

Remodeling Sperm Chromatin in *Xenopus laevis* Egg Extracts: The Role of Core Histone Phosphorylation and Linker Histone B4 in Chromatin Assembly

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Abstract. We find that the remodeling of the condensed *Xenopus laevis* sperm nucleus into the paternal pronucleus in egg extracts is associated with phosphorylation of the core histones H2A, H2A.X and H4, and uptake of a linker histone B4 and a HMG 2 protein. Histone B4 is required for the assembly of

chromosome structures in the pronucleus. However neither B4 nor core histone phosphorylation are required for the assembly of spaced nucleosomal arrays. We suggest that the spacing of nucleosomal arrays is determined by interaction between adjacent histone octamers under physiological assembly conditions.

XENOPUS *laevis* oocytes and eggs have proven useful in demonstrating the capacity of nuclear structure to be remodeled and thus acquire new functions, for example those of transcription and replication following the microinjection of somatic nuclei into oocytes and eggs, respectively (Gurdon, 1968, 1976; Merriam, 1969; Wakefield and Gurdon, 1983). In a natural context, the sperm nucleus will be rapidly remodeled by the egg cytoplasm following fertilization to form the paternal pronucleus (Poccia, 1986). The molecular details as to how this dramatic restructuring is accomplished have begun to be determined (Philpott et al., 1991; Philpott and Leno, 1992). Since the *X. laevis* paternal pronucleus is organized into a chromatin structure that resembles that of a normal somatic cell, whereas the sperm nucleus is not, the assembly of the pronucleus provides a convenient system for reconstructing the role of individual proteins in the assembly process.

X. laevis sperm nuclei contain histones H3 and H4, yet have reduced amounts of histones H2A and H2B, and lack histone H1 entirely (Risley and Eckhardt, 1981; Wolffe, 1989a,b; Philpott and Leno, 1992). Several sperm-specific basic proteins are associated with sperm chromatin and presumably function to compact DNA in the absence of a full complement of the histone proteins (Abé, 1987; Philpott and Leno, 1992; Risley, 1983). The molecular chaperone nucleoplasmin (Laskey et al., 1978) functions to exchange the sperm-specific basic proteins for histones H2A/H2B during the remodeling of *X. laevis* sperm nuclei in egg extracts (Philpott and Leno, 1992). However, sperm chromatin that is incubated in a purified solution of nucleoplasmin and his-

tone H2A/H2B does not acquire higher order aspects of chromatin structure (Philpott and Leno, 1992). Since incubation of sperm nuclei in a complete extract leads to the folding of nucleosomal arrays into higher-order chromatin structures that may require linker histones and eventually the assembly of nuclei, it is clear that proteins or activities in addition to the core histones and nucleoplasmin will be required.

The packaging of DNA by the histones within chromatin occurs through a series of ordered steps. These have been experimentally assessed using naked DNA and extracts of oocytes and eggs (Lohka and Masui, 1983, 1984; Glikin et al., 1984; Newport, 1987; Almouzni and Méchali, 1988a,b; Kleinschmidt and Steinbeisser, 1991). These extracts appear to reproduce the chromatin assembly process that follows the injection of DNA into oocyte nuclei or eggs (Wyllie et al., 1978; Forbes et al., 1983; Ryoji and Worcel, 1984; Almouzni and Wolffe, 1993). The first histones to stably associate with DNA in these extracts are H3 and H4 in the form of the tetramer (H3/H4)₂, deposition of histones H2A and H2B follow (Almouzni et al., 1990, 1991; Kleinschmidt et al., 1990). These events recapitulate the process of nucleosome assembly in vivo (Worcel et al., 1978), however, beyond the assembly of the histone core of the nucleosome little is known concerning the assembly of higher order structures (reviewed by Wolffe, 1992).

Worcel and colleagues discovered that ATP was required for the assembly of a physiologically spaced nucleosomal array (Glikin et al., 1984). This result led to the suggestion that topoisomerase II, which requires ATP as an energy source for activity might have a major role in the assembly process. However, subsequent work demonstrated that topoisomerase I has the predominant role in chromatin assembly (Almouzni and Méchali, 1988b; Wolffe et al., 1987; see also Annunzi-

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ato, 1989). Importantly hydrolysis of ATP or of other trinucleotides was found not to be necessary to provide an energy source for chromatin assembly (Almouzni and Méchali, 1988b). Other explanations for the ATP requirement in chromatin assembly include the stabilization of the phosphorylation state of particular proteins by inhibiting phosphatases, for example the maintenance of phosphorylation of histone H2A.X (Kleinschmidt and Steinbeisser, 1991), of nucleoplasmin (Almouzni et al., 1991) or of HMG 14/17-like proteins (Tremethick and Frommer, 1992). Importantly, exogenous ATP is not always required for the assembly of spaced chromatin, suggesting that any ATP requirement is an extract-dependent phenomenon, that may vary with preparation conditions (Crippa et al., 1993). Nevertheless, phosphorylated H2A.X and HMG 14/17-like proteins have been suggested to have important roles in generating nucleosomal arrays in which nucleosomes have physiological spacing (Kleinschmidt and Steinbeisser, 1991; Tremethick and Frommer, 1992). Phosphorylated nucleoplasmin is also more active in chromatin assembly than the unmodified protein (Sealy et al., 1986) and may more efficiently deposit histones H2A/H2B to complete the assembly of the histone octamer (Dilworth et al., 1987). Wrapping of DNA around the histone octamer is a necessary prerequisite for formation of a nucleosome, and it is possible that octamer-octamer interactions might directly lead to assembly of a physiologically spaced nucleosomal array (Almouzni et al., 1991; see also Hansen et al., 1989).

Other proteins in addition to the core histones may also contribute to the assembly of a chromatosome structure and spaced nucleosomal arrays. *Xenopus* oocytes, sperm, and eggs are severely deficient in normal somatic histone H1 and instead contain a cleavage stage linker histone B4 that has ~30% sequence identity to histone H1 (Smith et al., 1988; Wolffe, 1989a,b; Dimitrov et al., 1993; Hock et al., 1993). Histone B4 is an excellent candidate to replace histone H1 in normal embryonic chromosomes. Nevertheless, supplementation of oocyte or egg extracts containing histone B4 with exogenous histone H1 will increase the distance between nucleosomes from 180 to as much as 220 bp (Rodriguez-Campos et al., 1989). This suggests that exogenous histone H1 is stably incorporated into chromatin under these conditions, however, chromatosome structures were not defined in these experiments. Likewise, addition of exogenous HMG 14/17 to extracts (HMG 14/17 are deficient in *Xenopus* eggs and oocytes; Weisbrod et al., 1982; Crippa et al., 1993), can influence the apparent spacing of nucleosomes (Tremethick and Drew, 1993; Crippa et al., 1993). These supplementation experiments lead to the suggestion that histone H1-like proteins such as B4 or alternatively HMG 14/17-like molecules may have a key role in assembling both chromatosome structures and spaced nucleosomes.

In this work, we have directly examined the role of histone phosphorylation and of the linker histone B4 in chromatin structure during the remodeling of *Xenopus* sperm chromatin. We find that although major changes in histone phosphorylation occur concomitant with remodeling, these changes do not contribute to generating spaced (180 bp) nucleosomal arrays. We also find that although histone B4 has a role in generating a chromatosome-like particle within sperm chromatin, it does not contribute to the physiological spacing of nucleosomes.

Materials and Methods

Preparation of Eggs and Sperm

X. laevis frogs were purchased from Xenopus I (Ann Arbor, MI). Unfertilized eggs were obtained from *X. laevis* by injection of human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO). Eggs were collected in modified high salt Barth's saline (Gurdon and Wickens, 1983), dejellied with 2% cysteine at pH 7.8, rinsed several times, and sorted to remove all damaged or abnormal eggs.

