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Interaction of Nucleoplasmin with Core Histones*

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Nucleoplasmin is one of the most abundant proteins in *Xenopus laevis* oocytes, and it has been involved in the chromatin remodeling that takes place immediately after fertilization. This molecule has been shown to be responsible for the removal of the sperm-specific proteins and deposition of somatic histones onto the male pronuclear chromatin. To better understand the latter process, we have used sedimentation velocity, sedimentation equilibrium, and sucrose gradient fractionation analysis to show that the pentameric form of nucleoplasmin binds to a histone octamer equivalent consisting of equal amounts of the four core histones, H2A, H2B, H3, and H4, without any noticeable preference for any of these proteins. Removal of the histone N-terminal "tail" domains or the major C-terminal polyglutamic tracts of nucleoplasmin did not alter these binding properties. These results indicate that interactions other than those electrostatic in nature (likely hydrophobic) also play a critical role in the formation of the complex between the negatively charged nucleoplasmin and positively charged histones. Although the association of histones with nucleoplasmin may involve some ionic interactions, the interaction process is not electrostatically driven.

Under physiological conditions core histones interact with each other to form a heterotypic histone octamer consisting of a histone H3-H4 tetramer and two histone H2A-H2B dimers (1, 2), which constitute the protein core of the basic chromatin subunit, the nucleosome core particle (1, 2). In solution and in the absence of DNA the histone octamer exists in an equilibrium between its constitutive H2A-H2B dimers and the H3-H4 tetramer, which has been extensively characterized (3).

In contrast, nucleoplasmin is a pentameric acidic protein (4–8) that has been involved in the remodeling of the male pronuclear chromatin after fertilization of the oocyte in vertebrates (9–13) and invertebrate organisms (14). Nucleoplasmin can bind and remove the protamine complement of the male pronucleus and can also bind to maternal histones mediating the replacement of the former by the latter. The way in which all this occurs at the molecular level has proven to be more complicated than originally envisaged. For instance, the early hypothetical model in which the highly positively charged protamines electrostatically interact with the negatively charged nucleoplasmin (10) has been shown to be an oversimplification of the interactions involved (15), and a detailed physical characterization of the interaction of nucleoplasmin with histones is lacking. Thus the precise molecular mechanisms by which early developmental chromatin remodeling takes place are still poorly understood.

EXPERIMENTAL PROCEDURES

Recombinant Proteins and Histones—Recombinant nucleoplasmin was obtained by expression of plasmid pET20b containing the *Xenopus laevis* nucleoplasmin cDNA corresponding to the cDNA sequenced by Bürglin (15). Recombinant nucleoplasmin lacking the C-terminal main polyglutamic tract of the molecule (r-NP121) was prepared as described (15). Native and trypsinized histone octamers were obtained from chicken erythrocyte nucleosomes with or without treatment with immobilized trypsin (16) upon elution from a hydroxylapatite column using a 2 M NaCl, 100 mM potassium phosphate (pH 6.8) buffer (17).

Determination of Protein Concentrations—The concentration of nucleoplasmin was determined from the absorbance at 276 nm (13). The concentration of histone octamers was determined using an absorption coefficient at 230 nm of 4.2 $cm^2\ mg^{-1}$ (18). The extinction coefficient of the trypsinized histones was calculated from their respective amino acid sequences according to Gill and von Hippel (19) as described by Luger *et al.* (20).

Titration of Nucleoplasmin with Histone Octamers—Native or trypsinized histone octamers at a concentration of ~3–4 mg/ml in 2 M NaCl, 100 mM potassium phosphate (pH 6.8) buffer were rapidly mixed with a nucleoplasmin solution prepared in 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5) buffer. The mixture was carried out in such a way that the final concentration of nucleoplasmin in all the samples (~0.1–0.12 mg/ml) and the final buffer composition (0.24 M NaCl, 12 mM potassium phosphate, 8.8 mM Tris-HCl, 1.8 mM $MgCl_2$ (pH 7.5)) were kept the same in all the samples.

