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Magnesium-dependent Association and Folding of Oligonucleosomes Reconstituted with Ubiquitinated H2A*

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The $MgCl_2$-induced folding of defined 12-mer nucleosomal arrays, in which ubiquitinated histone H2A (uH2A) replaced H2A, was analyzed by quantitative agarose gel electrophoresis and analytical centrifugation. Both types of analysis showed that uH2A arrays attained a degree of compaction similar to that of control arrays in 2 mM $MgCl_2$. These results indicate that attachment of ubiquitin to H2A has little effect on the ability of nucleosomal arrays to form higher order folded structures in the ionic conditions tested. In contrast, uH2A arrays were found to oligomerize at lower $MgCl_2$ concentrations than control nucleosomal arrays, suggesting that histone ubiquitination may play a role in nucleosomal fiber association.

Although for many years histones were thought to be merely structural components of nucleosomes, the primary level of DNA organization required to compact the genome in the nucleus, they are now recognized as important players in the mechanisms underlying gene expression. One of the keys to chromatin's dynamic nature is post-translational modification of the flexible histone tails. These modifications include acetylation, phosphorylation, methylation, and ubiquitination (1–3). Ubiquitin is a small, mainly globular and highly conserved protein consisting of 76 amino acids found, as its name implies, in most living organisms. Ubiquitin has been found to be conjugated *in vivo* to histones H2A, H2B, H3, and H1 (4–7). Ubiquitin is reversibly attached to bovine H2A by means of an isopeptide bond between its terminal glycine and the ϵ amino group of H2A lysine 119 (8), which lies in the trypsin-accessible region of the carboxyl-terminal tail (9). Histones are among the most abundant ubiquitin-protein conjugates in higher eukaryotes, where 5–15% of the total H2A is ubiquitinated (10). The function of histone ubiquitination remains unclear. Although ubiquitin has been shown to play an important role in the degradation of many short-lived proteins (for reviews see Refs. 11, 12), two independent studies have shown that ubiquitination does not tag histones for degradation (13, 14). Nucleosomes can be reconstituted with two molecules of uH2A or uH2B[1] without obvious perturbation of the nucleosomal structure (15, 16). Although some studies have reported an enrichment of uH2A in transcriptionally poised or active chromatin (17, 18) others do not find this correlation (19–21). Moreover, inhibition of transcription does not alter the levels of uH2A in a variety of cell lines (22–24), whereas inhibitors of hnRNA synthesis were found to cause a decrease in uH2B levels (23, 24). Cell cycle studies have shown that, in cells undergoing mitosis, uH2A levels decrease progressively to non-detectable levels at metaphase but increase again in late anaphase (14, 25, 26). Based on these and other observations, several authors have proposed that H2A ubiquitination could perturb chromatin structure (*e.g.* Refs. 6, 17, 18), but until now this model has not been tested. In this report we analyze the folding of defined nucleosomal arrays containing uH2A in response to $MgCl_2$ using quantitative agarose gel electrophoresis and analytical centrifugation.

EXPERIMENTAL PROCEDURES

Materials—Fresh calf thymus and whole chicken blood were obtained from the local abattoir.

Ubiquitinated histone H2A was purified from calf thymus as described previously (16, 27). All chemicals were of reagent grade.

Preparation of Template DNA—The DNA template (208-12) consisting of 12 tandem repeats of a 208-bp sequence derived from *Lytechinus variegatus* 5 S rDNA was amplified and purified from plasmid p5S 208-12 (a kind gift from Dr. R. T. Simpson (28)). The plasmid was purified using Nucleobond (Machery-Nagel) columns followed by *Hha*I digestion. Template DNA thus excised was purified from the remainder of the plasmid by centrifugation through a linear 5–12% (w/v) sucrose gradient in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 16 h at 4 °C at 30,000 rpm in a Beckman SW 40 Ti rotor. Template DNA was concentrated from selected fractions by ethanol precipitation.

