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Article

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Nucleoplasmin Interaction with Protamines. Involvement of the Polyglutamic Tract†

Cèlia Prieto,‡ Núria Saperas,‡ Carme Arnan,‡ Melissa H. Hills,§ Xiaoying Wang,§ Manel Chiva,|| Rosa Aligué,⊥ Juan A. Subirana,‡ and Juan Ausió*,§

Departament d'Enginyeria Química, ETSEIB, Universitat Politècnica de Catalunya, Diagonal 647, Barcelona E-08028, Spain, Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada V8W 3P6, Departament de Ciències Fisiològiques II, Facultat de Medicina, Campus de Bellvitge, Universitat de Barcelona, L'Hospitalet de Llobregat, Barcelona E-08907, Spain, and Departament de Biologia Cellular, IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer), Facultat de Medicina, Universitat de Barcelona, Casanova 143, Barcelona E-08036, Spain

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ABSTRACT: Different recombinant forms of nucleoplasmin including truncations at the carboxyl-terminal end of the molecule (r-NP121, r-NP142) have been expressed and purified. All of them were found to oligomerize, forming pentameric complexes which, according to their hydrodynamic properties, can be modeled by oblate ellipsoids of constant width. In this model, the highly charged carboxyl ends appear to be arranged around a pentameric core along the plane defined by the major axes of the ellipsoid. Importantly, all the recombinant forms appear to be able to decondense protamine-containing sperm nuclei. However, although the stoichiometry with which protamines bind to these forms appears to be constant (2.5 mol of protamine/mol of nucleoplasmin pentamer), the efficiency with which they remove protamines from the sperm DNA decreases in the following order: o-NP > r-NP142 ≥ r-NP ≫ r-NP121. Therefore, the main polyglutamic tract of nucleoplasmin (which is absent in r-NP121), while enhancing the efficiency of protamine removal, is not indispensable for sperm chromatin decondensation in the biological model we have used.

Chromatin usually undergoes an important remodeling during spermatogenesis, changing from a somatic type organization to a highly condensed state in the mature sperm cell. This condensation is achieved through the replacement of somatic histones by sperm nuclear basic proteins (SNBPs), which include sperm-specific histones, protamine-like proteins, and protamines (*1*). Despite the compositional and structural diversity of these proteins (*2*−*4*), they all share the ability to efficiently neutralize the negative charge of the DNA phosphates which is ultimately responsible for the compact organization of sperm chromatin. Another characteristic feature of SNBPs is that of condensing DNA in a reversible way such that, after fertilization, the male pronucleus can regain the nucleosome organization which is characteristic of somatic chromatin. Nucleoplasmin is one of the better characterized proteins known to participate in this remodeling process.

Nucleoplasmin is an acidic and thermostable protein which is one of the most abundant proteins found in the nuclei of oocytes and nonfertilized eggs of *Xenopus laevis* and other amphibians (*5*, *6*). In *X. laevis*, sperm nuclear decondensation occurs in two stages; the first is differentiated by rapid and limited expansion, and the second is slower and membrane-dependent. It has been shown that the first stage of this transition is mediated by nucleoplasmin (*7*, *8*). The SNBPs of *X. laevis* consist of six different proteins (SP1−SP6) which coexist with a subset of core histones in which H3−H4 are present in larger amounts than H2A−H2B (*2*, *9*). Philpott and Leno (*8*) provided evidence that the nucleoplasmin-mediated chromatin remodeling process involves the removal of SNBPs and subsequent deposition of H2A−H2B histones (reviewed in *10*). The decondensation activity of *X. laevis* nucleoplasmin has been shown to be equally effective in decondensing sperm nuclei from heterologous systems, including other amphibians (*11*, *12*) as well as different vertebrate (*13*) and invertebrate organisms (*14*) with different SNBP compositions.

Nucleoplasmin has also been shown to facilitate assembly of nucleosome core particles. It was actually based on the role of this protein that the term "molecular chaperone" was first introduced (*15*). The studies carried out with *X. laevis* have shown that nucleoplasmin binds to histones H2A−H2B and together with protein N1/N2, which binds to histones H3 and H4, mediates nucleosome assembly (*16*). Thus, nucleoplasmin is able to participate in both chromatin assembly and disassembly processes.

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

‡ Universitat Politècnica de Catalunya.

§ University of Victoria.

|| Universitat de Barcelona, L'Hospitalet de Llobregat.

⊥ IDIBAPS, Universitat de Barcelona.

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Nucleoplasmin forms pentameric complexes (*17*) which result from the association of the protein monomers through their protease-resistant N-terminal regions (*18*). The monomers consist of 200 amino acids (*19*), and 2 slightly different sequences have been derived from cDNA information (*19*, *20*) (see also Figure 1).

One of the most remarkable structural features of nucleoplasmin is the existence of a long polyglutamic tract in the carboxy-terminal half of the molecule which has been assumed to be an obvious candidate for mediating its interactions with the basic chromosomal proteins (*10*, *19*, *20*). It has been suggested that nucleoplasmin could act as a "molecular hand" or a "five fingered grab" (*19*) in which the five acidic C-terminal regions that do not participate in pentamer formation could jointly participate in this process. However, experimental evidence is lacking. Also, despite some preliminary attempts to elucidate the binding stoichiometry (nucleoplasmin/basic chromosomal proteins) (*21*, *22*), conclusive results have not yet been produced. This work represents an attempt to experimentally address these two issues using several recombinant nucleoplasmin mutants and protamine-containing sperm nuclei.

