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Review

Remodeling of sperm chromatin induced in egg extracts of amphibians

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ABSTRACT Sperm nuclear basic proteins of *Bufo japonicus* consist of 2 distinct protamines, whereas those of *Xenopus laevis* consist of 6 sperm-specific basic proteins (SP1-6) in addition to H3, H4 and smaller amounts of H2A and H2B. Cloning of pertinent cDNAs and partial amino acid sequence studies suggested that these 6 sperm-specific proteins of *Xenopus* are encoded by 3 distinct genes. Despite differences in their initial compositions of chromatin, sperm nuclei exposed to amphibian egg extracts rapidly decondense, lose sperm-specific basic proteins, and concomitantly form an ordinary nucleosome core consisting of H2A, H2B, H3, H4, and cleavage-stage specific subtype H1X. In this remodeling process, nucleoplasmin plays dual roles as a molecular chaperone, selectively removing sperm-specific basic proteins from, and bringing H2A and H2B to, sperm DNA. Thus remodeling of chromatin is induced even in mammalian (human) sperm nuclei under defined conditions including nucleoplasmin and exogenous histones.

KEY WORDS: *sperm-specific basic nuclear proteins, cell-free system, nuclear reassembly, nucleoplasmin, molecular chaperone*

Introduction

Highly condensed nuclei of mature sperm of most animals contain a specific set of strongly basic, DNA-binding proteins, which are termed protamines or sperm-specific histones depending on their biochemical properties. This state of nuclei is achieved during spermatogenesis through the substitution or replacement of somatic histones by sperm-specific basic proteins, giving rise to transcriptionally inactive mature sperm. Because of this severe alteration of the chromatin state, the chromatin of a sperm nucleus must readjust to a more typical somatic state during fertilization in order to participate in the subsequent chromosomal activities in embryonic development. Thus sperm nuclei undergo two distinct, reverse types of dramatic remodeling of nucleoproteins during spermatogenesis and fertilization (cf., Poccia, 1986). The regulatory mechanisms underlying the remodeling leading to inactive chromatin during spermatogenesis have been explored to some extent (cf., Hecht, 1990). However, little is known about the process of remodeling to somatic conditions following fertilization.

The first events in fertilization that occur in sperm nuclei following their incorporation into the egg are the breakdown of nuclear envelopes, decondensation of highly compact chromatin, and the reassociation of the nuclear membrane to form swollen pronuclei leading to DNA synthesis (Longo, 1985). The decondensation of nuclei during pronuclear formation is thought to be associated with replacement of sperm-specific basic proteins by somatic histones. Biochemical analyses of this remodeling process, however, have so far been limited to studies on sea urchins, because a large number of pronuclei in a synchronous state can be obtained from moderately polyspermic sea urchin eggs (Poccia *et al.*, 1981). A cell-free system using amphibian egg extracts was originally developed to study the mechanisms involved in transformation of sperm nuclei after fertilization (Lohka and Masui, 1983; Iwao and Katagiri, 1984), and this system has been shown to induce the transformation of demembranated sperm nuclei into pronuclei and condensed chromosomes as successfully as in intact eggs (Lohka and Masui, 1983, 1984; Lohka and Maller, 1985; Ohsumi *et al.*, 1988). This system has also been used to study cell cycle regulation (Murray and Kirschner, 1989; Pfaller *et al.*, 1991), because of its obvious advantage for obtaining large quantities of cytoplasmic components from oocytes in synchronous stages of the cell cycle. We have been using the cell-free system of amphibian eggs for analyzing the post-fertilization remodeling of sperm nuclear chromatin, because of the ease with which large numbers of synchronously transformed nuclei with minimal contamination (10^{-4}) by female nuclei can be obtained at intervals of time after exposure to the egg cytoplasm.

Abbreviations used in this paper: AUT-PAGE, acid/urea/Triton X-100 polyacrylamide gel electrophoresis; PRA, protamine-removing activity; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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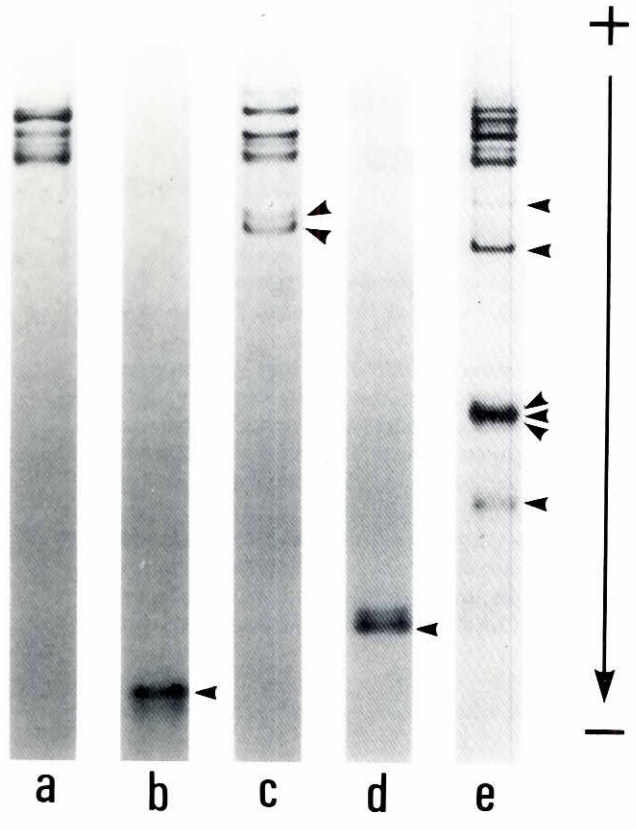

Fig. 1. AUT-PAGE profiles of sperm nuclear basic proteins (arrowheads) from salmon (b), bull frog *Rana catesbeiana* (c), toad *Bufo japonicus* (d) and clawed frog *Xenopus laevis* (e), in comparison with calf thymus histones (a).

