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Hybrid Nucleoprotein Particles Containing a Subset of Male and Female Histone Variants Form During Male Pronucleus Formation in Sea Urchins

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Abstract   To determine the changes in chromatin organization during male pronucleus remodeling, we have compared the composition of nucleoprotein particles (NP-ps) resulting from digestion with endogenous nuclease (ENase) and with micrococcal nuclease (MNase). Whole nuclei were isolated from sea urchin gametes and zygotes containing a partially decondensed (15 min postinsemination, p.i.) or a fully decondensed (40 min p.i.) male pronucleus and digested with nucleases. The NP-ps generated were analyzed in agarose gels, and their histone composition was determined. Sperm core histones (SpH) and cleavage stage (CS) variants were identified by Western immunoblots revealed with specific antibodies. A single NP-ps was generated after digestion of sperm nucleus with MNase, which migrated in agarose gels between DNA fragments of 1.78–1.26 Kb. Sperm chromatin remained undigested after incubation in ENases activating buffer, indicating that these nuclei do not contain ENases. One type of NP-ps was obtained by digestion of unfertilized egg nuclei, either with ENase or MNase; the NP-ps was located in the region of the agarose gel corresponding to DNA fragments of 3.4–1.95 Kb [Imschenetzky et al. (1989): Exp Cell Res 182:436–444]. When whole nuclei from zygotes containing the female pronucleus and a partially remodeled male pronucleus were digested with ENase, a single NP-ps was generated, which migrated between DNA fragments of 2.5–1.9 Kb. This particle contained only CS histone variants. Alternatively, when these nuclei were digested with MNase, two NP-ps were generated; the slower migrating NP-ps (s) was located in the same position of the agarose gel as those resulting from ENase digestion and the faster migrating NP-ps (f) migrated between DNA fragments of 1.95–1.26 Kb. It was found that Np-ps (s) contained only CS histone variants, whereas NP-ps (f) were formed by a subset of SpH and by CS histone variants. When nuclei from zygotes containing a fully decondensed male pronucleus were digested either with ENase or MNase, a single type of NP-ps was observed, which migrated in the same position as NP-ps (s) in agarose gels. This particle contained only CS histone variants. On the basis of the histone compositions and on electrophoretic similarities, it was concluded that NP-ps (s) originated from the female pronucleus and that NP-ps (f) were generated from the partially remodeled male pronucleus. Consequently, our results indicate that at an intermediate stage of male pronucleus remodeling the chromatin is formed by NP-ps containing a subset of both SpH and of CS histone variants, whereas at final stages of male pronucleus decondensation chromatin organization is similar to that of the female pronucleus.   © 1996 Wiley-Liss, Inc.

Key words: chromatin, pronucleus, nucleoprotein particles, sea urchins, zygotes

Following fertilization, the sperm nucleus transforms into a male pronucleus that fuses with the female pronucleus to reestablish the diploid genome of the embryos [Longo and Anderson, 1968]. Multiple studies, both in vivo and in vitro, have been directed to determine the changes in chromatin composition that accom-

pany male pronucleus remodeling. Cytochemical approaches have suggested the loss of the majority of sperm-specific basic proteins [Bloch and Hew, 1960; Vaughn, 1968; Das et al., 1975; Ecklund and Levine, 1975; Kopechy and Pavlok, 1975]. Biochemical studies in batracians have shown that during male pronucleus remodeling two sperm-specific basic proteins, X and Y, are replaced by maternally derived histones, H2A and H2B. These changes are promoted by nucleoplasmin [Philpott et al., 1991; Philpott and Leno, 1992].

In sea urchins, significant changes in chromatin structure occur during male pronucleus formation. Sperm nucleosomes are organized by sperm histone variants (SpH) interacting with 220–240-bp DNA fragments [Spadafora et al., 1976; Savic et al., 1981]. Unfertilized egg nucleosomes are formed by cleavage stage histone variants (CS) protecting 126 bp of DNA from nuclease digestion [Newrock et al., 1978; Imschenetzky et al., 1986, 1989]. At the end of the first cleavage cycle, nucleosomes present a shortened DNA repeat length when compared with sperm nucleosomes, and the overall histone composition is similar to that of unfertilized eggs [Imschenetzky et al., 1980; Savic et al., 1981; Shaw et al., 1981]. At present, the vast majority of information concerning histone transitions during male pronucleus remodeling is derived from polyspermic zygotes [Green and Poccia, 1985; Poccia, 1986; Cothren and Poccia, 1993; Cameron and Poccia, 1994]. These results indicate that the sperm-specific histones H1 and H2B (SpH1 and SpH2B) are released from the chromatin before amphimixis, whereas sperm-specific H2A (SpH2A) is lost after the fusion of both pronuclei, which occurs coincidentally with the onset of the first S phase [Longo and Kunkle, 1978; Green and Poccia, 1985]. Because male pronucleus remodeling is impossible to synchronize in polyspermic zygotes, the protein transitions responsible for the sperm chromatin remodeling are very difficult to interpret. Previously, we were able to discriminate in monospermic zygotes between male and female histone variants by using antibodies that recognize specifically sperm core histones (SpH). Our results indicated that all SpH were lost before the onset of the first S phase [Imschenetzky et al., 1991a]. Because of difficulties in the biochemical separation between the male and the female pronucleus, the definitive steps of chromatin remodeling responsible for male pronucleus formation are still unknown. By exploiting the differences in the overall chromatin structure between male and female pronuclei and of the lack of endogenous nuclease (ENase) activity in sperm nuclei, we could follow the changes in chromatin associated with male pronucleus remodeling in normal zygotes. Our results indicate that the female pronucleus can be followed by analyzing the nucleoprotein particles (NP-ps) derived from the zygote nucleus by digestion with ENase. Conversely, the fate of the male pronucleus can be investigated by ana-

lyzing one of the two NP-ps generated, followed by digestion of the zygote nucleus with micrococcal nuclease (MNase). This enzyme digests both male and female pronuclei, thus generating two types of NP-ps that can be resolved in agarose gels. By following this experimental strategy, we compared two distinct stages of male pronucleus remodeling: an intermediate stage and a final stage of decondensation.

