STRUCTURE OF CHROMATIN, PROTEIN TRANSITIONS, AND POST-TRANSLATIONAL HISTONE MODIFICATIONS IN SEVERAL SPERM MODELS

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Structure of Chromatin, Protein Transitions, and Post-translational Histone Modifications in Several Sperm Models.

PhD Thesis Kathryn Lucille Kurtz

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INTRODUCTION

RESULTS

SUMMARY

SUMMARY

STRUCTURE OF CHROMATIN, PROTEIN TRANSITIONS, AND POST-TRANSLATIONAL HISTONE MODIFICATIONS IN SEVERAL SPERM MODELS

The study of chromatin structure in several simple sperm models of increasing complexity was performed. Species demonstrating different types of sperm nuclear protein transitions and structural changes in spermatic chromatin during spermiogenesis were selected as models for comparison: "H" (non-histone proteins are removed), "H→P" (protamine displaces histones), and "H→Pp→P" (precursor protamine displaces histones, and subsequently is converted into the mature protamine). This study has an evolutionary focus, in which a primitive sperm model is identified, from which more complex models may have risen during evolution. The final sperm characteristics achieved are considered to be caused by the changes the immature sperm cell undergoes during the process of spermiogenesis, and are correlated to an adaptation to the fertilization biology of each species. A broader understanding of the variety of sperm shapes, their chemical variability, and the spermatic chromatin condensation patterns pertaining to species of these simple spermiogenic models has been achieved. In this study, the diversity in sperm characteristics is extrapolated to the function the sperm cell has to pass on its genetic material to achieve fertilization of the egg of its own species.

For three different models using four marine species, protein transitions, chromatin condensation, and acetylation patterns were described during spermiogeneis. Specifically, changes in chromatin architecture and its protein complement was extensively studied using mainly transmission electron microscopy, inmunocytochemistry using anti-histone, anti-precursor protamine, and anti-acetyl group antibodies, as well as high resolution polyacrylamide gel electrophoresis (PAGE) and western blotting.

A model of specialized sperm chromatin (crustacean type) has been included in this study, since for decades this type of chromatin has remained poorly understood. Crustacean type sperm, once believed to have nuclei void of basic DNA-associated proteins, was found to contain histones, and is considered a derivation of the "H" model. Three species of brachyuran crabs from two different families were used to compositionally and ultrastructurally study this unusually decondensed mature sperm chromatin. Characterization of the histones from these sperm using HPLC and amino acid analysis confirm that the basic proteins extracted from sperm of these crabs are indeed typical and canonical histones, though some appear modified by post-translational

modifications such as acetylation, which has never before been described in mature sperm. Additionally, in *Maja brachydactyla*, histones H3 and H2B appear in stoichiometric amounts different to what would be found in somatic chromatin. By performing micrococcal nuclease digestions, the presence of nucleosomes (or nucleosome-like particles) in the sperm of these species was confirmed, and demonstrated that histones are found interacting with the sperm DNA. Further, the histone/DNA ratio was evaluated in two *Cancer* species, and it was determined that these sperm only contain slightly over half the amount of basic protein per DNA unit compared to other sperm types. These results concerning the composition of the crustacean-type sperm chromatin help to explain its decondensed nature.

RESUM CATALÁ

I. INTRODUCCIÓ

El procés general de l'espermatogènesi comporta quatre principals passos: a) la divisió i proliferació de les cèl·lules mares o espermatogonis; b) la transformació d'alguns espermatogonis en espermatòcits; c) la divisió meiòtica dels espermatòcits per a produir espermàtides haploides, i d) la diferenciació composicional, morfològica i funcional de l'espermàtida per a produir l'espermatozoide o gàmeta masculí.

Aquesta última part s'anomena espermiogènesi i representa la part de l'espermatogènesi més sotmesa al canvi evolutiu. Les característiques dels espermatozoides son molt variables quan es comparen entre les diferents espècies, de manera que el nucli és possiblement l'únic orgànul que es troba present en tots els espermatozoides (per a revisions de morfologia dels espermatozoides, vegeu Franzen 1956, 1970, 1977, 1987, Baccetti i Afzelius 1976; Jamieson 1986a, 1996) En aquesta introducció i en el treball que es presenta aquí ens centrem exclusivament en el nucli espermiogènic/espermàtic.

La investigació del nucli en les espermiogènesis i en els espermatozoides ha estat efectuada des de diferents punts de vista, que resumim molt breument.

Pel que fa als nuclis espermiogènics s'han estudiat les particularitats dels mecanismes de regulació de l'expressió del gens i també dels seus RNA missatgers (revisat per Sasone-Corsi 2002), els canvis composicionals de les proteïnes nuclears al llarg de tot el procés espermiogènic (Balhorn et al., 1984; Govin 2004; Cáceres et al., 1999), i els canvis en els patrons de condensació de la cromatina espermiogènica (resumit en Harrison et al., 2005).

Pel que fa a les investigacions sobre els nuclis dels espermatozoides s'ha estudiat extensivament la ultraestructura a través de microscòpia electrònica (Baccetti i Afzelius 1976; Baccetti 1986; Jamieson 1981; 1984); els tipus de proteïnes que estan associades al DNA (Bloch 1969, 1976; Subirana 1983; Kasinsky 1989; Ausió 1995), les estructures dels complexes del DNA amb les proteïnes nuclears (Subirana 1975; Suzuki i Wakabayashi 1988; Balhorn 1982), i finalment l'arquitectura nuclear, és a dir, la posició en que els gens (o altres parts del DNA) es poden trobar en el nucli espermàtic madur (Ward i Coffey, 1991; Hazzouri et al., 2000).

Comprendre, integrar, i donar un significat evolutiu clar a partir de tota aquesta gran quantitat d'informació és actualment impossible per diverses raons entre les quals n'escollim les següents:

En primer lloc, en la Naturalesa no hi ha un model universal de la espermiogènesi o de la cèl·lula espermàtica. Tal i com hem dit abans, els processos espermiogènics són extraordinàriament variables. Tendeixen a divergir proporcionalment amb la distancia taxonòmica (i filogenètica) de les espècies, però també poden presentar processos de convergència evolutiva i mostrar molta similitud en espècies distants.

