Nucleoplasmin-Mediated Unfolding of Chromatin Involves the Displacement of Linker-Associated Chromatin Proteins†

Isbaal Ramos,‡ Adelina Prado,‡ Ron M. Finn,§ Arturo Muga,‡ and Juan Ausió*,§

Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada V8W 3P6, and Unidad de Biofisica (CSIC-UPV/EHU) y Departamento de Bioquímica y Biología Molecular, Universidad del País Vasco, Apdo. 644, 48080 Bilbao, Spain

Received March 1, 2005; Revised Manuscript Received April 12, 2005

ABSTRACT: We have previously characterized the interaction of nucleoplasmin with core histones and studied the possible involvement of this chaperone molecule in transcription. Here we study the interaction of nucleoplasmin with chromatin. We show that highly phosphorylated *Xenopus laevis* egg nucleoplasmin can unfold sperm and somatic chromatin in a way that involves the removal of chromosomal proteins from linker DNA regions without a stable interaction with the nucleosome. The complexes between egg nucleoplasmin and both somatic and sperm-specific linker proteins have been hydrodynamically characterized using sedimentation equilibrium in the analytical ultracentrifuge. The results are discussed within the context of the possible implication of nucleoplasmin in processes such as transcription and replication licensing which take place after egg fertilization at the onset of development.

Nucleoplasmin is a pentameric (*1*) chaperone (*2*) protein that has been identified in the eggs of *Xenopus* and in *Drosophila*, as well as in some other vertebrate organisms (*3*). The crystallographic structure of the core of this molecule has been recently elucidated (*4*, *5*). Nucleoplasmin is the single most abundant protein of the *Xenopus* egg, where it exists in part as a complex with histones H2A and H2B, as a chaperone (*2*, *6*−*10*).

The sperm chromatin of *Xenopus* consists of a complex mixture of SNBP[1] (called SP1/2, SP3, SP4, SP5, and SP6) (*10*) as well as histones H3 and H4 but is deficient in histones H2A and H2B (*11*). Therefore, it is believed that the chaperone activity of *Xenopus* nucleoplasmin provides the histone H2A and H2B complement to the male pronucleus upon replacement of the SNBP immediately after fertilization (*12*).

In addition to its active participation in the displacement of SNBPs from the male pronucleus (*12*, *13*), nucleoplasmin has also been involved in several other functions extending to the first stages of development, for instance, replication licensing (*14*, *15*), a process that has been shown to involve removal of histone H1 (*16*). However, the detailed molecular mechanisms involved in all these processes have just started to unravel (*3*).

In this paper, we study the interaction of highly phosphorylated active egg nucleoplasmin (eNP) with chromatin from two different types of quiescent cells: chicken erythrocytes and *Xenopus laevis* sperm. The results are discussed in the context of the functional implications for chromatin remodeling during spermatogenesis and DNA metabolism during early development.

MATERIALS AND METHODS

Proteins. Recombinant NP was prepared as described elsewhere (*17*). Native NP from *X. laevis* eggs was purified as described in ref *17*, and histone H5 was purified as described elsewhere (*18*). The concentration of nucleoplasmin was determined using an extinction coefficient of 7.1 $cm^2\ mg^{-1}$ at 230 nm. SNBPs from *X. laevis* testis tissue were extracted as described in ref *19*. The proteins were partially purified by reverse phase chromatography using a C_{18} Vydac column with a gradient from 0.1% TFA to 30% acetonitrile. The main SNBP peaks corresponding to sperm-specific proteins (SP) 6, 5-4-3, and 2 (*10*) were mixed, dried with a speed-vac, and resuspended in water.

Chromatin and Nucleosome Core Particle Preparation. Chromatin complexes and linker histones were prepared as described elsewhere (*18*, *20*). The concentration of DNA was determined using an extinction coefficient of 20 $cm^2\ mg^{-1}$ at 260 nm. DNA from the chromatin samples was obtained by proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation as described elsewhere (*21*).

Analytical Ultracentrifuge Analysis of Chromatin−*Nucleoplasmin Interactions*. Sedimentation velocity runs were

† This work was supported by Natural Science and Engineering Research Council of Canada Grant OGP 0046399-02 to J.A., grants from the University of the Basque Country (13505/2001) and MEC (BFU2004-03452/BMC) (to A.P. and A.M.), and travel fellowships from the Gobierno Vasco to A.P. and I.R.

* To whom correspondence should be addressed: Department of Biochemistry and Microbiology, University of Victoria, P.O. Box 3055, Petch Building, 220, Victoria, BC, Canada V8W 3P6. Phone: (250) 721-8863. Fax: (250) 721-8855. E-mail: jausio@uvic.ca.

‡ University of Victoria.

§ Universidad del País Vasco.

[1] Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; DTT, dithiothreitol; EM, EM buffer [100 mM KCl, 2 mM $MgCl_2$, and 10 mM Tris (pH 7.5)]; NCP, nucleosome core particle; NP, nucleoplasmin (eNP, egg nucleoplasmin); N_w, nucleosome weight average; SNBP, sperm nuclear basic protein; SP, sperm protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SNBP, sperm nuclear basic protein; TCA, trichloroacetic acid.

10.1021/bi050386w CCC: $30.25
Published on Web 05/21/2005

performed in a Beckman XL-A ultracentrifuge using an An-55 Al aluminum rotor. Samples were loaded in double-sector cells with aluminum-filled Epon centerpieces (*22*). UV scans were taken at 260 nm and analyzed by the van Holde and Weischet (*23*) method using the XL-A UltraScan II version 6.0 sedimentation data analysis software (Borries Demeler, Missoula, MT). Sedimentation velocity runs were performed at 20 °C.

Sedimentation equilibrium experiments were carried out using a Beckman XL-I analytical ultracentrifuge using an An-60 Ti (titanium) rotor. The samples were loaded on six-hole charcoal-filled Epon 12 mm cells. All runs were carried out at 7 °C. The scans were analyzed using XL-A UltraScan II version 6.0 sedimentation data analysis software (Borries Demeler) using global nonlinear, least-squares curve fitting (*24*, *25*). The protein samples were analyzed for equilibrium conditions achieved at different rotor speeds (see figure legends for more details). The partial specific volumes of the proteins were calculated from their amino acid compositions using the partial specific volumes provided in ref *26*.

Chromatin−eNP Interactions. Long chicken erythrocyte chromatin consisting of a nucleosome weight average number of approximately 40 nucleosomes along with nucleosome core particles was dialyzed against 50 mM NaCl, 1 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.5) and incubated for 3.5 h at 20 °C with different amounts of eNP. Different molar ratios of pentameric nucleoplasmin ($M_r = 110\,000$) per mole of nucleosome DNA ($M_r = 137\,900$) or mole of NCP DNA ($M_r = 96\,800$) were used as indicated in the corresponding figure legends.

