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ARTICLES

Immunobiochemical Evidence for the Loss of Sperm Specific Histones During Male Pronucleus Formation in Monospermic Zygotes of Sea Urchins

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Abstract To obtain information on the remodeling of sperm chromatin during male pronuclei formation, we have followed the sperm specific histones (SpH) that form the nucleosomal core by Western imunoblot analysis with policlonal antibodies directed against the core SpH. The results obtained indicate that the complete set of SpH is absent from zygote chromatin at the beginning of the first S phase. The disappearance of SpH is not coincidental for the five histone classes: SpH4 and SpH3 are lost 5–15 min post insemination (p.i.), SpH2B and SpH2A disappear 20–40 min p.i., and SpH1 is progressively diminished up to 30 min p.i. This order of sperm chromatin remodeling is not affected by the inhibition of protein synthesis by emetine, indicating that the factor(s) responsible for SpH disappearance are present in unfertilized eggs. The lost SpH's are not replaced by newly synthesized CS variants, since the basic proteins synthesized de novo during male pronuclei formation are not incorporated into chromatin remaining in the cytoplasm. These newly synthesized proteins are different from the CS variants as judged by their electrophoretic migration.

Key words: chromatin, chromatin remodeling, fertilization, embriogenesis

After fertilization, sperm nuclei are decondensed and transformed into male pronuclei that fuse with the female pronuclei re-establishing the diploid genome of embryos (Longo and Anderson, 1968). The biochemical transitions responsible for this remodeling are concerned with changes in the majority of sperm specific chromosomal proteins and the acquisition of chromosomal proteins that will induce a chromatin conformation compatible with fusion of male and female pronuclei.

Sea urchin sperm chromatin contains sperm specific histones (Sp H) (Imschenetzky et al., 1984; Strickland et al., 1977a, b, 1978a, b, 1980a–d; Subirana and Palau, 1968) resulting in a chromatin organization with the longest DNA repeat length yet determined, 240 to 260 b.p. of DNA, (Spadafora et al., 1976; Savic et al., 1981; Poccia, 1986). By contrast, egg chromatin is organized as atypical nucleoprotein particles, formed by seven CS chromosomal proteins, formerly CS histone variants (Newrock et al., 1978), interacting with 126 b.p. of DNA (Imschenetzky et al., 1989). The CS chromosomal proteins are homologous to histones in their function of maintaining chromosomal structural organization, but, differ from histones in their number and amino acid compositions (Imschenetzky et al., 1986). After fertilization sperm nucleosomes are remodeled to nucleoprotein particles exhibiting a shortened DNA repeat length (Savic et al., 1981; Shaw et al., 1981), for which the precise structural organization is still unknown. The histone composition of these nucleoprotein particles remains to be determined in normal embryos, since biochemical separation of male and female pronuclei is technically not feasible. To overcome this difficulty histone transitions have been investigated in polyspermic zygotes by electrophoretic analysis of sperm specific histones after fertilization. The results obtained from such studies indicated that shortly after fertilization (10 min p.i.) SpH1 is phosphorylated and then greatly diminished in the chromatin and the CS chromosomal protein that migrate in H1 region is recruited from maternal pool (Green and Poccia, 1985). The core histones appear to be similar to sperm histones before the beginning of the first S phase; then coincidental with

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the first replication wave, (30 min p.i.) the CS chromosomal proteins in the region of the gel where H2A, H2B, and H3 migrate become predominant by the end of the first cell cycle (Poccia, 1986). The CS variant that migrates in the H2A position is phosphorylated exclusively during the first S phase, and this phosphorylation is enhanced if DNA replication is inhibited (Green and Poccia, 1989). The fate of sperm specific core histones during male pronuclei formation remains unknown, since sperm histones are not clearly distinguishable from the CS variants in two-dimensional gels.