Octamer Reconstitution—Reconstitution of control or uH2A octamers containing either calf H2A or uH2A instead of H2A in the chicken erythrocyte octamer was carried out as described previously (16, 29).

Nucleosomal Array Reconstitution—Nucleosomal arrays were reconstituted from control or uH2A octamers and template DNA by salt gradient dialysis (30) as described previously (31). A ratio of 1.3 mol of histone octamer to 1 mol of 208-bp DNA was used to generate saturated nucleosomal arrays. Reconstitutes were stored at 4 °C no longer than 1 week.

Quantitative Agarose Gel Electrophoresis—A nine-lane multigel system as described by Hansen and co-workers (32–34) was used to determine the electrophoretic mobilities (μ) of reconstituted nucleosomal arrays in 0.2–3.0% (w/v) agarose. Running gels were prepared in E buffer (40 mM Tris-HCl, 0.25 mM EDTA, pH 7.8) containing a final concentration of 0 or 2 mM $MgCl_2$. Samples containing 0.6 μg of bacteriophage T3 standard and 0.5 μg of nucleosomal array were dialyzed for 4 h against running buffer prior to electrophoresis at 2.65 $V.cm^{-1}$ at 20 ± 2 °C for 8 h with buffer recirculation. Control and uH2A arrays were analyzed in parallel on each multigel. Samples were visualized by UV illumination after ethidium bromide staining. The gel free migration was calculated by extrapolation of the line fitted by linear regression to a plot of migration distance *versus* percentage agarose concentration (≤1% (w/v) agarose) to 0% agarose. The gel-free migration was converted to the gel-free mobility (μ_0'), which was then corrected for electro-osmosis and normalized as described previously (34) to obtain μ_0. The average gel pore radius (P_e) and effective radius (R_e) of nucleo-

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[1] The abbreviations used are: uH2A and uH2B, ubiquitinated histones H2A and H2B; bp, base pair(s); μ_0, gel-free electrophoretic mobility; R_e, effective macromolecular radius; P_e, effective gel pore size.

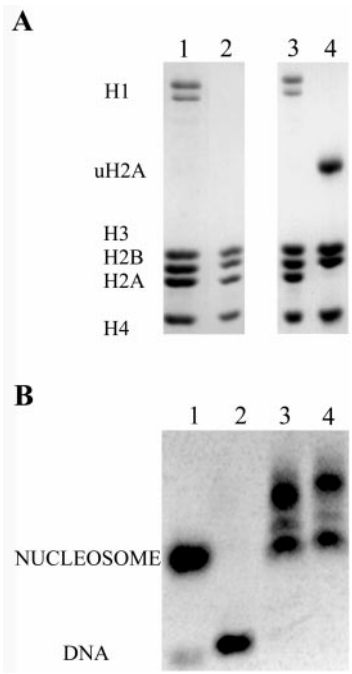

FIG. 1. **Reconstitution of control and uH2A nucleosomal arrays.** *A*, SDS-polyacrylamide gel electrophoresis analysis of reconstituted control and uH2A hybrid octamers (*lanes 2* and *4*). A total acid extract of calf thymus nuclei is included as a standard in *lanes 1* and *3*. Bands were visualized by staining with Coomassie Blue G-250. *B*, comparison of saturated control and uH2A nucleosomal arrays. Nucleoprotein gel of native 167-bp core particles (*lane 1*), *Ava*I digestion products of: naked 208-12 template DNA (*lane 2*), control nucleosomal arrays (*lane 3*), and uH2A nucleosomal arrays (*lane 4*). Bands were visualized under UV illumination after ethidium bromide staining.

somal arrays was determined as described previously (32–34).