In this paper, we report recent results obtained using an amphibian cell-free system to study the early events of sperm chromatin remodeling during fertilization. In view of their relevance to the mechanisms of chromatin remodeling, we also describe the molecular characteristics and synthesis of sperm-specific basic proteins during spermatogenesis in some amphibians. The implications of the finding of a unique chromatin composition of cleavage-stage nuclei and of the identification of a molecular chaperone that functions efficiently in the male chromatin remodeling process are discussed. There is a comprehensive review of how the *in vitro* system with amphibian eggs has contributed to the understanding of the mechanisms of chromatin assembly, DNA replication, and transcription (Almouzni and Wolffe, 1993).

Nuclear basic proteins in *Bufo* and *Xenopus* sperm

Sperm of anuran amphibians display wide ranges of nuclear basic proteins that differ in different genera. These proteins include protamines with an extremely high content of arginine, histone variants distinct from their somatic counterparts, and intermediates between them (Kasinsky *et al.*, 1978, 1985). As shown by acid/urea/Triton X-100 polyacrylamide gel electrophoresis (AUT-PAGE) in Fig. 1, the sperm-specific proteins of *Bufo japonicus* consist exclusively of fast-moving components without any somatic histones, while those of *Rana catesbeiana* include a slow-moving, sperm-specific histone variant in addition to a whole set of somatic core histones. The proteins from *Xenopus laevis* sperm consist of 6 different species of sperm-specific proteins (SP1-6) and core histones. Of the 4 core histones, the amounts of H2A and H2B are considerably less than those of H3 and H4 (Risley, 1990). Amino acid analyses indicated that the *Bufo* proteins contain 43.6% arginine and so are protamines, whereas those of *Rana catesbeiana* are more like H1 in having a high lysine content (Itoh, personal communication). Sperm-specific proteins in *Xenopus* are more complicated in that SP2 is similar to H4 in exhibiting a relatively high lysine/arginine ratio, while SP3-6 are unique in having a low lysine content and a high arginine content (Yokota *et al.*, 1991).

Information based on cloned cDNAs encoding sperm-specific proteins from *Bufo* and *Xenopus* has provided considerable insight into their exact molecular entities and interrelationships. The proteins from *Bufo* consist of two distinct components (P1 and P2) differing only in the 28th of their total 39 amino acid residues (P1, Asp; P2, Glu). This difference is due to a codon difference of GAT (Asp) and GAA (Glu) (Takamune *et al.*, 1991). Both P1 and P2 are

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trout(Iridine IA)     PRRRRSSSRPVRRRRRPRRVSRRRRRRGGRRRR
boar                  ARYRCCRSHSRSRCRPRRRRCRRRRRRCCPRRRRRAVCCRRYTVIRCRRC
mouse(mP1)            ARYRCCRSKSRSRCRRRRRRCRRRRRRCCRRRRRRCCRRRRSYTIRCKKY
Bufo(P1)              PPRRKRVSSAPRRRRRTYRRTTAHKHQDRPVHRRRRRRH
Bufo(P2)              PPRRKRVSSAPRRRRRTYRRTTAHKHQERPVHRRRRRRH
Xenopus(SP4)          SKVSGGSRRTRARRPMSNRRGRRSQSAAHRSRAQRRRRRTGTTRRARTSTARRARTRTARRSDLTRMMARDYGSDYRS
Xenopus(SP5)          SKMRGGSRRTRARRTMGNRRGRRSQTAAHRNRAQRRSRRTGTARRARTSTRRRRTSTAELTRMVSRAYGSEYRS
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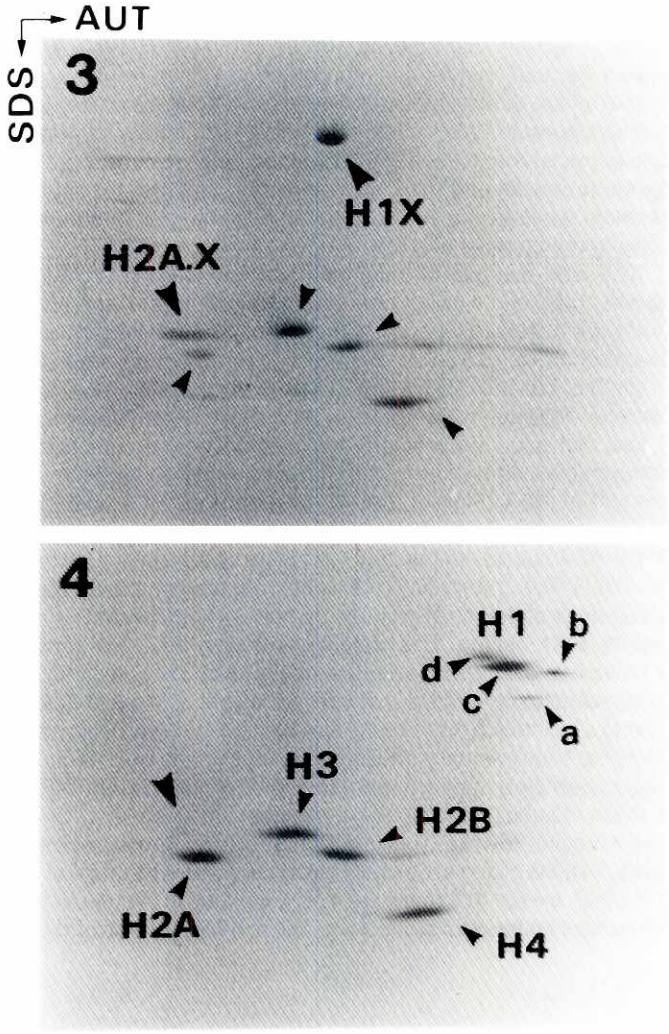
Fig. 2. Primary structures of sperm-specific nuclear basic proteins from *Bufo japonicus* and *Xenopus laevis*, in comparison with those of protamines from trout and mouse. *Hatched areas indicate arginine clusters. Sequences are those reported by Takamune et al. (1991) for Bufo, Hiyoshi et al. (1991) and Ariyoshi et al. (1993) for Xenopus, and Kleene et al. (1985) for others.*