Chromosomal Protein−eNP Interaction. For the interaction of histones H1 and H5 and *X. laevis* SP proteins with eNP, mixtures consisting of different molar amounts of these chromosomal proteins and eNP were made in 50 mM NaCl, 1 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.5). Upon incubation at room temperature for 1 h, the complexes thus obtained were subsequently analyzed in the analytical ultracentrifuge.

Nuclear Decondensation and Fluorescence Microscopy. Demembranated sperm nuclei from *X. laevis* were prepared as reported previously (*27*), and Triton X-100-permeabilized chicken erythrocyte nuclei were prepared as described in ref *20*. The nuclear density (nuclei per milliliter) of the sperm and erythrocyte nuclear samples was estimated from the total DNA nuclear content determined from the absorbance at 260 nm of a nuclear suspension lysed in the presence of SDS (*28*). A relationship of 3×10^4 nuclei per 0.14 μg (sperm nuclei) or 0.13 μg (erythrocyte) of DNA was used (*29*). Approximately 3×10^4 erythrocyte or sperm nuclei were incubated in a final volume of 33 μL consisting of 100 mM KCl, 2 mM $MgCl_2$, and 25 mM Tris-HCl (pH 7.5) at different eNP pentamer:nucleosome equivalent molar ratios, as indicated in the figure legends. These ratios were calculated using an M_r of 110 000 for the nucleoplasmin pentamer and an M_r of 132 600 for the approximate DNA fragment (200 bp) encompassing a nucleosome. Aliquots were taken at different incubation times and diluted with DAPI to stain the DNA, and the unfixed samples were immediately photographed.

Electrophoretic Analyses of Nuclear Decondensation. AU−PAGE (5% acetic acid−12% PAGE−2.5 M urea) and AUT−PAGE (5% acetic acid−12% PAGE−2.5 M urea−6 mM Triton X-100) (*30*) were used to characterize the chromosomal proteins removed from nuclear chromatin by egg nucleoplasmin. Briefly, the corresponding sperm or erythrocyte nuclei (approximately 7 μg per assay) were incubated in 100 mM KCl, 2 mM $MgCl_2$, and 25 mM Tris-HCl (pH 7.5) with increasing amounts of NP to reproduce the same nucleosome:NP pentamer molar ratio as in the nuclear decondensation experiments. The final volume of the incubation mixture was approximately 45 μL in each sample. Upon incubation at room temperature for 1 h, the nuclear suspension was centrifuged (16000*g* for 30 min at 4 °C). The resulting supernatants contain NP and basic proteins associated with NP, and the pellets contain the basic proteins that remained associated with DNA. Two methods were used in the recovery of the proteins from these two fractions. In the case of Figure 1, the supernatants were precipitated with 20% (w/v) trichloroacetic acid (TCA) and washed with cold acetone. Proteins from the pellets were extracted with 0.4 N HCl and then treated like the supernatants. For the experiments shown in Figure 4, the supernatants were dried with a speed-vac and the dry pellets thus obtained, as well as the pellet fractions, were resuspended in approximately 5 μL each of a sample buffer consisting of 5 M urea, 5% acetic acid, 1% protamine sulfate, and a mixture of 0.05% pyronin Y and pyronin B−ferric chloride complex, and incubated at 65 °C for 15 min prior to being loaded in the gel (*31*).

RESULTS

Egg Nucleoplasmin Removes X. laevis SNBP and Chicken Erythrocyte Linker Histones with the Highest Efficiency. The extent of nucleoplasmin phosphorylation increases dramatically during the developmental transition from the oocyte to the egg stage (*32*−*34*). Using *Xenopus* egg or oocyte extracts, it has been shown that this increase in the extent of phosphorylation presumably facilitates the sperm decondensation at fertilization through a process that involves an increased efficiency in removal of SNBPs (*35*). It has also been shown that incubation of egg extracts with *Xenopus* erythrocyte nuclei results in removal of the linker histones, a process that has been related to an increase in the level of replication licensing (*16*).

To follow up on these observations and in a first attempt toward the quantification of the molecular processes involved, we incubated demembranated mature sperm nuclei and permeabilized chicken erythrocyte nuclei with highly purified rNP, oNP, and eNp. The results of such experiments are shown in Figure 1. These results basically agree with those obtained with crude extracts and show that under the same experimental conditions only the fully phosphorylated eNP is able to completely remove the non-nucleosomal, chromosomal proteins bound to internucleosomal domains (linker proteins), in both sperm and erythrocytes. In fact, under these conditions, the recombinant version of NP (which lacks any phosphorylated residue) is almost completely inert (see Figure 1A,B, lane 1), while oNP displays an intermediate activity in good agreement with its degree of phosphorylation.

Nucleoplasmin Unfolds Chromatin but Does Not Stably Interact with Nucleosomes. To characterize the protein removal process at the chromatin level, we prepared a chicken erythrocyte chromatin fraction ($N_w = 40$) and

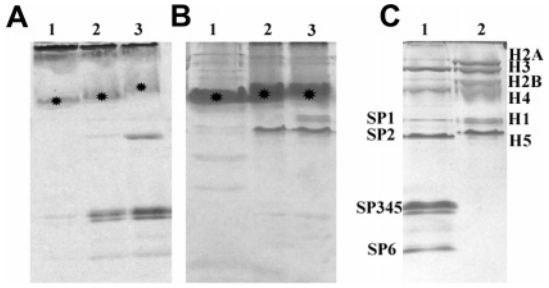

FIGURE 1: Removal of basic proteins from *X. laevis* sperm and chicken erythrocyte chromatin by recombinant (rNp), oocyte (oNp), and egg (eNp) nucleoplasmin. (A) Demembranated *X. laevis* sperm nuclei were incubated with 36 μM rNP, oNP, or eNP, and after centrifugation, the supernatants were loaded on an AUT−PAGE gel: lane 1, rNP; lane 2, oNP; and lane 3, eNP. (B) Permeabilized erythrocyte nuclei were incubated with 72 μM of different NP forms and treated as described for panel A: lane 1, rNP; lane 2, oNP; and lane 3, eNP. (C) Chromosomal proteins from the starting sperm (lane 1) and erythrocyte (lane 2) nuclei before incubation with nucleoplasmin. The identities of the different proteins bands are given. The asterisks denote the electrophoretic band corresponding to nucleoplasmin.