Microinjections were carried out by the method of Kay (1991). Usually 25 μ l of [³H]lysine (75–100 Ci/mmol; Amersham Corp., Arlington Heights, IL) and 300 μ l of [³H]arginine (35–70 Ci/mmol, Amersham Corp.) were dried and resuspended in 50 μ l of injection buffer (88 mM NaCl, 10 mM Hepes, pH 6.8) and 20 nl of this solution were injected in the cytoplasm of each oocyte, egg, or embryo.

X. laevis sperm nuclei were prepared by the method of Blow and Laskey (1986). Testes were homogenized in 2 ml SuNaSp (250 mM sucrose, 75 mM NaCl, 0.5 mM spermidine, and 0.15 mM spermine). Nuclei were pelleted by centrifugation at 1,000 g for 5 min and were resuspended in 0.5 ml SuNaSp. Demembration was performed by the addition of 20 μ l of a 1 mg/ml solution of lysolecithin. After 10 min, the reaction was stopped by the addition of 1 ml SuNaSp containing 3% bovine serum albumin at 0°C. The sperm nuclei (>98% pure) were washed three times in SuNaSp and finally resuspended in SuNaSp plus 30% glycerol.

Preparation of Low- and High-Speed Egg Extracts

Unfertilized eggs were dejellied and where necessary microinjected with [³H]lysine and [³H]arginine before extracts were prepared as previously described (Lohka and Masui, 1983, 1984). Briefly, dejellied eggs were disrupted by direct centrifugation (9,000 g for 30 min) at 4°C in a modified extraction medium (20 mM Hepes, pH 7.5, 70 mM potassium chloride, 1 mM DTT, 5% sucrose, 10 μ g/ml leupeptin). The supernatant after this centrifugation step is the low-speed extract. The low-speed extract was recentrifuged at 150,000 g for 60 min. The supernatant after this centrifugation step was the high-speed extract. All extracts were used immediately after preparation.

Phosphorylation of sperm chromatin components, of egg extract proteins or of decondensed sperm nuclei was examined by direct mixing of [³²P]ATP with the constituents. Normally 10⁶ nuclei, 10- μ l extract or the two combined were radiolabeled with 10 μ Ci of [³²P]ATP unless indicated otherwise.

Preparation of Antibodies to Histone B4, Immunoblotting, and Immunodepletion

All cloning steps for the production of B4 as a fusion protein were done using standard methodology (Maniatis et al., 1982). The B4.2 clone (provided as the kind gift of R. Smith, Eli Lilly, Indianapolis, IN) was used to produce a DNA fragment containing the entire B4 coding sequence by PCR using the following primers: 5'GGT GAT TCT CCC ATG GCT CCT-3' and 5'CCC CGG ATC CTC GAG TAT ATC AGC CTA-3'. The former primer introduced a NcoI restriction site at the ATG encoding the translational start of B4 and the latter primer introduced a XhoI site nine nucleotides beyond the TAG translational termination codon (Smith et al., 1988). The resulting fragment was restricted with these two enzymes and ligated into NcoI-XhoI-restricted pGEX-KG (Guan and Dixon, 1991), giving a construct that fused the B4 coding sequence in-frame to the coding sequence of glutathione-S-transferase. This construct was transformed into the *Escherichia coli* strain BL21 (DE3) pLysS, from which the fusion protein was isolated after induction with isopropyl- β -D-thiogalactopyranoside (IPTG)¹.

To obtain a large amount of the fusion protein for immunization of rabbits to produce polyclonal antibodies, inclusion bodies were isolated from induced bacteria as follows: the cells from 250 ml of bacterial culture were thawed and resuspended in 5 ml of buffer A (2.4 M sucrose, 40 mM Tris-HCl, pH 8.0, 10 mM EDTA) and allowed to sit on ice for 30 min. 20 ml of buffer B (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 100 μ g/ml lysozyme, 75 μ g/ml PMSF) was then added and the incubation on ice continued for an additional 80 min. DNase I (20 μ g/ml), 10 mM MgCl₂, and 0.1% deoxycholate were added and the incubation on ice continued until the viscosity of the mixture decreased (~30 min). The preparation was then centrifuged in a rotor (SW28; Beckman Instruments, Palo

1. Abbreviation used in this paper: IPTG, isopropyl- β -D-thiogalactopyranoside.

Alto, CA) for 30 min at 8,000 rpm to collect the inclusion bodies. The pellets were carefully resuspended in wash buffer (buffer B plus 10 mM EDTA and 0.5% Triton X-100) and allowed to sit at room temperature for 10 min. The inclusion bodies were centrifuged again in an SW28 rotor, as above, and then solubilized in 10 ml of 10 mM Tris-HCl, pH 8.0, 1 mM DTT, and 6 M urea. The preparation was incubated at 37°C for 30 min and then centrifuged at 10,000 rpm in a rotor (SS34; Sorvall, Newton, CT) for 20 min to remove bacterial debris. The urea was removed by stepwise dialysis in buffer C (25 mM Tris-HCl, pH 8.0, 25 mM NaCl, 1 mM DTT, and 75 µg/ml PMSF) with 4, 2, and 0 M urea. A final dialysis was performed against 25 mM Tris-HCl, pH 8.0, 25 mM NaCl, 1 mM DTT, and 10% glycerol, and the dialyzed preparation was spun in an SS34 rotor at 10,000 rpm for 20 min. The fusion protein constituted the major protein in supernatant from the inclusion body preparations. Rabbit anti-B4 sera were produced at Spring Valley Farms (Sykesville, MD) using 175 µg of protein from the inclusion body preparations for the initial injection and subsequent boosts.

To affinity purify anti-B4 antibodies, soluble fusion protein was obtained as follows: bacteria were induced and treated as described above for the preparation of inclusion bodies through the SW28 centrifugation. The supernatant from this centrifugation was incubated with rotation for 1 h at 4°C with 1 ml of glutathione-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) that had previously been washed extensively with buffer D (10 mM Hepes-KOH, pH 8.0, 1 mM DTT, 75 µg/ml PMSF). After this incubation, the supernatant was removed and the beads were washed extensively with buffer D plus 150 mM NaCl. The fusion protein was eluted from the beads using buffer D plus 5 mM glutathione, and then it was dialyzed into coupling buffer (0.1 M NaHCO₃, pH 8.3, 0.5 M NaCl). This purified soluble fusion protein was coupled to CNBr-activated Sepharose 6B according to the manufacturer's suggestions and antibodies directed against the protein were then purified from the sera by standard methods (Harlow and Lane, 1988). All Western blots using this antibody were made by standard methods (Harlow and Lane, 1988), and in most cases they were visualized using India ink before incubation with the antibody to ascertain transfer and to visualize the protein molecular weight standards that were also electrophoresed on the gels.

Immunodepletion of B4 from *Xenopus* extracts was performed essentially as described by Dasso et al., (1992). Briefly protein A-Sepharose beads (Sigma Chemical Co.) were blocked by two 15-min room temperature incubations with 3.5 vol of bovine serum albumin (10 mg/ml) in PBS. They were then washed three times in 3.5 vol of PBS. After washing, 600 µl of purified anti-B4 antibodies (350 µg/ml) was added to 100 µl of packed beads, and the mixture was allowed to incubate at room temperature for 1 h with rotation. To prepare beads for mock treatment of extracts, preimmune serum containing an approximately equivalent amount of immunoglobulin G was diluted with PBS to 600 µl, and then incubated with 100 µl of packed beads. After incubation with antibodies, the beads were washed three times in 400 µl of PBS. To deplete the cytosol fraction, 4 vol of cytosol and 1 vol of packed beads were incubated at 4°C for 1.5 h with rotation. The beads were pelleted by centrifugation, and the cytosol was removed and reincubated for an additional 1.5 h with 1 vol of fresh beads. PBS contains 125 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8.1 mM Na₂HPO₄. XB contains 100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 50 mM sucrose, and 10 mM Hepes (pH 7.7).