Analytical Ultracentrifuge Analysis—Sedimentation velocity and sedimentation equilibrium analyses were carried out with a Beckman XL-A analytical ultracentrifuge (Beckman-Coulter, Inc., Fullerton, CA) using An-55 AL 9 aluminum and An-60 Ti (titanium) rotors, respectively. The samples were loaded on Kel-F 12-mm double sector cells. Experiments were carried out at 20 °C at different speeds as indicated in the figure legends. Sedimentation velocity scans were analyzed using XL-A Ultra Scan version 4.1 sedimentation data analysis software (Borries Demeler, Missoula, MT), which employs a published method of boundary analysis (21). Sedimentation equilibrium scans were analyzed using the XL-A data software analysis. The molecular weight average was calculated from the best fitting slope of ln of absorbance at 230 nm *versus* the square of the radial distance. The partial specific volume of nucleoplasmin was calculated from its amino acid composition following the method of Cohn and Edsall (22) using the amino acid partial specific volumes from Perkins (23). The value calculated in this way was $\bar{v}$ = 0.734 cm^3/g. A value of $\bar{v}$ = 0.753 cm^3/g was used for the histone octamer (3).

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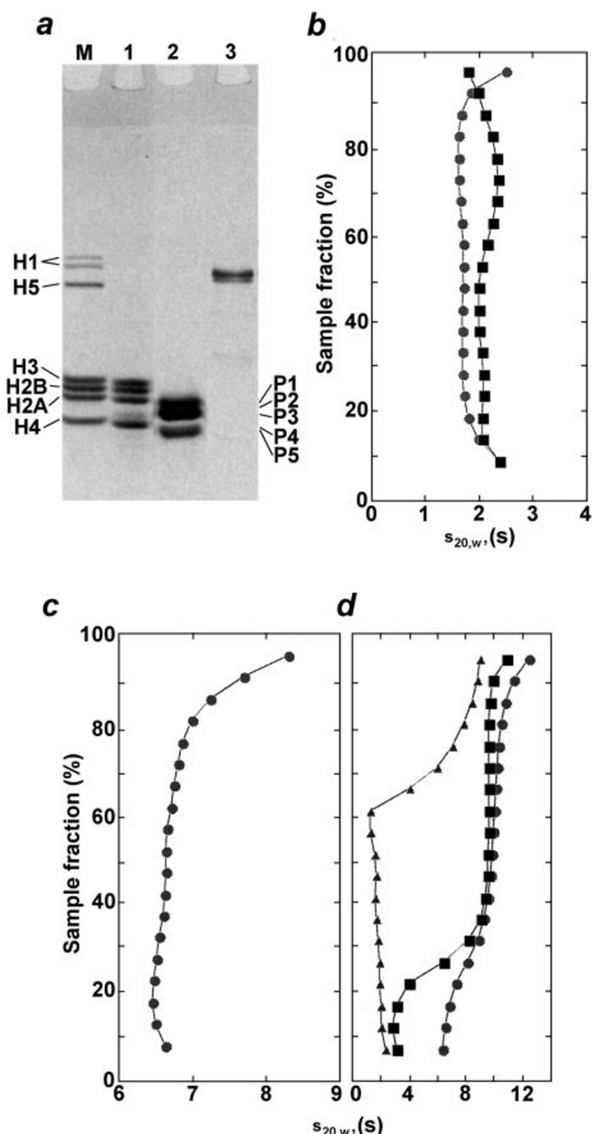

FIG. 1. **Sedimentation velocity analysis of nucleoplasmin, histones, and histone-nucleoplasmin complexes.** *a*, SDS-PAGE of the samples analyzed. *Lane 1*, chicken erythrocyte core histones; *lane 2*, trypsinized core histones (the trypsin-resistant peptides P1–P5 are indicated (38)); *lane 3*, nucleoplasmin. M is a chicken erythrocyte histone marker. *b*, *c*, and *d*, integral distribution plots of the sedimentation coefficient of native (*circles*) and trypsinized (*squares*) core histones (*b*), nucleoplasmin (*c*), and nucleoplasmin in the presence of 0.5 (*circles*), 1.0 (*squares*), and 3.0 (*triangles*) mol of histone octamer/mol of nucleoplasmin pentamer (*d*). The runs were carried out at 20 °C at 40,000 rpm. The buffer used in all these experiments was 240 mM NaCl, 12 mM potassium phosphate, 1.8 mM $MgCl_2$, 8.8 mM Tris-HCl (pH 7.5). The boundaries were analyzed according to the method of van Holde and Weischet (21).

