulatory proteins and was characterized by a marked tendency for histone octamers to entirely or partially dissociate spontaneously (8–10). These studies focused on the events leading to a loss of nucleosomal structural integrity. The experimental work was performed using a wide array of biochemical and biophysical methods (6,7,11–13).

Historically, the initial experiments assessing the issue of nucleosomal stability have mostly relied on nuclease sensitivity or restriction enzyme accessibility assays to determine histone-DNA interactions (14–16). Biophysical methods such as analytical ultracentrifugation (12) and circular dichroism (CD) melting (17), despite their requirements for large amounts of purified material, have significantly contributed to the determination of the effects of pH, temperature, and salt concentration on nucleosome stability. On careful reading, it appears that the experimental conditions used may have promoted spontaneous nucleosome dissociation. Recently, investigations of concentration effects on chromatin stability using in vitro reconstituted mononucleosomes have been performed. Nucleosomal integrity was assessed using a combination of techniques such as electrophoretic mobility shift assay and nuclease footprinting experiments (16). This study concluded that nucleosome core particles were unstable at concentrations lower than 5 ng/μL but did not allow direct conclusions to be drawn about the stability of longer arrays of nucleosomes. The effects of molecular crowding and electrostatic effects linked to histone tail interactions in *cis*- or *trans*- was not addressed. Recently, single-molecule studies have yielded information about the energetic requirement for nucleosome dissociation (18,19). This approach has allowed a more precise assessment of the mechanism of nucleosome disassembly at low concentration. In this study, which is an attempt to extend our understanding of the stability of nucleosome arrays as a function of concentration, we have used a gel electrophoresis method that has been specifically developed to study structural and compositional changes of chromatin or nucleosomal arrays under various conditions. The Quantitative

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Agarose Gel Electrophoresis method (referred to hereafter as QAGE (20)) uses multiple lanes of agarose at different percentages (from 0.2% to 1%) poured within the same frame (referred to as multigels) and is well suited to monitor the integrity and composition of nucleosomal arrays. The analysis is achieved by measuring the electrophoretic mobility of the samples, allowing the determination of both the surface-charge density and the effective radius of the investigated chromatin samples. These physical measurements can then be used to determine the number of nucleosomes present on a given chromatin sample, even at very low concentrations (after hybridization and Southern blotting (21)). This method of analysis has been calibrated using the well-characterized 5S rDNA 208-12 nucleosomal array model system (20,22,23). To perform our study, we used DNA templates consisting of 12 tandemly repeated 5S rDNA from *Lytechinus variegatus* positioning sequences (24) and purified core histones for in vitro reconstitution of the components into chromatin by salt dialysis (23). The QAGE results obtained with the in vitro-assembled chromatin templates were compared and calibrated against corresponding values determined experimentally by analytical ultracentrifugation (20) and atomic force microscopy (AFM) (25), demonstrating the viability and reliability of the electrophoretic method. In the experiments described in this study, chromatin samples were run in multigels at concentrations ranging from 20 to 1.25 ng/μL. Because the preparation and use of multigels is fairly time-consuming, and to maximize the use of samples and gels, QAGE multigels were loaded with samples and a T3 phage internal marker several times at regular time intervals (multiple-loading method (26)). Up to four DNA or chromatin samples of identical or different concentrations could be run on the same multigel without loss of resolution (26). Using this method, we have investigated the stability and composition of nucleosomal arrays as a function of concentration. After the determination of nucleosomal occupancy as a function of concentration of saturated nucleosomal arrays (NA), we performed a similar analysis on sub-saturated nucleosomal arrays. Finally, we investigated whether individual histones or entire octamers were lost and, if so, the location of the loss.

MATERIALS AND METHODS

Preparation of DNA template

The DNA used for the multigel and AFM experiments consists of 12 repeats of the 208 5S-rDNA from the *Lytechinus variegatus* (sea urchin) nucleosome positioning sequence isolated from the plasmid pPolI 208-12 (22) using the procedure described in Adkins et al. (26).

In vitro reconstitution of nucleosomal arrays

Histones were purified from chicken erythrocytes as described by Hansen et al. (27). Saturated nucleosomal arrays were reconstituted by salt dialysis (28) using a molar ratio of 1:1 (histone octamer:208 bp of DNA), leading to an average of 11–12 nucleosomes per template. Subsaturated NA were assembled at molar ratios (histone octamer:208 bp of DNA) of 0.8, 0.6, and 0.4, resulting in an expected 9, 6, and 4 nucleosomes (respectively) for each 208-12 DNA template (28). After reconstitution, all samples were dialyzed against 10 mM Tris, 1 mM EDTA plus 2.5 mM NaCl. The concentration of reconstituted NA was adjusted to 20 ng/μL. Samples were serially diluted as necessary. In all cases, the efficiency of reconstitution was verified by sedimentation velocity (data not shown) using an XL-A analytical ultracentrifuge (Beckman, Fullerton, CA) and analyzed using Ultrascan software (Demeler, B, UTHSCSA, San Antonio, TX).

