Eur. J. Biochem. 240, 186–194 (1996)

Dissociation of protamine-DNA complexes by *Xenopus* nucleoplasmin and minichromosome assembly *in vitro*

Simón A. RUIZ-LARA, Luís CORNUDELLA and Antonio RODRíGUEZ-CAMPOS

Departamento de Biología Molecular y Celular, Centro de Investigación y Desarrollo del CSIC y Unitat de Biologia Molecular del Centre de Referència en Biotecnologia de la Generalitat de Catalunya, Barcelona, Spain

(Received 9 April/4 June 1996) – EJB 96 0525/1

Nucleoplasmin, an acidic thermostable protein abundant in the nucleus of *Xenopus laevis* oocytes, has been found to dissociate complexes of pUC19 DNA and protein ϕ1, an intermediate protamine present in ripe sperm from the mollusc *Mytilus edulis*. Cruder preparations of nucleoplasmin, such as the amphibian oocyte S150 extract and its thermostable fraction, also dissociate the heterologous DNA-ϕ1 complexes and, in addition, promote the assembly of plasmid DNA into a minichromosome displaying regular nucleosomal periodicity, as revealed by micrococcal nuclease digestion. In contrast, purified nucleoplasmin complemented with rat hepatocyte core histone octamers in the presence of DNA topoisomerase I, although capable of inducing nucleoprotein formation onto the complexed DNA, fails to position nucleosomes at the native spacings seen in chromatin *in vivo*. These data favour the existence of a general mechanism to bring about, in a concerted manner, removal of sperm-specific nuclear proteins and reconstitution of somatic chromatin following fertilization.

Keywords: chromatin assembly; nucleoplasmin; oocyte S150 extract; sperm decondensation; sperm-protein–DNA interactions.

The process of fertilization is a temporal sequence of events involving the species-specific fusion of the plasma membranes of the sperm and the egg. Upon fertilization, the sperm chromatin is transformed into the male pronucleus through a series of steps including loss of the sperm envelope, chromatin dispersion and formation of a pronuclear envelope (Longo and Kunkle, 1978). During echinoderm embryogenesis, decondensation of sperm chromatin is associated with the replacement of sperm-specific nucleoproteins by postfertilization transitional histones of the egg cytoplasm and final transition to somatic nucleohistone (Longo, 1985). From the inability of oocyte histone protein pools to displace *in vitro* sperm DNA-bound proteins, it has been suggested that egg cytoplasmic and/or nuclear factors may be required to promote nucleoprotein exchange reactions initiating soon after sperm entry (for reviews, see Longo and Kunkle, 1978; Poccia, 1986).

In contrast to the evolutionary conservative somatic histones, chromatin-condensing proteins in mature sperm cells appear to exhibit a vast structural diversity. The variety of sperm nucleoproteins is such that attempts to cluster them into groups have yielded solely loose categorizations of limited specificity (Bloch, 1976; Poccia, 1986; Oliva and Dixon, 1991). Nonetheless, more recent evidence has revealed that constant structural motifs can be sorted out amongst these diverse nucleoproteins (Kasinsky, 1995).

Correspondence to L. Cornudella, Dept. of Molecular & Cell Biology, Centro de Investigación y Desarrollo del CSIC, Jordi Girona, 18–26, E-08034 Barcelona, Spain

Fax: +34 3 204 5904.

Abbreviations. $PheMeSO_2F$, phenylmethanesulfonyl fluoride; MN, micrococcal endonuclease.

Enzymes. Deoxyribonuclease I (EC 3.1.21.1); micrococcal endonuclease (EC 3.1.31.1).

Nucleoplasmin is an acidic thermostable protein most abundant in the nuclei of *Xenopus* oocytes, that interacts with histones to form specific complexes (Laskey et al., 1978; Earnshaw et al., 1980; Mills et al., 1980). Nucleoplasmin in extracts of unfertilized *Xenopus* eggs promotes nucleosome assembly under physiological conditions of histones on exogenously added DNA templates, yielding nucleosomal arrays evenly spaced at about 200 bp (reviewed by Laskey and Earnshaw, 1980; Dilworth and Dingwall, 1988). The active form of nucleoplasmin is a pentamer (Earnshaw et al., 1980) which selectively binds H2A and H2B histones (Kleinschmidt et al., 1985; Dilworth et al., 1987). It has been shown that there exists more than a single species of nucleoplasmin. Furthermore, differences in the degree of phosphorylation of nucleoplasmin from unfertilized eggs and oocytes have been found (Sealy et al., 1986; Cotten et al., 1987), reporting that phosphorylation of nucleoplasmin enhances the capacity to assemble chromatin *in vitro*. Recent work has indicated that nucleoplasmin is capable of initiating decondensation of chromatin from *Xenopus* sperm (Philpott and Leno, 1992) as well as from human sperm (Itoh et al., 1993) facilitating substitution of histones for sperm-specific proteins. The selective removal of sperm-specific proteins appears to be closely coupled to the deposition of histones onto DNA. Consequently, nucleoplasmin-containing complexes have been considered as being bifunctional, mediating both sperm chromatin dissociation as well as somatic nucleosome assembly (Philpott et al., 1991; Philpott and Leno, 1992). Chromatin-assembly factors mimicking nucleoplasmin ability have been observed in a variety of animal species and tissues (Schmidt-Zachman et al., 1987; Ishimi et al., 1984; Stillman, 1986; Cotten and Chalkey, 1987; Ulitzur and Gruenbaum, 1989; Berrios and Avilion, 1990).

In this work, we have approached the chromatin assembly pathways by examining the ability of nucleoplasmin to dissociate *in vitro* heterologous complexes of pUC19 DNA and the

basic nucleoprotein φ1, an intermediate protamine present in ripe sperm cells from the common clam *Mytilus edulis* (Subirana, 1983; Ausio, 1986; Ruiz-Lara et al., 1993). This sperm-specific protein defines one of the five major categories in the classification of sperm nuclear proteins proposed by Bloch (1976), known as the *Mytilus* class. We have also assayed the capability of *Xenopus* oocyte extracts to rearrange these formed complexes and to assemble somatic-like chromatin. Finally, we have examined whether the formed complexes are remodelled to chromatin when mixed with nucleoplasmin and core histone octamers from rat liver. We have found that either the *Xenopus* oocyte S150 extract (Shimamura et al., 1988), the thermostable fraction thereof, or purified nucleoplasmin, mediate dissociation of the complexes containing *Mytilus* sperm nucleoprotein φ1. In addition, when nucleoplasmin is incubated with rat liver core histone octamers, the mixture dissociates DNA-φ1 complexes with subsequent formation of nucleosomal structures yielding DNA repeats of about 150 bp upon micrococcal nuclease (MN) digestion. Inference is made from these results about the concurrent mediation of nucleoplasmin in the tightly coupled processes of disassembly of the sperm nucleus and reconstitution of somatic chromatin.

EXPERIMENTAL PROCEDURES

Preparation of the oocyte S150 extract. Oocyte S150 extracts were prepared from adult *Xenopus laevis* female frogs essentially as described previously (Shimamura et al., 1988). The final oocyte high-speed supernatant was stored frozen in 300-μl aliquots at −80°C until used.

