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Nucleosome assembly *in vitro*: separate histone transfer and synergistic interaction of native histone complexes purified from nuclei of *Xenopus laevis* oocytes

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High speed supernatants of *Xenopus laevis* oocyte nuclei efficiently assemble DNA into nucleosomes *in vitro* under physiological salt conditions. The assembly activity cofractionates with two histone complexes composed of the acidic proteins N1/N2 in complex with histones H3 and H4, and nucleoplasmin in complex with histones H2B and H2A. Both histone complexes have been purified and their nucleosome assembly activities have been analysed separately and in combination. While the histones from the N1/N2 complexes are efficiently transferred to DNA and induce supercoils into relaxed circular plasmid DNA, the nucleoplasmin complexes show no supercoil induction, but can also transfer their histones to DNA. In combination, the complexes act synergistically in supercoil induction thereby increasing the velocity and the number of supercoils induced. Electron microscopic analysis of the reaction products shows fully packaged nucleoprotein structures with the typical nucleosomal appearance resulting in a compaction ratio of 2.8 under low ionic strength conditions. The high mobility group protein HMG-1, which is also present in the soluble nuclear homogenate from *X.laevis* oocytes, is not required for nucleosome core assembly. Fractionation experiments show that the synergistic effect in the supercoiling reaction can be exerted by histones H3 and H4 bound to DNA and the nucleoplasmin complexes alone. This indicates that it is not the synchronous action of both complexes which is required for nucleosome assembly, but that their cooperative action can be resolved into two steps: deposition of H3 and H4 from the N1/N2 complexes onto the DNA and completion of nucleosome core formation by addition of H2B and H2A from the nucleoplasmin complexes.

Key words: nuclear proteins/histone complexes/chromatin assembly/nucleosome assembly factors

Introduction

Histones and DNA contain the information necessary for nucleosome core formation in a self-assembly reaction, provided that the assembly pathway is carefully controlled (for review, see Laskey and Earnshaw, 1980; Dilworth and Dingwall, 1988). This control includes the promotion of correct histone-histone and histone-DNA interactions in order to minimize the formation of non-specific insoluble aggregates. Such interactions can be promoted in the purified histone-DNA system by salt gradient dialysis (Axel *et al.*, 1974; Oudet *et al.*, 1975), by slow addition of renatured histone pairs to an excess of DNA at near physiological ionic strength (Ruiz-Carillo *et al.*, 1979) or by the addition of polyglutamic acid, which probably shields the positive charges of the histones (Stein *et al.*, 1979).

A number of different factors derived from cellular extracts have been characterized which also mediate nucleosome core assembly when added to purified histones and DNA at physiological salt concentrations. These assembly factors include nucleoplasmin (Laskey *et al.*, 1978a), a certain RNA (Nelson *et al.*, 1981), high mobility group protein 1 (HMG-1) (Bonne-Andrea *et al.*, 1984), a nucleoplasmin-like protein from *Xenopus* tissue culture cells (nucleoplasmin S; Cotten and Chalkley, 1987) and a 53 kd protein from mammalian cells (Ishimi *et al.*, 1983). Recently, Smith and Stillman (1989) described an assembly factor, derived from a nuclear extract, which promotes nucleosome assembly on replicating SV40 DNA using the soluble histones provided in the cytosolic replication extract.

In an alternative approach, the soluble non-chromatin bound histones present in oocyte- or egg-homogenates have been used for chromatin reconstitution *in vitro* (Laskey *et al.*, 1977; Nelson *et al.*, 1979; Glikin *et al.*, 1984; for review see Dilworth and Dingwall, 1988). When examining the organization of the soluble histones present in *Xenopus* oocyte nuclei, we observed that the non-chromatin bound histones are stored in two defined complexes with acidic non-histone proteins (Kleinschmidt and Franke, 1982; Kleinschmidt *et al.*, 1985). Histones H3 and H4 are associated with proteins N1/N2, whereas histones H2A and H2B are in a complex with nucleoplasmin. The primary structure of both histone binding proteins contains extended acidic domains, which are probably involved in the interaction with the histones (Kleinschmidt *et al.*, 1986, 1988; Bürglin *et al.*, 1987; Dingwall *et al.*, 1987). HMG-1, which also contains an acidic domain (Walker *et al.*, 1980), is not associated with the soluble histones, although it is stored in the oocyte nuclei in a similar pool to the histones (Kleinschmidt *et al.*, 1983). The histones derived from the two histone complexes perform nucleosome assembly with plasmid DNA at physiological ionic strength in a partially fractionated nuclear extract (Kleinschmidt *et al.*, 1985). Dilworth *et al.* (1987) identified these histone complexes in *Xenopus* eggs and showed by immunodepletion that the complexes are required for nucleosome assembly in *Xenopus* egg homogenates. In the present study we describe the purification of the two histone complexes from *Xenopus* oocyte nuclei and their interaction with DNA separately and in combination. Both complexes are able to transfer their histones independently to the DNA and they show a strong synergism when they are present together in the assembly reaction. The steps of cooperation between the complexes have been analysed. HMG-1 is not required for nucleosome core formation in this system.

Results

Fractionation of the nucleosome assembly activity in nuclear supernatants of X.laevis oocytes

Fractionation of a high speed supernatant of *Xenopus* oocyte nuclei by sucrose gradient centrifugation and analysis of the fractions for their nucleosome assembly activity showed that this activity co-fractionates with two proteins, which we have previously shown to be associated with the stored histones (Figure 1; Kleinschmidt *et al.*, 1985). Proteins N1/N2 present in fractions 4–10 (Figure 1a and c, arrowheads) are associated with the histones H3 and H4 (Figure 1a, dots), whereas nucleoplasmin (Figure 1a and c, open circles) is complexed with histones H2B and H2A (Figure 1a, dots). The two complexes overlap in fractions 7–10. Nucleosome assembly activity, however, measured as supercoiling of a relaxed circular plasmid DNA (Figure 1b), is not restricted to those fractions but is also observed in the fractions containing only N1/N2 and histones H3 and H4 (Figure 1b, fractions 4 and 6). No DNA supercoiling was induced by any of the other fractions. In order to analyse whether the histone complexes alone perform the nucleosome assembly reaction or if this process requires other factors, we have purified both complexes by preparative methods.

Purification and characterization of the histone complexes

The critical step in the purification of the two histone complexes was their separation under native conditions. This was achieved by reverse $(NH_4)_2SO_4$ gradient chromatography on Sepharose B6, where both complexes elute at different $(NH_4)_2SO_4$ concentrations (Figure 2a, lane 2; 2b, lane 2). The nucleoplasmin complexes elute as a highly enriched single peak at the beginning of the gradient (Figure 2b, lane 2) and can be further purified by sucrose gradient centrifugation (Figure 2b, lane 3). The centre peak fractions of the complexes are electrophoretically homogeneous (Figure 2b, lane 4), whereas the nucleoplasmin pool fraction contains a minor amount of HMG-1 (Figure 2b, lane 3, compare with 2c), which was identified by two-dimensional tryptic peptide mapping of the excised polypeptide band (data not shown). The polypeptide of 68 kd in the nucleoplasmin pool fraction (Figure 2b, lane 3) is bovine serum albumin (BSA), which was used to coat the collodium bags in which the complexes were concentrated after purification. The nucleoplasmin complexes contain three major histone polypeptides as was expected from immunoprecipitation studies with nucleoplasmin antibodies (Kleinschmidt *et al.*, 1985; Dilworth *et al.*, 1987). Histones H2B and H2A co-migrate in SDS–PAGE with the respective calf thymus histones (Figure 2b) and give nearly identical two-dimensional tryptic fingerprints in comparison with calf thymus histones (data not shown). The prominent component slightly above the position of calf thymus histone H3 (Figure 2b, denoted H2A.X; designated H3* in Kleinschmidt *et al.*, 1985) behaves similarly to H2A in acid–urea–Triton gel electrophoresis (Dilworth *et al.*, 1987; our own observation) and produces a tryptic peptide map which is similar, although not identical to histone H2A from calf thymus (data not shown). This histone therefore probably represents a replacement variant of H2A designated H2A.X (West and Bonner, 1980).