MATERIALS AND METHODS

Recombinant Proteins. Plasmid pET16b-NED containing the *X. laevis* nucleoplasmin cDNA [corresponding to the cDNA sequenced by Bürglin (*20*, *23*)] was kindly provided to us by Dr. J. F. Kalinich (Armed Forces Radiology Research Institute, Bethesda, MD). Deletion mutants r-NP121 and r-NP142 were created by introducing stop codons at positions 122 or 143 from the N-termini either by using conventional PCR or by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). To prevent incorporation of the N-terminal histidine tag encoded by the pET16b-NED plasmid, the different recombinant forms were subcloned into a *Nde*I/*Bam*HI site of pET20b. A full-length recombinant nucleoplasmin (r-NP) and two deletion mutants (r-NP121 and r-NP142) were prepared in this way. Confirmatory DNA sequence analyses of all the constructs were carried out on an ABI Prism 310 Genetic Analyzer (ABI) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Corp., Foster City, CA).

Transformed *E. coli* BL21(DE3)pLysS were grown at 37 °C and 250 rpm in LB medium supplemented with 50 μg/mL ampicillin. The culture was induced for 4 h in the presence of 1 mM IPTG. Cells were harvested by centrifugation at 4000*g* for 5 min at 4 °C. Pellets from r-NP and r-NP142 were resuspended in 10 mM K_2HPO_4, 20 mM HEPES (pH 7.5) buffer, and those from r-NP121 were resuspended in 0.1 M NaCl, 25 mM Tris-HCl, 1 mM EDTA (pH 7.5) buffer. In addition, all buffers contained a mixture of protease inhibitors (1 mM PMSF and 1 μg/mL each of pepstatin A, leupeptin, and aprotinin). After sonication (3 times for 20 s, setting 7 microtip) in a Sonifer cell disruptor (Branson Sonic Power Co., Danbury, CT), the insoluble material was removed by centrifugation (16000*g*, 20 min at 4 °C), and the supernatant was heated at 80 °C for 10 min. The cloudy suspension thus obtained was centrifuged at 16000*g* for 20 min at 4 °C in order to remove the denatured insoluble proteins. The highly enriched nucleoplasmin-containing supernatants obtained in this way were subjected to further chromatographic purification. The r-NP and r-NP142 heat-soluble extracts were purified by hydroxyapatite (Bio-Gel HTP, Bio Rad, Hercules, CA) using an elution gradient of 0.1−0.3 M K_2HPO_4 in 20 mM HEPES (pH 7.5) buffer, and the r-NP121 extracts were purified by anion exchange chromatography using a DEAE-52 resin (Whatman International Ltd., Maidstone, England) eluted with a 0.1−0.4 M NaCl gradient in 25 mM Tris-HCl, 1 mM EDTA (pH 7.5) buffer. Occasionally the chromatographic fractions obtained from the hydroxyapatite purification showed some contamination with DNA which could be easily removed by chromatography in DEAE.

Purification of Native Nucleoplasmin. Sexually mature adult female *X. laevis* frogs were purchased from the Centre de Recherches de Biochemie Macromoléculaire (CNRS, Montpellier, France). Oocytes were obtained by dissection of the gonadal tissue, and nucleoplasmin was purified as described previously (*13*).

Isolation of Sperm Nuclei and Protamine Purification. Sperm cells were obtained from sexually mature *Dicentrarchus labrax* (sea bass) fish by abdominal massage. This species was chosen because its sperm cells consist of a single protamine component and thus provide a simple model to study nuclear decondensation. Sperm nuclei were prepared as described elsewhere (*13*). Nuclear density (nuclei/mL) was estimated from the total DNA nuclear content as determined from the absorbance at 260 nm of nuclear suspensions lysed in the presence of SDS (*24*). A relation of 5×10^6 sperm nuclei per 21 μg of DNA was used (*14*). Protamines were extracted from sperm nuclei and purified as described previously (*25*).

Determination of Protein Concentrations. The concentrations of native nucleoplasmin and the different nucleoplasmin constructs studied in this paper were determined from the absorbance of their solutions at 276 nm. The extinction coefficients of the different proteins analyzed were calculated from their respective amino acid sequences according to Gill and von Hippel (*26*). A UV spectrum of the purified proteins was routinely carried out to ensure that the purified samples were not contaminated by residual RNA or DNA, which would result in the presence of a major absorption peak at 260 nm.

The concentration of *D. labrax* protamine was determined from amino acid analysis of aqueous solutions of known absorbance at 230 nm which also contained known quantities of a Norleucin standard. An $A_{230} = 2.12$ for a water solution containing 1 mg/mL protein was determined in this way.

Circular Dichroism. Circular dichroism (CD) spectra were recorded at 20 °C on a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) as described elsewhere (*27*). The proteins were extensively dialyzed against 100 mM KCl, 2mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4) buffer (EM buffer), and the spectra were recorded in this buffer. The mean residue molar ellipticity [θ] was calculated using an amino acid molecular weight average of $M_r = 111.3$ for r-NP, $M_r = 112.5$ for r-NP142, and $M_r = 111.6$ for r-NP121.

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 and 40 000 rpm. 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 (*28*, *29*). Sedimentation equilibrium scans were analyzed using a nonlinear, least-squares, curve-fitting algorithm (*30*) contained in the XL-A data software analysis.

The partial specific volumes of the different proteins were calculated from their amino acid composition following the method of Cohn and Edsall (*31*) using the amino acid partial specific volumes from (*32*). The values calculated in this way were as follows: $\bar{v} = 0.734$ cm^3/g for r-NP, $\bar{v} = 0.731$ cm^3/g for r-NP142, and $\bar{v} = 0.742$ cm^3/g for r-NP121.