METHODS AND MATERIALS
Gametes and Zygotes

Sea urchins, *Tetrapygus niger*, were collected from the bay of Concepción and maintained at room temperature in an aquarium containing natural sea water under constant aeration. Unfertilized eggs, sperm, and zygotes were obtained as described previously [Imschenetzky et al., 1984, 1986, 1991a].

Nuclei Isolation and Digestion With Nucleases

Unfertilized egg nuclei were isolated by an aqueous ethanol/Triton X-100 procedure according to Poccia et al. [1981] and digested as described by Savic et al. [1981]. To activate the ENase, the isolated nuclei were washed twice with 10 vol of 0.01 M NaCl, 2.5 mM MgCl$_2$, 0.03 mM CaCl$_2$, and 0.01 M Tris-HCl, pH 7.6 (buffer RSB). The nuclei were then suspended in 5 vol of the same buffer and digested for different times at 37°C for the time indicated in each experiment. The digestions with MNase were performed with isolated nuclei that were suspended in 5 vol of buffer RSB with 1 vol of MNase at 360 units/ml (Worthington) in a medium containing 0.01 M NaCl, 0.2 mM CaCl$_2$, 0.01 M Tris-HCl, pH 8.0, and incubated at 37°C for the time indicated in each experiment. These digestions were stopped by adding 0.05 vol of 0.01 M Na$_2$ EDTA (Ethylendinitrilo tetraacetic acid), pH 8, at 4°C. Then the soluble chromatin was released by overnight dialysis at 4°C against 1 mM EDTA, pH 8, as described by Fisher and Felsenfeld [1986]. The nuclear debris were removed by low speed centrifugation, and the soluble chromatin obtained was electrophoresed on 1% agarose gels to analyze the NP-ps.

Electrophoresis of NP-ps

Electrophoresis of the NP-ps was performed by using horizontal 1% agarose gels in a solution containing 0.04 M Tris-acetate and 1 mM EDTA, as described by Weintraub [1984]. For visualiza-

tion of DNA, the gels were stained with ethidium bromide at 1 μg/ml, transilluminated by short wave UV light, and photographed through a wratten 23 A filter onto Polaroid Type 665 film. For visualization of proteins, the gels were stained with Coomassie blue R-250 as described previously [Imschenetzky et al., 1989]. To compare more precisely the patterns of DNA fragments derived from nuclease digestion, the gels were photographed and the negatives scanned in a microdensitometer (Joyce Loebl, model MK III CS). Lamda DNA digested with restriction nucleases Hind III and Eco RI was used as standard for electrophoretic migration.

Isolation of Sperm and CS Histone Variants

Sperm-specific (SpH) and native-cleavage-stage (CS) histone variants were isolated from sperm, eggs, and zygotes as described previously [Imschenetzky et al., 1984, 1986]. Core sperm histones were isolated as described by Liao and Cole [1981]. To remove the poly(ADP-ribose) moiety from the native CS histone variants, these proteins were treated with phosphodiesterase linked to Sepharose 4B [Imschenetzky et al., 1996].

Preparation of Antibodies Against Nucleosomal Core Sperm Histones

Core sperm histones were used as antigen. An initial dose of 1.2 mg of core SpH in 1 ml of 0.1 M sodium phosphate buffer, pH 7.4, and emulsified with 1 ml of complete Freund's adjuvant was used to immunize rabbits. Inoculations were given biweekly with the same doses of antigen but dissolved in incomplete Freund's adjuvant [Imschenetzky et al., 1991b]. IgG antibodies were purified from rabbit immune sera by affinity chromatography on protein A–Sepharose 4B (Sigma Chemical Company, St. Louis, MO, USA) as described by Tijssen [1988]. The antibodies were eluted with 0.1 M sodium citrate, pH 3.0, and neutralized immediately to pH 8.0 with 1 M Tris HCl, pH 8.0. A titer of 1:640 of the antibodies against core SpH was determined by ELISA (Enzyme-linked immunosorbent assay) with an alkaline phosphatase antibody conjugate [Voller and Bidwell, 1986]. The antibodies against core SpH used in excess, were diluted 1:50 in a Tris buffered saline solution (TBS) containing 0.02 M Tris, pH 7.5, 0.5 M NaCl and 1% (w/v) gelatine to incubate the nitrocellulose membranes for the Western immunoblot analysis.

Preparation of Antibodies Against CS Histone Variants

Whole native CS histone variants were used as antigen. An initial dose of 2.5 mg of CS histone variants dissolved in 1 ml of 0.1 M sodium phosphate buffer, pH 7.4, and emulsified with 1 ml of complete Freund's adjuvant was used to immunize rabbits. Inoculations were given subcutaneously at days 20, 40, and 60 with the same dose of antigen dissolved in incomplete Freund's adjuvant. Then the same dose of antigen was injected intraperitoneally once a month for 4 months. IgG antibodies were purified from rabbit immune sera by affinity chromatography as described in the previous paragraph. We found that CS histone variants are extensively poly(ADP-ribosylated) in unfertilized eggs and in zygotes [Imschenetzky et al., 1991b, 1993, 1996]. Because the polymers of ADP-ribose are antigens, we eliminated the fraction of the antibodies that were directed against the poly(ADP-ribose) moiety of CS variants by adsorption of ADP-ribose polymers linked to Sepharose 4B. Polymers of ADP-ribose were synthesized and purified from a rat-liver cell-free system as described previously [Burzio et al., 1979; Imschenetzky et al., 1991b]. The linking of these polymers to Sepharose 4B was performed by following the procedure recommended by Pharmacia (Pharmacia Uppsala, Sweden). A titer of 1:2,000 of antibodies against native CS histone variants was determined by ELISA by using an alkaline phosphatase antibody conjugate, as described by Voller and Bidwell [1986]. These purified antibodies used in excess were diluted 1:200 in a TBS containing 0.02 M Tris, pH 7.5, 0.5M NaCl, and 1% (w/v) gelatine to incubate the nitrocellulose membranes for the Western immunoblot analysis.