The N1/N2-containing fractions obtained by the reverse ammonium sulphate gradient chromatography (Figure 2a, lane 2) was further purified by gel filtration on Superose 6 (Figure 2a, lane 3) and DEAE anion exchange chromatography (Figure 2a, lane 4). If necessary, minor impurities could be removed by further chromatography on a Mono

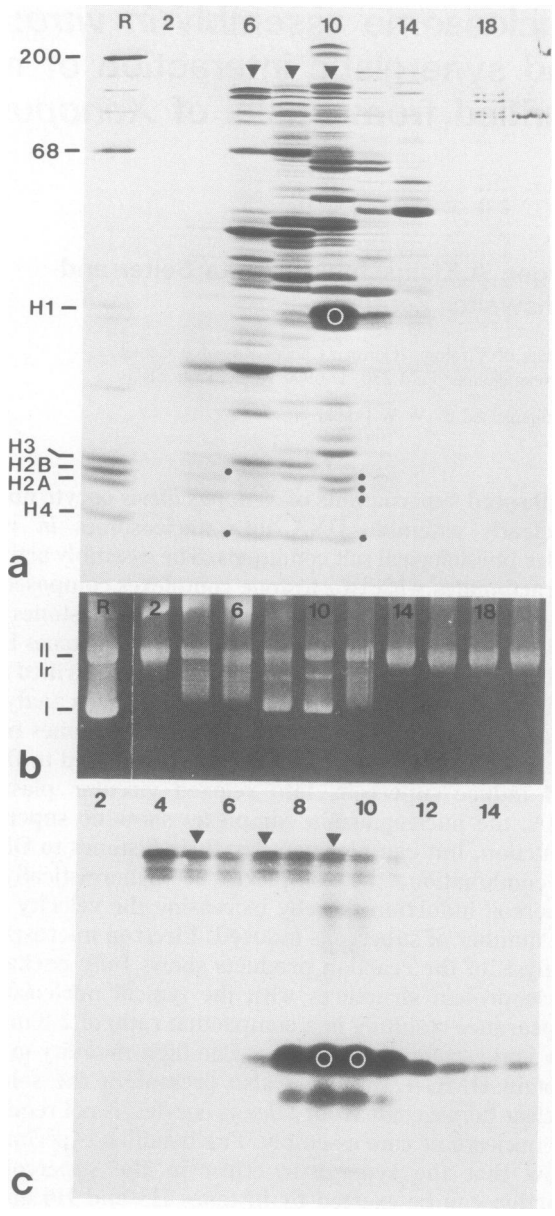

Fig. 1. Co-fractionation of histone binding proteins N1/N2 and nucleoplasmin with the nucleosome assembly activity in nuclear extracts of *X.laevis* oocytes. Total proteins of a 100 000 *g* supernatant of nuclei of *X.laevis* oocytes were fractionated by sucrose gradient centrifugation (see Materials and methods) and analysed by SDS–PAGE and Coomassie blue staining (**a**) for induction of DNA supercoiling (**b**), and by immunoblotting with antibodies against N1/N2 and nucleoplasmin (**c**). Positions of polypeptides N1/N2 (▼), nucleoplasmin (○) and the histones (dots) are indicated. Lane R shows the reference proteins myosin (200 kd), BSA (68 kd) and calf thymus histones. The nucleosome assembly activity shown in (b) was measured as supercoiling of relaxed circular plasmid DNA by an aliquot of the same sucrose gradient fractions as shown in (a). The resultant DNA was extracted, separated on 1.2% agarose gels and stained with ethidium bromide. The position of form I (fully supercoiled), form II (nicked circular) and form I_r (closed circular, relaxed) DNA is indicated at the left side of lane R, which contained supercoiled reference plasmid DNA. The sedimentation positions of proteins N1/N2 (▼) and nucleoplasmin (○) were determined in a parallel gradient by immunoblotting with antibodies specific for N1/N2 and nucleoplasmin (c). Fraction numbers are indicated at the top of the lanes.

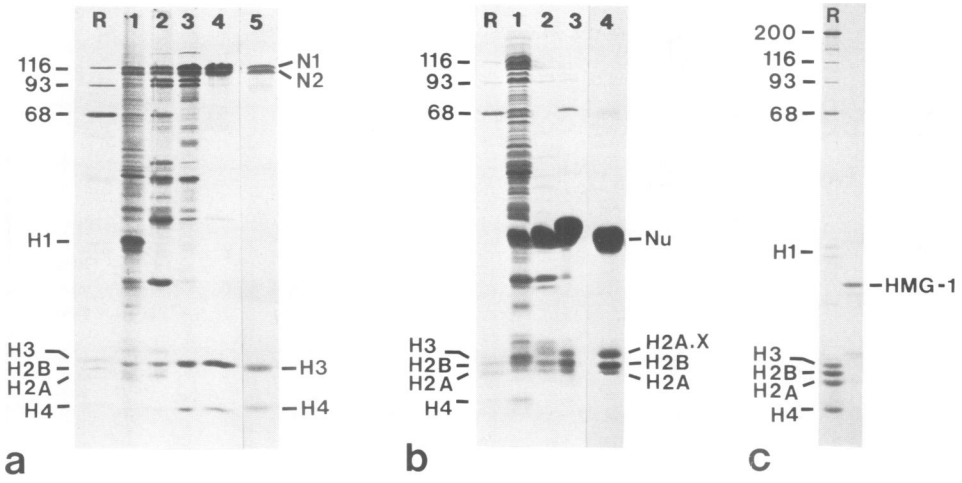

Fig. 2. Polypeptide composition of fractions obtained at different purification steps of the histone complexes from *Xenopus* oocyte nuclei. The polypeptides present in fractions obtained during purification of the N1/N2−histone complexes are shown in (**a**). Lane 1, nuclear 100 000 *g* supernatant; lane 2, pool fraction after reverse $(NH_4)_2SO_4$ gradient chromatography; lane 3, pool fraction after Superose 6 chromatography; lane 4, pool fraction after DEAE−Sephacel chromatography; lane 5, single fraction obtained by chromatography of a Superose 6 pool fraction on a Mono Q column. The polypeptide composition of fractions obtained during purification of the nucleoplasmin complexes is shown in (**b**; Nu, nucleoplasmin). Lane 1, nuclear 100 000 *g* supernatant; lane 2, pool fraction after reverse ammonium sulphate gradient chromatography; lane 3, pool fraction after sucrose gradient centrifugation; lane 4, single peak fraction obtained by sucrose gradient centrifugation as shown in lane 3. The HMG-1 containing pool fraction separated from the nucleoplasmin complexes by sucrose gradient centrifugation is shown (**c**). Polypeptides were separated by polyacrylamide gel electrophoresis according to Thomas and Kornberg (1975) and stained by Coomassie blue. Reference proteins (lanes R) are myosin (200 kd), β-galactosidase (116 kd), phosphorylase a (93 kd), BSA (68 kd) and calf thymus histones. The polypeptide band of 68 kd seen in (b) lane 3 is BSA which enters the preparation during the final concentration step (see Materials and methods).

Q column (Figure 2a, lane 5). The purified complexes contained only histones H3 and H4 in association with proteins N1 and N2 (Figure 2a, lanes 4, 5; Figure 3a, lane 1). We always recovered both polypeptides N1 and N2 in the histone complex preparations in agreement with our immunoprecipitation results (Kleinschmidt *et al.*, 1985). The polypeptides of 43 and 50 kd repeatedly found in immunoprecipitates with the N1/N2 antibodies (Kleinschmidt *et al.*, 1985) were separated from the histone complexes during the purification procedure. Since they were also not recovered by crosslinking of the complexes of intact nuclei (Kleinschmidt *et al.*, 1985), we believe that they are not tightly associated with the complexes and probably represent unspecific co-precipitates.

