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Purification of a novel, nucleoplasmin-like protein from somatic nuclei

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We have purified a nucleoplasmin-like protein from the nuclei of somatic *Xenopus laevis* cells. This protein possesses a number of the distinctive features of nucleoplasmin isolated from oocytes or unfertilized eggs. The protein is recognized by both monoclonal and polyclonal antisera raised against egg nucleoplasmin. The protein has an oligomeric structure, which must be heated in SDS to completely dissociate, is acidic, phosphorylated and efficiently promotes the *in vitro* formation of chromatin. We have partially characterized this novel protein and because of its resemblance to nucleoplasmin isolated from oocytes or unfertilized eggs we have named this protein nucleoplasmin S.

Key words: chromatin/histone/nucleoplasmin/phosphorylation/somatic

Introduction

Nucleoplasmin and N1/N2 are acidic, phosphorylated proteins which accumulate in association with histones in amphibian oocytes (Kleinschmidt and Franke, 1982; Kleinschmidt *et al.*, 1985). These proteins promote the formation of soluble chromatin structures *in vitro*, at physiological ionic strengths, from purified histones and DNA, the property for which nucleoplasmin was originally isolated (Laskey *et al.*, 1978; Mills *et al.*, 1980; Earnshaw *et al.*, 1980). The notion that these proteins may play a role in the transport and/or deposition of histones is supported by the observation that shortly before fertilization, which triggers a period of very rapid chromatin assembly, the oocyte form of nucleoplasmin becomes massively phosphorylated (Sealy *et al.*, 1986; Cotten *et al.*, 1986; Burglin *et al.*, 1987). This modification profoundly enhances the chromatin assembly activity of this protein (Sealy *et al.*, 1986; Cotten *et al.*, 1986). Both nucleoplasmin and N1/N2 are karyophilic proteins (De Robertis *et al.*, 1978; Dabauvalle and Franke, 1982) and the active process of transport into the nucleus has been studied in detail with nucleoplasmin (Dingwall *et al.*, 1982; Feldherr *et al.*, 1984; Newmeyer *et al.*, 1986a,b). In addition, there is evidence that nucleoplasmin may be involved in RNA metabolism. The protein has been found associated with actively transcribed regions of lampbrush chromosomes (Moreau *et al.*, 1986). Immunological evidence suggests that both nucleoplasmin (Krohne and Franke, 1980a,b) and N1/N2 (Krohne, 1985) are present in the nuclei of a range of vertebrate cell types and are not a peculiar feature of amphibian oogenesis. However, there are no published reports of the purification of nucleoplasmin from a non-gamete cell type.

We are studying the process of chromatin assembly and gene activation in eukaryotic cells and are interested in further defining the role that nucleoplasmin might play in these processes in somatic cell types. We set out to determine if a nucleoplasmin-like molecule could be isolated from the nucleus of a somatic cell. Consistent with previous immunological observations (Krohne and Franke, 1980a,b), we have identified a small amount of a protein in a *Xenopus* kidney cell line which resembles the oocyte form of nucleoplasmin. This protein is either present in the cytoplasm or, more likely, it rapidly leaks out of the nucleus during isolation procedures. We have also identified and purified to homogeneity, a much more abundant, nucleoplasmin-like protein that is present in the nucleus of somatic cells. This second protein has a slightly greater mol. wt than oocyte nucleoplasmin but it possesses a number of distinctive nucleoplasmin-like features. Like oocyte nucleoplasmin, this protein exists as an oligomer (most likely either a pentamer or a hexamer) in solution, and requires boiling in SDS for complete dissociation to the monomer. The protein is recognized on Western blots by both an affinity-purified polyclonal and a monoclonal antibody raised against egg nucleoplasmin. The protein binds histones *in vitro* and possesses potent *in vitro* chromatin assembly activity. The protein has a similar amino acid composition to oocyte nucleoplasmin, with a slightly lower glutamic acid content, and the protein is phosphorylated.

This second form of nucleoplasmin is present in all tissues of *Xenopus* that we have examined. A similar protein is present in chicken embryos, calf thymus, rat liver and HTC cells, a rat hepatoma cell line. We suggest that this protein is common to all somatic cell types and because of the protein's strong resemblance to oocyte nucleoplasmin, we tentatively name this protein nucleoplasmin S (for nucleoplasmin simulacrum, should the protein be positively identified as a nucleoplasmin homologue, the S can then signify somatic). We have yet to determine the function of this protein *in vivo*. However, because the protein is as abundant in non-replicating cells as it is in replicating cells, the protein's function is probably not limited to replication.

Results

Identification of a nucleoplasmin-like molecule in somatic cells

We initiated our search for a somatic form of nucleoplasmin in XLA cells, a cell line derived from *Xenopus* kidney. A 500 mM whole cell extract was prepared and partially fractionated by ammonium sulfate precipitation and elution from phenyl-Sepharose. This material was analyzed by Western blotting, using an affinity-purified, polyclonal, anti-nucleoplasmin antibody. When oocyte or egg nucleoplasmin are analyzed with this antibody preparation an immunoreactive species of ~150 kd is observed if the sample is not boiled in SDS prior to electrophoresis (Figure 1A, lane 2). However, if the sample is boiled in SDS in order to disrupt the oligomeric structure of the protein, then the resulting signal of the monomer is at least 20-fold diminished in intensity (Figure 1A, lane 1). The antibody is apparently recognizing some feature of the oligomeric nucleoplasmin structure, relatively little of which is preserved in the conformation of the dissociated

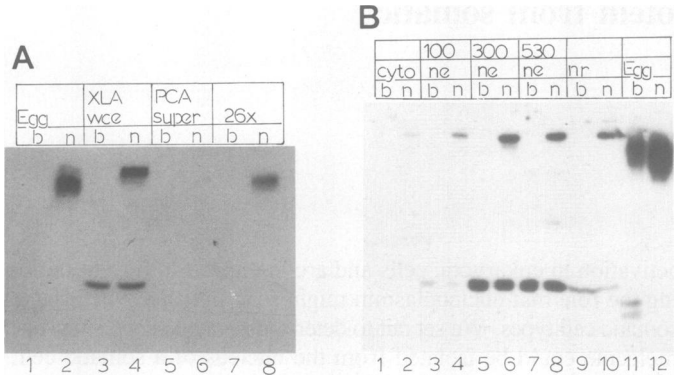

Fig. 1. Panel A: nucleoplasmin S in whole cell extracts. XLA cells were homogenized in 500 mM NaCl, 20 mM Tris, pH 8, 7 mM β-mercaptoethanol, 5 mM EDTA, 1 mM benzamidine, 0.1 mM PMSF, 0.5% Triton X-100, and centrifuged for 30 min at 10 000 r.p.m. in a Sorvall HB-4 rotor. The supernatant was adjusted to 35% saturation in ammonium sulfate, centrifuged again and this supernatant was applied directly to a phenyl-Sepharose column equilibrated with 1.5 M ammonium sulfate, 20 mM Tris, pH 8. The column was washed with three column volumes of this buffer and the bound protein was eluted with 20 mM Tris, pH 8. The eluted protein was analyzed by Western blotting. Bound antibody was localized by ^{125}I-labeled protein A incubation followed by autoradiography at −70°C. **Lanes 1** and **2**, 0.1 μg egg nucleoplasmin (purified as described by Sealy *et al.*, 1986); **lanes 3** and **4**, XLA whole cell extract; **lanes 5** and **6**, 5% perchloric-acid-soluble protein from the XLA whole cell extract after dialysis to remove the acid; **lanes 7** and **8**, the material described for lanes 5 and 6 precipitated overnight at −20°C with 4 vol ethanol and dissolved in 1/26 the initial volume. The samples in odd-numbered lanes were heated in a boiling water bath for 10 min in SDS application buffer (b); the samples in even-numbered lanes were applied directly to the gel in SDS application buffer, without heating (n). **Panel B**: screening somatic cell extracts for nucleoplasmin. XLA cells were fractionated into cytosolic and 100, 300 and 530 mM nuclear extracts as described in Materials and methods. The extracts were dialyzed into np storage buffer and aliquots were resolved by SDS−acrylamide electrophoresis, transferred to nitrocellulose and probed with affinity-purified anti-nucleoplasmin antibody. Bound antibody was localized by ^{125}I-labeled protein incubation followed by autoradiography at −70°C. **Lanes 1** and **2**, cytoplasm; **lanes 3** and **4**, 100 mM nuclear extract; **lanes 5** and **6**, 300 mM nuclear extract; **lanes 7** and **8**, 530 mM nuclear extract; **lanes 8** and **9**, material remaining with the nucleus following 530 mM extraction; **lane 11**, 1 μg purified egg nucleoplasmin; **lane 12** 0.1 μg egg nucleoplasmin. Samples in odd-numbered lanes were heated in a boiling water bath for 10 min in SDS application buffer before electrophoresis (b), samples in even-numbered lanes were applied in application buffer without boiling (n).

monomer. In XLA cell extracts we find an immunoreactive, >200-kd protein (Figure 1A, lane 4). Similar to egg nucleoplasmin, the reactivity of this XLA protein is diminished when the sample is heated in SDS (Figure 1A, lane 3). However, unlike oocyte nucleoplasmin, a lower mol. wt, presumably monomeric, signal is present and its immunoreactivity is not diminished by boiling in SDS (Figure 1A, lanes 3 and 4). That a direct precursor−product relationship exists between these two mol. wt forms is demonstrated below.