In normal diploid embryos the strong similarity of the amino acid compositions of the histones in eggs and zygotes entering the first S phase suggests the loss of the complete set of sperm histones before the first S phase in sea urchin embryos (Imschenetzky et al., 1980). As was previously shown in *Tetrapygus niger* sperm histones differ in their amino acid composition from the CS variants that are found in unfertilized eggs (Imschenetzky et al., 1984, 1986). It could, therefore, be expected that the antibodies against the core SpH will not cross react with the CS variants. Consequently, this approach was utilized to directly examine the fate of sperm specific core histones after fertilization by Western immunobolt analysis. As shown in this report, the antibodies directed against the core SpH did not cross react with CS variants, these antibodies were then utilized to determine the fate of the core SpH after fertilization. To investigate the potential need of proteins synthesized after fertilization in sperm chromatin remodeling, the fate of core SpH was also determined in emetine treated zygotes. Alternatively, the incorporation of newly synthesized acidic proteins into chromatin was determined from fertilization until the beginning of the first S phase. The results reported here demonstrate that the complete set of sperm specific histones that are form the nucleosomal core disappears from chromatin before the first S phase in normal diploid zygotes of sea urchins. This sperm chromatin remodeling is not dependent on the proteins synthesized after fertilization.

METHODS AND MATERIALS

Gametes and Zygotes

Sea urchins (*Tetrapygus niger*) were collected from the bay of Concepcion 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., 1988a, 1989).

Isolation of Chromosomal Proteins and Gel Electrophoresis

Sperm histones and the CS chromosomal proteins from eggs and zygotes were isolated as described previously (Imschenetzky et al., 1984, 1986). Core sperm histones and SpH1 were isolated as described by Liao and Cole (1981). Sample preparation and uni- and two-dimensional gel electrophoresis were performed as described previously (Alfageme et al., 1974; Laemmli, 1970; Imschenetzky et al., 1984).

Preparation of Antisera and Western Immunoblots Analysis

Core sperm histones were utilized as antigen. Briefly, an initial dose of 1.2 mg of core SpH in 1 ml of buffer 0.1 M sodium phosphate, pH 7.4 was emulsified with 1 ml of complete Freund adjuvant and was used to immunize rabbits. Inoculations were given biweekly with the same dose of antigen dissolved in incomplete Freund adjuvant. The titer of the serum against core SpH was determined by ELISA, using an alkaline phosphatase antibody conjugate, essentially as described by Voller and Bidwell (1986). When the titer of the antiserum was 1/640, the serum used in excess was diluted 1/50 in a Tris buffered saline solution (TBS) containing 0.02 M Tris, pH 7.5, 0.5M NaCl, and 1% (w/v) gelatin to incubate the nitrocellulose membranes for the Western immunoblot analysis. The procedures followed for the electrophoretic transfer, blotting, and immunodetection were essentially those described by Towbin et al., 1979), modified as described previously (Imschenetzky et al., 1988b).

Determination of Proteins Synthesized de novo After Fertilization

Zygotes were incubated continuously from 3 to 30 min after insemination in a mixture of ^{3}H amino acids (algal protein hydrolyzate, NEN, Boston, MA) at a concentration of 10 mCi/ml. At 30 min the zygotes were harvested and processed to obtain the CS chromosomal proteins and the acidic chromosomal proteins as described previously (Imschenetzky et al., 1986, 1988a). Then, the cytoplasmic soluble fraction was obtained after a centrifugation at 30,000*g*

for 30 min and the proteins soluble in 0.25 M HCl were isolated. The radioactivity associated with newly synthesized polypeptides was measured by the paper filter disc method described by Bollum (1969) and the identification of radioactive basic cytoplasmic proteins was performed by fluorography of the two-dimensional gels in which these proteins were separated. Gels were prepared for fluorography as described by Chamberlain (1979) and exposed to Kodak X-OMAT AR films as described by Laskey and Mills (1975).

RESULTS

Electrophoretic Analysis of Histones During Male Pronucleus Formation

To obtain information about the complement of histones in diploid zygotes during male pronucleus formation, the total acid soluble chromosomal proteins were isolated from zygotes harvested at different stages of male pronucleus decondensation, 5 min p.i., 15 min p.i. (initial and intermediate decondesation stages), 30 min p.i., and 40 min p.i. (final decondesation stage and the beginning of the first S phase). In Figure 1 the electrophoretic profiles of these proteins are compared under two different denaturing conditions with those obtained from male and female gametes. Figure 1B shows that sperm histone H1 (SpH1) is resolved better in 12% polyacrylamide SDS gels than in Urea/Triton DF-16 gels (Fig. 1A). SpH1 was detected in zygotes until 30 min p.i. with the intensity of H1 band decreasing as time passed, suggesting that SpH1 is released from sperm chromatin according to its decondensation stage. The sperm core histones co-migrate with unfertilized egg derived CS chromosomal proteins in both Triton DF-16/urea and SDS gels in the first dimension (Fig. 1A,B), but in two-dimensional analysis, sperm H2A (SpH2A) is clearly distinguished from the CSF chromosomal protein that migrates in the SpH2A region in the uni dimensional gels (Fig. 1C,D). By contrast to Sp H1 and SpH2A, the other core histones SpH3, SpH2B, and SpH4 are not electrophoretically distinguishable from the CS variants derived from maternal chromatin either by uni- or two-dimensional electrophoretic analysis.