The HMG-1 containing fraction is separated from the nucleoplasmin complexes by sucrose gradient centrifugation (Figure 2c). This fraction contains in addition several low mol. wt non histone polypeptides.

To determine the stoichiometry of the histones and the binding proteins in the complexes we have also prepared histone free binding proteins by ion exchange chromatography in the presence of 4 M urea in the case of the N1/N2 complexes (Figure 3a), and by binding of the histones to DNA−cellulose in the case of the nucleoplasmin complexes (Figure 3d). Since we were unable to determine differences in mol. wt between the complexes and the free binding proteins using gel filtration and sedimentation velocity analysis we performed crosslinking with 1% formaldehyde (Jackson, 1978).

Comparison of the results of crosslinking experiments of purified N1/N2 histone complexes with those of histone free N1/N2 showed crosslink products of the complexes between 130 and 150 kd (Figure 3b, lane 1) which are not formed with the histone free N1/N2 (Figure 3b, lane 2). These correspond to the values obtained by crosslinking of the complexes in intact nuclei (not shown). Assuming an association of both histones H3 and H4 together with N1 or N2, they could perform a $(H3)_2(H4)_2$ tetramer with the binding protein. If, however, they associate separately with the binding protein N1 or N2 one can calculate a 1:2 to 1:4 stoichiometry of N1 or N2 to histone H4 and a stoichiometry of 1:1 to 1:3 for N1 or N2 to histone H3. A very large product of ~380 kd was also observed (Figure 3b) and may result from N1/N2 aggregations, which are also observed in sedimentation experiments (Figure 1c). All crosslink products contain histones as shown by the cleavage of the crosslink products and second dimension gel electrophoresis (Figure 3c).

The crosslinking products of the nucleoplasmin histone complexes reveal a relative mol. wt difference of ~10 000 in comparison to the histone free nucleoplasmin pentamer (Figure 3e) which suggests a molar histone:nucleoplasmin-pentamer ratio of 1:1, although a 2:1 ratio cannot be excluded. Second dimension analysis of the crosslink products after cleavage showed that the crosslinked complexes contained histones H2B, H2A and H2A.X (Figure 3f).

Nucleosome assembly activity of the purified histone complexes

Interaction of the separately purified histone complexes with DNA was tested in a DNA supercoiling assay and by DNA retardation using non-denaturing gel electrophoresis. As shown in Figure 4a (lanes 1−3) the purified N1/N2 histone complexes are able to transfer the histones H3 and H4 to the DNA as measured by supercoil induction into a relaxed circular plasmid DNA. This result confirms our earlier observation with crude preparations, that the N1/N2 complexes can form nucleosome-like structures at physiological ionic strength without the aid of nucleoplasmin

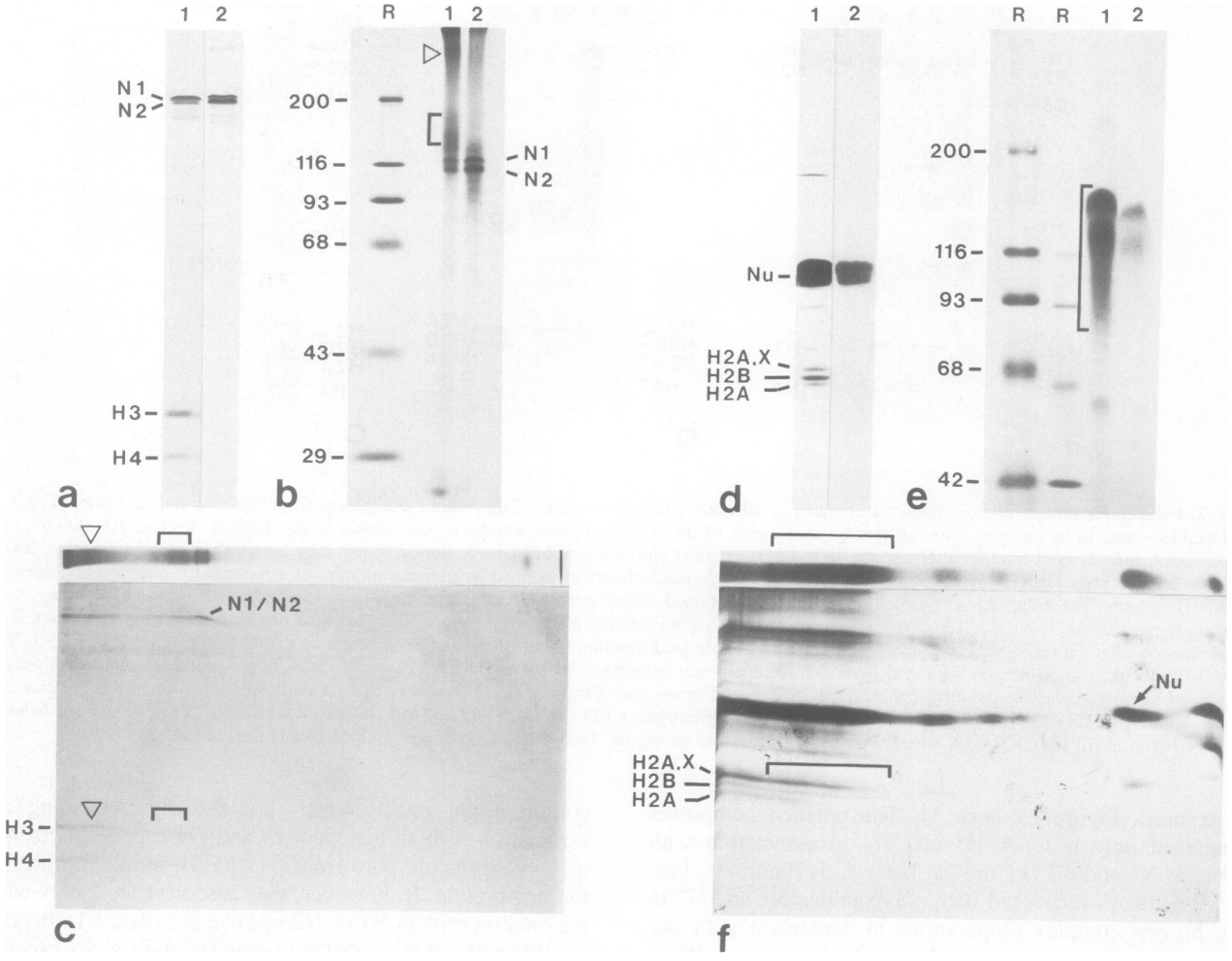

Fig. 3. Estimation of the size of the purified histone complexes by chemical crosslinking. Polypeptides N1/N2 were purified in complex with histones H3 and H4 (**a**, lane 1) and without histones (a, lane 2; both SDS−PAGE; 18% polyacrylamide, Coomassie blue staining) and used for chemical crosslinking with formaldehyde (**b**, lane 1 with histones; lane 2, without histones; SDS−PAGE; 12% polyacrylamide, Coomassie blue staining). The histone content of the crosslinking products shown in (b) lane 1 (⌐¬ and ▽) is demonstrated by the second dimension analysis, after cleavage of the crosslinks (**c**, SDS−PAGE, 18% polyacrylamide, Coomassie blue staining). The first dimensional gel used for cleavage of the crosslinks and the second dimension analysis is aligned in the upper part of (c). Similarly, nucleoplasmin (Nu) was purified with and without histones (**d**, lanes 1 and 2; SDS−PAGE, 18% polyacrylamide; Coomassie blue staining) and used for chemical crosslinking with formaldehyde (**e**, lane 1 with histones, lane 2 without histones; SDS−PAGE; 12% polyacrylamide; Coomassie blue staining). The crosslinking products of a parallel experiment with nucleoplasmin containing histones (⌐¬) was analysed by second dimension gel electrophoresis after cleavage of the crosslinks (**f**; SDS−PAGE; 18% polyacrylamide silver stained gel). The top lane in (f) shows the first dimensional gel used for the two dimensional analysis. Reference proteins (lanes R) are myosin (200 kd), β-galactosidase (116 kd), phosphorylase a (93 kd), BSA (68 kd), actin (43 kd) and carbonic anhydrase (29 kd). The polypeptide band above nucleoplasmin in (d) lane 1 is BSA which enters the complex preparation during the final concentration step (see Materials and methods).