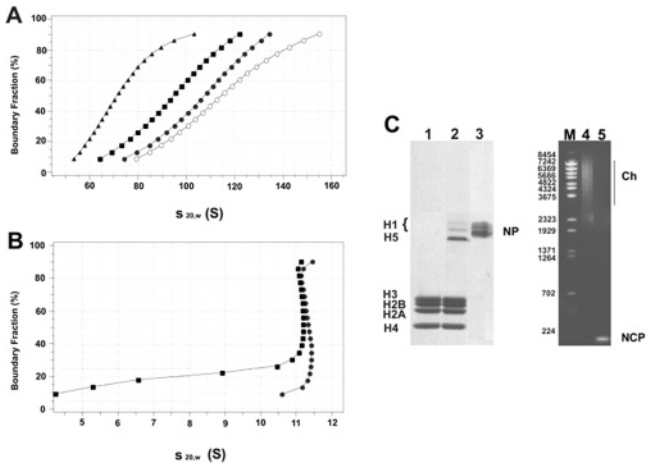

FIGURE 2: Integral distribution plots of the sedimentation coefficient of chicken erythrocyte chromatin and nucleosomes in the presence of nucleoplasmin. (A) Long chicken erythrocyte chromatin incubated in the presence of different molar eNP:nucleosome equivalent ratios: 0:1 (white circles), 0.6:1 (gray circles), 1.2:1 (black squares), and 4.8:1 (black triangles). (B) Chicken erythrocyte nucleosome core particles without (black circles) or after incubation with 0.5 mol of egg nucleoplasmin per mole of nucleosome core particle. The analytical ultracentrifuge runs were carried out at 20 °C and 22 000 rpm in panel A and 40 000 rpm in panel B. (C) Electrophoretic characterization of the protein (left-hand side, SDS−PAGE) and DNA (right-hand side, 1% agarose−PAGE) components of the samples analyzed in panels A and B: lane 1, histones from nucleosome core particles; lane 2, histones from chromatin; and lane 3, nucleoplasmin. The broad appearance (heterogeneity) of the band is due to different extents of phosphorylation which are present in the egg form of the protein (*62*). Lane M is a λDNA *Bst*EII digest used as a marker. The sizes of the different electrophoretic bands (in base pairs) are given at the left-hand site. Lane 4 is the DNA from the chromatin fraction, and lane 5 is the 146 bp DNA from the nucleosome core particles: Ch, chromatin; NCP, nucleosome core particles; and NP, nucleoplasmin.

incubated it with eNP at different eNP:nucleosome ratios. Figure 2A shows the variation of the integral distribution of the sedimentation coefficient of this chromatin fraction as the eNP pentamers per nucleosome ratio increases from 0 to approximately 5. In the absence of NP, the pattern exhibits a characterstic broad distribution of $s_{20,w}$ values due to the

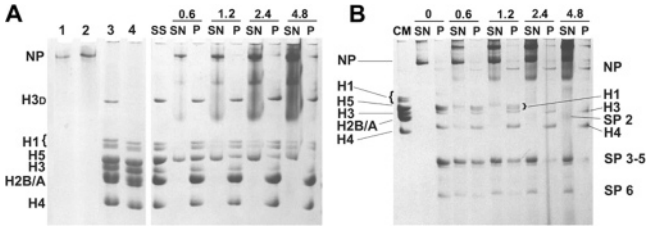

FIGURE 3: Nucleoplasmin solubilization of chromosomal proteins from nuclei. (A) AU−PAGE analysis of the proteins remaining in the nuclear pellet (P) and those associated with NP in the supernatant (SN) after incubation of chicken erythrocyte nuclei with increasing amounts of nucleoplasmin for 1 h at room temperature. Lanes 1 and 2 correspond to different amounts of eNP. Lanes 3 and 4 are histones extracted from nuclei before (lane 3) or after (lane 4) treatment with β-mercaptoethanol to indicate the position of the oxidized histone H3 dimer (H3D). SS denotes the starting sample (without NP treatment). (B) Same as panel A for *X. laevis* sperm nuclei. CM denotes the chicken erythrocyte histone marker. The molar ratios (moles of NP pentamer per mole of nucleosome equivalent) are indicated at the top of the corresponding lanes.

intrinsic heterogeneity of the chromatin fraction (*36*). The midpoint $s_{20,w}$ value of the distribution decreases as the eNP pentamer per nucleosome ratio increases up to a value of 4.8 eNP, where it reaches a value similar to that of the same fraction upon artificial depletion of linker histones (*20*, *37*).

Therefore, the above-mentioned nuclear decondensation is the result of chromatin unfolding due to nucleoplasmin-induced removal of linker histones, a process that is reminiscent of that observed in the nucleus of the H1 knockout strain (delta H1) of *Tetrahymena thermophila* (*38*).

Figure 2B shows the integral distribution of the sedimentation coefficient of individual nucleosomes in the absence and presence of nucleoplasmin. In the absence of eNP, NCPs exhibit their characteristic highly homogeneous distribution centered at ~11.5 S (*37*, *39*). This distribution is basically unaffected by the presence of eNP, since the small sample fraction sedimenting at a value similar to that found for free nucleoplasmin roughly equals the amount of added protein. Therefore, removal of linker histones from the chromatin described above does not involve a stable interaction of eNP with the chromatin fiber, a result that would be expected from the negatively charged nature of both the eNP itself and the chromatin fiber.

Removal of Linker Chromosomal Proteins Requires Approximately Five eNP Pentamers per Nucleosome. The results from the previous section suggest that five nucleoplasmin pentamers per nucleosome are required to completely unfold the chromatin fiber. To further assess this observation, we incubated demembranated sperm nuclei and permeabilized chicken erythrocyte nuclei with increasing amounts of eNP corresponding to different eNP:nucleosome equivalent molar ratios (Figure 3). As shown in this figure, by the time a ratio of 4.8 was reached, almost all the SP proteins from sperm chromatin and most of the linker histones (H1 and H5) from the erythrocyte nuclei had been removed. In the latter instance, it is important to note that histone H5 is removed more efficiently by nucleoplasmin than histone H1, a fact that may reflect either a differential accessibility of linker histones in the erythrocyte chromatin fiber (*40*) or a different affinity for eNP. This result is also in very good agreement with previous experimental data that showed a preferential removal of H1° (an amphibian equivalent of