Purification of nucleoplasmin and sperm protein φ1. Nucleoplasmin was purified as described (Laskey et al., 1978; Zucker and Worcel, 1990) with slight modifications. The whole S150 extract was heated to 80°C for 10 min, then quickly chilled on ice. The heat-denatured extract was clarified by centrifugation at 12000 rpm in a microfuge for 30 min, then fractionated in linear 5−20% sucrose gradients made in 20 mM Hepes/NaOH, pH 7.5, 0.1 mM EDTA, 2 mM $MgCl_2$ (buffer A) containing phenylmethanesulfonyl fluoride ($PhMeSO_2F$) (0.1 μg/ml). Centrifugation was carried out for 24 h at 40000 rpm in the Beckman SW41 rotor at 4°C. Fractions (0.5 ml) were collected and the location of nucleoplasmin along the gradient was determined by SDS/PAGE of 50 μl of each fraction on 18% slab gels. Sucrose gradient fractions containing nucleoplasmin were pooled and applied directly to a 1-ml hydroxylapatite column (Bio-Gel HTP, Bio-Rad) equilibrated with 0.4 M NaCl in 20 mM Hepes/NaOH, pH 7.5, 0.1 mM EDTA, 10 μg/ml $PhMeSO_2F$ (buffer B). The column was washed with 5 ml 2 M NaCl in buffer B, then re-equilibrated with 10 mM KH_2PO_4 in buffer B. Contaminant proteins were eluted off the column with 5 ml 0.1 M KH_2PO_4 in buffer B. Nucleoplasmin was finally eluted with 10 ml 0.4 M KH_2PO_4 in buffer B and the eluate extensively dialyzed against distilled water overnight at 4°C. The purified nucleoplasmin was vacuum concentrated, brought to 0.1 M in NaCl and stored at −80°C.

Protein φ1 was extracted from ripe sperm of *M. edulis* by a procedure previously described (Ausio and Subirana, 1982) and purified to electrophoretic homogeneity.

Preparation of DNA-φ1 complexes. Purified sperm protein φ1 was allowed to associate to pUC19 DNA, either relaxed or supercoiled, over a range of mass ratios. Incubations were carried out for 30 min at room temperature. The DNA-φ1 mass ratios assayed, varied from 1:0.1 to 1:10 in a final volume of 10 μl sterile distilled water.

Nuclease digestion analyses. DNA-φ1 complexes at different mass ratios were probed with DNase I and MN. Following complex formation, the reaction mixtures were made 0.1 mM in $CaCl_2$ and digested at 37°C with either DNase I (20 ng/μl) or MN (0.3 U/μl) for various times, as indicated. Digestion reactions were halted by chilling on ice after addition of 100 mM EDTA, pH 7.0, to 20 mM and 2.5% sarkosyl to 0.5%. Digests were then treated with 1 mg/ml proteinase K for 1 h at 60°C in the presence of 0.1% SDS. After incubation, samples were deproteinized with an equal volume of saturated phenol and the DNA precipitated with 2.5 volumes ethanol upon standing at −20°C. Control digestions of free DNA in the absence of protein φ1 were carried out in parallel, exactly in the manner described. Analytical electrophoresis of DNA fragments isolated from digests was performed at room temperature on 1% agarose gels cast and run in 90 mM Tris/H_3BO_3, pH 8.3, 2.5 mM EDTA (TBE buffer), together with DNA size markers.

Chromatin assembly reactions. The chromatin assembly reactions on formed DNA-φ1 complexes at unit mass ratio (1.5 μg each component) using oocyte S150 extracts, were carried out for 6 h at 37°C essentially as described (Shimamura et al., 1988). Mock reconstitutions were performed in parallel over free plasmid DNA and by adding protein φ1 (1.5 μg) to the oocyte S150 at the onset of assembly. All reconstitutions were tested for the extent of chromatin assembly by MN digestion analysis.

For the reconstitution assays using purified components, pure nucleoplasmin (4.5 μg) was mixed with rat hepatocyte core histone octamers (2.24 μg) in 50 μl assembly buffer (160 mM NaCl, Tris/HCl, pH 7.5, 0.3 mM EDTA, 1 mM dithiothreitol, 1 mM 2-mercaptoethanol, 0.01% Nonidet P40) and incubated for 45 min at 37°C. Simultaneously, relaxed pUC19 DNA and protein φ1 (1.5 μg each) were complexed under standard conditions. Both reactions were subsequently mixed and incubated further for 4 h at 37°C after addition of calf thymus DNA topoisomerase I (10 U). 10% of the final mixture was removed to analyze the topology of the DNA following deproteinization and the remainder subjected to MN digestion.

Isolation of minichromosomes and analysis of DNA topology. Minichromosomes reconstituted either without or in the presence of protein φ1 were isolated by sedimentation through linear 15−30% sucrose gradients made in 100 mM NaCl, 5 mM Hepes/NaOH, pH 7.5, 0.2 mM EGTA, in a Beckman SW41 rotor at 22000 rpm for 16 h at 4°C. The sedimentation position of the assembled minichromosomes was determined by electrophoresis of 50 μl of each gradient fraction on 1.5% agarose gels containing ethidium bromide (0.5 μg/ml).

Gradient-purified minichromosomes were deproteinized with 1 mg/ml proteinase K for 1 h at 60°C in the presence of 0.2% SDS and 20 mM EDTA. After incubation, samples were extracted with saturated phenol. Aqueous phases were made 3 M in ammonium acetate, and the DNA was precipitated with three volumes ethanol. DNA was recovered by centrifugation, washed and resedimented twice with cold 80% ethanol, dried under reduced pressure, and finally dissolved in 10 mM Tris/HCl, pH 8.0, 1 mM EDTA (TE buffer). 50% of each DNA sample was electrophoresed in a 1% agarose gel in TBE buffer, together with relaxed and supercoiled forms of plasmid DNA as references. The other halves of the DNA samples were resolved on parallel 1% agarose gels cast and run in TBE containing 4 μM chloroquine in the dark with buffer recirculation. After electrophoresis, chloroquine was removed by washing with distilled water and the gels were next stained with ethidium bromide.

Protein analysis. Aliquots of pooled gradient fractions containing the minichromosomes assembled without or with protein φ1, were layered over a 1-ml cushion of 25% sucrose in gradient buffer and sedimented in a Beckman SW60 rotor at 30000 rpm for 16 h at 4°C. After centrifugation, supernatants were drained

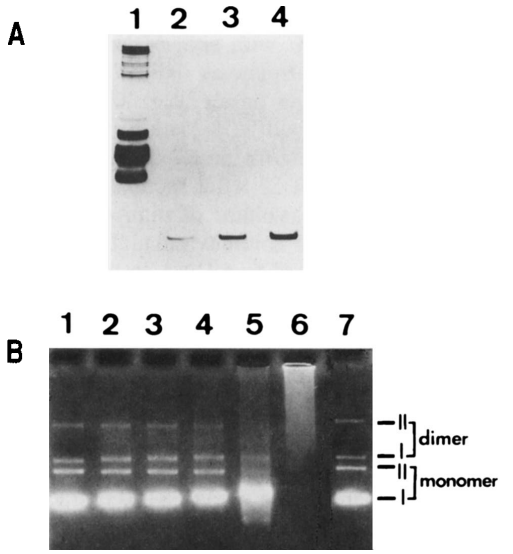

Fig. 1. Interaction of sperm protein ϕ1 with plasmid DNA. (A) Electrophoretic analysis of nuclear basic protein ϕ1 extracted from *M. edulis* ripe sperm, in an acid/urea/polyacrylamide slab gel containing 15% acrylamide, stained with Coomassie blue. Lane 1, chicken erythrocyte histones (1 μg); lanes 2–4, purified sperm protein ϕ1 (10, 20 and 40 μg, respectively). (B) Agarose (1%) gel electrophoretic patterns of supercoiled pUC19 DNA (0.3 μg) complexed under standard conditions (see Experimental Procedures) with the following amounts of purified protein ϕ1: lane 2, 0.03 μg; lane 3, 0.06 μg; lane 4, 0.12 μg; lane 5, 0.24 μg; lane 6, 0.3 μg. Leftmost and rightmost lanes show supercoiled plasmid DNA (0.3 μg). The gels were stained with ethidium bromide, transilluminated with ultraviolet light and photographed. The mobilities of the supercoiled form of DNA (I) and the relaxed form (II) are indicated. m, monomer; d, dimer.