En segon lloc, les diferents aproximacions al seu estudi que hem esmentat s'han fet gairebé sempre de manera independent les unes de les altres. Per exemple, es coneix en moltes espècies el tipus de condensació de la cromatina però no se sap a quin tipus de proteïna unida al DNA correspon, i a l'inrevés. No obstant, aquí hem d'excloure la espermiogènesi dels mamífers on s'ha estudiat més la correlació entre estadis espermiogènics, activitat genètica, proteïnes nuclears i cromatina condensant (Balhorn et a., 1984; Hazzouri et al., 2000; Yu et al., 2000; Zao et al., 2001, 2004; Adham et al., 2001).

En el treball que presentem en aquesta memòria intentem fer un estudi que correlacioni simultàniament els canvis de condensació de la cromatina espermiogènica amb els canvis de proteïnes nuclears i també amb els canvis en l'acetilació de les histones.

Amb aquest objectiu, a partir de la bibliografia coneguda, hem definit els següents *models* de transicions en els nuclis espermiogènics:

I.- Model histones → histones [H → H]

Aquest grup el formen les espècies en les que el nucli espermàtic madur conté histones unides al DNA. Aquestes histones son iguals (o molt similars) a les histones somàtiques. Això implica que en el decurs de la espermiogènesi els canvis de les proteïnes nuclears es basa únicament en la pèrdua de les proteïnes "no-histones" de l'espermàtida primerenca, sense que hi hagi canvis en les proteïnes bàsiques principals (histones).

Les espècies amb el tipus de transició [H → H] es troben entre els invertebrats, com els nemertins *Cerebratulus californiensis* i *C. lacteus* (Wang i Ausió 2001), en el mixínid *Petromyzon marinus* (Saperas et al., 1994), i entre els peixos (*Opsanus tau*: Casas et al., 1981; *Carassius auratus*: Muñoz-Guerra et al., 1982).

En alguns cassos s'ha estudiat la ultraestructura del nucli al llarg de la espermiogènesi (Casas et al., 1981), i s'ha trobat que la cromatina de les espermàtides

intermèdies s'organitza en gràmuls (estructures fibrogranulars) de 20-25 nm de diàmetre, i que aquests gràmuls es disposen més apretadament en la cromatina espermàtica madura. També en alguns cassos (Muñoz-Guerra et al., 1982) s'ha demostrat que la cromatina d'aquestes espècies està organitzada en nucleosomes.

II.- Model histones → protamina [H → P]

En les espermiogènesis que conformen aquest model, les histones (que sempre es troben presents en el nucli de l'espermàtida primerenca) son substituïdes per un tipus de proteïna molt bàsica anomenada protamina. En aquest model, la substitució de les histones per la protamina és directe, és a dir, no s'efectua a través de cap altre tipus de proteïna. Al final de la espermiogènesi la única molècula de proteïna bàsica que es troba unida al DNA és la protamina. Hi han moltes espècies de peixos (revisat en Saperas et al., 1994) que pertanyen a aquest model, així com amfibis (Kasinsky et al., 1999), aus (Oliva i Dixon 1991), i moltes espècies d'invertebrats (Subirana et al., 1973; Zalensky i Zalenskaya 1980, Daban et al.,1991).

En algunes espècies pertanyents a aquest model també han estat estudiades la transició de proteïnes nuclears espermiogèniques (Sautière et al., 1991; vegeu la revisió d'Oliva i Dixon 1991) i l'acetilació de les histones (Christensen et al., 1984; vegeu també la revisió d'Oliva i Dixon 1991), i en moltes espècies s'ha examinat el tipus de condensació de la cromatina espermiogènica a través de microscòpia electrònica (Billard 1983; Healy 1987, 1989; Saperas et al., 1993; Giménez eta al., 2002a) però sempre independentment dels estudis bioquímics anteriorment esmentats.

En resum, malgrat que els nuclis pertanyents a aquest model estan ben caracteritzats des dels punts de vista bioquímic i morfològic no es coneix quina relació existeix entre la composició de la cromatina i la seva estructura.

III.- Model histones → precursor de protamina → protamina [H → Pp → P]

Aquest model representa el nivell de complexitat química immediatament superior al model de transició [H → P]. Està representat en els mol·luscs cefalòpodes decàpodes (calamars, sèpies, sepioles, etc) (Kadura i Khrapunov 1988; Wouters-Tyrou et al., 1995)

Rousseau-Prévost et al., (1988), Martin-Ponthieu et al., (1991) i Wouters-Tyrou (1991) varen demostrar que les histones de les espermàtides són desplaçades del nucli per una proteïna de 77-78 residus aminoacídics. El conjunt dels primers 21 residus (N-

terminals) eren moderadament bàsics, mentre que els 56-57 residus C-terminals eren extraordinariament rics en arginina. En les fases finals de la espermiogènesi, aquesta molècula de 77-78 residus pateix un trencament enzimàtic i una eliminació dels 21 residus N-terminals, i només la última part (56-57) residus C-terminals molt rics en arginina) romanen units al DNA espermàtic madur. La molècula de 96-100 residus va ser anomenada "precursor de la protamina", i la molècula de 56-57 residus va ser anomenada "protamina".

Aquest és doncs un model de transició en que les histones no són desplaçades per la protamina que es troba en els nuclis dels espermatozoides, sinó per un precursor seu (una molècula menys bàsica que després es transforma en protamina).

Hi han estudis sobre la condensació de la cromatina espermiogènica d'animals corresponents a aquest model (Maxwell 1975; Fields i Hompson 1976; Hou i Maxwell 1992). No obstant, són incomplets i no donen informació de quina relació hi ha entre les estructures de la cromatina i el tipus de proteïna que interactua amb el DNA.

Per altra cantó hi ha un únic estudi sobre les modificacions postraduccionals de les histones. Coupez et al. (1987) varen demostrar que la histona H4 és acetilada d'una manera seqüencial en la gònada de *Sepia officinalis*, seguint el següent ordre: lis (12) → lis (5) → lis (16) →lis (8).