Figs. 3 and 4. Two-dimensional electrophoresis of basic proteins *from male pronuclei after 60 min incubation with egg extracts* **(3)** *and from erythrocyte nuclei* **(4)** *of Bufo, showing the presence of specific variants of H1 and H2A in pronuclei. From Ohsumi and Katagiri (1991a).*

classified as protamines, since they have small molecular weights and possess plural arginine clusters, a property shared with the protamines of fishes, birds and mammals (Fig. 2). The protamines of *Bufo* and fishes differ from those of mammals in lacking a cysteine residue.

Of the 6 SPs in *Xenopus* sperm, SP4 with 78 amino acid residues and SP5 with 74 amino acid residues have been completely sequenced based on their cDNAs (Fig. 2) (Hiyoshi *et al.*, 1991; Ariyoshi *et al.*, 1994). The arginine contents (36-37%) and the presence of one arginine cluster, as well as the relatively larger molecular weights of both SP4 and SP5, indicate that they are intermediates between somatic histones and protamines. At present, only partial amino acid sequence data for 23-30 N-terminal regions of the other 4 SPs are available. SP1 and SP2 have identical 23 N-terminal amino acid sequences, and at least the 30- and 28 N-terminal sequences of SP6 and SP3, respectively, are the same as that of SP4 (Ariyoshi *et al.*, 1994). Thus we tentatively propose that the 6 SPs in *Xenopus* sperm are encoded by 3 different genes: those for SP1 and -2, SP3, -4 and -6, and SP5. Confirmation of this assumption awaits further complete sequencing to determine whether these sperm-specific proteins represent post-translational products derived from three mRNAs.

Northern blot analyses and *in situ* hybridization studies indicate that the *Bufo* protamine genes are first expressed in round spermatids, like their mammalian and avian counterparts (Hecht, 1990), whereas the *Xenopus* SP4 and SP5 genes are expressed earlier in pachytene stage spermatocytes, and the mRNAs are then post-transcriptionally processed (Hiyoshi *et al.*, 1991; Mita *et al.*, 1991; Ariyoshi *et al.*, 1994). Despite this difference in the stage of commencement of transcription, both *Bufo* protamines and *Xenopus* SP4 begin to accumulate in nuclei of late spermatids concomitant with condensation of elongating nuclei (Moriya and Katagiri, 1991). Fluorographic tracings of [^{14}C]Arg taken up by spermatogenic cells of *Xenopus* indicated that all the histones in mature sperm have been synthesized in primary spermatocytes. The synthesis of SPs in nuclear elongating spermatids occurs in the order, SP1 and SP3-5, then SP2, and finally SP6 (Yokota *et al.*, 1991). Studies are needed to examine the state of phosphorylation and/or dephosphorylation of amphibian sperm-specific proteins in relation to their role in condensation of sperm nuclei. A portion of protamine 2 (P2) of *Bufo* is known to be phosphorylated in mature sperm (Takamune *et al.*, 1991).

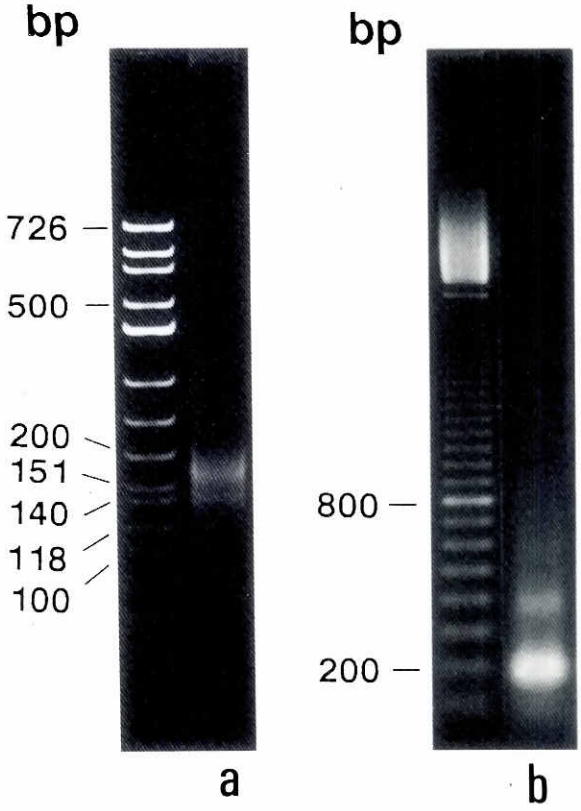


Fig. 5. Micrococcal nuclease digestion of *Xenopus* sperm nuclei after incubation with egg extracts for 30 min. *Nuclei were digested extensively* **(a)**, *or mildly* **(b)**. *Base pair (bp) ladder markers are shown on the left. Electrophoresis was carried out in polyacrylamide-* **(a)** *and agarose* **(b)** *gels.*