Sucrose Gradients—Sucrose gradients (5–20% sucrose) were prepared in 240 mM NaCl, 12 mM potassium phosphate, 1.8 mM $MgCl_2$, 8.8 mM Tris-HCl (pH 7.5) buffer. Native or trypsinized histones at ~2.5 mg/ml in 2 M NaCl, 100 mM potassium phosphate buffer (pH 6.8) were rapidly mixed with a 0.25 mg/ml nucleoplasmin solution in 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5) buffer at different stoichiometric ratios and were immediately loaded on the sucrose gradients. The gradients were run for 21.5 h at 103,800 × *g* at 4 °C. Electrophoretic analysis of the proteins was carried out by SDS-PAGE (15% polyacrylamide) (24).

RESULTS

The Pentameric Form of Nucleoplasmin Binds Core Histones in an Amount Equivalent to One Histone Octamer—Native core histones (see Fig. 1*a*, *lane 1*) at elevated ionic strength (≥2 M NaCl) and at high concentrations of protein exist as a globular histone octamer (25) held together by specific interactions between the "histone fold" (2, 26) domains of these proteins. Under these high ionic strength conditions the histone octamer (M_r = 108,000) sediments with an $s_{20,w}$ = 3.9 ± 0.1 S (27–30). At lower ionic strengths, near physiological conditions the octamer is fully dissociated into a histone H3-H4 tetramer (M_r = 53,200) and two histone H2A-H2B dimers (M_r = 27,700 × 2). This heterogeneous mixture exhibits a sedimentation coefficient of 1.6–2.0 S for native histones and 2.0–2.4 S in the case of the trypsinized histones (see Fig. 1, *a* and *b*). The slight increase in $s_{20,w}$ despite the decrease in mass observed in the latter case emphasizes the extended conformation of the histone domains (tails) removed by trypsin. It is not possible with the current technology to accurately define the individual sedimentation coefficients of the H3-H4 tetramer and the H2A-H2B dimer from a mixture of both complexes.

In contrast, nucleoplasmin forms under physiological conditions a stable pentamer (M_r = 110,000), which has a sedimentation coefficient of 6.5 ± 0.1 S (see Fig. 1*c*) (15). Although the preparation of recombinant nucleoplasmin used by us is very pure as judged by SDS-PAGE (see Fig. 1*a*, *lane 3*), it contains ~15% slightly faster (6.5–8.4 S) sedimenting material (see Fig. 1*c*). This is most likely the result of some nonspecific association between pentamers and some improperly folded monomers.

When a nucleoplasmin solution was mixed with 0.5 mol of histone octamer/mol of nucleoplasmin pentamer, this resulted in a broader sedimentation coefficient distribution spanning from 6.5 to 12.5 S (see Fig. 1*d*). Further addition of histone octamer to a ratio of 1 mol of octamer/mol of nucleoplasmin pentamer produced a bimodal distribution of the sedimentation coefficients in which about 85% (after correction for the difference in extinction coefficients of nucleoplasmin and the histone octamer) of the sample sedimented with a narrow sedimentation coefficient distribution centered at around 9.5 S, whereas the remainder sedimented as histones (see Fig. 1*d*). Sedimentation equilibrium analysis of the sample consisting of 1:1 molar complex gave an average molecular weight of 201,700, which is in very good agreement with that expected (219,000).

Addition of histones to a 3:1 molar ratio to nucleoplasmin resulted again in a bimodal distribution in which the fraction of material sedimenting as free histones comprised 50% (see Fig. 1*d*) (after correction for the difference in extinction coefficients of free nucleoplasmin and histone-nucleoplasmin complex). This value is in very good agreement with the theoretically expected value (47%) calculated in the assumption that 1 mol of nucleoplasmin binds to 1 mol of histone octamer.