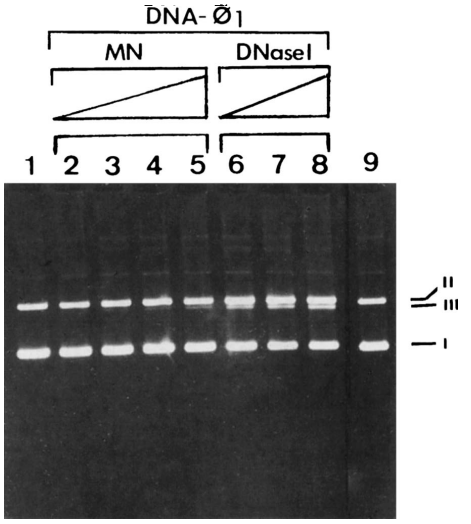

Fig. 2. Kinetics of nuclease digestions of DNA-ϕ1 complexes. Agarose gel electrophoresis of a formed DNA-ϕ1 complex at mass ratio of 1, digested with MN (0.3 U/μl) for the following times (lanes 2–5): 0, 2.5, 5, and 10 min, respectively. Sample duplicates were digested in parallel with DNase I (20 ng/μl) for 2.5, 5 and 10 min (lanes 6–8). Supercoiled pUC19 DNA (170 ng) was run as reference in lanes 1 and 9. The supercoiled (I), relaxed (II) and linear (III) forms of plasmid DNA are indicated in this and in Figs 3 and 7.

off the tubes and the pelleted minichromosomes were suspended in loading buffer for electrophoresis (8 M urea, 0.9 M acetic acid, 5% 2-mercaptoethanol) containing 1% Pyronine Y as tracking dye. The solubilized proteins were resolved on acid/urea/polyacrylamide slab gels containing 15% acrylamide, which had been electrophoresed for 4 h. Gels were visualized by staining with silver (Wray et al., 1981).

Dissociation of DNA-ϕ1 complexes by nucleoplasmin. DNA-ϕ1 complexes at unit mass ratio were formed under the standard conditions described above. The formed complexes (in 10 μl distilled water) were reacted with varying amounts of purified nucleoplasmin in a final volume of 40 μl, adjusted with buffer A. After 4 h incubation at 37°C, sample volumes were adjusted to the required strength with tenfold concentrated restriction buffer, brought to 50 μl, and digested with *Bam*HI (10 U) for 2 h at 37°C. After digestion, samples were extensively deproteinized, the DNA recovered by ethanol precipitation and electrophoresed in 1% agarose gels in TBE buffer.

RESULTS

Association of sperm protein ϕ1 to DNA. We were interested in studying the association of DNA with a protein of extreme basicity such as sperm-specific nucleoprotein ϕ1 from the mussel *M. edulis*, reasoning that this strongly basic protein would display high affinity for the negatively charged DNA template. To this effect, the intermediate protamine ϕ1 was extracted from mature sperm and purified to electrophoretic homogeneity (Fig. 1A). Subsequently, the latter was incubated in the presence of supercoiled pUC19 DNA at increasing mass ratios and the mode of interaction was assessed by mobility shift gel electrophoresis (Fig. 1B). At a mass ratio of plasmid DNA to ϕ1 of 1, both components formed a complex unable to enter the gel. The same result was obtained at mass ratios higher than 1 (not shown).

The next experiments aimed to evaluate the extent of protection conferred on the DNA by the attachment of protein ϕ1, monitoring the actions of DNase I and MN on newly formed complexes at unit mass ratio (Fig. 2). The action with time of these two enzymes on DNA-ϕ1 complexes was analyzed electrophoretically following extensive deproteinization of digest aliquots at each digestion interval. MN was unable to degrade within the pUC19-ϕ1 complex (lanes 2–5 in Fig. 2). In contrast, the ϕ1-containing complex appeared to be gradually accessible to DNase I (Fig. 2, lanes 6–8) by the appearance of bands corresponding to linearized plasmid DNA increasing in intensity with digestion time.

***In vitro* chromatin reconstitution in the presence of ϕ1.** We also verified whether protein ϕ1 either alone or previously complexed to plasmid DNA, affected reconstitution of chromatin *in vitro* mediated by *Xenopus* oocyte S150 extract, which can assemble closed circular DNA molecules into minichromosomes (Glikin et al., 1984). These can be characterized under appropriate conditions by the linking number change and the generation of MN resistance (Laskey et al., 1978).

Three chromatin-assembly reactions on pUC19 DNA relaxed with topoisomerase I were prepared. A formed plasmid DNA-ϕ1 complex at unit mass ratio was used as template in the first reconstitution whereas in the second an equivalent amount of purified nucleoprotein was exogenously added at the onset of the assembly reaction. The third was processed as a reconstitution standard in the absence of nucleoprotein ϕ1. After incubation, the assembly mixtures were tested for their chromatin reconstitution capacity by MN digestion and DNA supercoiling analyses. MN digestions, in all cases, generated sets of DNA fragments which resolved electrophoretically into comparable nucleosomal ladder patterns displaying a mean periodicity of 170 bp (Fig. 3) in agreement with previous spacings reported

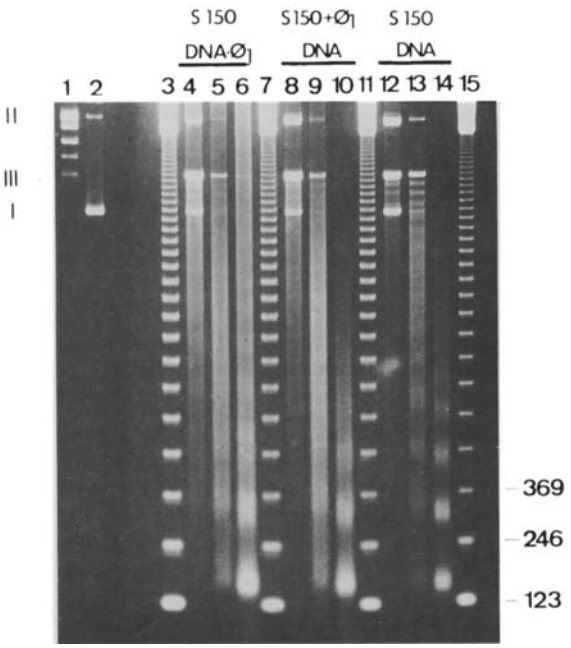

Fig. 3. MN digestion analysis of chromatin assembled on DNA-ϕ1 complexes *in vitro*. Relaxed pUC19 DNA (lane 1) was complexed to protein ϕ1 at unit mass ratio (1.5 μg each reactant) under standard conditions and incubated with oocyte S150 extract (300 μl) in a final volume of 0.5 ml for 6 h at 37°C. 10% of the assembly reaction was set aside for the analysis of DNA topology following deproteinization (lane 2) and the remainder subjected to MN digestion for 2, 8 and 32 min (lanes 4–6). Two mock-assembly reactions were carried out under exactly the same conditions on naked plasmid DNA, adding protein ϕ1 (1.5 μg) to the S150 extract at the onset of assembly (lanes 8–10), or in the absence of added sperm protein (lanes 12–14). All samples were deproteinized and the DNA fragments resolved on a 1.5% agarose gel containing ethidium bromide. A 123-bp ladder of DNA fragments (Gibco-BRL) (lanes 3, 7, 11 and 15, respectively) was run in parallel as size marker.