Electrophoresis of Proteins and Analysis of Western Immunoblots

Sample preparation and electrophoresis of proteins in one- or two-dimensional sodium dodecyl-sulfate polyacrylamide gels (SDS-PAGE) were performed as described by Laemmli [1970]. For the two-dimensional analysis, the first dimension was a lane from 1% agarose gels containing the NP-ps derived from digestion with nucleases. The lane was cut and layered onto 18% (w/v) SDS-PAGE and subjected to electrophoresis in the second dimension. After electrophoresis, the gels were transferred to nitrocellulose

membranes and analyzed by Western immunob-
lots. The procedures followed for the electropho-
retic transfer, blotting, and immunodetection
were essentially those described by Towbin et al.
[1979] and modified as described in Ims-
chenetzky et al. [1991a]. One lane containing
whole SpH or the native CS histone variants
was included in each SDS-PAGE as a control.

RESULTS
Analysis of NP-ps

Nuclei were isolated from sperm, unfertilized
eggs, and zygotes harvested at two stages of
male pronucleus remodeling: an intermediate
(15 min postinsemination, p.i.) and a terminal
(40 min p.i.) stage of decondensation. NP-ps
were obtained after digestion with ENase or
with MNase. The results obtained are shown in
Figures 1 and 2. As shown, the electrophoretic
profiles revealed a broad and uniform distribu-
tion of discrete NP-ps. To confirm the nucleopro-
tein composition, these NP-ps were stained with
both ethidium bromide and Coomassie blue. The
electrophoretic migration of these NP-ps was
not altered by increasing the incubation time
with the nucleases (Fig. 1A,B). As reported pre-
viously, no differences were observed in the mi-
gration profiles by decreasing the incubation
time or by lowering the temperature of incuba-
tion [Imschenetzky et al., 1989], suggesting that

these NP-ps represent discrete domains of chro-
matin organization.

Similar NP-ps were obtained after digestion
of unfertilized egg nuclei with ENase and MNase
[Imschenetzky et al., 1989]. The NP-ps derived
from unfertilized egg nuclei migrated between
DNA fragments of 1.95–3.4 Kb (Fig. 1A, lanes
a–d). Sperm chromatin remained undigested af-
ter 180 min of incubation in appropriate condi-
tions for ENase activation (results not shown),
indicating that ENase is absent from this
nucleus. However, when sperm nuclei were di-
gested with MNase, one major type of NP-ps
was obtained, which was located in the region of
the gel corresponding to DNA fragments of 1.26–
1.78 Kb (Fig. 1B, lanes d–f). Clearly defined
NP-ps were obtained from sperm nuclei only
after 60 min of digestion (Fig. 1B, lane d),
whereas these NP-ps were generated before 10
min of digestion of the nuclei from unfertilized
eggs and zygotes (Fig. 1, lanes a–d; Fig. 2A–D).
At shorter times of digestion of sperm nuclei
with MNase, a smear of poorly defined chroma-
tin digest products was observed (Fig. 1B, lanes
a–c), suggesting that sperm chromatin is less
accessible to digestion with MNase than those
from unfertilized eggs or zygotes.

When the nuclei isolated from zygotes contain-
ing the female pronucleus and a partially decon-
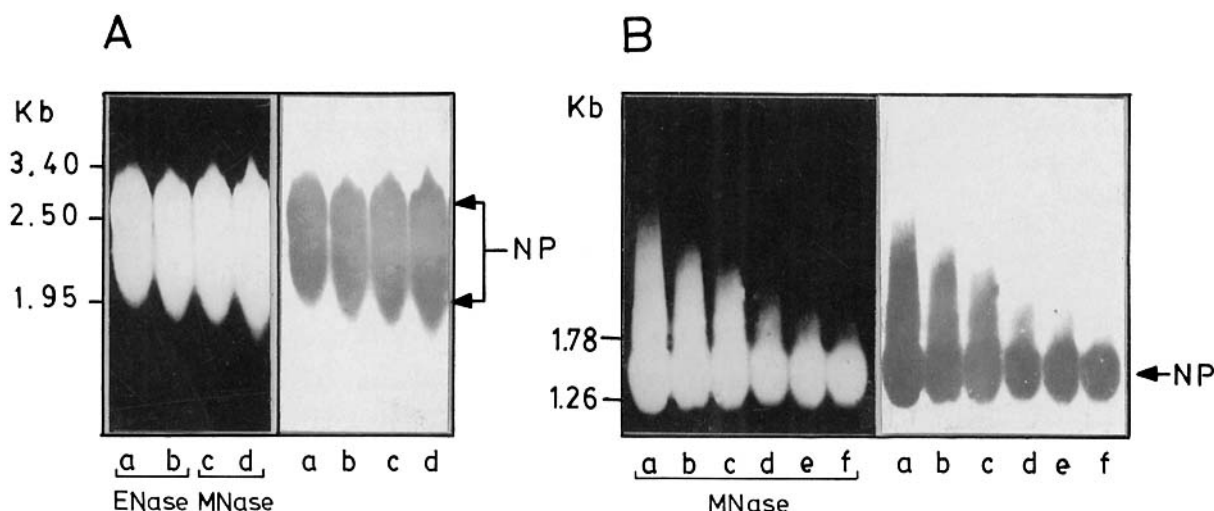densed male pronucleus (15 min p.i.) were sub-