(Kleinschmidt *et al.*, 1985; see also Figure 1). A band shift assay with purified mononucleosomal DNA supports the interpretation that histones H3 and H4 are efficiently transferred from the N1/N2 complexes to the DNA (Figure 4c, lane 2). Two-dimensional analysis of the band shift gels confirmed that the retardation resulted from the deposition of the histones onto the DNA (not shown). In addition, analysis of *in vitro* assembled minichromosomes shows that N1/N2 and nucleoplasmin do not bind to DNA (Dilworth *et al.*, 1987; Shimamura *et al.*, 1988; our own observation).

In contrast, the nucleoplasmin complexes containing histones H2B and H2A are not able to induce supercoiling of DNA (Figure 4a, lanes 4−6). A weak supercoiling activity found in the pool fraction of nucleoplasmin complexes (Figure 4b, lanes 1−3) is due to the residual amount of HMG-1 present in these fractions (see Figure 2b, lane 3). Addition of the separated HMG-1 fraction (Figure 2c) to the centre peak fraction of nucleoplasmin (Figure 2b, lane 4) gives the same weak supercoiling (Figure 4b, lanes 4−6). This supercoiling was also generated by the HMG-1 fraction alone without nucleoplasmin and histones (not shown), which is in agreement with earlier observations (Javaherian *et al.*, 1978; Bonne *et al.*, 1980). However, histone transfer from the nucleoplasmin complexes to DNA alone can be demonstrated by the band shift assay (Figure 4c, lane 1). Consistently the two retardation complexes seen in Figure 4c, lane 1, were observed after incubation of the DNA with the nucleoplasmin complexes and occasionally a rather weak band shift product was also seen slightly above the position of the mononucleosomal DNA (Figure 4c, lanes

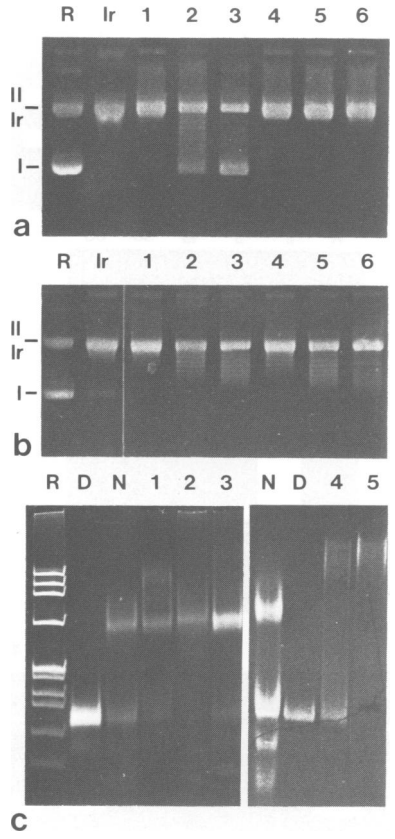

Fig. 4. Histone transfer to DNA from single purified histone complexes as measured by DNA supercoiling and a DNA mobility shift assay. The purified histone complexes were incubated with relaxed circular plasmid DNA (I_r) and the products of the supercoiling reaction were separated by agarose gel electrophoresis (1.2%) and stained with ethidium bromide. (**a**) Lanes 1–3, N1/N2 histone complexes at different histone to DNA ratios (lane 1, 0.4:1; lane 2, 2:1; lane 3, 4.8:1). Lanes 4–6, nucleoplasmin histone complexes (peak fraction, see Figure 2b, lane 4) at different histone to DNA ratios (lane 4, 0.3:1; lane 5, 1.6:1; lane 6, 4:1). (**b**) Lanes 1–3, nucleoplasmin histone complexes (pool fraction, see Figure 2b, lane 3) containing minor amounts of HMG-1 incubated with relaxed plasmid DNA at different histone to DNA ratios (lane 1, 0.6:1; lane 2, 1.5:1; lane 3, 3:1). Lanes 4–6 show nucleoplasmin histone complexes (peak fraction, see Figure 2b, lane 4) to which HMG-1 has been added in a quantity similar to the histones and incubated at different histone to DNA ratios (lane 4, 0.6:1; lane 5, 1.6:1; lane 6, 3.3:1). Lanes R show fully supercoiled reference DNA. The migration positions of form I, form I relaxed and form II DNA are indicated on the left. (**c**) The mobility shifts induced by incubation of the histone complexes (lanes 1–3) and HMG-1 (lanes 4 and 5) with mononucleosomal DNA (D) in comparison with purified nucleosome cores (N, upper band) after gel electrophoresis under non-denaturing conditions. Lane 1, nucleoplasmin complexes (pool fraction; histone:DNA ratio 3:1), lane 2, N1/N2 histone complexes (histone:DNA ratio 2:1); lane 3, N1/N2 complexes plus nucleoplasmin complexes (histone:DNA ratio 1:1); lanes 4 and 5, HMG-1 fraction (HMG-1:DNA ratio of 1:1 and 2:1). R: mol. wt standard: ϕX174 digested with *Hae*III.

D). Addition of HMG-1 to the nucleoplasmin complexes results in a different band shift pattern, which is similar to that produced by HMG-1 alone (Figure 4c, lanes 4 and 5).

Addition of both histone complexes together to relaxed circular plasmid DNA at increasing concentrations progressively increased the supercoil induction (Figure 5a). The nucleoprotein complexes formed after a 2 h incubation of the histone complexes with the DNA at physiological salt concentrations, and a histone:DNA ratio of 3.3:1 (Figure 5a, lane 6) were prepared for electron microscopy by the