Micrococcal Nuclease Digestion of Chromatin

X. laevis sperm nuclei isolated as described by Blow and Laskey (1986) were taken immediately after isolation or were incubated in the low or high speed egg extracts as described. The nuclei (generally 100-µl vol containing 10⁷ nuclei) were diluted into 700 µl of buffer XN (50 mM Hepes-KOH, pH 7.0, 250 mM sucrose, 75 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine) and pelleted at 3,000 g. The pelleted nuclei were further washed twice in 500 µl of buffer XN, before resuspension in micrococcal nuclease digestion buffer (10 mM Tris HCl, pH 7.5, 80 mM NaCl, 2 mM CaCl₂, 25% glycerol). Micrococcal nuclease (1–10 U per 10⁶ nuclei) was added and digestion allowed to occur at room temperature for 5 min. To stop the reaction 30 mM EDTA was added to the samples and contaminating RNA removed through digestion with RNase A. After addition of 0.5% SDS and digestion with proteinase K the samples were deproteinized by extraction with phenol/chloroform. After ethanol precipitation DNA fragments were analyzed by electrophoresis.

Electrophoretic Analysis of the Proteins

SDS-polyacrylamide (18%) gel electrophoresis was carried out as described by Laemmli (1970). Two-dimensional electrophoresis was per-

formed as described by Russanova et al., (1980, 1989). The proteins were first separated in a 15% polyacrylamide slab gel containing 7 M urea and 5% acetic acid (Panyim and Chalkley, 1969). The strip with the separated proteins was then cut out from the gel and placed on the top of a second gel, which was made of a 2–3 cm 5% stacking gel and a 12–15 cm separating gel, containing 0.4% Triton X-100, and 6 M urea (West and Bonner, 1980). The gels were stained either with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Cambridge, MA) or with silver nitrate as described by Wray et al. (1981).

For identification of the tritium-labeled or -phosphorylated histones the gels were stained with Coomassie to determine the position of unlabeled carrier histones that served as markers, destained, treated with Amplify (Amersham Corp.) as recommended by the manufacturer, dried, and autoradiographed.

Results

Changes in Protein Composition of Sperm Chromatin during Decondensation and Replication

Xenopus sperm nuclei undergo a defined series of morphological changes during the remodeling process. The first stage of nuclear decondensation occurs very rapidly (<10 min) in a high speed (150,000 g) supernatant of eggs (see Materials and Methods) and requires only nucleoplasm (Philpott et al., 1991). The second stage of decondensation leading to pronuclear formation (~30 min) requires the assembly of a nuclear envelope, and occurs only in low speed egg extracts (9,000 g) in which the nuclear membranes are present (Lohka and Masui, 1984). Finally, the fully assembled pronucleus has the capacity to replicate its DNA and duplicate its chromosomal structures (Lohka and Masui, 1983; Blow and Laskey, 1986; Wolffe, 1993). Chromatin decondensation and pronuclear assembly were monitored by light microscopy using both fluorescence and phase contrast to image the nuclei (see Wolffe, 1989a). We examined the proteins present in sperm nuclei at each stage of this process using two-dimensional gel electrophoresis (Russanova et al., 1980, 1989; Dimitrov et al., 1993). In each case we carried out a parallel electrophoretic analysis of histones isolated from the nucleated erythrocytes of *Xenopus* (RBC). There are several unusual features of *Xenopus* sperm chromatin (Fig. 1 A). The linker histone H1 is severely reduced in abundance, an observation confirmed using H1-specific antibodies (data not shown; Wolffe, 1989a; Dimitrov et al., 1993; Philpott and Leno, 1992). Histones H2A and H2B are also reduced in abundance compared to histones H3 and H4, however, they are not completely absent (Risley, 1983; Wolffe, 1989a; Philpott and Leno, 1992). The identities of each of the core histones were confirmed by transfer to nitrocellulose and gas phase sequencing (data not shown). With respect to histone acetylation, histones H4 and H2B are predominantly deacetylated. The level of histone acetylation in *Xenopus* sperm chromatin is even less than that found within the transcriptionally repressed, nonreplicating *Xenopus* erythrocyte chromatin (Fig. 1 A, compare RBC with Sperm nuclei). This is in contrast to the hyperacetylation of histone H4 that occurs during trout spermiogenesis (Christensen et al., 1986). Three new proteins appear in sperm nuclei compared to RBC, these are labeled x, y, and z in Fig. 1 A. Each of these peptides was microsequenced. Protein x was identical to histone H3 and subsequent elution and reduction followed by electrophoresis revealed it to be a dimer of histone H3, probably formed through cross-linking of H3 molecules mediated by oxidation of the -SH

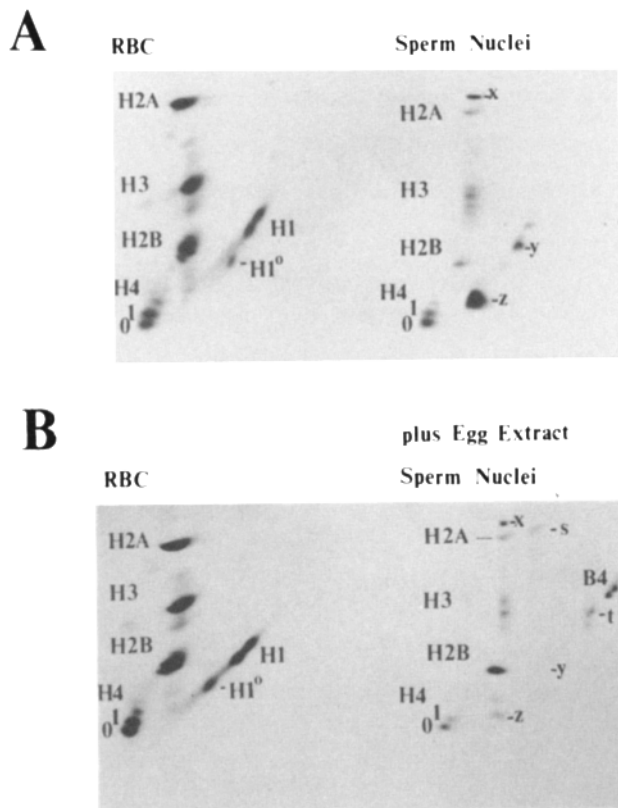


Figure 1. The protein composition of *X. laevis* sperm chromatin before (A) and after (B) remodeling in the *X. laevis* high speed egg extract. (A) Two-dimensional gel electrophoretic patterns of proteins after staining with Coomassie Blue are shown of *X. laevis* erythrocyte chromatin (RBC) or *X. laevis* sperm nuclei. The core histones H2A, H2B, H3, H4 are indicated as are the linker histones H1 and H1°. The deacetylated (O) and monacetylated (I) forms of H4 are indicated. Within sperm nuclei are three novel proteins indicated by x, y, and z (see text for details). (B) As in A except the sperm nuclei have been decondensed in high speed egg extract. The positions of the core histones and of proteins x, y, and z are indicated. The position of three new proteins: s, t, and B4 that accumulate in sperm nuclei in the extract are shown.

group of cysteine 110 (data not shown). This accounts for the apparent depletion of histone H3 relative to H4. Protein z has the partial NH₂-terminal peptide sequence Ala.Ser.Pro.Pro.Ala.Arg.Lys.Lys.Arg.Lys.Arg.Asn.Gln.Pro.Gly.Tyr.Ser.Gln.Leu.Val.Val.Lys.Thr.Ile, which has limited (45%) identity to the NH₂-terminal tail of histone H2B (Wells and McBride, 1989). It did not prove possible to obtain any peptide sequence from protein y, however, it is likely that both proteins z and y are similar to the family of *X. laevis* sperm-specific proteins described by Abé and colleagues (Abé and Hiyoshi, 1991; Hiyoshi et al., 1991).