Analytical Ultracentrifugation—Sedimentation velocity analyses (35) were carried out on a Beckman XL-A analytical centrifuge using an An-55 Al (aluminum) rotor and double sector cells with aluminum-filled Epon centerpieces as described elsewhere (36, 37). Runs were routinely carried out at 20 °C in 10 mM Tris-HCl (pH 7.5) 0.1 mM EDTA or in E buffer. The UV scans (260 nm) were analyzed using XL-A Ultra Scan version 4.1 sedimentation data analysis software (Borries Demeler, Missoula, MT). The average sedimentation coefficient ($s_{20,w}$) values were determined by second moment analysis (38).

Differential Centrifugation Assay for Nucleosomal Array Oligomerization—The solubility of nucleosomal arrays at an A_{260} of 1.6 in increasing $MgCl_2$ concentrations was determined as described previously (39).

RESULTS

Reconstitution of Control and uH2A Nucleosomal Arrays—Ubiquitinated H2A (uH2A) and H2A were purified from an acid extract of calf thymus and reconstituted with equimolar quantities of chicken H2B, H3, and H4 to form uH2A and control octamers, respectively (Fig. 1*A*). The integrity of the octamers was confirmed by dimethylsuberimidate cross-linking (29) (not shown). Control and uH2A nucleosomal arrays were prepared by salt gradient dialysis reconstitution (30) of control or uH2A octamers onto a 208-12 DNA template (28). The DNA template is a tandem array of 12 copies of a 208-bp DNA fragment from the *L. variegatus* 5 S rRNA gene (28). Nucleosomal arrays must be saturated with nucleosomes to fold maximally in response to $MgCl_2$, because previous studies have shown that the extent of folding decreases significantly as the number of nucleosomes per array decreases (32). Arrays are considered to be saturated when 12 nucleosomes are reconstituted, because each 208-bp repeat contains a nucleosome-positioning sequence. The degree of array saturation was determined by analysis of the products of *Ava*I digestion of nucleosomal arrays (Fig. 1*B*). The *Ava*I site has been shown to flank the principal nucleosome-positioning site on this template (40, 41). Thus digestion of saturated arrays should give rise to mononucleosomes whereas 208-bp free DNA monomers are also produced upon digestion of subsaturated arrays. No free DNA was detected following *Ava*I digestion of either nucleosomal array. Densitometric quantitation of Fig. 1*B* showed that mononucleosomes constituted 45 and 42% of total reconstituted species in control and uH2A arrays, respectively, indicating saturation of both arrays (31). uH2A nucleosomes exhibited reduced mobilities compared with that of control nucleosomes in agreement with previous observations (15, 18). The oligomers of slower migration seen in Fig. 1*B* are the result of nucleosomes not being uniquely positioned on 208-12 DNA and some minor positions blocking the *Ava*I restriction site (41). Micrococcal nuclease digestion of saturated control and uH2A arrays gave rise to a well-defined nucleosomal ladder at intermediate stages of digestion (not shown). There was no significant difference in the rate of digestion of the two arrays, and extensive digestion produced mononucleosomes in both cases.