Immunobiochemical Analysis of the Histone Complement during Male Pronucleus Formation

To detect the sperm specific histones that form the nucleosomal cores in zygotes at different stages of male pronucleus decondensation, we determined the representation of these proteins with antibodies directed against core SpH by Western immunoblot analysis. Total acid soluble chromosomal proteins were obtained from both gametes and zygotes at different times p.i. (5 min, 15 min, 30 min, and 40 min) and resolved by electrophoresis in uni- and two-dimensional gels. These gels were subsequently transferred to nitrocellulose membranes and reacted with antibodies against the core SpH. Figure 2A shows that anti-core SpH does not cross react with CS chromosomal proteins from unfertilized eggs, with exception of the antiSpH2A which exhibits a cross reaction with CSF that migrates into the SpH2A region in uni-dimensional gels. This cross reaction was resolved by two-dimensional gels, as shown in Figure 2B, since SpH2A and CSF migrate into a different position in these gels.

The fate of sperm specific core histones after fertilization was analyzed by Western blots with antisera directed against core SpH. As shown in Figure 3A, SpH4 ceased to be a component of zygotic chromatin before 15 min p.i., while SpH3 and SpH2A were slightly detectable at that time. At the final stages of male pronucleus decondensation (30 min p.i.), SpH2B is still detectable, but is no longer present in chromatin at the beginning of the first S phase (40 min p.i.). The band observed in SpH2A position in the uni-dimensional gels, corresponds to CSF, as is shown in the two-dimensional analysis (Fig. 3B), since SpH2A was detected in the chromatin of zygotes 15 min p.i., but disappeared afterwards.

This pattern of sperm chromatin remodeling was not altered when the zygotes were continuously exposed to emetine at a concentration of 10^{-4} M, from fertilization until the beginning of the first S phase. As shown in Figure 4, the Western immunoblots analyses are similar for the core SpH isolated from zygotes that were cultured in the presence (Fig. 4A) or in the absence of emetine (Fig. 4B).

Proteins Synthesized De Novo During Male Pronucleus Formation

To examine the newly synthesized proteins during male pronucleus formation, zygotes were continuously incubated in a mixture of ^{3}H amino acids from 3 min p.i. to 30 min p.i. The acid soluble proteins were then isolated from the chromatin and from the cytoplasmic fraction (S 30), and the radioactivity associated with these