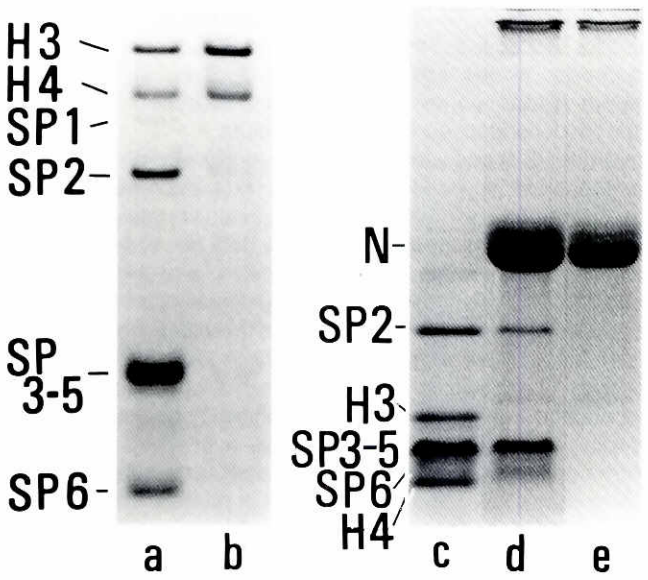


Fig. 6. AUT-PAGE (a,b) and SDS-PAGE (c-e) profiles of *Xenopus* sperm nuclear basic proteins *before* **(a,c)** *and after incubation with ion-exchange chromatographed egg extracts* **(b)** *or nucleoplasmin* **(d)**, *showing selective removal of sperm-specific proteins (SP1-6) in* **(b and d). (e)** *Nucleoplasmin (N).*

In summary, despite large differences in the composition and molecular entities of nuclear basic proteins, the mature sperm nuclei of *Bufo* and *Xenopus* are similarly highly condensed and transcriptionally inactive. Their chromatins are completely resistant to micrococcal nuclease digestion, and hence are not nucleosomal. However, because of their difference in chromatin composition, these sperm provide useful models for comparative analysis of the mechanisms of remodeling to somatic type chromatin induced by egg extracts, as discussed below.

Remodeling of chromatin in sperm nuclei exposed to egg cytoplasm

For preparation of active egg extracts for induction of sperm nuclear transformation *in vitro*, mature eggs collected from the uterus (ovisac) are dejellied, packed in test tubes with a minimum amount of ice-cold extraction medium (100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4), and centrifuged at 10,000xg for 10 min (2°). The semitransparent layer between the lipid (top) and the yolk (bottom) layers is used as active, low-speed egg extract. Before incubation with the egg extract, sperm are briefly treated with lysolecithin. Thus, in this assay system the breakdown of nuclear envelopes, the initial step of nuclear transformation in fertilizing sperm, is mimicked. When these lysolecithin-permeabilized sperm are incubated at 20-22°C with the egg extract obtained by low-speed centrifugation, the sperm nuclei undergo decondensation within 1 min, transform into well-developed pronuclei in 60 min, and form fully condensed chromosomes in 90 min *(Xenopus)* or 120-180 min *(Bufo)*. The preparation of egg extracts by the egg lysis procedure mimics the egg activation process in that it induces release of intracellular Ca^{2+} stores, allowing the progress of the state of egg cytoplasm from the M to S phase of the cell cycle. The addition of EGTA to chelate Ca^{2+} at the time of preparation of the extract prevents this transition of the cytoplasmic state, and so sperm-derived condensed chromosomes instead of swollen pronuclei are directly induced *in vitro* ("mitotic egg extracts"). Egg extracts prepared after centrifugation at >100,000xg (high-speed egg extracts) retain the ability to support initial decondensation of sperm pronuclei and their nuclear protein remodeling, but fail to promote well-developed pronuclei due to the lack of membrane components (Lohka and Masui, 1984; Newport, 1987).