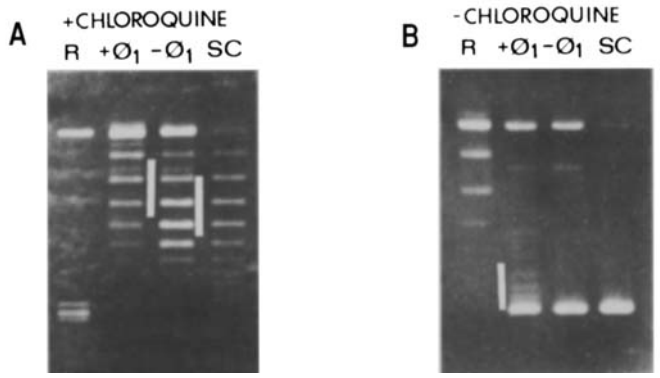

Fig. 4. Topological analysis of DNA from minichromosomes assembled with and without protein ϕ1 and purified by sucrose gradient centrifugation. Two identical minichromosome assembly reactions with S150 extract were performed under standard conditions. Relaxed pUC19 (3 μg) previously complexed to protein ϕ1 at unit mass ratio was used as DNA template in the first reaction, while protein-free plasmid DNA was used in the second assembly. After reconstitution, the products of both reactions were sedimented in parallel through 15–30% sucrose gradients as described in Experimental Procedures. Gradient fractions containing minichromosomes assembled in the presence and absence of protein ϕ1 were pooled, extensively deproteinized and the final DNA samples divided in half. Both halves of each DNA sample were independently resolved on 1% agarose gels in the absence (B) and presence (A) of 4 μM chloroquine, together with relaxed pUC19 DNA (R) and supercoiled plasmid DNA (SC) (1.5 μg each DNA form). $+\phi$1, DNA topoisomers from minichromosomes assembled on the formed DNA-ϕ1 complex. $-\phi$1, DNA topoisomers from control minichromosome (without protein ϕ1). Vertical bars encompass the regions of distribution of DNA topoisomers.

elsewhere (Shimamura et al., 1988; Sapp and Worcel, 1990). The nucleosome banding patterns resulting from MN digestions in the presence of nucleoprotein ϕ1, either complexed to DNA or added at time zero of reconstitution, appeared somewhat blurred with no concomitant alteration of the 170-bp repeat size which remained unchanged. To further corroborate these results, the hydrodynamic behaviour of the minichromosome assembled in the presence of protein ϕ1 was analyzed by sedimentation through linear isotonic sucrose gradients which prevent chromatin unfolding as well as aggregation and hence, allow for purification of the assembled minichromosomes from other soluble components present in the oocyte supernatant (Shimamura et al., 1988). The sedimentation velocity of assembled minichromosomes in these gradients is a function of their mass and degree of nucleohistone compaction (Worcel et al., 1978).

Gradient fractions containing the control minichromosome lacking protein ϕ1 and those of the minichromosome assembled on the formed DNA-ϕ1 complex were separately pooled (Fig. 4). Aliquots of each pool were extensively deproteinized and the DNA subjected to electrophoretic analysis. DNA from duplicate aliquots was resolved on parallel 1% agarose gels, one of them cast in the presence of 4 μM chloroquine (Fig. 4A). The DNA from both minichromosomes displays a sizable degree of negative supercoiling as compared to the initial state of relaxation of the DNA. As seen in the presence of chloroquine, the centre of the topoisomer distribution of the DNA from the minichromosome assembled on the ϕ1-containing complex does not exactly coincide with that of the DNA topoisomer ladder from the control minichromosome without protein ϕ1. The distribution centre of the latter appears displaced by 1 or 2 bands. Considering that the formation of each nucleosome introduces a linking number change of one in the covalently closed circular DNA (Germond et al., 1975), the shift observed between the two topoisomer ladders reveals a relative difference of 1 or 2 in the number of nucleosomes in minichromosomes assembled with protein ϕ1 in respect to the 18 regularly spaced nucleosomes reported for a fully loaded minichromosome assembled in the absence of exogenous protein (Rodriguez-Campos et al., 1989). These results are consistent with those of the MN digestion analysis as well as with the notion that the interaction of protein ϕ1 with DNA somehow hinders the process of minichromosome assembly.

Protein composition of minichromosomes assembled on DNA-ϕ1 complexes. To determine the fate of protein ϕ1 during minichromosome formation, aliquots of pooled gradient fractions containing the assembled minichromosomes were pelleted through a 25% sucrose cushion. The resulting nucleoprotein sediments were dissolved in 8 M urea, 0.9 M acetic acid and the solubilized proteins characterized by electrophoresis in acid/urea/polyacrylamide slab gels containing 15% acrylamide (Fig. 5). The electrophoretic analysis revealed no change between the protein complement of the minichromosome control lacking protein ϕ1 (Fig. 5, lane $-\phi$1) and the content of the minichromosome assembled on preformed ϕ1-containing complexes at unit mass ratio (lane $+\phi$1). In addition, the proteins present in both minichromosomes comigrated with the nucleosomal histones run in parallel as reference (Fig. 5, lane His). There was no evidence of the presence of protein ϕ1 among proteins recovered from the minichromosome assembled on the preformed DNA-ϕ1 complex, even after resorting to a sensitive

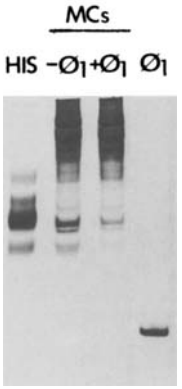

Fig. 5. Protein analysis of assembled minichromosomes. Sucrose gradient sedimented minichromosomes (MCs), assembled in the absence (−ϕ1) and presence (+ϕ1) of protein ϕ1 were pelleted through a 25% sucrose cushion and their proteins characterized in acid/urea/polyacrylamide gels containing 15% acrylamide. 1 μg of chicken erythrocyte histones (HIS) and 10 μg purified sperm protein ϕ1 (ϕ1) were co-electrophoresed as reference proteins. Gels were visualized by silver staining.

silver staining technique (Wray et al., 1981) to enhance protein detection. The slowly migrating material in the minichromosome lanes (MCs) corresponds to some contaminating proteins of the S150 extract known to cosediment with assembled minichromosomes and which resolve on the high-molecular-mass range of the SDS-gels (Shimamura et al., 1988, 1989; Zucker and Worcel, 1990). The protein composition found correlates further to the preceding results concerning the MN digestion and DNA topology analyses as well as those of the hydrodynamic behaviour of the minichromosome formed over the DNA-ϕ1 complex.

Nucleoplasmin in the S150 extract. The identification of protein factors in *Xenopus* (Philpott et al., 1991; Philpott and Leno, 1992) and *Bufo japonicus* (Oshumi and Katagiri, 1991) egg extracts as well as in *Drosophila* embryo extracts (Ulitzur and Gruenbaum, 1989; Berrios and Avilion, 1990), capable of decondense sperm chromatin, prompted an examination of the effect of purified nucleoplasmin on heterologous complexes of pUC19 DNA and sperm-specific protein ϕ1. To this effect, nucleoplasmin was isolated from the S150 extract by zonal centrifugation and purified further by hydroxylapatite chromatography as described in Experimental Procedures. Fractions from the 5–20% sucrose gradient containing nucleoplasmin were pooled and the protein subjected to further purification on hydroxylapatite. Distribution of proteins along the gradient was analyzed by SDS/PAGE (Fig. 6B) and compared to that of a similarly fractionated, untreated S150 extract (Fig. 6A). The purified nucleoplasmin was used as electrophoretic reference marker.