Conformational Parameters. By combining the known molecular masses of the protein complexes and sedimentation velocity results, it was possible to determine the conformational parameters of the pentameric complexes formed by the different nucleoplasmin constructs. The frictional coefficients and asymmetry of the complexes were calculated as described previously (*33*) assuming an oblate ellipsoid shape of semiaxes a and b ($\gamma = a/b$) with (*34*)

$$f/f_0 = \frac{(\gamma^2 - 1)^{1/2}}{\gamma^{2/3} \tan^{-1}[(\gamma^2 - 1)^{1/2}]} \quad (1)$$

where

$$f = \frac{M}{sN}(1 - \bar{v}\rho) = 6\pi\eta R_s \quad (2)$$

and

$$f_0 = 6\pi\eta R_0 \quad (3)$$

with

$$R_0 = [3M(\bar{v} + \epsilon_1)/4\pi N]^{1/3} \quad (4)$$

M = the molecular weight of the complex, s = the sedimentation coefficient, N = Avogadro's number, $\bar{v}$ = the partial specific volume, ρ = the solution density, η = the viscosity of the solvent, $R_s = (f/f_0)R_0$ = Stokes radius, R_0 = the radius of a sphere corresponding to the volume of the macromolecule, and ϵ_1 = the preferential hydration parameter. A value $\epsilon_1 = 0.22$ g of H_2O/g of protein was used in the calculations (*35*). The ratio $f/f_0 = 1$ corresponds to a perfectly spherical molecule, and higher values are indicative of the extent of departure from the spherical shape. From γ, the values for the semiaxes a and b were determined for a hydrated volume, V_h, of the protein equivalent to an oblate:

$$V_h = \frac{M}{N}(\bar{v} + \epsilon_1) = (4/3)\pi a^2 b \quad (5)$$

The radius of gyration can then be calculated as follows (*36*):

$$R_G{}^2 = (b^2 + 2a^2)/5 \quad (6)$$

Sperm Chromatin Decondensation Assay. Demembranated *D. labrax* sperm nuclei were incubated in the presence of nucleoplasmin, and its chromatin decondensing activity was monitored by fluorescence microscopy (*14*). In brief, 2 μL of nuclear suspension containing approximately 3.6×10^5 nuclei in EM buffer was mixed with an equal volume of Hoechst 33258 and placed under a coverslip. Then, 5 μL of 5.8×10^{-5} M nucleoplasmin in the same buffer was allowed to diffuse under the coverslip. The fluorescence resulting from the dye intercalation into DNA was monitored at $\lambda =$ 360 nm. Control experiments were carried out using 5 μL of EM buffer. In addition, samples containing 20×10^6 nuclei were incubated in 70 μL of EM buffer in the absence (control) or in the presence of 5.8×10^{-5} M nucleoplasmin for 90 min. at room temperature. After incubation, the samples were centrifuged at 16000*g* in an Eppendorf microfuge (Briknman Instruments Inc., Westbury, NY), and the proteins from the supernatants and pellets were electrophoretically analyzed using acetic acid/urea/polyacrylamide gel electrophoresis as described in (*13*).

Determination of the Nucleoplasmin/Protamine Stoichiometry. The stoichiometry of the nucleoplasmin/protamine complexes was determined by fluorescence spectroscopy and by electrophoretic analysis.

In the first instance, the change in fluorescence emission resulting from the titration of a nucleoplasmin solution with increasing amounts of protamine was analyzed in a PTI spectrofluorimeter (Photon Technology International Inc., London, Ontario, Canada). Monochromators were set at 295 nm for excitation and at 340 nm for emission, and the slit was set at 4 nm. Fluorescence emission was measured from 300 to 450 nm at 25 °C with continuous stirring. The concentration of nucleoplasmin was 1 μM in EM buffer, and its fluorescence emission was allowed to stabilize for 30 min before proceeding with the protamine titration. Control experiments were also carried out by performing an identical titration with buffer lacking protamine. Corrections were done to account for the dilution factor introduced during the titration. Mathematical analyses of the spectral signals were performed with FELIX software (PTI, London, Ontario).

For the electrophoretic analysis, 2 μg of protamine was incubated in EM buffer with increasing amounts of nucleoplasmin for 30 min at room temperature. The excess of protamine not bound to nucleoplasmin was determined by analysis from neutral pH polyacrylamide gels (see below).

Polyacrylamide Gel Electrophoresis (PAGE). Sperm nuclear basic proteins were analyzed using acetic acid (5%)/urea (2.5 M)/15% polyacrylamide gels (*37*) or by neutral pH, 15.9% polyacrylamide gels in the absence of urea (*38*). In both instances, the polymerization of the gels was carried out using thiourea/H_2O_2 as described in (*39*).

Nucleoplasmin was analyzed using either SDS−PAGE (15% polyacrylamide) (*40*, *41*) or by native PAGE (10% polyacrylamide) (*42*).

RESULTS

Nucleoplasmin Mutants. In addition to the recombinant version of Bürglin's *Xenopus* nucleoplasmin (*20*), we were also able to express two C-termini deletion mutants of the same protein. r-NP142 is a nucleoplasmin version truncated at amino acid 142 (see Figures 1 and 2), in which the main polyglutamic tract has been preserved. The deletion would be expected to enhance the exposure of this highly charged region to the buffer and hence affect the sperm nuclear decondensing activity of the molecule. In r-NP121, the entire

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NPB  1          NTSKVEKPVSLIWGCELNEQNKTFAFKIEDEEEKCEHQLALRTV
NPD  1    MASTVSNTSKLEKPVSLIWGCELNEQDKTFEFKVEDDEEKCEHQLALRTV
                ****  *************** *** ** ** *************

NPB  45   CLGDKAKDEFHIVEIVTQEEGKEKPVPIASLKPSILPMATMVGIELTPPV
NPD  51   CLGDKAKDEFNIVEIVTQEEGAEKSVPIATLKPSILPMATMVGIELTPPV
          ********** ********** ** **** ********************

                                ▼
NPB  95   TFRLKAGSGPVYISGQHVAMEEDYSWAEEEDEGE----EEEEEEEDPESP
NPD  101  TFRLKAGSGPLYISGQHVAMEEDYSWAEEEDEGEAEGEEEEEEEEDQESP
          ********** ********************     ******** ***

          ▼
NPB  141  PKAVKRPAATKKAGQAKKKKLDKEDESSEEDSPTKKGKGAGRGRKPAAKK
NPD  151  PKAVKRPAATKKAGQAKKKKLDKEDESSEEDSPTKKGKGAGRGRKPAAKK
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FIGURE 1: Primary structure of *Xenopus laevis* nucleoplasmin. The amino acid sequences from the two cDNA clones determined by Bürglin et al. (*20*) (*NPB*) and by Dingwall et al. (*19*) (NPD) are aligned for comparison. Identical amino acids are indicated by an asterisk. Note that most of the variation between the two sequences involves only conservative changes. Acidic amino acids are in boldface, and the arrowheads point to the C-terminal end of the deletion mutants shown in Figure 2.