Preparation of multigels and QAGE analysis

Agarose multigels were prepared as described by Georgel and Hansen (21). The 1.5% agarose frame initially prepared allows for 9 to 18 individual resolving agarose lanes to be poured (at agarose concentrations ranging from 0.2% to 1%). After setting for 1 h, each lane of the gels was loaded with 10–20 μL of DNA or NA samples plus 500 ng of T3 phage (the internal marker) plus glycerol (to a final concentration of 10%). For single loading experiments, the gels were run at 1.33 V/cm for 6 h in 40 mM Tris base, pH 8.0, 20 mM sodium acetate, 1 mM EDTA. For multiple loading, the samples were diluted just before electrophoresis and loaded at 1-h intervals (times 0 h, 1 h, 2 h, and 3 h). The DNA, NA, and T3 phage were detected by either SYBR green staining (for concentrations down to 2.5 ng/μL) or Southern blot (for gel containing DNA or NA at concentrations below 2.5 ng/μL (26)). The probe used to recognize the 208 DNA (5′CGA ACC CGT GGC CAG GAC CC 3′) was biotynilated at the 5′ end and detected using the Pierce North2South kit. A T3 phage probe was prepared using a similar method (26)

Sample preparation for AFM imaging

All nucleosomal arrays used for AFM experiments (50 μL at 20 ng/μL and 50 μL at 1.25 ng/μL) were prepared as indicated in the reconstitution section (see above) and then dialyzed against 10 mM Hepes, 1 mM EDTA before cross-linking in the presence of 0.1% glutaraldehyde (EM grade) plus 1 mM EDTA for 6 h at room temperature, followed by dialysis against 1 L of 1 mM EDTA for 12 h. The cross-linked NAs were deposited on 3-aminopropyltriethoxysilane-treated mica slides (25). A few microliters of the prepared chromatin samples were deposited on the treated mica slides and incubated for 40 min at room temperature. The surface was then rinsed three times with 1 mL of water.

Precipitation of Mg^{2+}-dependent nucleosomal arrays

Self-association of oligonucleosomes was induced by increasing Mg^{2+} concentration to 30 mM, and incubating at room temperature for 10 min (as described (29)). The nucleosomal arrays, at concentrations ranging from 20 ng/μL to 0.625 ng/μL, were precipitated by centrifugation at 13,500 g for 10 min in a standard tabletop centrifuge. The DNA template does not precipitate under these conditions. The pelleted chromatin samples were collected and resuspended in 1× SDS-PAGE loading dye. All samples were electrophoresed in 18% SDS gels for 2 h at 150 V. The gels were Coomassie-stained followed by a brief silver-staining enhancement (30) to compare the ratio of the core histones.

RESULTS

Validation of multiple loading QAGE method to study chromatin stability

To assess the effect of low concentration on the stability of nucleosomal arrays, we made use of the well-characterized 5S rDNA 208-12 DNA model system (22,24) after in vitro

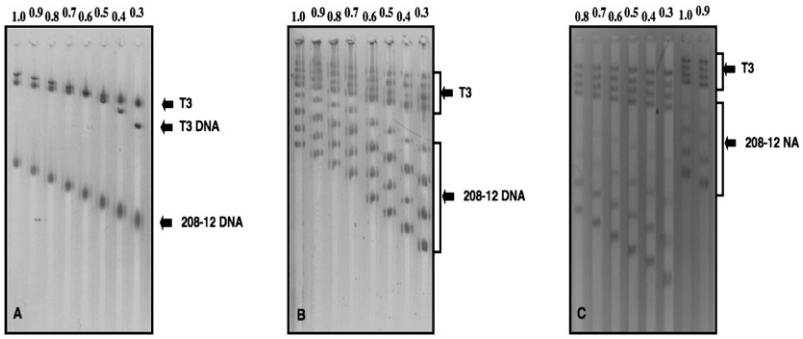

FIGURE 1 Multigel analysis after single and multiple loading. (*A*) Single loading of 208-12 DNA. The DNA (20 ng/μL) and T3 phage, used as internal marker, were electrophoresed using a standard eight-lane gel. The percentages of agarose are indicated above the lanes. The T3 phage can partially dissociate, liberating its genomic DNA (indicated as T3 DNA). Migration distances were measured and used to determine the P_e, R_e, and μ'_0. (*B*) Multiple-loading of multigel. A similar gel setup was used to analyze 208-12 DNA, at a concentration of 20 ng/μL, sequentially loaded with T3 phage at 1-h intervals. (*C*) Multiple-loading of multigel. 208-12 NA was electrophoresed at concentrations ranging from 20 to 2.5 ng/μL after sequential loading with internal control T3 phage at 1-h intervals. Note: the loading order was different from that of *A* and *B*. Migration distances were recorded and used as described above. All gels were stained with SYBR green.