One distinctive feature of oocyte nucleoplasmin is its solubility in 5% perchloric acid (Kleinschmidt *et al.*, 1985), a property shared with histone H1 and the HMG proteins. To determine if the XLA signal possesses this characteristic, the XLA extract was adjusted to 5% PCA and the supernatant was analyzed for nucleoplasmin content by Western blotting. We find no signal in the unconcentrated sample (Figure 1a, lanes 5 and 6). If this extract is concentrated 26-fold we still find none of the higher mol. wt signal, but an oocyte nucleoplasmin-sized signal can now be observed (Figures 1A, lanes 7 and 8). Like the oocyte protein, this material has an immunoreactive high mol. wt signal which diminishes with boiling in SDS. The immunoreactivity of these XLA proteins appears to be authentic. No signal is observed when the Western is probed with normal rabbit serum nor is there a signal when the antibody is omitted and the Western is incubated only with labeled protein A (results not shown). Furthermore, a similar pattern of reactivity is observed if the Western is probed with a monoclonal antibody prepared against egg nucleoplasmin (results not shown).

Thus, consistent with previous analyses (Krohne and Franke, 1980a,b) protein immunoreactive to antibodies prepared against oocyte nucleoplasmin is present in somatic cells. However, we have observed at least two types of signal: an abundant signal with a greater mol. wt than oocyte nucleoplasmin, which is not soluble in perchloric acid, and a 26-fold less abundant, perchloric-acid-soluble species with a mol. wt that is similar to oocyte nucleoplasmin. To clarify the relation between these proteins we proceeded to purify the more abundant immunoreactive species.

Screening cell fractions for nucleoplasmin S

XLA cells were divided into a series of subcellular fractions to determine the location of nucleoplasmin S. Cells were homogenized in a Triton-containing buffer to prepare nuclei, these nuclei were washed once with the Triton-containing buffer. The initial cytoplasmic material was pooled with this Triton wash of nuclei. It is quite possible, of course, that easily extracted nuclear proteins may be present in this fraction. The nuclei were then extracted sequentially with buffers containing 100, 300 and 530 mM NaCl. Finally, the remaining nuclear proteins were released by sonicating the nuclear residue in the presence of SDS. The proteins of these extracts were separated by SDS−acrylamide electrophoresis, transferred to nitrocellulose and probed with an affinity-purified, polyclonal anti-nucleoplasmin antibody. We find that the bulk of the major immunoreactive protein in XLA cells, nucleoplasmin S, is released from the nucleus at 300 mM and higher ionic strength (Figure 1B, lanes 5−8). Although it is not shown here, if we extract with PCA and concentrate the samples as described above, the oocyte nucleoplasmin-sized signal can be found only in the cytoplasmic fraction, consistent with reports that this protein is nuclear but rapidly leaks out of the nucleus during fractionation (Krohne and Franke, 1980a,b). It appears that only a portion of the higher mol. wt signal is extracted from the nucleus with a single 300 mM salt wash. We have found that repeated extractions with buffers containing 300 mM NaCl can remove substantially all of the immunoreactive species while repeated washings with 100 mM NaCl, even in the presence of 0.5% Triton X-100, do not remove the protein (unpublished data). The amount of material remaining with the nucleus after 530 mM extraction appears to be a significant fraction of the total signal in this experiment (Figure 1B, lanes 9 and 10) because the nuclei were extracted with 530 mM NaCl only once. Nearly quantitative removal of this material can be obtained by repeating the 530 mM extraction. Furthermore, if chromatin is prepared by lysing nuclei in 10 mM EDTA, the bulk of the immunoreactive protein remains associated with the chromatin unless the ionic strength is raised above 300 mM NaCl (unpublished data).

Purification of nucleoplasmin S

The purification of nucleoplasmin S is initiated by preparing nuclei as described above and extracting them twice with 530 mM NaCl (without the lower ionic strength extractions). This nuclear extract is adjusted to 35% saturation in ammonium sulfate, the supernatant of this preparation is applied directly to a phenyl-