The acid extracts of chromatin proteins were collected from sperm nuclei after various periods of incubation with egg extracts, and were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) or two dimensional electrophoresis in combination with AUT-PAGE (Ohsumi and Katagiri, 1991a). The nuclei of *Bufo* sperm completely lost protamines on incubation for 5 min, but acquired a whole set of nucleosomal core histones comparable to those found in erythrocyte nuclei, except for a small amount of H2A (Figs. 3 and 4). In pronuclei, H2A was mostly replaced by a putative variant H2A.X that has been shown to be a major H2A species in the egg cytoplasm of *Xenopus* (Dilworth *et al.*, 1987). The chromatin proteins of pronuclei exhibited a striking difference from those of erythrocyte nuclei in possessing a unique variety of H1 histone. This protein unique to pronuclei is similar to H1 histones in showing selective solubility in 5% perchloric acid, being extractable with NaCl at concentrations of below 0.7 M, and having a lysine-rich amino acid composition, but was not regarded as a post-translationally modified form of somatic type H1 judging from its peptide mapping patterns using V8 proteases. On the basis of these criteria, this H1 subtype was designated as H1X (Ohsumi and Katagiri, 1991a). The male pronuclei of *Xenopus* possess basic proteins giving essentially the same pattern as those of *Bufo* pronuclei, except that there are 4 H1X variants in *Xenopus*. Thus, the nuclear histones of pronuclei consist of H2B, H3, H4 and H2A.X

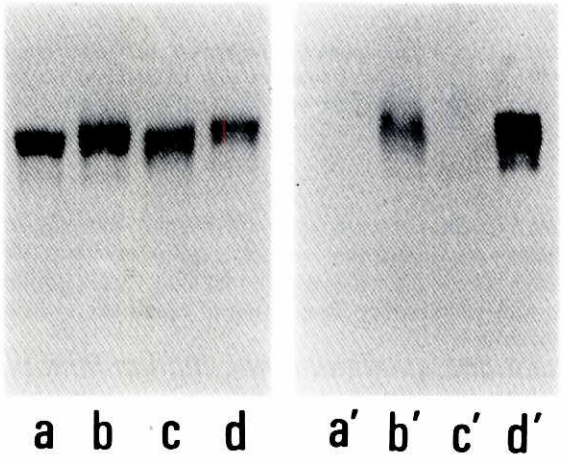


Fig. 7. Coomassie blue-stained native PAGE profiles of nucleoplasmin (a) and supernatants after incubation with nucleoplasmin from dithiothreitol-reduced human (b), -unreduced human (c), and *Bufo* (d) sperm nuclei. (a'-d') *Western blot analyses using rabbit anti-Bufo protamine serum of the gels in* **(a-d)**. *Note that protamines released from human* **(b')** *and Bufo* **(d')** *sperm nuclei are associated with nucleoplasmin, exhibiting slightly lower mobilities than free nucleoplasmin* **(a,c).** *The antibodies used were cross-reactive with human protamines. From Itoh et al. (1993).*

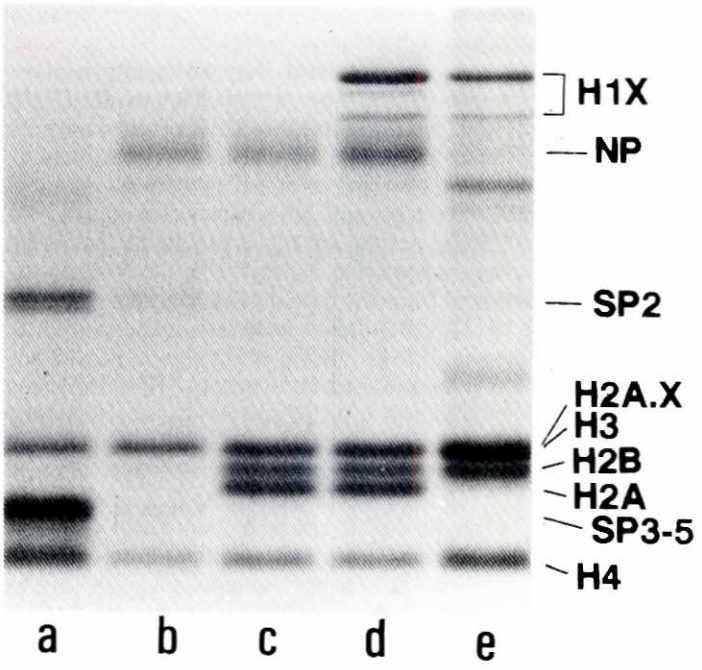


Fig. 8. SDS-PAGE profiles of acid-extracted proteins *of Xenopus sperm nuclei* **(a)** *and those after incubation with nucleoplasmin* **(b)**, *nucleoplasmin plus H2A and H2B* **(c)**, *nucleoplasmin plus H2A, H2B and H1X* **(d)**, *and egg extracts* **(e)**, *showing removal of SPs and assembly of added histones. NP, coprecipitated nucleoplasmin.*

predominating over H2A, as well as H1X in place of other H1 subtypes. During embryogenesis, this histone pattern is retained until the late blastula (stage 9), but at the midgastrula stage (stage 11) the amount of H1X decreases concomitantly with the appearance of a predominant H1 subtype similar to that in erythrocytes (Fig. 4). The relative amounts of H2A and H2A.X are also reversed during the gastrulation period resulting in H2A predominance over H2A.X. Thus the typical nuclear histone pattern of differentiated somatic cells is attained at the tailbud stage.