Basic protein profile of the unheated S150 extract subjected to zonal centrifugation is presented (Fig. 6A). The untreated extract contained a protein sedimenting along the fractions 11–15 that made up the central portion of the gradient, whose electrophoretic migration closely matched that of the nucleoplasmin standard. Concomitantly, a highly stainable protein migrating slightly ahead of nucleoplasmin, cosedimented along gradient fractions 3–8 with the bulk of histones present in the S150 extract. It should be noted that the protein bands positioned near the centre of the gradient stained poorly with Coomassie blue as did the nucleoplasmin standard (Fig. 6A, lane 1). The heated S150 extract (Fig. 6B) showed some differences in protein distribution. The bulk of the nucleoplasmin-like protein sedimented again half way down the gradient in four fractions (Fig. 6A, 9–12), but was preceded by trace amounts of a comigrating protein

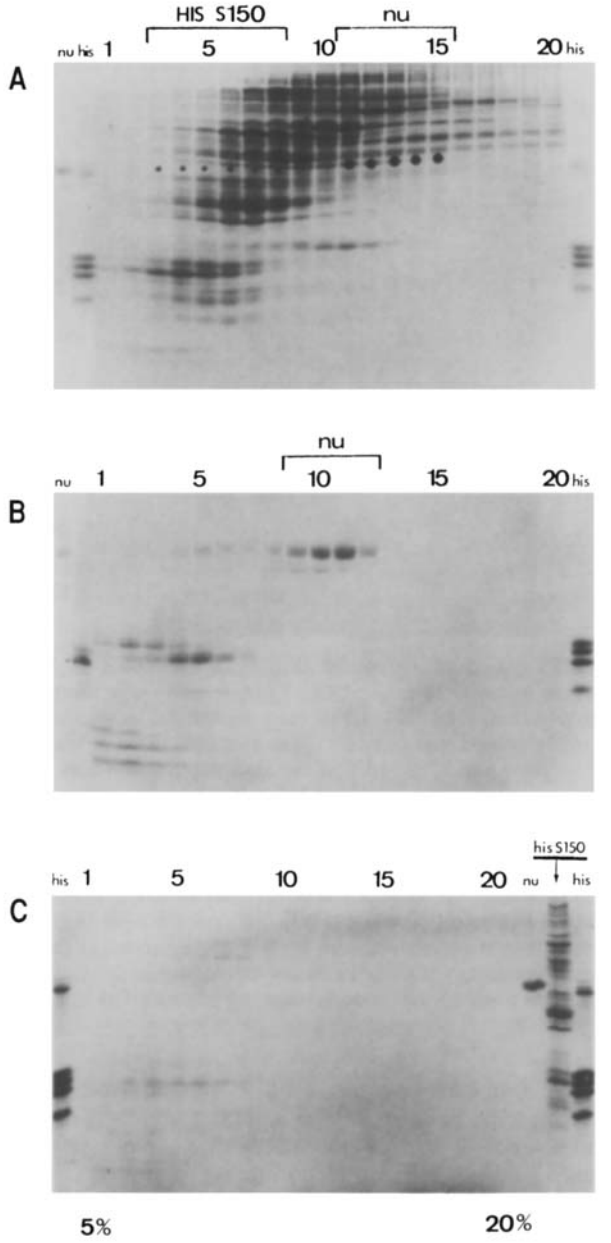

Fig. 6. Sucrose gradient fractionation of oocyte S150 extract. Aliquots (2.5 ml) of the whole S150 extract were fractionated by centrifugation in linear 5–20% sucrose gradients for 24 h in a Beckman SW41 rotor as described in Experimental Procedures. Fractions of 0.5 ml each were collected and the distribution of proteins along the gradients analyzed by SDS/PAGE of 50 μl of each fraction on 18% acrylamide gels (A) Protein composition of gradient fractions from a native S150 extract. Note that nucleoplasmin (filled dots) sediments apart from the bulk of oocyte histones (HIS S150). In turn, a highly stainable protein (stars) migrating slightly ahead of nucleoplasmin (nu), cosediments with oocyte endogenous histones. (B) Exactly as in (A), except that the S150 extract was heated to 80°C for 10 min and clarified in a microfuge prior to gradient centrifugation. The bulk of thermostable nucleoplasmin sediments along fractions 9–12 (enclosed in the horizontal bracket). (C) Histone-enriched fractions from the unheated S150 extract (denoted HIS S150 in A) were pooled and dialyzed. An aliquot was set aside for electrophoretic analysis and the remainder heated to 80°C for 10 min and processed as described in (B). Note that no nucleoplasmin is detected in any gradient fraction, not even in the aliquot removed from the pooled fractions before the 80°C heating step (see lane labelled his S150). Marker lanes are as follows: nu, purified nucleoplasmin; his, chicken erythrocyte histones. Gels were stained with Coomassie blue. Sedimentation direction is from left to right.

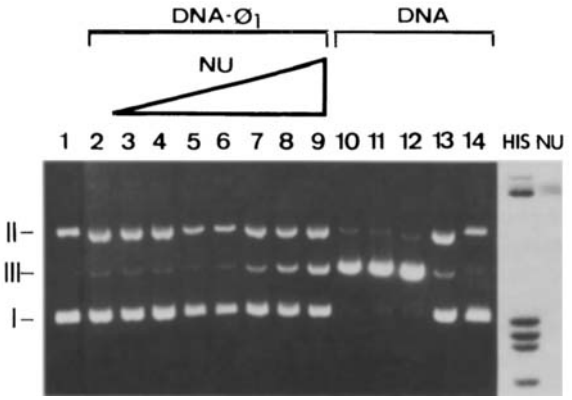

Fig. 7. Displacement of protein ϕ1 complexed to DNA by nucleoplasmin. The product of a standard reaction of supercoiled pUC19 DNA with protein ϕ1 at unit mass ratio was dispensed in eight identical aliquots containing 0.17 μg each reactant, to which the following amounts of purified nucleoplasmin were added: 0, 10, 20, 40, 100, 200, and 400 ng (lanes 2–9). After 4 h incubation, *Bam*HI (10 U/aliquot) was added and the samples digested for 2 h. Following restriction, all samples were deproteinized and the restricted DNA analyzed in 1% agarose gels containing ethidium bromide. For calibration, the following DNA markers were co-electrophoresed: 0.17 μg supercoiled pUC19 DNA (lanes 1 and 14); plasmid DNA digested with *Bam*HI in restriction buffer (lane 10) and in buffer A (lane 11); plasmid DNA restricted in the presence of 0.4 μg purified nucleoplasmin (lane 12), and with 0.17 μg protein ϕ1 (lane 13). The inset at the right shows reference proteins resolved in an acid/urea/polyacrylamide gel/chicken erythrocyte histones (His) and purified nucleoplasmin (Nu).

distinguishable from fraction 4 on. In addition, this latter set of gradient fractions contained a group of proteins resolving electrophoretically within the migration range of core histones and which, in turn, exhibited the typical light-scattering effect of histones on oblique illumination (Kleinschmidt et al., 1985).

To assess in a more direct way the behaviour of histones in the S150 extract, histone-containing fractions of the untreated extract sedimented in sucrose gradients were pooled (fractions 3–8, Fig. 6A) and extensively dialyzed. An aliquot was saved for subsequent electrophoretic analysis (penultimate lane, Fig. 6C) and the remainder heated at 80°C for 10 min, chilled on ice and centrifuged as described before. The ensuing heat-stable supernatant was next fractionated in a parallel sucrose gradient and the protein content electrophoretically analyzed (Fig. 6C). It can be clearly observed that no heat-stable protein is detectable across the gradient running in the position of nucleoplasmin, not even in those fractions containing thermostable proteins displaying histone-like mobility (Fig. 6C, fraction 2–8). Furthermore, nucleoplasmin was absent from the electrophoretic pattern corresponding to the aliquot of the untreated histone-containing pool from the fractionated S150 extract, which was spared the 80°C heating step. The slow-moving bands which sedimented in the lighter portion of the gradient upon heating of the S150 extract (fraction 4–8, Fig. 6B) might well arise from complexes containing nucleoplasmin in the untreated S150 extract sedimenting at higher S values (fractions 11–15, Fig. 6A), which become dissociated upon heating. The results of the hydrodynamic analyses suggest that the bulk of nucleoplasmin is not largely associated to histone molecules in the S150 extract.