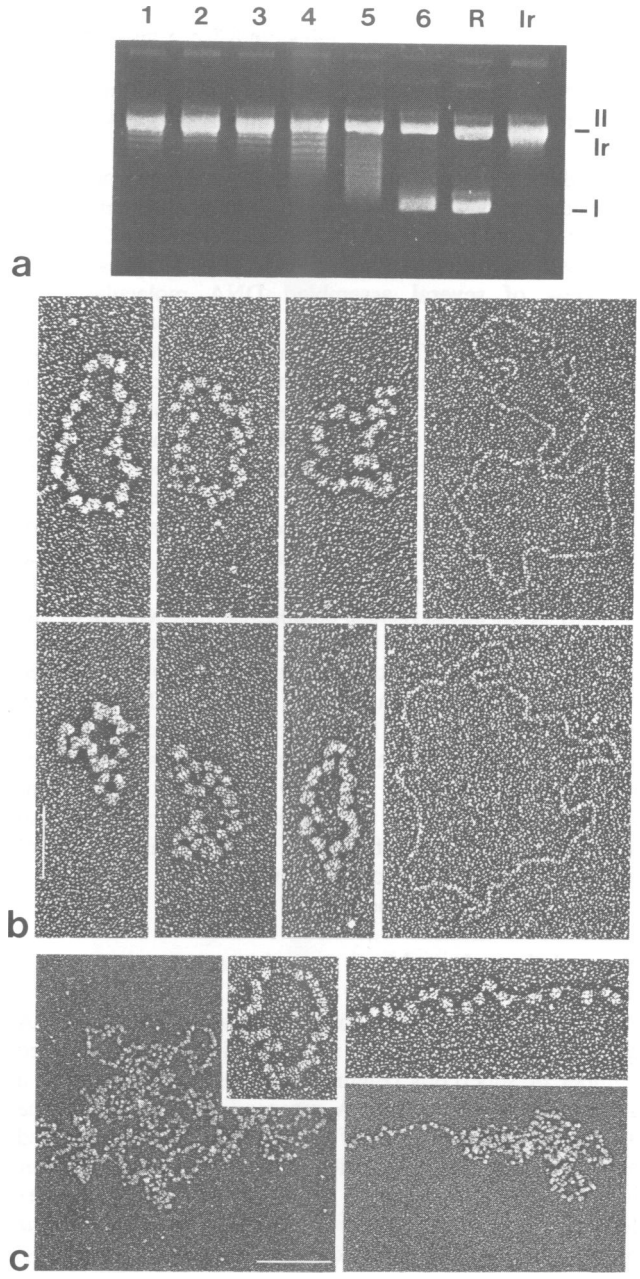

Fig. 5. Analysis of nucleoprotein complexes reconstituted by incubation of both purified histone complexes with DNA. Relaxed circular plasmid DNA was incubated with both histone complexes at different histone to DNA ratios (**a**, lane 1, 0.2:1; lane 2, 0.3:1; lane 3, 0.4:1; lane 4, 0.8:1; lane 5, 1.6:1; lane 6, 3.2:1) and analysed for supercoil induction after 2 h by gel electrophoresis on a 1.2% agarose gel. For reference, fully supercoiled plasmid DNA is shown in lane R and relaxed input DNA in lane I_r. The migration positions of form I, form I relaxed and form II DNA are indicated on the right. The reaction products were also prepared for electron microscopic analysis by the spreading procedure. (**b**) Examples of the typical beaded appearance of the nucleosomal chain obtained by reconstitution with both histone complexes at a histone:DNA ratio of 3.2:1. For estimation of the DNA foreshortening ratio due to the nucleosome formation the deproteinized DNA used for the reconstitution experiments has been prepared in the same way (b, right panels). Areas of aggregated reconstituents shown in (**c**) demonstrate the nearly complete packaging into nucleoprotein complexes which are mostly beaded. Sometimes the chromatin strand shows a filamentous appearance due to the close packaging of individual nucleosomes (insert in c). Magnifications in (b) and inserts in (c) are 100 000-fold (bar = 0.1 μm); magnification in (c) is 40 000-fold (bar = 0.25 μm).

spreading technique. Figure 5b shows the normal appearance of the reconstituted nucleoprotein complexes, which probably represent true nucleosomes according to their size and frequency on the DNA ring. An average of 23 beads per ring with a mean diameter of 100 nm has been determined on the 5.2 kb long DNA circle. The compaction ratio of 2.8 in this extended configuration is in good agreement with values determined for SV40 minichromosomes under similar spreading conditions (Griffith, 1975; Crémisi *et al.*, 1976). The lower magnification in Figure 5c gives a representative overview of several assembled DNA molecules, and demonstrates the overall dense packaging of the DNA into nucleosomes. The nucleosomal areas are not always unravelled into a beads on a string like structure, but are sometimes still more condensed (see inserts in Figure 5c).

The histone:DNA ratios given for the assembly reactions are likely to be too high as regards the number of histone molecules actually involved in the assembly reaction. Since the purified histone complexes used for this reaction did not contain all four histones in equal amounts (see Figure 2) the histone species present at the lowest level limits the assembly capacity of the added histone complexes. Independent of this constraint, we observed that addition of the nucleoplasmin complex to the N1/N2 complex induced a greater degree of supercoiling at significantly lower N1/N2 complex concentrations than the N1/N2 histone complexes alone. This suggested a contribution of the nucleoplasmin complexes which by themselves did not show any supercoil induction. An increased activity by the combined complexes was also observed in the band shift assay (Figure 4c, lane 3), where only one-fifth of each complex was needed to induce a complete retardation when added together to the DNA.

Synergistic action of both complexes in nucleosome core assembly

Addition of both histone complexes together to the DNA at concentrations where they separately induce only a limited number of supercoils (Figure 6a, lanes 1–3) results in a synergistic increase of DNA supercoiling (Figure 6a, lane 4). Synergistic induction of supercoiling is also observed after adjusting the final histone concentrations of all four histones to the same level as the separate histone pairs (compare Figure 5a, lane 5 with Figure 6a, lane 1). The presence of HMG-1, added to the nucleoplasmin complexes, leads to some supercoiling by this fraction alone (Figure 6a, lane 3) but does not stimulate or significantly inhibit the cooperative action of both histone complexes (Figure 6a, lane 5). Limited histone availability is not the reason for the reduced supercoil formation observed with N1/N2 complexes alone (Figure 6a, lane 1), since prolonged incubation of these complexes at the same histone:DNA ratio leads to further supercoiling as shown in Figure 6b, lane 1. This result suggests that not only the absolute number of supercoils is elevated when both complexes act together, but also the velocity of supercoiling is increased. This conclusion has been directly tested by comparing the kinetics of DNA supercoiling with N1/N2 complexes alone and in combination with nucleoplasmin (Figure 6c and d). The acceleration of supercoiling by the addition of nucleoplasmin complexes to the N1/N2 complexes is evident: the amount of supercoiled DNA that was observed after 30 min in the presence of both histone complexes (Figure 6c) required an overnight incubation when only the N1/N2 complexes were added (Figure 6d). The kinetics of supercoil induction by

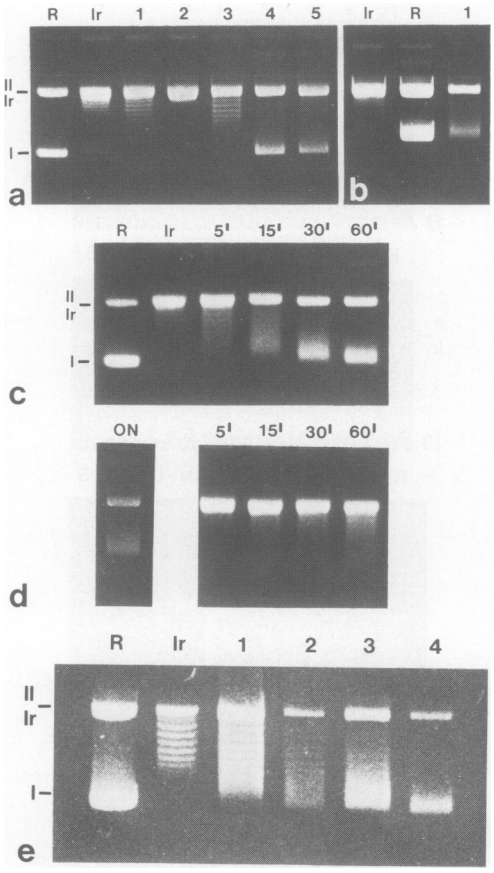

Fig. 6. Synergism between N1/N2– and nucleoplasmin–histone complexes in the nucleosome assembly reaction. The synergistic influence of both histone complexes together on the supercoiling reaction results in an increase in the number of superhelical turns and the velocity of supercoiling (**a**–**d**) and can be separated into two steps (**e**). The purified histone complexes were incubated separately with relaxed circular plasmid DNA at a concentration where they induce supercoiling only weakly or not at all (a, lane 1, N1/N2 complexes, histone:DNA ratio of 1.5:1; lane 2, nucleoplasmin complexes, histone:DNA ratio 2.6:1; lane 3, nucleoplasmin complexes plus HMG-1 fraction, histone:HMG-1:DNA ratio 2.6:2.6:1). Combined incubation of N1/N2 complexes with nucleoplasmin complexes (a, lane 4) or N1/N2 with nucleoplasmin complexes and HMG-1 (a, lane 5) strongly increased supercoil induction. Final histone:DNA ratios were in (a) lane 4, 4:1 and in (a) lane 5, histone:HMG-1:DNA 4:2.6:1. An overnight incubation of N1/N2–histone complexes alone (b, lane 1, histone:DNA ratio 1.5:1) leads to a considerable increase in supercoiling in comparison to the 2 h incubation (a, lane 1). The time course of supercoiling of relaxed plasmid DNA by the action of both purified histone complexes combined (c) was compared with that of the N1/N2–histone complexes alone (d). The time points (minutes) at which the reaction was stopped are indicated at the top (ON: overnight incubation). The purified histone complexes were added in histone:DNA ratios of 4:1 (c) and 1.5:1 (d), meaning that both reactions contained the same amount of N1/N2–histone complexes. For separation of the assembly reaction into two steps, relaxed circular plasmid DNA has been incubated with N1/N2–histone complexes for 2 h at a concentration which leads to partial supercoil induction (histone:DNA ratio 1.5:1) and sedimented by centrifugation at 150 000 *g* for 1 h (e, lane 1 supernatant, lane 2 sediment). After removal of the supernatant, the sediment was redissolved in assembly buffer and incubated with nucleoplasmin–histone complexes (histone:DNA ratio ~2.0:1) for 1 h and analysed for supercoil induction (e, lane 4). The supernatant was also mixed with nucleoplasmin complexes (histone:DNA ratio ~1:1) and analysed for an increase in supercoil induction after 1 h incubation. The DNA was isolated and analysed on 1.2% agarose gels and stained with ethidium bromide. Supercoiled reference DNA (R) and relaxed form I DNA (I_r) are run in parallel. The migration positions of form I, form II and relaxed form I DNA (I_r) are indicated at the left.

