Turnover of Basic Chromosomal Proteins in Fertilized Eggs: A Cytoimmunochemical Study of Events In Vivo

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ABSTRACT The chromosomal complements of mouse oocytes, ova, and fertilizing sperm have been studied by immunofluorescence with specific antisera to the basic protein fraction of sperm nuclei and to histones H_{2b} and H_4, and by staining with ethidium bromide. These studies support the hypothesis, previously proposed (Rodman and Barth, 1979, *Dev. Biol.* 68:82–95), that the chromosomes of the oocyte in maturation incorporate unique basic protein(s) similar to those incorporated during spermiogenesis. That similarity is characterized, here, by immunologic cross-reactivity. The basic proteins of the fertilizing sperm nucleus and the cross-reactive moiety of the two haploid complements of the ovum are displaced simultaneously, shortly after sperm entry. However, because the unique basic proteins incorporated into the oocyte chromosomes do not, as in the spermatogenic sequence, entirely replace the histones, the maternal chromosomes display histones H_{2b} and H_4 at all postfertilization stages examined, whereas the decondensing paternal complement, for an interval before maturation of the pronuclei, contains neither sperm basic chromosomal proteins nor histones. Sequential staining of the same specimens with ethidium bromide revealed well-organized nuclear morphology of the residual DNA complex. Those observations suggest that, for an as yet undefined period in the transformation from spermatozoal to embryonic genome, the chromatin is devoid of a complement of basic proteins.

During maturation, the chromosomal complements of the mammalian male and female gametes are reduced to a transcriptionally inert state and, after fertilization, are transformed to a transcriptionally competent state. It is reasonable to assume that the genesis of each of those states is related to alterations in the composition and organization of the chromatin. The available information, however, of the molecular events associated with the induction of either inertness in the gametic genomes or competence in the embryonic genome is limited, for the most part, to those occurring in spermatogenesis. The restriction obviously arises from the difficulty of obtaining mammalian oocytes or one- to two-cell embryos in adequate quantity for biochemical study of the nucleoproteins. To circumvent that restriction, therefore, we have developed methodology for cytoimmunochemical study of the chromosomal constituents of mouse oocytes and fertilized ova. We report here data obtained by application of that methodology.

Termination of RNA synthesis occurs, in the mouse, at midspermiogenesis (1, 2) and is believed to be related to the progression of turnover of basic chromosomal proteins. That progression includes displacement of histones and, from an early stage in spermiogenesis, incorporation of small, cysteine- and arginine-rich basic polypeptides accompanied by increasingly greater nuclear condensation (3). Although a similar correlation between molecular changes and cessation of transcription in the oocyte complement has yet to be made, the time at which RNA synthesis ceases has been identified in the mouse as just before germinal vesicle breakdown (4). The observation that the metaphase II meiotic chromosomes of the oocyte display arm ratios and banding patterns differing from those of their mitotic counterparts (5), followed by evidence that the oocyte complement undergoes two events of incorporation of (presumably) basic proteins, one in the terminal period of the dictyotene and the other after the first meiotic division (6), has led to the speculation that the complement of the oocyte, as that of the sperm, may have a unique nucleopro-

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tein composition. Specifically, it has been proposed (6) that the chromosomal proteins, incorporated during oocyte maturation, may be similar or identical to those incorporated during spermiogenesis. We here provide data in support of that hypothesis.

With regard to transformation of the parental genomes, it is known that, shortly after sperm entry into the ooplasm, decondensation of the sperm nucleus is initiated and the second meiotic division of the oocyte complement is completed. Then, the second polar body is extruded, and the maternal haploid set of chromosomes initiates metaphase-interphase transition. Finally, the parental complements form pronuclei, asynchronously, because the male pronucleus is seen while the female chromosomes are still identifiable (7, 8). Kopecny and Pavlok (9) have observed the loss from fertilizing mouse sperm of labeled arginine incorporated during spermiogenesis. Other than that, we know of no studies of the mechanisms of progression of events in the postfertilization restoration of transcriptional competence to the parental genomes. It is clear that not only must the gamete-unique proteins be deleted, but for the sperm, at least, histones must be introduced and nucleosomal organization be established *de novo*.

To probe those events, we have extended the cytoimmunochemical study of the chromosomal proteins of oocytes and fertilized eggs to determine when the nucleosomal core histones, H_{2b} and H_4, are present. Thus, we show that, after sperm entry, the displacement of the gamete-unique basic proteins takes place simultaneously from both maternal and paternal complements. However, unlike the sperm, where the spermiogenetic basic proteins have replaced the histones entirely, H_{2b} and H_4 have been retained in the oocyte chromosomes and, consequently, are present in the maternal complement. On the other hand, the decondensed fertilizing sperm nucleus, for an interval before formation of the binucleate zygote, appears to be devoid of either gamete basic proteins or nucleosomal histones. An in vitro study of the progression of decondensation and basic protein displacement of sperm nuclei[1] demonstrated that swollen sperm heads, denuded of membranes and depleted of basic chromosomal proteins, retain DNA organization and intact chromatin boundaries, as delineated by ethidium bromide staining. The in vivo study of this report shows similar retention of DNA organization and intact chromatin boundaries in the swollen intra-ooplasmic sperm, which are inferred to contain neither sperm basic nuclear proteins nor histones. Although that interpretation should be considered preliminary and requires confirmation by further experimental evidence, the implications are provocative: for a short period in transformation the sperm DNA is not complexed with a complement of basic proteins.

MATERIALS AND METHODS

Unfertilized oocytes and fertilized ova were obtained from ICR mice induced to superovulate by the administration of gonadotrophic hormones as described (4) and, as required, mated 16–20 h before sacrifice.

Cytologic Preparations

Considerable preliminary work was carried out to devise a cytologic preparation of oocyte or zygote in which the chromosomal elements could be identified and the various cytochemical and immunochemical procedures carried out. The following conditions were established: (*a*) Because it was found that the fluorochromes complexed with and irreversibly bound to the zona pellucida or its fragments, it was necessary to remove the zona before placing the oocyte or ovum on the slide. (*b*) Specific immunofluorescence of the chromosomal elements appeared to be inhibited or quenched by undispersed overlying ooplasm and by some ooplasmic particles. Also, some ooplasmic particles (lipid granules?) stained nonspecifically with the fluoresceinated reagent. It was necessary, therefore, to cause the oocyte or ovum to burst and the chromosomal elements to be dispersed, free of ooplasm, while still retaining their spatial context. The zona was removed by incubation in hyaluronidase (10 IU/ml) for 5 min (protease inhibitor added) followed by vigorous pipetting. The specimen with zona removed was placed in a drop of water on a slide, and the water was "chased" within 5–10 s by a drop of methanol–acetic acid (9:1), which was immediately blown dry. In every instance, a single specimen was placed on a slide and attempt was made to include some of the cells of the cumulus oophorus (derived from the ovarian follicle) as "somatic cell controls." The enzymatic and hypotonic treatment resulted in distortion of the morphology of those cells, but some identifiable cells could be found in most preparations.