Nucleoplasmin-mediated dissociation of DNA-ϕ1 complexes. To unambiguously ascertain whether purified nucleoplasmin could mimic the dissociation of plasmid DNA-ϕ1 complexes induced by the S150 extract, formed complexes were incubated with purified protein alone. Protein ϕ1 was allowed to react at unit mass ratio with supercoiled pUC19 DNA at room temperature. After 30 min incubation, the reaction mixture was dispensed in eight identical aliquots to which increasing amounts of purified nucleoplasmin were added (0–400 ng) and incubations carried out for 4 h at 37°C. After incubation, all samples were restricted with *Bam*HI to test the accessibility to the single restriction site in the plasmid DNA. Digests were then treated with Proteinase K and fully deproteinized with phenol prior to electrophoresis of the DNA (Fig. 7). The restriction analysis revealed a gradual trend of the supercoiled DNA complexed to protein ϕ1 to linearize with the increase in nucleoplasmin concentration (Fig. 7, lanes 3–9), whereas in the absence of added nucleoplasmin the superhelicity was undistinguishable from that of the supercoiled DNA control (compare Fig. 7, lanes 1 and 2). The extent of linearization was not complete but reached about 25% of the total mass of the complexed supercoiled DNA incubated with the highest amount of nucleoplasmin assayed (Fig. 7, lane 9). In contrast, plasmid DNA alone was easily digested by *Bam*HI under all conditions tested, except when protein ϕ1 was present at the onset of the restriction, which suppressed enzymatic activity (Fig. 7, lane 13). Thus, the endonuclease fully linearized naked DNA (Fig. 7, lane 10), even when digestion was performed with the buffer used in the assembly of the DNA-ϕ1 complex (lane 11). Likewise, the addition of nucleoplasmin during restriction did not interfere with the enzyme (Fig. 7, lane 12).

Since nucleoplasmin does not seem to bind to DNA to any extent and accounting for its ability to remove protein ϕ1 from the formed complex, as reflected in the cleared access of *Bam*HI to DNA (compare lanes 9 and 12 in Fig. 7), it can be reasonably presumed that nucleoplasmin seizes the displaced protein involving a reciprocal attachment comparable to the reported association with H2A and H2B in *Xenopus* egg extracts (Dilworth et al., 1987).

***In vitro* chromatin formation on DNA-ϕ1 complexes using nucleoplasmin and pure histones.** Several *in vitro* chromatin assembly systems essentially involving defined DNA templates and isolated histones have been developed. Such methods, however, used to work inefficiently and most often resort to unusual DNA structures (Stein and Bina, 1984; Stein and Mitchell, 1988) or fail to meet physiological ionic strength requirements (Axel et al., 1974; Germond et al., 1975). An additional drawback of these procedures is that nucleosomes become improperly packed with average repeats of 150 bp or even lower values, unlike the physiological spacings found in native chromatin. In contrast, the ability of purified nucleoplasmin to promote nucleosome assembly *in vitro* onto closed circular DNA plasmids from histones in the presence of DNA topoisomerase I (Earnshaw et al., 1980), provided a reference for assaying chromatin formation on formed DNA-ϕ1 complexes using purified nucleoplasmin, together with core histone octamers prepared from rat liver and DNA topoisomerase I, under physiological ionic strength.

Purified nucleoplasmin was mixed with core histone octamers extracted from rat hepatocytes in 0.2 M NaCl and incubated for 45 min. Simultaneously, relaxed pUC19 DNA was allowed to associate to protein ϕ1 at unit mass ratio for 30 min in the usual conditions. Components of both reactions were subsequently mixed and incubated further to 4 h after addition of DNA topoisomerase I. 10% of the incubated sample was set aside for topological analysis and the remainder subjected to MN digestion for various times. A mock assembly lacking complexed ϕ1 was prepared in parallel and processed in an identical manner. The respective topological state of the DNA in both

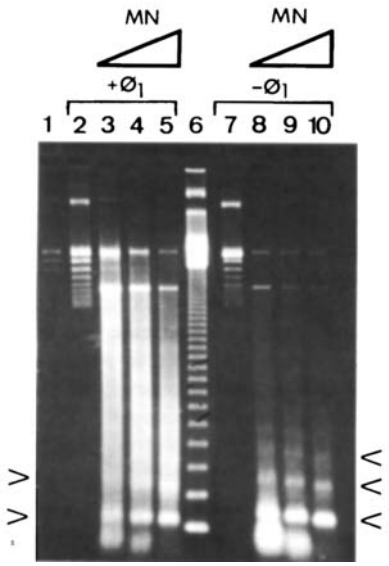

Fig. 8. Chromatin assembly on the DNA-ϕ1 complex by nucleoplasmin supplemented with histones. A mixture of purified nucleoplasmin (4.5 μg) and rat hepatocyte core histone octamers (2.24 μg) preincubated as described in Experimental Procedures, was pooled with a formed complex of relaxed pUC19 DNA with protein ϕ1 at unit mass ratio (1.5 μg each) and the final mixture incubated for 4 h with addition of calf thymus DNA topoisomerase I (10 U). A reference reaction was carried out in parallel in identical conditions, except that the template used was naked plasmid DNA. 10% of both reaction mixtures (0.15 μg DNA) was removed for analysis of DNA topology (lanes 2 and 7). The remainders of the two reactions were made 3 mM in $CaCl_2$ and subsequently digested with MN for 2, 8 and 16 min at room temperature. Following digestion, all samples were deproteinized and the DNA fragments resolved on a 1.5% agarose gel in TBE buffer. The left panel (lanes 3–5) shows DNA digestion patterns in order of increasing digestion times from chromatin assembled on the formed DNA-ϕ1 complex, whereas those from chromatin assembled on naked plasmid DNA without protein ϕ1 are shown on the right panel (lanes 8–10). Marker lanes 1 and 6 contained relaxed pUC19 DNA (0.15 μg) and a 123-bp ladder of DNA (Gibco-BRL), respectively. Open arrowheads at the sides point to the DNA oligomers produced by the partial MN digestions.

reconstituted minichromosomes was examined (Fig. 8, lanes 2 and 7). The incubated mixture of nucleoplasmin and core histone octamers was capable of assembling chromatin, regardless of the presence of protein ϕ1 during the reconstitution reactions. At each digestion interval, MN generated comparable sets of DNA fragments resolving electrophoretically into ladder-like nucleosomal patterns with an average repeat length of about 150 bp, thereby confirming previous work on *in vitro* chromatin reconstitutions facilitated by endogenous components isolated upon fractionation of whole cell extracts of *Xenopus* oocytes (Zucker and Worcel, 1990). This notwithstanding, the assembly process over naked DNA was more efficient than that carried in the presence of protein ϕ1 as inferred from the lower resolution of the corresponding MN ladders and their blurred appearance (Fig. 8, lanes 3–5), thus corroborating the tendency noted in chromatin reconstitutions on DNA-ϕ1 templates promoted by the *Xenopus* S150 extract already described.