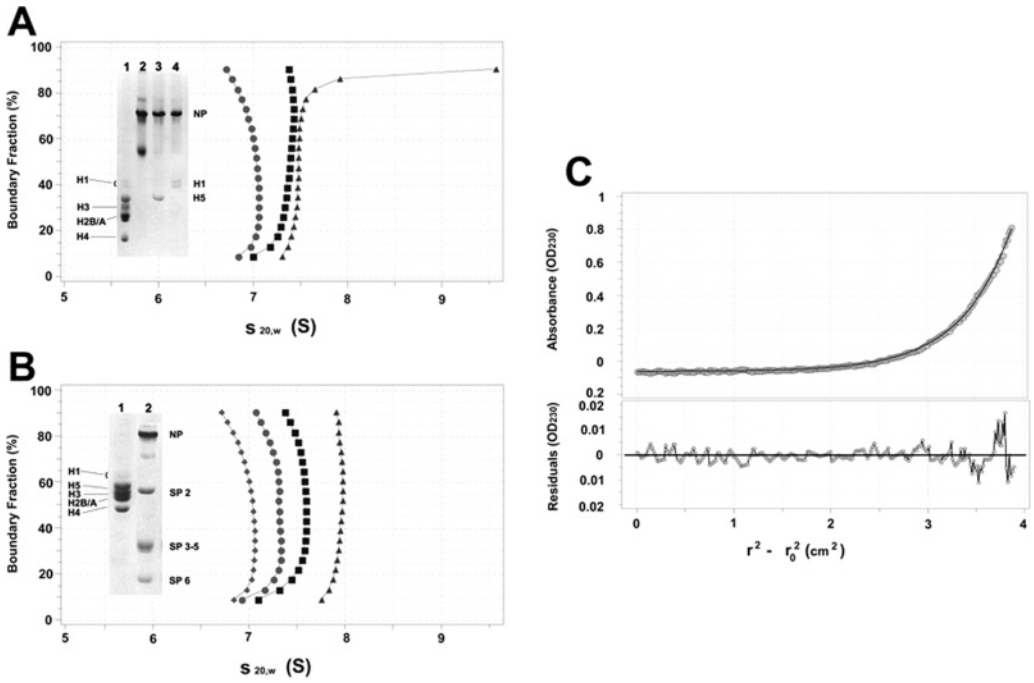

FIGURE 4: Analytical ultracentrifuge characterization of nucleoplasmin complexes with different chromosomal proteins. (A) Integral distribution plots of the sedimentation coefficient of eNP (circles), the eNP−chicken erythrocyte histone H5 complex (1 mol of eNP pentamer per 2.8 mol of H5) (triangles), and the eNP−chicken erythrocyte histone H1 complex (1 mol of eNP pentamer per 3.7 mol of H1) (squares). The inset is an AU−PAGE gel of the proteins. (B) Integral distribution plots of the sedimentation coefficient of eNP (diamonds) and eNP−*Xenopus* SP complexes at different eNP pentamer:SP proteins molar ratios: 1:2.8 (circles), 1:4.1 (squares), and 1:5.5 (triangles). The inset shows an AU−PAGE gel of the proteins in the eNP−*Xenopus* SP (10) complex (lane 2) in comparison to a chicken erythrocyte histone marker (lane 1). (C) Sedimentation equilibrium analysis of the eNP−*Xenopus* SP (1 mol of eNP pentamer per 5.5 mol of SP) complex. The top plot shows the absorbance at 230 nm as a function of the square of the radial distance of the sample at any position within the cell (r) minus the square of the radial position at a reference point (r_0) ($r^2 - r_0^2$). The continuous line in this plot was obtained by fitting the experimental data (circles) to a single ideal species with an M_r of 164 000. The bottom plot shows χ^2 residuals as a function of $r^2 - r_0^2$ for the best fit (solid line). The run was performed at 4 °C and 14 000 rpm.

Table 1: Hydrodynamic Parameters and Stoichiometry Values of NP and Its Complexes with Different Chromosomal Proteins

	H5	H1	SP	eNP	rNP
$s_{20,w}$ (S)	7.5	7.4	8.0	6.9	6.5
$[\text{protein/eNP}]_{in}$[a]	2.8[e]	3.7[f]	5.5[g]	−	−
M_r[b]	156000	192000	164000	110200	110200
$[\text{protein/eNP}]_{det}$[c]	2.4[e]	3.7[f]	5.4[g]	−	−
f/f_o[d]	1.07	1.48	1.23	1.08	1.15

[a] Initial protein:eNP (moles of protein per mole of eNP pentamer) ratio. [b] M_r experimentally determined by sedimentation equilibrium. [c] Protein:eNP (moles of protein per mole of eNP pentamer) ratio determined from the experimental M_r values. [d] Calculated as described in ref *43*. [e] Assuming M_r(H5) = 20 600 (*59*). [f] Assuming M_r(H1) = 22 000. [g] Assuming an average M_r(SP1/2−3/5−6) = 10 000.

avian H5) when *X. laevis* erythrocyte nuclei were incubated in the presence of egg extracts (*41*).

Characterization of the Complexes between eNP and Linker Histones and Sperm SP. We finally characterized the interaction between *X. laevis* NP and linker histones in solution. The results of this analysis are shown in Figure 4 and Table 1. In a preliminary titration experiment, it was found that H1−eNP, H5−eNP, and SP−eNP complexes precipitated at 4.0−4.5:1, 3.0−3.5:1, and 6.0−6.5:1 basic ligand:eNP pentamer molar ratios, respectively. Figure 4A shows the sedimentation velocity analysis of H1−eNP and H5−eNP complexes close to these saturation values. In all instances, the input protein:eNP ratios and the protein:eNP ratios determined from sedimentation equilibrium analysis of the resulting complexes are very similar or slightly lower (see Table 1), suggesting that under these conditions very little precipitation had occurred. From these results, it is thus possible to estimate the saturation ratios of the protein−eNP complexes: 3.7 mol of H1/mol of eNP, 2.4 mol of H5/mol of eNP, and 5.4 mol of SP/mol of eNP (Table 1). The value determined for the *X. laevis* SP−eNP complex is very similar to the value of 5.0 previously reported by Iwata et al. using fluorescence spectroscopy and salmine protamine (*42*). It is higher than the value of 2.5 reported for similar experimental conditions using recombinant NP (rNP) (i.e., in the absence of any phosphorylation) (*43*). This underscores the relevance of NP phosphorylation in the process of protamine (and most likely linker histones H1 and H5) removal in agreement with Figure 1, and points to the importance of electrostatics in the interaction of NP with these proteins, in contrast to what has been reported for core histones (*4*, *44*). In this regard, the ability to bind more H1 than H5 most likely reflects the lower charge density of the former when compared to the latter, and might also be related to the difference in the relative affinity of oNP and eNP for these histones (Figure 3). However, a difference in the accessibility of H1 versus H5 within the chromatin fiber cannot be disregarded (*40*).

