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Massive Phosphorylation Distinguishes *Xenopus laevis* Nucleoplasmin Isolated from Oocytes or Unfertilized Eggs†

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ABSTRACT: Nucleoplasmin isolated from unfertilized *Xenopus laevis* eggs possesses an in vitro chromatin assembly activity which is superior to nucleoplasmin isolated from oocytes. It is demonstrated here that the two forms of the protein differ in the amount of attached phosphate, with the egg protein possessing nearly 20 phosphate groups per protein monomer and the oocyte protein possessing less than 10 phosphate groups per monomer. A kinase preparation from unfertilized eggs is shown to be capable of modifying oocyte nucleoplasmin so that it displays the electrophoretic heterogeneity of egg nucleoplasmin. Furthermore, when the egg protein is treated with phosphatase and repurified, the chromatin assembly activity deteriorates to the level of the oocyte protein.

Nucleoplasmin is a pentameric, acidic protein which can be isolated from the oocytes or eggs of *Xenopus laevis* (Laskey et al., 1978; Mills et al., 1980). It is a major component of the oocyte nucleus, comprising 7–10% of the nuclear protein (Mills et al., 1980; Krohne & Franke, 1980). An immunologically similar protein is present in the nuclei of a number of other higher eukaryotic cell types (Krohne & Franke, 1980). Complexes containing nucleoplasmin and histones have been isolated from *Xenopus* oocyte nuclei (Kleinschmidt et al., 1985), and the protein effectively promotes the formation of nucleosomes in vitro (Laskey et al., 1977, 1978; Earnshaw et al., 1980; Sealy et al., 1986). For these reasons, nucleoplasmin is thought to play a role in the process of storage, transport, or deposition of histones onto DNA.

Nucleoplasmin isolated from oocytes is much inferior to egg nucleoplasmin in an in vitro chromatin assembly assay (Sealy et al., 1986). The two forms of the protein display different mobilities when assayed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis ($NaDodSO_4$–PAGE). The egg form of the protein has a more acidic p*I* than the oocyte form when assayed by isoelectric focusing (Sealy et al., 1986). It is unlikely that the egg protein is a different gene product than the oocyte protein because radioiodinated oocyte nucleoplasmin can be converted into the egg form of the protein when it is microinjected into hormonally stimulated oocytes (M. Cotten, unpublished results), indicating that a modification of the oocyte form is sufficient for the conversion.

Thus, the most likely explanation for the differences between the oocyte and egg forms of nucleoplasmin lies in some form of posttranslational modification. There is substantial evidence in the literature of phosphorylation altering the mobility of protein on $NaDodSO_4$–PAGE (Ahmad et al., 1982; Shih et

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al., 1979; Stadel et al., 1983; Wegener & Jones, 1984; Zoller et al., 1979). The transition between the oocyte and egg form of the protein occurs during the first meiotic division of the oocyte (M. Cotten, unpublished results), a period of development marked by large changes in protein phosphorylation activity (Maller et al., 1977; Maller & Smith, 1985). It has been shown that a protein with the molecular weight and charge characteristics of nucleoplasmin is phosphorylated during oocyte maturation (Maller & Smith, 1985). Furthermore, we have recently shown that phosphatase treatment of native oocyte and egg nucleoplasmin can remove much of the electrophoretic difference between the two forms of the protein (Sealy et al., 1986). However, a significant difference in electrophoretic mobility remained so that the difference in assembly activity could not be ascribed with assurance to phosphorylation.

We now present evidence that the major difference between the two proteins is solely in the extent of phosphorylation. We demonstrate that treatment of denatured oocyte and egg nucleoplasmin with phosphatase produces proteins that comigrate on an acid–urea–acrylamide electrophoresis system, which separates peptides primarily on the basis of their charge. On the basis of the mobility changes that occur upon phosphatase treatment, we calculate that the egg form of nucleoplasmin possesses nearly 20 phosphate groups per 30 000 molecular weight monomer, whereas the oocyte form of the protein contains less than 10 phosphate groups per monomer. We also demonstrate that a protein kinase containing extract from unfertilized *Xenopus* eggs is capable of phosphorylating oocyte nucleoplasmin. Oocyte nucleoplasmin modified with this extract in vitro displays the electrophoretic heterogeneity of egg nucleoplasmin, consistent with the notion that phosphorylation is responsible for this heterogeneity. Furthermore, we show that when egg nucleoplasmin is treated with phosphatase and then repurified, the resulting protein displays a decreased chromatin assembly activity that is similar to that of nucleoplasmin isolated from oocytes.

Experimental Procedures

Nucleoplasmin Isolation. Nucleoplasmin was isolated from *Xenopus laevis* oocytes or eggs as previously described (Sealy et al., 1986). Nucleoplasmin was repurified after phosphatase treatment by chromatography on DEAE-cellulose. The proteins were applied to a 2-mL column equilibrated with 25 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), and 100 mM NaCl. Under these conditions, calf intestinal phosphatase does not bind the resin, and after the column was washed with 3 column volumes of the above buffer, nucleoplasmin was eluted from the column with 25 mM Tris, pH 7.5, 1 mM EDTA, and 400 mM NaCl. Fractions containing nucleoplasmin (as assayed by $NaDodSO_4$–PAGE) were pooled and concentrated with an Amicon Centricon.