During the first stage of sperm decondensation in the high speed supernatant of *Xenopus* eggs, proteins y and z are rapidly removed from sperm probably by interaction with nucleoplasmin (Philpott and Leno, 1992). Several new proteins appear concomitant with the decondensation process (Fig. 1 B) including more H2B (deacetylated), and the proteins s and t. Protein s is H2A.X (see later; Dilworth et al., 1987; Mannironi et al., 1989; Kleinschmidt and Steinbeisser, 1991), and microsequencing established that t is a

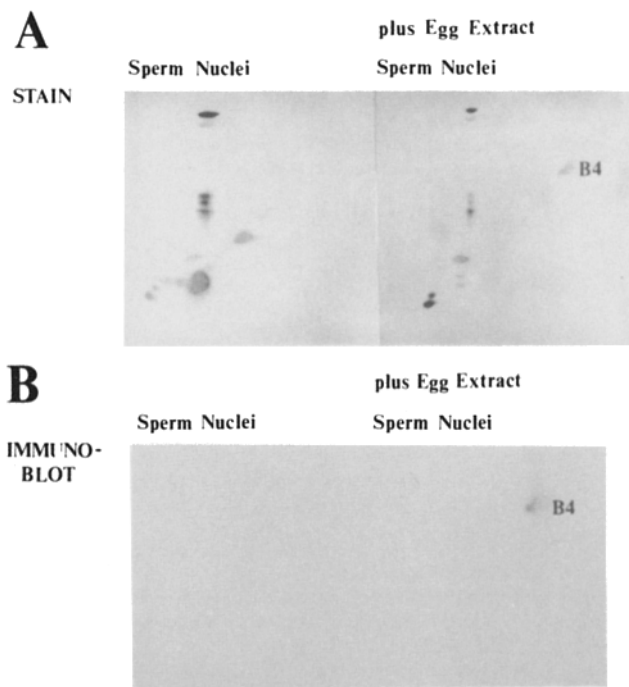


Figure 2. Immunoblot identifying histone B4 as accumulating in *Xenopus* sperm chromatin following incubation in high speed egg extract. (A) Two-dimensional electrophoretic analysis of proteins in *Xenopus* sperm nuclei alone (left) or following incubation in high speed egg extract. The position of B4 is indicated. Two filters stained with Indian ink after Western transfer are shown. (B) Immunodetection of histone B4 on these filters after reaction with polyclonal antibodies specific for B4 (Dimitrov et al., 1993).

Xenopus homolog of HMG2. The partial NH₂-terminal peptide sequence of protein t is Gly.Lys.Gly.Asp.Pro.Asn.Lys.Pro.Arg.Gly.Lys.Met.Ser.Ser.Tyr.Ala.Tyr.Phe.Val.Gln.Tyr.Cys.Arg.Glu.Glu.His.Lys.Lys.Lys.Phe, which is identical to the NH₂-terminal sequence of the chicken HMG2 protein (Davis and Burch, 1992) and distinct from the human HMG1 protein (Shirakawa et al., 1990; Tsuda et al., 1988). The histone B4 also accumulates in decondensed sperm nuclei (Fig. 1 B), the identity of B4 was confirmed using specific antibodies (Fig. 2; Dimitrov et al., 1993). All of the major components stably incorporated into the decondensed sperm nucleus have thus been identified.

We extended our analysis of protein incorporation into sperm chromatin during the first stage of decondensation by making use of high-speed egg extracts prepared from eggs in which the histones had been previously radiolabeled with [³H]lysine and [³H]arginine (Materials and Methods; Dimitrov et al., 1993). This enables the distinction to be made between proteins that originate entirely from stores sequestered in the oocyte, and proteins (radiolabeled) that originate in part from the translation of stored maternal mRNA in the egg. The newly synthesized proteins taken up at this time are histones H2A, H2B, HMG2 (protein t) and B4 (Fig. 3). Histone H2A.X is not apparent using this assay (Fig. 3). This is in contrast to results obtained when total protein is stained (Fig. 1 A). This difference presumably reflects incorporation of H2A.X into sperm chromatin from stores laid down in the oocyte and what is probably a low rate of H2A.X

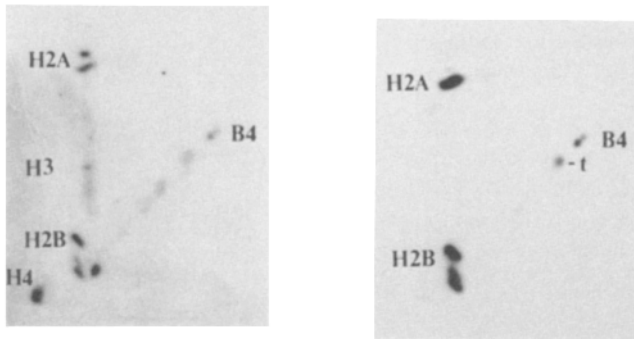


Figure 3. Newly synthesized proteins taken up by sperm chromatin during decondensation in radiolabeled high speed extract. Left hand panel shows total proteins in decondensed sperm nuclei following staining with Coomassie Blue. (Note: the spot immediately to the right H2B is some incompletely depleted protein z.) Right hand panel shows radiolabeled proteins synthesized in the eggs before extract preparation, that are taken up by sperm chromatin. The positions of histones H2A, H2B, B4, and protein t are indicated.

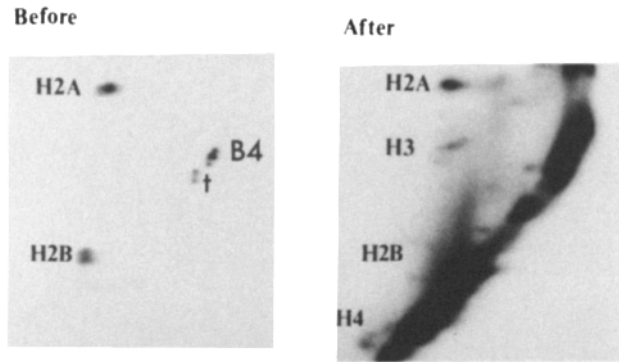


Figure 4. Newly synthesized proteins taken up by sperm chromatin during complete pronuclear assembly in the radiolabeled low speed egg extract before and after replication of the pronuclei. (Left) Radiolabeled proteins synthesized in the eggs before extract preparation, that are taken up by sperm chromatin during pronuclear assembly. The positions of histones H2A, H2B, B4, and protein t are indicated. In this experiment aphidicolin (125 $\mu\text{g/ml}$) is added to the extract to inhibit DNA replication. (Right) Radiolabeled proteins synthesized in the eggs before extract preparation that are taken up by replication sperm pronuclei. The positions of the core histones are indicated. No aphidicolin is added to this extract.

synthesis from endogenous mRNA in the egg (see later). The histone H2A.X that was previously synthesized during oogenesis is stored as a complex with nucleoplasmin (Dilworth et al., 1987).