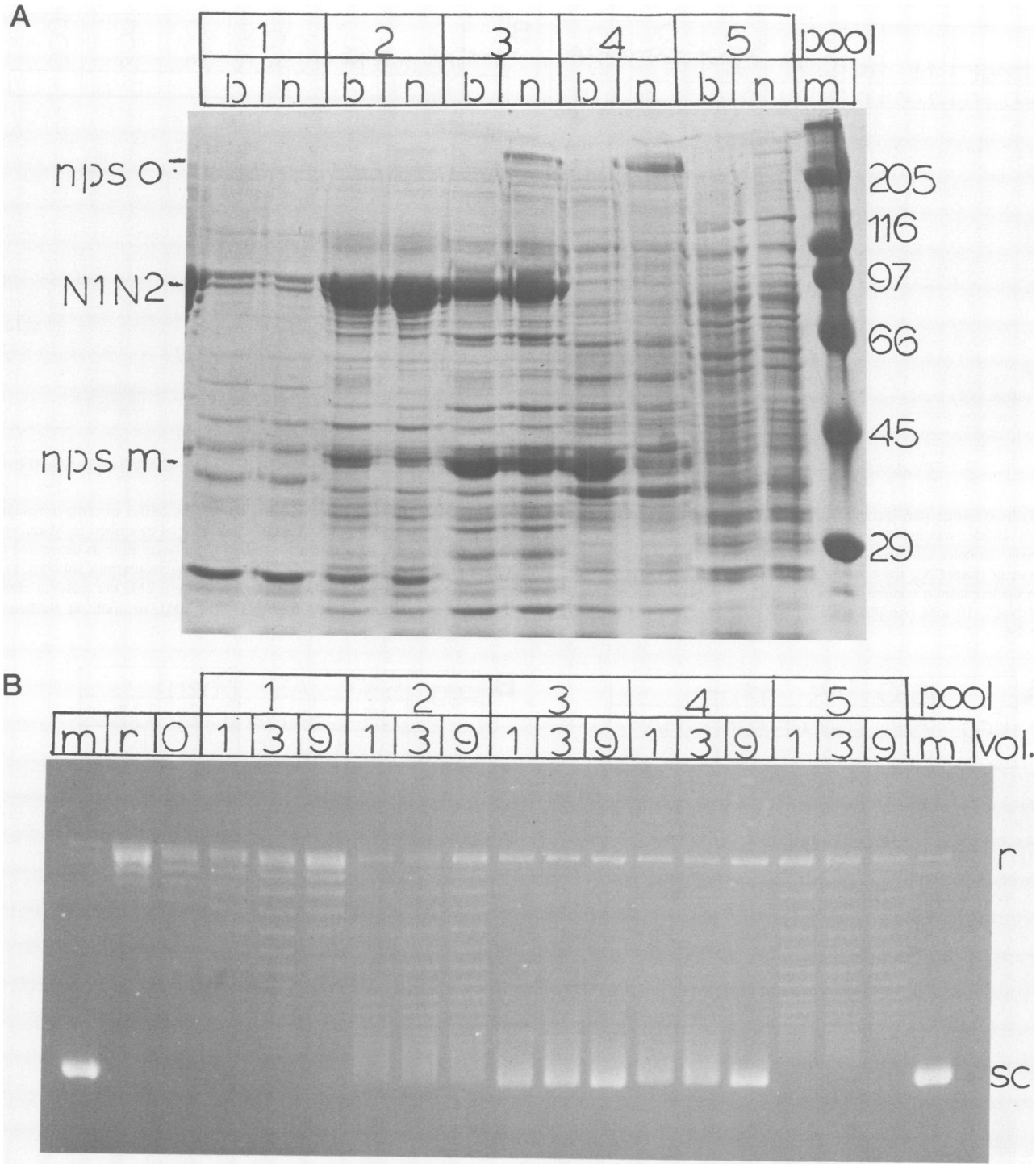

Fig. 2. Phenyl-Sepharose fractionation of chromatin assembly activity. Fractions containing proteins eluted by reverse salt gradient from phenyl-Sepharose chromatography were pooled and dialyzed into NP storage buffer. **Panel A.** Aliquots of 20 μl (containing ~5 μg total protein) were analyzed for protein content by SDS–acrylamide electrophoresis followed by staining with Coomassie Blue. The positions of the nucleoplasmin S oligomer (npsO) monomer (npsM) and of N1/N2 are indicated; m, mol. wt standards of carbonic anhydrase, egg albumin, bovine serum albumin, phosphorylase b, β-galactosidase and myosin with approximate mol. wts indicated in kilodaltons. **Panel B.** The phenyl-Sepharose pools assayed for chromatin assembly activity using 10, 30 or 90 μl of each pool (indicated by 1, 3 and 9 at the top of the figure). Assembly assays were performed as described in Materials and methods using 0.3 μg relaxed pBR322 per assay and a histone to DNA ratio of 1. The DNA from the assembly was resolved on a 2% agarose TPE gel and stained with ethidium bromide. M, Supercoiled pBR322 marker; r, the relaxed pBR322 DNA upon which the assembly was performed.

Sepharose column and eluted with a reverse salt gradient. The proteins present in pooled fractions from the phenyl-Sepharose column are shown in Figure 2A. Both nucleoplasmin S and a pair of ~100 000-kd proteins, which could be related to the oocyte N1/N2 proteins (see below) elute early in the gradient. In contrast, histone H1 (the only histone likely to have been extracted from nuclei under these ionic conditions) is not eluted until late in the gradient and will not affect the assembly assays to be described below. The monomeric/oligomeric behavior of nucleoplasmin S is most apparent in pool 4 (Figure 2A).

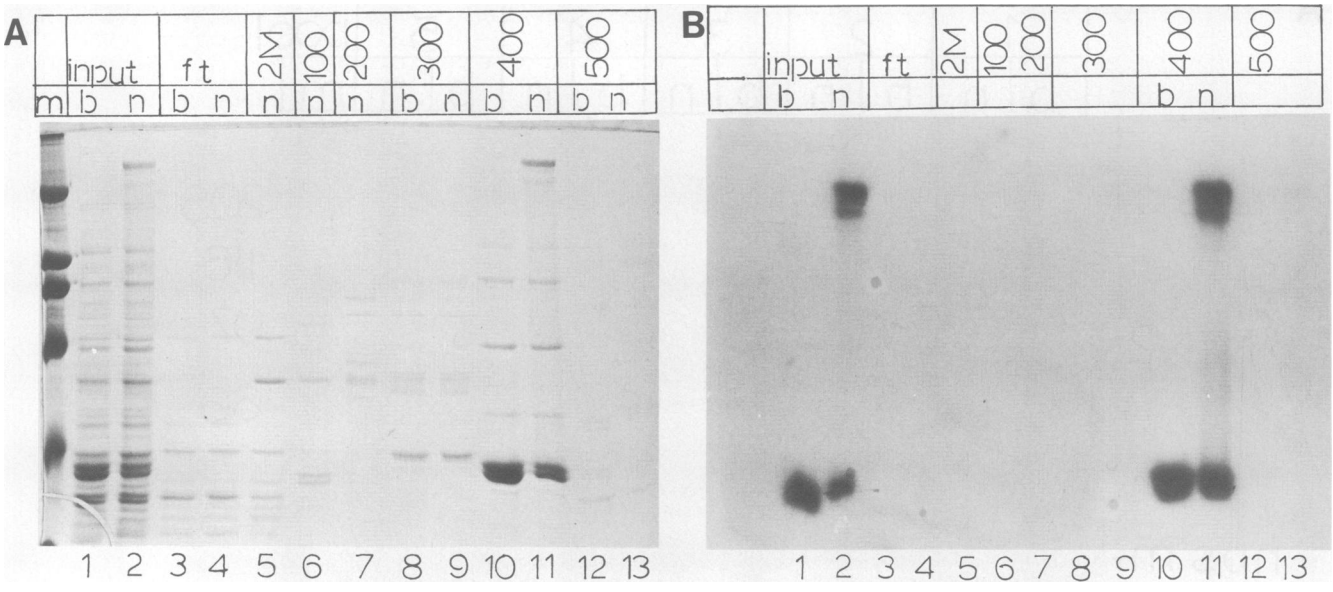

Fig. 3. Hydroxyapatite purification of somatic nucleoplasmin. The appropriate phenyl-Sepharose pool, after dialysis into np storage buffer to remove residual ammonium sulfate, was adjusted to 1 M NaCl, 10 mM sodium phosphate, pH 7, and resolved by hydroxyapatite chromatography as described in Materials and methods. Samples eluting in each step were resolved on duplicate 8% acrylamide–SDS gels. One gel was stained with Coomassie Blue to determine total protein content (**panel A**); the contents of the second gel were transferred to nitrocellulose and probed with affinity-purified anti-nucleoplasmin antibody to determine nucleoplasmin content (**panel B**). Input, phenyl-Sepharose pool 4 (see Figure 2A); ft, hydroxyapatite column flowthrough; 2M, the material eluted with 2 M NaCl, 10 mM phosphate pH 7; 100 etc., the material eluted with the indicated concentration of phosphate, pH 7 (in mM); m, mol. wt markers as in Figure 2A.