In these titration experiments, the apparent average sedimentation coefficient of the fastest sedimenting fraction corresponding to the histone-nucleoplasmin complex exhibits a trend to decrease as the histone/nucleoplasmin ratio increases from 0.5 to 3.0 (mol of equivalent histone octamer/mol of nucleoplasmin pentamer) (see Fig. 1*d*). This is most likely due to the presence of free sedimenting histones. Regardless of this, the results of the histone/nucleoplasmin titration shown in Fig. 1*d* clearly indicate that each nucleoplasmin pentamer is able to bind approximately one histone octamer equivalent.

Binding of Core Histones to Nucleoplasmin Does Not Exhibit Any Specific Core Histone Preference—The results above do not allow us to determine whether the different histone domains (H2A-H2B dimer and H3-H4 tetramer) bind to the nucleoplasmin pentamer with the same efficiency or whether there is any preference for either one of them when the nucleoplasmin pentamer is exposed to an excess of histone octamers. To analyze this and to corroborate the previous analytical ultracentrifuge data, we carried out a histone to nucleoplasmin titra-

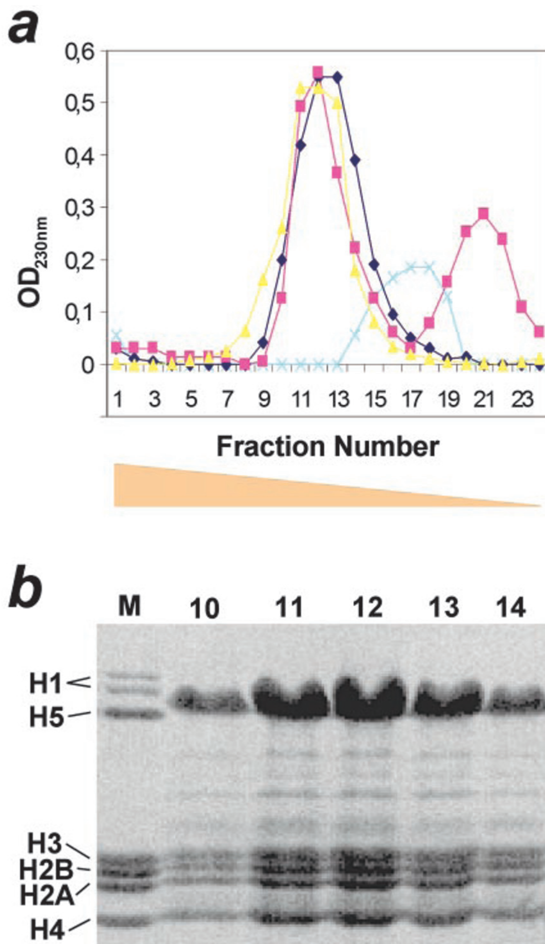

FIG. 2. **Sucrose gradient analysis of histone-nucleoplasmin complexes.** *a*, sucrose gradient fractionation profile of nucleoplasmin (X *symbol*), nucleoplasmin-core histone complex (1:1, mol/mol) (*diamonds*), and nucleoplasmin-core histone complex (1:2, mol/mol) (*squares*) in comparison with nucleosome core particles (M_r = 204,800) (*triangles*). The *triangle* at the *bottom* indicates the gradient direction of the sucrose gradient. Sucrose gradients (5–20% sucrose) were prepared in 240 mM NaCl, 12 mM potassium phosphate, 1.8 mM $MgCl_2$, 8.8 mM Tris-HCl (pH 7.5) buffer. *b*, SDS-PAGE analysis of several of the fractions for nucleoplasmin-core histone complex (1:1, mol/mol). The *numbers above* the gel indicate the fractions of the sucrose gradient. *NP*, nucleoplasmin.