The preparation of spermatogenic cells was made by lightly pressing the cut surface of a mouse testis on a slide, flooding the smear with methanol–acetic acid (9:1), and allowing it to air-dry.

Characterization of Antiserum to Sperm Basic Nuclear Proteins (SBNP)

A solution of the SBNP of mouse sperm was prepared and used to immunize a rabbit as previously described (3). The antibody content of the antiserum was identified as follows: A 4 M guanidinium chloride (GuCl) solution of SBNP was alkylated by treatment with ethylenimine (10) to allow separation of the two protamines of mouse sperm chromatin (3). The components of the SBNP were separated by elution with ascending concentrations of GuCl from a Bio-Rex 70 column (Bio-Rad Laboratories, Richmond, Calif.) (11). The elution fractions (Fig. 1) were grouped into three pools: (1) fractions 1–8, (2) fractions 12–18, (3) fractions 30–46. Pools 2 and 3 were electrophoresed on both SDS and acetic acid-urea polyacrylamide gels. The respective mobilities of the protein contents of each pool indicated that pool 2 consisted of a group of polypeptides (Fig. 2) less basic than those of pool 3. The components of pool 3 resolved into two bands on the acetic acid–urea PAGE (Fig. 3) representing the two protamines previously identified in mouse sperm chromatin (3). An unstained duplicate of the SDS PAGE of pool 2 (Fig. 2) and one of the acetic acid–urea PAGE of pool 3 (Fig. 3) were transferred to nitrocellulose paper (12), treated with the SBNP antiserum and ^{125}I-protein A, and a radioautogram was prepared of the immunologic reactivity (Figs. 2*b* and 3*b*).

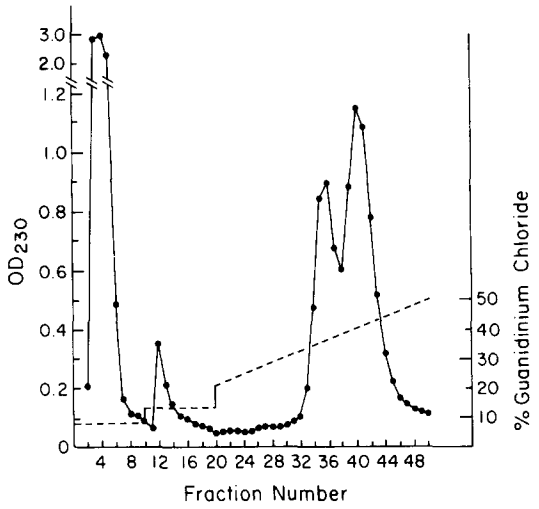


FIGURE 1 Chromatogram (OD 230 nm [●]) of GuCl (---) elution of mouse SBNP from an ion-exchange Bio-Rex 70 column. The elution fractions were grouped into three pools. Because pool 1 (fractions *1–8*) contained only trace amounts of TCA-precipitable material, the yield from a series of chromatographic separations, representing 4.8×10^9 sperm, was electrophoresed on a single lane of an SDS gel, transferred, and tested (12) with the SBNP antiserum; no immunologic reactivity was detected. The components of pool 2 (fractions *12–18*), representing 10^6 sperm, were separated on an SDS PAGE (Fig. 2) and those of pool 3 (fractions *30–46*), representing 10^6 sperm, were separated on an acetic acid–urea PAGE (Fig. 3).

[1] Rodman, T. C., F. H. Pruslin, and V. G. Allfrey. Mechanisms of displacement of sperm basic nuclear proteins in mammals. An in vitro simulation of post-fertilization events. Manuscript submitted for publication.

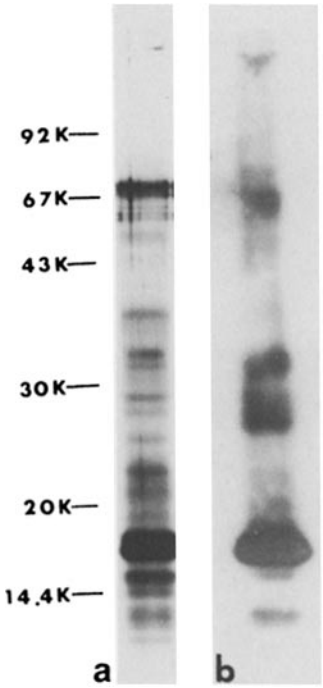


FIGURE 2 (*a*) Silver-stained SDS PAGE of pool 2 (Fig. 1) representing the nonprotamine class of mouse SBNP. (*b*) Radioautogram of a duplicate of the stained gel, transferred and treated with the antiserum to SBNP, showing that the antiserum contains antibodies to the two major components (16,000–17,000 mol wt) and to several of the minor components, some of which are too close to be separately resolved on the radioautogram.

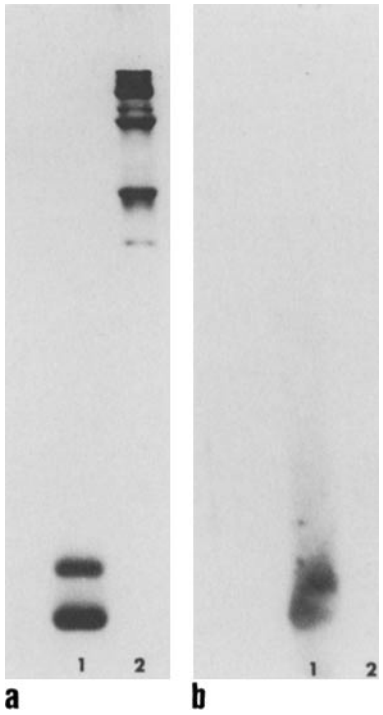


FIGURE 3 (*a*) Coomassie Blue–stained acetic acid–urea PAGE; lane *1*, pool 3 of chromatogram (Fig. 1); lane *2*, calf thymus histones. (*b*) Radioautogram of the immunologic reactivity of the SBNP antiserum with a duplicate of the gel of *a*. By virtue of their aminoethylation (see Materials and Methods), the two protamines of mouse SBNP (3) are well separated and antibodies to both are shown to be present in the antiserum. Although the highly positive protamines move too quickly for the histones to be well separated on the same gel (cf. Fig. 11), the absence of antibodies to histones is clearly demonstrated, confirming observations of a previous study using the SBNP antiserum (15).

Characterization of Antisera to Histones H_{2b} and H_4

Purified histones from calf thymus nuclei were prepared (13) and immunization of a separate rabbit with each histone was carried out by the method of Stollar and Ward (14) with modifications of the immunization schedule (H. P. Hoffmann, unpublished observations). The specificity of each antiserum was demonstrated by the gel transfer method (12) as described above and its titer of activity determined by solid phase radioimmunoassay (15).