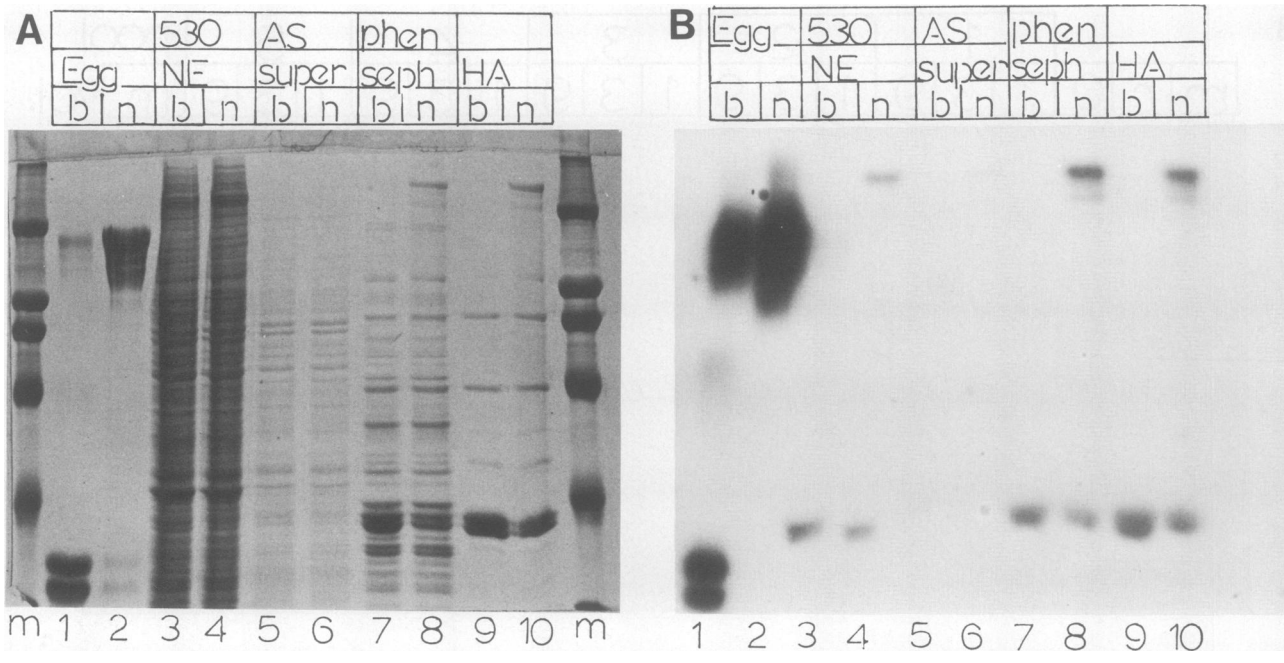

Fig. 4. A summary of somatic nucleoplasmin purification. Aliquots of material at each stage of the somatic nucleoplasmin purification were resolved by SDS–acrylamide electrophoresis on duplicate 8% acrylamide gels. One of the gels was stained with Coomassie Blue to determine total protein content (**panel A**), the second gel was transferred to nitrocellulose and probed with affinity-purified anti-nucleoplasmin antibody to determine nucleoplasmin content (**panel B**). Egg, Egg nucleoplasmin, 0.8 μg per lane in panel A, 0.8 μg (boiled) and 0.08 μg (not boiled) in panel B; 530 NE, 530 mM nuclear extract of XLA cells; AS super, 35% saturated ammonium sulfate supernatant of a 530 mM nuclear extract of XLA cells; phen-Seph, the somatic nucleoplasmin-containing pool from phenyl-Sepharose chromatography; HA, material eluted from hydroxyapatite by 400 mM phosphate; b, n, samples in SDS application buffer were either heated for 10 min in a boiling water bath (b) before electrophoresis or applied to the gel in application buffer without the boiling water bath treatment (n); m, mol. wt markers as in Figure 2A.

At this point in the purification, the *in vitro* chromatin assembly activity of the partially purified fractions can be assayed (Figure 2B). We find that a certain amount of assembly activity is associated with the phenyl-Sepharose pool containing primarily the N1/N2-like protein (pool 2). This is consistent with previous reports of the interaction of this protein with histones (Kleinschmidt *et al.*, 1985). However, a more potent assembly activity is found in those fractions which contain both the N1/N2-like protein and nucleoplasmin S (pool 3) and nucleoplasmin S without the N1/N2-like proteins (pool 4). Fractions enriched in other pro-

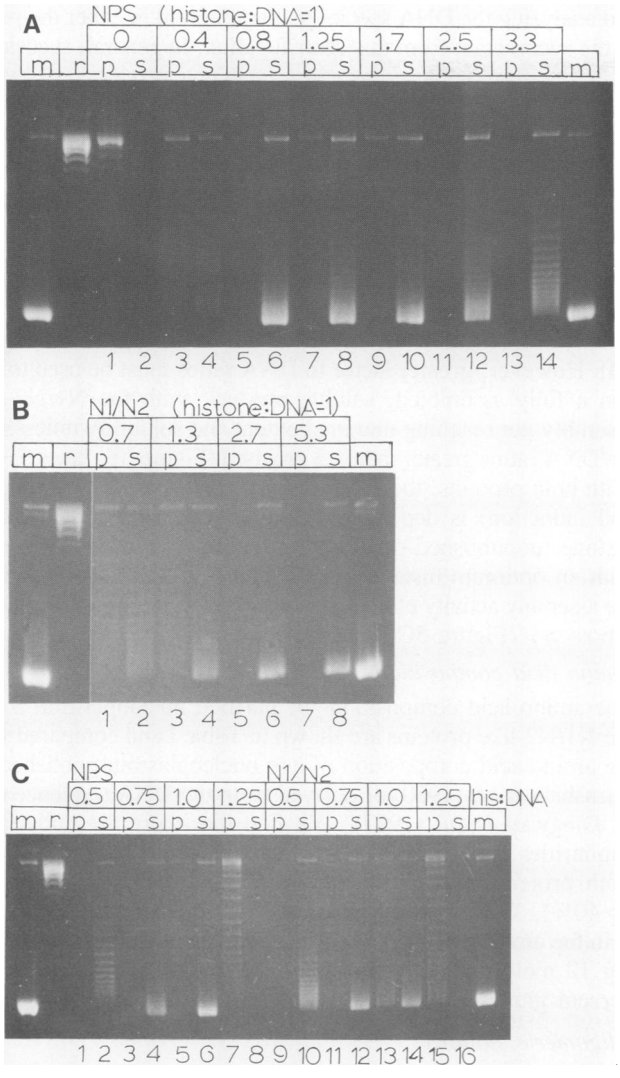

Fig. 5. Chromatin assembly with purified nucleoplasmin S and N1/N2. Chromatin assembly was performed as described in Materials and methods using 0.3 μg relaxed pBR322 per assay. Each reaction was centrifuged for 3 min at 15 000 *g* to identify precipitated material; p, the material present in the pellet from this centrifugation; s, the material present in the supernatant. M, Supercoiled pBR322 marker; R, the relaxed pBR322 used as the substrate for the assembly reaction. In all panels, DNA from the assembly assays was recovered by ethanol precipitation and resolved in 2% agarose TPE gels. The resulting DNA pattern was visualized by staining with ethidium bromide. **Panel A**: assembly with no added nucleoplasmin S (**lanes 1** and **2**) or increasing quantities of nucleoplasmin S (**lanes 3–14**) expressed as nucleoplasmin S to DNA ratios above the figure. The histone to DNA ratio was 1. **Panel B**: assembly with purified N1/N2-like proteins at the indicated N1/N2 to DNA ratios. The histone to DNA ratio was 1. **Panel C**: assembly with either nucleoplasmin S (NPS) or N1/N2 as a function of histone concentration. The indicated histone to DNA ratios were used with a nucleoplasmin S to DNA ratio of 1.25 or an N1/N2 to DNA ratio of 2.7.

teins showed no appreciable assembly activity (pools 1 and 5).

To determine if the chromatin assembly activity in these fractions is indeed a function of nucleoplasmin S and of the N1/N2-like proteins, a further purification of these proteins was obtained using hydroxyapatite chromatography. We have observed that both oocyte and egg nucleoplasmin bind hydroxyapatite efficiently and require high phosphate concentrations for elution, with the more highly phosphorylated egg nucleoplasmin requiring higher (up to 500 mM) phosphate concentrations for elution than the less phosphorylated oocyte protein (eluted by 400 mM phosphate, unpublished observation). Both nucleoplasmin S and

Table I. Amino acid composition of XLA nucleoplasmin S and N1/N2 compared with that of *X.laevis* egg nucleoplasmin (Earnshaw *et al.*, 1980) and *X.laevis* oocyte N1/N2 (derived from the amino acid sequence published by Kleinschmidt *et al.*, 1986)

Amino acid	Egg NP	NPS	Oo N1/N2	XLA 'N1/N2'
Asn	9.2	8.2	11.4	12.1
Gln	20.0	12.5	21.9	16.8
Ser	6.7	8.1	9.0	6.6
Gly	8.2	7.2	4.4	11.2
His	2.2	1.3	1.5	0.3
Arg	2.2	3.7	1.9	4.1
Thr	5.4	6.4	5.9	4.6
Ala	7.2	6.6	9.0	8.0
Pro	7.3	7.2	3.4	4.9
Tyr	2.1	1.9	1.4	1.3
Val	6.2	6.2	4.2	4.1
Met	1.0	1.4	2.4	1.2
Cys	–	1.4	0.8	2.0
Ile	3.2	4.3	3.1	6.0
Leu	6.2	8.9	6.8	4.3
Phe	2.3	2.5	1.0	3.7
Lys	11.3	12.1	10.7	12.0

Samples of eletroeluted nucleoplasmin S (Figure 6A, lanes 3 and 4) or hydroxyapatite-purified N1/N2 (Figure 6A, lane 5) were analyzed on a Waters Pico-tag system. Values are expressed as moles percent.

the N1/N2-like proteins bind to hydroxyapatite in 10 mM phosphate in the presence of up to 2 M NaCl. The protein-bound resin is then washed with a step gradient of increasing phosphate concentration. Both nucleoplasmin S and the N1/N2-like proteins, but very few other proteins, remain bound in 300 mM phosphate and are eluted with 400 mM phosphate. The hydroxyapatite elution profile of phenyl-Sepharose pool 4 (substantially free of the N1/N2-proteins; see Figure 2A) is shown in Figure 3, demonstrating the purification of nucleoplasmin S. When the nucleoplasmin-S-containing or N1/N2-containing phenyl-Sepharose pools are chromatographed on hydroxyapatite, essentially pure (>95% by protein staining) nucleoplasmin S or N1/N2-like proteins can be obtained in the 400 mM phosphate eluent. Nucleoplasmin S purified in this manner can be seen in Figure 3A. Western analysis of the hydroxyapatite purification of nucleoplasmin S (Figure 3B) demonstrates that the immunoreactive species is indeed the protein observed by Coomassie Blue staining.