Altres models. El cas dels crustacis decàpodes braquiürs

Hi han models molt més complexos de condensació de la cromatina espermiogènica. Per exemple el nucli espermiogènic del mol·lusc *Murex brandaris* pateix un bon nombre de transicions estructurals (Amor i Durfort 1990) i de recanvis de proteïnes nuclears (Cáceres et al., 1999, 2000). L'exemple conegut més complex és el dels mamífers (Alfonso et al., 1993; Oko et al., 1996, Meistrich et al., 2003). Finalment també hi ha altres models que sense ser més complexos representen casos especials.

Entre aquests últims hem escollit el cas dels crustacis decàpodes braquiürs, ja que diversos autors (Bloch 1969, Chevaillier 1966; Langreth 1969, Vaughn i Hinsch 1972) van observar que el nucli espermàtic d'aquestes espècies no es trobava en estat de condensació i que no contenia proteïnes bàsiques unides al DNA.

Aquest model ofereix una especial importància i mereix ser re-examinat, tal i com hem fet en el treball que presentem.

II. OBJECTIUS

En general doncs, els estudis sobre espermiogènesis/espermatozoides s'han enfocat en la bioquímica del nucli, o sobre la seva morfologia, però hi ha molt pocs estudis que integrin aquests aspectes. En el treball que presentem intentem caracteritzar bioquímicament i estructuralment la cromatina espermiogènica i espermàtica. Els objectius concrets són els següents:

- Relacionar els canvis en la estructura de la cromatina, els canvis en la composició de proteïnes i l'acetilació de les histones en els tres models de transicions següents.
  - Model [H → H]
  - Model [H → P]
  - Model [H → Pp → P]
- Identificar les característiques comuns a aquests tres tipus d'espermiogènesis.
- Estudiar la composició i estructura de la cromatina espermàtica de dos crustacis decàpodes braquiürs, prèviament descrits sense proteïnes bàsiques nuclears.

III.RESULTATS

Capítol 1.- Models de transicions de proteïnes nuclears espermàtiques i de condensació de la cromatina

Correspon a l'article: Spermiogenic nuclear protein transitions and chromatin condensation. Proposal for an ancestral model of nuclear spermiogenesis. J. Exp. Zool. (sotmès a publicació).

I.- Resultats del model [H → H] (*Sparus aurata*)

La cromatina espermàtica madura està organitzada per histones (Figura 1 B), les quals presenten una composició aminoacídica igual que les histones somàtiques, inclosa la histona H1 (taula 1). Aquestes histones organitzen la cromatina en nucleosomes amb un DNA de 214 parells de bases (Figura 1 D). En el decurs de la espermiogènesi, l'espermàtida perd les proteïnes no histones, que amb molta probabilitat contribueixen a la formació d'heterocromatina (compareu les figures 1 A i 1 B). Els canvis estructurals de la cromatina inclouen la transformació de la cromatina de tipus somàtics de l'espermàtida primerenca en una organització en grànuls de 20 nm de diàmetre, els quals es troben regularment distribuïts en el nucli (Figura 3: IA→IB). Aquesta transformació és acompanyada d'una ona d'acetilació moderada de les histones del tetràmer H3 i H4 (Figura 3: IIIA→IIIB, i Figuras 6 i 7A, B). La compactació de la cromatina final s'efectua simultàniament a la desacetilació de les histones (Figura 3: IIIB→IIIC i Figura 6 A, B), de manera que els grànuls de 20 nm pateixen agregació entre ells i una coalescència parcial (Figura 3: IIB→IID).

II.- Resultats del model [H → P] (P representa una protamina típica en el cas de *Dicentrarchus labrax* i una gran protamina, o SNBP, en el cas de *Monodonta turbinata*)

En aquest següent model de complexitat major, les histones son directament reemplaçades per protamines. Per estudiar aquest model, hem utilitzat dues espècies que es troben en posicions taxonòmiques molt distants (el peix ossi *D. labrax* i el mol·lusc gastròpode *M. turbinata*). Les protamines d'aquestes espècies son diferents per la seva mida (34 i 106 residus aminoacídics respectivament) i per la seva estructura primària, i han sorgit independentment en l'evolució. No obstant mantenen característiques químiques similars com son una gran riquesa en residus aminoacídics bàsics, la disposició de les arginines en grups homogenis i la presència de serines com a

residus fosforilables (Figura 2C). Els canvis estructurals de la cromatina espermiogènica inclouen la transformació de la cromatina de tipus somàtic de la espermàtida primerenca en cromatina disposada en grànuls de 20 nm molt homogenis en la seva mida i en la seva distribució (Figura 4: IIA). El canvi esmentat està acompanyat d'una acetilació de les histones tetramèriques (Figura 4: IIIA→IIIB i Figura 5: IIIA→IIIB,C, i Figura 6 i 7) El desplaçament de les histones per la protamina es correlaciona amb la transformació de la cromatina cap a grànuls progressivament majors i relativament irregulars (Figura 4: IIB2→IIC,D→IIE; Figura 5: IID1→IID2; Figures 6,7). Els grànuls formats per la unió del DNA amb les protamines tenen un diàmetre aproximat de 80-90 nm en el cas de *D. labrax* i de 60nm en *M. turbinata* (Figura 4: IIE; Figura 5: IID2). Es coneix que les histones que estan essent desplaçades per la protamina es troben en un estat elevat d'acetilació (Christensen et al., 1984; Oliva et al., 1987; Oliva i Dixon 1991). Això és evident en les imatges de la cromatina presentades a les figures 4 (IIIC, D) i 5 (IIID1). Malgrat que en el passat no s'hi ha prestat massa atenció, els nostres resultats mostren que la acetilació de la histona H3 acompanya a l'acetilació de la histona H4 durant ambdues espermiogènesis, tant la que correspon al model [H → H], com la corresponent al model [H → P] (Figures 6 i 7).