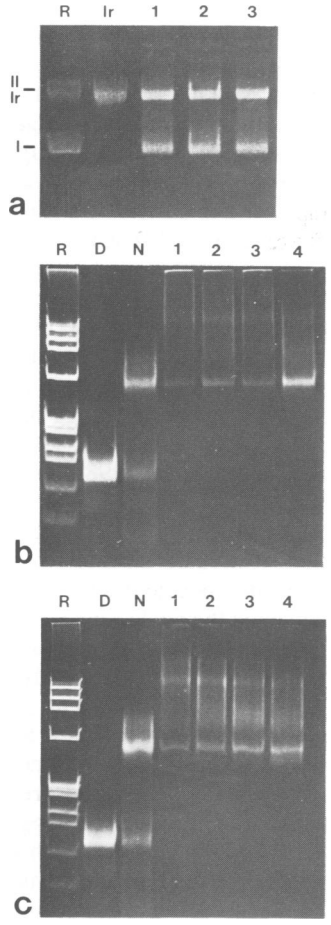

Fig. 7. Influence of sequential addition of the histone complexes to the DNA on nucleosome core formation. Both histone complexes were added to the DNA in a different sequential order and analysed for supercoil induction (**a**) and DNA mobility shift induction (**b** and **c**). Relaxed circular plasmid DNA (I_r) was first incubated for 2 h with the N1/N2 complexes (a, lane 1) or with nucleoplasmin complexes (a, lane 2) and then complemented for 1 h with the corresponding histone complex. Lane 3 in (a) shows the reaction products formed when both complexes were added at the same time to the DNA. Migration positions of form I, form II and relaxed form I (I_r) DNA are indicated at the left. R, supercoiled reference plasmid. Mononucleosomal DNA (b and c, lane D) was first incubated for 2 h with N1/N2−histone complexes (b) and then complemented with nucleoplasmin−histone complexes for different times and analysed by non-denaturing gel electrophoresis (b, lane 4, 1 min; lane 3, 15 min; lane 2, 30 min; lane 1, 60 min). Conversely mononucleosomal DNA was first incubated for 2 h with nucleoplasmin complexes and then complemented for different times with N1/N2−histone complexes (c, lane 4, 1 min; lane 3, 15 min; lane 2, 30 min; lane 1, 60 min). Migration positions of mononucleosomes is shown in lanes N. R: mol. wt standards: ϕX174 digested with *Hae*III.

nucleoplasmin− and N1/N2−histone complexes together correspond to the kinetics observed with unfractionated high speed supernatant of *Xenopus* oocyte nuclei, whereas the 5S fraction which contains only N1/N2−histone complexes (see Figure 1) shows a similar delayed time course of supercoiling to purified N1/N2−histone complexes (data not shown).

The synergistic action of both complexes can be resolved in two steps

One of the key questions in resolving the mechanism of cooperation between the two histone complexes in the nucleosome assembly process is whether both histone binding proteins must be present to promote a concerted transfer of the histones to the DNA or if it is possible to resolve the assembly process into two steps. In order to test this possibility, we partially preassembled DNA with N1/N2−histone complexes for 2 h, sedimented the assembly products by centrifugation at 150 000 *g* for 1 h and removed the unassembled histone complexes and free N1/N2 proteins. The sediment was redissolved in physiological ionic strength buffer and reacted with nucleoplasmin complexes. As shown in Figure 6e the incubation of the preformed 'H3−H4-containing chromatin' (Figure 6e, lane 2) with the nucleoplasmin complexes increased the supercoil induction without the aid of N1/N2 and in the absence of soluble histones H3 and H4 (Figure 6e, lane 4). A stimulation of supercoiling was also observed in the supernatant fraction after nucleoplasmin had been added (Figure 6e, lanes 1 and 3).

Are the histones from the two complexes transferred to the DNA in a preferred order?

The separate complexing of histones H3 and H4 with N1/N2 and H2B and H2A with nucleoplasmin raises the question of whether they are required to enable an ordered sequence of histone deposition onto the DNA during the assembly process. In an attempt to analyse this question we changed the order of histone complex addition to the DNA and analysed the products of nucleosome core formation by supercoil induction and the band shift assay. With the supercoil assay no significant difference in the quantity of supercoil induction was observed, independently of whether the DNA was preincubated for 2 h with nucleoplasmin or with N1/N2−histone complexes respectively (Figure 7a). This indicates that the final degree of supercoil induction is not dependent on a defined order of histone deposition onto DNA, at least not as long as both histone complexes or histone binding proteins are present in solution. Using the band shift assay, however, we observed additional products at non-nucleosomal positions when the DNA was preincubated with nucleoplasmin complexes (Figure 7c), which were less prevalent following preincubation with N1/N2 complexes (Figure 7b). This result suggests that predisposition of H3 and H4 facilitates correct nucleosome core formation, whereas predisposition of H2B and H2A leads to more incorrect products which are not visualized with the supercoil assay.

Discussion

The histones used for nucleosome assembly *in vivo* are synthesized in the cytoplasm and provided in a soluble form to the cell nucleus primarily during the S phase of the cell cycle. However, histone synthesis also occurs uncoupled from the cell cycle (Adamson and Woodland, 1974; Wu and Bonner, 1981; Wu *et al.*, 1982; Waithe *et al.*, 1983), which in the case of *Xenopus* oocytes leads to a histone pool which exceeds the DNA level by up to 1000-fold (reviewed by Woodland, 1980). These histones are used for chromatin assembly during DNA replication, after they have been released into the cytoplasm of the egg and reaccumulated into the small nuclei of the developing embryo. We have purified for the first time under native conditions such physiologically non-DNA bound histones from the germinal vesicles of the *Xenopus* oocytes in order to analyse their organization and their function in the nucleosome assembly process.

Laskey *et al.* (1978b) first observed that the stored, non-chromatin bound histones present in *Xenopus* egg homogenates have a net negative charge, which suggested the association of the histones with negatively charged cellular components. We have shown that the stored histones in the oocyte nuclei are not randomly associated with negatively charged molecules but exist in two defined complexes with acidic, karyophilic proteins: histones H3 and H4 are associated with proteins N1 and N2 and histones H2B and H2A are associated with nucleoplasmin (Kleinschmidt and Franke, 1982; Kleinschmidt *et al.*, 1985). Two further histones, designated as H3* and H4* due to their electrophoretic behaviour, were also noted in the nucleoplasmin complexes (Kleinschmidt *et al.*, 1985). Dilworth *et al.* (1987) have confirmed the existence of the two histone complexes in *Xenopus* eggs. However, several deviating observations concerning the histone complex composition have been emphasized by these authors: (i) only N1 is part of the H3−H4-containing complex; (ii) H3* is a variant form of H2A and (iii) H4* is a degradation product and not present in egg extracts. Our results continue to dispute some of these points, while we are in agreement with others.