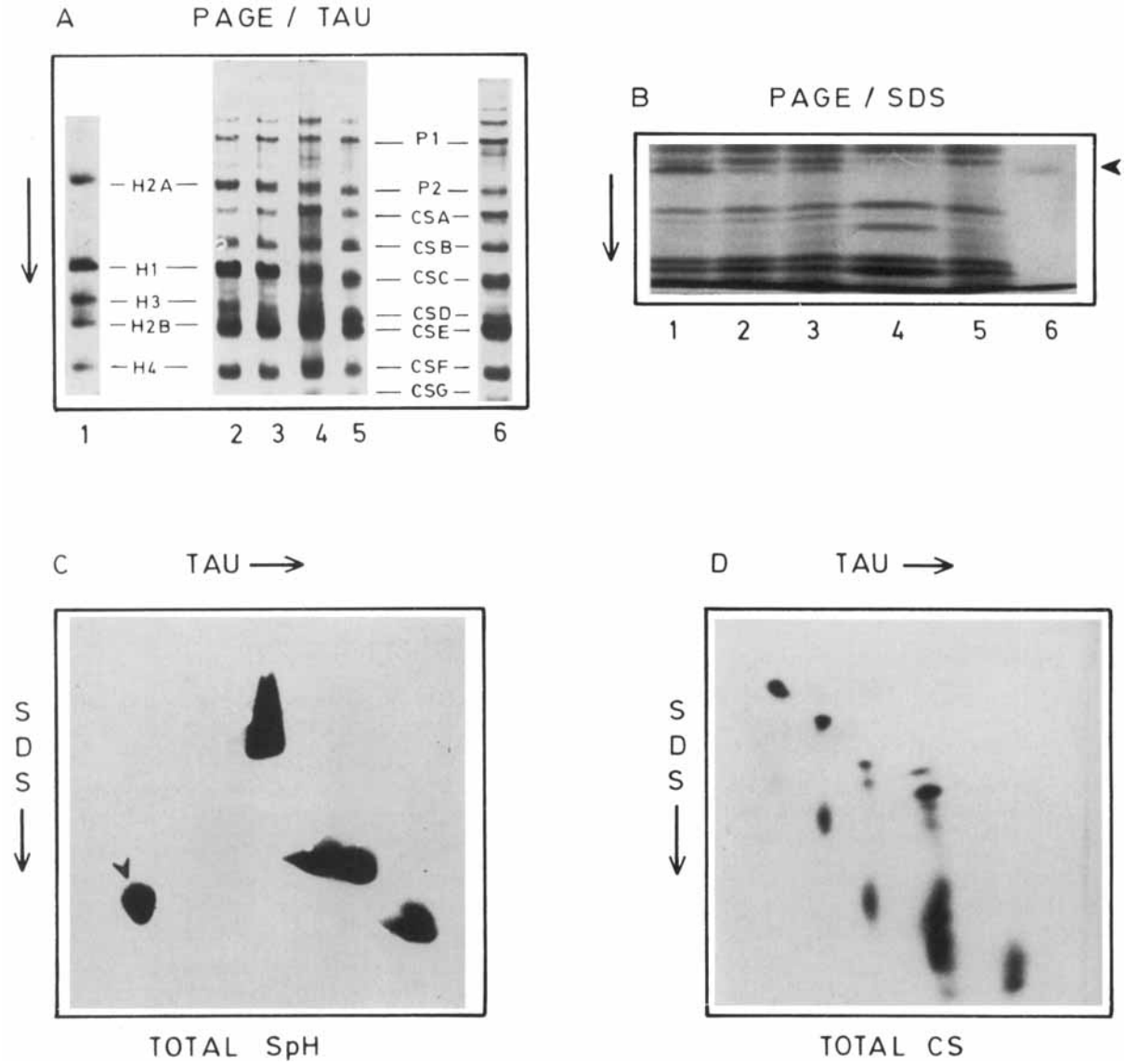

Fig. 1. Electrophoretic analysis of total basic chromosomal proteins obtained from gametes and from zygotes. Acid soluble proteins were obtained from chromatin and electrophoresed under different denaturing conditions. **A:** 15% acrylamide gels containing Triton DF-16, acetic acid, and urea (TAU). **Lane 1** shows sperm histones; **lanes 2–5** show basic chromosomal proteins isolated from zygotes harvested 5 min p.i., 15 min p.i., 30 min p.i., 40 min p.i., respectively, and **lane 6** shows CS variants from unfertilized eggs. **B:** 12% acrylamide gels containing SDS. The region of the gel corresponding to SpH1 is indicated by an arrowhead. Basic chromosomal proteins isolated from zygotes collected at 5 min p.i. (**1**); 15 min p.i. (**2**); 30 min p.i. (**3**); 40 min p.i. (**4**); CS variants from unfertilized eggs (**5**); and sperm histone H1 (SpH1) (**6**). Two-dimensional electrophoretic analysis of histone variants isolated from gametes; 15% acrylamide/TAU gels were used as the first dimension gels and 18% acrylamide/SDS gels as the second dimension. **C:** Sperm histones. **D:** CS variants from unfertilized eggs. The proteins were stained with Coomasie blue and the direction of electrophoretic migration is indicated by arrows.