Similar results are obtained using low speed extracts of *Xenopus* eggs in which the histones have been radiolabeled. This experiment examines protein uptake under conditions where the possibility of DNA replication is eliminated through the addition of aphidicolin, which inhibits DNA polymerase α . Thus, before replication (Fig. 4, *Before*) only H2A, H2B, HMG2, and B4 are incorporated into sperm chromatin. Analysis of paternal pronuclei following replication in the radiolabeled extract (Blow and Laskey, 1986; Wolffe, 1993; in the absence of aphidicolin), reveals that all four core histones plus a great many other radiolabeled proteins are incorporated into nuclei (Fig. 4, *After*). Replication was monitored by incorporation of radiolabeled dCTP into DNA, more extensive analysis has shown efficient semi-conservative DNA replication (Wolffe, 1993). Thus the replication process makes use of both stored proteins and those proteins that are being synthesized within the egg, these include histones H3 and H4. We conclude that during both the first and second stages of sperm decondensation in the assembly of a replication competent paternal pronucleus, there is only the incorporation of a limited number of proteins into the remodeled sperm chromatin in stoichiometric amounts. These proteins include core histones H2A, H2A.X, and H2B, HMG2 and the histone B4. Following replication, a much broader spectrum of proteins undergoing active synthesis in the egg are incorporated into nuclei. It is surprising that relatively large quantities of nonhistone proteins are incorporated into replicating nuclei compared to the histones (Fig. 4, *After*). This may reflect a greater reliance on de novo synthesis of nonhistone proteins to assemble nuclei in the egg following fertilization than of the core histones. We next examined what changes in chromatin structure occur concomitant with these major changes in protein composition.

The Assembly of Nucleosomal Arrays during the Remodeling of Sperm Chromatin

Previous work has suggested that although sperm chromatin has unusual sensitivity to micrococcal nuclease compared to somatic nuclei, a repeating structure similar to that of a nucleosomal array could be visualized following resolution of DNA fragments derived from micrococcal nuclease digestion (Wolffe, 1989a). The DNA fragments of nucleosomal size (multiples of ~ 180 bp) within sperm chromatin are not as clearly resolved as those resulting from digestion of somatic (RBC chromatin) especially on extended micrococcal nuclease digestion (Fig. 5 A). This could be explained by a reduction of the differential accessibility of micrococcal nuclease to linker DNA compared to DNA in the "nucleosomal core" within sperm chromatin compared to somatic chromatin. In somatic chromatin the linker DNA between nucleosomal cores is much more accessible to micrococcal nuclease than the DNA within the core (van Holde, 1988), hence, the linker DNA is digested before DNA within the core (Noll and Kornberg, 1977). Exposure of sperm chromatin to increasing concentrations of micrococcal nuclease leads to a heterogeneous distribution of DNA fragment sizes. Moreover, in contrast to the clear resolution of chromatosome (168 bp; Simpson, 1978) and core particle (146 bp) length DNA fragments on extensive micrococcal nuclease digestion of somatic (RBC) chromatin, no significant selective accumulation of fragments of this length is clearly resolved on digestion of sperm chromatin (Fig. 5 B). The chromatosome is a 168-bp long DNA fragment that interacts with both core histones and a single molecule of linker histone, it accumulates as a kinetic intermediate during micrococcal nuclease digestion of normal somatic cell chromatin (Simpson, 1978). This failure to accumulate chromatosome or core length DNA fragments indicates that DNA in the "linker" has comparable accessibility to micrococcal nuclease in sperm chromatin to that associated with the core his-

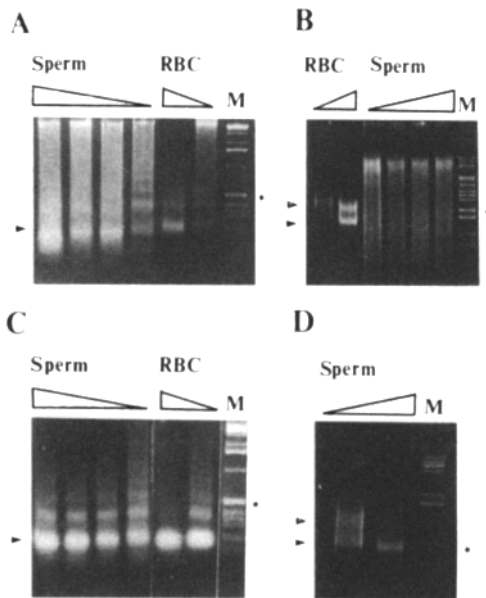


Figure 5. Remodeling of nucleosomal arrays during sperm nuclear decondensation in the high speed egg extract. Markers are for *A* and *C*, a 1-kb ladder (GIBCO-BRL, Gaithersburg, MD), the marker DNA fragment indicated by the asterisk is 517 bp in length. Markers for *B* and *D* are the same 1-kb ladder (GIBCO-BRL), however the DNA fragment indicated by the asterisk is 154 bp in length. (*A*) Micrococcal nuclease digestion of sperm chromatin alone is shown (*Sperm*), together with *Xenopus* erythrocyte chromatin (*RBC*). DNA is shown after deproteinization resolved on a 1.5% agarose gel. The size of nucleosome core length DNA is indicated by the horizontal arrow. (*B*) Micrococcal nuclease digestion of sperm chromatin alone does not lead to significant accumulation of stable nucleosome core (146 bp) or chromatosome (168 bp) length DNA fragments. Extensive micrococcal digests of sperm chromatin (*Sperm*) and of erythrocyte chromatin (*RBC*) are shown resolved on a nondenaturing 10% polyacrylamide gel. Nucleosome core length DNA is indicated by the closed horizontal arrow, chromatosome length DNA by the open horizontal arrow. (*C*) As in *A* except the sperm chromatin has been decondensed in the presence of the high speed egg extract. (*D*) As in *B* except the sperm chromatin has been decondensed in the presence of the high speed egg extract.

tones. In contrast, sperm nuclei that have been incubated in the high-speed egg extract prior to micrococcal nuclease digestion, generate a stable nucleosomal array following digestion (Fig. 5 *C*, Wolffe, 1989a; Philpott and Leno, 1992). Importantly, more extensive micrococcal digestion of remodeled sperm chromatin reveals the presence of clearly resolved chromatosome and core particle length kinetic intermediates (Fig. 5 *D*). The presence of core particle length DNA indicates association of all of the core histones. The presence of chromatosome length DNA indicates that a protein is acting like a true linker histone in the presence of the core histone within the egg extract, in order to assemble the chromatosome. We suggest that the remodeling of *Xenopus* sperm chromatin involves structural transitions from a structure in which nucleoprotein complexes distinct from nucleosomes are regularly arrayed along the DNA molecule (Fig. 5 *A*) to one in which a more conventional nucleosome organization exists (Fig. 5, *C* and *D*). This transition involves the loss of proteins *z* and *y* and the accumulation of H2A,

H2A.X, H2B, HMG2, and B4 (Fig. 1). We next asked what role the histone B4 had in the chromatin structural transitions observed.

Histone B4 Is Necessary for the Formation of Chromatosome Structures during the Remodeling of Sperm Chromatin

We depleted *Xenopus* egg extracts of the B4 protein using specific antibodies (Fig. 2). Serial dilution of the extract, followed by slot blotting and immunodetection revealed that B4 concentrations were severely reduced by immunodepletion (see Fig. 6 *B*). We then allowed sperm nuclei to be remodeled in mock- and B4-depleted extracts. Depletion of histone B4 has no effect on sperm chromatin decondensation at the light microscopic level (not shown). Sperm nuclear chromatin was then digested extensively with micrococcal nuclease. Chromatosome size DNA fragments (168 bp) only accumulated in the egg extracts containing the histone B4. Thus incorporation of histone B4 stabilizes histone-DNA contacts that are resistant to micrococcal nuclease from 146 to 168 bp (Fig. 6 *A*, note that equal amounts of DNA are loaded in lanes 4 and 5). We conclude that histone B4 is functioning like a linker histone in the assembly of a chromatosome (Simpson, 1978; Hayes and Wolffe, 1993). These results were confirmed with a range of digestion conditions (not shown). Our next experiments examined whether depletion of the histone B4 from egg extracts would influence the spacing of nucleosomes, which has been suggested as a function for linker histones (Rodriguez-Campos et al., 1989). Remarkably we find that depletion of B4 has no effect at all on the spacing of nucleosomal arrays (~ 180 bp) within the resolution of this assay (± 5 bp) (see Fig. 10 *A*). We conclude that B4 is not important in determining the separation of histone octamers along the DNA molecule.