Table 1: Conformational Parameters of the Pentameric Complexes of the Different Nucleoplasmin Constructs

parameter	r-NP	r-NP142	r-NP121
molecular mass[a]	110229	79864	67502
$\bar{v}$ (cm^3/g)	0.734	0.731	0.742
$s_{20,w}$ (S)	6.4	5.4	4.8
f/f_0	1.17	1.14	1.09
R_0 (Å)	34.7	31.3	29.6
R_s (Å)	40.6	40.7	32.3
a/b	4.1	3.5	2.8
a (Å)	54.2	47.5	41.8 (36.4)[b]
b (Å)	13.6	13.6	14.9 (13.0)[b]
R_G (Å)	34.8	30.7	27.3

[a] The molecular masses of the pentameric forms were calculated from the molecular masses of the monomer as deduced from the amino acid sequences (M_r = 22 046, 15 973, and 13 501 for r-NP, r-NP142, and r-NP121, respectively). [b] Assuming a cylinder shape of radius = *a* and height = 2*b*.

C-terminal main polyglutamic tract of the molecule has been removed. This was expected to abolish the nuclear decondensing activity of nucleoplasmin. By using this mutant, we wanted to check if the C-terminal polyglutamic tracts are indeed responsible for the sperm chromatin decondensation as previously hypothesized (*10*, *19*, *20*). Figure 2A schematically summarizes the different recombinant nucleoplasmin constructs used in this work.

A commonly encountered problem with recombinant nucleoplasmin expression in *E. coli* has to do with the coexpression of a variable amount of anomalously truncated forms that often interfere with the purification of the full-length molecule (*13*, *23*, *43*). However, our purification strategy allowed us to eliminate this problem as seen from the electrophoretic homogeneity of the proteins shown in Figure 2B.

Conformation of the Nucleoplasmin Complex. Previous work with recombinant nucleoplasmin proteins suggested that the presence of histidine tags at their amino termini could lead to improper oligomerization of these molecules promoting the formation of larger association complexes (*13*). To avoid this problem, all the recombinant nucleoplasmin constructs used in this work were expressed without any tagging and were tested for their oligomerization ability before proceeding with any further characterization.

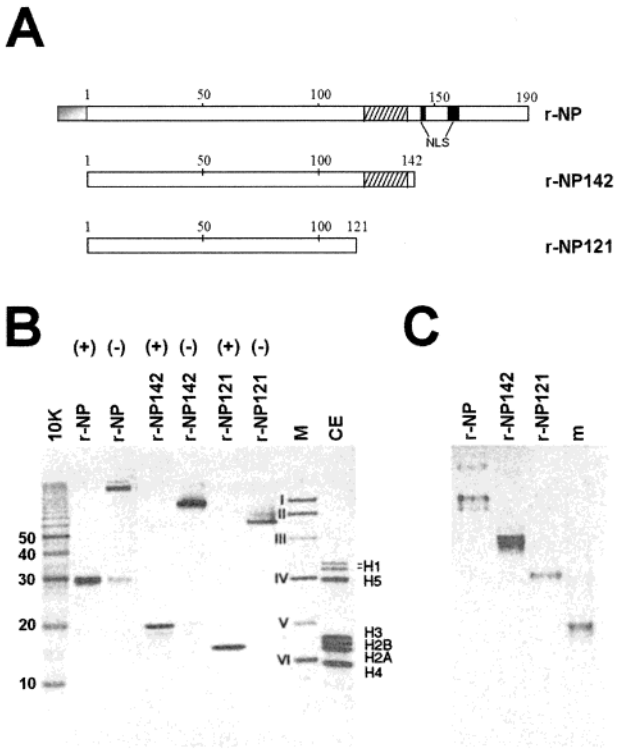

FIGURE 2: Recombinant nucleoplasmin and deletion mutants. (A) Schematic representation of the full-length recombinant nucleoplasmin (r-NP) and the two deletion mutants (r-NP142, r-NP121; see arrowheads in Figure 1) produced by overexpresssion in *E. coli* using different Bürglin's cDNA constructs. Amino acid numbering is as in Figure 1 (*NPB*). The first eight N-terminal amino acids of r-NP (ARIRAQFR, shadowed box) are a result of the cloning procedure used in the construction of the pET16b-NED plasmid provided to us by Dr. Kalinich (*23*). NLS indicates the nuclear localization signal, and the main acidic (polyglutamic) tract of nucleoplasmin is shown by the hatched box. (B) SDS−PAGE analysis of the different r-NP constructs with (+) or without (−) boiling. Note that in the absence of boiling the samples retain their pentameric association even in the presence of the SDS contained in the electrophoresis loading buffer. Used as markers in these gels were the following: CE, chicken erythrocyte histones, 10 K, 10 kDa peptide ladder (Gibco BRL, Life Technologies, Grand Island, NY); and M, low molecular weight protein marker (Bio-Rad, Hercules, CA) (the M_rs are I, 97 400; II, 66 200; III, 45 000; IV, 31 000; V, 21 500; VI, 14 400). (C) Native PAGE analysis of the recombinant nucleoplasmin constructs. m, nucleoplasmin monomer (r-NP142).