proteins was determined. The results are shown in Table I as compared with proteins that are insoluble in 0.25 M HCl. As shown in Table I the major fraction of newly synthesized proteins were acidic and only a minor fraction of cytoplasmic basic proteins were observed, whereas no synthesis de novo of chromosomal basic proteins was detected. The cytoplasmic basic proteins were further analyzed by two-dimensional gel electrophoresis and the radioactivity associated with the newly synthesized polypeptides was determined by fluorography (Fig. 5). As shown in Figure 5 the basic polypeptides synthesized from 3 min p.i. until 30 min p.i. differ from

Fig. 2. Western blot analysis of basic chromosomal proteins isolated from gametes using antibodies to sperm core histones. Acid soluble proteins were isolated from gametes and electrophoresed in 18% acrylamide gels containing SDS, then transferred to nitrocellulose membranes and revealed using antibodies to sperm core histones (anti-cSpH). **A: Lane 1** shows the CS variant from unfertilized eggs cross reacting with the serum anti-cSpH; **lane 2** shows standard sperm core histones stained with Coomasie blue; **lane 3** shows the Western blot of the sperm core histones **B:** Total CS variants from unfertilized eggs were electrophoresed in two-dimensional gels, the first dimension was performed in 15% acrylamide/TAU gels, and the second dimension in 18% acrylamide/SDS gels, which were then transferred to nitrocellulose membranes and revealed using antibodies to cSpH. The Coomasie stained slot of the first dimension gel, containing the CS variants from unfertilized eggs, is included in the top of the two-dimensional Western blot as a reference of electrophoretic migration of these proteins. The direction of electrophoretic migration is indicated by arrows.

the CS chromosomal proteins, as well as from the SpH, indicating that these chromosomal proteins are not synthesized during male pronucleus remodeling.

DISCUSSION

The fate of sperm histones during male pronucleus formation was determined by an immunobiochemical analysis of the core SpH in normal diploid zygotes of sea urchins. As shown in this report the total complement of SpH is lost from sperm chromatin during male pronucleus remodeling. The core sperm histones disappeared noncoincidentally, since SpH4 and SpH3 were lost in the early and intermediate stage of male pronuclei decondensation (5 to 15 min p.i.), whereas SpH2B and SpH2A disappeared in the final stage of nuclear decondensation (20 to 30 min p.i.) (Fig. 3). These results are complemented by the finding that the basic proteins synthesized de novo from fertilization until the onset of the first S phase are not bound to chromatin and remain in the cytoplasm of zygotes (Table I and Fig. 5). These newly synthesized basic proteins are electrophoretically different from both the CS variants and the SpH when analyzed in a two-dimensional high-resolution gel (Fig. 5). Previously, these basic proteins were not resolved in uni-dimensional gels, thus leading to misinterpretations concerning the cytoplasmic proteins migrating in the upper and lower regions of acetic acid urea gels (Imschenetzky et al., 1983).

The results reported here extend previous findings concerning the mechanisms of male pronucleus formation, since the cytochemical approaches to this problem have led to the idea of the loss of sperm specific basic proteins in all species thus far investigated. However, the cytochemical analysis lacks the molecular detail provided in this report (Vaughn, 1968; Bloch and Hew, 1960; Ecklund and LeVine, 1975; Das et al., 1975; Kopecny and Pavlok, 1975). The biochemical evidence obtained from the studies of polyspermic zygotes provides the schedule of the acquisition of different CS variants after fertilization, but no direct evidence can be inferred concerning the disappearance of the nucleosomal core SpH, since these components of sperm chromatin are not electrophoretically distinguishable from the maternal CS proteins (Poccia et al., 1981; Green and Poccia, 1985, 1989). The results reported here are in agreement with the general idea of a non-coordinated replacement of the SpH by maternally-derived CS variants, although the order of this replacement is