Immunofluorescence

The presence or absence of chromosomal proteins identical to or cross-reactive with mouse SBNP or calf thymus histone H_{2b} or histone H_4 was demonstrated on gamete or zygote complements by indirect immunofluorescence, using the specific rabbit antiserum (undiluted or diluted 1:5 with phosphate buffer) and fluoresceinated goat anti-rabbit IgG, according to the procedure previously described (3). For each series of preparations, specimens representative of all stages were selected and carried through the procedure with preimmune serum substituted for antiserum. No fluorescence of chromosomal elements was observed in any preimmune serum-treated specimen. The preparations were mounted in phosphate buffer and photographed with phase-contrast optics on high-contrast film before immunofluorescence, and in the fluorescence microscope, with the Zeiss fluorescein isothiocyanate (FITC) exciter filter and 530-nm barrier filter on Tri-X film, after immunofluorescence.

Ethidium Bromide Staining for DNA

After immunofluorescence photography, the preparations were stained with ethidium bromide[1] and photographed on Tri-X film with the 546-nm interference filter for excitation and the 580-nm barrier filter, thus excluding emission from the FITC complex.

Detection of Sulfhydryl Groups in Ooplasm

Specimens selected for representative stages of oocyte maturation and postfertilization were incubated in a solution of tritium-labeled *N*-ethylmaleimide (NEM) in phosphate buffer (0.1 mC/ml) for 30 min, washed, and processed for radioautography as described (6).

RESULTS

Ooplasm as a Reducing Medium

It has been shown (3) that the spermatozoal basic chromosomal proteins, which are incorporated during spermiogenesis, may be displayed in spermatids and spermatozoa by immunofluorescence. Those proteins were revealed in spermatids of stages 1–14 by simple application of immunofluorescence to untreated testicular smears, while their display in spermatids of stages 15 and 16 and spermatozoa required treatment with a reducing reagent. It was concluded, therefore, that an oxidative event taking place in the spermatid nucleus results in compaction of the chromatin to a configuration wherein the antigenic sites of the basic proteins are "masked," i.e., unavailable for immunologic recognition and, conversely, are made available by treatment with a reducing agent. A dominant mechanism of that compaction is, presumably, the production of disulfide bonds between the copious cysteinyl residues of those proteins (16).

Because the hypothesis of this study proposes that the chromosomes of the metaphase II fertilizable oocyte contain proteins similar to those of the spermatozoal chromatin, it was essential to determine whether reducing treatment before immunofluorescence was necessary to display the putative oocyte

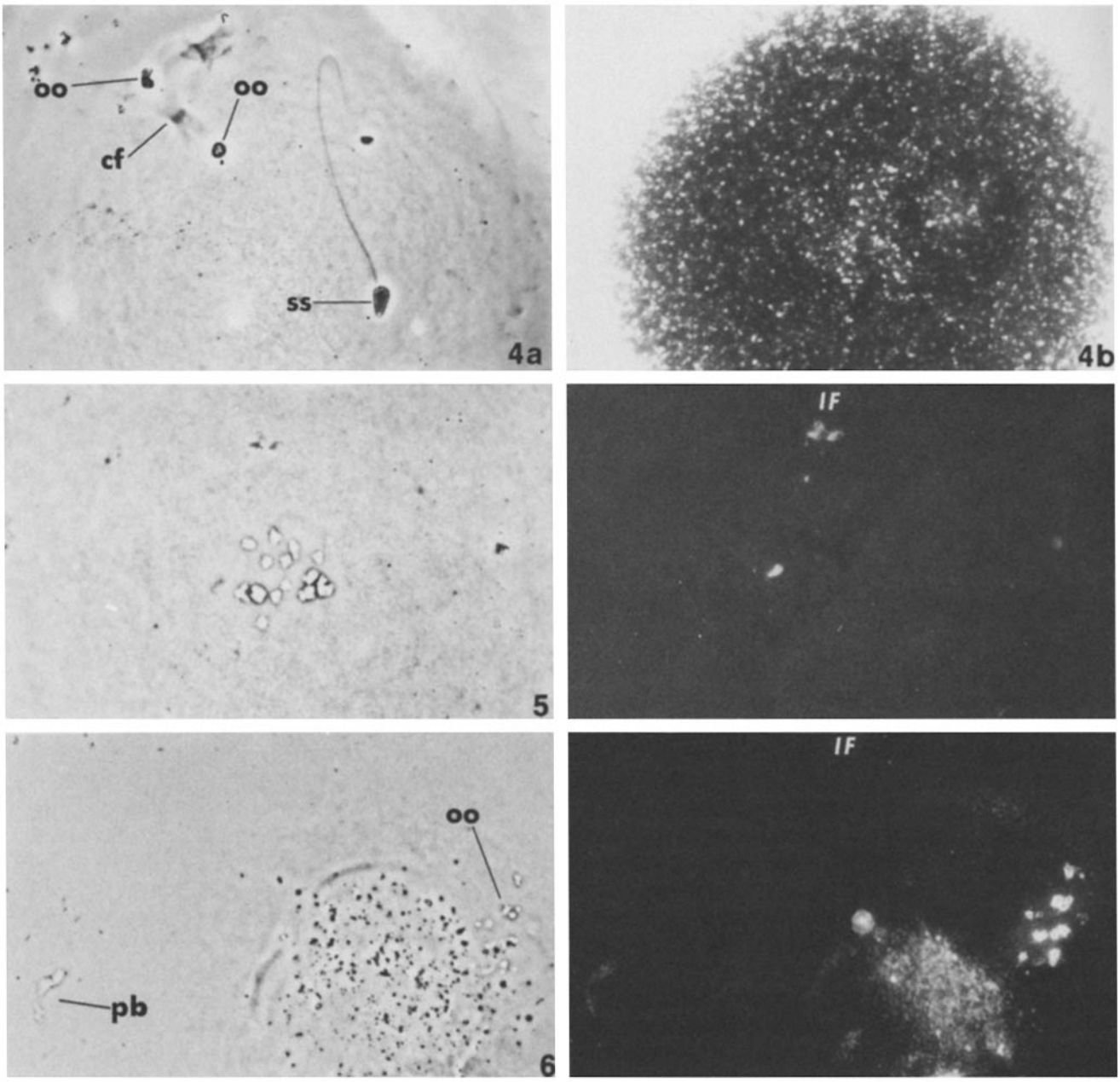


FIGURE 4 (*a*) Fertilized ovum, with gametic complements in situ; (ss) swollen fertilizing sperm; (*oo*) haploid complement of ovum at telophase II; (*cf*) site of cleavage furrow. (*b*) Radioautogram of NEM incorporation, showing high concentration of SH groups in ooplasm. × 400.

FIGURE 5 Phase contrast and immunofluorescence (IF) with SBNP antiserum, of oocyte at diakinesis. The chromosomes are not immunofluorescent. × 720. (For Figs. 5–10 and 13–18, the phase-contrast micrograph of unstained specimen is to the left of the IF).