Acid–Urea Electrophoresis. The acid–urea electrophoresis system used was a modification of the system described by Panyim and Chalkley (1969). Electrophoresis was through a 15% acrylamide–0.4% bis(acrylamide) gel containing 8 M urea and 0.9 N acetic acid. The gel contained a 5% acrylamide stacking gel in 8 M urea–0.9 N acetic acid, and the gel was preelectrophoresed at 10 V/cm for at least 7 h. One hour before application of the samples, free radicals in the gel were scavenged by applying 10 μL of 0.6 M cysteamine, 20% sucrose, and 0.9 N acetic acid to each lane and continuing the electrophoresis for 30 min before application of the samples. The gel buffer was 0.9 N acetic acid–8 M urea for preelectrophoresis. Before the scavenging solution was applied, the top chamber buffer was replaced by 0.9 N acetic acid and remained so for the subsequent electrophoresis. Samples for electrophoresis contained freshly added 8 M urea, 0.9 N acetic acid, 10% glycerol, and 0.005% methyl green. The samples were resolved by electrophoresis at 150–190 V for 12–15 h (15-cm gel). Gels were stained in 0.25% Coomassie blue (Bethesda Research Laboratories), 10% acetic acid, and 40% methanol.

$NaDodSO_4$–PAGE. $NaDodSO_4$–PAGE was performed with a 15% acrylamide–0.4% bis(acrylamide) gel as previously described (Sealy et al., 1986). Because of the resistance of pentameric nucleoplasmin to dissociation in the presence of calcium, trypsin digestion samples contained 20 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and 4 M urea in addition to the other components during the preparation for electrophoresis. Gels were stained with Coomassie blue as described above.

Protease Digestions. Nucleoplasmin was digested with trypsin in 10 mM $CaCl_2$, 150 mM NaCl, and 25 mM Tris, pH 8.0, at a ratio of 0.1 μg of trypsin per 10 μg of nucleoplasmin at 37 °C for 30 min. Reactions were stopped by adding phenylmethanesulfonyl fluoride (to 0.1 mM) before electrophoresis. Nucleoplasmin was digested with pepsin in 100 mM HCl at a ratio of 3 μg of pepsin/μg of nucleoplasmin at 37 °C for 45 min.

Phosphatase Treatment. Nucleoplasmin samples were incubated with calf intestinal phosphatase (New England Nuclear), 10 mM $MgCl_2$, and 100 mM Tris, pH 8.8, at 37 °C for 30 min (10 units of enzyme/μg of nucleoplasmin). This was followed by adding an additional 10 units of phosphatase/μg of nucleoplasmin for a further 60 min at 37 °C.

Where indicated, nucleoplasmin samples were denatured by adding $NaDodSO_4$ to 1% and heating the samples to 100 °C for 10 min. The $NaDodSO_4$ concentration was diluted to 0.25%, and a first aliquot of phosphatase (10 units/μg of nucleoplasmin) was added for 30 min at 37 °C. The $NaDodSO_4$ concentration was then diluted to 0.06%, and a second equal aliquot of phosphatase was added for 60 min at 37 °C.

Nucleoplasmin samples were treated with bacterial alkaline phosphatase (Bethesda Research Laboratories) in the same buffer as the calf intestinal phosphatase reaction. Thus, nucleoplasmin (1 μg) was treated with 150 units of the phosphatase for 30 min followed by an additional 150 units of the enzyme for 60 min. Bacterial alkaline phosphatase digestions were performed at either 37 or 65 °C as indicated in the figure legends.

Preparation of Egg Extract. An egg extract for modifying oocyte nucleoplasmin was prepared essentially as described by Wu and Gerhart (1980) for the extraction of a maturation promoting factor (MPF) from unfertilized *Xenopus* eggs. The procedure, in brief, is as follows. Unfertilized eggs were dejellied in 2% cysteine, pH 7.0, rinsed numerous times in Barth's saline, and then rinsed twice with extraction buffer (EB: 80 mM 2-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.3). The eggs were then homogenized in an equal volume of EB and centrifuged at 40 000 rpm in an SW50.1 rotor (150000*g*) for 35 min at 4 °C. The clear layer between the fat and yolk was removed and fractionated by ammonium sulfate precipitation. The extract was adjusted to 1.1 M ammonium sulfate in EB, incubated on ice for 10 min, and centrifuged for 10 min at 15000*g*, 4 °C. The precipitated material was suspended in 1.1 M ammonium sulfate in EB and centrifuged again at 15000*g* for 10 min, and the supernatant was discarded. This ammonium sulfate wash, which removes any adventitiously bound nucleoplasmin, was

repeated once more. The final pellet was dissolved in 0.1 volume of DB (DB is EB diluted to two-thirds concentration), dialyzed extensively against DB at 4 °C, and stored at −70 °C. This preparation has a protein concentration of approximately 5 mg/mL.

Antibody Techniques. The preparation of a monoclonal antibody to nucleoplasmin has been described (Sealy et al., 1986). Antibody precipitations of nucleoplasmin were performed by incubating the nucleoplasmin sample with antibody-containing culture medium in modified Puck's saline (350 mM NaCl, 6 mM dextrose, 5 mM KCl, 4 mM $NaHCO_3$, and 0.5 mM EDTA) plus 0.5% NP40 (v/v) and 1% Triton X-100 (v/v) for 2 h on ice. Formaldehyde-fixed *Staphylococcus aureus* (Staph A) (Pansorbin, Calbiochem) was added, the incubation was continued for 20 min, on ice, and the antibody–Staph A complex was harvested by centrifugation, washed twice with modified Puck's saline plus detergents and twice with Puck's saline, and resolved, after boiling in 1% $NaDodSO_4$, by $NaDodSO_4$–PAGE.