chromatin assembly. To validate the use of multiple loading in multigels (26) and demonstrate that the method is suitable for our quantification analysis, we first calibrated our electrophoretic analytic method using 208-12 DNA at 20 ng/μL after regular single loading (Fig. 1 *A*), or multiple loading (Fig. 1 *B*). After 6 h of migration at 48 V, the multigels were either stained with SYBR green (Molecular Probes, Eugene, OR) or Southern blotted and probed with DNA fragments hybridizing respectively with the 3′ end of the 208-12 DNA fragment and the origin of replication of the T3 phage (see sequence in Material and Methods). The distances of migration of both the T3 phage internal marker and 208-12 DNA molecules were recorded. The surface-charge density values were calculated as described in Adkins et al. (26) (see Table 1) from these data using Eq. 1:

$$\mu/\mu'_0 = (1 - R_e/P_e)^2, \quad (1)$$

where μ represents the mobility, μ'_0 is the surface-charge density, R_e is the effective radius, and P_e is the pore size of the agarose gels. The results confirm that, as previously reported (26), the multiple loading strategy did not affect the sensitivity of the QAGE method for DNA molecule analysis.

Saturated nucleosomal arrays are stable at 20 ng/μL

A similar set of calibration experiments were performed using in vitro-reconstituted NA to confirm the validity of our multiple-loading procedure using chromatin samples instead of DNA. Before the electrophoresis experiments, 208-12 nucleosomal arrays (27) were reconstituted using the salt dialysis method (23) at a 1:1 ratio of histone to DNA to generate saturated nucleosomal arrays at a final concentration of 20 ng/μL. The NAs were analyzed by analytical ultracentrifugation to assess the level of nucleosome saturation of the DNA template. The Svedberg values (S-value) for the samples were calculated to be 28–29 S, corresponding to an average of 11–12 nucleosomes assembled per DNA template (28). A multiple loading QAGE experiment was then performed (in triplicate), using the internal control T3 phage and 208-12 NA at 20 ng/μL (26). The calculation confirmed the S-values calculated from the analytical ultracentrifugation experiments. The loading order of the samples did not affect the calculations (data not shown (26)). AFM images were prepared, and subsequent counting of nucleosomes present on the NA at 20 ng/μL matched and validated our QAGE results (Fig. 2). Of 115 NA scored, 97 had 10–12 nucleosomes assembled on the 208-12 DNA template. The number of nucleosomes observed per NA template varied from 7 to 12.

TABLE 1 QAGE analysis: μ'_0 and R_e from multiple loading of 208-12 DNA

DNA concentration (ng/μL)	Surface-charge density (μ'_0)	Effective radius (nm)
20	-2.36×10^{-4} ($\pm$ 0.01)	42 $\pm$ 0.3
10	-2.34×10^{-4} ($\pm$ 0.06)	44 $\pm$ 0.6
5	-2.35×10^{-4} ($\pm$ 0.01)	42 $\pm$ 0.5
2.5	-2.23×10^{-4} ($\pm$ 0.1)	42 $\pm$ 0.2

Saturated nucleosomal arrays experience histone loss at concentrations lower than 2.5 ng/μL

The stability of saturated 208-12 NAs was tested by QAGE as a function of concentration. As described in the previous section, in vitro-assembled saturated NA were electrophoresed after multiple loading in 0.2% to 1% agarose multigels, using samples ranging in concentration from 20 to 1.25 ng/μL (prepared by serial dilution, Fig. 3). After measurements of the electrophoretic mobilities (μ as described by Fletcher and co-workers (20)), and calculations of the surface-charge density (μ'_0) for each sample including the internal control T3 phage (Fig. 3 *A*), we determined the average number of nucleosomes per template (using Eq. 1). The calculated number of nucleosomes was graphed as a function of DNA concentration for each tested sample (Fig. 4 *A*). The results indicate that saturated NAs appeared fairly stable down to 5 ng/μL, but further dilution to 2.5 ng/μL resulted in an average loss of one or two nucleosomes per DNA molecule (Fig. 4 *A*) as monitored by measurements of surface-charge density (μ'_0). An additional serial dilution to a final concentration of 1.25 ng/μL leads to an estimated global loss of four or five nucleosomes per chromatin sample (Fig. 4 *A*). AFM