Capítol 2.- Transicions estructurals, i químiques en una cromatina pertanyent a un model més complex (model [H → Pp → P])

Correspon als tres articles que segueixen:

I.- Article: Sperm nucleomorphogenesis in the cephalopod *Sepia officinalis*. Tissue and Cell 39: 99-108, 2007

Els canvis estructurals de la cromatina al inici de la espermiogènesi es basen en una transició de la cromatina de tipus somàtic de l'espermàtida més primerenca a una cromatina de nou organitzada en grànuls de 20 nm molt homogenis tant en el seu diàmetre com en la seva distribució (Figura 1 A→B). Aquest canvi estructural no està correlacionat amb canvis importants en la forma ni en el volum nuclear. En el estadi següent (estadis B → C en la figura 1) la cromatina granular és remodelada cap a fibres de 25-30 nm de diàmetre (figura 1C), la qual cosa es produeix simultàniament amb un canvi en la forma nuclear. Posteriorment, la cromatina es transforma globalment en fibres més gruixudes de 40-45 nm de diàmetre (estadis C → D a la figura 1). Durant la transició

de fibres de 25-30 nm a fibres de 40-45 nm, la forma i volum nuclears no presenten canvis significatius. A partir de l'estadi D, les fibres de 40-45 nm no pateixen cap més remodelació, i en canvi es van ajuntant progressivament (fases E → G en la figura 1). Durant aquestes fases de condensació, la cromatina va adquirint una condensació uniforme (figura 1G). En els estadis següents (Figures 10 – 12) les fibres de cromatina son reclutades progressivament en direcció a la membrana nuclear dorsal i s'ajunten definitivament.

II.- Article: Transition of nuclear proteins and chromatin structure in spermiogenesis of *Sepia officinalis*. Mol. Reprod. Develop. 74: 360-370, 2007

Les transicions de proteïnes durant la espermiogènesi inclouen la substitució directa de les histones (de tipus somàtic, vegeu taula 1) per un precursor de la protamina (Figura 2 A, B). La primera remodelació cap a gränuls de 20 nm de diàmetre no sembla estar associada a cap tipus de transició de proteïnes bàsiques (Figura 4). La segona remodelació, en la que els gränuls de 20 nm es transformen en fibres de 30 nm, és deguda a la entrada del precursor de la protamina i a la seva interacció amb el complex DNA-histones (Figura 4 C). La tercera remodelació (fibres de 30 nm que es converteixen en fibres de 40-45 nm) té lloc simultàniament a la desaparició de les histones de la cromatina (Figura 5 F). En la fase de condensació, les fibres de 45 nm presenten coalescència entre elles per a formar agregats de cromatina progressivament més grans (Figura 4 E). En aquesta fase no hi ha variació substancial de proteïnes nuclears (Figura 4 E, F) de manera que la condensació de la cromatina ha de correspondre a canvis postraduccionals com és la pèrdua de l'extrem N-terminal i la possible desfosforilació de les seves serines.

III.- Article: Acetylation of histone H4 in complex structural transitions of spermiogenic chromatin. J. Cell. Biochem. 102: 1434-1441, 2007.

El patró d'acetilació de la histona H4 en la espermiogènesi de *S. officinalis* es troba correlacionat amb dos diferents canvis estructurals de la cromatina espermiogènica. A través de la utilització de anticossos específics per a les formes monoacetilades en lisines diferents de la histona H4 (anti H4-acK12 i anti H4-acK16) (Figures 2 i 3 respectivament) hem mostrat que la primera remodelació de la cromatina (de la cromatina semblant a la somàtica de l'espermàtida més primerenca a la cromatina en gränuls de 20 nm) es correlaciona amb una monoacetilació de la histona H4 (concretament una

monoacetilació en la lisina 12 (Figura 4 B), mentre que la remodelació estructural de les fibres de 30-35 nm a les fibres de 40-45 nm es produeix simultàniament amb una hiperacetilació de la histona H4 (Figura 5 C, D), i simultàniament amb la expulsió de les histones del nucli espermiogènic (Figura 6 D)

Aquests tres treballs ens han permès comprendre amb certa profunditat quin efecte fan els canvis de proteïnes i el procés d'acetilació de les histones sobre les diverses formes que la cromatina es va organitzant al llarg de l'espermiogènesi, i també el seu efecte permissiu sobre la nucleomorfogènesi espermàtica d'aquesta espècie. Per altre cantó també destaquem que el pas de cromatina de tipus somàtic a grànuls de 20 nm amb histones moderadament acetilades és un procés que es repeteix en totes les espècies estudiades independentment de la seva posició taxonòmica.

Capítol 3. Cromatina espermàtica especialitzada: el model dels espermatozoides de crustacis decàpodes braquiürs

Correspon als dos articles que segueixen

I.- Article: Histones and nucleosomes in *Cancer* sperm, previously described as lacking basic DNA-associated proteins. A new model of sperm chromatin. J. Cell. Biochem. (en prensa, es pot obtenir *on line*)

La cromatina espermàtica de *C. Pagurus* conté histones (Figura 2, Figura 3A, Figura 5, Taula 2). Aquestes histones interactuen amb el DNA formant nucleosomes de 170 parells de bases (Figura 3 B, Taula 1). La relació [histones/DNA] en el nucli espermàtic de dues espècies de *Cancer* és de 0,5 a 0,6 (pes/pes), relació que és molt menor que la proporció [proteïna/DNA] trobada en altres nuclis espermàtics que contenen histones o protamina, les quals presenten valors de 1,0 a 1,2 (pes/pes) (Taula 3). La baixa relació entre histones i DNA de *Cancer* es correlaciona molt bé amb la gran sensibilitat d'aquesta cromatina a la digestió amb nucleasa micrococal, reflectida per la gran proporció de DNA soluble en PCA que apareix després d'un curt període de digestió (Figura 4). Una important característica és que la histona H4 es troba en un estat molt elevat d'acetilació en la cromatina espermàtica madura (Figura 6), la qual cosa també ajuda a comprendre perquè la cromatina de l'espermatozoide no es troba condensada com en la major part de les altres espècies

II. Article: Preliminary study of sperm chromatin characteristics of the brachyuran crab *Maja brachydactyla*. Histones and nucleosomes in crustacean sperm nuclei previously described without SNBPs. Tissue and Cell. (Sotmés)

En el decurs de la espermiogènesi de *M. Brachydactyla* es produeix en el nucli una pèrdua de les àrees de cromatina condensada, de manera que les espermàtides intermèdies la cromatina, observada al microscopi electrònic, apareix uniforme i molt poc electrodensa (Figura 1 C). Addicionalment, el nucli desenvolupa una gran capacitat per canviar de forma (Figura 2, A, B, C). En la cromatina espermàtica madura, les histones es troben associades al DNA (Figura 3 A, Figura 4). Per a demostrar aquests fets, vam efectuar digestions dels espermatozoides madurs amb nucleasa micrococal. Les digestions van originar una sèrie de partícules discretes de cromatina composades per DNA i histones (A, B en la figura 4 A). El fragment límit de DNA en aquestes digestions tenia aproximadament 100 parells de bases (Figura 4 B), mentre que la histona H3 apareixia en molt baixa quantitat en aquesta cromatina (Figura 4 A). Aquests resultats suggereixen que la cromatina espermàtica de *M. Brachydactyla* es troba organitzada en unitats similars, però no idèntiques als nucleosomes.