Figure 2B and 2C show that all the nucleoplasmin constructs used by us exhibit a molecular mass which is larger than that of their corresponding monomeric forms. The pentameric nature of the complexes was confirmed by analytical ultracentrifuge analysis using both sedimentation equilibrium (results not shown) and sedimentation velocity analysis (Figure 3). Table 1 summarizes the conformational parameters of the different nucleoplasmin complexes. The oblate shape was chosen based on the shape available from the crystallographic structure of the nucleoplasmin core pentamer (*44*).

At the secondary structural level, the CD spectra of both o-NP and r-NP exhibited a large peak with a minimum at 198 nm which is in agreement with our previously published results (*13*). In contrast, the CD spectra of r-NP121 and r-NP142 exhibit a minimum at about 215 nm (data not shown) which is characteristic of the β-pleated sheet conformation (*45*). The displacement toward higher wave-

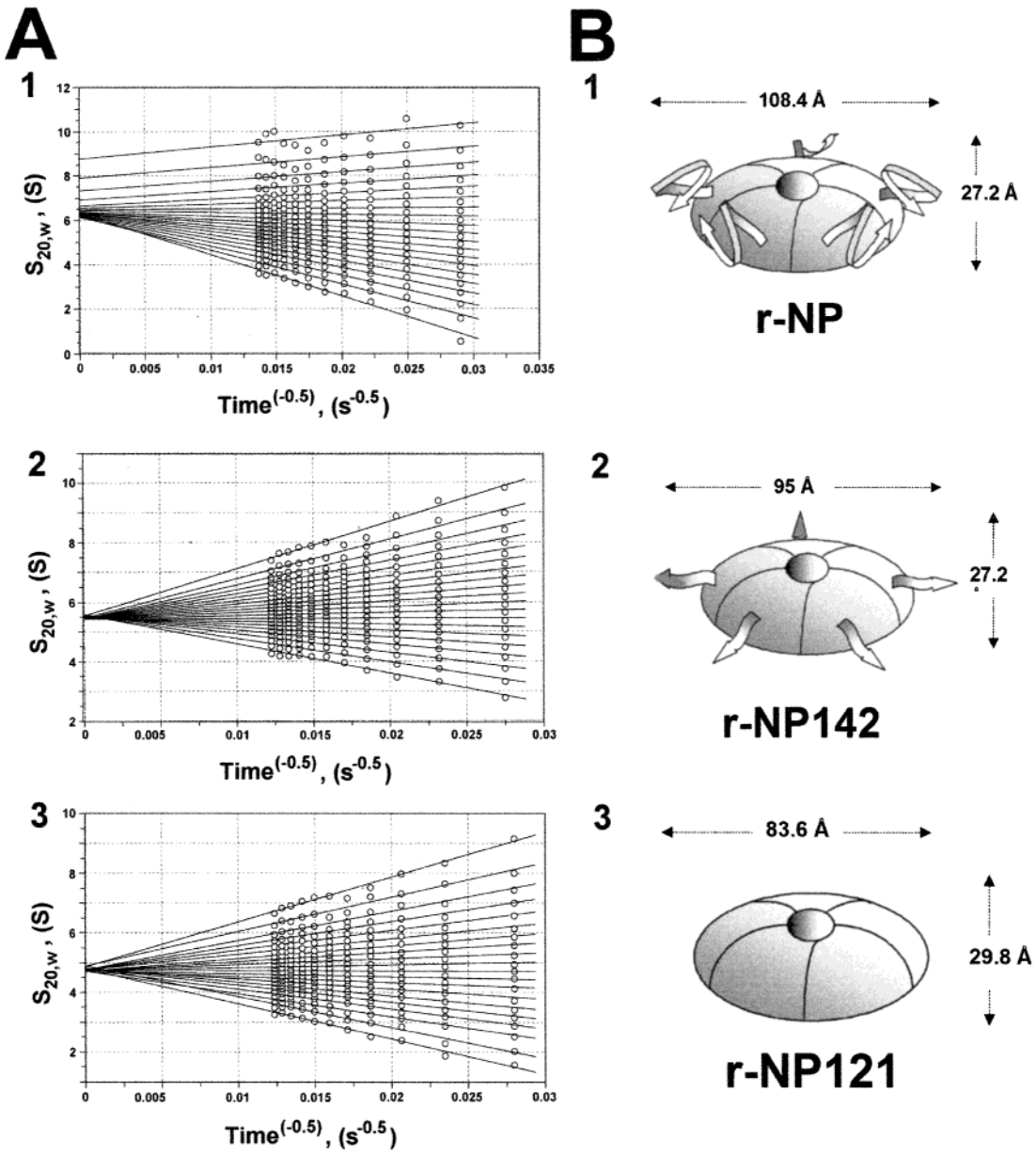

FIGURE 3: Sedimentation velocity analysis of different nucleoplasmin constructs (A) and models proposed from the conformational parameters shown in Table 1 (B) for r-NP (1), r-NP142 (2), and r-NP121 (3). The runs were performed at 20 °C and 40 000 rpm in 100 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4) (EM) buffer. The sedimentation velocity analysis was carried out according to van Holde and Weischet (*28*). In the "fan plots" obtained with this analysis, each of the lines converging toward a common $s_{20,w}$ value (in Svedberg units, S) is proportional to the fraction of sample represented. Time is shown in seconds. In the models shown in (B), the ribbons represent the highly charged carboxyl-terminal domain of nucleoplasmin, and the arrowheads represent the C-termini.

lengths of the minimum exhibited by the C-termini truncated mutants reflects a larger contribution of the β-pleated sheet structure to the N-terminal portion of nucleoplasmin, in agreement both with recent crystallographic data (*44*) and with other previously published results using recombinant nucleoplasmin from Dingwall's clone (*43*). The loss of random coil structure and the increase in β structure as the C-terminal domains are removed support the notion that these domains exhibit a rather extended conformation which will be discussed later.