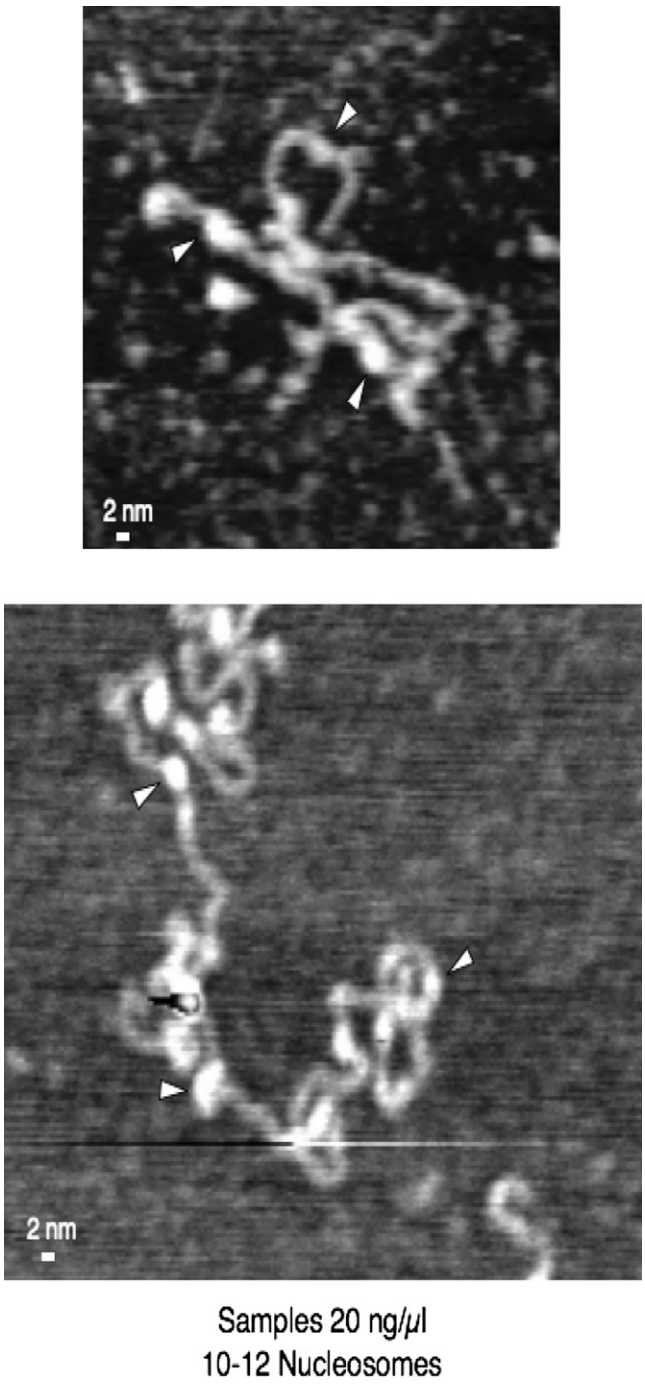

FIGURE 2 AFM images of saturated NA at 20 ng/μL. Reconstituted NAs were deposited on 3-aminopropyltriethoxysilane-treated mica slides and imaged by AFM. Individual nucleosomes are indicated by white arrowheads. The nucleosomes were counted on each individual NA. The average number of nucleosomes was determined to be 10–11 per NA template.

image analysis of NAs at a concentration of 1.25 ng/μL was performed to estimate the number of nucleosomes. Of 53 NA scored, 47 NA contained six to eight nucleosomes (examples of NA at 1.25 ng/μL are shown in Fig. 4), confirming the QAGE results. The number of nucleosomes observed per template varied from 4 to 10.

Nucleosome stability as a function of chromatin saturation: occupancy versus concentration

To determine the influence of nucleosome density (chromatin saturation) on the stability of the NAs, we performed the same type of QAGE experiments on 208-12 NAs reconstituted at a histone:DNA ratio (R) <1:1, leading to the formation of subsaturated NA. The ratios used for in vitro assembly varied by 0.2 value decrements from $R = 0.8$ to $R = 0.4$. The final NA concentration was adjusted to 20 ng/μL. After assembly, the resulting average number of nucleosomes per 208-12 DNA template was confirmed by analytical sedimentation velocity measurements (data not shown) performed under conditions similar to those described by Hansen and Lohr (28). The calculated values corresponded to an average of nine ($R = 0.8$), six ($R = 0.6$), and four ($R = 0.4$) nucleosomes per NA. The results were confirmed by QAGE measurements (Fig. 5, 20 ng/μL bars). As with the saturated NAs, serial dilutions were performed to reach concentrations of 10, 5, 2.5, and 1.25 ng/μL. The samples were electrophoresed in multigels using the multiple-loading method (in triplicate). The measurements of mobility (μ) and calculated μ_0' allowed for the determination of an average number of nucleosomes per 208-12 DNA template (Fig. 5). In contrast to observations of saturated NAs, the chromatin samples displayed an increased sensitivity to concentration. A gradual loss of histones was observed from 10 ng/μL to 2.5 ng/μL. The samples at $R = 0.4$ appeared to retain an average of two nucleosomes per template at concentrations lower than 2.5 ng/μL. The overall results indicate lower nucleosome stability for subsaturated NAs.