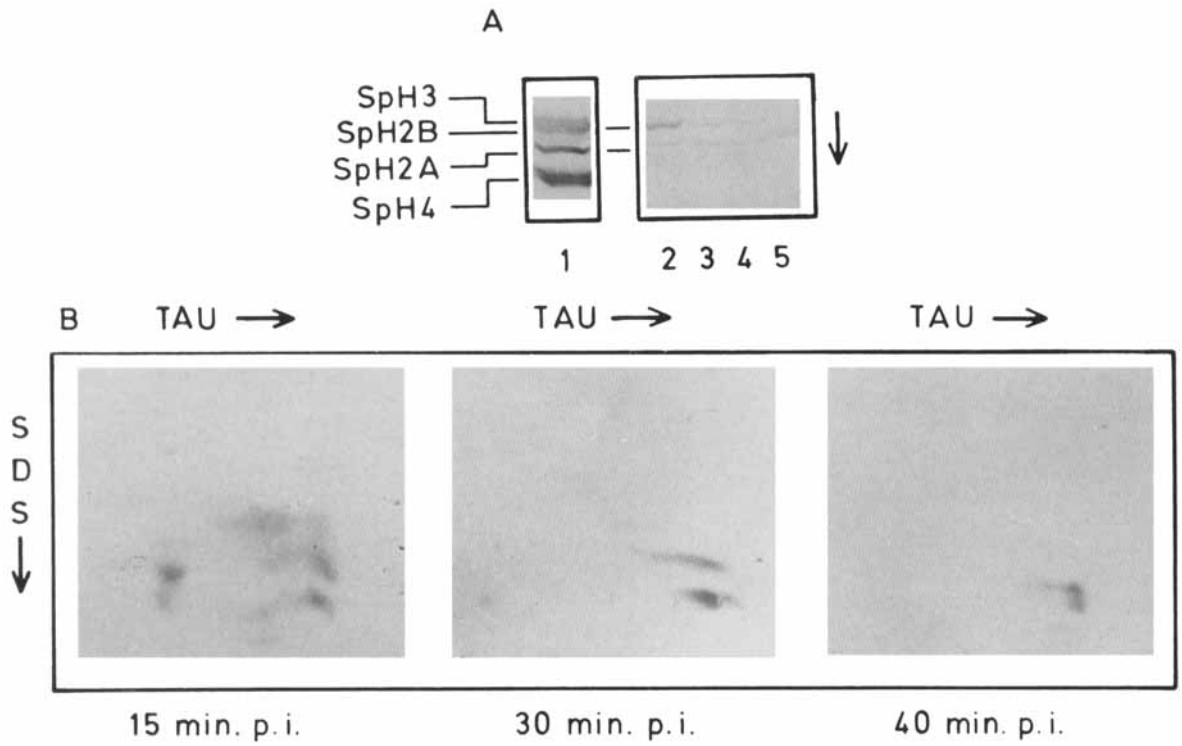

Fig. 3. Western blot analysis of basic chromosomal proteins isolated from zygotes using antibodies to sperm core histones. **A:** Total basic proteins were isolated from the chromatin of zygotes and electrophoresed in 18% acrylamide gels containing SDS, and were then transferred to nitrocellulose membranes and revealed using antibodies to sperm core histones (anti-cSpH). The Western blot of sperm core histones is shown in **lane 1; lanes 2–5** show the Western blots corresponding to the basic chromosomal proteins isolated from zygotes 5 min p.i.; 15 min p.i.; 30 min p.i., and 40 min p.i., respectively. **B:** Whole basic chromosomal proteins from zygotes were harvested 15 min p.i., 30 min p.i., and 40 min p.i., respectively, and were electrophoresed in two-dimensional gels; 15% acrylamide/TAU gels were used as the first dimension and 18% acrylamide/SDS gels as the second dimension. They were then transferred to nitrocellulose membranes and revealed using antibodies to cSpH. The time of zygote harvesting is indicated and the direction of electrophoretic migration is indicated by arrows.

not strictly coincident in polyspermic and normal zygotes. Total loss of the SpH was observed in normal zygotes entering the first S phase, whereas Sp2HA and Sp2HB are still present in polyspermic conditions (Poccia, 1986). The interpretations of these differences is very difficult as the identification of male and female basic protein complements in polyspermic zygotes, based only on changes of electrophoretic mobilities might be misleading since ambiguities can reflect major changes of the electrophoretic migration of CS variants induced by post-translational modifications such as ADPribosylations and ubiquitinilations (Boulikas et al., 1990) or minor changes of migration induced by acetylations, methylations, or phosphorylations. Therefore, the loss of a spot in a gel or its appearance does not strictly indicate the replacement of a specific SpH for its maternal counterpart. A complete loss of the sperm histones complement at the beginning of S phase, demonstrated here by an immunobiochemical approach, is consistent with our previous report indicating that the amino acid composition of the whole complement of basic chromosomal proteins from eggs and zygotes at the beginning of the first S phase are almost identical (Imschenetzky et al., 1980).