Fig. 1. Nucleoprotein particles obtained by digestion of nucleus from gametes with nucleases. The nuclease
digestion products were separated by electrophoresis in 1% (w/v) agarose gels and stained with ethidium bromide
(left) and Coomassie blue (right). A: Nuclei from unfertilized eggs were digested with endogenous nuclease (ENase)
for (lane a) 5 min and (lane b) 10 min and with micrococcal nuclease (MNase) for (lane c) 5 min and (lane d) 10 min.
B: Nuclei from sperm were digested with MNase for (lane a) 10 min, (lane b) 20 min, (lane c) 40 min, (lane d) 60 min,
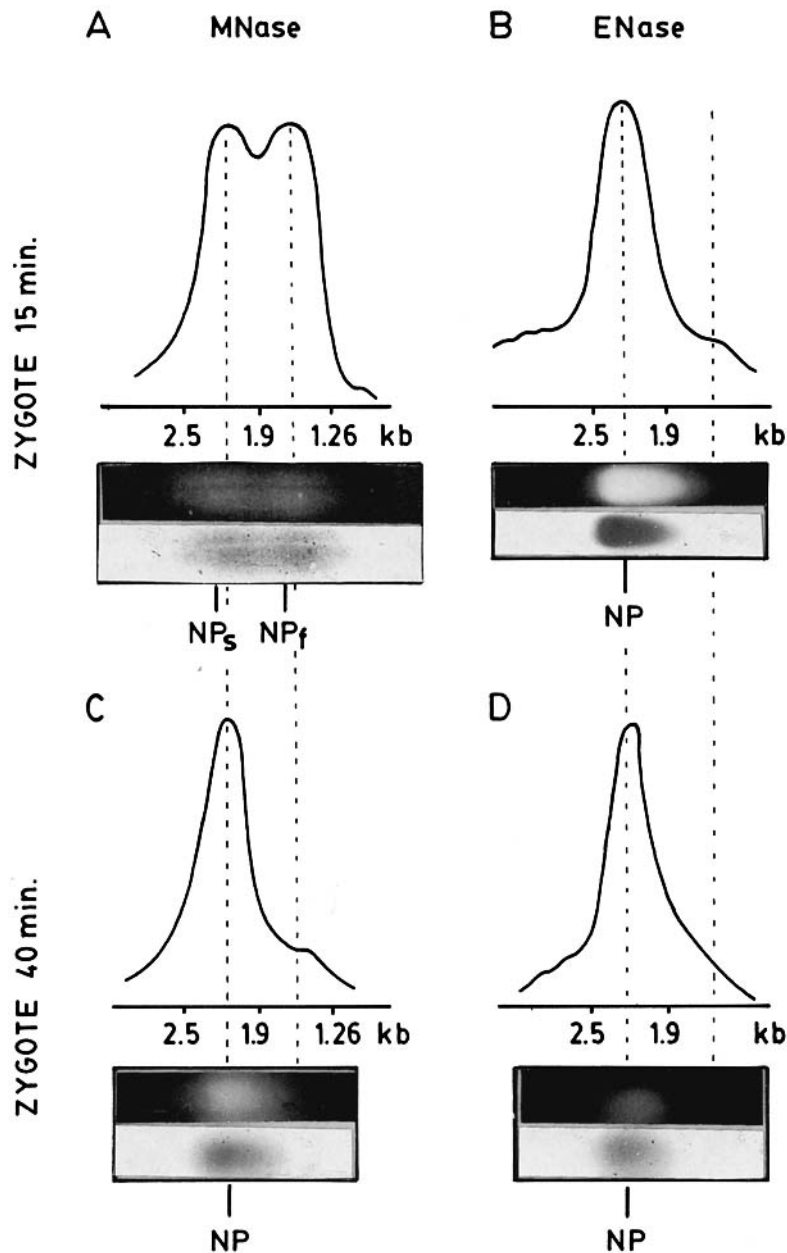(lane e) 80 min, and (lane f) 90 min.

Fig. 2. Nucleoprotein particles obtained by digestion of nucleus from zygotes with nucleases. The nuclease digestion products were separated by electrophoresis in 1% (w/v) agarose gels, stained with ethidium bromide (top) and Coomassie blue (bottom), photographed, and scanned in a microdensitometer. Nuclei from zygotes obtained 15 min p.i. were digested for 10 min with MNase (A) and with ENase (B). Nuclei from zygotes obtained 40 min p.i. were digested for 10 min with MNase (C) and with ENase (D). Lamda DNA digested with restriction nucleases Hind III and Eco RI were run in parallel as standards for electrophoretic migration.

jected to ENase digestion, a single NP-ps was found, which migrated between DNA fragments of 1.9–2.5 Kb. Conversely, when these nuclei were digested with MNase, two NP-ps were generated. The slowly migrating NP-ps are designated NP-ps (s), and the faster migrating NP-ps are designated NP-ps (f) in Figure 2A. The NP-ps

(s) were localized in the region of the gel corresponding to DNA fragments of 1.9–2.5 Kb, which is identical to the position of the unique NP-ps obtained as a product of ENase digestion (Fig. 2B) and closely resembles the migration of those derived from unfertilized eggs. It was also found that NP-ps (f) were observed in the gel in a

position between DNA fragments of 1.26–1.95 Kb, with a migration closely resembling those obtained for sperm-derived NP-ps.

When the nucleus isolated from zygotes harvested at the final stages of male pronucleus decondensation (40 min p.i.) was digested with ENase, a single NP-ps was found. A similar type of particle was also found after MNase digestion. This particle migrated as DNA fragments of 1.9–2.5 Kb (Fig. 2C,D), thus resembling the NP-ps (s) and those derived from unfertilized eggs.

Protein Composition of NP-ps

To determine the histone components that constitute the NP-ps generated by nuclease digestion, these particles were subjected to a two-dimensional electrophoresis and subsequently to Western immunoblot analysis. The identification of male and female histone variants was performed with antibodies against core SpH or against CS histone variants. The specificity of these antibodies is shown in Figure 3. The antibodies against core SpH exhibited a positive reaction with the core SpH (Fig. 3A, lane b) and

a negative reaction with the CS chromosomal proteins (Fig. 3A, lane c). Similarly, the antibodies against the CS histone variants recognized the whole set of CS histone variants (Fig. 3B, lane b) and did not cross react with the SpH (Fig. 3B, lane a). Because native CS histone variants are extensively poly(ADP-ribosylated) [Imschenetzky et al., 1996], the anti-CS antibodies were further purified to avoid an undesirable reaction with this posttranslational modification. The resulting antibodies exhibited a positive reaction with CS histone variants that were treated with phosphodiesterase-Sepharose and were not reactive against isolated polymers of ADP-ribose (Fig. 3C).