Quantitative Agarose Gel Electrophoresis—$MgCl_2$-induced folding of nucleosomal arrays was analyzed by quantitative agarose gel electrophoresis (32, 33). Nucleosomal arrays were electrophoresed in multigels of concentrations ranging from 0.2 to 3% (w/v) agarose in E buffer or E buffer containing 2 mM free Mg^{2+}. Data from multigels were used to generate Ferguson plots, which were convex in shape (32, 34) for both control and uH2A arrays (Fig. 2, *A* and *B*). The data from the linear portion of the Ferguson plots were used to calculate μ_0, the gel free mobility (Table I), which is a measure of the average electrical surface charge density of a macromolecule (42). The extent of array saturation has been shown to influence μ_0 values. The μ_0 value obtained for control arrays in low salt buffer (Table I) lies within the range of -1.82 ± 0.04 to $-1.92 \pm 0.02 \times 10^{-4}$ $cm^2.V^{-1}.s^{-1}$ previously reported for saturated arrays reconstituted on the same DNA template (31, 34, 43, 44) thereby providing further confirmation that array saturation was achieved. In the presence of 2 mM Mg^{2+}, the gel free mobility of control arrays decreased by 45% as the arrays adopted a more compact structure (32). The gel free mobility of uH2A arrays was 10% lower than that of control arrays both in the absence and in the presence of 2 mM free Mg^{2+}. In E buffer, this reduction in μ_0 corresponds to either an increase of 25 to 30 positive charges per octamer (32) or to the shielding of an equivalent number of charges per octamer or a combination of both effects. The later explanation is more likely, because at pH 7.0, ubiquitin has 11 acidic and 11 basic residues, of which only three of the seven lysine residues are not involved in intramolecular contacts and are fully exposed on the surface of the molecule (45). The 44% reduction of μ_0 values observed for uH2A arrays in E buffer + 2 mM Mg^{2+} was comparable to that of control arrays.

Quantitative agarose gel electrophoresis data can also be used to determine an average R_e, which can be correlated to the surface area of a rod-like nucleosomal array at low agarose concentrations (<0.6% w/v) (32, 34) as well as to the frictional coefficient derived from the average sedimentation coefficient (46). The effective radii (R_e) of control and uH2A arrays remained essentially constant at all agarose concentrations in E buffer without and with 2 mM $MgCl_2$ (Table II), whereas naked template DNA (data not shown) was found to reptate at smaller pore sizes as reported previously (32). The R_e values obtained for control arrays at pore sizes ≥200 nm (Table II) correlate well with previous estimates of 26–28 nm in E buffer and 20.5–22 nm in the presence of 2 mM $MgCl_2$ (32, 34, 43, 44) for equivalent arrays under the same electrophoretic conditions. No significant difference in R_e values of control and uH2A

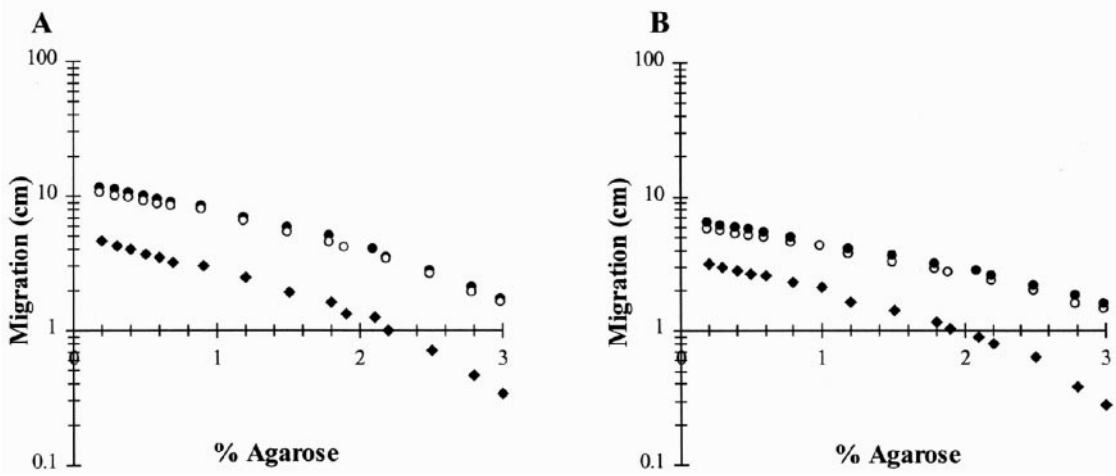

FIG. 2. **Ferguson plots of bacteriophage T3 (♦), saturated control (●) and uH2A (○) nucleosomal arrays in E buffer (*A*) and E buffer containing 2 mM free $MgCl_2$ (*B*).** The gel free migrations were converted to gel free mobilities to determine μ_0 as described under "Experimental Procedures."