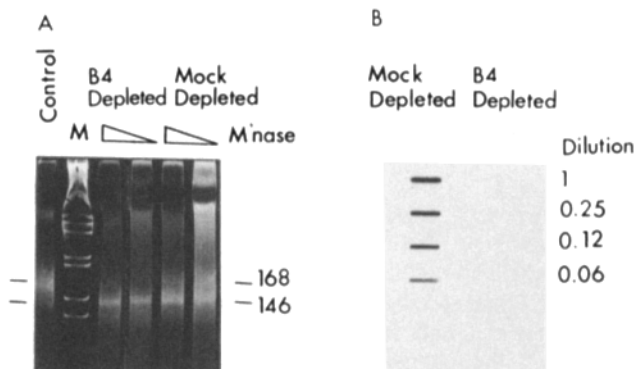


Figure 6. Histone B4 is essential for the assembly of chromatosomes in the egg extract. *Xenopus* high speed egg extract was depleted of histone B4 using polyclonal antibodies (Materials and Methods). (*A*) *Xenopus* sperm chromatin was allowed to decondense in high speed egg extract that was depleted for B4, or that was mock depleted. The chromatin was then extensively digested with micrococcal nuclease, DNA was deproteinized and fragments resolved on a nondenaturing 8% polyacrylamide gel. As a control sperm chromatin that had been decondensed in untreated extract containing B4 protein was also digested (*Control*). Markers are a 1-kb ladder (GIBCO-BRL). (*B*) Depletion was assayed by slot blotting serial dilution of mock depleted and depleted extract, and then immunoblotting using antibodies against histone B4.

Histone Phosphorylation during the Sperm Chromatin Remodeling Process

We wished to examine whether the phosphorylation of histones might have a role in the sperm chromatin remodeling process. Several correlations have been made between histone phosphorylation and biological processes. For example, phosphorylation of histone H2A.X has been proposed as a major determinant of nucleosomal spacing during chromatin assembly on small plasmid DNA molecules (Kleinschmidt and Steinbesser, 1991). Phosphorylation of histone H3 has been correlated with transcriptional activation (Mahadevan et al., 1991). Linker histone phosphorylation has been paradoxically associated with both mitotic chromosome condensation (Roth and Allis, 1992), and with chromosome decondensation and transcriptional activity (Aubert et al., 1992). We initially incubated *Xenopus* sperm nuclei with [γ^{32} P]-ATP in isolation in order to determine whether any sperm chromatin associated histone kinases might exist. We detect the specific phosphorylation of sperm histone H2A (Fig. 7, 32 P-labeled). As a control we incubated the egg extract alone with [γ^{32} P]ATP and failed to observe any histone phosphorylation (Fig. 7, Extract 32 P-labeled). We have not yet identified the spots reflecting radiolabeling within the egg extract alone. Our next experiments involved the incubation of *Xenopus* sperm nuclei at decreasing ratios of nuclei to egg extract in the presence of [γ^{32} P]ATP (Fig. 5). As previously reported (Kleinschmidt and Steinbesser, 1991), H2A.X is phosphorylated in chromatin, however we find that histone H2A is also phosphorylated. The proportion of H2A.X that is phosphorylated compared to H2A increases as the ratio of sperm nuclei to cytoplasm decreases, until the equivalent amounts of phosphorylated H2A and H2A.X are present (Fig. 8, A-C). Thus, both H2A.X incorporation into chromatin and H2A.X phosphorylation might appear to be a consequence of the more effective decondensation of sperm chromatin observed under these conditions and may have a role in the assembly of nucleosomal arrays (Kleinschmidt and Steinbesser, 1991; but see Figs. 9 and 10 later). Surprisingly histone H4 is also phosphorylated in sperm nuclei (Fig. 8). This phosphorylation probably derives from kinases present within the egg extract, since H4 is not phosphorylated in sperm in the absence of extract. Thus both histones H2A and H4 which are already within sperm chromatin, and

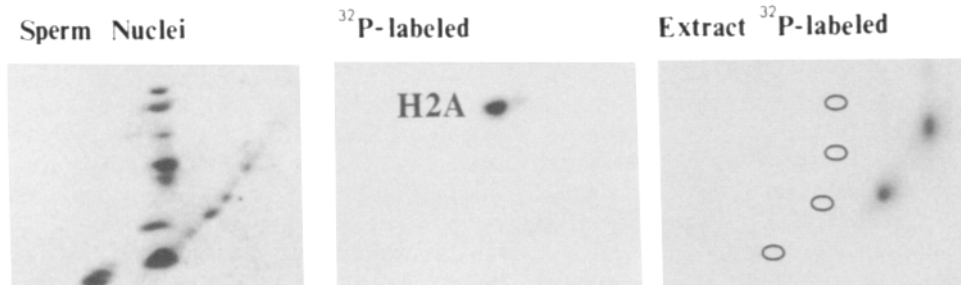


Figure 7. Phosphorylation of proteins in sperm chromatin alone, on in egg extract alone. Coomassie Blue stained sperm nuclear proteins are shown for reference (*Sperm Nuclei*). An autoradiogram is shown of sperm nuclear proteins incubated with [γ^{32} P]ATP (32 P-labeled) or of high speed egg extract incubated with [γ^{32} P]-ATP (*Extract 32 P-labeled*). The position of H2A in radiolabeled sperm chromatin is indicated, as are the approximate mobilities of the core histones (*open circles*) in the radiolabeled extract.



Figure 8. Phosphorylation of proteins in sperm chromatin incubated in high speed egg extract. Sperm nuclei were incubated in high speed egg extract in the presence of [γ^{32} P]ATP at three ratios: (A) 2×10^6 nuclei per μ l extract; (B) 5×10^5 nuclei per μ l extract; and (C) 2×10^5 nuclei per μ l extract. The stained gels of marker histones from *Xenopus* erythrocyte nuclei are shown in the lower panel, radiolabeled proteins in sperm chromatin in the upper panel. The positions of core histones H2A, H2A.X, and H4 are indicated.

histone H2A.X which is assembled into sperm chromatin from a storage form in egg cytoplasm are phosphorylated. Quantitation of the levels of phosphorylation (not shown) suggest that >50% of these histones are stably phosphorylated in a decondensed sperm nucleus.

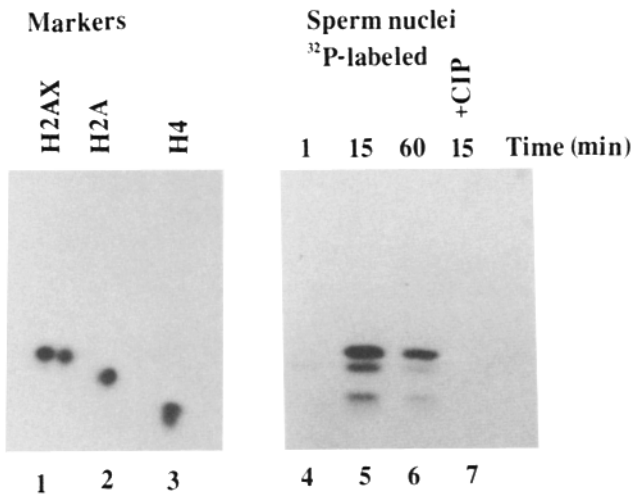


Figure 9. Kinetics of histone phosphorylation during sperm chromatin remodelling in high speed egg extracts and inhibition of phosphorylation by calf intestinal phosphatase (*CIP*). Markers are of the phosphorylated histones shown in Fig. 8 resolved on two-dimensional gels, that have been excised, eluted and resolved on an 18% polyacrylamide gel containing SDS (lanes 1-3). (Note: the two spots for H2A.X are due to the loading of two adjacent gel wells.) The kinetics of phosphorylation of sperm chromatin proteins during decondensation in high speed egg extract is shown, proteins were isolated after 1, 15, or 60 min of incubation plus [γ - 32 P]ATP. In lane 7, both sperm chromatin and egg extract were incubated with calf intestinal phosphatase (*CIP*) (20 U) before mixing with [γ - 32 P]ATP.

