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 chromatosome 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; Wolfe, 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 histone 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), 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; Wolfe et al., 1987; see also Annunzi-
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 by treatment with protein 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 μl of [3H]lysine (75–100 Ci/mmol; Amersham Corp., Arlington Heights, IL) and 300 μl of [3H]arginine (35–70 Ci/mmol, Amersham Corp.) were dried and resuspended in 50 μl of injection buffer (88 mM NaCl, 10 mM Hepes, pH 6.9) and 20 μl 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. Demembranation was performed by the addition of 20 μl 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 μg/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 [γ-32P]ATP with the constituents. Normally 106 nuclei, 10-μl extract or the two combined were radiolabeled with 10 μCi of [γ-32P]ATP unless indicated otherwise.

Preparation of Antibodies to Histone B4, Immuno blotting, 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 AGT 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 134 and the latter primer introduced a Xhol site nine nucleotides beyond the TAG translational termination codon (Smith et al., 1988). The resulting fragment was restric ted with these two enzymes and ligated into NcoI-XhoI-restricted pGEX-KG 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) pLYS, from which the fusion protein was isolated after induction with isopropyl-β-D-thiogalactopyranoside (IPTG).

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 μg/ml lysosome, 75 μg/ml PMSF) was then added and the incubation on ice continued for an additional 80 min. DNase I (20 μg/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 Alto, 1989). Importantly hydrolysis of ATP or of other nucleotides was found not to be necessary to provide an energy source for chromatin assembly (Almouzni and Mechali, 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 (Tremethick and Frommer, 1992). Importantly, exogenous ATP is not always required for the assembly of spaced nucleosomes. Xenopus oocytes, sperm, and eggs are severely deficient in normal somatic histone HI and HMG 14/17-like molecules may have a key role in chromatin assembly (Almouzni et al., 1991) or of HMG 14/17-like proteins (Tremethick and Frommer, 1992).

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 HI and instead contain a cleavage stage linker histone B4 that has ~30% sequence identity to histone HI (Smith et al., 1988; Wolfe, 1989a,b; Dimitrov et al., 1993; Hock et al., 1993).

Histone B4 is an excellent candidate to replace histone HI in normal embryonic chromosomes. Nevertheless, supplementation of oocyte or egg extracts containing histone B4 with exogenous histone HI will increase the distance between nucleosomes from 180 to as much as 220 bp (Rodriguez-Campos et al., 1989). This suggests that exogenous histone HI 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 HI-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.
pellets were carefully resuspended in wash buffer (buffer B plus 10 mM EDTA, 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 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 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 buffer D plus 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 the inclusion body preparations for the initial injection and subsequent boosts.

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 specific activity nucleosome peptide mix (10% 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 100 μl 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 ethanolic 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 performed 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 Dimitrov et al., (1981).

For identification of the tritium-labeled or -phosphorylated histones the gels were stained with Coomassie to determine the position of unlabelled 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 nucleoplasmin (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 chromosomnal 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. 1.4). 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 to 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 spermigenesis (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.
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 (0) and monacetylated (1) 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.

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.Tyr.Ala.Tyr.Phe.Val.Gln.Tyr.Cys.Arg.Glu.Glu.His.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 sequested 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
Xenopus 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 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; Wolfe, 1993; in the absence of aphidicolin), reveals that all the 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 extensive 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 heterogenous 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 histone.
Chromatosome. We suggest that the remodeling of core historic within the egg extract, in order to assemble the protein is acting like a true linker histone in the presence of the presence of chromatosome length DNA indicates that a pro-
modeled sperm chromatin reveals the presence of clearly re-
aldes (Fig. 5 C, Wolffe, 1989a; Philpott and Leno, 1992). The presence of core particle length core particles. In contrast, sperm nuclei that have been incubated in the high-speed egg extract prior to micrococcal nuclease di-
gestion, generate a stable nucleosomal array following di-
gestion (Fig. 5 C, Wolffe, 1989a; Philpott and Leno, 1992). Importantly, more extensive micrococcal digestion of re-
modeled sperm chromatin reveals the presence of clearly re-
solved chromatosome and core particle length kinetic inter-
mediates (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 pro-	ein 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 nucleo-
somes are regularly arrayed along the DNA molecule (Fig. 5 A) to one in which a more conventional nucleosome orga-
nization exists (Fig. 5, C and D). This transition involves the loss of proteins z and y and the accumulation of H2A,
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 [γ32P]-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, 32P-labeled). As a control we incubated the egg extract alone with [γ32P]ATP and failed to observe any histone phosphorylation (Fig. 7, Extract 32P-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 [γ32P]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 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 [γ32P]ATP. In lane 7 both sperm chromatin and egg extract were incubated with calf intestinal phosphatase (CIP) (20 U) before mixing with [γ32P]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 (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

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 Steinbeisser, 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): 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 nucleoplasmmin, 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 synthesis 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 HMG2 (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 Steinbeisser, 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 $[^{32}\text{P}]	ext{ATP}$ (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 NH$_2$-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 Steinbeisser, 1991). However our experiments differ in that we make use of sperm chromatin as a template whereas Kleinschmidt and Steinbeisser (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; Wolfe, 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 crosslinking reveal 180 bp of contacts between the histone octamer and DNA (Pruss and Wolfe, 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 HIX (Ohsumi and Katagiri, 1991) in chromosome condensation in Xenopus mitotic extracts (Ohsumi et al., 1993). The authors conclude that HIX 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 HIX 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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