A summary of the purification of nucleoplasmin S is displayed in Figure 4. A substantial purification is obtained with the ammonium sulfate fractionation. The separation of the N1/N2-like proteins from nucleoplasmin S is achieved by judiciously pooling phenyl-Sepharose fractions (see Figure 2A; only the pool containing nucleoplasmin S alone is shown in Figures 3 and 4) and a substantial purification of both proteins is obtained by hydroxyapatite chromatography. From the results of a number of preparations we estimate that both nucleoplasmin S and the N1/N2-like proteins are present in XLA cells at 0.1–0.5% of the histone (or DNA) mass.

Chromatin assembly activity

The chromatin assembly activity of these purified nuclear proteins can now be tested (Figure 5). We have previously found that a major problem interfering with *in vitro* chromatin assembly at physiological ionic strengths is the formation of high mol. wt protein–DNA aggregates (Cotten and Chalkley, 1985). The formation of these aggregates can be monitored by centrifuging assembly reactions for a brief period at the end of the reaction

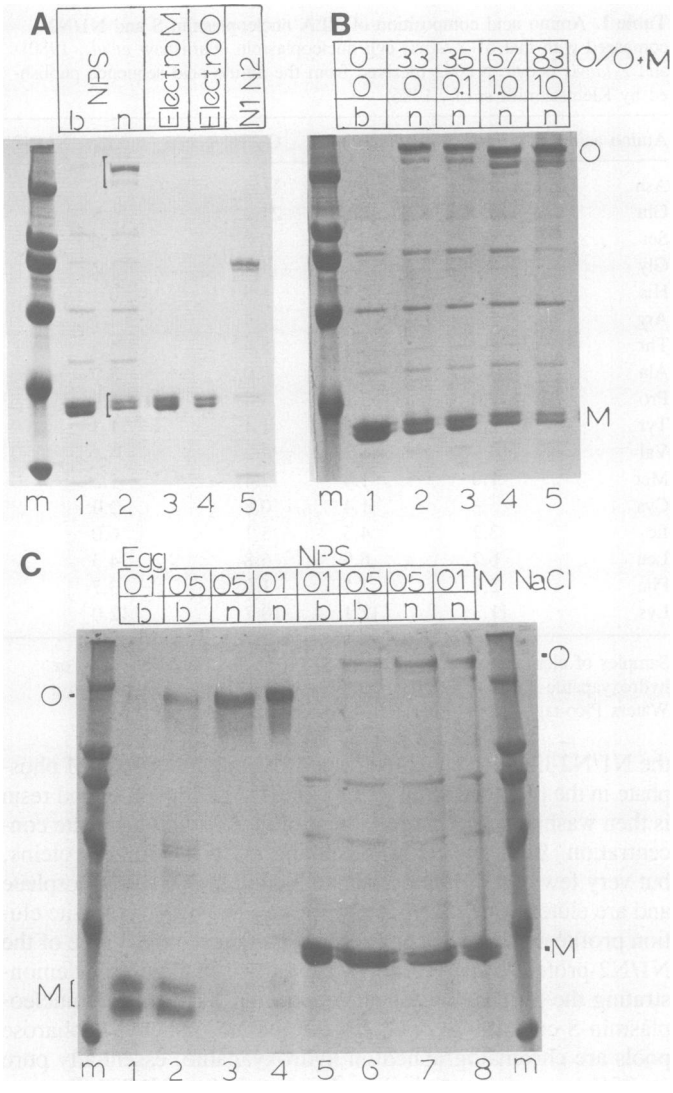

Fig. 6. Chemical features of nucleoplasmin S and N1/N2. **Panel A**: electroelution of nucleoplasmin S oligomer and monomer. Hydroxyapatite-purified nucleoplasmin S were resolved on a preparative SDS–acrylamide gel. The monomeric and oligomeric nucleoplasmin regions of the gel (indicated by brackets) were excised and the protein eletroeluted as described by Hunkapillar *et al.* (1983). The material in each of these samples was analyzed by SDS–acrylamide electrophoresis after heating in SDS. Also shown is hydroxyapatite-purified N1/N2-like protein. NPS, Hydroxyapatite-purified nucleoplasmin S either heated in a boiling water bath (b) or not (n) before electrophoresis; electro M, the material electroeluted from the monomeric band; electro O, the material electroeluted from the oligomeric band; N1/N2, hydroxyapatite-purified N1/N2; m, mol. wt markers as in Figure 2A. The gel was stained with Coomassie Blue after electrophoresis. **Panel B**: calcium stabilizes the nucleoplasmin S oligomer. Hydroxyapatite-purified somatic nucleoplasmin was incubated with 0, 0.1 mM 1 mM and 10 mM $CaCl_2$ for 30 min. The samples were analyzed by SDS–acrylamide electrophoresis and the gel was stained with Coomassie Blue after electrophoresis. b, n and m, Boiled, not boiled and mol. wt markers as described above. The amount of nucleoplasmin S oligomer divided by the total amount of nucleoplasmin S (oligomer plus monomer) in each sample as determined by densitometric scanning of the stained gel is indicated at the top of the figure. **Panel C**: 500 mM NaCl stabilizes the oligomer. Egg nucleoplasmin (Egg) and nucleoplasmin S (NPS) were analyzed by SDS–acrylamide electrophoresis. Samples were either in 0.1 M NaCl containing np storage buffer (see Materials and methods) or in np storage buffer with the NaCl concentration adjusted to 0.5 M as indicated above the figure. Samples were either heated for 10 min at 100°C in a boiling water bath in SDS application buffer before electrophoresis (b) or applied directly to gel in application buffer without heating (n). m, Mol. wt markers as in Figure 2A.

and analyzing the DNA species that are present in either the pellet or the supernatant from this centrifugation. In general, successful assembly is accompanied by a largely soluble product. At physiological ionic strengths, in the absence of some mediating factor, little assembly occurs (monitored by the induction of supercoils in a relaxed circular DNA molecule; Germond *et al.*, 1975) and the resulting DNA–protein species are found in the pellet (Figure 5A, lanes 1 and 2). When the assembly activity of the nucleoplasmin S is assayed we find that at nucleoplasmin S to DNA ratios (mass/mass) of 0.8 and above, a completely soluble, fully assembled product is obtained (Figure 5A, lanes 5–14). A similar activity is observed with the purified N1/N2-like proteins (Figure 5B). However, greater factor to DNA ratios must be used to obtain a fully assembled, soluble product, with the N1/N2-like assembly not reaching maximal extent and solubility unless factor/DNA ratios greater than 1.3 are used (Figure 5B, lanes 3–8). With both proteins, the assembly activity (as assayed by supercoil induction) is dependent upon the concentration of added histone (unpublished data). Furthermore, the assembly occurs with an optimum histone to DNA ratio of 1 (Figure 5C) with the assembly activity of both proteins inhibited by histone to DNA ratios >1 (Figure 5C, lanes 7, 8 and 15, 16).

Amino acid composition

The amino acid compositions of purified nucleoplasmin S and the N1/N2-like proteins are shown in Table I and compared with the amino acid composition of egg nucleoplasmin published by Earnshaw *et al.* (1980) or derived from the DNA sequence data of Dingwall *et al.* (1987). We find that there are substantial similarities between egg nucleoplasmin and nucleoplasmin S. Both proteins possess a high degree of charged amino acids (>40%). The major difference in the amino acid composition is in the amount of glutamic acid, with the somatic protein having 12 mole percent while oocyte nucleoplasmin has 20 mole percent glutamic acid.

Oligomeric stability

That the monomeric form of nucleoplasmin S is indeed derived from the oligomer was documented after gel purification of the higher mol. wt form. Hydroxyapatite-purified nucleoplasmin S is shown in Figure 6A (lanes 1 and 2). When either the oligomer (upper bracket) or the monomer (lower bracket) are electroeluted from gel slices and analyzed by electrophoresis after denaturation, an identical protein doublet of 40 kd is obtained (Figure 6A, lanes 3 and 4).