The disruption of nucleosomal arrays at very low concentrations suggests the loss of entire histone octamers

The multigel analysis provides information about both structural (through the calculation of the radius, R_e, of the NA) and the overall surface charge density (μ_0'). The μ_0' values indicate the presence of positively charged histones but cannot be used for precise quantification of loss of dimers of H2A-H2B or tetramers of H3-H4. To determine whether the observed changes in μ_0' are associated with the loss of individual histone dimers, tetramers, or entire octamers, we took advantage of the ability to precipitate chromatin samples by use of the oligomerization assay described by Schwarz et al. (29). Briefly, the concentration of $MgCl_2$ was raised to 30 mM, which causes spontaneous self-assembly of nucleosomal arrays. After a brief period of centrifugation, the chromatinized material can be pelleted. The pellet fractions were recovered and analyzed for content by SDS-PAGE followed by Coomassie and silver staining enhancement. It was noted that histones H2B and H2A comigrated. To evaluate the effect of concentration on the loss of individual histones, the experiment was performed using NAs at concentrations ranging

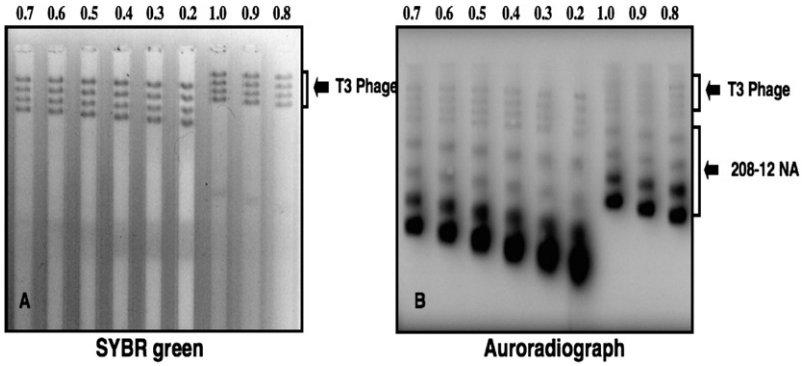

FIGURE 3 QAGE analysis of saturated NA stability as a function of concentration. (*A*) SYBR green staining of the multigel revealed the location of the internal marker T3 phage. The migration distances were used to determine the P_e of the various gels. The agarose percentages are indicated above the lanes. (*B*) Multiple loading of saturated NAs at concentrations ranging from 20 to 1.25 ng/μL. After electrophoresis, the DNA was transferred to a membrane and probed by Southern hybridization (using probes recognizing the T3 phage DNA and the 208 DNA sequence). Distances of migration of the sequentially loaded samples were used to calculate the R_e and μ'_0.

from 20 ng/μL to 0.625 ng/μL (see *lanes 1* to *4*; note that histones H2A and H2B tend to comigrate in our gel). The signal for the core histones did not significantly decrease at concentrations down to 2.5 ng/μL, indicating the presence of NA (*lanes 1* and *2*, Fig. 6) and matching our results shown in Fig. 4 *A*. The relative intensity of the bands did not change, suggesting no obvious loss of individual histones. At concentrations of 1.25 and 0.625 ng/μL (*lanes 3* and *4*, Fig. 6), at which NA are destabilized, the intensity of signal decreased as expected by undergoing a transition from NA to DNA. The observed signals for each of the bands were still indicative of equal ratios of each of the core histones, suggesting dissociation of entire octamers rather than sequential loss of H2A-H2B dimers or H3-H4 tetramers.