We wished to determine whether the phosphorylation of histones during the remodeling of *Xenopus* sperm chromatin was necessary for the assembly of nucleosomal arrays. Our first experiments were to definitively attribute the identity of the phosphorylated proteins visualized on the two-dimensional gels. The proteins provisionally identified as phosphorylated H2A, H2A.X, and H4 were resolved on two-dimensional gels, excised from the gel, and resolved on an 18% polyacrylamide gel containing SDS (Laemmli, 1970). As previously reported (Mannironi et al., 1989; Kleinschmidt and Steinbesser, 1991) histone H2A.X has a mobility less than that of histone H2A in this type of electrophoresis system (Fig. 9, lane 1). The kinetics of histone phosphorylation when sperm nuclei are incubated in the high speed extract are rapid (Fig. 9, lanes 4-6). Histone H2A is phosphorylated within 1 min, and all three core histones, H2A, H2A.X, and H4 are modified within 15 min. In these experiments constant amounts of total sperm nuclear proteins were loaded in every gel lane. The rapid phosphorylation of histone H2A is probably due to the histone and histone kinase already being present in sperm chromatin (Fig. 7). Stable phosphorylation of the core histones can be inhibited by incubation of sperm nuclei and high speed egg extract with calf intestinal phosphatase (Fig. 9, lane 7, *CIP*). Therefore we next asked whether nucleosomal arrays could be assembled in the absence of stable histone phosphorylation.

The assembly of nucleosomal arrays during sperm chromatin remodeling is unaffected by the absence of stable histone phosphorylation (Fig. 10 B). Therefore, we propose that there is no requirement for stable phosphorylation of the histones in order for nucleosome assembly to occur or for

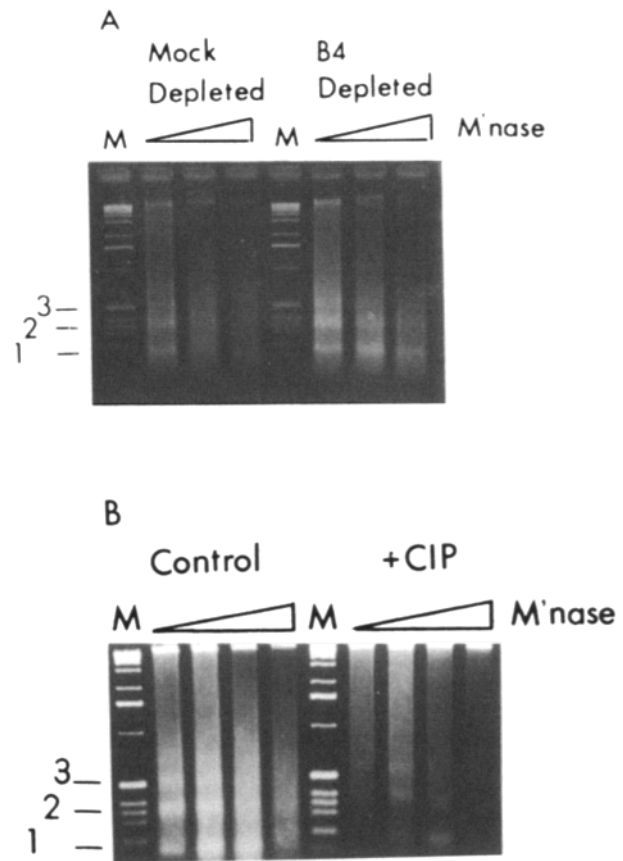


Figure 10. Neither histone B4 nor stable histone phosphorylation are required for the assembly of spaced nucleosomal arrays. (A) Histone B4 was either depleted or mock depleted from high speed egg extracts before sperm nuclear decondensation in these extracts (see Fig. 6). The decondensed sperm chromatin was digested with micrococcal nuclease, digestion products were deproteinized and resolved on a 1.5% agarose gel before staining with ethidium bromide. The numbers and horizontal lines refer to DNA fragments corresponding to one (1), two (2), or three (3) nucleosomes. Markers are a 1-kb ladder (GIBCO-BRL). The asterisk indicates a DNA fragment size of 517 bp. (B) As in A except decondensation of sperm nuclei was in the presence (+ *CIP*) or absence (*Control*) of nuclei and extract treated with calf intestinal phosphatase (see Fig. 9).

the nucleosome to be physiologically spaced (~180-bp per nucleosome) during the assembly of the paternal pronucleus from sperm chromatin.

Discussion

We have determined the major changes in chromosomal protein composition during the transition from a condensed sperm nucleus to the paternal pronucleus. In agreement with previous work, histones H2A and H2B (Philpott and Leno, 1992), and H2A.X (Dilworth et al., 1987; Mannironi et al., 1989; Kleinschmidt and Steinbesser, 1991) are incorporated into the chromatin of the paternal pronucleus. Here we demonstrate that HMG2 and histone B4 are also incorporated into the paternal pronucleus. Sperm-specific proteins z and y are lost from chromatin during this remodeling process (Abé, 1987; Philpott and Leno, 1992). We have also found

that major transitions occur in core histone phosphorylation during sperm chromatin remodeling. These include not only phosphorylation of histone H2A.X, as previously reported for chromatin assembly on naked DNA (Kleinschmidt and Steinbesser, 1991) but also phosphorylation of histones H2A and H4.

The Transition from the Condensed Sperm Nucleus to the Paternal Pronucleus

X. laevis sperm nuclei retain a full complement of histones H3 and H4 which form the arginine-rich kernel of the nucleosome core (Fig. 1 A; Camerini-Otero et al., 1976; Hayes et al., 1991). The presence of the (H3/H4)₂ tetramer in *X. laevis* sperm chromatin probably accounts for the pre-existing ladder of spaced nucleoprotein complexes following micrococcal nuclease digestion (Fig. 5 A). The nucleosome is built around the (H3/H4)₂ tetramer, histones H2A/H2B can only bind to DNA if the tetramer has wrapped 120-bp DNA around it, and histone H1 can only bind once histones H2A/H2B are present (van Holde, 1988; Hayes et al., 1991).

In contrast to the full complement of histones H3 and H4, histones H2A and H2B are relatively deficient in *X. laevis* sperm chromatin (Risley, 1981; Wolffe, 1989b), and histone H1 is absent from sperm (Wolffe, 1989a). This is consistent with the lack of protection of core and chromatosome length DNA following extended micrococcal nuclease digestion (Fig. 5 A). Although the molecular mechanisms leading to histone H2A, H2B, and H1 displacement during spermatogenesis and spermiogenesis are unknown, it is likely that these proteins are replaced with sperm-specific proteins (Abé 1987, Fig. 1 A, proteins z and y). These proteins are removed remarkably rapidly on incubation of sperm nuclei in *Xenopus* egg extract (Fig. 1 B). This removal is believed to be catalyzed by nucleoplasmin, the molecular chaperone that also directs the sequestration of histones H2A.X and H2B in the oocyte (Dilworth et al., 1987; Philpott and Leno, 1992).