Egg nucleoplasmin exists in solution as a pentamer (Earnshaw *et al.*, 1980) of subunits which migrate with the apparent mol. wt of 33 kd. We have observed that a number of factors influence the stability of this pentamer. At 150 mM ionic strength, pH 8.0, egg nucleoplasmin must be boiled in SDS to obtain free subunits (Figure 6C, lane 1, boiled and lane 4, not boiled). We have found that the presence of higher concentrations of NaCl (up to 500 mM) stabilize the egg pentamer so that it is stable to boiling in SDS (Figure 6, lane 2; an alternate, but unlikely possibility is that the pentamer still dissociates in high ionic strength but the salt facilitates the renaturation when the sample is cooled for electrophoresis). We have found that nucleoplasmin S behaves in a similar fashion. Boiling in SDS produces a complete dissociation of the protein to the monomer (Figure 6C, lane 5). However, in the presence of 500 mM NaCl, a significant proportion of the material remains as an oligomer after boiling in SDS (Figure 6C, lane 6). A similar proportion of the material remains as an oligomer in the absence of boiling in SDS, either in 500 mM NaCl (Figure 6C, lane 7) or in 100 mM NaCl (Figure 6C, lane

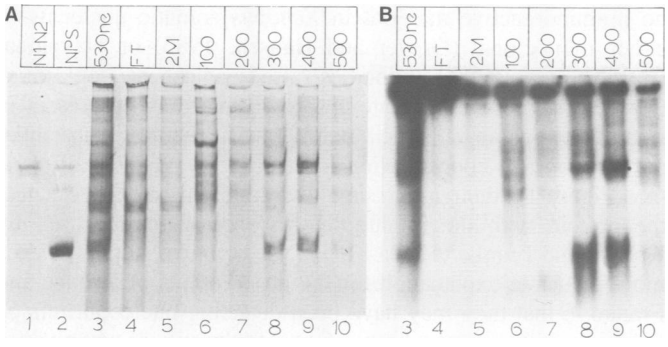

Fig. 7. Phosphorylation of nucleoplasmin S. XLA cells were labeled with 0.1 mCi/ml [^{32}P]orthophosphate in phosphate-free Waymouth's medium for 5 h. The labeled cells were harvested, nuclei were prepared and extracted twice with 530 mM NaCl as described in Materials and methods. This extract was fractionated on a hydroxyapatite column as described in Materials and methods. After dialysis into np storage buffer, the fractions were resolved by electrophoresis on an 8% acrylamide, acid–urea gel (Panyim and Chalkley, 1969). The gel was stained with Coomassie Blue (**panel A**), dried and exposed to X-ray film (**panel B**). N1/N2, Hydroxyapatite-purified N1/N2-like proteins; NPS, hydroxyapatite-purified nucleoplasmin S; 530ne, the 530 mM nuclear extract of labeled XLA cells. The remainder of the notation is the same as in Figure 3.

8). However, in general the nucleoplasmin S oligomer appears to be less stable than the egg nucleoplasmin pentamer. More than 90% of the egg nucleoplasmin sample is present as a pentamer in the absence of boiling (Figure 6C, lanes 3 and 4) whereas the nucleoplasmin S oligomer makes up ~30% of the material (Figure 6C, lanes 7 and 8). We do not know the chemical nature of this instability. One possibility is that the hydrophobic interactions that stabilize the oligomeric structure are less extensive in nucleoplasmin S. A second possibility is that the presence of large amounts of phosphate on the molecule destabilizes the oligomer by charge–charge repulsion. If this is so, then one might predict that calcium ions might bind these phosphate groups, partially neutralize the charge and produce a stabilization of the nucleoplasmin S oligomer.

To test this idea, nucleoplasmin S was incubated with 0, 0.1, 1 and 10 mM calcium chloride for 30 min, SDS was added and the samples were resolved by electrophoresis. Consistent with our hypothesis, we find that the presence of calcium produces a stabilization of the oligomer in the presence of SDS. In the absence of calcium, 33% of the material is present as an oligomer (Figure 6B, lane 2) while increasing amounts of calcium yield an increasingly stable oligomer (up to 83% oligomer in 10 mM calcium; Figure 6B, lane 5). We have observed similar results with magnesium, manganese and zinc (results not shown).

Nucleoplasmin S is phosphorylated in vivo

The results of the calcium stabilization experiment suggest that nucleoplasmin S might be phosphorylated. To test this directly, nucleoplasmin S was isolated from XLA cells grown in the presence of [^{32}P]orthophosphate for 5 h. The 530 mM nuclear extract from these cells was loaded on to a hydroxyapatite column and eluted with the standard phosphate gradient. When the proteins in the hydroxyapatite fractions are resolved by electrophoresis on an acid–urea gel (Figure 7A). We find that nucleoplasmin and the N1/N2-like proteins are two of the most abundant acidic proteins extracted from the nucleus by this ionic strength. The basic proteins in this extract, predominantly histone H1, migrate off the bottom of the gel under conditions which resolve the acidic proteins. Consistent with the hydroxyapatite fractionations shown above, both nucleoplasmin S and the N1/N2-like

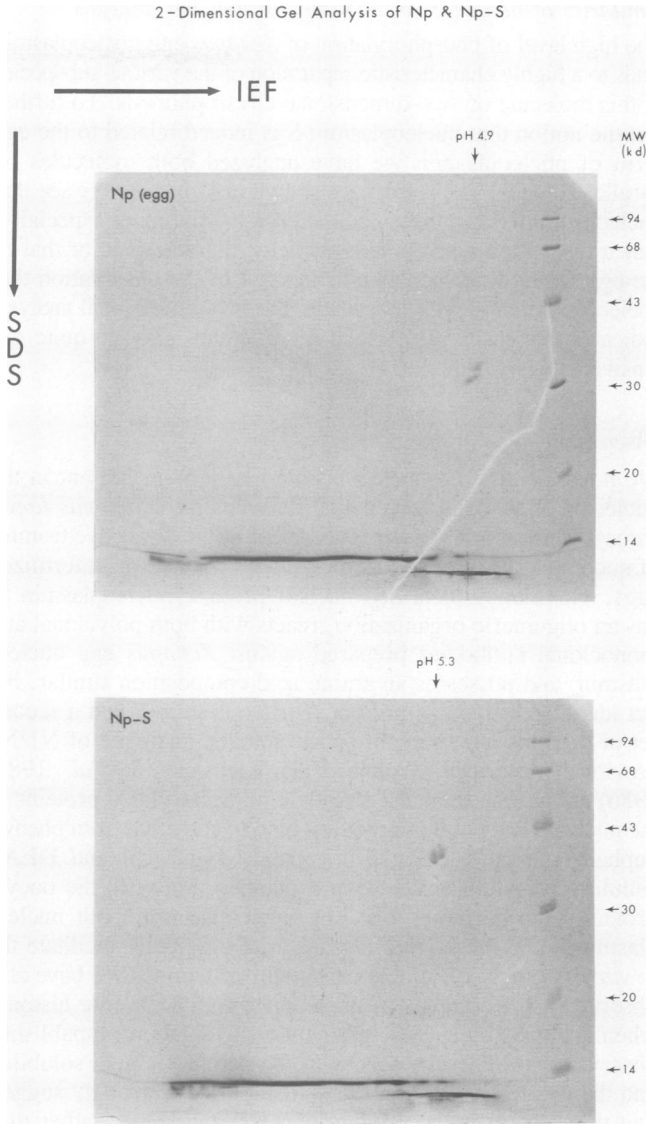

Fig. 8. Two-dimensional gel electrophoresis of nucleoplasmin S. Samples were subjected to isoelectric focussing (pH 3–9) in the presence of 4 M urea. The gels were then electrophoresed on 16% polyacrylamide gels in the presence of 1% SDS along with the standards as indicated. In a parallel run lacking protein, the gel was dissected into small fractions and the pH gradient determined directly.

proteins are eluted from the resin by high phosphate concentrations (Figure 7, lanes 8–10). When this gel is exposed to X-ray film (Figure 7b), we find labeled material comigrating with purified nucleoplasmin S (lane 1) and the N1/N2-like material (lane 2). We have further purified the material eluting from hydroxyapatite at 400 mM phosphate by ammonium sulfate precipitation and elution from phenyl-Sepharose. We continue to find labeled material comigrating with the nucleoplasmin S and N1/N2-like bands (unpublished data). Thus we are confident that the labeled material we are observing is nucleoplasmin S and N1/N2 rather than a comigrating labeled contaminant. Furthermore, calf intestinal phosphatase treatment of nucleoplasmin S and the N1/N2-like proteins, performed as previously described for egg nucleoplasmin (Cotten *et al.*, 1986), increases the mobility of both nucleoplasmin S and N1/N2 on acid–urea gels (unpublished data).