The loss of histone octamers is statistically more likely to happen through the ends of linear DNA

To evaluate the likelihood of the loss of histone octamers as a function of their relative position on the 208-12 DNA template, we counted the nucleosomes on AFM images prepared from 1.25 ng/μL NA samples. We arbitrarily assigned nucleosome positioning sequences 1 to 3 as ''End 1'' and 10 to 12 as ''End 2'' and positions 4 to 9 as ''Middle.'' The analysis of 51 separate NAs yielded a distribution that clearly indicates a higher stability of nucleosomes deposited in the ''Middle'' position. The mean occupancy was calculated to be 7 nucleosomes per NA, with 4.5 nucleosomes in the ''Middle'' and a combined 2.5 nucleosomes located on positions ''End1'' and ''End 2.'' Paired *t*-tests were used to analyze the differences between the number of nucleosomes in the ''Middle'' versus ''End'' positions (Table 2). The calculated *p*-value was 8.235×10^{-8}, indicative of a significant difference between loss of nucleosomes from the ends rather than the middle positions. The mean difference between ''Middle'' and ''End'' positions was 2.137, with a 95% confidence interval of 1.469 to 2.805. These results clearly indicated a preferential loss of histone octamers from the ends of our linear DNA template.

DISCUSSION

This work demonstrates the utility of the multiple-loading QAGE method for the determination of NA stability. One caveat of the QAGE method is its limitation in the range of salt concentrations that can be used. The presence of monovalent cations such as K+ or Na+, even at fairly low concentrations (60–100 mM range), generates excessive temperatures and current that can induce spontaneous dissociation of nucleosomes and electrophoretic migration artifacts. In addition, that same monovalent cation concentration range has been shown to promote histone loss from nucleosome core particles (16). We decided to perform our experiments at a low NaCl concentration (2.5 mM) to permit direct comparison with a vast array of chromatin-related publications pertaining to NCP and NA stability using analytical ultracentrifugation (2,3,7,13,19–23) and/or QAGE methods (28,29,31). The study focused on the effect of concentration and histone octamer saturation of the DNA templates on the stability of chromatin. It also describes the importance of the location of the nucleosome on the linear chromatin, indicating a higher propensity for losing histones from linear samples through an ''end effect.''

Our results indicate that saturated NAs are fairly stable down to a concentration of 5 ng/μL but then start losing histones if further diluted, as indicated by a more negative surface-charge density and as confirmed by AFM images (Fig. 4 *B*). At 2.5 ng/μL, the average number of nucleosomes drops from 10–11 down to 8–9, and subsequent dilution to 1.25 ng/μL equates to an additional loss of one nucleosome per template on average. By comparing those nucleosome occupancy numbers to those obtained studying nucleosome core particle (NCP) dissociation experiments (16), the results suggest that saturated NAs are more stable than NCP. Godde and co-workers demonstrated that NCP's dissociation was facilitated by $MgCl_2$ (up to 5 mM) and KCl (60 mM) (16). The results clearly indicate that NCP assembled on *Xenopus* 5S rDNA were displaying significant histone disassembly at concentrations below 4 ng/μL. KCl concentrations >70 mM significantly affected the percentage of nucleosome disruption (36% disruption reported at 70 mM KCl (16)). The difference in stability is likely to be attributed to electrostatic interactions between adjacent nucleosomes (3), with a potential additional stabilizing effect associated with eventual chromatin folding. As has been previously reported, even at the low salt concentration used for this study, a moderate