Sperm Nuclear Decondensation Activity. The chromatin of the sperm from the fish *Dicentrarchus labrax* consists of a single homogeneous protamine which tightly compacts the DNA within the sperm nucleus. This protamine has a typical fish protamine primary structure with the sequence: $P(R)_4QASRPV(R)_5T(R)_2STAE(R)_5(V)_2(R)_4$ (*25*) that closely resembles that of the protamines found in the sperm of some amphibians such as the toad *Bufo japonicus* [P1 = $(P)_2$-$(R)_2KRV(S)_2AP(R)_5TY(R)_2(T)_2AHKHQDRPVH(R)_6H$] (*46*). Thus, in contrast to *Xenopus laevis*, in which spermatozoa consist of six SNBPs plus histones (see the introduction), the single protamine component of the sperm nuclei of *D. labrax* provides a more simple model for the analysis of sperm chromatin decondensation.

Incubation of *D. labrax* nuclei with equivalent molar concentrations of the different nucleoplasmin molecules described in this paper (see Materials and Methods) produced the results shown in Figure 4. We observed that while the swelling of nuclei induced by native o-NP was faster and more extensive, all the other recombinant forms (including r-NP121, which lacks the main polyglutamic tract) were able to promote swelling of the nuclei (see Figure 4A). These

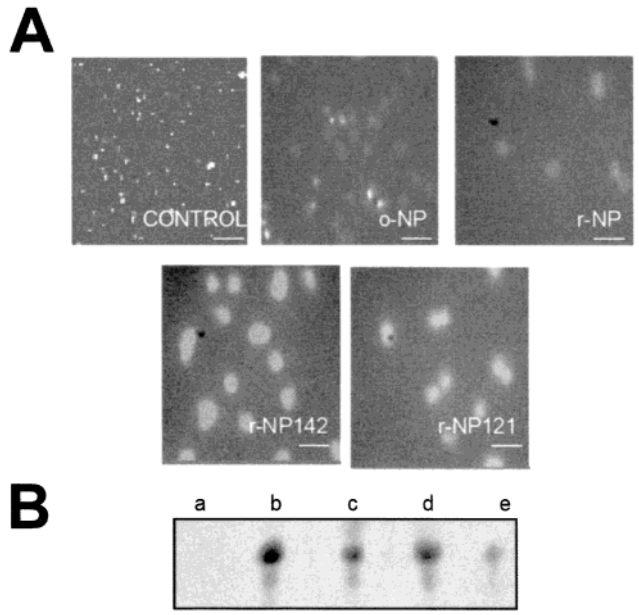

FIGURE 4: Nucleoplasmin nuclear sperm decondensing activity. (A) Fluorescence micrographs of the protamine-containing sperm nuclei from the fish *Dicentrarchus labrax* after incubation with EM buffer (control) and with either native oocyte (o-NP) or different recombinant nucleoplasmin constructs (r-NP, r-NP142, r-NP121) (see text for details). The bar corresponds to 50 μm. (B) Acetic acid/urea/PAGE analysis of the protamines removed from *D. labrax* sperm nuclei after incubation in the presence of (a) EM buffer (control), (b) o-NP, (c) r-NP, (d) r-NP142, and (e) r-NP121.

results came as a surprise as we were expecting that r-NP121 would be unable to remove protamines due to the lack of the main polyglutamic stretch. To ensure that the nuclear swelling observed was the result of protamine removal, sperm nuclei incubated with the same nucleoplasmin forms were centrifuged, and the proteins of both the pellets and the supernatants were electrophoretically analyzed (see Materials and Methods for more details). As shown in Figure 4B, native oocyte nucleoplasmin (o-NP) was found to be the most effective in removing protamines. All the recombinant nucleoplasmin constructs exhibited a similar albeit slightly reduced activity, with r-NP142 and r-NP121 being the most and least effective, respectively. Therefore, r-NP121, the mutant lacking the main C-terminal polyglutamic tract, while still able to promote nuclear swelling, exhibits a significantly impaired protamine removal efficiency when compared to the other recombinant forms.

Stoichiometry of the Nucleoplasmin/Protamine Complexes. The next step was to determine the binding stoichiometry of the nucleoplasmin/protamine complexes. We used two different approaches.

In a first attempt, we used the fluorescence spectrophotometry approach described by Iwata et al. (*22*). With this technique, the intrinsic fluorescence of the nucleoplasmin molecule gradually decreases upon sequential addition of protamine up to a point where a plateau is reached when a fully saturated nucleoplasmin/protamine complex is formed (*22*). Our attempts to reproduce these data showed that it was possible to observe two types of fluorescence decrease. The first one occurs prior to the addition of protamine and stabilizes after approximately 30 min at room temperature. The nature of this decrease is presently unknown. A second decrease occurs upon further sequential addition of protamine. The inflection point obtained before the second plateau was reached corresponds to a ratio of 2.5 mol of protamine/mol of pentameric nucleoplasmin (see Figure 5A1).

To corroborate this value, an electrophoretic approach was used which utilizes neutral PAGE (see Materials and Methods and also legend to Figure 5A2). Under the experimental conditions used in this approach, only the protamine molecules in excess of a stoichiometric protamine/nucleoplasmin complex were able to enter the gel. Therefore, as the titration of a constant amount of protamine by increasing amounts of nucleoplasmin takes place, the amount of free protamine entering the gel decreases until a saturated complex is formed. Beyond this point, and maintaining the same amount of nucleoplasmin, if the amount of protamine is increased in the titration, it will then be able to freely enter the gel again. The inflection point determined in this way (see Figure 5A2) corresponds to a stoichiometric value for the saturation of the complex which is identical with that obtained from fluorescence determination (Figure 5A1). Using this approach, the same protamine binding stoichiometry for the r-NP121 and r-NP142 truncated forms of nucleoplasmin was found (Figure 5B1,2).