La cromatina espermàtica de *M. brahydactyla* presenta altres característiques inusuals en la cromatina somàtica: deficient quantitat de histona H3 (Figura 3, Figura 4 A), un alt nivell d'acetilació de la histona H3 remanent (Figura 6 B, Figura 6 D) i una quantitat d'H2B superior a l'altra histona del dímer (H2A) .

IV. DISCUSSIÓ

A.- Models de transició de proteïnes i d'estructura de la cromatina espermiogènica

I.- Model [H → H]

El model més senzill que hem estudiat, correspon al peix ossi *S. aurata*. En aquesta espermiogènesi hem observat els següents fets:

La cromatina es diferencia des d'un estadi de tipus somàtic (espermàtida primerenca) a una cromatina granular de 20 nm (espermàtida intermèdia), i finalment a una cromatina compacta per agregació i coalescència parcial dels grànuls (espermatozoide).

La cromatina organitzada homogèniament en grànuls de 20 nm conté histones, i a més, presenta les histones del tetràmer (H3 i H4) amb un estat moderat d'acetilació. Donat que l'acetilació d'histones impedeix la formació d'estructures d'ordre superior de la cromatina (Calestagne-Moreli i Ausió 2006; Tremethick 2007) és molt probable que el limitat grau d'acetilació de les H3 i H4 afavoreixi la disposició granular (cada grànul ha de contenir de 4 a 6 nucleosomes), però inhibeixi la formació d'esructures més compactes.

La cromatina de l'espermatozoide conté histones desacetilades, i la seva unitat estructural és el nucleosoma. És lícit interpretar que la desacetilació de les histones H3 i H4 en aquesta última fase permeti l'aproximació dels nucleosomes i la formació d'estructures més compactes.

És important insistir que, contràriament al que havia estat proposat (Christensen et al., 1984), en aquest model hi ha activitat acetilasa, i que aquesta activitat acetilasa no està relacionada amb l'expulsió de les histones de la cromatina, sinó en l'adquisició d'una estructura (grànuls de 20 nm) que es reparteix homogèniament en els nuclis durant les primeres fases de la espermiogènesi.

II.- Model [H → P]

Aquest model estava estudiat amb cert detall en espècies que contenien protamina "típica" (Billard 1983; Saperas et al., 1993, 1994; Frehlick et al., 2006; vegeu la revisió d'Oliva i Dixon 1999). Nosaltres hem intentat relacionar els fets bioquímics amb l'estructura de la cromatina (*D. labrax)*, i addicionalment hem examinat tot el procés en

una espècie (*M. turbinata*) que presenta una molècula de "protamina" molt major que la protamina típica (i sense cap relació evolutiva).

En els dos casos estudiats, en el decurs de la espermiogènesi la cromatina presenta tres tipus de canvis: el primer és el canvi de cromatina de tipus somàtic a cromatina organitzada uniformement en grànuls de 20 nm. Morfològicament, aquesta cromatina correspon a la seva anàloga de *S. aurata*. Les característiques de la composició proteínica i de l'acetilació també són les mateixes que hem trobat en el model anterior: la cromatina de 20 nm està constituïda per histones, de les quals la H3 i la H4 es troben en un estat limitat d'acetilació. Les consideracions que fem a aquests fets són idèntiques a la que hem fet en el cas anterior.

El segon canvi estructural és la remodelació dels grànuls de 20 nm a grànuls majors i més irregulars de 80-90 nm (*D. labrax*), i 60 nm (*M. turbinata)*. Aquesta remodelació es produeix quan les histones acetilades són reemplaçades per la protamina, és a dir, els grànuls majors (90 i 60 nm) són complexos de DNA i protamina (probablement fosforilada: Oliva i Dixon 1991). Això es demostra pel fet que els anticossos anti-histones no marquen aquests grànuls, i per altra banda la purificació d'aquests grànuls a través de sonicació de les espermàtides mostra que estan composats per DNA i protamina.

L'últim canvi observable al microscopi electrònic és la coalescència parcial d'aquests grànuls majors. Aquest fet és anàleg a la coalescència dels grànuls menors en *S. aurata*, i encara que no ho hem estudiat específicament, ha de correspondre a la desfosforilació de la protamina.

Model [H → Pp → P]

L'espècie estudiada en aquest cas és la sèpia (*Sepia officinalis*). Els estudis presentats en aquest treball mostren les següents transicions de la cromatina espermiogènica.

Igual que es produïa en els casos anteriors es produeix una reordenació de la cromatina cap a un estadi de grànuls de 20 nm uniformement distribuïts en el nucli de les espermàtides joves. Aquests grànuls contenen histones i en concret la histona H4 presenta monoacetilació (en aquesta espècie no hem estudiat l'acetilació de la histona H3). A partir dels grànuls de 20 nm la cromatina es remodela cap a fibres de 25-30 nm. En aquestes fibres s'hi troben a més del DNA, les histones acetilades i el precursor de la protamina.

La fibra de 25-30 és una estructura que immediatament es transforma en fibres de 40-45 nm ja que les histones continuen patint acetilació i són desplaçades de la cromatina. Les fibres de 40-45 nm estan constituïdes per DNA més precursor de la protamina (més una petita quantitat d'histones remanents molt acetilades que encara no han estat desplaçades de la cromatina). Finalment, les fibres de 40-45 nm es van ajuntant entre elles. Això correspon a la transformació del precursor en protamina (i molt possiblement la desfosforilació d'aquesta molècula).