Initially, we analyzed the NP-ps derived from MNase digestion of nuclei from zygotes containing partially decondensed male pronuclei. The Western blot analysis revealed that the proteins contained in both NP-ps (s) and NP-ps (f) were recognized by the anti-CS antibodies (Fig. 4A). It was also observed that only the proteins contained in NP-ps (f) were recognized by the anti-SpH antibodies (Fig. 4B). This result indicates that NP-ps (s) are formed solely by CS histone
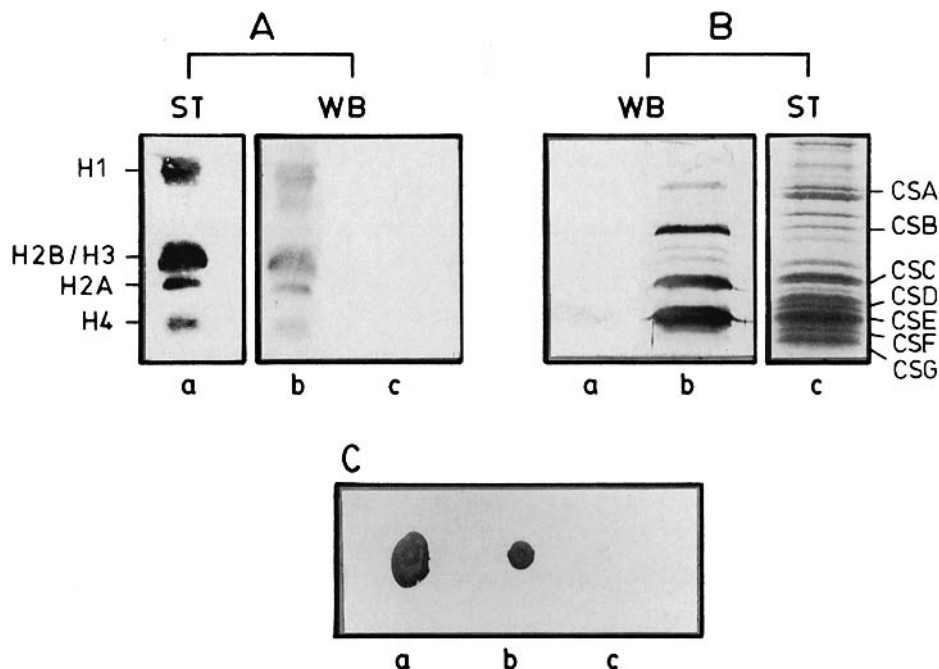

Fig. 3. Specificity of antibodies. Sperm histones (SpH) and cleavage-stage (CS) histone variants were analyzed by SDS-PAGE, transferred to nitrocellulose membranes, and revealed with antibodies anti-SpH (A) and anti-CS (B). A: Lane a: 40 μg of SpH variants stained with Coomassie blue; lane b: SpH variants revealed with antibodies anti-SpH; and lane c: CS variants revealed with antibodies anti-SpH. B: Lane a: SpH variants revealed with antibodies anti-CS; lane b: CS variants revealed with antibodies anti-CS; and lane c: 60 μg of CS variants stained with Coomassie blue. C: Dot blot analysis revealed with antibodies anti-CS: (lane a) 50 μg of native CS histone variants, (lane b) 30 μg of CS histone variants treated to remove the ADP-ribose moiety, and (lane c) 50 μg of (ADP-ribose) polymer.
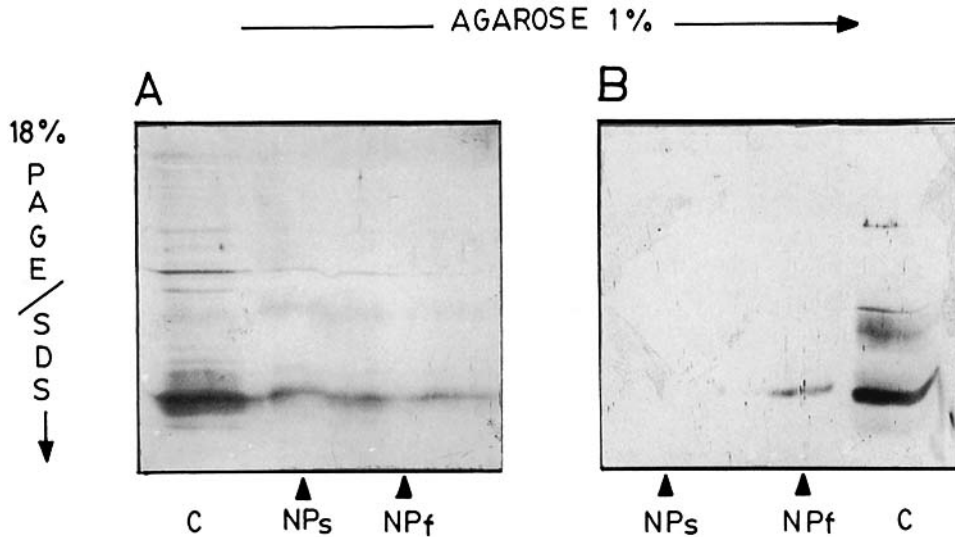
Fig. 4. Protein composition of NP-ps obtained by MNase digestion of the nucleus from zygotes containing a partially decondensed male pronucleus. The lane of the first-dimension agarose gel containing the NP-ps generated by MNase digestion of the nucleus from zygotes harvested 15 min p.i. was run on a second dimension on an 18% SDS-PAGE, transferred to nitrocellulose, and revealed with antibodies anti-CS (A) and anti-SpH

(B). A: 50 μg of native CS histone variants were run in the same gel as the controls for immunodetection (lane C). The position of the NP-ps (s) and (f) are indicated by arrowheads (lanes NPf, NPs). B: 40 μg SpH were run in the same gel as the control for immunodetection (lane C). The position of the NP-ps (s) and (f) are indicated by arrowheads (lanes NPf, NPs).

variants, whereas NP-ps (f) are composed not only of CS but also of SpH histone variants. Similarly, when the NP-ps obtained from the same zygotes following ENase digestion were analyzed, it was found that the histones present in these particles were recognized by the anti-CS antibodies, and no detectable reaction was observed for the anti-SPH antibodies (Fig. 5A,B). These results indicate that NP-ps (s) originated from the female pronucleus.