FIGURE 6 IF, with SBNP antiserum, of oocyte after first meiotic division. The metaphase II chromosomes of the oocyte (*oo*) are immunofluorescent; the complement of the first polar body (*pb*) is not. × 280.

chromosomal proteins. Radioautograms of NEM activity (e.g., Fig. 4) confirmed that, throughout maturation and postfertilization, the ooplasm is sulfhydryl rich and that, therefore, extraneous reducing treatment was not required. Those observations also fortify the conclusion that postfertilization decondensation of the sperm nucleus takes place in a reducing medium (17).

Because the NEM-treated specimens were not subjected to removal of zona and treatment to disperse the ooplasm (see Materials and Methods), Fig. 4*a* serves to show the chromosomal complements *in situ*, demonstrating that swelling of the sperm head is well under way before abscission of the second polar body takes place and, thus, while the two haploid complements of the ovum are in compact telophase mass.

Immunofluorescence with Antiserum to SBNP

Figs. 2 and 3 show that the antiserum contains antibodies to the two protamines that are the major components of mouse SBNP and to the principal components of the nonprotamine

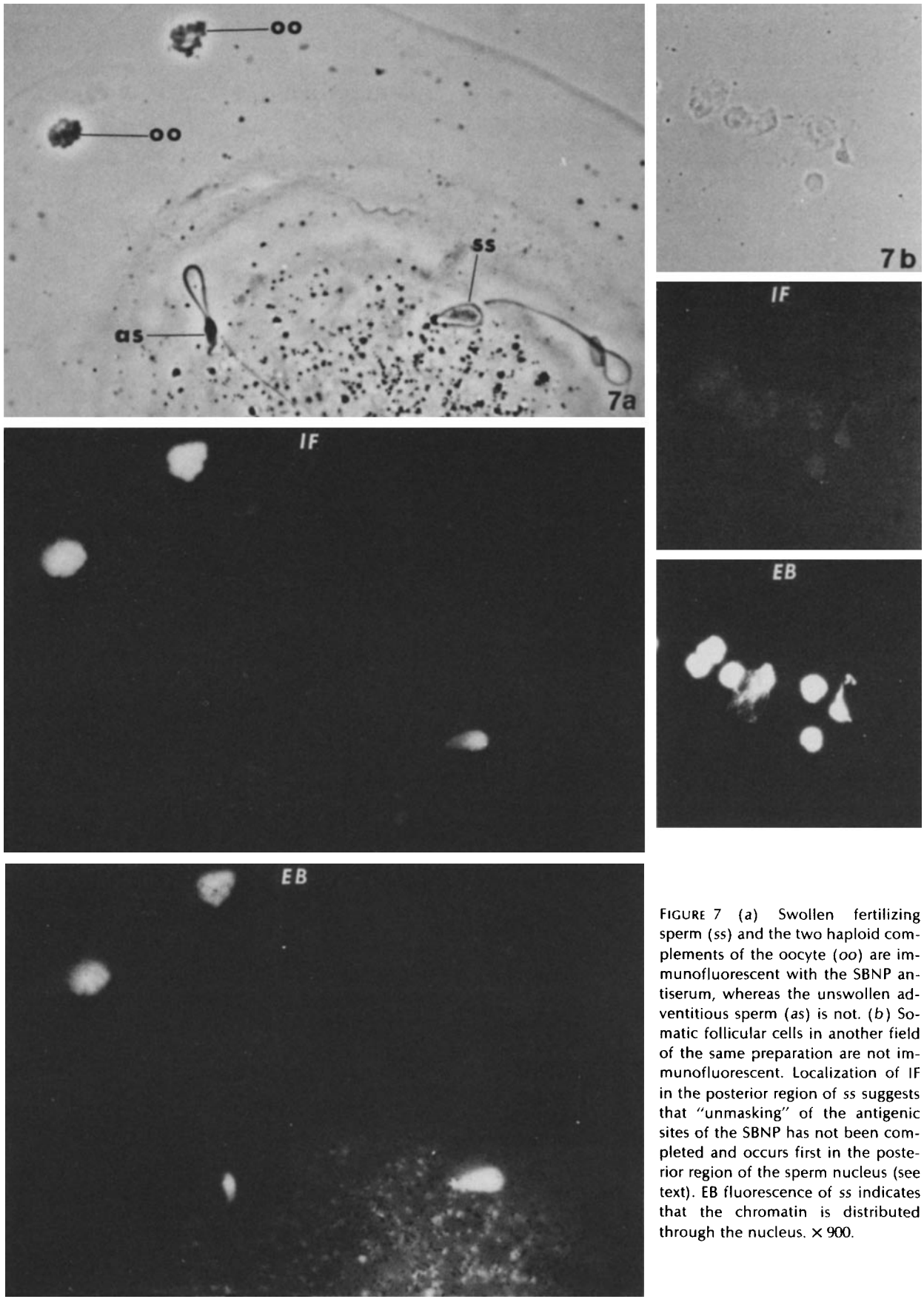
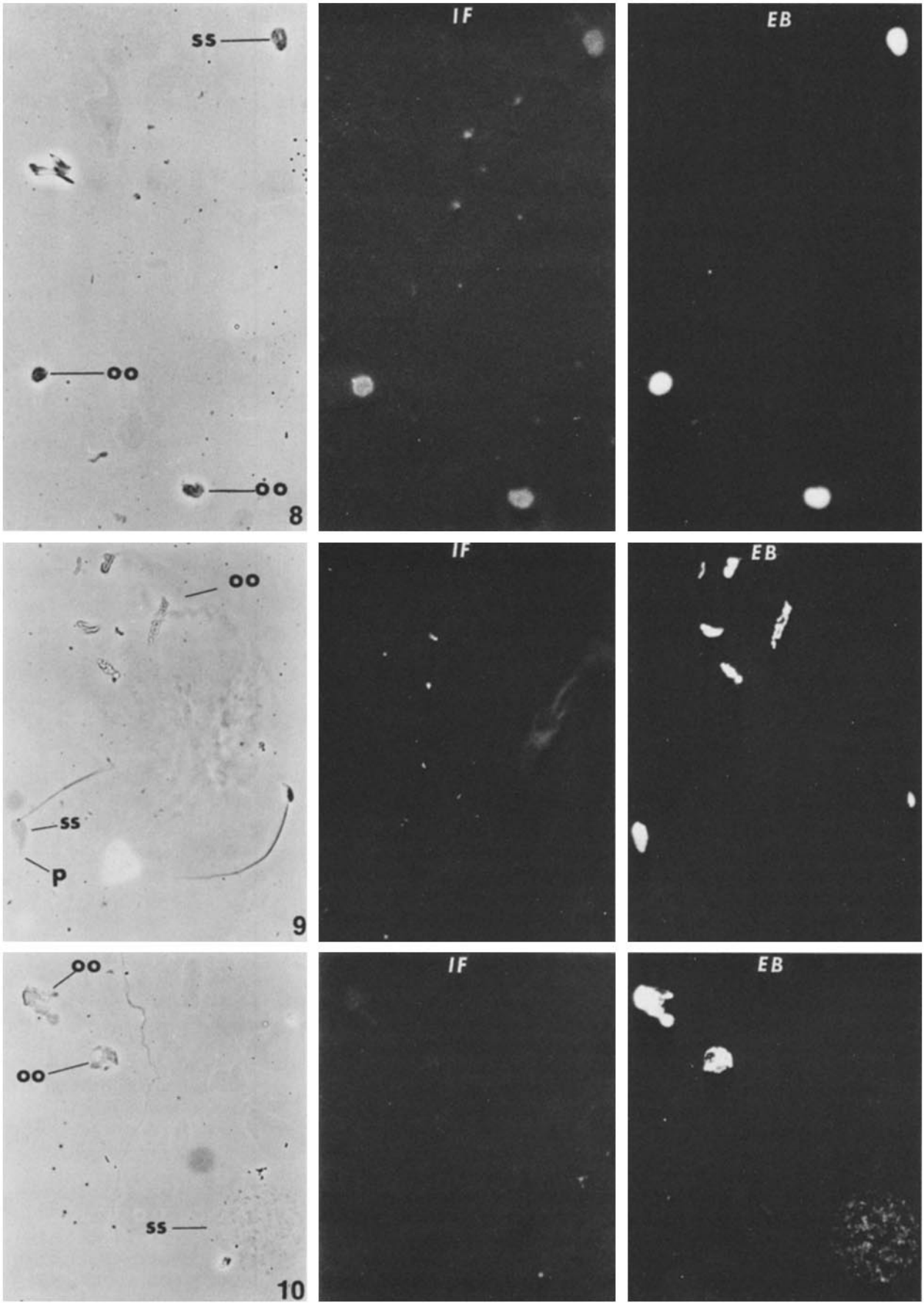