Aquest model ens permet comprendre dos fets principals. Primer, que el pas inicial de la espermiogènesi (equivalent a la formació de grànuls de 20 nm amb histones moderadament acetilades) és un pas que es dona en molts tipus d'espermiogènesis i en moltes espècies que no tenen una relació taxonòmica propera (veure també Ribes et al., 2001). Segon, que a través d'introduir molècules i modificacions postraduccionals entre les histones inicials i la protamina (o SNBP) final, la cromatina espermiogènica pot anar adquirint una gran variació en les estructures que adopta al llarg del procés.

Quin sentit evolutiu pot tenir l'estadi de grànuls de 20 nm?

Donat que és l'únic estadi intermedi en la transició que es dona en les espermiogènesis més senzilles (entre l'espermàtida primerenca i l'espermatozoide), i a més es dona en moltes espermiogènesis molt diferents entre sí, aquest podria ser un caràcter ancestral en l'espermiogènesis. Les espècies més primitives de la classificació taxonòmica presenten aquest tipus de transició.

És possible que en les espermiogènesis ancestrals es formés la cromatina granular de 20 nm gràcies a una acetilació moderada de les histones. Aquesta cromatina no presenta diferencies zonals en la seva estructura (i molt possiblement tampoc diferencies funcionals), de manera que representa un substrat homogeni per a ser empaquetat simplement per desacetilació.

Una possibilitat addicional és que aquest substrat estructural homogeni sigui molt adequat per a que en algunes línies evoltives, s'introduís la protamina, la qual desplaçaria amb facilitat unes histones ja preparades (acetilades)

Quin sentit pot tenir l'aparició de la protamina ([model H → P])?

Aquest punt sembla molt clar, en les figures 3, 4, i 5 de l'article 1, es mostra que si bé l'aparició de la cromatina de 20 nm i la subseqüent desacetilació de les histones

provoquen una reducció del volu nuclear, la interposició de la protamina en el procés, provoca una reducció addicional del volum nuclear. Els nuclis am protamina són menors i el DNA més fortament empaquetat. Això afavoreix la protecció del DNA, fa que es pugui aumentar el nombre d'espermatozoides que pot emmagatzemar una gònada, o estructura (epidídim, deferent, espermatòfor...), disminueix la despesa enegètica del espermatozoide, i pot afavori la comptitivitat dels espermatozoides

Quin sentit pot tenir que la cromatina espermiogènica vagi canviant de manera complexa la seva estructura fins a l'espermatozoide?

Segons la nostra opinió una de les possibles funcions d'aquests patrons complexos de cromatina condensant espermàtica és que permet que (degut a forces generalment originades en el citoplasma com microtúbuls, etc.), el nucli vagi adoptant formes molt més elaborades i complicades, i adaptades a la biologia de la fertilització de l'espècie. En general, aquests tipus de transicions estructurals i de composició es donen en espècies amb nuclis espermàtics de forma complicada És interessant citar, entre molts altres, el cas del mol·lusc *Murex brandaris*, en el que es pot comparar la complexitat del patró de condensació de la cromatina: (Amor i Durfort 1990) amb la complexitat de les transicions de proteïnes nuclears (Càceres et al., 1999) i amb la forma especial del nucli espermàtic. En canvi, els nuclis espermàtics que provenen dels models [H → H] i [H → P] corresponen a espècies de fertilització externa que presenten nuclis arrodonits, ovalats o lleugerament modificats.

B.- Model especial dels crustacis decàpodes braquiürs

Malgrat que molts autors havien descrit l'absència de proteïnes bàsiques en aquests nuclis no condensats, el nostre estudi ha posat de manifest uns quants fets interessants.

Per començar, els nuclis espermàtics madurs no exhibeixen un volum major que el nucli de l'espermàtida, amb la qual cosa es pot dir que si bé no estan condensats, tampoc es troben en estat de descondensació.

El DNA d'aquests nuclis està estabilitzat per histones, de les quals la H4 (*Cancer*) i la H3 (*Maja*) esta molt acetilades. Possiblement aquest estat d'acetilació impedeix el que anomenem "la condensació de nucli".

Les característiques especials d'aquestes cromatines (que per la seva peculiaritat, mereixen ser estudiades encara amb més profunditat) representen una adaptació indispensable pel tipus de fertilització d'aquestes espècies. Quan l'espermatozoide pren contacte amb l'embolcall de l'oòcit, el seu acrosoma reacciona amb una eversió que impulsa el nucli a través d'un canal (creat per la pròpia eversió acrosòmica) en direcció a l'interior del citoplasma oocitari (Hinsch, 1971; Goudeau, 1982; Medina i Rodríguez, 1992) Aquest canal és estret i requereix que el nucli es deformi (tal i com ho faria un eritròcit passant per un capil·lar més estret). El nucli espermàtic ha de ser deformable, ja que en cas contrari no podria travessar el canal acrosòmic i entrar en el citoplasma.

V. CONCLUSIONS

1.- L'increment en la complexitat de les transicions de proteïnes nuclears espermiogèniques afecta el patró de condensació de la cromatina, la forma del nucli espermàtic i el seu volum. Hem de pensar que aquests fets formen part de les adaptacions evolutives de cada espècie al seu tipus de fertilització.

2.- Un fet comú a moltes espermiogènesis és la desaparició de les regions d'eucromatina i heterocromatina de les espermàtides primerenques cap a una cromatina consistent en gràmuls de 20 nm homogèniament distribuïts en el nucli cel·lular. Aquesta transició estructural té lloc independentment del tipus de transició de proteïnes posterior. Els gràmuls de 20 nm han de contenir de 4 a 6 nucleosomes, i les seves histones estan parcialment acetilades. A partir d'aquest estadi, cada model d'espermiogènesi segueix el seu propi patró de condensació. Els gràmuls de 20 nm podrien representar un estadi primitiu en l'evolució de l'espermiogènesi.