By following the same criteria, we analyzed the unique type of NP-ps derived from nuclease digestion of nuclei from zygotes containing a fully decondensed male pronuclei. We found that these NP-ps were composed of only CS histone variants. The proteins contained in these NP-ps were positively recognized by the anti-CS antibodies and were not recognized by the anti-SpH antibodies (Fig. 6A,B). These results indicate that these NP-ps are very similar to those present in the female pronucleus before amphimixis.

DISCUSSION

By analyzing the products of nuclease digestion of the nuclei from sea urchin zygotes containing female pronuclei and partially decondensed male pronuclei, we could identify two types of NP-ps. One type was the particle desig-

nated NP-ps (s) containing CS histone variants and the other a hybrid particle designated NP-ps (f) containing a subset of sperm histone variants (SpH2A–SpH2B) [Imschenetzky et al., 1991a] and components of the CS histone variants from unfertilized egg (CS D–E). At present, it is impossible to determine precisely the individual CS variants that are contained in NP-ps (f) except for their designation according to their electrophoretic migration [Imschenetzky et al., 1986]. As reported, the CS variants are found in a poly(ADP-ribosylated) form in unfertilized eggs and zygotes; therefore, their electrophoretic profiles are very difficult to interpret unambiguously [Imschenetzky et al., 1996]. In contrast with the heterogeneous NP-ps (s) and (f), one homogeneous type of NP-ps was generated from zygotes containing a fully decondensed male pronucleus. This type was formed only by CS histone variants and exhibited an electrophoretic migration similar to that of NP-ps (s). The similarity of the electrophoretic migration of the NP-ps derived from MNase digestion of sperm nuclei with the NP-ps (f) from zygotes strongly suggests that this type of particle originates from male pronucleus. The detection of sperm histones as components of the protein core of these particles confirms that these NP-ps were derived from the partially remodeled male pro-
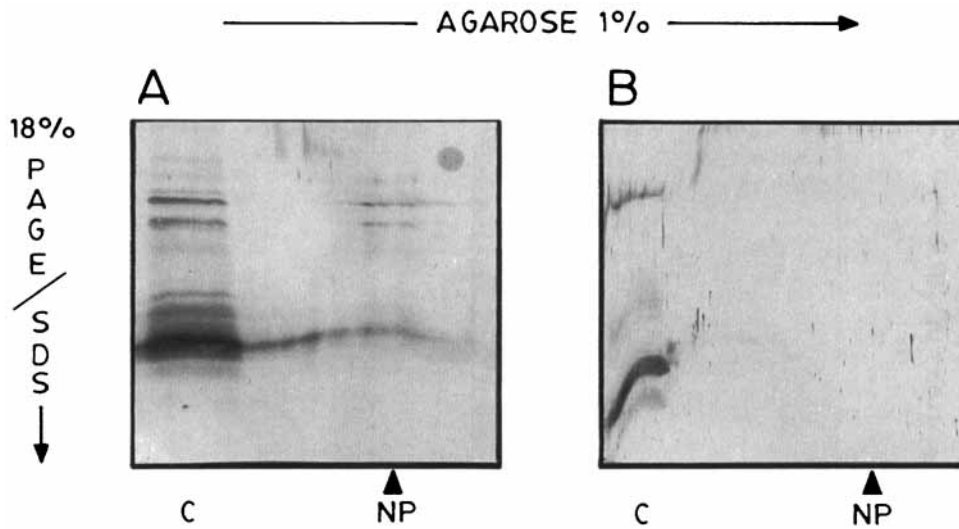
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                       NP   NP

Fig. 5. Protein composition of NP-ps obtained by ENase digestion of the nucleus from zygotes containing a partially decondensed male pronucleus. The lane of the first-dimension agarose gel containing the NP-ps resulting from ENase digestion of the nuclei from zygotes obtained 15 min p.i. was run on a second-dimension SDS-PAGE, transferred to nitrocellulose, and revealed with antibodies anti-CS (A) and anti-SpH (B). **A:** 50 μg

of native CS histone variants were run in the same gel as the control for immunodetection (*lane C*). The position of the single NP-ps is indicated by an arrowhead (*lane NP*). **B:** 40 μg SpH were run in the same gel as the control for immunodetection (*lane C*). The position of the NP-ps is indicated by an arrowhead (*lane NP*).
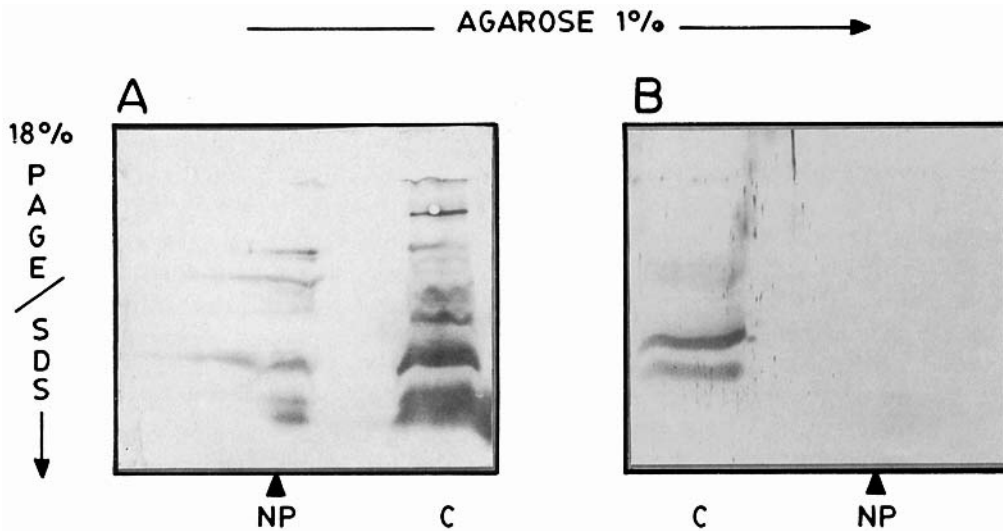
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        NP   NP