TABLE I
Gel free mobility, μ_0 ($\times 10^{-4}$ $cm^2 \cdot V^{-1} \cdot s^{-1}$) values of nucleosomal arrays in low and high salt buffer

Array	E buffer	E buffer + 2 mM $MgCl_2$
Control	−1.88 ± 0.04	−1.052 ± 0.02
uH2A	−1.62 ± 0.02	−0.94 ± 0.01

TABLE II
R_e values of nucleosomal arrays in low and high salt buffer
Values represent the mean ± S.E. of eight determinations.

Array	E buffer	E buffer + 2 mM $MgCl_2$	R_e Mg^{2+}/R_e[a]
		nm	
Control	26.4 ± 3.8	22.4 ± 0.5	0.85 ± 0.14
uH2A	25.0 ± 2.7	22.0 ± 0.3	0.88 ± 0.1

[a] The S.E. was calculated according to the equation: % = [(%S.E. R_e Mg^{2+})^2 + (%S.E. R_e)^2]$^{1/2}$ (32).

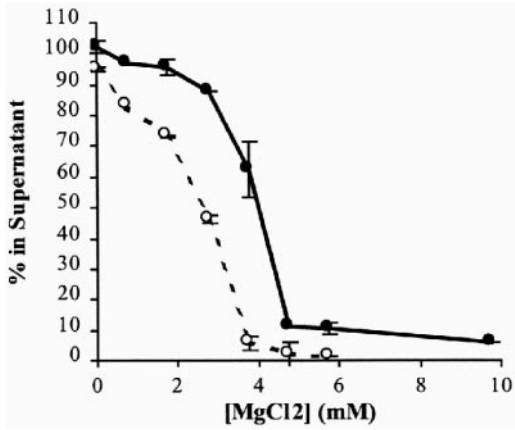

FIG. 3. **Mg^{2+}-dependent oligomerization of saturated control (●) and uH2A (○) nucleosomal arrays.** Arrays were incubated in increasing concentration of $MgCl_2$ for 10 min at room temperature before centrifugation. Unaggregated arrays remain soluble in the supernatant. Each point represents the average of two to three determinations.

arrays was observed (Table II), suggesting that uH2A has little effect on the compaction of nucleosomal arrays under these experimental conditions.

Oligomerization—Nucleosomal arrays have been shown to oligomerize rapidly in response to increasing concentrations of divalent salts (39, 47). This association is reversible upon removal of the salt by extensive dialysis (39) and is distinct from the folding process (46). Some evidence suggests that the results obtained from *in vitro* oligomerization of relatively short chromatin fragments may be significant regarding the *in vivo* interaction of chromatin fibers during chromosomal condensation (39). Histone tails have been shown to play an important role in this process as their absence (39, 48, 49) or acetylation (43) hinder Mg^{2+}-induced oligomerization of nucleosomal arrays. Fig. 3 shows that uH2A arrays oligomerized at lower Mg^{2+}concentrations than control arrays. Control arrays were 50% oligomerized at ~4 mM $MgCl_2$ in close agreement with previous results (48), whereas uH2A arrays were almost fully oligomerized at this concentration. Because the gel free mobility of uH2A arrays was 10% lower than that of control arrays (Table I), ubiquitin may thus shield some of the DNA charge, thereby facilitating the aggregation process. Ubiquitin itself may also provide additional surfaces for inter-array contacts.