Chromatin Assembly. Chromatin assembly assays were performed as previously described (Sealy et al., 1986). Core histones (control or hyperacetylated) were obtained by salt extraction of hydroxyapatite-bound chromatin from either control HTC cells or HTC cells grown in the presence of sodium butyrate to induce histone hyperacetylation (Cotten & Chalkley, 1985). Histone preparations were quantitated by densitometric scanning of Coomassie blue stained gels standardized with known quantities of histones. The supercoiled DNA species produced by chromatin assembly reactions were resolved on 2% agarose gels in 30 mM Tris, pH 7.7, 36 mM NaH_2PO_4 and 1 mM EDTA (Shure & Vinograd, 1976) with recirculating buffer, and the DNA pattern was located by staining with ethidium bromide. For densitometric analysis, stained gels were photographed with UV illumination using Polaroid type 55 film, and the resulting negatives were scanned with a densitometer.

RESULTS

Effect of Phosphatase Treatment on Nucleoplasmin Heterogeneity. Oocyte and egg nucleoplasmin can be distinguished on an acid–urea–acrylamide gel system, which is capable of resolving proteins in a manner which depends upon their charge and molecular weight. The two forms of nucleoplasmin display differences in mobility that are consistent with the egg form having an acidic modification (Figure 1, lanes 2 and 3). The oocyte form of the protein is resolved into a pair of species while the egg form displays a slower overall mobility with much greater heterogeneity than the oocyte form. Phosphatase treatment alters the mobility of both the egg and the oocyte protein on an acid–urea gel (Figure 1, lanes 4 and 5). The phosphatase-treated oocyte protein shows a small increase in mobility. In contrast, not only has the phosphatase-treated egg nucleoplasmin lost the bulk of its additional heterogeneity but also it has developed a substantially increased mobility that is comparable to, though not identical with, that of the oocyte protein.

We wondered if the difference in mobility between oocyte and egg nucleoplasmin which remains after phosphatase treatment is due to phosphate groups that are masked by some feature of nucleoplasmin secondary or tertiary structure. That this is probably correct is demonstrated by the following experiment. Egg and oocyte nucleoplasmins were heated at 100 °C in 1% $NaDodSO_4$ to denature the proteins. This solution was then diluted to a $NaDodSO_4$ concentration permissive to phosphatase activity, and the phosphatase was added. The products of this reaction were resolved on an acid–urea gel

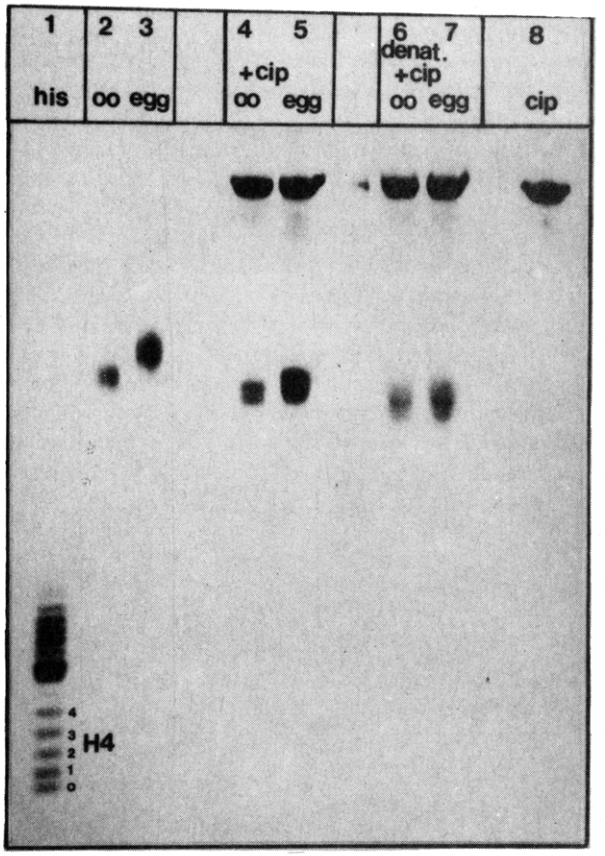

FIGURE 1: Oocyte and egg nucleoplasmin resolved by acid–urea electrophoresis before and after phosphatase treatment. Oocyte or egg nucleoplasmin (1-μg samples) was treated with calf intestinal phosphatase as described under Experimental Procedures. Lane 1, hyperacetylated core histones (the numbers refer to the acetylation state of histone H4); lane 2, oocyte nucleoplasmin; lane 3, egg nucleoplasmin; lane 4, oocyte nucleoplasmin treated with calf intestinal phosphatase; lane 5, egg nucleoplasmin treated with calf intestinal phosphatase; lane 6, oocyte nucleoplasmin which had been exposed to 1% $NaDodSO_4$ at 100 °C, treated with calf intestinal phosphatase; lane 7, egg nucleoplasmin which had been exposed to 1% $NaDodSO_4$ at 100 °C, treated with calf intestinal phosphatase; lane 8, calf intestinal phosphatase. All samples were dissolved in 0.9 N acetic acid–8 M urea and resolved by electrophoresis on an acid–urea gel as described in the text.