Our two-dimensional gel system clearly resolves the incorporation of both histones H2A and H2A.X (protein s) into sperm chromatin during the remodeling process (Fig. 1 B). H2A.X is absent from sperm chromatin before incubation in the egg extract (Fig. 1, compare A with B). Furthermore, examination of newly synthesized histones in the egg that are incorporated into sperm pronuclei, does not reveal any H2A.X incorporation, only that of H2A and H2B (Figs. 3 and 4). Thus the H2A.X must derive from protein synthesized and stored in the developing oocyte (Woodland and Adamson, 1977). Incorporation of core histones H2A and H2B, together with pre-existing histones H3 and H4 accounts for the protection of nucleosome core length (146 bp) DNA fragments on extended micrococcal nuclease digestion (Fig. 5 D).

We demonstrate that two other proteins aside from the core histones are incorporated into sperm chromatin during the assembly of the pronucleus, the linker histone B4 (Figs. 1 and 2) and HMG2 (Fig. 1). Both of these proteins are present in amounts such that the majority of nucleosomes would be expected to contain a molecule of B4 and/or HMG 2 (Fig. 1 B). The expression of the histone H1-like protein, B4 is restricted to oogenesis and early embryogenesis (Smith et al., 1988; Dimitrov et al., 1993; Hock et al., 1993; Cho and Wolffe, 1994). Depletion of B4 protein from the *Xenopus* egg

extract prevents the accumulation of chromatosome length DNA (~168 bp) during micrococcal nuclease digestion of pronuclear sperm chromatin (Fig. 6). However, chromatosome length DNA accumulates in normal decondensed sperm chromatin containing B4 (Figs. 5 D and 6). Since the formation of a chromatosome requires the presence of a linker histone molecule (Simpson, 1978; Hayes and Wolffe, 1993) we propose that histone B4 is functioning as a true linker histone in the paternal pronucleus.

The presence of HMG 1- and 2-like molecules has been previously reported in *X. laevis* oocytes (Kleinschmidt et al., 1983; Weisbrod et al., 1982). It has also been suggested that HMG 1 might be capable of replacing histone H1 in chromatin (Jackson et al., 1979). However, normally HMG 1 or 2 is associated with a relatively minor fraction of chromatin <5% (Goodwin et al., 1977; Isackson et al., 1980). Within sperm chromatin, the stoichiometry of the protein would suggest that an HMG 2-like molecule would potentially be associated with ~50% of the chromatin. Such an abundant protein might be involved in nucleosome assembly (Bonne-Andrea et al., 1984), or the assembly of nucleosomal arrays. The *Xenopus* sperm pronucleus has a functional requirement for rapid replication and chromatin assembly, but is normally transcriptionally quiescent (Wolffe, 1989a). Future experiments will explore the role of *Xenopus* HMG 2 in these nuclear functions.

Replication of the paternal pronucleus is necessary for incorporation of newly synthesized histones H3 and H4 in the sperm pronucleus (Fig. 4). This result demonstrates that histones H3 and H4 within the sperm pronucleus do not readily exchange with histones H3 and H4 in the low speed extract in the absence of replication. This is consistent with the results in mammalian cells dividing in culture (Louters and Chalkley, 1985; Jackson, 1990). During replication newly synthesized histones H3 and H4 will be assembled into nucleosomes together with nascent DNA.

The Assembly of Nucleosomal Arrays

Several hypotheses have been proposed to account for the assembly of nucleosomal arrays with a physiological spacing of approximately 170–180 bp (reviewed by Wolffe, 1992). These include interaction of the core histones alone with DNA (Almouzni et al., 1991), interaction of core histones plus phosphorylated histone H2A.X with DNA (Kleinschmidt and Steinbesser, 1991), interaction of core histones plus linker histones with DNA (Rodriguez-Campos et al., 1989), and interaction of core histones plus HMG 14/17-like proteins with DNA (Tremethick and Frommer, 1992). We do not detect HMG 14/17-like proteins in *Xenopus* sperm chromatin (Fig. 1) or in egg extracts (Crippa et al., 1993; Weisbrod et al., 1982). This would appear to exclude a direct structural role for these proteins in establishing spaced nucleosomal arrays in *Xenopus* sperm chromatin. More surprisingly there is no histone H1 in *Xenopus* eggs or sperm (Wolffe, 1989a,b), however, histone B4 is necessary for the assembly of chromatosomes in the sperm pronucleus (Fig. 5). Our results demonstrate the depletion of histone B4 from the egg extract does not prevent the assembly of nucleosomal arrays with a physiological spacing of ~180 bp (Fig. 10 A). This implies that the assembly of chromatosomes is not necessary to assemble a physiologically spaced nucleosomal array.

We also examined the phosphorylation of histones during sperm chromatin remodeling. We find that histones B4, H3, and H2B are not phosphorylated during the remodeling process. However a histone H2A kinase is associated with sperm chromatin, and the histone H2A in sperm chromatin is phosphorylated in the presence of [γ ³²P]ATP (Fig. 7). Histones H4 and H2A.X are also phosphorylated within sperm nuclei in the presence of the high speed egg extract. The *in vivo* phosphorylation of H4 that has been documented is at the NH₂-terminal serine residue, this occurs in the cytoplasm shortly after histone synthesis (Sung and Dixon, 1970; Ruiz-Carillo et al., 1975; Jackson et al., 1976). Like H2A, no functional significance has been attached to the phosphorylation of histone H4. In contrast, the phosphorylation of histone H2A.X has been correlated with the reconstitution of physiologically spaced chromatin *in vivo* (Kleinschmidt and Steinbesser, 1991).

We find that histone H2A.X incorporation and phosphorylation correlates with the efficiency of decondensation of sperm chromatin into pronuclei (Fig. 8). However, we also find that complete elimination of stably phosphorylated amino acids using calf intestinal phosphatase (Fig. 2) does not influence nucleosome spacing (Fig. 10). These results contrast with those of Kleinschmidt and Steinbesser (1991) who observed an elimination of nucleosome alignment completely at high alkaline phosphatase concentrations (cited as data not shown; Kleinschmidt and Steinbesser, 1991). However our experiments differ in that we make use of sperm chromatin as a template whereas Kleinschmidt and Steinbesser (1991) examine *de novo* nucleosome assembly on naked duplex DNA. Nevertheless it seems unlikely that histone H2A.X which constitutes at most 50% of the H2A in sperm chromatin (Fig. 1) could account for 100% of nucleosome spacing. This observation coupled to the physiological spacing of nucleosomes in the absence of significant phosphorylation of H2A.X lead us to suggest that histone phosphorylation is not essential for the assembly of canonical nucleosomes or nucleosomal arrays within the paternal pronucleus.

We conclude that the core histones themselves, in particular histones H2A and H2B play the major role in nucleosome spacing (see Almouzni et al., 1991). We note that a nucleosome-like ladder is pre-existing in *Xenopus* sperm (Fig. 5 A; Wolffe, 1989a), perhaps this acts as a framework for subsequent assembly of true nucleosomal arrays. Considerable evidence supports interactions of the core histone with 160–180 bp of DNA. Hydroxyl radical footprinting of DNA associated with the histone octamer reveals ~180 bp of protection (Hayes et al., 1990). Likewise histone–DNA cross-linking reveal 180 bp of contacts between the histone octamer and DNA (Pruss and Wolffe, 1993). Our results strengthen the hypothesis that the assembly of physiologically spaced nucleosome arrays depends on the core histones alone, and their interactions during the assembly of the nucleosome core.

Note

While this work was in preparation, Ohsumi and colleagues reported the role of histone H1X (Ohsumi and Katagiri, 1991) in chromosome condensation in *Xenopus* mitotic extracts (Ohsumi et al., 1993). The authors conclude that H1X is not required for mitotic chromosome condensation or the

assembly of nucleosomal arrays, but is required for the assembly of a chromatosome. While it is possible that H1X is identical to histone B4 based on our observations in this manuscript, such an identity has not been established at this time.

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