Interestingly, with the exception of histone H1, none of the other nucleoplasmin-interacting partners studied here causes a large change in the frictional parameters of the resulting complexes (see Table 1), an indication that these proteins bind quite tightly to the nucleoplasmin pentamer.

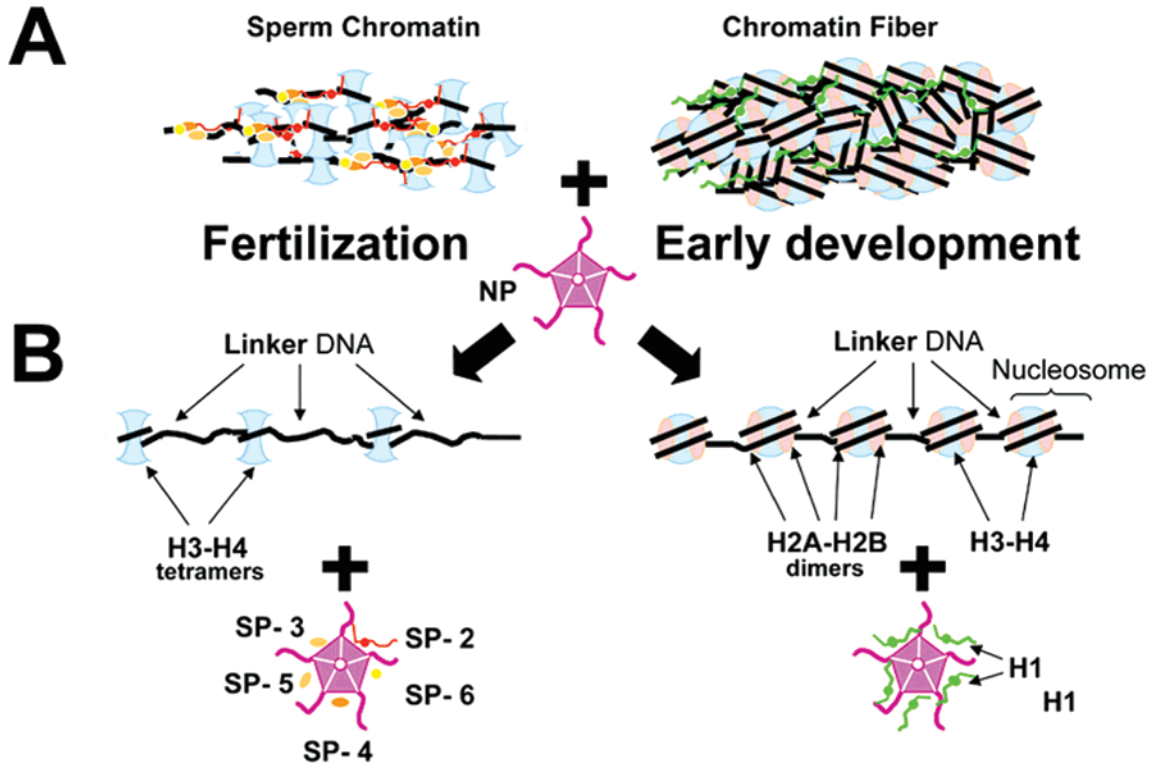

FIGURE 5: Nucleoplasmin-mediated chromatin unfolding. (A) This process plays an important role both during fertilization when the SNBPs (SP-2 and SP-6) from the male nucleus are removed immediately after fertilization (*12*, *13*) and in chromatin remodeling during the early stages of development (*14*, *15*). (B) In both instances, the unfolding is the result of the removal of the chromosomal proteins from the linker DNA domains. Neither the H3−H4 tetramers associated with *X. laevis* sperm chromatin (*11*) nor the histones from the histone octamer in the somatic chromatin fiber are removed by nucleoplasmin during this process. In addition to SNBP removal, nucleoplasmin can also add the histone H2A−H2B complement to the H2A−H2B deficient sperm chromatin during fertilization (*3*) (not shown here). This process may also be facilitated by the transient unfolding of the sperm chromatin fiber upon removal of the SP proteins. Whether deposition of H2A and H2B and removal of SPs are carried out simultaneously by the same NP molecule or are the result of two independent events is not currently known.

DISCUSSION

When taken together, the results presented in this paper clearly indicate that nucleoplasmin can unfold both sperm and somatic chromatin (Figures 2 and 3), through a process that involves removal of chromatin linker-associated proteins without any physical interaction with the nucleosomes (Figure 2). Figure 5 presents a model for the NP-mediated unfolding of sperm and somatic type chromatin. Phosphorylation plays a critical role in the process (Figure 1), adding to the notion that factors other than the polyglutamic tracts present in the primary structure of the NP molecule are very important for its functional activity (*3*, *43*, *44*). Although this finding has been proposed previously (*18*, *39*), the results presented here directly link differences in phosphorylation with selective removal of specific linker histones from DNA.

Nucleoplasmin participates in one of the more drastic chromatin remodeling processes in the eukaryotic cell, the remodeling of the male pronucleus after egg fertilization. In *X. laevis*, the sperm chromatin consists of an SP protein complement in a chromatin complex that is deficient in histones H2A and H2B (*10*). The SP components are removed during fertilization by NP which then replaces them with H2A and H2B (*12*). In contrast to other chromatin remodeling complexes such as SWI/SNF (*45*), NP does not require energy from ATP. Importantly though, and in contrast to these latter types of remodeling complexes, nucleoplasmin can have access to chromatin proteins from only the linker domains (bound to linear DNA) but not from the nucleosome-constrained regions. Access to the NCP histones requires the unwrapping of nucleosomal DNA before dissociation of histones from the complex can take place, a process that most likely involves the use of chemical energy from ATP and/or physical energy from DNA supercoiling.

The interaction of nucleoplasmin with "linker" histones, and especially the fact that phosphorylation can regulate the repertoire of linker histones that NP can extract from DNA, is very interesting and has several important implications. Notably, several linker histone-binding proteins (histones of the H1 family) have recently been described which either have chaperone activity such as NASP (*46*) or can remodel chromatin in a derepressive way targeted to specific genes such as PELP1 (*47*). Whether NP acts in a manner similar to that of this latter protein but in a more generalized nonspecific way remains to be established.