(Figure 1, lanes 6 and 7) to yield essentially identical patterns for both egg and oocyte proteins. It is apparent that the residual level of modification is removed by the phosphatase treatment when egg nucleoplasmin is denatured before the enzyme is added. The egg sample contains three protein species that now comigrate with the oocyte form of the protein. The upper two forms in each sample are probably due to incompletely removed phosphate. Although we have never been able to produce a dephosphorylated nucleoplasmin preparation containing only the fastest migrating species, we have noted variability in the ratio of the three forms which may reflect varying degrees of phosphatase treatment.

Estimate of the Number of Phosphate Groups Attached to Nucleoplasmin. The number of phosphate groups attached to egg or oocyte nucleoplasmin can be estimated by analyzing the changes in mobility on an acid–urea gel that occur when either oocyte or egg nucleoplasmin is treated with phosphatase. In this electrophoresis system, the difference in mobility between parental histone H4 (28 positive charges) and monoacetylated H4 (27 positive charges) is, as expected, approximately 3% [Figure 1, lane 1; see also Panyim & Chalkley (1969)].

When the mobility of the band center of the control egg

nucleoplasmin sample (lane 3) is compared to the band center of the calf intestinal phosphatase (CIP)-treated, denatured egg nucleoplasmin (lane 7), we find that there is a change in mobility of 21%. Since the charge on nucleoplasmin at this pH (pH 2) is +45 [calculated from the amino acid composition reported by Earnshaw et al. (1980)], a 21% change in mobility corresponds to an actual charge modification of 9.5 units of negative charge. Because the pH of the gel system is approximately the same as the pK of the most acidic phosphatate hydroxyl, each phosphate group has an effective charge of 0.5. Therefore, this charge difference of 9.5 must be multiplied by a factor of 2.0 to determine the phosphate content. This results in a value of some 19 phosphates responsible for the change in mobility between fully phosphorylated and phosphatase-treated egg nucleoplasmin. A similar calculation indicates that there are roughly seven phosphate groups associated with the phosphorylated form of oocyte nucleoplasmin. We estimate that there is approximately 10–20% error in these numbers, reflecting the determination of the mobility shift combined with uncertainty about the pK of the phosphate hydroxyl associated with a protein in a urea environment. Calculations based on the changes in mobility that occur when denatured nucleoplasmin is treated with phosphatase indicated that there are approximately five additional phosphate groups on the egg nucleoplasmin and two additional phosphate groups on the oocyte nucleoplasmin which are made available to the phosphatase by denaturation.

Phosphatase Controls. A stoichiometric amount of calf intestional phosphatase is required to dephosphorylate these molecules. Although the charge changes that are observed support the idea that it is only phosphate that is being removed by these enzyme preparations, it is possible that some contaminating enzymatic activity, present in the phosphatase preparation, could be contributing in some way to the changes in mobility that are being observed. That this is unlikely is indicated by the observation that the phosphatase-induced mobility shift is blocked in the presence of the phosphatase inhibitor zinc (10 mM, Figure 2, lane 6). We have noted an identical result using 15 mM sodium molybdate (Sealy et al., 1986) or 10 mM β-glycerophosphate (M. Cotten, unpublished results). Similar shifts in egg nucleoplasmin mobility can also be produced by bacterial alkaline phosphatase at 65 °C, where most proteases are likely to be much less active (Figure 2, lane 9) [although the mobility shift with this enzyme preparation is only partial at 37 °C (Figure 2, lane 7)]. Furthermore, combined digestions with both calf intestinal phosphatase and bacterial alkaline phosphatase do not produce any additional mobility shift (Figure 2, lanes 8 and 10), suggesting that both enzymes are removing the same moieties.

Location of Phosphate Groups. It is possible to use selective protease digestion to determine if certain portions of the protein molecule contain differing degrees of phosphorylation. Trypsin and pepsin digestions have provided evidence that the amino-terminal portion of nucleoplasmin possesses the histone binding properties of the intact molecule, whereas the carboxy-terminal portion contains the signal required for nuclear localization of nucleoplasmin (Dingwall et al., 1982). Accordingly, it was of interest to ask which overall domain of nucleoplasmin was associated with phosphatase-sensitive modifications. Oocyte nucleoplasmin treated with trypsin yields a 23-kilodalton (kDa) trypsin-resistant core representing the amino-terminal portion of the molecule (Dingwall et al., 1982; Sealy et al., 1986). Unfortunately, the remaining portion of the molecule is quickly digested to fragments that are too small for analysis by gel electrophoresis. When the trypsin-resistant cores of oocyte and egg nucleoplasmins are compared, the egg peptide shows much of the heterogeneity of the intact molecule [Figure 3A, lanes 1 and 2; see Sealy et al. (1986)] whereas the oocyte peptide displays much less heterogeneity. This heterogeneity is lost when the proteins are treated with phosphatase before trypsin cleavage (Figure 3A, lanes 3 and 4).