(i) the biochemically purified complexes described in this study fully confirm our immunoprecipitation data (Kleinschmidt *et al.*, 1985), in that we always recover two polypeptides of M_r 105 000 and 110 000 (e.g. N1 and N2) in association with H3 and H4. In all immunoprecipitates shown by Dilworth *et al.* (1987) two polypeptides in the same mol. wt range are also visible. Certainly, the relationship of the polypeptides which we call N1 and N2 remains to be clarified.

(ii) A major component of the nucleoplasmin complex, designated H3* (Kleinschmidt *et al.*, 1985) behaves like a H2A variant (West and Bonner, 1980) when analysed in an acid−urea−Triton gel system (Dilworth *et al.*, 1987; our own observation). Although there is no further evidence for including this polypeptide in the histone H2A group, we also prefer this interpretation, since it balances the stoichiometry of histone H2A, which is otherwise underrepresented in the nuclear extracts of *Xenopus* oocytes and in the reconstituted nucleosomes.

(iii) A very minor histone component at the H4 position in SDS−PAGE was observed in immunoprecipitates and after native isoelectric focusing of the nucleoplasmin complexes, and was designated H4* due to its isoelectric point which is different to that of H4 (Kleinschmidt *et al.*, 1985). In two preparations of biochemically purified histone complexes we observed a polypeptide with these properties by one-dimensional SDS−PAGE, when a large amount of protein was applied. Two-dimensional tryptic peptide analysis unequivocally identified it as a histone H4 (data not shown), which excludes the interpretation that it results from degradation of another histone. Since it represents a very minor component and we cannot completely exclude a redistribution between the histone complexes, although we have never observed a mixed distribution of histones between the two binding proteins, we have not analysed this question any further.

The stoichiometry of the histones to their binding proteins could not be determined by gel filtration or sedimentation analysis, due to the small mol. wt difference between the free binding proteins and the histone complexes. The crosslinking data suggest a 1:1 or 2:1 stoichiometry of the histones to the histone-binding proteins which is consistent with only a small mol. wt difference. In the case of N1/N2 complexes the crosslink products of M_r 150 000 could theoretically account for a 4:1 stoichiometry of histones H3 and H4 to N1/N2. Higher mol. wt aggregates of proteins N1/N2 with the histones H3 and H4 of up to 6.8S and a crosslink product of ~350 kd were also observed; however, their subunit composition is difficult to evaluate. In addition, it is not clear in the case of a 2:1 stoichiometry of the histones to the binding proteins whether the histones are present in homo- or heterodimers. There were no uncomplexed core histones detectable in the nuclear extracts of *X.laevis* oocytes and in particular no indications for a preformed octamer. The controversial question of whether there is a type of histone H1 present in the oocyte or egg homogenate (von Dongen *et al.*, 1983; Dilworth *et al.*, 1987; Smith *et al.*, 1988; Rodriguez-Campos *et al.*, 1989) was not addressed in this study.

Since nucleosome assembly *in vivo* occurs exclusively in the cell nucleus it seemed valuable to use the soluble supernatant of *Xenopus* oocyte nuclei to characterize the components involved in the nucleosome assembly reaction. Microinjection experiments have shown that the components needed for nucleosome formation are already present in these nuclei (Wyllie *et al.*, 1978; Zentgraf *et al.*, 1979), although they are used later in early embryogenesis. Immunodepletion experiments with antibodies against N1 and nucleoplasmin indicated that the histone complexes are necessary for nucleosome assembly in *Xenopus* egg homogenates (Dilworth *et al.*, 1987). Using the purified histone complexes we could now demonstrate that the complexes are also sufficient for nucleosome core assembly *in vitro*, as determined by supercoil induction and electron microscopic analysis of the reaction products. HMG-1, also described as an assembly factor (Bonne-Andrea *et al.*, 1984), is not required for this reaction and is not associated with the histones (Kleinschmidt *et al.*, 1983). Interestingly, the HMG-1 fraction alone shows supercoil induction (see also Javaherian *et al.*, 1978; Bonne *et al.*, 1980) and HMG-1 is rapidly bound to the minichromosomes during nucleosome assembly in total nuclear extracts (unpublished results).

The ability to transfer separately the histones from the two complexes to DNA suggests that a coordinated transfer of all four histones is not necessary for nucleosome core assembly. Separation of assembly into two independent steps is most clearly shown by the increase of supercoil induction upon addition of nucleosome complexes to preformed 'H3−H4-containing nucleoprotein complexes'. Since the nucleoplasmin complexes themselves do not supercoil DNA, they have to interact with the histones H3 and H4 already bound to the DNA, thereby increasing the supercoiling. N1/N2, soluble H3 and H4 or soluble N1/N2 histone complexes are not required for this step. The role of N1/N2 seems to be to efficiently transfer H3 and H4 to the DNA. These assembly intermediates can be used by the nucleoplasmin complexes to complete nucleosomal cores. Such a mechanism would be in good agreement with the sequence of histone deposition deduced from *in vivo* observations, which demonstrated a stepwise association of first histones H3 and H4 with the DNA followed by histones H2B and H2A after DNA replication (Worcel *et al.*, 1978; Crémisi and Yaniv, 1980; Jackson and Chalkley, 1981). However, this sequence of histone deposition seems not to

be fixed in the *in vitro* system used in this study since the separate, sequential addition of the two histone complexes to the DNA led to the same degree of supercoil induction irrespective of their sequence of addition, which indicates a high degree of flexibility in handling the already transferred histone pairs. The supercoil induction assay, however, may not detect qualitative differences in the nucleosomes formed by different sequential addition of the histone complexes to the DNA, which is actually suggested by the appearance of band shift products at non-nucleosomal positions.

It has been claimed that the *Xenopus* oocyte or egg system reflects a specialized example of chromatin assembly that occurs outside of S phase. It is obvious that the histone complexes described in this and earlier studies are used *in vivo* for chromatin assembly in connection with DNA replication during early embryogenesis. The coupling of DNA synthesis with nucleosome assembly in the *Xenopus* egg system has also been demonstrated *in vitro* (Dilworth *et al.*, 1987; Almounzi and Mechali, 1988). In general, however, DNA replication seems not to be a prerequisite for nucleosome assembly, as shown in somatic cells *in vivo* with SV40 DNA (Cereghini and Yaniv, 1984). Although the large excess of histones over DNA in the *Xenopus* oocyte probably represents a specialized case, it at least provides a model system for how a living cell handles the highly positively charged histones in solution and subsequently assembles them into nucleosomes. It also poses a larger fascinating question of whether mammalian cells have developed other proteins and mechanisms to facilitate chromatin formation (Ishimi *et al.*, 1984, 1987; Smith and Stillman, 1989).