FIGURE 7 (*a*) Swollen fertilizing sperm (*ss*) and the two haploid complements of the oocyte (*oo*) are immunofluorescent with the SBNP antiserum, whereas the unswollen adventitious sperm (*as*) is not. (*b*) Somatic follicular cells in another field of the same preparation are not immunofluorescent. Localization of IF in the posterior region of *ss* suggests that "unmasking" of the antigenic sites of the SBNP has not been completed and occurs first in the posterior region of the sperm nucleus (see text). EB fluorescence of *ss* indicates that the chromatin is distributed through the nucleus. × 900.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class and confirm (15) that the antiserum does not contain antibodies to histones. Also demonstrated (see legend to Fig. 1) was the absence of antibodies to other moieties of mouse sperm that might have been present in trace amounts in the SBNP used for immunization. The immunofluorescence of the mouse oocyte or zygote chromosomes complements of this series, therefore, represents one or more components of mouse SBNP or a protein with sufficient molecular similarity to be immunologically cross-reactive.

PREFERTILIZATION STAGES: It has been shown that the metaphase II chromosomes of the secondary oocyte incorporate a (presumably) basic protein, whereas the sister complement, that of the first polar body, does not (6). The immunofluorescence procedure, therefore, was carried out on ovarian oocytes at successive stages of meiosis to determine whether the putative unique chromosomal protein of this study is represented by that incorporation. Of 27 preparations of oocytes at diakinesis–metaphase I, none showed immunofluorescence (e.g., Fig. 5). Because, as noted in Materials and Methods, it was necessary to remove the zonae, the polar bodies were lost and, of more than 40 trials, only two preparations were obtained in which both oocyte and first polar body complements could be identified. In both, the oocyte complements were stained, whereas the polar body complements were not (e.g., Fig. 6). Of 26 preparations of ovulated oocytes in which follicular cells were retained, 21 metaphase II complements were immunofluorescently reactive with the antiserum, whereas all somatic cell complements were nonreactive.

FERTILIZED OVA: After entry into the ooplasm, the fertilizing sperm nucleus swells and undergoes a succession of morphologic changes as chromatin decondensation to pronucleus proceeds. To make certain identification of the transforming sperm nucleus and of the variably compacted haploid complements of the oocyte, we stained the preparations with ethidium bromide (EB) after photography of the immunofluorescence. The EB identification of four gametic chromosomal complements in Fig. 7 *a* is clear: two oocyte sets at telophase, the moderately swollen fertilizing sperm, and an adventitious unswollen sperm. Of those, the two oocyte complements and the fertilizing sperm nucleus are immunofluorescently reactive with the antiserum; predictably (3), the unswollen sperm is not. The specificity of the immunofluorescence reaction is confirmed by the lack of staining in the metaphase and interphase complements of the somatic cells (Fig. 7 *b*).

As the transformation progresses, the immunofluorescence reactivity is lost simultaneously from the maternal and paternal complements (Figs. 8–10). Of 37 fertilized eggs in which those complements were clearly displayed, nine were immunofluorescently positive. In every instance, the paternal and maternal complements were similarly stained or unstained (e.g., Figs. 7–10). We conclude, therefore, that the SBNP and the cross-reactive oocyte chromosomal proteins are deleted from their respective complements at the same time. As noted in the Discussion, the low percentage of positively stained preparations indicates that the displacement of those gamete-specific proteins is completed within a short interval after sperm entry.

Immunofluorescence with Antisera to Histones

The antibody content of each of the antisera to histones H_{2b} and H_4 is characterized in Figs. 11 and 12. The competence of the antisera to recognize, with high specificity, isolated histones H_{2b} and H_4, respectively, is demonstrated in the radioautogram of the immunologic reactivity of each antiserum with its corresponding antigen (Fig. 11) and is verified in the radioimmunoassay (Fig. 12). The competence of the antisera to recognize those histones in spermatogenic cell (Fig. 13) and somatic cell (Figs. 14 and 17) chromatin *in situ* is shown. Fig. 12 indicates that each of the antisera displays considerable immunologic activity at the 100-fold dilution. Because the antiserum was used undiluted or diluted 1:5 for the immunofluorescence preparations, it is reasonable to equate negative fluorescence with absence of the antigen. The presence of each histone was separately tested for by immunofluorescence with its specific antiserum on a series of oocytes and ova at successive maturation and postfertilization stages. For each antiserum, >50 preparations were tested: all maternal and somatic cell complements were positive (e.g., Figs. 14–17), whereas no immunologic reactivity with either anti-H_{2b} or anti-H_4 was displayed by the fertilizing sperm at any of the stages or degree of decondensation represented by Figs. 14–17. Immunofluorescence with antiserum to H_{2b} was displayed by both pronuclei in the specimen of Fig. 18. Figs. 14–18 represent the H_{2b} antiserum series. Similar results at all stages were observed in the H_4 antiserum series.