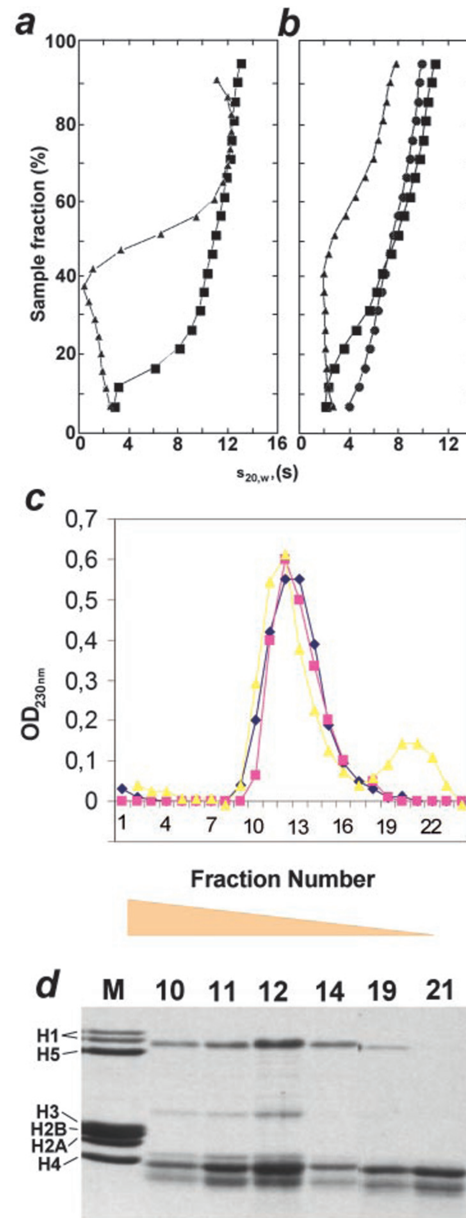

FIG. 3. **Interaction of full-length and C-terminal truncated nucleoplasmin with trypsinized and native core histones.** *a*, sedimentation velocity analysis of full-length recombinant nucleoplasmin titrated with 1.0 (*squares*) and 2.0 (*triangles*) mol of trypsinized histone octamer/mol of nucleoplasmin, respectively. *b*, sedimentation velocity analysis of recombinant nucleoplasmin lacking the main polyglutamic tract of the molecule titrated with 0.5 (*circles*), 1.0 (*squares*), and 2.0 (*triangles*) mol of histone octamer/mol of nucleoplasmin, respectively. The experimental conditions and analysis of the sedimentation velocity experiments were as described in Fig. 1. *c*, sucrose gradient fractionation profile of nucleoplasmin-trypsinized core histone complex (1:1, mol/mol) (*pink*) and nucleoplasmin-trypsinized core histone complex (1:2, mol/mol) (*yellow*) in comparison with nucleoplasmin-core histone complex (1:1, mol/mol) (*dark blue*). The *triangle* indicates the gradient direction of the sucrose gradient. The sucrose gradients were run under the same experimental conditions described in Fig. 2. *d*, SDS-PAGE analysis of several of the fractions of the sucrose gradient corresponding to the nucleoplasmin-trypsinized core histone complex (1:2, mol/mol). The *numbers above* the gels indicate the fractions of the sucrose gradient.

tion using sucrose gradients (Fig. 2*a*) and analyzed the protein composition of the peaks corresponding to the resulting complexes using SDS-PAGE (Fig. 2*b*). As can be seen, titration of nucleoplasmin pentamers with a stoichiometric amount (1:1, mol/mol) of histone octamers resulted in a single symmetrical peak (Fig. 2*a, dark blue diamonds*) that sediments slightly slower than nucleosome core particles ($s_{20,w}$ = 11.0 ± 0.2 S, M_r = 204,800) (16) run under the same conditions (Fig. 2*a, yellow triangles*) and faster than the nucleoplasmin pentamer (Fig. 2*a*, *light blue* x *symbols*). When the same experiment was carried out using a larger ratio of histone octamers (2 mol of histone octamer/mol of nucleoplasmin pentamer), two symmetrical peaks were obtained (Fig. 2*a*, *pink squares*). The faster moving one overlaps with the peak corresponding to the stoichiometric complex. After correction for the difference in extinction coefficient of the histone octamer and nucleoplasmin, the area of the slow moving peak was determined to be 29.7% of that of the faster moving peak (31% expected on the basis of 1 mol of nucleoplasmin binding 1 mol of histone octamer). More importantly, the relative stoichiometry of the core histones was the same in both peaks (results not shown). Neither the histone tails nor the main polyglutamic tract of nucleoplasmin affects the binding of histones to nucleoplasmin.