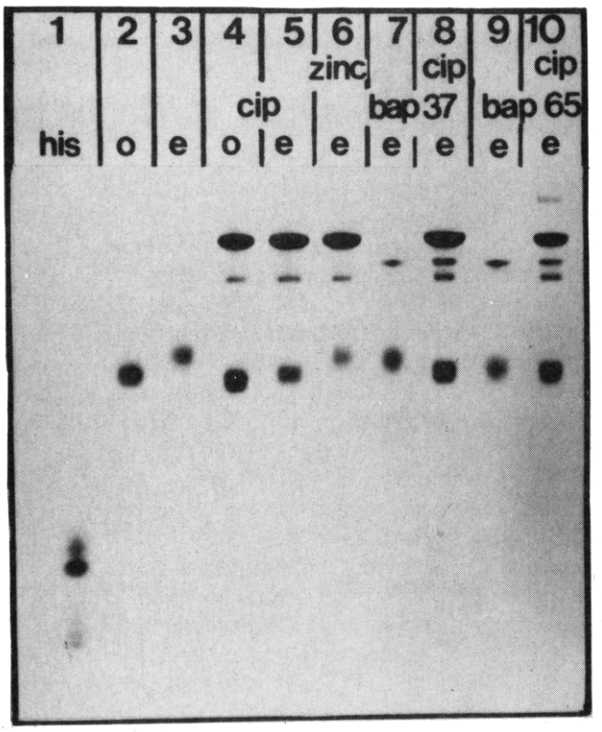

FIGURE 2: Phosphatase controls. Samples of nucleoplasmin were treated with either calf intestinal phosphatase or bacterial alkaline phosphatase as described under Experimental Procedures, resolved on an acid–urea electrophoresis gel, and located by Coomassie blue staining. Lane 1, hyperacetylated core histones; lane 2, oocyte nucleoplasmin; lane 3, egg nucleoplasmin; lane 4, oocyte nucleoplasmin treated with calf intestinal phosphatase; lane 5, egg nucleoplasmin treated with calf intestinal phosphatase; lane 6, egg nucleoplasmin treated with calf intestinal phosphatase in the presence of 10 mM $ZnCl_2$; lane 7, egg nucleoplasmin treated with bacterial alkaline phosphatase at 37 °C; lane 8, egg nucleoplasmin treated with calf intestinal phosphatase followed by treatment with bacterial alkaline phosphatase at 37 °C; lane 9, egg nucleoplasmin treated with bacterial alkaline phosphatase at 65 °C; lane 10, egg nucleoplasmin treated with calf intestinal phosphatase followed by treatment with bacterial alkaline phosphatase at 65° C.

The digestion of nucleoplasmin with pepsin produces a 17-kDa peptide containing the amino terminus and a 16-kDa carboxy-terminal peptide which is further cleaved to 12- and 4-kDa peptides (Dingwall et al., 1982; Sealy et al., 1986). The pepsin digestion products of oocyte and egg nucleoplasmin show a remarkable degree of heterogeneity when analyzed on an acid–urea gel, reflecting the large degree of charge modification on these proteins. The amino-terminal portion of the molecules gives rise to a set of seven different peptides when the egg molecule is digested with pepsin whereas the oocyte protein produces four such fragments (Figure 3B, lanes 1 and 2; see also Figure 3C for an expanded electrophoretic pattern of the oocyte and egg core peptides). The set of amino-terminal egg peptides displays a retarded mobility relative to the oocyte peptides, again demonstrating an acidic modification. There are also clear differences in the mobilities of the carboxy-terminal peptides, with peptides derived from the egg protein reflecting the additional degree of modification.

When the pepsin digestion products of oocyte or egg nucleoplasmin are treated with phosphatase (Figure 3B, lanes