3.- Una segona ona d'acetilació s'observa en les espermiogènesis en les que les histones són desplaçades. El grau d'acetilació és major que en la primera ona i es troba relacionada amb la sortida de les histones de la cromatina espermiogènica.

4.- Comparativament amb els models [H → H] i [H → P], el (relativament) petit increment en complexitat química que mostra l'espermiogènesi de la sèpia, introduint una molècula precursora de la protamina, involucra un fort increment de la complexitat en el patró de condensació de la cromatina espermiogènica.

5.- El DNA des nuclis dels espermatozoides dels crustacis estudiats, està organitzat per histones formant nucleosomes (o estructures similars) de mida petita. L'acetilació de les histones (que inclou hiperacetilació de H4 en *Cancer*, i elevada acetilació de H3 en *Maja*), i la baixa proporció proteïnes bàsiques/DNA, ajuden a comprendre l'estat de "no condensació" dels nuclis espermàtics d'aquestes espècies. L'acetilació de les histones H4 i H3 han d'estar involucrades en la inhibició de la formació d'estructures compactes i rígides de la cromatina dels nuclis espermàtics de crustacis. Aquest fet és una adaptació obligatòria al tipus de biologia de la fertilització d'aquestes espècies.

6.- Pot ser que el nucleosoma no sigui l'únic component estructural dels nuclis espermàtics dels crustacis, ja que la cromatina es digereix amb una gran rapidesa per la nucleasa micrococal. Poden existir regions relativament grans del genoma estabilitzades per altres tipus de ions.

Nota sobre la bibliografia

Les cites bibliogràfiques que apareixen en aquest resum es poden consultar a la bibliografia general del final de la memòria d'aquest treball de tesi.

INTRODUCTION

I. INTRODUCTION

I.1- The sperm cell

Sperm genome protection is essential for preserving genetic information provided by the male gamete, as well as facilitating transport and long-term storage of this information in the absence of DNA-repair mechanisms. The process of maturation of the male gamete, called spermiogenesis, involves events leading to the simplification of the sperm cell. Three aspects of this include the loss of nearly all the cytoplasmic contents, the appearance of a few specialized organelles, and the reduction of nuclear volume. This final aspect is directly related to the type of basic proteins associated to the DNA which are involved in packaging the sperm genome.

Aside from the nucleus, the important main components of the sperm cell include the acrosome, which contains enzymes necessary for penetration of the sperm cell into the outer layers of the oocyte, flagella used to propel the cell toward the female gamete, and mitochondria, which provide ATP for flagellar movement (Fawcett 1975). The sperm nucleus has been found to be a quite variable organelle among different species in terms of its protein composition and morphology, and therefore most elaboration and focus will be placed on this component of the sperm cell.

I.1.1 Categories of sperm cells

When characterizing sperm cell types, some considerations are made about the correlation among changes of different levels of (1) chromatin condensation patterns during spermiogenesis, (2) morphology of sperm, and (3) fertilization biology (Daban et al., 1991).

The term *primitive sperm* (Retzius 1906; Franzén 1956, 1970, 1977, 1987) has been used to describe simple sperm cells, meaning that (1) the head (consisting of the acrosome and nucleus) is fairly short (4-6μm), (2) the midpiece contains 4-5 unmodified mitochondria clustered in a ring around two centrioles at the base of the head, and (3) the distal centriole is anchored to the plasma membrane and gives rise to the tail (a typical 9+2 axonome surrounded by the plasma membrane). Examples are seen in many polychaetes and molluscs (Jamieson 1991, 1986a). In a further categorization of primitive sperm, Jamieson distinguished *ect-aquasperm*, *ent-aquasperm*, and *introsperm* (Jamieson 1991). Ect-aquasperm is a term for externally fertilizing sperm of the primitive type, while Ent-aquasperm (modified type) corresponds to sperm of animals exhibiting internal fertilization (Jamieson 1991) in which the sperm has been shed into ambient water prior

to being drawn in by the female, and fertilizes the egg internally. Introsperm types never enter the water; the sperm cell is transferred directly from the male to the female. *Ent-aquasperm* and *introsperm* show different modifications to the primitive (*ect-aquasperm*) type, mainly in their differences in sperm morphology, which determine the category the sperm fits into. Such morphological differences usually include the presence or absence of an acrosome. The degree of acrosomal development also tends to differ, usually depending on the mode of fertilization and the necessity (or lack thereof) of an acrosome for penetration of the external egg coat (Amanze and Iyengar 1990).

I.2. The sperm nucleus

The sperm nucleus is the most important component of the sperm cell; the chemical composition which is species-unique, as well as the chromatin packaging particular to the sperm nucleus, contribute to an extremely reduced nuclear volume. Chromatin is the complex composed of DNA and associated basic proteins, histones being the characteristic protein component in somatic chromatin. In contrast with somatic histones which are a remarkably conserved class of proteins, the chemical composition of DNA-associated proteins in germ cells is quite diverse (Subirana et al., 1983; Saperas et al., 1993) showing high variability among different taxomomic groups (Ecklund and Levine 1975; Oliva and Dixon 1991; Daban et al., 1991). The first discovery of basic proteins associated to sperm chromatin was achieved by Meischer in 1874, when he described the basic "protamine" from isolated sperm heads of salmon. Since then, it has become evident that an increase in basicity of the proteins associated with spermatic chromatin involves a more efficient DNA packaging, contributing to the reduction of the nuclear volume.