It is possible to remove the highly positively charged N-terminal domains of histones by treatment of nucleosome particles with immobilized trypsin (16) followed by hydroxylapa-

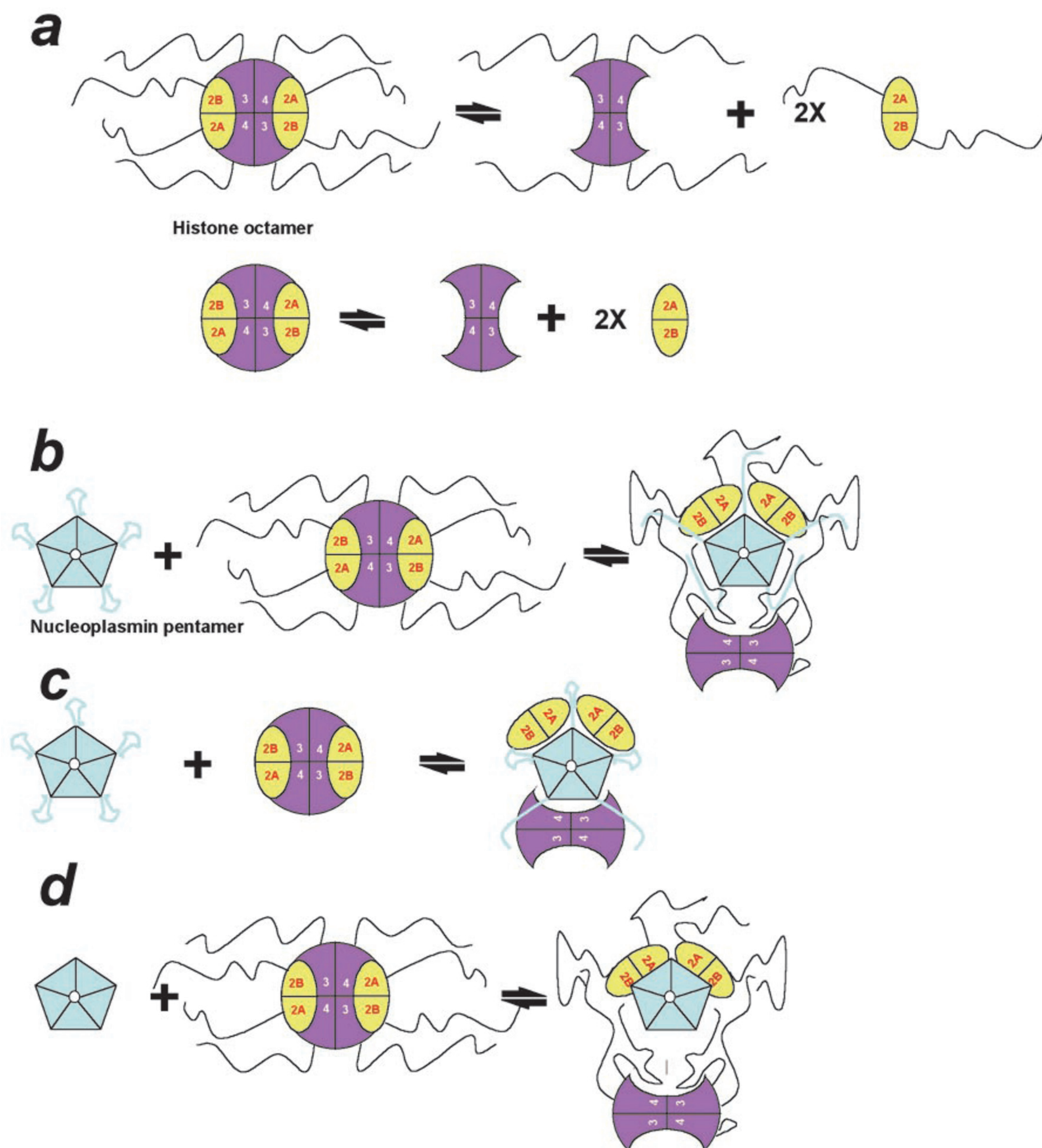

FIG. 4. **Schematic representation of the histone-histone and histone-nucleoplasmin interactions.** *a*, histone octamer in the presence (3) or absence (39) of histone tails. *b*, *c*, and *d*, model proposed for the association between core histones and nucleoplasmin in solution in the presence of histone tails (*b* and *d*) and in the absence of either histone tails (*c*) or the C-terminal polyglutamic tract of nucleoplasmin (*d*).

tite chromatography to remove the DNA. The treatment does not affect the histone fold, which is responsible for the maintenance of the octameric histone complex, and hence it does not have much influence on the stability of the histone octamer.

When a nucleoplasmin solution was titrated with increasing amounts of trypsinized histone octamers, the sedimentation behavior of the complexes (see Fig. 3*a*) was very similar to that observed for native histone octamers (see Fig. 1*d*). A boundary corresponding to ~34–36% (after correction for difference in extinction coefficient) of free histones is observed when a starting ratio of 1 mol of nucleoplasmin was titrated with 2 mol of trypsinized histone octamers (see Fig. 3*a*), which is only slightly higher than the 29% expected. Similarly, when this complex was analyzed in a sucrose gradient (see Fig. 3*c*), ~27.5% of the mixture sedimented as a slowly moving peak. The rest sedimented as a symmetrical peak for which the mobility overlapped with that of a complex consisting of 1:1 mol of nucleoplasmin/mol of trypsinized histones and exhibited an apparent molecular weight by sedimentation equilibrium of 149,800. The trypsinized histone nature of the slowly sedimenting peak and the histone composition of both peaks was visualized by SDS-PAGE analysis (see Fig. 3*d*). This analysis revealed that as with the native histones, the faster sedimenting peak (corresponding to the 1:1 nucleoplasmin/trypsinized histones) has no apparent histone preference.