Indeed, the involvement, if any, of nucleoplasmin in transcription still remains controversial. Using a heterologous system consisting of reconstituted nucleosomes and several transcription factors (GAL4-AH, USF, and Sp1), Chen et al. (*48*) described a role of nucleoplasmin in stimulating binding of the transcription factor to nucleosomes. In the presence of the ternary complex consisting of transcription factor(s), reconstituted nucleosomes, and *Xenopus* nucleoplasmin, it was shown that the process involved the displacement of histones H2A and H2B. Partly on the basis of these results, it has recently been proposed that during transcription nucleoplasmin can operate as a "histone sink" (*49*) that

mediates the dynamic exchange of histones (*50*) that takes place during transcription of certain genes by RNA polymerase II (*51*). However, when a more homologous system was used, consisting of nucleosomes reconstituted onto a 5S rRNA gene fragment, *Xenopus* TFIIIA and *Xenopus* nucleoplasmin, no effect was observed in the enhancement of binding of TFIIIA to nucleosomes reconstituted onto the 5S DNA template (*52*). While the acquisition of transcriptional competence by somatic nuclei using *Xenopus* extracts has been described previously (*41*), the involvement of nucleoplasmin in that process remains unknown. Nevertheless, it is thus possible that after fertilization, NP exerts a generalized derepressive role. The results presented in this paper would lend support to this notion rather than to the histone sink hypothesis (*49*).

Within the same context, the finding that nucleoplasmin preferentially removes only linker-associated chromosomal proteins also has implications for the role of this protein in those vertebrate organisms that retain a canonical somatic-like core histone complement in their sperm, which occurs in several species of fish and amphibians (*53*, *54*). Interestingly, a nucleoplasmin protein has been identified and characterized in the carp (*Cyprinus carpio*) (*55*) which strongly resembles *X. laevis* nucleoplasmin in sequence. The main role of this protein in such organisms may be that of sperm histone H1 removal immediately after fertilization. Binding to histone H1 in the early developmental stages may play a critical role in DNA replication licensing (*14*, *16*). Also, if the SNBPs of the PL/P type (*54*) can be operationally considered linker binding chromosomal proteins (like linker histones) (*56*), the remodeling of the male pronucleus after fertilization could be considered an extrapolation of this role. It is important to point out here that the situation in mammals may be more complicated. Two nucleoplasmin-like proteins, NPM2 and NPM3, have been described in these organisms. While NPM3 (*57*) is possibly related to sperm decondensation, NPM2 is crucial for histone deacetylation and heterochromatin formation surrounding nucleoli in oocytes and early zygotes (*58*).

The analysis of the complexes between NP and different linker-binding chromosomal proteins using purified components, and the characteristic properties of each of these components, may help in the understanding of the differential ability of NP to dissociate these proteins from the chromatin linker DNA domains. As shown here, this activity is strongly dependent on the extent of NP phosphorylation. Such analysis can also help to explain the different stoichiometry with which these chromosomal proteins bind to NP.

Inspection of the sequences and amino acid composition of the linker chromosomal proteins studied here, in combination with the stoichiometries experimentally determined for their saturating complexes with eNP (Table 1), shows that these proteins all contain a similar number of basic residues that could be involved in ionic interactions with acidic NP. This in turn emphasizes the importance of electrostatic interactions in the stabilization of these complexes. In the case of histone H5 [which has 189 amino acids and 36 mol % basic residues (*59*)], it is possible to estimate that approximately 163 basic residues could potentially participate in complex stabilization. While this is likely an overestimate (as not all the positive charges in the chromosomal proteins may participate in the interaction), this value is strikingly similar to that calculated with *X. laevis* SP (*11*, *53*). In this instance, it is possible to estimate from the amino acid analysis of these proteins (*53*) an average positive charge of approximately 30 mol %. Taking an average amino acid length of 100 amino acids for the SP mixture and from Table 1, we can calculate an average value of ~160 ionic interactions. Despite the approximations used in the latter calculation, the value thus obtained is very similar to that calculated for H5 and that of 210 calculated for the histone H1 mixture [which has an average of 220 amino acids and 26 mol % basic residues (*60*)]. This higher estimate is presumably due to the less clustered distribution of positive charges in these molecules which results in a large number of molecules binding to the eNP pentamer, and which also results in a more asymmetric conformation of the resulting complex (see Table 1). Regardless of the details, the similarity of all these values despite the different conformation and size of the molecules compared suggests that the interaction between these molecules and eNP is electrostatically driven.

The eNP pentamer is highly phosphorylated (Figure 2C, lane 3) and contains an average of 50 (*17*) to 100 phosphates (*35*). The largest polyglutamic A2 track of NP located at the C-terminal unstructured end of the molecule has 14 glutamic acids (18 including the short A3 track closer to the C-terminus) (*3*, *43*). This means that this region, which had been initially involved in binding to basic chromosomal proteins (*61*), contains a similar number of negative charges contributed by the phosphates. Furthermore, it only accounts for approximately half of the values described in the previous section. As already indicated, it is difficult to determine the precise number of the ionic interactions involved (and most likely the above numbers are overestimated). Nevertheless, the electrostatic nature of the interaction and the contribution of the phosphates outside of the C-terminal domain [at the "distal face" of the pentameric NP core (*62*)] may explain the lower stoichiometric values observed for the interaction of protamines with recombinant NP (*60*) and the higher efficiency of binding to linker histones observed in Figure 1.

Thus, while the chaperone activity of NP appears to be driven by stereospecific recognition of the core histones (mainly H2A and H2B) (*4*, *44*), the chromatin decondensing activity studied here appears to involve a strong electrostatic component with phosphorylation of the molecule being an important part of it.

REFERENCES

1. Earnshaw, W. C., Honda, B. M., Laskey, R. A., and Thomas, J. O. (1980) Assembly of nucleosomes: The reaction involving *X. laevis* nucleoplasmin, *Cell 21*, 373−383.
2. Laskey, R. A., Honda, B. M., Mills, A. D., and Finch, J. T. (1978) Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA, *Nature 275*, 416−420.
3. Prado, A., Ramos, I., Frehlick, L. J., Muga, A., and Ausió, J. (2004) Nucleoplasmin: A nuclear chaperone, *Biochem. Cell Biol. 82*, 437−445.
4. Dutta, S., Akey, I. V., Dingwall, C., Hartman, K. L., Laue, T., Nolte, R. T., Head, J. F., and Akey, C. W. (2001) The crystal structure of nucleoplasmin-core: Implications for histone binding and nucleosome assembly, *Mol. Cell 8*, 841−853.
5. Namboodiri, V. M. H., Dutta, S., Akey, I. V., and Head, J. F. (2003) The crystal structure of *Drosophila* NLP-core provides insight into pentamer formation and histone binding, *Structure 11*, 175−186.