Fig. 6. Protein composition of NP-ps obtained by ENase digestion of the nucleus from zygotes containing a totally decondensed male pronucleus. The lane of the first-dimension agarose gel containing the NP-ps resulting from ENase digestion of the nuclei from zygotes obtained 40 min p.i. was run on a second-dimension SDS-PAGE, transferred to nitrocellulose, and revealed with antibodies anti-CS (A) and anti-SpH (B). **A:** 50 μg

of native CS histone variants were run in the same gel as the control for immunodetection (*lane C*). The position of the unique type of NP-ps is indicated by an arrowhead (*lane NP*). **B:** 40 μg SpH were run in the same gel as the control for immunodetection (*lane C*). The position of the single NP-ps is indicated by an arrowhead (*lane NP*).

nucleus. In addition, the increased sensitivity to MNase digestion of the chromatin from zygotes as compared with that of sperm is consistent with a more decondensed state of male pronucleus as compared with sperm nuclei. Further-

more, by digesting the nucleus from this zygotes with ENase, a single type of maternally derived NP-ps was observed. The obvious interpretation of these results is that MNase digests both male and female pronuclei, whereas ENase is present

only in female pronuclei. As shown in this report, ENase was absent from sperm nuclei, thus suggesting that the ENase present in female pronuclei before fertilization is not translocated to male pronuclei during its remodeling. Consequently, by comparing the NP-ps generated from ENase and MNase digestion, it is possible to follow the changes in chromatin organization of male pronucleus independently from female pronucleus.

The hybrid histone composition of the NP-ps from zygotes containing a partially remodeled male pronucleus is consistent with previous reports that have indicated that core sperm histones are lost in a noncoordinated manner during male pronucleus remodeling [Poccia et al., 1981; Imschenetzky et al., 1991a]. It was previously postulated that sperm histones could be replaced by CS histone variants recruited from a maternal pool [Green and Poccia, 1985; Imschenetzky et al., 1991a]. Based on the results reported here, we conclude that the replacement of sperm histones by CS histone variants occurs at an intermediate stage of male pronucleus remodeling. In addition, the protein composition of NP-ps from zygotes containing a fully decondensed male pronucleus further supports the similarity of male and female chromatin at the time of amphimixis. These results strengthen our previous conclusion concerning the loss of the whole set of sperm core histones at the time of the fusion of both pronuclei [Imschenetzky et al., 1991a].

The changes in chromatin organization during male pronucleus remodeling described in this report are in agreement with the steps for male pronuclei remodeling postulated for sea urchins by Cothren and Poccia [1993]. In operative terms, these steps were defined based on morphological observations as an initial or conical condensed male pronucleus (state I), an intermediate state of pronucleus decondensation that exhibits an ovoid shape (state II), and a fully decondensed spherical male pronuclei (state III). Accordingly, the hybrid NP-ps formed by a subset of sperm histones and CS histone variants and generated by MNase digestion from a partially decondensed male pronucleus defines the chromatin composition of the ovoid-shaped state II male pronuclei. In addition, the single NP-ps found as the unique product of nuclease digestion of zygote nucleus, which contained a fully decondensed male pronucleus, defines in bio-

chemical terms the structure of the spherical male pronucleus (state III).

The mechanism responsible for male pronucleus formation is not yet fully understood. In sea urchins, it was reported that transformation from state I to state II requires factors that are absent from oocyte cytoplasm before maturation. It was also demonstrated that this transformation may be blocked by protein kinases inhibitors and by N-ethylmaleimide [Longo and Kunkle, 1978; Luttmer and Longo, 1987; Cothreen and Poccia, 1993; Cameron and Poccia, 1994]. It was also reported that the progression from state II to state III is promoted by the rise of intracellular pH that occurs at fertilization [Carron and Longo, 1980; Cothreen and Poccia, 1993; Cameron and Poccia, 1994]. In batracians, it was reported that nucleoplasmin with an as yet undefined cytoplasmic factor participates in male pronucleus remodeling [Philpott et al., 1991; Philpott and Leno, 1992]. As reported by Dilworth et al. [1987], nucleoplasmin plays an essential role in the physiological assembly of nucleosomes by adding the H2A–H2B dimers to the tetramer formed by histones H3–H4, initially organized by proteins N1–N2. Both factors were found in significant amounts in *Xenopus laevis* egg extracts [Dilworth et al., 1987; Philpott and Leno, 1992]. Interestingly, in sea urchin zygote, sperm histone disappearance appears to occur in a noncoordinated manner during male pronucleus remodeling. Initially, the H3–H4 tetramer is removed and then the SpH2A–SpH2B dimers are released [Imschenetzky et al., 1991a]. In this context, it is tempting to speculate that factors such as nucleoplasmin and proteins N1–N2 participate in male pronucleus remodeling. Nevertheless, factors that could mimic these functions in sea urchins have not been identified yet.

ACKNOWLEDGMENTS

REFERENCES

Bloch DP, Hew H (1960): Schedule of spermatogenesis in the pulmonate snail Helix aspersa with special reference to histone transition. J Biophys Biochem Cytol 8:69–81.

Burzio LO, Riquelme PT, Koide SS (1979): ADP-ribosylation of rat liver nucleosomal core histones. J Biol Chem 254:3029–3037.

Cameron LA, Poccia DL (1994): In vitro development of the sea urchin male pronucleus. Dev Biol 162:568–578.

Carron CP, Longo FJ (1980): Relation of intracellular pH and pronuclear development in the sea urchin, Arbacia punctulata. Dev Biol 79:478–487.