DISCUSSION

Mode of interaction of sperm-specific protein ϕ1 with DNA. The assessment of the mode of association between the intermediate protamine ϕ1 from *M. edulis* sperm and pUC19 DNA indicates that the protein displays high affinity for DNA, yielding a closely packed nucleoprotein complex effectively shielded from MN access. On the basis of the progressive albeit limited linearization of complexed DNA produced upon DNase I digestion with time, it appears unlikely that the formation of the complex can be accounted for by a mere and unstructured aggregation due to a random binding of the highly basic protein to DNA. In contrast, it affords rather compelling evidence that the DNA in the complex may be conformationally organized around a core of protein, leaving preferentially exposed DNase I sensitive sites in a manner comparable to that of nucleosomal chromatin.

Dissociation of sperm protein ϕ1 complexed to DNA. At the onset of fertilization, a dramatic remodelling of the male germinal cell occurs entailing decondensation of chromatin through the displacement of sperm-specific basic proteins and/or protamines, and concurrent deposition onto DNA of maternal histones accumulated in the egg during the process of oogenesis. It has been shown that nucleoplasmin is necessary and sufficient to initiate decondensation of the homologous sperm chromatin and promote somatic-type chromatin assembly (Philpott et al., 1991; Philpott and Leno, 1992).

We have extended these reports examining the dissociation *in vitro* of formed complexes between the highly basic *Mytilus* sperm protein ϕ1 and pUC19 DNA by either the *Xenopus* oocyte S150 extract or nucleoplasmin purified therefrom. Our data with the S150 extract demonstrate that the heterologous DNA-ϕ1 complexes undergo full dissociation and that the henceforth denuded DNA is subsequently used to assemble nucleosomal chromatin regularly spaced. The dislodged protein ϕ1 does not appear to be bound to the ensuing minichromosome, as judged from the analysis of its protein composition, not even when the former is added to free DNA at the start of the assembly reaction. Dissociation of the DNA-ϕ1 complex is not arrested when purified nucleoplasmin substitutes for the whole S150 extract. However, the reaction progresses with a substantial loss of efficiency. About 25% of the complexed DNA undergoes linearization upon restriction with *Bam*HI in the presence of pure nucleoplasmin suggesting that the latter, besides removing sperm protein ϕ1 from the complex, tends to sequester the displaced protein. It is worth noting that ϕ1 completely inhibits restriction of DNA in the absence of nucleoplasmin. The higher efficiency of dissociation displayed by the S150 extract in comparison to that of purified nucleoplasmin can be accounted for assuming the existence in the whole extract of factor(s) potentiating the dissociation process mediated by nucleoplasmin. However, the eventual presence in the S150 extract of a protease activity specific for sperm proteins should not be excluded. This activity could gradually degrade sperm proteins once sequestered by nucleoplasmin upon removal from DNA, and hence keep nucleoplasmin-binding capacity unsaturated. This enzymic activity cannot be assigned to nucleoplasmin since no degradation of protein ϕ1 has been noticed in the *in vitro* assays performed (not shown).

Sedimentation behaviour of nucleoplasmin. The fractionation of *Xenopus* S150 extract by zonal centrifugation in linear sucrose gradients has confirmed previous observations indicating that nucleoplasmin is mostly free rather than bound to histones in this extract (Zucker and Worcel, 1990). In opposition, a 7S complex of nucleoplasmin with histones H2A and H2B has also been reported (Kleinschmidt et al., 1985). The most likely explanation for this dissimilarity may be tentatively attributed to the actual differences between the experimental conditions used in each case, such as substantially differing concentrations of the whole oocyte extract. Dissociation of the 7S complex at low concentrations of extract would be feasible under the extended

sedimentation conditions used here considering that histones are weakly bound to nucleoplasmin (Dilworth et al., 1987). In contrast, higher concentrations of extract would likely promote aggregation of the components, thereby favouring their cosedimentation. Our experiments do not permit to discriminate between different causes, nor they rule out the binding of nucleoplasmin to histones *in vivo*. Their association might be largely dependent on the relative concentrations of both components determined by the own concentration of the extract itself. Likewise, the activation of enzymic mechanisms such as phosphorylation might modify native molecules rendering them associable. There is evidence that extracts from unfertilized eggs are more active in assembling chromatin than those from oocytes (Cotten et al., 1987). In analogy, the more highly phosphorylated forms of nucleoplasmin are also more efficient for assembling histones on DNA than unphosphorylated molecules. The former are more abundant in unfertilized eggs than in oocytes (Sealy et al., 1986). The different forms of nucleoplasmin found *in vivo* might well be the reason why its association with histones is elusive in the oocyte as compared to unfertilized eggs.

Nucleosomal-like assembly with purified components. Our *in vitro* assays of chromatin formation on formed DNA-ϕ1 complexes using purified nucleoplasmin and isolated rat liver core histone octamers have shown that the sperm protein ϕ1 undergoes displacement from its complex with DNA. Concomitantly, nucleosome structures are assembled on the denuded DNA as revealed by digestions with MN despite the presence of protein ϕ1. These results confirm the ability of nucleoplasmin to dissociate the DNA-ϕ1 complex and the capacity, when complemented with octameric histones, to lay them down in the freed DNA leading to formation of nucleosomes. Likewise, the DNA fragments of about 150 bp and multiples thereof generated by MN digestion, argue that the *in vitro* chromatin reconstitution yields nucleosomal structures packed together very closely. It can be also inferred that nucleoplasmin and core histone octamers together fail to form nucleosomes spaced at the physiological periodicities of most cellular chromatins. This inference is consistent with similar observations concerning *in vitro* chromatin reconstitution studies using fractionated components (Zucker and Worcel, 1990).

Our data demonstrate that the heterologous DNA-ϕ1 complex is rearranged on incubation either with the oocyte S150 extract or nucleoplasmin complemented with octameric histones, leading to the assembly of nucleosomal particles as deduced from nuclease digestions. Protein analysis has revealed that the dissociated sperm protein does not become bound to the resulting minichromosome nor it is degraded by nucleoplasmin. A lesser efficiency of nucleosome formation over the DNA-ϕ1 complex has been observed in both types of nucleosome assembly reactions conducted, in comparison to the extent of asssembly on naked DNA. This lower strength in chromatin reconstitution can be accounted for, provided that the basic properties of protein ϕ1 together with the results of protein analysis are considered. It is conceivable that nucleoplasmin at least transiently associates with the sperm protein during the removal from DNA. The high affinity of protein ϕ1 for DNA due to its strong basicity can be expected to undermine the sequestration capacity of nucleoplasmin and consequently disturb to some extent the process of dissociation. The nuclease digestion patterns of chromatin reconstituted on DNA-ϕ1 complexes lend support to this explanation.

The evidence reported here concerning the dissociation of the heterologous complex of pUC19 DNA and *Mytilus* sperm protein ϕ1 promoted by nucleoplasmin and the coupled formation of chromatin upon exogenous core histone supplementation or mediation of oocyte S150 extract, is in close agreement with the suggestion that nucleoplasmin is instrumental in the remodelling of sperm chromatin. This proposal is based on the findings that this protein facilitates the removal of *Xenopus* sperm-specific proteins from the homologous sperm DNA, as well as the concerted deposition of histones to assemble somatic-type chromatin (Philpott and Leno, 1992). Our demonstration that the role of nucleoplasmin can be closely reproduced in an heterologous complex such that formed by sperm-specific protein ϕ1 with a plasmid DNA, argues for the existence of a general mechanism to effect the coupled processes of decondensation of the sperm nucleus and reconstitution of somatic chromatin at fertilization.

This work was supported in part by grant PB94-0042 from the Spanish *Dirección General de Investigación Científica y Técnica* and by the NATO Scientific Affairs Division grant CRG93-1146 (to Luís Cornudella). We thank E. Jiménez for kindly providing core histone octamers prepared from rat liver hepatocytes. Simón A. Ruiz-Lara was a recipient of a fellowship for Foreign Scholars from the Ministry of Education and Science of Spain.