Thus far, we have not succeeded in obtaining satisfactory preparations of the stages intervening between those of the specimens of Figs. 17 and 18. The specific immunofluorescence of the condensing chromatin of the pronuclei in Fig. 18 is distinguishable from the nonspecific fluorescence of the cytoplasmic granules, whereas that distinction is less firm in pronuclei with more diffuse chromatin. The degree of condensation of the chromatin in Fig. 18 suggests that the pronuclei of that one-cell embryo may have completed DNA synthesis (19). Methods for studying the earlier pronuclear stages and the events associated with introduction of histones into the paternal genome are in process of development.

Inspection of the immunofluorescence data, as illustrated by Figs. 5–10 and 14–18 show that the chromosomal proteins of the oocyte or zygote complements are accessible for immunologic reactivity at all stages. Absence of immunofluorescence with any of the antisera, therefore, may not be attributed to lack of accessibility for the antibody at certain states of chromatin.

FIGURE 8 IF with antiserum to SBNP showing that the basic proteins are still present in the swollen sperm nucleus (*ss*) and in the two complements of the ovum (*oo*). All complements have been scattered free of ooplasm, allowing the low level of IF to be detectable. × 500.

FIGURE 9 The proteins immunoreactive with SBNP antiserum have been displaced from the much swollen sperm (*ss*) and from the decompacting chromosomes of the ovum (*oo*). Note retention of perforatorium (*p*). × 400.

FIGURE 10 IF shows that the SBNP immunoreactive proteins are present in neither the fertilizing sperm chromatin (*ss*), which has decondensed to the configuration of a pronucleus, nor in the two complements of the ovum (*oo*), which have begun decompaction from the telophase mass. × 400.

DISCUSSION

The observations of this study indicate that the metaphase II chromosomes of the mature secondary oocyte of the mouse contain a protein that is immunologically cross-reactive with some component(s) of the SBNP fraction of mouse spermatozoa. The antiserum used to obtain those data has been shown, by immunochemical fractionation, to contain antibodies to the

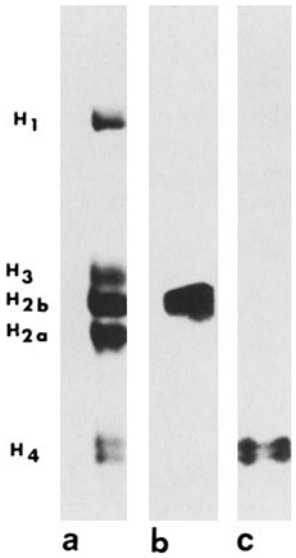

FIGURE 11 (*a*) Acetic acid-urea PAGE of calf thymus histones, Coomassie-Blue stained; the two major forms of H_4, monoacetylated and nonacetylated, are separately resolved (18). (*b*) Radioautogram of duplicate gel transferred and tested with the antiserum to H_{2b}. (*c*) Another duplicate similarly treated and tested with the antiserum to H_4; both forms are recognized by the antibody. The specificity of each antiserum is demonstrated.

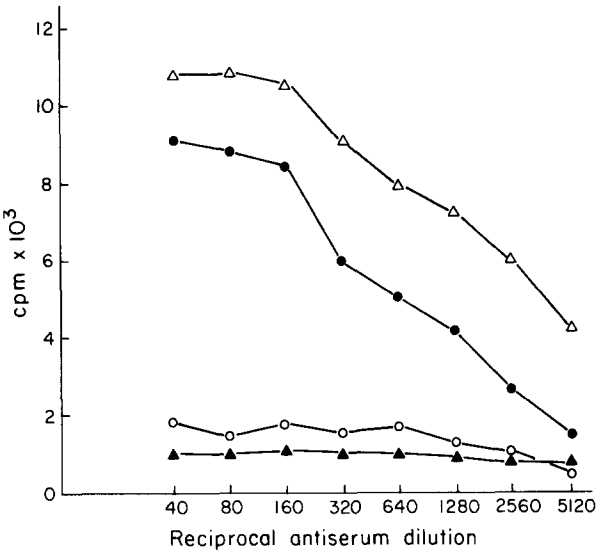

FIGURE 12 Radioimmunoassay of the reactivity of the two histone antisera with each of the purified histones. △ = anti-H_4 vs. H_4; ▲ = anti-H_4 vs. H_{2b}; ● = anti-H_{2b} vs. H_{2b}; ○ = anti-H_{2b} vs. H_4. The high specificity of each antiserum shown in Fig. 11 is verified and the titer of immunologic activity at 100-fold dilution supports the interpretations of the IF data of Figs. 13–18.

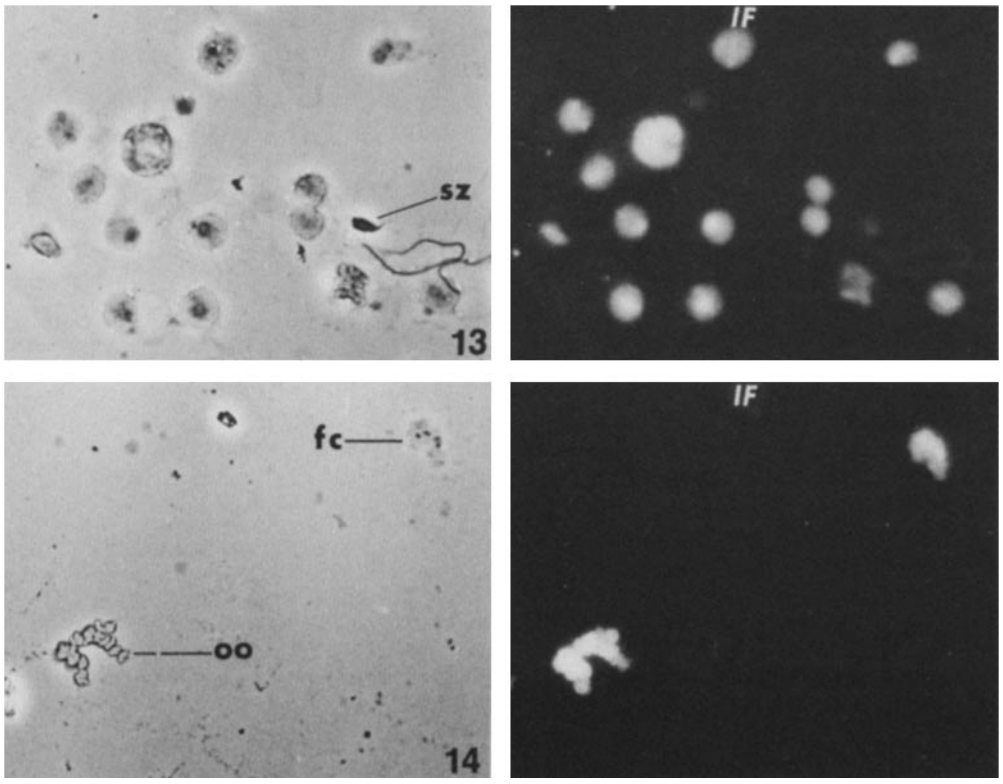

FIGURE 13 IF with antiserum to histone H_{2b} of testicular smear, demonstrating the presence of H_{2b} in all spermatogenic cells other than the spermatozoon (*sz*). × 400.