Conceivably our H1X has already been reported in *Xenopus* in two previous papers: (a) as an early embryonic, H1-like protein detected by its electrophoretic mobility (Koster *et al.*, 1979), and (b) as the B4 protein defined by its immunochemical localization and cDNA-based amino acid composition (Smith *et al.*, 1988). Very recent immunochemical observations using monoclonal antibodies detecting B4 protein (Hock *et al.*, 1993) showed that the ontogenic emergence and localization of the B4 protein are essentially the same as those of our H1X. The study by Hock *et al.* (1993) lends support to the notion that H1X (B4 protein) is a unique maternally stored H1 that is integrated into the chromosomes of early embryonic nuclei. Histone subtypes specific to pregastrula stage embryos have also been found in sea urchins (for review see Poccia, 1986). The cleavage-specific H1 subtype, CSH1, could be a sea urchin counterpart of H1X because it has a larger molecular weight and relatively smaller proportion of lysine residues than other H1 subtypes. H2A.X in amphibians is also expected to be a functional homolog of the sea urchin CSH2A. Replication dependent synthesis of this subtype needs to be studied in the amphibian system to confirm this. The finding of transitions of H1X and H2A.X to H1 and H2A, respectively, during the blastula stage deserves attention because of its possible relevance to the regulation of chromatin activities in relation to the "midblastula transition" in embryogenesis, which includes dramatic changes in the rates of DNA replication, transcription, cleavage cycles and cell motility (cf., review by Kirschner *et al.*, 1985).

The primary chromatin structures of pronuclei and condensed chromosomes induced by incubation of *Xenopus* sperm with homologous egg extracts were analyzed by micrococcal nuclease digestion, followed by electrophoresis of DNA in agarose or polyacrylamide gel. Extensive nuclease digestion of pronuclear chromatin induced by incubation for more than 10 min with the egg extract resulted in formation of DNA fragments of approximately 170 base pairs, a size comparable to that of chromatosome monomers in somatic chromatin digests (Fig. 5). The same sized fragments of nuclease-protected DNA were obtained when *in vitro*-induced chromosomes derived from sperm were digested with micrococcal nuclease (Ohsumi *et al.*,1993). In addition, DNA fragments in a ladder of approximately 200 base pairs were obtained by mild digestion of these sperm-derived chromosomes, as in the digests of pronuclear chromatin (Philpott and Leno, 1992), indicating that the nucleosome monomers are regularly spaced at similar intervals to those in ordinary somatic chromatin. Thus, regardless of whether it is in a pronuclear or condensed chromosomal state, the sperm chromatin is evidently reconstituted to be of the somatic type in both nucleosome core structures and spacing in a regular array in DNA.

The involvement of DNA topoisomerase II (topo II), which is known to participate in organizing chromatin loops, was studied by incubation of sperm nuclei with the egg extracts containing a specific inhibitor of this enzyme (ICRF-193) (Ohsumi, Kishimoto

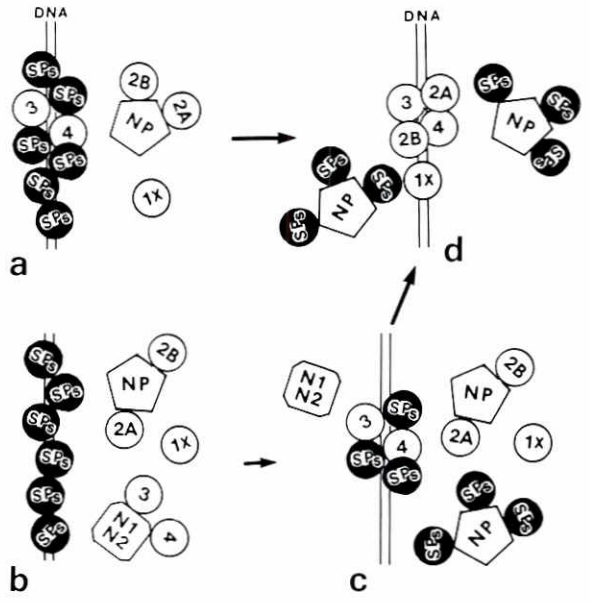


Fig. 9. Schematic illustration of nucleoplasmin (NP)-mediated reassembly of sperm chromatin (a,b) into somatic nucleosome structure (d) through decondensation (thick arrows). (a,b) *Initial states of chromatin in Xenopus* **(a)** *and Bufo or mammalian* **(b)** *sperm; N1N2, chromatin assembly proteins other than NP; Sps, sperm-specific basic proteins; 1X, 3, 4, 2A and 2B, histones.*

and Ando, unpublished). In the presence of this inhibitor, the nuclei exhibited normal decondensation, but persisted in this state without further chromatin dispersion or swelling. The chromosome condensation observed in the mitotic egg extracts also did not occur in the presence of this inhibitor. Nuclease digestion indicated, however, that under these conditions chromatin reconstitution was unaffected, giving rise to the normal nucleosome core with normal spacing. These results are consistent with the notion that topo II is involved in the assembly of higher order chromatin structures associated with DNA synthesis or chromosome condensation.

The successful induction of the somatic type chromatin structure by sperm DNA in a cell-free system prompted us, in collaboration with T. Kishimoto, to employ the same system to examine the role of H1 in mitotic chromosome condensation. A number of previous studies have suggested an essential role of H1 in chromosome condensation (Bradbury, 1992) and stability of 30 nm chromatin fibers (Thoma *et al.*, 1979). For examination of the necessity of H1X in mitotic chromosome condensation, "mitotic egg extracts" of *Xenopus* were immunodepleted of H1X by treatment with rabbit anti-H1X antibodies. Even in these H1X-depleted extracts, sperm nuclei were transformed to condensed chromosomes indistinguishable from control chromosomes containing H1X. Micrococcal nuclease digestion further revealed that in the absence of H1 binding to nucleosomes, H1X-free chromosomes exhibited the same nucleosome repeat length of approximately 200 base pairs as control chromosomes with H1X (Ohsumi *et al.*, 1993). These observations strongly argue against the essential role of H1 in the structural organization required for condensed metaphase chromosomes, although a role of H1 in modulating the functions of a putative chromosome condensing factor(s) cannot be ruled out. The role of H1 (H1X) in the structural organization and functions of chromatins thus merits further investigation.