The factor(s) involved in male pronucleus formation is (are) present in the cytoplasm of fertilized eggs, since the sperm histone disappearance was not affected by the inhibition of protein synthesis by emetine, either in diploid zygotes (Fig. 4) or polyspermic zygotes (Poccia et al., 1981). This capacity to promote sperm chromatin remodeling develops during oocyte meiotic maturation and subsequent egg activation in sea urchins (Longo and Kunkle, 1978), amphibians (Moriya and Katagiri, 1976; Lohka and Masui, 1983, 1984a,b), and mammals (Usui and Yanagimachi, 1976; Iwamatsu and Chang, 1972;

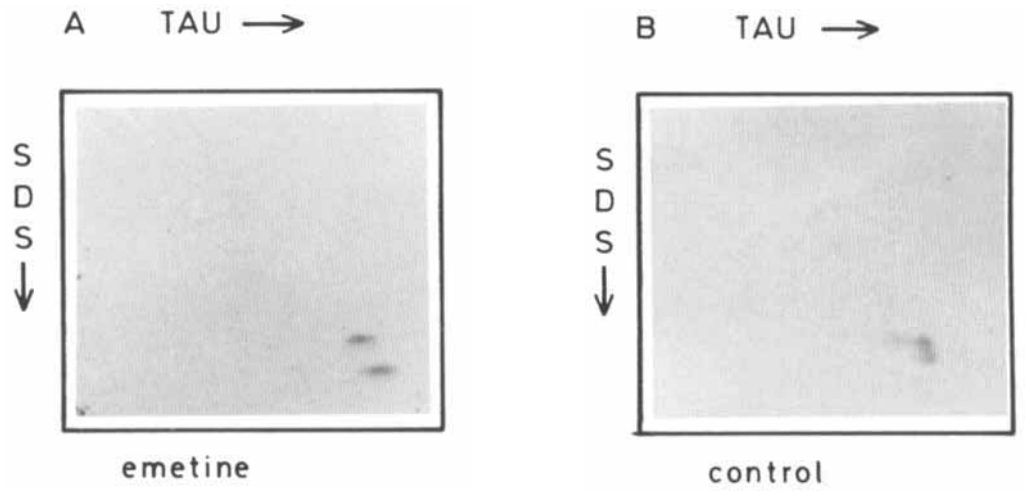

Fig. 4. Western blot analysis of basic chromosomal proteins isolated from zygotes 40 min p.i. using antibodies to sperm core histones. Basic chromosomal proteins were isolated from normal zygotes and from zygotes that were continuously exposed to emetine from insemination until 40 min p.i.; these proteins were electrophoresed in two-dimensional gels using 15% acrylamide/TAU gels as the first dimension gels and 18% acrylamide/SDS gels as the second dimension. They were then transferred to nitrocellulose membranes and revealed using antibodies anti-cSpH. **A:** Shows the Western blot corresponding to the basic chromosomal proteins isolated from zygotes exposed to emetine; **B:** shows the Western blot of the basic chromosomal proteins isolated from normal zygotes. The direction of electrophoretic migration is indicated by arrows.

Niwa and Chang, 1975) or soon after the germinal vesicle breakdown in Pectinaria (Hylander et al., 1981) and starfish (Schuetz and Longo, 1981; Hirai et al., 1981). The nature of the factor(s) participating is not yet understood, nor the molecular mechanisms involved. However, postranslational modifications of the SpH, for example, phosphorylation of SpH1 and SpH2B that has been described for polyspermic sea urchin zygotes (Green and Poccia, 1985).