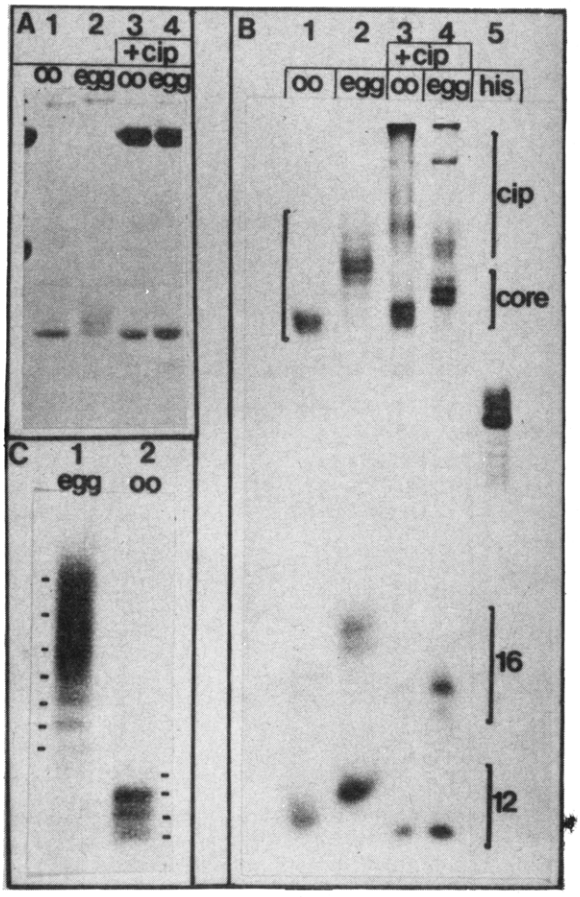

FIGURE 3: (A) Trypsin digestion of normal and CIP-treated nucleoplasmin. Lane 1, oocyte nucleoplasmin digested with trypsin as described in the text; lane 2, egg nucleoplasmin digested with trypsin; lane 3, CIP-treated oocyte nucleoplasmin digested with trypsin; lane 4, CIP-treated egg nucleoplasmin digested with trypsin. Trypsin digestion and CIP treatment were performed as described under Experimental Procedures. Samples were resolved by $NaDodSO_4$–PAGE. (B) Pepsin digestions of nucleoplasmin resolved by acid–urea gel electrophoresis. Lane 1, oocyte nucleoplasmin digested with pepsin; lane 2, egg nucleoplasmin digested with pepsin; lane 3, CIP treatment of oocyte nucleoplasmin pepsin digestion products; lane 4, CIP treatment of egg nucleoplasmin pepsin digestion products; lane 5, hyperacetylated core histones. The brackets indicate peptides derived from the pepsin activity upon the phosphatase (cip), derived from the amino-terminal core of nucleoplasmin (core), and derived from the 16- and 12-kDa carboxy-terminal tail of nucleoplasmin (16 and 12) as verified by second-dimension electrophoresis in $NaDodSO_4$–PAGE. (C) Expanded version of the amino-terminal pepsin fragments resolved on an acid–urea gel. Lane 1, egg nucleoplasmin core peptides; lane 2, oocyte nucleoplasmin core peptides.

3 and 4), both the amino-terminal and carboxy-terminal peptides adopt similar mobilities. The carboxy-terminal peptides become clearly identical in mobility while the amino-terminal peptides show the same difference in mobility seen with native nucleoplasmin molecules which have been treated with phosphatase, with a strong doublet present in both the oocyte and egg samples and the egg doublet migrating with a reduced mobility (Figure 1, lanes 4 and 5). Evidently, the phosphate groups which are protected against phosphatase action are located in the amino-terminal (histone binding) portion of the molecule. In addition, when the amino-terminal pepsin fragments of oocyte or egg nucleoplasmin are resolved on a longer acid–urea gel, the pattern of heterogeneity is consistent with the presence of a large number of charge modifications (Figure 3C). If each peptide band represents the presence of an additional phosphate group, counting the number of bands in the amino- and carboxy-terminal pepsin fragments provides evidence that a large number of phosphate

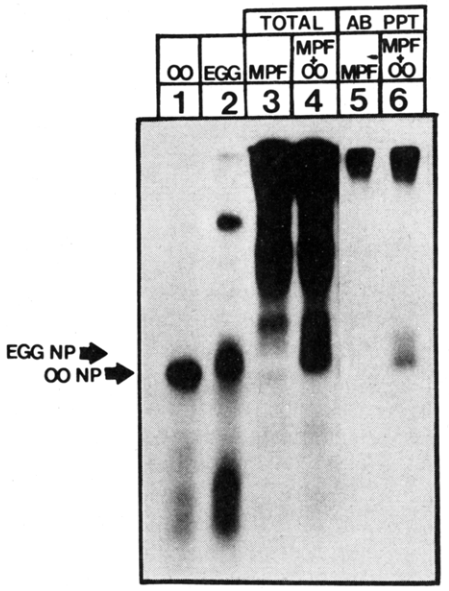

FIGURE 4: Phosphorylation of oocyte nucleoplasmin by an egg extract. Purified oocyte nucleoplasmin (120 ng/sample) was incubated with 5 μL of ammonium sulfate precipitated MPF preparation in the presence of 10 μM [γ-^{32}P]ATP (17 Ci/mmol) for 90 min at room temperature. The nucleoplasmin in each sample was either applied directly to the gel (lanes 3 and 4) or purified by antibody precipitation (lanes 5 and 6) before resolution by $NaDodSO_4$–acrylamide electrophoresis. The phosphorylated protein pattern was identified by autoradiography. Lanes 1 and 2 contain ^{125}I-labeled oocyte or egg nucleoplasmin; lanes 3 and 5 display the kinase activity in the absence of added oocyte nucleoplasmin; lanes 4 and 6 display the kinase activity in the presence of oocyte nucleoplasmin. The locations of egg or oocyte nucleoplasmin (monomers) are indicated at the side of the gel.

groups are present on the egg form of nucleoplasmin.

The migration of the amino-terminal and carboxy-terminal fragments reveals a startling asymmetry of charge. Removal of 16 kDa of protein from nucleoplasmin leaves a 17-kDa fragment which migrates only very slightly faster than the intact 33-kDa molecule, indicating that the 17-kDa fragment is highly negatively charged (even after phosphatase treatment). In contrast, the carboxy-terminal material migrates twice as rapidly as histone H4 even though one of the fragments is probably of comparable molecular weight to the histone. Thus, the carboxy-terminal portion of nucleoplasmin must be exceedingly basic.

Modification of Oocyte Nucleoplasmin in Vitro. A kinase-containing extract from unfertilized *Xenopus* eggs has been described (Wu & Gerhart, 1980). This extract, termed the maturation promoting factor (MPF), induces meiosis when injected into stage VI oocytes, and it is thought that protein phosphorylation may play a role in this process [reviewed in Kirschner et al. (1985)]. Since the transition between the oocyte and egg forms of nucleoplasmin also occurs during this meiotic period (M. Cotten, unpublished results), we wondered if the kinase activity associated with MPF might be capable of modifying oocyte nucleoplasmin to the extent that changes in electrophoretic mobility could be identified.