Similarity of behavior on two-dimensional gel analysis

The high level of phosphorylation of *Xenopus* egg nucleoplasmin leads to a highly characteristic separation of the various subspecies of this molecule on two-dimensional electrophoresis. To further test the notion that nucleoplasmin S is indeed related to the egg form of nucleoplasmin we have analyzed both molecules on parallel 2D gels. The results are shown in Figure 8. We see that nucleoplasmin S is a highly acidic protein, and more especially, that it displays a type of heterogeneity characteristic of that of the egg form of nucleoplasmin. In view of the observation that nucleoplasmin S is phosphorylated, the two-dimensional analysis indicates that the level of such modification may be quite extensive.

Discussion

We have identified a nucleoplasmin-like protein present in the nuclei of XLA cells, a cell line derived from *Xenopus laevis* kidney. This protein possesses a number of the distinctive features of nucleoplasmin isolated from *X. laevis* oocytes or unfertilized eggs. This newly identified nuclear protein, nucleoplasmin S, has an oligomeric organization, reacts with both polyclonal and monoclonal antibodies prepared against *Xenopus* egg nucleoplasmin, and possesses an amino acid composition similar, but not identical to the egg protein. Nucleoplasmin S and a second set of proteins which might be the somatic analogue of N1/N2 previously described (Krohne, 1985; Kleinschmidt *et al.*, 1985, 1986) are among the most abundant phosphorylated proteins in the nucleus. Both of these proteins bind to and elute from phenyl-Sepharose (Figure 2), hydroxyapatite (Figure 3) and DEAE cellulose (unpublished data) in a manner similar to the oocyte proteins. Furthermore, like egg nucleoplasmin, both nucleoplasmin S and the N1/N2-like material efficiently facilitate the *in vitro* assembly of soluble chromatin structures. We have also observed a 1:1 complex of nucleoplasmin S with core histones when analyzed by sucrose gradient centrifugation (unpublished observations). The differences in the perchloric acid solubility and the greater mol. wt of the somatic protein strongly suggest that this protein is the product of a separate gene, rather than a post-translationally modified form of the oocyte protein. However, because of the numerous similarities between this somatic protein and nucleoplasmin, we have tentatively named this protein nucleoplasmin S. We stress that this nomenclature is purely one of convenience and must wait until detailed sequence information on the new protein is available for confirmation.

It is apparent that nucleoplasmin S is present as an oligomer in solution (see Figure 6). The apparent mol. wt of the oligomer on SDS–acrylamide gels is 220–250 kd, consistent with an oligomer comprising five or six 40-kd subunits. We have attempted crosslinking experiments similar to those used by Earnshaw *et al.* (1980) to demonstrate that egg nucleoplasmin is a pentamer. However, heterogeneity in the higher mol. wt products made it difficult to distinguish a pentamer from a hexamer. Considering the other similarities between nucleoplasmin and nucleoplasmin S described in this paper it seems likely that nucleoplasmin S is a pentamer in solution; however, we cannot rule out the possibility that it exists as a hexamer.

There are conflicting immunological data in the literature concerning the presence of nucleoplasmin in somatic nuclei. Krohne and Franke have reported that the nuclei of a number of somatic cell types contain a protein that is recognized by anti-nucleoplasmin polyclonal antibodies (Krohne and Franke, 1980a,b). However, other investigators have reported that they could find no immunoreactive material in *Xenopus* somatic nuclei using either monoclonal (Dreyer and Hausen, 1983) or polyclonal (Burglin *et al.*, 1987) antibodies raised against nucleoplasmin. Our data suggest that oocyte nucleoplasmin may be present in small amounts in somatic nuclei; but it requires substantial purification and concentration to detect it by Western analysis (see Figure 1), though conceivably one might be able to detect the protein with the immunofluorescence technique used by Krohne and Franke (1980a,b). However, our results provide a more plausible explanation for the observations of Krohne and Franke in that they may have been detecting nucleoplasmin S, rather than the oocyte form of nucleoplasmin occurring in a widespread fashion in a range of non-oocyte cells. It is possible that the antisera of Dreyer and Hausen and of Burglin *et al.* recognize epitopes that are unique to oocyte nucleoplasmin and might not recognize the more widespread nucleoplasmin S.

We have assayed other tissues for the presence of nucleoplasmin S. When a 530 mM nuclear extract is fractionated by hydroxyapatite or phenyl-Sepharose chromatography we find proteins with a similar mol. wt and oligomeric/monomeric behavior in frog kidney, frog lung, rat liver, calf thymus, chicken embryos and in HTC cells, a rat hepatoma line (results not shown). The quantity of this material is quite constant throughout the tissues that we have surveyed. We find that in all tissues analyzed, nucleoplasmin S is present at 5–10 μg/mg DNA. The replicational activity of the tissues has no apparent relationship to the quantity of nucleoplasmin present. This suggests that whatever functions nucleoplasmin S may have in the cell they are not limited to replication.

The *in vitro* chromatin assembly activity of both nucleoplasmin S and egg nucleoplasmin is highly efficient, with these proteins functioning at a 1:1 mass ratio with histones. This does not, of course, necessarily mean that these proteins necessarily play a role in assembly in the cell (or that they are restricted to such a role). Indeed, oocyte nucleoplasmin can be found associated with specific sites in lampbrush chromosomes, with the localization sensitive to actinomycin D, suggesting a role for the protein in the transcription process or in the packaging of transcription products. It is quite possible that these phosphorylated, acid proteins may play a general role in mediating nucleic acid–basic protein interactions such as chromatin assembly as well as RNP assembly and the transport of basic proteins throughout the nucleus. The *in vitro* activity of these proteins as mediators of histone–DNA interactions may reflect this more general activity in the cell.

The gene for nucleoplasmin (Burglin *et al.*, 1987; Dingwall *et al.*, 1987) and N1/N2 (Kleinschmidt *et al.*, 1986) has been cloned. Although nucleoplasmin S bears a striking resemblance to oocyte nucleoplasmin at the protein level we do not yet know what similarities they possess at the genetic level. We are currently pursuing the cloning of the nucleoplasmin S gene. Certainly information at the DNA sequence level will clarify the molecular relationship between these two proteins. The levels of nucleoplasmin messenger RNA decrease to an undetectable level during early embryogenesis (Burglin *et al.*, 1987). It would be of interest to determine if concomitant with this decrease in oocyte-form nucleoplasmin expression there is an appearance of nucleoplasmin S. Clearly one approach to determining the *in vivo* role of these proteins involves genetic methods with which one might be able to disrupt the genes for these proteins and monitor the effect on the cell. We are currently testing these ideas.

Finally we comment upon the N1/N2-like proteins which co-isolate through so many steps with nucleoplasmin S. Such co-

isolation, in association with their invariable doublet nature on gels and their mol. wt and acidic nature recall the behavior of oocyte N1/N2 proteins. Further support for such a tentative assignment comes from their amino acid composition, the observation that they are heavily phosphorylated *in vivo*, soluble after heating to 80°C, form 1:1 complexes with histones and are capable of directing efficient *in vitro* chromatin assembly. Further tests of this identity must await the use of specific antibodies or the application of the techniques of molecular cloning.

During the final stages of preparation of this manuscript, Schmidt-Zachmann *et al.* (1987), reported the identification of a member of the nucleoplasmin family isolated from nucleoli of somatic frog cells. They have tested nucleoplasmin S with their monoclonal antibody preparations which are specific to the nucleolar nucleoplasmin. Nucleoplasmin S is recognized strongly by these materials, supporting our contention that it is indeed a member of the nucleoplasmin family, and probably indicating an identity with the protein identified by Schmidt-Zachmann *et al.*.