Cothren CC, Poccia DL (1993): Two steps required for male pronucleus formation in the sea urchin egg. Exp Cell Res 205:126–133.

Das NK, Micou-Eastwood J, Alfert M (1975): Cytochemical studies on the protamine-type protein transition in sperm nuclei after fertilization and early embryonic histones of Urechis caupo. Dev Biol 43:333–339.

Dilworth SM, Black SJ, Laskey RA (1987): Two complexes that contain histones are required for nucleosome assembly in vitro: Role of nucleoplasmin and N1 in Xenopus egg extracts. Cell 51:1009–1018.

Ecklund PS, Levine L (1975): Mouse sperm basic nuclear protein electrophoretic characterization and fate after fertilization. J Cell Biol 66:251–262.

Fisher EA, Fensenfeld G (1986): Comparison of the folding of b-globin and ovalbumin gene containing chromatin isolated from chicken oviduct and erythrocytes. Biochemistry 25:8010–8016.

Green GR, Poccia D (1985): Phosphorylation of sea urchin sperm H1 and H2B histones precedes chromatin decondensation and H1 exchange during male pronuclear formation. Dev Biol 108:235–245.

Imschenetzky M, Puchi M, Massone R (1980): Histone analysis during the first cell cycle of development of the sea urchin Tetrapygus niger. Differentiation 17:111–115.

Imschenetzky M, Puchi M, Oyarce A, Massone R, Inostroza D (1984): A comparative study of the histones isolated from sperm of the sea urchin Tetrapygus niger. Comp Biochem Physiol 78B:393–399.

Imschenetzky M, Puchi M, Massone R, Ramirez P (1986): Characterization of cleavage stage chromosomal proteins from zygotes of the sea urchin Tetrapygus niger. Comp Biochem Physiol 84B:23–27.

Imschenetzky M, Puchi M, Gutierrez S, Massone R (1989) Analysis of supranucleosomal particles from unfertilized eggs of sea urchin. Exp Cell Res 182:436–444.

Imschenetzky M, Puchi M, Pimentel C, Bustos A, Gonzales M (1991a): Immunobiochemical evidence for the loss of sperm specific histones during male pronucleus formation in monospermic zygotes of sea urchins. J Cell Biochem 47:1–10.

Imschenetzky M, Montecino M, Puchi M (1991b): Poly(ADP-ribosylation) of atypical CS histone variants is required for the progression of S phase in early embryos of sea urchins. J Cell Biochem 46:234–241.

Imschenetzky M, Montecino M, Puchi M (1993): Temporally different poly (adenosine diphosphate-ribosylation) signals are required for DNA replication and cell division in early embryos of sea urchins. J Cell Biochem 51:198–205.

Imschenetzky M, Morin V, Carvajal N, Montecino M, Puchi M (1996): Decreased heterogeneity of CS histone variants after hydrolysis of the ADP-ribose moiety. J Cell Biochem 61:109–117.

Kopechy V, Pavlok A (1975): Autoradiographic study of mouse spermatozoan arginine rich nuclear protein in fertilization. J Exp Zool 191:85–96.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.

Liao LW, Cole RD (1981): Condensation of dinucleosomes by individual subfractions of H1 histone. J Biol Chem 256:10124–10128.

Longo F, Anderson F (1968): The fine structure of pronuclear development and fusion in sea urchin Arbacia punctulata. J Cell Biol 39:335–368.

Longo FJ, Kunkle M (1978): Transformations of sperm nuclei upon insemination. Curr Topics Dev Biol 12:149–184.

Luttmer SJ, Longo FJ (1987): Rates of male pronuclear enlargement in sea urchin zygotes. J Exp Zool 243:289–298.

Newrock RM, Alfageme ECR, Nardi, RV, Cohen, LH (1978): Histone changes during chromatin remodeling in embryogenesis. Cold Spring Harbor Symp Quant Biol 42:421–431.

Philpott A, Leno GH (1992): Nucleoplasmin remodels sperm chromatin in Xenopus egg extracts. Cell 69:759–767.

Philpott A, Leno GH, Laskey RA (1991): Sperm decondensation in Xenopus egg cytoplasm is mediated by nucleoplasmin. Cell 65:569–578.

Poccia D (1986): Remodeling of nucleoproteins during gametogenesis, fertilization, and early development. In Bourne C, Jeon K, Friedlander M (eds): "International Review of Cytology, vol. 105." New York: Academic Press, pp 1–65.

Poccia D, Savic J, Krystal G (1981): Transitions in histone variants of the male pronucleus following fertilization and evidence for a maternal store of cleavage-stage histone in the sea urchin eggs. Dev Biol 82:287–296.

Savic A, Richman P, Williamson P, Poccia D (1981): Alterations in chromatin structure during early sea urchin embryogenesis. Proc Natl Acad Sci USA 78:3706–3710.

Shaw B, Cognetti G, Sholes WM, Richards R (1981): Shift in nucleosome populations during embryogenesis: Microheterogeneity in nucleosomes during development of the sea urchin embryos. Biochemistry 20:4971–4978.

Spadafora C, Bellard M, Compton JL, Chambon P (1976): The DNA repeat lengths in chromatins from sea urchin sperm and gastrula cells are markedly different. FEBS Lett 69:281–285.

Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Tijssen P (1988): Affinity chromatography of immunoglobulins or antibodies In Burdon RH, Knippenderg PH (eds): "Laboratory Techniques in Biochemistry and Molecular Biology, vol. 15." New York: Elsevier, pp 105–114.

Vaughn JC (1968): Changing nuclear histone patterns during development. 1. Fertilization and early cleavage in the crab Emerita analoga. J Histochem Cytochem 16:473–479.

Voller A, Bidwell D (1986): Enzyme-linked immunosorbent assay. In Rose N, Friedman H, Fahey J (eds): "Manual of Clinical Laboratory Immunology." Washington, DC: American Society for Microbiology, pp 99–109.

Weintraub H (1984): Histone-H1-dependent chromatin superstructures and the suppression of gene activity. Cell 38:17–27.