We next decided to analyze the role of the C-terminal polyglutamic tract of nucleoplasmin in the formation of the nucleoplasmin-histone complex. As this region of nucleoplasmin is highly negatively charged under physiological conditions, it would be expected to play a major role in the formation of the complex with the positively charged histones. As shown in Fig. 3*b*, removal of the polyglutamic tract had little if any effect on histone binding. Truncation of nucleoplasmin does not affect the formation of a pentameric complex, which sediments with

an average sedimentation coefficient of 4.8 ± 0.1 S (15). Upon addition of histone octamers, the complexes thus formed behave in a way that is also very similar to that of the native complexes. Approximately 40% of the histones sediment as free histones when 2 mol of histones are mixed with 1 mol of truncated nucleoplasmin (see Fig. 3*b*), a value that is in good agreement with that expected (36%). The complex sediments with an average sedimentation coefficient of 10 ± 0.1 S, which is almost indistinguishable from that of the native complex, a fact that in this instance most likely reflects the decrease in the frictional properties (asymmetry) of the nucleoplasmin pentamer upon removal of the C-terminal domains of the monomer (15).

DISCUSSION

In the pioneering nucleoplasmin work of Laskey and coworkers (31), it was shown that this molecule formed a histone storage complex with histones H2A and H2B. The dual functional involvement of this protein in removal of the sperm-specific proteins and assembly of the male pronuclear chromatin was later established (for review, see Refs. 9 and 10). A model was proposed in which histone H2A-H2B dimers were added to the histone H2A-H2B-deficient sperm chromatin of *X. laevis* (32) upon removal of the sperm-specific (SP1–6) proteins.

However, SP1–6 proteins represent an important sperm chromatin component that needs to be replaced by complete nucleosomes (consisting of a whole set of storage histones H2A, H2B, H3, and H4). Also, nucleoplasmin has been shown to be the major protein of the egg of other amphibians, such as the toad *Bufo japonicus*, whose sperm protein composition consists only of protamines (12). Furthermore, amphibian nucleoplasmin alone in the presence of added histones has been shown to be able to produce chromatin remodeling of protamine containing sperm chromatin (33) or protamine-reconstituted DNA complexes (34). These studies suggest that in addition to providing a chaperone role for histones H2A and H2B, nucleoplasmin may also be able to bind to a full core histone complement and participate in this way in the process of nucleosome assembly during early development.