DISCUSSION

Defining the Model. About 20 years ago, a cell-free system was originally developed using amphibian egg extracts in an attempt to study the mechanisms involved in the transformation of the sperm nuclei upon fertilization (*47*, *48*). This system mimics the events that take place in the intact egg (*47*, *49*−*51*, reviewed in *12*). It was later demonstrated that nucleoplasmin was one of the proteins that plays an important role in these events. It was shown that nucleoplasmin is necessary and sufficient for the initial decondensation events of the male pronucleus, acting as a disassembly and assembly factor for chromatin remodeling during fertilization (*7*, *8*).

Several sperm decondensation factors have been shown to exhibit a broad range of interspecific activity. For instance, *Xenopus* sperm can be decondensed by *Drosophila* embryo extracts (*52*−*54*) while *Xenopus* egg extracts can remodel sperm from organisms as diverse as mussels (*14*) and humans (*51*, *55*). Furthermore, purified nucleoplasmin is by itself able not only to decondense but also to reassemble sperm chromatin into nucleosomes in a similar interspecific way (*8*, *11*).

The primary structure of nucleoplasmin revealed the existence of several polyglutamic acid tracts (*19*, *20*). Those tracts, and, more specifically, the almost uninterrupted run of acidic amino acids from residues 122−138 (Figure 1, *NPB*), were immediately thought to be good candidates to explain the chromatin remodeling activities of the molecule. One of the initial purposes of our work was to provide experimental support to this hypothesis. To this end, we have constructed and bacterially expressed different truncated forms (r-NP121, r-NP142) of *X. laevis* nucleoplasmin lacking different portions of the C-terminal end of the molecule which contains the main acidic tract. With them, we have assessed their ability to decondense the sperm nuclei of the fish *D. labrax* whose major sperm nuclear basic protein belongs to the protamine type (*1*). The ability of *X. laevis* nucleoplasmin to decondense fish sperm nuclei has already been determined (*13*).

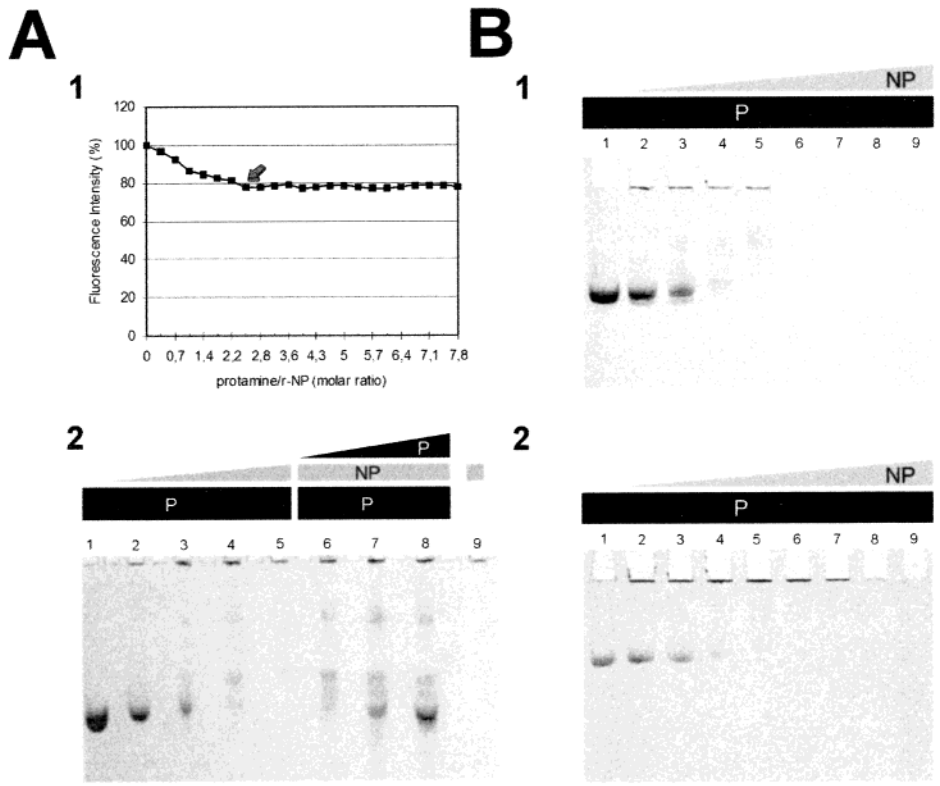

FIGURE 5: Determination of the protamine/nucleoplasmin stoichiometry. (A1) Analysis of the nucleoplasmin intrinsic fluorescence variation upon addition of increasing amounts of *D. labrax* protamine. A stable fluorescence plateau is reached upon addition of 2.5 mol of protamine/mol of whole (r-NP) nucleoplasmin pentamer as indicated by the arrow. (A2) Neutral pH-PAGE analysis of the binding stoichiometry of *D. labrax* protamine to r-NP. This approach is based on the fact that under the experimental conditions used for these neutral gels (which ran from + to −) only protamines which are positively charged (but not nucleoplasmin or the insoluble protamine/nucleoplasmin complexes) can enter the gels. As schematically shown on top of the gel, a fixed amount of *D. labrax* protamine is initially incubated with increasing amounts of nucleoplasmin until a fully saturated complex is formed and hence no protamine is able to enter the gel. Further addition of protamine beyond this point does not participate in the complex providing the gel with its symmetric appearance. The (mol of protamine/mol of r-NP pentamer) ratios for the different lanes were (1) 2.5/0, (2) 10, (3) 5, (4) 3.3, (5) 2.5, (6) 3.1, (7) 3.75, (8) 4.37, and (9) 0/3.75. (B) Shows the same analysis as in A2, for (1) r-NP142 and (2) r-NP121. In these two instances, only the first part of the titration is shown. The mol of protamine/mol of NP (r-NP142 or r-NP121) pentamer ratios for the different lanes were (1) 2.5/0, (2) 10, (3) 5, (4) 3.3, (5) 2.5, (6) 2, (7) 1.7, (8) 1.4, and (9) 1.25.