Taken together, the information derived from this report and others (Poccia et al., 1981, 1986; Shaw et al., 1981; Imschenetzky et al. 1980, 1983, 1984, 1986, 1988a,b, 1989) suggests that during male pronuclei formation the whole complement of SpH is lost, as well as the majority of sperm specific non-histone chromosomal proteins (Imschenetzky et al., 1988b). This remodeling of sperm chromatin is independent of proteins synthesized after fertilization, suggesting maternal origin for the factor(s) involved in male pronucleus formation. Synthesis de novo of cleavage stage variants was not detected during the time of male pronuclei formation, adding further support to the replacement of SpH by maternally stored CS chromosomal proteins. At the time of amphimixis, estimated to occur at the beginning of the first S phase (Longo and Kunkle, 1978), the chromosomal protein complement of the male pronuclei is similar to that of the female pronuclei, establishing a compatibility between male and female genomes in terms of their protein composition. Taken together, these results are consistent with male pronucleus formation resulting from a total remodeling of chromatin protein composition, so, the factor(s) involved in male pronuclei formation should be clearly differentiated from others that may also participate in chromatin condensation-decondensation mechanisms occurring in mitosis or meiosis in sea urchin eggs (Arion and Meijer, 1989; Meijer and Pondaven, 1988) or other cells (Kishimoto, 1988), since the mitosis-dependent condensation-decondensation cycles

TABLE I. Proteins Synthesized During Male Pronucleus Formation*

Basic proteins	$\overline{cpm}$
S_{30} fraction	30545 ± 1431
Chromatin	non-detectable
Acidic proteins	**cpm**
S_{30} fraction	156253 ± 19006
Chromatin	244720 ± 25350

*Radioactivity associated with basic proteins isolated from chromatin and cytoplasmic fraction (S 30) as compared with acidic proteins. Average data of four independent experiments.

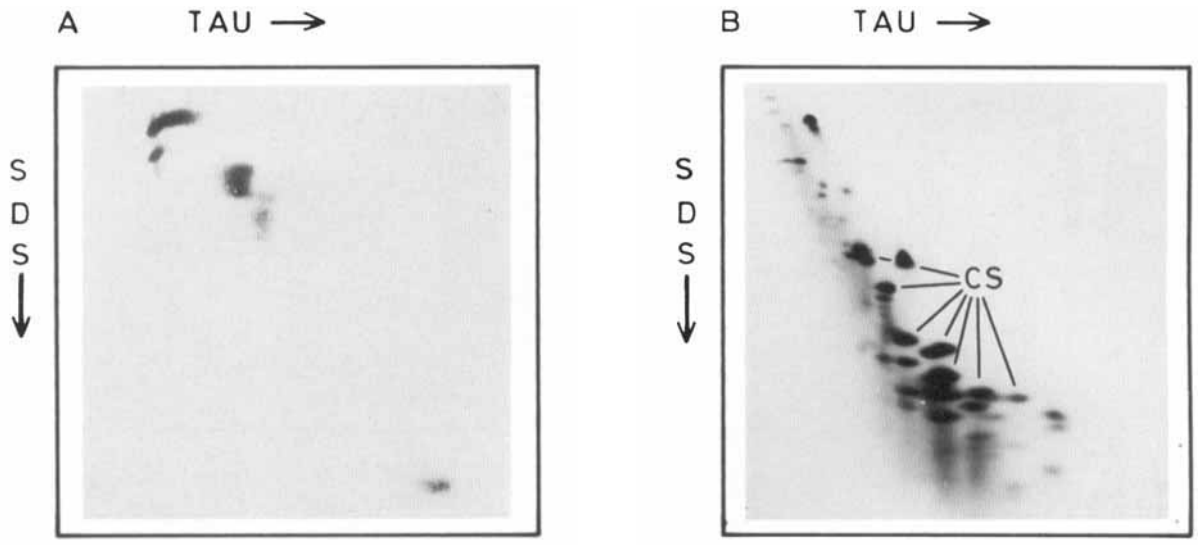

Fig. 5. **A:** Fluorograph of the basic cytoplasmic proteins synthesized during male pronuclei remodeling. Zygotes were labeled with a mixture of ^{3}H amino acids (algal protein hydrolyzate); the radioactive acid soluble proteins were obtained from the cytoplasmic fraction (S 30 fraction), electrophoresed in two-dimensional gels, and subjected to fluorography. The first dimension gels were 15% acrylamide/TAU and the second dimension were 18% acrylamide/SDS. The Coomasie blue stained two-dimension electrophoresis of total basic chromosomal proteins isolated from zygotes harvested 40 min p.i. is included as a standard of electrophoretic migration of the CS variants in **B.** The direction of electrophoretic migration is indicated by arrows.

do not induce the dramatic changes of chromosomal protein composition observed during male pronucleus formation.

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