REFERENCES

Ausio, J. (1986) Structural variability and compositional homology of the protamine-like components of the sperm from the bivalve molluscs, *Comp. Biochem. Physiol. 85B*, 439–449.

Ausio, J. & Subirana, J. A. (1982) Nuclear proteins and the organization of chromatin in spermatozoa of *Mytilus edulis*, *Exptl Cell Res. 141*, 39–45.

Axel, R., Melchior, W., Sollner-Webb, B. & Felsenfeld, G. (1974) Specific sites of interaction between histones and DNA in chromatin, *Proc. Natl Acad. Sci. USA 71*, 4101–4105.

Berrios, M. & Avilion, A. A. (1990) Nuclear formation in a *Drosophila* cell-free system, *Exptl Cell Res. 191*, 64–70.

Bloch, D. P. (1976) Histones of sperm, in *Handbook of genetics* (King, R. C., ed.) vol. 5, pp. 139–149, Plenum Press, New York.

Cotten, M. & Chalkley, R. (1987) Purification of a novel, nucleoplasmin-like protein from somatic nuclei, *EMBO J. 6*, 3945–3954.

Dilworth, S. M. & Dingwall, C. (1988) Chromatin assembly *in vitro* and *in vivo*, *BioEssays 9*, 44–49.

Dilworth, S. M., Black, S. J. & Laskey, R. A. (1987) Two complexes that contain histones are required for nucleosome assembly *in vitro*: role of nucleoplasmin and N1 in *Xenopus* egg extracts, *Cell 51*, 1009–1018.

Earnshaw, W. C., Honda, B. M., Laskey, R. A. & Thomas, J. O. (1980) Assembly of nucleosomes: the reaction involving *X. laevis* nucleoplasmin, *Cell 21*, 373–383.

Germond, J. E., Hirt, B., Oudet, P., Gross-Bellard, M. & Chambon, P. (1975) Folding of the DNA double helix in chromatin-like structures from simian virus SV40, *Proc. Natl Acad. Sci. USA 72*, 1843–1847.

Glikin, G., Ruberti, I. & Worcel, A. (1984) Chromatin assembly in *Xenopus* oocytes: *in vitro* studies, *Cell 37*, 33–41.

Ishimi, Y., Hirosumi, J., Sato, W., Sugasawa, K., Yokota, S., Hanaoka, F. & Yamada, M. (1984) Purification and initial characterisation of a protein which facilitates assembly of nucleosome-like structures from mammalian cells, *Eur. J. Biochem. 142*, 431–439.

Itoh, T., Ohsumi, K. & Katagiri, C. (1993) Remodelling of human sperm chromatin mediated by nucleoplasmin from amphibian eggs, *Dev. Growth Differ. 35*, 59–66.

Kasinsky, H. E. (1995) Evolution and origins of sperm basic proteins, in *Advances in spermatozoal taxonomy and phylogeny* (Jamieson, B. G. M., Ausio, J. & Justine, J. L., eds) pp. 463–473.

Kleinschmidt, J. A., Fortkamp, E., Krohne, G., Zentgraf, H. & Franke, W. W. (1985) Co-existence of two different types of soluble histone complexes in nuclei of *Xenopus laevis* oocytes, *J. Biol. Chem. 260*, 1166–1176.

Laskey, R. A. & Earnshaw, W. E. (1980) Nucleosome assembly, *Nature 286*, 763–767.

Laskey, R. A., Honda, B. M., Mills, A. D. & Finch, J. T. (1978) Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA, *Nature 275*, 416–420.

Longo, F. J. (1985) *Biology of fertilization*, vol. 3, Academic Press, New York.

Longo, F. J. & Kunkle, M. (1978) Transformations of sperm nuclei upon insemination, *Curr. Topics Dev. Biol. 12*, 149–184.

Mills, A. D., Laskey, R. A., Black, P. & De Robertis, E. M. (1980) An acidic protein which assembles nucleosomes *in vitro* is the most abundant protein in *Xenopus* oocyte nuclei, *J. Mol. Biol. 139*, 561–568.

Ohsumi, K. & Katagiri, C. (1991) Characterization of the ooplasmic factor inducing decondensation of and protein removal from toad sperm nuclei: involvement of nucleoplasmin, *Dev. Biol. 148*, 295–305.

Oliva, R. & Dixon, G. H. (1991) Vertebrate protamine genes and the histone-to-protamine replacement reaction, *Prog. Nucleic Acid Res. Mol. Biol. 40*, 25–93.

Philpott, A. & Leno, G. H. (1992) Nucleoplasmin remodels sperm chromatin in *Xenopus* egg extracts, *Cell 69*, 759–767.

Philpott, A., Leno, G. H. & Laskey, R. A. (1991) Sperm decondensation in *Xenopus* egg cytoplasm is mediated by nucleoplasmin, *Cell 65*, 569–578.

Poccia, D. (1986) Remodelling of nucleoproteins during gametogenesis, fertilization and early development, *Int. Rev. Cytol. 105*, 1–65.

Ruiz-Lara, S., Prats, E., Casas, M. T. & Cornudella, L. (1993) Molecular cloning and sequence of a cDNA for the sperm-specific protein ϕ1 from the mussel *Mytilus edulis*, *Nucleic Acids Res. 21*, 2774.

Sapp, M. & Worcel, A. (1990) Purification and mechanism of action of a nucleosome assembly factor from *Xenopus* oocytes, *J. Biol. Chem. 265*, 9357–9365.

Schmidt-Zachman, M. S., Hügle-Dörr, B. & Franke, W. W. (1987) A constitutive nucleolar protein identified as a member of the nucleoplasmin family, *EMBO J. 6*, 1881–1890.

Sealy, L., Cohen, M. & Chalkley, R. (1986) *Xenopus* nucleoplasmin: egg vs. oocyte, *Biochemistry 25*, 3064–3072.

Shimamura, A., Tremethick, D. & Worcel, A. (1988) Characterization of the repressed 5S DNA minichromosomes assembled *in vitro* with a high-speed supernatant of *Xenopus laevis* oocytes, *Mol. Cell. Biol. 8*, 4257–4269.

Shimamura, A., Sapp, M., Rodríguez-Campos, A. & Worcel, A. (1989) Histone H1 represses transcription from minichromosomes assembled *in vitro*, *Mol. Cell. Biol. 9*, 5573–5584.

Stein, A. & Bina, M. (1984) A model chromatin assembly system: factors affecting nucleosome spacing, *J. Mol. Biol. 178*, 341–363.

Stein, A. & Mitchell, M. (1988) Generation of different nucleosome spacing periodicities *in vitro*. Possible origin of cell type specificity, *J. Mol. Biol. 203*, 1029–1043.

Stillman, B. (1986) Chromatin assembly during SV40 DNA replication *in vivo*, *Cell 45*, 555- 565.

Subirana, J. A. (1983) Nuclear proteins in spermatozoa and their interaction with DNA, in *The sperm cell* (André, J., ed.) pp. 197–213, Martinus Nijhoff, The Hague.

Ulitzur, N. & Gruenbaum, Y. (1989) Nuclear envelope assembly around sperm chromatin in cell-free preparations from *Drosophila* embryos, *FEBS Lett. 259*, 113–116.

Wray, W., Boulikas, T., Wray, V. P. & Hancock, R. (1981) Silver staining of proteins in polyacrylamide gels, *Anal. Biochem. 118*, 197–203.

Worcel, A., Han, S. & Wong, M. L. (1978) Assembly of newly replicated chromatin, *Cell 15*, 969–977.

Zucker, K. & Worcel, A. (1990) The histone H3/H4-N1 complex supplemented with histones H2A-H2B dimers and DNA topoisomerase I forms nucleosomes on circular DNA under physiological conditions, *J. Biol. Chem. 265*, 14487–14496.