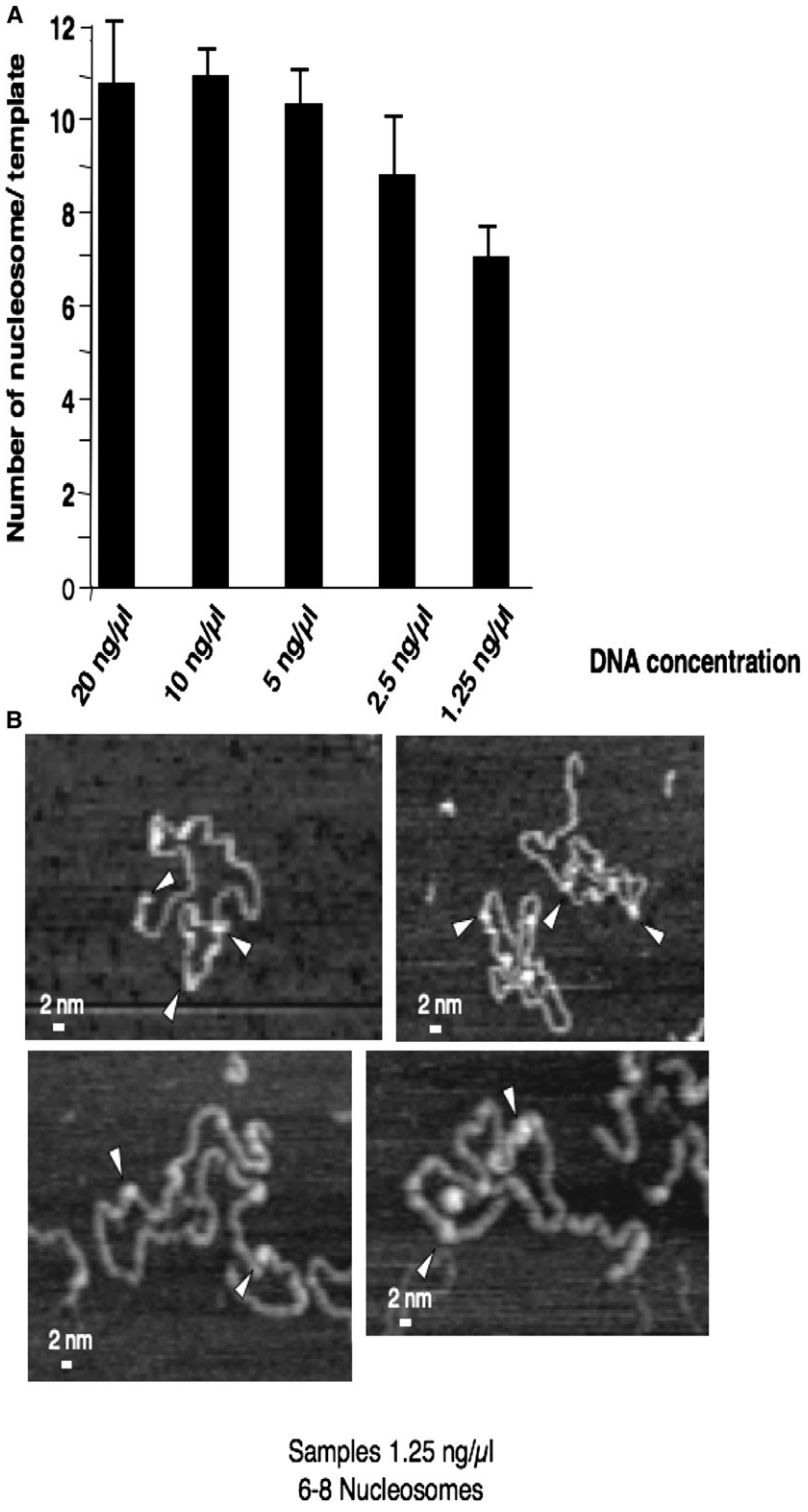

FIGURE 4 Stability of saturated NAs as a function of concentration. (*A*) The number of nucleosomes present on the NA at various concentrations was derived from the multigel analysis. The DNA concentrations are indicated on the *x* axis. The number of nucleosomes per NA is indicated on the *y* axis. (*B*) AFM images of saturated NA at 1.25 ng/μL. The average number of nucleosomes per DNA template was estimated to be between six and eight per individual NA.

amount of folding can occur, possibly contributing by promoting stability of the nucleosome-nucleosome interactions and providing an additional level of protection. These multiple layers of shielding are the most likely contributors to the difference in stability observed between the NCP and the saturated NAs. The importance of the charge density was confirmed by our analysis of NAs assembled under conditions favoring subsaturation (28). The lower nucleosome density of the subsaturated NAs was expected to correspond to a higher sensitivity to low concentrations, and our results (Fig. 5) confirmed our hypothesis. As the subsaturated NAs were exposed to low concentrations through serial

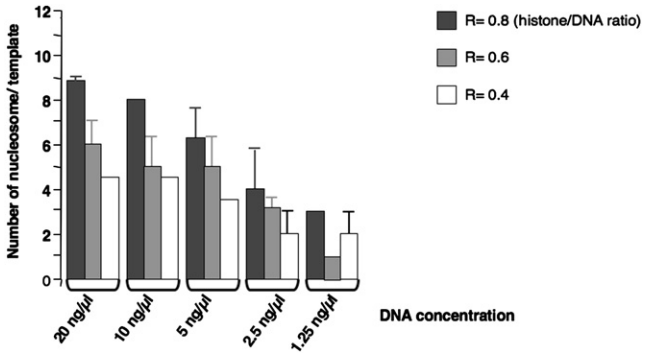

FIGURE 5 Stability of subsaturated NAs as a function of concentration. As for saturated NAs, chromatin samples assembled at ratios $R = 0.8$ (*black bars*), $R = 0.6$ (*gray bars*), and $R = 0.4$ (*white bars*) were sequentially diluted and electrophoresed in multigels. DNA concentrations are indicated on the *x* axis. The number of nucleosomes per NA is indicated on the *y* axis.

dilution, an obvious increase in loss of histones was observed (as indicated by the changes in surface-charge density). The increase in levels of subsaturation of the NAs (nine versus six versus four nucleosomes per template) exacerbated the sensitivity to dilution. The only exception that we noticed was when using NAs reconstituted at an $R = 0.4$ (four nucleosomes per DNA molecule on average at 20 ng/μL) at lower concentrations. In this case, no real difference in nucleosome occupancy was observed at the 2.5 to 1.25 ng/μL transition. One possible explanation for this result might come from the location of the remaining nucleosomes clustering toward the center of the DNA template and shielding each other, preventing histone loss. It is important to note that multiple factors such as temperature, salt concentration (16,32,33), and presence of detergent (NP-40) (19) have been reported to significantly affect stability.