FIGURE 14 IF with antiserum to histone H_{2b} of metaphase II oocyte (*oo*) and somatic follicular (*fc*) cell chromosomes, showing the presence of the histone in both complements. × 400.

major components, two protamines (Fig. 3), and to some of the minor components (Fig. 2), the nonprotamine basic proteins of mouse sperm nuclei.[1] The immunochemical methods of this study do not designate the components(s) of the SBNP with which the oocyte chromosomes are cross-reactive. Those methods do, however, distinguish the reactive component as gamete-specific and suggest that it is incorporated into the oocyte complement after the first meiotic division.

Thus far, our attempts to identify that protein by biochemical analyses of oocyte chromosomes have been unsuccessful. Those analyses showed, as does the other series of immunofluorescence data in this study, that the oocyte metaphase II chromosomes contain nucleosomal core histones. A single mouse sperm, in which all histones have been replaced by the SNBPs contains 3.5 pg of the protamines and 0.062 pg of the nonprotamine group (F. H. Pruslin, unpublished observations). Even though the complement of the secondary oocyte is diploid, that complement might, with histones retained, contain an even smaller quantity of the unique basic protein(s), and the collection of an adequate number of ovulated mammalian oocytes for biochemical identification of that protein may not be feasible.

The cytochemical procedure allows the separate display of the basic chromosomal proteins by immunofluoresence and of

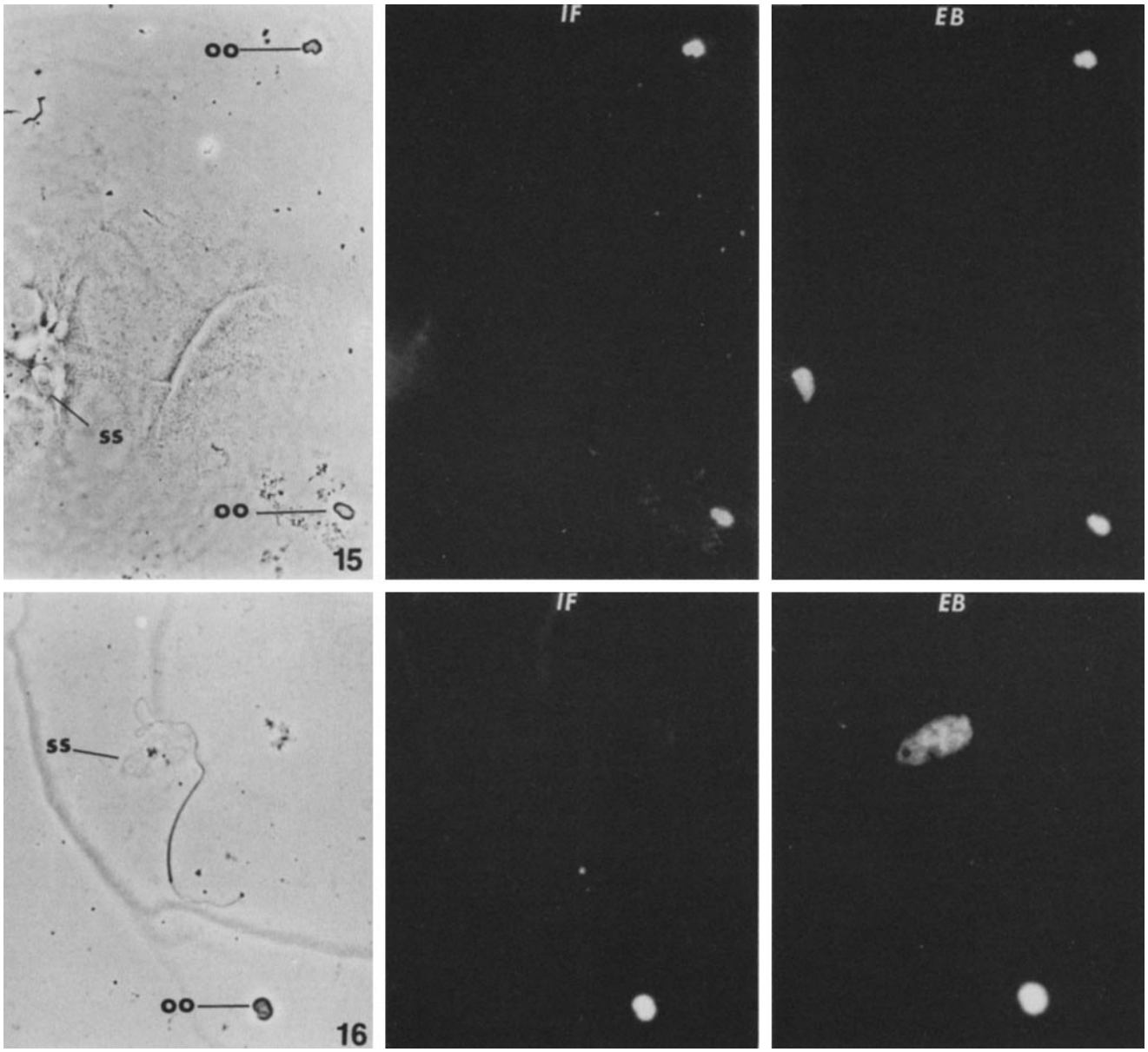

FIGURE 15 IF with antiserum to histone H_{2b}, where both complements of the ovum (*oo*) are immunofluorescent, whereas that of the swollen fertilizing sperm (*ss*) is not. The degree of decondensation of the sperm nucleus, revealed by EB staining, shows that this is a later stage than that represented by Fig. 9, where SBNP are absent, thus indicating that the sperm complement at this stage contains neither SBNP nor histones. × 400.

FIGURE 16 IF with antiserum to histone H_{2b} where the complement of the ovum (*oo*) is immunofluorescent, whereas the much-decondensed sperm nucleus (*ss*) is not. The degree of decondensation of the sperm chromatin, revealed by EB, indicates that this is a still later stage than that of the fertilized ovum of Fig. 15; SBNP have been deleted and histones have not yet been incorporated into the paternal complement. × 400.