Participation of nucleoplasmin as a molecular chaperone in sperm nuclear remodeling

The reconstitution of somatic chromatin in fertilized sperm nuclei discussed above must be preceded by the removal of protamines or sperm-specific basic proteins. The acquisition of exactly the same chromatin basic proteins in *Bufo* and *Xenopus* pronuclei after incubation with the egg extracts, despite large differences in the protein compositions in their original condensed state of chromatin, suggests that there must be a unique mechanism in the egg cytoplasm by which sperm-specific chromatin proteins are selectively removed. This removal of sperm nuclear proteins is a quick process, as demonstrated by immunocytochemical observations using antiserum against protamines in *Bufo* (Ohsumi and Katagiri, 1991b), which showed that (a) sperm nuclei lose protamines within 5 min after their entrance into the egg, when the nuclei are still located just under the sperm entrance cone, and (b) lysolecithin-permeabilized sperm incubated with the egg extracts lose protamines within 1 min in association with marked nuclear decondensation. The removal of protamines and concomitant decondensation of sperm nuclei are induced on incubation with the cytosol from growing and mature oocytes and blastulae, but not with those from postneurula embryos and adult tissues. This removal of nuclear proteins is not restricted to homologous species: egg extracts from *Bufo* selectively remove sperm-specific proteins (SP1-6) and sperm-specific H1 subtype histones, but not the core histones from sperm nuclei of *Xenopus* and *Rana catesbeiana*, respectively.

Recent studies have confirmed that an acidic protein, "nucleoplasmin", known to be present at high concentrations in the amphibian oocytes, plays a major role in removal of sperm-specific nuclear proteins. We fractionated the activity in egg extracts for removal of protamines from sperm nuclei by conventional ion-exchange chromatographies and gel-filtration procedures. We measured this unique activity in egg extracts for removing protamines ("protamine-removing activity": PRA) by scoring the ratios of SP3-5/histone H3 in the SDS-PAGE images before and after incubation of *Xenopus* sperm with test fractions (Fig. 6) (Ohsumi and Katagiri, 1991b). Taking advantage of its unusual thermostability and large quantity in egg extracts, PRA was purified as a protein with mobilities of 140 kDa and 36 kDa on native- and SDS-PAGE, respectively, and an isoelectric point in the range of 4.2-4.5. The purified PRA had a surprisingly similar amino acid composition to that reported for nucleoplasmin (Earnshaw *et al.*, 1980). In fact, a physiological concentration (700 μg/ml) of nucleoplasmin purified from *Bufo* or *Xenopus* egg extracts induced nuclear decondensation and selective removal of sperm-specific proteins of both species of the sperm (Ohsumi and Katagiri, 1991b; Philpott and Leno, 1992). Using another approach, Philpott *et al.* (1991) showed that immunodepletion of nucleoplasmin from *Xenopus* egg extracts abolished the initial rapid decondensation of sperm nuclei, and that readdition of purified nucleoplasmin rescued the activity of depleted extracts.

Very recent experiments (Itoh *et al.*, 1993) have extended the previous finding that amphibian egg extracts induce decondensation of human sperm nuclei (Brown *et al.*, 1987; Ohsumi *et al.*, 1988), by demonstrating that nucleoplasmin purified from *Bufo* egg extracts also selectively removes protamines from human sperm nuclei, provided the latter have been pretreated with the disulfide-reducing agent dithiothreitol. These studies suggest that, besides the well-documented ability of disulfide-reduction of protamines by glutathione (cf., Perreault *et al.*, 1988), the mammalian egg cytoplasm may contain macromolecular mechanisms that mimic that of amphibian nucleoplasmin for removing protamines from sperm nuclei.

An intriguing extension of the finding that nucleoplasmin removes sperm-specific proteins was the demonstration that nucleoplasmin functions by binding to these proteins, but not by degrading them. This was evidenced by the coprecipitations of nucleoplasmin and sperm-specific proteins in Western blots (Ohsumi and Katagiri, 1991b; Itoh *et al.*, 1993) or two-dimensional PAGE (Philpott and Leno, 1992) analyses of supernatants after incubation of sperm nuclei with nucleoplasmin (Fig. 7). Thus the removal of sperm-specific proteins during the initial pronuclear decondensation does not involve any enzymatic nor energy-dependent process. Conceivably the removal of sperm-specific proteins is accomplished by affinity-based, stoichiometric binding of nucleoplasmin to sperm proteins. This is supported by our observation (unpublished) that nucleoplasmin that had been preincubated with protamines at the protamine/nucleoplasmin ratios exceeding 1/10 by weight did not induce sperm nuclear decondensation. Another finding to support this view is the dependence of protamine-removing activity on the phosphorylated state of nucleoplasmin (Ohsumi *et al.*, in preparation). Previous studies (Cotten *et al.*, 1986; Sealy *et al.*, 1986) showed that nucleoplasmin from mature eggs is more highly phosphorylated