6. Mills, A. D., Laskey, R. A., Black, P., and De Robertis, E. M. (1980) An acidic protein which assembles nucleosomes in vitro is the most abundant protein in *Xenopus* oocyte nuclei, *J. Mol. Biol. 139*, 561−568.
7. Kleinschmidt, J. A., Fortkamp, E., Krohne, G., Zentgraf, H., and Franke, W. W. (1985) Co-existence of two different types of soluble histone complexes in nuclei of *Xenopus laevis* oocytes, *J. Biol. Chem. 260*, 1166−1176.
8. Laskey, R. A., Honda, B. M., Mills, A. D., Morris, N. R., Wyllie, A. H., Mertz, J. E., De Roberts, E. M., and Gurdon, J. B. (1978) Chromatin assembly and transcription in eggs and oocytes of *Xenopus laevis*, *Cold Spring Harbor Symp. Quant. Biol. 42* (Part 1), 171−178.
9. Dilworth, S. M., Black, S. J., and Laskey, R. A. (1987) Two complexes that contain histones are required for nucleosome assembly in vitro: Role of nucleoplasmin and N1 in *Xenopus* egg extracts, *Cell 51*, 1009−1018.
10. Mann, M., Risley, M. S., Eckhardt, R. A., and Kasinsky, H. E. (1982) Characterization of spermatid/sperm basic chromosomal proteins in the genus *Xenopus* (Anura, Pipidae), *J. Exp. Zool. 222*, 173−186.
11. Risley, M. S., Eckhardt, R. A., Mann, M., and Kasinsky, H. E. (1982) Determinants of sperm nuclear shaping in the genus *Xenopus*, *Chromosoma 84*, 557−569.
12. Philpott, A., and Leno, G. H. (1992) Nucleoplasmin remodels sperm chromatin in *Xenopus* egg extracts, *Cell 69*, 759−767.
13. Philpott, A., Leno, G. H., and Laskey, R. A. (1991) Sperm decondensation in *Xenopus* egg cytoplasm is mediated by nucleoplasmin, *Cell 65*, 569−578.
14. Gillespie, P. J., Li, A., and Blow, J. J. (2001) Reconstitution of licensed replication origins on *Xenopus* sperm nuclei using purified proteins, *BMC Biochem. 2*, 15.
15. Gillespie, P. J., and Blow, J. J. (2000) Nucleoplasmin-mediated chromatin remodelling is required for *Xenopus* sperm nuclei to become licensed for DNA replication, *Nucleic Acids Res. 28*, 472−480.
16. Lu, Z. H., Xu, H., and Leno, G. H. (1999) DNA replication in quiescent cell nuclei: Regulation by the nuclear envelope and chromatin structure, *Mol. Biol. Cell 10*, 4091−4106.
17. Hierro, A., Arizmendi, J. M., De Las Rivas, J., Urbaneja, M. A., Prado, A., and Muga, A. (2001) Structural and functional properties of *Escherichia coli*-derived nucleoplasmin. A comparative study of recombinant and natural proteins, *Eur. J. Biochem. 268*, 1739−1748.
18. Garcia Ramirez, M., Leuba, S. H., and Ausió, J. (1990) One-step fractionation method for isolating H1 histones from chromatin under nondenaturing conditions, *Protein Expression Purif. 1*, 40−44.
19. Wang, X., and Ausió, J. (2001) Histones are the major chromosomal protein components of the sperm of the nemerteans *Cerebratulus californiensis* and *Cerebratulus lacteus*, *J. Exp. Zool. 290*, 431−436.
20. Ausió, J., Dong, F., and van Holde, K. E. (1989) Use of selectively trypsinized nucleosome core particles to analyze the role of the histone "tails" in the stabilization of the nucleosome, *J. Mol. Biol. 206*, 451−463.
21. Rabbani, A., Iskandar, M., and Ausió, J. (1999) Daunomycin-induced unfolding and aggregation of chromatin, *J. Biol. Chem. 274*, 18401−18406.
22. Garcia Ramirez, M., Dong, F., and Ausió, J. (1992) Role of the histone "tails" in the folding of oligonucleosomes depleted of histone H1, *J. Biol. Chem. 267*, 19587−19595.
23. van Holde, K. E., and Weischet, W. O. (1978) Boundary analysis of sedimentation velocity experiments with monodisperse and paucidisperse solutes, *Biopolymers 17*, 1387−1403.
24. Straume, M., and Johnson, M. L. (1992) Analysis of residuals: Criteria for determining goodness-of-fit, *Methods Enzymol. 210*, 87−105.
25. Straume, M., and Johnson, M. L. (1992) Monte Carlo method for determining complete confidence probability distributions of estimated model parameters, *Methods Enzymol. 210*, 117−129.
26. Perkins, S. J. (1986) Protein volumes and hydration effects. The calculations of partial specific volumes, neutron scattering matchpoints and 280-nm absorption coefficients for proteins and glycoproteins from amino acid sequences, *Eur. J. Biochem. 157*, 169−180.
27. Lohka, M. J. (1998) Analysis of nuclear envelope assembly using extracts of *Xenopus* eggs, *Methods Cell Biol. 53*, 367−395.
28. Wang, X., Moore, S. C., Laszckzak, M., and Ausió, J. (2000) Acetylation increases the α-helical content of the histone tails of the nucleosome, *J. Biol. Chem. 275*, 35013−35020.
29. Rice, P., Garduno, R., Itoh, T., Katagiri, C., and Ausió, J. (1995) Nucleoplasmin-mediated decondensation of *Mytilus* sperm chromatin. Identification and partial characterization of a nucleoplasmin-like protein with sperm-nuclei decondensing activity in *Mytilus californianus*, *Biochemistry 34*, 7563−7568.
30. Zweidler, A. (1978) Resolution of histones by polyacrylamide gel electrophoresis in the presence of nonionic detergents, *Methods Cell Biol. 17*, 223−233.
31. Richards, R. G., and Shaw, B. R. (1982) In situ protamine release: A versatile sample preparation method for the electrophoretic analysis of nuclear proteins on acid/urea-based gels, *Anal. Biochem. 121*, 69−82.
32. Laskey, R. A. (1983) Phosphorylation of nuclear proteins, *Philos. Trans. R. Soc. London, Ser. B 302*, 143−150.
33. Cotten, M., Sealy, L., and Chalkley, R. (1986) Massive phosphorylation distinguishes *Xenopus laevis* nucleoplasmin isolated from oocytes or unfertilized eggs, *Biochemistry 25*, 5063−5069.
34. Sealy, L., Cotten, M., and Chalkley, R. (1986) *Xenopus* nucleoplasmin: Egg vs oocyte, *Biochemistry 25*, 3064−3072.
35. Leno, G. H., Mills, A. D., Philpott, A., and Laskey, R. A. (1996) Hyperphosphorylation of nucleoplasmin facilitates *Xenopus* sperm decondensation at fertilization, *J. Biol. Chem. 271*, 7253−7256.
36. Ausió, J., Borochov, N., Seger, D., and Eisenberg, H. (1984) Interaction of chromatin with NaCl and $MgCl_2$. Solubility and binding studies, transition to and characterization of the higher-order structure, *J. Mol. Biol. 177*, 373−398.
37. Ausió, J. (2000) Analytical ultracentrifugation and the characterization of chromatin structure, *Biophys. Chem. 86*, 141−153.
38. Shen, X., Yu, L., Weir, J. W., and Gorovsky, M. A. (1995) Linker histones are not essential and affect chromatin condensation in vivo, *Cell 82*, 47−56.
39. Ausió, J., Seger, D., and Eisenberg, H. (1984) Nucleosome core particle stability and conformational change. Effect of temperature, particle and NaCl concentrations, and cross-linking of histone H3 sulfhydryl groups, *J. Mol. Biol. 176*, 77−104.
40. Rabbani, A., Finn, R. M., Thambirajah, A. A., and Ausió, J. (2004) Binding of antitumor antibiotic daunomycin to histones in chromatin and in solution, *Biochemistry 43*, 16497−16504.
41. Dimitrov, S., and Wolffe, A. P. (1996) Remodeling somatic nuclei in *Xenopus laevis* egg extracts: Molecular mechanisms for the selective release of histones H1 and H1(0) from chromatin and the acquisition of transcriptional competence, *EMBO J. 15*, 5897−5906.
42. Iwata, K., Hozumi, K., Itoh, T., Sakairi, N., Tokura, S., Katagiri, C., and Nishi, N. (1997) Conformation of nucleoplasmin and its interaction with DNA-protamine complex as a simple model of fish sperm nuclei, *Int. J. Biol. Macromol. 20*, 171−178.
43. Prieto, C., Saperas, N., Arnan, C., Hills, M. H., Wang, X., Chiva, M., Aligue, R., Subirana, J. A., and Ausió, J. (2002) Nucleoplasmin interaction with protamines. Involvement of the polyglutamic tract, *Biochemistry 41*, 7802−7810.
44. Arnan, C., Saperas, N., Prieto, C., Chiva, M., and Ausió, J. (2003) Interaction of nucleoplasmin with core histones, *J. Biol. Chem. 278*, 31319−31324.
45. Wolffe, A. P. (1994) Transcriptional activation. Switched-on chromatin, *Curr. Biol. 4*, 525−528.
46. Richardson, R. T., Batova, I. N., Widgren, E. E., Zheng, L. X., Whitfield, M., Marzluff, W. F., and O'Rand, M. G. (2000) Characterization of the histone H1-binding protein, NASP, as a cell cycle-regulated somatic protein, *J. Biol. Chem. 275*, 30378−30386.
47. Nair, S. S., Mishra, S. K., Yang, Z., Balasenthil, S., Kumar, R., and Vadlamudi, R. K. (2004) Potential role of a novel transcriptional coactivator PELP1 in histone H1 displacement in cancer cells, *Cancer Res. 64*, 6416−6423.
48. Chen, H., Li, B., and Workman, J. L. (1994) A histone-binding protein, nucleoplasmin, stimulates transcription factor binding to nucleosomes and factor-induced nucleosome disassembly, *EMBO J. 13*, 380−390.
49. Loyola, A., and Almouzni, G. (2004) Histone chaperones, a supporting role in the limelight, *Biochim. Biophys. Acta 1677*, 3−11.