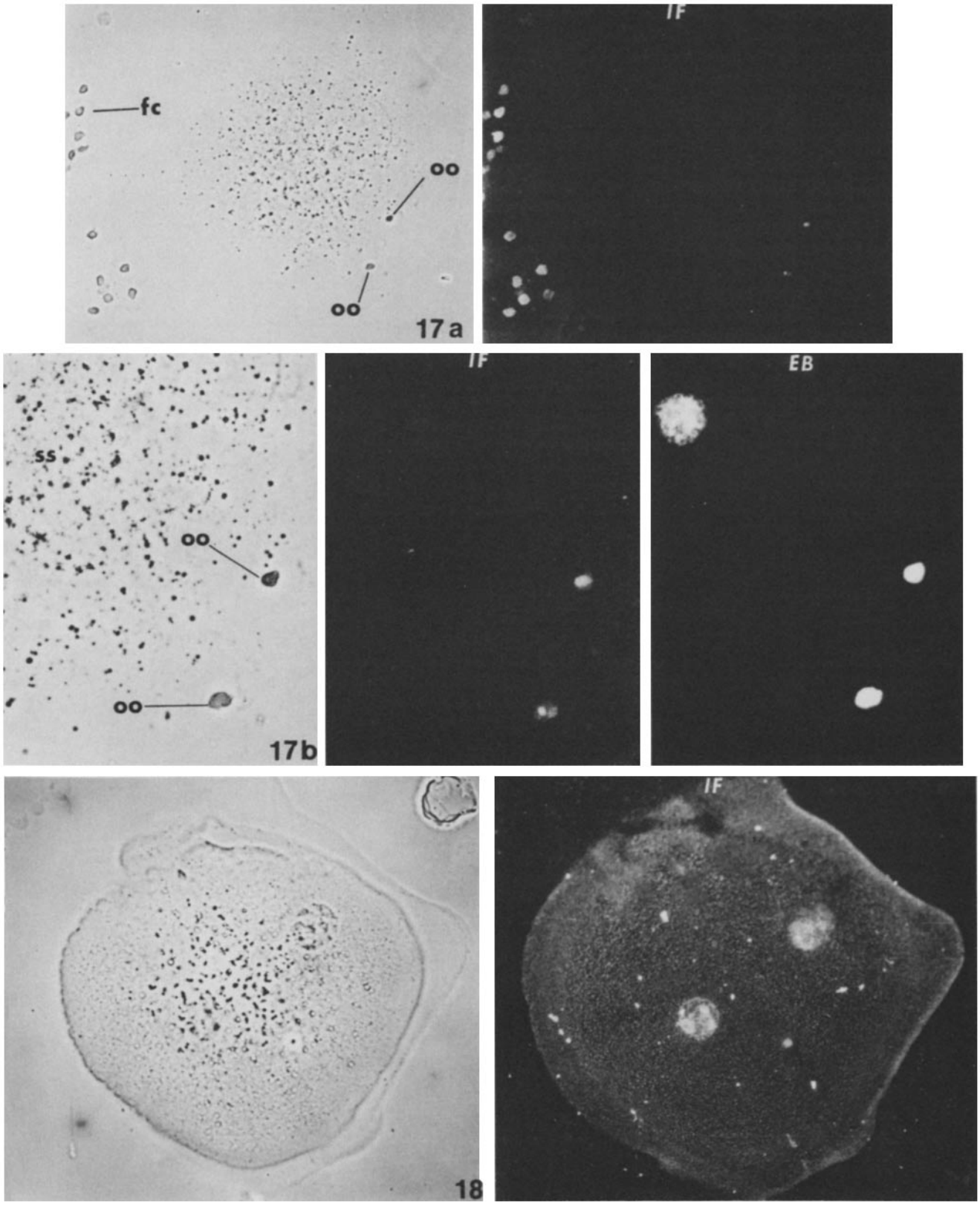

FIGURE 17 IF with antiserum to H_{2b}. (*a*) IF is displayed on the somatic cells (*fc*) and on the two complements of the ovum (*oo*), but not on the sperm chromatin (*ss*). × 100. (*b*) Higher magnification, showing absence of IF on the greatly decondensed chromatin of the male pronucleus. × 400.

FIGURE 18 IF with antiserum to histone H_{2b} of a one-cell embryo. The two pronuclei are immunofluorescent, indicating that H_{2b} is present in both maternal and paternal complements. × 400.

the DNA by EB fluorescence on the same specimen. With that resolution, it is particularly interesting to note the different distribution of fluorescence revealed by each of the two reagents in the fertilizing sperm of Fig. 7 *a*. The immunofluorescence is localized in the posterior region of the slightly swollen sperm head, whereas the entire nucleus is EB-stained. An in vitro study[1] of the displacement of the basic nuclear proteins from mouse sperm nuclei has demonstrated that, in the early period of the reducing-swelling treatment designed to simulate the in vivo intra-ooplasmic events, the posterior region of the nucleus displays immunofluorescence, whereas the anterior region is still unstained. Presumably, "unmasking" of the antigenic sites of the basic proteins occurs first in the posterior region. Because the moderate degree of swelling of the fertilizing sperm of Fig. 7 suggests that entry had occurred shortly before the specimen was collected, a significant correlation between the progression defined in the in vitro system and that observed in Fig. 7 of this in vivo study is implied.

In later stages of sperm swelling, the immunofluorescence of the sperm nucleus and of both telophase complements of the oocyte is similar in intensity (Fig. 8) and, still later, when the swollen sperm appears to be depleted of its basic proteins, the chromosomes of the ovum display no immunofluorescence (Figs. 9 and 10). The very swollen sperm of Fig. 9 is, once again, correlative with sperm of the in vitro study[1] from which the basic protein fraction has been completely extracted: the morphologic contours are retained, the perforatorium persists, and, as demonstrated by EB staining, the chromatin boundaries are intact. In view of the correspondence in immunofluorescence staining of sperm and both complements of the ovum, it is possible that similar ooplasmic factors are operative in the basic protein displacement of all three. Thus far, those factors have not been defined.

Because the previously reported (6) incorporation of basic proteins in the oocyte complement takes place in condensed metaphase chromosomes, it was not unexpected to find that those chromosomes had retained histones. Our immunofluorescence data do not disclose whether the nucleosomal assembly of the retained H_{2b} and H_4 is conserved as the unique protein is added to those chromosomes nor whether postfertilization deletion of that protein requires disassembly. Both unexpected and clearly resolved, however, are the observations that no moiety cross-reactive with somatic histones H_{2b} and H_4 could be detected in the fertilizing sperm nuclei at stages where the SBNPs were no longer present. The EB staining of the sperm at those stages displays the residual DNA complex as organized chromatin in successively more decondensed states. Thus, the maintenance of that organization must be dependent upon chromatin moieties other than the unique basic proteins or histones.

There is evidence from in vitro studies of somatic cells (20) and sperm[1] that the spatial organization of the DNA complex of chromatin is retained after treatment to remove the basic proteins. The observations of this study indicate that a similar residual DNA complex occurs in vivo in the special instance of the transforming sperm nucleus. The interval between sperm entry and appearance of both pronuclei has been estimated as 3–4 h with pronuclear DNA synthesis detected 4–8 h later (19, 21). That immunofluorescence with the antiserum to SBNP was displayed on the complements of <25% of the zygote preparations that were suitable for analysis suggests that the gamete basic proteins are displaced very soon after sperm entry. Thus, if histone incorporation does not precede pronuclear DNA synthesis, the sperm chromatin may be in its basic protein–depleted state for a minimum period of 3–4 h and for as long as 11 h. The question then arises whether that constitutes a period of high risk for the paternal contribution to the embryonic genome.

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