Analytical Ultracentrifugation—Sedimentation velocity experiments were next used to monitor the effect of H2A ubiquitination on nucleosomal array folding in response to increasing $MgCl_2$ concentrations (Fig. 4). The 208-12 oligonucleosome complexes used in these experiments consisted of 11–11.5 nucleosomes per DNA template determined as described elsewhere (35). The histone loading was kept slightly under-saturated because of the oligomerization problems discussed in the previous section. The sedimentation coefficients of uH2A arrays obtained in this way were on average 11% lower than that of control arrays (Fig. 4*B*) regardless of whether the experiments were carried out in 10 mM Tris-HCl (pH 7.5 buffer) (Fig. 4, *A* and *B*) or in E buffer (40 mM Tris-HCl) (results not shown). This could be due to the lesser histone loading of the DNA template (50) and/or of a slight increase in the frictional parameters of uH2A arrays due to the presence of two ubiquitin molecules per nucleosome. Nevertheless, increases in $s_{20,w}$ values of uH2A arrays in response to $MgCl_2$ paralleled those of control arrays, and in 2 mM $MgCl_2$ uH2A arrays attained a degree of compaction similar to that of control arrays (Fig. 4*C*) (31) where $s^{+MgCl_2}/s^{-MgCl_2} = 1.35$. This 35–40% increase in the sedimentation coefficient has been correlated with the formation of an intermediately folded species such as an open helix (31, 37, 47). The results presented in Figs. 2 and 4 show that the presence of ubiquitin attached to H2A does not hinder this degree of nucleosomal array compaction.

DISCUSSION

Histone modifications such as acetylation and phosphorylation mediate changes in chromatin largely through alteration of the charge of amino acid residues in the amino-terminal