50. van Holde, K. E., Lohr, D. E., and Robert, C. (1992) What happens to nucleosomes during transcription? *J. Biol. Chem. 267*, 2837−2840.
51. Baer, B. W., and Rhodes, D. (1983) Eukaryotic RNA polymerase II binds to nucleosome cores from transcribed genes, *Nature 301*, 482−488.
52. Howe, L., Itoh, T., Katagiri, C., and Ausió, J. (1998) The histone binding protein nucleoplasmin does not facilitate binding of transcription factor IIIA to nucleosomal *Xenopus laevis* 5S rRNA genes, *Biochemistry 37*, 1174−1177.
53. Kasinsky, H. E. (1989) *Specificity and distribution of sperm basic proteins*, CRC Press, Boca Raton, FL.
54. Ausió, J. (1999) Histone H1 and evolution of sperm nuclear basic proteins, *J. Biol. Chem. 274*, 31115−31118.
55. Sato, K., IIhara, A., Sakai, N., Ito, F., Nomizu, M., and Nishi, N. (2002) Purification and characterization of a nucleolasmin-like protein from carp (*Cyprinus carpio*) eggs, *Fish. Sci. 68*, 904−912.
56. Lewis, J. D., and Ausió, J. (2002) Protamine-like proteins: Evidence for a novel chromatin structure, *Biochem. Cell Biol. 80*, 353−361.
57. McLay, D. W., and Clarke, H. J. (2003) Remodelling the paternal chromatin at fertilization in mammals, *Reproduction 125*, 625−633.
58. Burns, K. H., Viveiros, M. M., Ren, Y., Wang, P., DeMayo, F. J., Frail, D. E., Eppig, J. J., and Matzuk, M. M. (2003) Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos, *Science 300*, 633−636.
59. Briand, G., Kmiecik, D., Sautiere, P., Wouters, D., Borie-Loy, O., Biserte, G., Mazen, A., and Champagne, M. (1980) Chicken erythrocyte histone H5. IV. Sequence of the carboxy-termined half of the molecule (96 residues) and complete sequence, *FEBS Lett. 112*, 147−151.
60. Coles, L. S., Robins, A. J., Madley, L. K., and Wells, J. R. (1987) Characterization of the chicken histone H1 gene complement. Generation of a complete set of vertebrate H1 protein sequences, *J. Biol. Chem. 262*, 9656−9663.
61. Dingwall, C., Dilworth, S. M., Black, S. J., Kearsey, S. E., Cox, L. S., and Laskey, R. A. (1987) Nucleoplasmin cDNA sequence reveals polyglutamic acid tracts and a cluster of sequences homologous to putative nuclear localization signals, *EMBO J. 6*, 69−74.
62. Banuelos, S., Hierro, A., Arizmendi, J. M., Montoya, G., Prado, A., and Muga, A. (2003) Activation mechanism of the nuclear chaperone nucleoplasmin: Role of the core domain, *J. Mol. Biol. 334*, 585−593.

BI050386W