The r-NP142 construct has a deleted C-terminal region ranging from amino acid 143 to the end of the molecule (see Figures 1 and 2). This deletion mutant corresponds to the trypsin-resistant core of nucleoplasmin (*19*) and contains the main polyglutamic tract at the very C-terminal end. We reasoned that this structural characteristic might confer an enhanced mobility (accessibility) to this polyacidic domain that would alter the decondensation properties of the native molecule. Most relevant was deletion mutant r-NP121 which lacks the C-terminal end of nucleoplasmin from amino acid 122. This mutant lacks the main polyglutamic tract of the native form (see Figure 2A) and according to the current hypothesis should lack sperm nuclei decondensing activity.

The Conformation of Nucleoplasmin. The crystallographic data (*44*) show that the nucleoplasmin core pentamer has an average diameter of 60 Å and is 40 Å tall, in agreement with earlier electron micrographs (75 Å diameter) (*17*). The conformational parameters determined by us in solution (Table 1) coincide with these values. Indeed, the r-NP121 pentamer, which represents the closest approximation to the crystallographic structure of the pentameric core, can be modeled according to our data either by an oblate 83.6 Å in diameter and 29 Å tall (see Figure 3B3) or by a cylinder 73 Å in diameter and 26 Å tall (see Table 1). Significantly, the whole nucleoplasmin molecule exhibits an important increase in diameter (108 Å) without any major departure in its height (27 Å) as sketched in our model (Figure 3B1). The apparent disagreement with the diameter obtained by electron microscopy (*17*) may possibly reflect the fact that the C-terminal domains protruding beyond the core exhibit a rather extended conformation and a reduced level of tertiary structure which would make them invisible by electron microscopy.

Role of the Carboxyl-Terminal Polyglutamic Tracts of Nucleoplasmin in the Sperm Decondensing Activity of the Molecule. Our sperm decondensation experiments (Figure 4A) show that all the recombinant NP constructs are efficient in this process. However, the ability to remove protamines (Figure 4B) was lower for any of the recombinant forms as compared to their native o-NP counterpart (Figure 4B, lane b). This difference in activity can be accounted for in part by the lack of phosphorylation in the bacterially expressed constructs. Phosphorylation of o-NP has been shown to play a very important role in the nuclei decondensing activity of the molecule (*56*, *57*). The substantially lower efficiency of r-NP121 to remove protamines (Figure 4B, lane e) contrasts with its ability to decondense sperm nuclei (Figure 4A) and with the constant stoichiometry (2.5 mol of protamine/mol of NP pentamer) with which all the r-NP forms bind to protamines. Thus, while it appears that the C-terminal

polyglutamic tracts of nucleoplasmin are not indispensable for sperm nuclei decondensation, they do enhance its protamine removal efficiency. The lack of effect of the polyglutamic tract on sperm nuclei decondensation is reminiscent of the results obtained with NAP-1 where deletion of the most negatively charged part of the molecule was shown to be dispensable for nucleosome assembly (*58*). It is possible that the more exposed polyglutamic tract of mutant r-NP142 may account for the slightly greater activity of this form. The results obtained by Hierro (*59*) using a Dingwall's nucleoplasmin clone and *X. laevis* sperm also point to an increased nucleoplasmin decondensation activity resulting from higher exposure of the main polyglutamic tract. The swelling effect of nucleoplasmin on *D. labrax* sperm nuclei is more conspicuous than that observed by these authors on *X. laevis* (*59*). This is probably so because nucleoplasmin does not remove all the SNBP from the *X. laevis* nuclei while it removes the single protamine component in *D. labrax*.

The results of the protamine/nucleoplasmin stoichiometry also have important implications. It has been proposed that the polyacidic regions of each monomeric subunit in the nucleoplasmin pentamer could act in a concerted way like a "hand" or a "five fingered grab" producing a large negatively charged region that would operate as a single binding site for histones (*19*). However, our results using nucleoplasmin and protamines showed that all the recombinant constructs used by us exhibit the same stoichiometry of 2.5 molecules of protamine per molecule of nucleoplasmin pentamer. This result suggests that at least when it comes to interacting with fish protamines, nucleoplasmin may rather operate as two pentamers ("two hands") acting together to "grab" five molecules of protamine. This could be accomplished either by an "internal grab" (i.e., five protamines sandwiched between two nucleoplasmin pentamers) or through external interactions. Alternatively, the stoichiometry found may represent an average of the number of protamine molecules bound per nucleoplasmin pentamer, which may be variable.

The stoichiometry results described in this paper are in contradiction with previous reports by Iwata and co-workers (*22*), who also used a fluorescence spectroscopy approach to the problem. Their results indicate a 1:1 ratio of protamine per nucleoplasmin monomer. However, no indication is given in their paper regarding the intrinsic fluorescence decay observed by us in the absence of protamine and which could have resulted in a higher stoichiometry ratio estimate.

In summary, the results presented here show the following: (a) both full-length nucleoplasmin and all our deletion mutants present an overall shape that can be modeled by an oblate ellipsoid formed by five nucleoplasmin monomers; no evidence of a decamer particle (*44*) has been found; (b) all the recombinant nucleoplasmin forms produced (even the one lacking the main polyglutamic tract) are able to decondense sperm nuclei by removing its protamine; (c) the main acidic tract of nucleoplasmin, although not essential, highly enhances protamine removal from sperm chromatin; and (d) the stoichiometry of the nucleoplasmin/protamine complexes corresponds to a value of 2.5 mol of protamine/mol of pentameric nucleoplasmin. Further work remains to be done to elucidate the elusive molecular aspects involved in the mechanisms of sperm chromatin remodeling by this fascinating molecule.

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