We tested this idea by preparing MPF through the ammonium sulfate precipitation step (which removes endogenous egg nucleoplasmin) and assaying the capacity of the extract to phosphorylate added oocyte nucleoplasmin. We find that in the absence of added oocyte nucleoplasmin, there is little phosphorylated protein migrating in the nucleoplasmin region of the gel (Figure 4, lane 3) and that when an antibody precipitation with a monoclonal antibody to nucleoplasmin is performed, there is no detectable material with nucleoplasmin mobility (Figure 4, lane 5). However, if oocyte nucleoplasmin

is added to the MPF preparation, substantial phosphorylation in the nucleoplasmin region of the gel is now detected (Figure 4, lane 4), and antibody precipitation reveals nucleoplasmin with the increased electrophoretic heterogeneity of egg nucleoplasmin (Figure 4, lane 6). Although the possibility remains that some form of modification other than phosphorylation is responsible for the altered mobility of nucleoplasmin added to this extract, the labeling of the protein by the MPF with [γ-^{32}P]ATP, coincident with the altered mobility, provides further support for the notion that phosphorylation is responsible for the electrophoretic heterogeneity.

Phosphatase Treatment Inactivates Egg Nucleoplasmin in Assembly Assays. If the major chemical difference between oocyte and egg nucleoplasmin lies in the level of phosphorylation, then egg nucleoplasmin treated with phosphatase should exhibit the reduced chromatin assembly activity of oocyte nucleoplasmin. We have assayed the in vitro chromatin assembly activity of various nucleoplasmin samples using an assay system that measures the induction of supercoils in a circular DNA molecule when a nucleosome is formed (Germond et al., 1975). Furthermore, since the assembly activity of histones varies with the acetylation state of the proteins (Cotten & Chalkley, 1985), we have assayed the assembly using both control and hyperacetylated histones in order to study assembly under a broad range of conditions.

We have chosen to assay the chromatin assembly activity of egg nucleoplasmin treated with phosphatase under nondenaturing conditions. It is clear that these conditions fail to remove all of the attached phosphate but the difficulties inherent in producing a correctly renatured, pentameric protein after denaturation by boiling in $NaDodSO_4$ precluded the use of fully dephosphorylated nucleoplasmin for this study. The fact that the dephosphorylated nucleoplasmin monomer retains at least 5 of its 19 attached phosphate groups must be taken into account when interpreting the results of these experiments.

Figure 5 shows the assembly activity of egg nucleoplasmin (lanes 1 and 4), egg nucleoplasmin treated with phosphatase (lanes 2 and 5), and oocyte nucleoplasmin (lanes 3 and 6) using either hyperacetylated histones (lanes 1–3) or control histones (lanes 4–6). It is apparent that the egg nucleoplasmin assembles material much more effectively than the oocyte nucleoplasmin. There are very few DNA molecules with intermediate levels of supercoils (which would indicate incomplete assembly) and very little DNA present in the pellet (the presence of DNA in the pellet indicates the formation of insoluble histone–DNA species). In contrast, the assemblies performed with oocyte nucleoplasmin produce large amounts of DNA species with intermediate levels of supercoiling (lanes 3 and 6). The pattern of supercoiled DNA species produced by assembly with phosphatase-treated egg nucleoplasmin (lanes 2 and 4) is strikingly similar to that produced by oocyte nucleoplasmin.