A crystallographic structure of nucleoplasmin from *X. laevis* and a nucleoplasmin-like protein from *Drosophila* has been recently published, and a model has been proposed based on this structural information for the possible interactions with the histone octamer (6–8). According to this model, five histone octamers bind to a nucleoplasmin decamer (two pentamers). However, a nucleoplasmin decamer has never been observed in solution (15). Furthermore, neither under the ionic strength conditions chosen in those works to prepare their histone-nucleoplasmin complexes (1 M NaCl) nor under those used to purify them (100 mM NaCl) (6, 8) do core histones exist as an octamer. Therefore, for that model to be true it would also need to be argued that nucleoplasmin stabilizes the structure of the histone octamer under those ionic conditions, a fact that remains yet to be experimentally determined.

The results presented in this paper, under ionic physiological conditions where the histone octamer is in equilibrium with the histone H3-H4 tetramer and the histone H2A-H2B dimer (3) (see Fig. 4*a*), indicate that one complete histone octamer equivalent binds a nucleoplasmin pentamer. The lack of a bimodal distribution at ratios of histone/nucleoplasmin ≤ 0.5 (mol/mol) in all of the complexes studied argues that the histone octamers do not bind like intact octamers but rather as H2A-H2B dimers and H3-H4 tetramers (see Fig. 4, *b–d*).

The persistence of the stoichiometry of interaction observed both in the absence of the histone tails and in the absence of the polyglutamic rich C-terminal domain of nucleoplasmin shows that these highly charged domains play a minor role in the interaction and that the recognition must take place between the folded structure of the nucleoplasmin pentamer (6) and the histone fold (2, 26) as indicated in Fig. 4, *b–d*. As can be seen in Fig. 3*a*, the complex formed between the nucleoplasmin pentamer and the truncated histones exhibits a slightly higher sedimentation coefficient value (11.2 ± 0.2 S) (despite the decrease in molecular weight) than that of the complex formed with intact histones, a fact that reflects the higher symmetry of the complex resulting from the removal of the histone tails (see Fig. 4*c*).

Therefore, the *in vitro* analysis of the interaction between nucleoplasmin and core histones does not show any preference of nucleoplasmin by any particular set of core histones, which are all present in stoichiometric amount in the resulting complexes.

Xenopus oocytes as many other vertebrate and invertebrate oocytes store large amounts of histones in their nuclei. These histones presumably are involved in the chromatin remodeling that takes place immediately after fertilization in those organisms in which nuclear sperm chromatin consists of protamines and non-histone sperm-specific proteins. In *Xenopus*, two soluble complexes have been isolated from oocytes consisting of H2A-H2B and nucleoplasmin and histone H3-H4 in association of a pair of distinctive polypeptides (N1 and N2) (35). However, nucleoplasmin is by far the most abundant protein in *Xenopus* oocytes (10), and a fraction of H3-H4-like histones has also been found to be associated to it in 7 S complexes purified from these oocytes by sucrose gradients (36). Although the relation, if any, of the 10–11 S histone-nucleoplasmin complexes studied in this work to the 7 S complexes identified by Kleinschmidt *et al.* (36) is not clear, the presence of four distinctive nucleosomal core histones in this fraction provides additional support to the notion that nucleoplasmin can participate in the full assembly of complete nucleosomes *in vitro* (33, 37). The results described in this paper using mixtures of H2A-H2B and histone H3-H4 tetramers and the stoichiometry with which they bind to nucleoplasmin have implications for the way nucleoplasmin functions at the level of nucleosome/chromatin assembly *in vivo*. They raise the possibility that nucleoplasmin alone may, in certain instances, participate in the full nucleosome assembly after removal of the sperm-specific proteins from the male pronuclear chromatin fibers.

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