Materials and methods

Sequential extraction of cells

XLA cells were grown at 25°C in Hepes-buffered Waymouth's medium supplemented with 10% fetal calf serum. Cells were harvested by exposure to 0.5 mM EDTA, were washed twice with 150 mM NaCl, 20 mM Tris, pH 8.0 (TBS) and either used directly or stored frozen at −20°C. Extraction of cells was performed as described by Sealey and Chalkley (1987). Cells were lysed by gently suspending them in 10 vol 10 mM Hepes, pH 8.0, 0.5 M sucrose, 50 mM NaCl, 1 mM EDTA, 0.25 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 0.5% Triton X-100, 7 mM β-mercaptoethanol, 1 mM PMSF, 5 μg/l leupeptin, 0.1 μM pepstatin (buffer a). The cells were gently stirred with a pipette and the nuclei were collected by centrifugation at 1000 *g* for 5 min. The nuclei were washed once in buffer a, the supernatant was combined with the initial nuclear supernatant (this fraction is termed cytoplasm) and the nuclei were then gently suspended in 4 vol of 10 mM Hepes, pH 8.0, 25% glycerol, 100 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 7 mM β-mercaptoethanol, 10 mM benzamidine, 1 mM PMSF, 5 μg/l leupeptin, 0.1 μM pepstatin (100 M buffer b). The nuclei were incubated on ice in this solution for 15 min with frequent gentle agitation. The nuclei were then collected (100 *g*, 5 min) the supernatant (100 mM nuclear extract) was removed and the nuclei were extracted in a similar fashion with buffer b containing 300 or 530 mM NaCl. All extracts were dialyzed extensively against 100 mM NaCl, 20 mM Tris, pH 8.0, 13 mM β-mercaptoethanol, 10 mM benzamidine, 1 mM EDTA, 0.1 mM PMSF and 0.01% NP40. The nuclear pellet remaining after the 530 mM extraction was vigorously sonicated in 4 vol 0.1% SDS before application to SDS electrophoresis gels.

Because of the large number and expense of tissue culture flasks required for these studies, we have developed a method for reutilizing these disposable flasks. After harvesting the cells with EDTA, the flasks were washed three times with deionized water and stored in a 65°C incubator, 24 h with the caps removed, to dry the flasks, followed by 2 weeks with the caps loosely installed. This dehydration is sufficient for sterilization of the flasks but is cool enough that the plastic does not melt. If care is taken to completely remove cells before allowing the flask to dry, there appears to be no interference in the subsequent growth of cells in these recycled flasks. We have T-150s that have been in use since 1982.

Western blotting analysis

Protein samples, resolved on 8% acrylamide−SDS gels, were transferred to nitrocellulose filters (S&S, 0.45 μm) at 100 V, <250 mA for 1.5 h. The filters were blocked in 3% non-fat dry milk (Johnson *et al.*, 1984), 150 mM NaCl, 20 mM Tris, pH 8.0, 0.01% azide, 0.01 mM PMSF (TBS-milk) for at least 1.5 h and incubated with affinity-purified antibody (see below) in TBS-milk overnight. Filters were then washed for 1.5 h with multiple changes of TBS-milk, incubated with [^{125}I]protein A (NEN, Bolton Hunter labeled, 0.5 μCi/10 × 15 cm filter) for 1.5 h in <0.5 ml TBS-milk followed by washing as above. Dried filters were exposed to X-ray film at −70°C with intensifying screens.

Preparation of antiserum

A rabbit polyclonal serum was prepared against purified *Xenopus* egg nucleoplasmin. Because this polyclonal serum recognized several nuclear proteins, the serum was affinity purified essentially as described by Heller *et al.* (1986). A 55% ammonium sulfate supernatant, 80°C heat supernatant of *Xenopus* egg extract was prepared (Moreau *et al.*, 1986) containing essentially pure egg nucleoplasmin. This preparation was resolved on an 8% acrylamide gel and transferred to nitrocellulose as described above. A strip of the filter corresponding to one lane of the gel was cut off and stained with amido black to locate the egg nucleoplasmin pentamer. This region of the filter was removed from the remaining portion of the filter, cut into 2-mm square bits and blocked as described above. These bits were then incubated overnight with rabbit polyclonal serum and washed for at least 3 h with multiple changes of TBS-milk. The bound antibody was eluted by exposing the gel bits to 0.2 M glycine, pH 2.5 for 2 min with vigorous vortexing. The eluent was removed and to it was added 1/4 vol of 1 M Tris, pH 8.0. This elution was repeated twice more, the eluents were pooled and stored at 4°C, without dialysis, in the presence of 0.01% azide.

Purification of nucleoplasmin S

Nuclei were prepared from the XLA cell contents of 20−25 T-150 (~250 million cells/flask) as described above. The nuclei were extracted twice with buffer B containing 530 mM NaCl, the extracts were pooled (~50 ml) and diluted to 150 ml with 500 mM NaCl, 20 mM Tris, 1 mM benzamidine and 0.1 mM PMSF and ammonium sulfate was added to 35% saturation. The material was allowed to incubate on ice for 30 min, centrifuged at 10 000 r.p.m. in a Sorvall HB-4 rotor and the supernatant, containing nucleoplasmin, was applied directly to a 7-ml phenyl-Sepharose column equilibrated with 1.5 M ammonium sulfate, 20 mM Tris, pH 8.0 at 4°C. The column was washed with three column volumes of this buffer and then eluted with a 30-ml reverse salt gradient of 1.5−0 M ammonium sulfate in 20 mM Tris pH 8.0. Fractions of the gradient eluent were assayed by gel electrophoresis and the nucleoplasmin-S-containing fractions were pooled accordingly and dialyzed extensively against 100 mM NaCl, 20 mM Tris, pH 8, 7 mM β-mercaptoethanol, 1 mM EDTA, 1 mM benzamidine, 0.1 mM PMSF and 0.01% (v/v) NP-40 (np storage buffer).

Hydroxyapatite chromatography

Samples to be resolved on hydroxyapatite were adjusted to 10 mM sodium phosphate, pH 7, 1 M NaCl and applied to a column of hydroxyapatite (HTP, Bio-Rad) equilibrated with 10 mM sodium phosphate, pH 7, 2 M NaCl. The column was washed with three column volumes of 2 M NaCl, 10 mM sodium phosphate, pH 7 and then with three column volumes each of 100, 200, 300, 400 and 500 mM sodium phosphate, pH 7. The hydroxyapatite chromatography was performed at room temperature. Nucleoplasmin S and N1/N2 remain bound to hydroxyapatite in 300 mM phosphate but are eluted with 400 mM phosphate. Column eluents were promptly dialyzed at 4°C against np storage buffer.

Chromatin assembly assay

The chromatin assembly activity was assayed by following the insertion of supercoils into relaxed circular DNA (Germond *et al.*, 1975) essentially as previously described (Sealy *et al.*, 1986). Supercoiled pBR322 was incubated with 2 units topoisomerase I (wheat germ, Promega Biotec) per μg DNA for 40 min, room temperature in 150 mM NaCl, 20 mM Tris, pH 8.0, 1 mM DTT. Meanwhile, hyperacetylated core histones (Cotten and Chalkley, 1985) were incubated with nucleoplasmin S or N1/N2 preparations in 150 mM NaCl, 20 mM Tris, pH 8.0, 1 mM EDTA, 7 mM BME, 0.1 mM PMSF, 1 mM benzamidine, 0.01% NP40 at a histone concentration of 3 μg/ml. At the end of this incubation, relaxed DNA (0.3 μg in 5 μl) was added to the histone sample and the assembly reaction was allowed to proceed for 2 h at room temperature. The assembly samples were then centrifuged for 3 min at 15 000 *g* at room temperature, the supernatant was removed and the pellet was suspended in an equal volume of the assembly reaction buffer. SDS and proteinase K were added to both pellet and supernatant fractions (0.1 and 0.02 μg/ml), the samples were incubated at 65°C for 20 min and the DNA was precipitated with 3 vol −20°C absolute ethanol. The precipitated DNA was recovered by centrifugation, resolved by electrophoresis on 2% agarose gels in 30 mM Tris, 36 mM phosphate, 1 mM EDTA (Shure and Viograd, 1976) and stained with ethidium bromide to identify the DNA pattern.

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