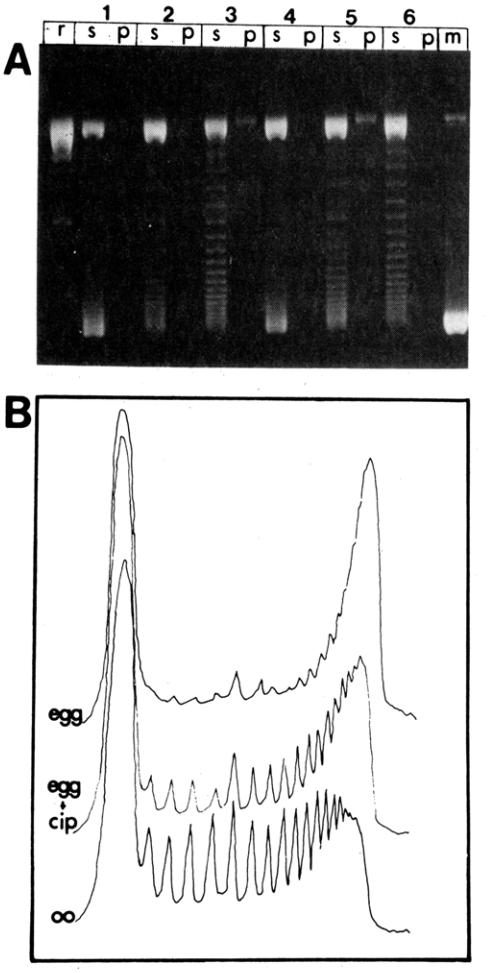

FIGURE 5: Chromatin assembly activity of dephosphorylated egg nucleoplasmin. Egg nucleoplasmin was treated with calf intestinal phosphatase and repurified by chromatography on DEAE-cellulose as described under Experimental Procedures. Chromatin assembly was assayed by the induction of supercoils in a relaxed circular DNA (pBR322) using either hyperacetylated core histones at a histone to DNA ratio of 1 or control core histones at a histone to DNA ratio of 2 as previously described (Sealy et al., 1986) with a nucleoplasmin to histone ratio of 1.4 for both types of histones. Following the assembly period (2 h, room temperature, in the presence of topoisomerase I), the reactions were centrifuged at 15000*g* for 5 min to identify precipitated products, and the material in the pellet and supernatant was adjusted to 0.3 M sodium acetate, 0.2 mg/mL proteinase K, and 0.5% $NaDodSO_4$, and incubated for 40 min at 37 °C, and the DNA was recovered by ethanol precipitation. The resulting DNA samples were resolved by electrophoresis on a 2% agarose gel in 36 mM Tris, 30 mM sodium phosphate, and 1 mM EDTA, pH 7.7, and the DNA pattern was located by staining with ethidium bromide. (A) Lane r, relaxed, form I DNA (the starting material for the assembly reaction); lanes p and s, the pellet and supernatant fractions, respectively, from each reaction; lane 1, egg nucleoplasmin assembly with hyperacetylated histone; lane 2, phosphatase-treated egg nucleoplasmin assembly with hyperacetylated histones; lane 3, oocyte nucleoplasmin assembly with hyperacetylated histones; lane 4, egg nucleoplasmin assembly with control histones; lane 5, phosphatase-treated egg nucleoplasmin assembly with control histones; lanes 6, oocyte nucleoplasmin assembly with control histones. (B) The Polaroid type 55 negative of the assembly reaction products (supernatant fractions) from control histone assembly was scanned with a densitometer. Egg, the DNA species produced by assembly with egg nucleoplasmin; egg + cip, the DNA species produced by assembly with egg nucleoplasmin treated with calf intestinal phosphatase; oo, the DNA species produced by assembly with oocyte nucleoplasmin.

Discussion

There are two major differences between nucleoplasmin isolated from oocytes and nucleoplasmin isolated from unfertilized eggs. The egg form of nucleoplasmin exhibits a more acidic p*I* than the oocyte form, and not only does it display a greater amount of heterogeneity but also it migrates more slowly when resolved by electrophoresis on both $NaDodSO_4$ (Sealy et al., 1986) and acid–urea gels (Figures 1 and 2). Second, the egg form is superior to the oocyte form in in vitro chromatin assembly assays (Sealy et al., 1986). We demonstrate here that both of these differences can be accounted for by differences in the level of phosphorylation. Both the electrophoretic heterogeneity (Figure1) and the chromatin assembly activity (Figure 4) of egg nucleoplasmin are reduced by treatment with phosphatase. Furthermore, oocyte nucleoplasmin can be modified in vitro so that it displays the electrophoretic heterogeneity of egg nucleoplasmin (Figure 4).

Coincident with this modification is the attachment of phosphate to the molecule, further suggesting that phosphorylation is responsible for the electrophoretic heterogeneity of egg nucleoplasmin.

In the most fully phosphorylated form isolated, oocyte nucleoplasmin contains approximately 35 phosphate groups per (pentamer) molecule whereas the egg nucleoplasmin molecule contains on the order of 100 phosphate groups. This observation satisfactorily accounts for both the more acidic p*I* of egg nucleoplasmin and also the decreased mobility of egg nucleoplasmin, in SDS gels, the latter presumably reflecting the increased molecular weight due to all the extra phosphate groups. Approximately 30% of the phosphate groups associated with nucleoplasmin cannot be removed unless the nucleoplasmin is first denatured, indicating that these groups are sterically protected in some way. The protected phosphate groups are all found in the highly negatively charged amino-terminal two-thirds of nucleoplasmin. The protection may well reflect an interaction between the highly positively charged carboxy-terminal fragment and the negatively charged amino-terminal portion of the molecule. Since this latter fragment not only is the most extensively modified portion of nucleoplasmin but also is the site of histone binding and organization, it is not surprising that nucleoplasmin-mediated histone assembly onto DNA should be dependent upon phosphorylation.

The conclusion that successful chromatin assembly depends upon the extensive phosphorylation of egg nucleoplasmin is based upon the loss of some of the assembly activity after phosphatase treatment. Ideally, we would have liked to assay assembly after removing all of the phosphate following denaturation, but our lack of knowledge about nucleoplasmin renaturation precluded this study. Although phosphatase treatment of the native nucleoplasmin reduced the electrophoretic mobility to a value close to that of the phosphatase-treated oocyte nucleoplasmin, the decrease in assembly activity does not fully shift to that of the oocyte form. This may reflect the survival of key phosphate groups in a protected form in the amino-terminal portion of the egg molecule.

Although the basis for the enhanced assembly activity is clearly the level of nucleoplasmin phosphorylation, the actual molecular mechanism of this enhancement is not yet known. The two forms of nucleoplasmin are capable of binding similar quantities of histones, as assayed by sedimentation of histone–nucleoplasmin complexes through sucrose gradients (L. Sealy, unpublished results). It seems likely that the egg form of nucleoplasmin, with its bank of associated phosphate groups, may be more capable than oocyte nucleoplasmin of organizing these bound histones into a (presumably octamer) form that is necessary for correct deposition onto DNA.

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