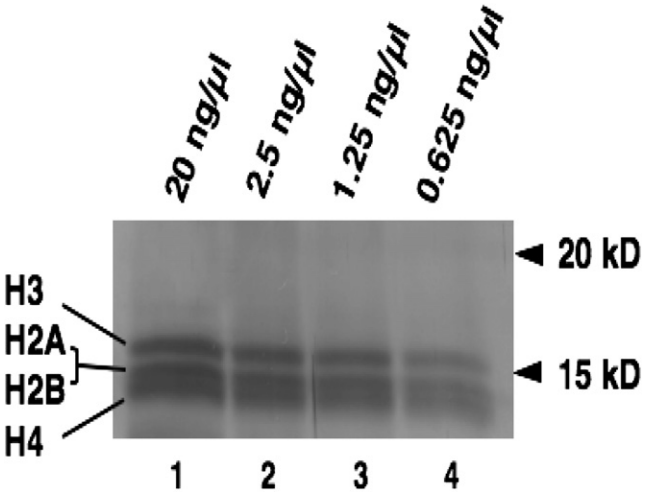

FIGURE 6 Histone composition of NAs at high and low concentrations. Coomassie staining enhanced with silver of NAs at concentrations ranging from 20 ng/μL to 0.625 ng/μL recovered after $MgCl_2$ precipitation. The location of the core histones is indicated on the left side. Note that histones H2A and H2B tend to comigrate. The black arrowheads indicate the position of the molecular weight markers.

TABLE 2 Statistical analysis of nucleosomal positions on nucleosome arrays at 1.25 ng/μL

	Nucl. in "middle" position	Nucl. at positions "end 1" + "end 2"	Total
Total number of nucleosomes	234	125	359
Mean (nucleosome/ DNA template)	4.588	2.451	7.039
Standard deviation	1.205	1.539	1.311

Nucl., Nucleosome; "middle" position refers to 5S rDNA repeats 4 to 9; "end 1" and "end 2" respectively refer to repeats 1 to 3 and 10 to 12.

In conclusion, all the results obtained with subsaturated NAs appear to confirm the link between nucleosome stability in NAs and electrostatic shielding. It is also important to note that subsaturated NAs do not fold very efficiently (28) and therefore do not provide any additional stabilizing effect through chromatin compaction. Our AFM data assessing the location of nucleosomes along the NAs at low concentration (1.25 and 0.625 ng/μL) reflected a statistically significant propensity for nucleosomes to be dissociated and lost at the end of the linear chromatin templates (Table 2).

The apparent loss of stoichiometric amounts of individual core histones suggests that the steady state, measured by SDS-page after NA precipitation and analysis of the histone composition of both pellet and supernatant, favored disassembly of histone octamers. The ratio of the individual histones associated with the chromatin in both the pellet and supernatant did not appear to vary significantly. Under our experimental conditions, the actual order of loss could not be assessed. Despite ionic conditions that do not favor sliding, the loss of nucleosomal structure may be related to histone octamer mobility (34,35). The sliding toward the end(s) of our linear template would certainly shift the equilibrium toward loss of octamers, as opposed to the sequential loss of dimers of H2A-H2B followed by H3-H4 tetramers. The DNA ends may also become partially unwound and promote loosening of the histone-DNA interactions (36). As chromatin is being remodeled near a DNA double-strand break (37), the loss of histone octamers from the ends may be a relevant issue in the case of DNA double-strand breaks. The rate of recruitment of the various proteins (DNA damage sensor MRX (38); the chromatin remodeling complex INO80 (39); Rad51 (40)) involved in DNA repair may be influenced by core histone loss.

In conclusion, our data clearly indicate the importance of using saturated NAs at a concentration greater than 5 ng/μL to prevent spontaneous loss of nucleosomes. This information can become critical when assessing loss of histones or nucleosomes through active mechanisms of chromatin remodeling. The potentially observed histone depletion from NAs may be directly linked to spontaneous loss and may not involved any other proteins. In addition, we have demonstrated that subsaturated NAs are even more sensitive to low concentrations than their saturated counterparts. The increased instability should be taken into account in

determining the experimental conditions. It also implies that the level of saturation of the chromatin used for any experiments should be tested and should remain above 20 ng/μL. The location of nucleosome depletion or loss may also be addressed in a different manner as we observed that loss of histones is also significantly more likely to occur at the ends of linear chromatin templates than at the center.

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