Materials and methods

Purification of histone complexes, nucleoplasmin and proteins N1/N2

Both histone complexes were purified from 80 ml of a 1 h × 100 000 *g* supernatant of homogenized, mass-isolated germinal vesicles of *X.laevis* oocytes (Kleinschmidt and Franke, 1982) in buffer A [83 mM KCl, 17 mM NaCl, 10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$ and 0.5 mM phenyl methyl sulphonyl fluoride (PMSF)] with 2.5 mM D-dithiothreitol at a protein concentration of 1–2 mg/ml. Proteins were precipitated at 4°C with 70% $(NH_4)_2SO_4$ (corrected for 0°C) for 1 h and then adsorbed by end-over-end rotation for 30 min at 4°C onto 10 g Sepharose B6 (Pharmacia, Uppsala, Sweden), which was equilibrated in a saturated $(NH_4)_2SO_4$ solution and then applied to a 1.5 × 20 cm column. The adsorbed proteins were eluted and fractionated by a 70–20% gradient of $(NH_4)_2SO_4$ in buffer A (200 ml) at 4°C with a flow rate of 0.5 ml/min. Fractions containing nucleoplasmin and proteins N1/N2 were identified by gel electrophoresis and separately pooled. The proteins of the pooled fractions were precipitated by $(NH_4)_2SO_4$ (~90% final concentration at 0°C) assuming a $(NH_4)_2SO_4$ concentration of 55% in the nucleoplasmin pool fraction and of 45% in the N1/N2 pool fraction, collected by centrifugation and further purified separately. The nucleoplasmin containing sediment was dissolved in 1.8 ml of buffer A, dialysed for 30 min against buffer A, loaded onto 5–30% sucrose gradients (in buffer A) and centrifuged for 18 h at 36 000 r.p.m. (4°C) in a Beckman SW40-type rotor. The fractions were analysed by gel electrophoresis and the histone complexes (present in the major peak of absorbance at 280 nm) were immediately concentrated to ~1 mg/ml by vacuum dialysis in collodion bags (Sartorius, Göttingen, FRG), which were previously coated with a 0.1% BSA solution in buffer A, to prevent losses of the histones. The ammonium sulphate sediment containing proteins N1/N2 was solubilized in 0.9 ml buffer A and chromatographed on a Superose 6 column (Pharmacia, Uppsala, Sweden; 1.6 × 60 cm) in buffer A with a flow rate of 0.3 ml/min at 4°C. Fractions containing N1/N2 were pooled and immediately applied to a DEAE–Sephacel column (Pharmacia, Uppsala, Sweden; 1 × 5 cm), equilibrated in buffer A and the histone complexes were eluted in a single peak with buffer A containing additional 0.2 M KCl. The eluate was dialysed against 10 mM $(NH_4)HCO_3$, pH 7.8, for 2 h at 4°C, lyophilized, redissolved in buffer A at a concentration of ~1 mg/ml and again dialysed against buffer A at 4°C. If necessary, the DEAE eluate was rechromatographed on a Mono Q ion exchanger (Pharmacia, Uppsala, Sweden) from which it was eluted by a salt gradient of 0–1 M KCl in buffer A and further processed as described for the DEAE–Sephacel eluate. The purified fractions were kept at −70°C. Histone free nucleoplasmin was prepared by adsorption of the histones on DNA cellulose (Sigma, München, FRG) in buffer A, with a 5-fold excess of DNA over the histones. For preparation of histone free N1/N2 the Superose eluate fraction was adjusted to 4 M urea and chromatographed on a Mono Q column (Pharmacia, Uppsala, Sweden) using a linear gradient of 0–1 M KCl in buffer A, containing 4 M urea. Protein N1/N2 was lyophilized after buffer exchange against 10 mM $(NH_4)HCO_3$, pH 7.8, and redissolved in a 20 mM triethanolamine buffer, pH 7.4, containing the same salts as buffer A.

The histone content of the histone complexes has been estimated by coelectrophoresis of purified complexes with a concentration series of standard calf thymus histones. The histone:DNA ratios are given as weight ratios of total histones (e.g. H3 and H4 for the N1/N2 complexes and H2B and H2A for the nucleoplasmin complexes) to DNA.

In vitro nucleosome assembly and related reactions

Plasmid DNA (Bluescribe containing a 1.95 kb insert; Kleinschmidt *et al.*, 1986) was prepared by conventional methods (Maniatis *et al.*, 1982) and relaxed by the action of topoisomerase I (Gibco-BRL) in a buffer containing a final concentration of 75 mM KCl, 10 mM Tris, pH 8, 0.5 mM EDTA and 2.5% glycerin with 3 U/μg DNA. The standard assembly reaction mixture contained 150 ng relaxed form I DNA, 0.5 U topoisomerase I and 3.7 mM EDTA. The different reaction components were added in buffer A and the final volume was adjusted to 10 μl by the addition of assembly buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF). Some experiments were done in a 15 μl reaction volume containing 300 ng DNA and 1 U topoisomerase. The reaction was performd in siliconized tubes for 2 h at 26°C unless otherwise stated and terminated by addition of an equal volume of phenol. DNA was extracted by phenol, followed by chloroform and electrophoresed on a 1.2% agarose gel (Seakem) in a Tris–acetate–EDTA buffer (Maniatis *et al.*, 1982). DNA was visualized by staining with ethidium bromide and illumination with UV light at 254 nm. The band shift assay was performed essentially as described by Aragay *et al.* (1988). Nucleosomal core DNA was prepared from chicken erythrocyte chromatin (Zentgraf and Franke, 1984) and DNA–histone complex formation was assayed by incubation of the different histone complex fractions (in buffer A) with 500 ng mononucleosomal DNA (145 bp) in the presence of 3.7 mM EDTA for 2 h at 26°C. The final volume was adjusted to 10 μl with buffer A. DNA–histone complexes were analysed on the higher ionic strength gels (1 × TBE, Aragay *et al.*, 1988) containing 6% polyacrylamide, stained with ethidium bromide and photographed under UV light.

In order to prepare nucleoprotein complexes containing only histones H3 and H4, relaxed form I DNA was incubated with purified N1/N2–histone complexes in a standard assembly assay for 2 h at a histone:DNA ratio of 1.7:1 to obtain only partially supercoiled DNA. The resulting nucleoprotein complexes were sedimented by centrifugation in an airfuge (Beckman) at 150 000 *g* for 1 h and the supernatant was carefully removed. The sediment was redissolved in assembly buffer, nucleoplasmin complexes were added to the sediment and the supernatant to achieve a histone:DNA ratio of 2:1 for H2B and H2A histones in the sediment fraction and incubated again for 1 h. The assembly products were analysed as described above.

Gel electrophoresis, immunoreactions and sedimentation analysis

SDS–PAGE was performed according to Thomas and Kornberg (1975) using 18% polyacrylamide, and gels were stained with Coomassie blue R-250 (Serva, Heidelberg, FRG) or with silver as described by Blum *et al.* (1987).

Antibodies against nucleoplasmin and N1/N2, and conditions for immunoblotting and immunoprecipitation are described in Kleinschmidt *et al.* (1985). Sedimentation analysis of histone complexes was performed with a 10-fold concentrated 100 000 *g* supernatant of mass isolated germinal vesicles for 18 h at 4°C in a SW40 rotor (Beckman, USA) at 36 000 r.p.m. using a 5–30% sucrose gradient in buffer A. Fractions of 0.4 ml were concentrated 5-fold by centrifugation through Ultra free-MC filter units (Millipore) before analysis of polypeptide composition and nucleosome assembly.

Crosslinking experiments

Crosslinking was performed essentially as described by Jackson (1978). The purified histone complexes or histone free binding proteins were dialysed against TEA buffer (87 mM KCl, 17 mM NaCl, 20 mM triethanolamine–HCl, pH 7.4) at a concentration of ~0.1 mg/ml and

reacted with 1% formaldehyde (v/v) for 45 min at room temperature. The reaction products were concentrated to dryness by vacuum dialysis against buffer A and solubilized in SDS-containing sample buffer without mercaptoethanol (60 mM Tris–HCl, pH 6.8, 5 mM EDTA, 1% SDS, 10% glycerin). Crosslinking products were analysed by SDS–PAGE (12% acrylamide) in the first dimension. After staining, gel tracks were excised and prepared for second dimension as described by Jackson (1978), except that the buffer for cleavage of the crosslinks was adjusted to pH 7.4.

Electron microscopy

For electron microscopy, spread preparations of chromatin assembled *in vitro* and deproteinized DNA were made essentially as described elsewhere (Müller *et al.*, 1978). Rotary shadow-casting was performed with platin:palladium (80:20) at an angle of 8°. Electron micrographs were taken with a Zeiss electron microscope (EM 10A) at 40 kV. The magnification indicator was routinely controlled by comparison with a grating replica. Length measurements, calculations and countings were done as described earlier (Scheer and Zentgraf, 1978). For contrast enhancement, micrographs were printed in reverse contrast.

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