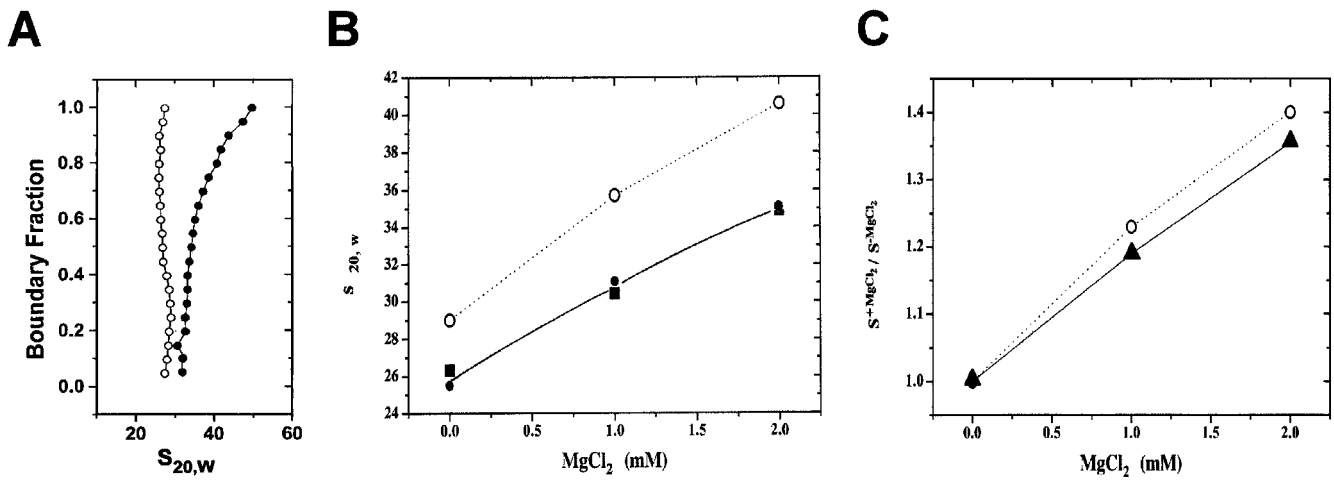

FIG. 4. **Magnesium dependence of the sedimentation coefficient ($s_{20,w}$) of 208-12 nucleosome arrays.** *A*, integral distribution of the sedimentation coefficient of uH2A containing nucleosome arrays in the absence (○) or in the presence of 2 mM $MgCl_2$ (●). The integral distributions were obtained after analysis of the sedimentation boundaries using the method of van Holde and Weischet (58). *B*, uH2A-containing nucleosome arrays (●, ■) are compared with nucleosome arrays reconstituted with native histones (○) (32). The data shown by (●, ■) were obtained from two completely independent reconstitution experiments. *C*, same results as in *panel B* plotted as in (32). In this representation, the sedimentation coefficient of the arrays at a given magnesium concentration (S^{+MgCl2}) are plotted relatively to the sedimentation coefficient of the arrays in the starting buffer in the absence of magnesium (S^{-MgCl2}). Nucleosome arrays reconstituted with native histones (○) average of the two sets of experimental data for nucleosome arrays reconstituted with uH2A (▲). All experiments were carried out at 20 °C and at 18,000 r.p.m. (●) or 20,000 r.p.m. (■). The composition of the buffer was 10 mM Tris-HCl (pH 7.5) 0.1 mM EDTA.

histone tails. Ubiquitination is, by comparison, a bulky modification that has led researchers to postulate its function to lie in hindering chromatin folding (*e.g.* Refs. 6, 17, 18). This postulate has been difficult to confirm *in vivo*, because the enzymes involved in conjugating ubiquitin to histones are also required for the ubiquitination of many other proteins that may directly or indirectly affect chromatin folding. We have therefore used an *in vitro* model system to assay the impact of histone H2A ubiquitination on the Mg^{2+}-induced folding and oligomerization of nucleosomal arrays. Moreover, the extent of H2A ubiquitination used in this study was far greater than that *in vivo* where it is more common for only one H2A molecule to be ubiquitinated per nucleosome (51). In the absence of linker histones, nucleosomal arrays equilibrate between moderately folded and extensively folded structures in buffers containing 2 mM $MgCl_2$ (31, 37, 47). The data obtained from quantitative agarose gel electrophoresis (Tables I and II) and analytical ultracentrifugation (Fig. 4) show that uH2A and control arrays attained a similar extent of compaction in 2 mM $MgCl_2$ relative to low salt conditions. This indicates that uH2A does not affect this degree of nucleosomal array folding. Thus, although the tail domains of histones are crucial for the salt-induced folding of nucleosomal arrays (37, 48, 49, 52), the carboxyl-terminal tails of H2A can be ubiquitinated without much impact on the folding process. Furthermore, the results shown in Fig. 4*A* in 2 mM $MgCl_2$ are almost identical to those previously reported for unmodified arrays (see Fig. 3*A* of Ref. 47). Therefore, it is possible to conclude that histone H2A ubiquitination neither affects the 28–40 S folding transition, which is characteristic of the histone H1-depleted chromatin in either the presence of monovalent (31, 37) or low concentrations of divalent ions (47), nor the maximum folding (40–55 S transition), which occurs at higher levels of histone saturation in the presence of $MgCl_2$ (43, 44, 47, 49).

Although support for uH2A playing a role in hindering the final stages of chromatin compaction has been provided by reports of the loss of the uH2A ubiquitin moiety at metaphase (25, 26), not all compact chromatin structures are devoid of ubiquitin. In mice spermatocytes, uH2A has been associated with the inactive sex body that contains the heterochromatic X and Y chromosomes (53), and in *Drosophila* ubiquitin has been shown to be mainly associated with the band domains of polytene chromosomes (54). Further investigations are required to determine if uH2A affects the higher degree of folding attained by nucleosomal arrays containing linker histones in response to elevated salt concentrations (44). It also remains to be investigated if ubiquitination of H2A could affect the binding of other proteins involved in the formation of mitotic chromosomes. Finally, it has been suggested that histone ubiquitination could label specific chromatin regions (26, 55) and as such could be part of the "histone code" (56). This ubiquitin tag could direct as yet unidentified or known cellular machinery such as chromatin remodeling complexes (57) to uH2A-enriched chromatin regions such as the 5′-end of the mouse dihydrofolate reductase gene (17) or the *copia* and hsp 70 genes in *Drosophila* (18).

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