than that in premature oocytes. Comparison of protamine-removing activities by incubating *Xenopus* sperm nuclei with various concentrations of nucleoplasmins from premature and mature eggs revealed that the mature egg nucleoplasmin had about twice the activity of that of premature eggs. Similar assays of the cdc2/cyclin B kinase-induced phosphorylation of premature egg nucleoplasmin and alkaline phosphatase-induced dephosphorylation of mature egg nucleoplasmin showed increase and decrease, respectively, of the protamine-removing activity, again suggesting charge-dependent interactions of nucleoplasmin and sperm-specific basic proteins. All the sperm-specific proteins (SP1-6), but not somatic histones of *Xenopus* sperm nuclei, are readily phosphorylated on incubation with cdc2/cyclin B kinase (Shimada, personal communication). Interestingly, the resulting phosphorylated SPs are more easily released from nuclei than nonphosphorylated ones, as determined by their differential solubilities in NaCl solution. This possibly facilitates their removal from chromatin during initial decondensation steps, although at present there is no evidence for phosphorylation of sperm-specific proteins in the chromatin remodeling process of amphibian sperm. In sea urchins, two sperm-specific histones are specifically and highly phosphorylated before pronounced pronuclear decondensation (Green and Poccia, 1985), although phosphorylation of these histones is not sufficient for nuclear decondensation (Poccia *et al.*, 1990).

Nucleoplasmin is a highly acidic protein and possesses an unusual stretch of long polyglutamic acid residues towards its C-terminal region (Dingwall *et al.*, 1987), suggesting that it functions in charge-dependent disruption of the protamine-DNA complex. Perhaps relevant to this function of nucleoplasmin is a previous report by Dean (1983) of decondensation of reduced and alkylated mouse sperm nuclei on incubation with polyglutamic acid, associated with protamine removal as judged by induction of sensitivity of DNA to nuclease digestion. Polyglutamic acid can also induce nuclear decondensation of amphibian sperm under physiological ionic conditions, but unlike nucleoplasmin it removes both SPs and core histones from *Xenopus* sperm chromatin (unpublished observation). It is noteworthy that nucleoplasmin specifically removes sperm-specific proteins of a variety of amphibian groups despite their wide variations in primary structure and resulting charge differences *(loc. cit.)*. Taken together, these findings indicate that the action of nucleoplasmin in removing sperm-specific proteins should be effected not simply by charge-dependent disruption of the protamine-DNA complex, but also by other specific domains of this acidic protein, which may bind stoichiometrically with sperm-specific proteins. Thus the exact molecular basis for association of sperm-specific proteins and nucleoplasmin poses intriguing questions for future studies. Taking advantage of information on the complete primary structure of nucleoplasmin and the availability of its cDNA (Burglin *et al.*, 1987; Dingwall *et al.*, 1987), it will be possible to determine the actual functional sites of this protein by quantifying the protamine-removing activities of "mutated" nucleoplasmins or their digested fragments.

Previous analyses of nucleosome assembly using plasmid DNA and *Xenopus* egg extracts have shown that nucleosome core formation is aided by the karyophilic chromatin assembly factors N1/N2 and nucleoplasmin (Kleinschmidt *et al.*, 1986; Dilworth *et al.*, 1987). These factors facilitate orderly and synergistic assembly, *viz.*, first disposition of H3 and H4 from N1/N2 complexes onto DNA, and then additions of H2A and H2B from nucleoplasmin complexes (Kleinschmidt *et al.*, 1990). In this context the ability of nucleoplasmin to remove sperm-specific basic proteins highlights its dual functions as a molecular chaperone in the remodeling process of sperm chromatin. This role of nucleoplasmin is of adaptive value, particularly in chromatin reassembly in *Xenopus* sperm which already have H3 and H4 and small amounts of H2A and H2B. Thus for reassembly of the somatic nucleosome core from *Xenopus* sperm, SPs must be removed and H2A and H2B deposited, both processes being accomplished by nucleoplasmin. These processes do indeed occur under defined conditions, as shown in Fig. 8, and are associated with nucleosome core formation (cf., Philpott and Leno, 1992). The dual roles of nucleoplasmin proposed here may conceivably proceed in a concerted fashion by individual nucleoplasmin molecules, provided there is a stronger affinity between nucleoplasmin and SPs than between nucleoplasmin and H2A/H2B, as well as a stronger affinity between DNA and H2A/H2B than nucleoplasmin and H2A/H2B (Fig. 9). The co-immunoprecipitations of nucleoplasmin-H2A/H2B and nucleoplasm-SPs indicate that the latter is stronger than the former (Philpott and Leno, 1992).

Similar mechanisms may operate in the remodeling of other types of sperm chromatins that contain no somatic histones (see Fig. 9b,c,d). Nucleosomal repeat units, as defined by the appearance of nuclease protected DNA, have been reassembled even from human sperm chromatin under defined conditions containing nucleoplasmin and somatic core histones, although the unit fragments obtained were slightly shorter than those produced in the egg extracts (Itoh *et al.*, 1993). Perhaps under these experimental conditions the reassembly process was less efficient, due to the lack of N1/N2 and/or other reassembly factors. This experimental system also provides a means for analyzing the fine structural significance of chromatin possessing H2A.X and H1X instead of H2A and H1, respectively. Further *in vitro* chromatin remodeling studies using sperm lacking core histones, e.g. *Bufo* or mammalian sperm, should be effective for determining appropriate conditions including putative chromatin assembly factors that support reconstitution of fully formed somatic type chromatins.

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