I.3. Sperm Nuclear Basic Proteins (SNBPs)

Less than a century following the discovery of basic proteins unique to sperm, sperm nuclear basic proteins were broken down into five major categories, classified by Bloch (1969, 1976):

Type 1: True protamines (small, arginine rich) (salmo type)

Type 2: Stable protamines or keratinous protamines (arginine-rich, also contain cysteine) (grass hopper type)

Type 3: Intermediate sperm histones (*Xenopus*, *Bufo*)

Type 4: Somatic-like histone category (*Rana*)

Type 5: No basic proteins detected in nucleus of mature sperm (ameboid shape)

Since the establishment of this classification, much work has been dedicated to the detailed characterization of some proteins belonging to each class (Zalensky and Zalenskaya 1980, 1981; Ausió and Subirana 1982; Kasinsky 1989; Subirana 1983; Daban et al., 1991). Later, Subirana described an alternative classification, dividing sperm proteins into protamines and sperm histones (1975, 1983; reviewed in Oliva and Dixon 1991):

1.- Variations to nucleosomal histones: Sperm histones

a) no detectable change in the family of very lysine-rich H1 histones

b) slight changes in the family of H1 histones

c) additional sperm specific basic proteins

d) Considerable changes in histones H1 and H2B

2.- Lower molecular weight proteins, more basic protamine rich in arginine

3.- Cystine-containing protamines

4.- Intermediate proteins containing lysine, arginine, and sometimes histidine

5.- Absence of basic nuclear proteins in sperm

Since the turn of the century, a broader perspective exists concerning the types of sperm nuclear basic proteins (SNBPs), which are classified into three general groups: Histone (H), Protamine (P), and Protamine-Like Protein (PL) (Ausió 1999; see Fig. I.7.1). Of these three general groups, the H type comprises a highly evolutionarily conserved group of chromosomal proteins that are similar to the main histone constituents of somatic chromatin, which would be analogous to Boch's *Rana* type. An example of this category is sperm chromatin of the gold fish, *Carassius auratus* (Munoz-Guerra et al, 1982), in which sperm histones retain compositional identity with the somatic counterpart. There exist several examples of sperm containing histones that replace somatic histones of stem cells with a complement often consisting of highly differentiated sperm-specific variants, such as in echinoderms (Casas et al., 1989) and other invertebrates (Zalenskaya et al, 1981).

The protamine type consists of usually small proteins that are rich in arginine and cysteine. Examples can be found in both vertebrate and invertebrate organisms (Subirana and Colom 1987; Rooney et al., 2000; Giménez-Bonafé et al., 2002a, b). Generally, protamines replace somatic histones of the stem cells during spermiogenesis (reviewed in Oliva and Dixon 1991), though in complex systems such as mammals, protamine-associated sperm chromatin coexists with 10-15% chromatin organized by histones (Gatewood et al., 1987).

The PL type is an intermediate type between histones and protamines in bacisity, originally described and characterized in bivalve molluscs, such as *Mytilus edulis* (Ausió 1986, 1992).This type of protein consists of a highly heterogeneous group of SNBPs which are rich in arginine and lysine, and phylogenetically related to somatic-type linker histones (the H1 family) (Eirin-Lopez et al., 2006a), such as the presence of a trypsin-resistant globular core (Ausió and Subirana 1982). There are essentially two main subtypes of protamine-like proteins: those containing a winged helix domain, a feature which supports its relation to histone H1, are referred to as PL-I. The other subtype lacks this domain, and is therefore smaller in size, with a higher electrophoretic mobitlity and a structural and phylogenetic relation to the amino- and carboxy-terminal domains of linker histone H1 (Eirín-Lopez et al., 2006b). PL proteins often co-exist with variable portion of histones in the mature sperm. For example, a bony fish, the red mullet *Mullus surmuletus* has been described as having nearly only PL proteins organizing its sperm chromatin (Saperas et al., 2006), where PL accounts for at least 90% of the protein component of mature sperm.

I.3.1. General characteristic features of sperm nuclear proteins

Extensive study has been done on sperm from several animal classes including a variety of vertebrates such as fish (Gimenez-Bonafé et al., 2000), amphibian (Kasinsky et al., 1985), rooster (Okamura and Nishiyama 1976; Sprando and Russel 1988), mammals (Ecklund and Levine 1975; Zao et al., 2001), and several invertebrates species such as echinoderms (Cornudella and Rocha 1979; Marzluff et al., 2006) and molluscs (Daban et al., 1991; Ausio 1992 a,b; Cáceres et al., 2000). Based on early studies, in 1975 Subirana drew the following conclusions concerning features which should be thereon considered characteristic of basic proteins from sperm:

1.- In the majority of species studied which contain basic proteins, one or more typical components appear in sperm, even if all somatic histones have been conserved.

2.- The degree of conservation of somatic histones varies in different species.

3.-The components typical for spermatozoa are always more basic than the somatic histones, usually with a high arginine content. A high percentage of serine (5 to 18%) is also found, the significance of which is evident from the work of Ingles and Dixon (1967) suggesting these residues as phosphorylation sites.

4.- Tyrosine and/or cysteine are amino acids common to many highly basic sperm proteins.

I.4 Sperm chromatin

Specialized sperm proteins create the necessary architectural features of sperm chromatin through their high affinity to bind DNA tightly, and therefore have an important function in the sperm cell. Protection of the male gamete genome is a primary role. For example, the protamine-DNA complex may create such a stabilized chromatin network that enzyme, bacteria, and other chemical agents do not have easy access to DNA (Subirana and Puigjaner 1973). This protection is essential when it is considered that, not only does the gene content need to be preserved, but this genetic information must be transported and often stored for considerable lengths of time in the absence of DNA-repair mechanisms. Inhibition of transcription is another important role of sperm nuclear basic proteins, since the genome of the sperm cell is in an inert state. A final role of sperm chromatin in many cases is the importance of the condensation process in achieving the final sperm shape. The transitions of SNPB organizing spermatic DNA during different stages of spermiogenesis have a global effect on the patterns developed during sperm chromatin condensation, and an important influence over the final morphology of the sperm cell (Daban et al., 1991).

I.4.1 Biological significance of specialized condensed chromatin architecture

The acquired chromatin structure, one which is quite compact, resulting in an inert genome, must be easily reversed back into an active state upon fertilization. Therefore, the specialized chromatin packaging of the male germ cell must be compatible with the oocyte machinery for it to be able to unpackage and reprogram the male genome for successful embryonic development (Philpott et al., 2000; McLay and Clarke 2003; Tamada et al., 2006). This implies that there may be a specific organization to the packaging process of the sperm genome, an order related to the area of the nucleus occupied by each chromosome, chromatin domain, and gene. Otherwise, the reversal of the inert and compact chromatin to begin embryonic development would likely not be achieved. In mammals, the reversal involves a timely decondensation of the male genome, achieved by factors found in the female gamete, which are necessary for complete fertilization (Perreault et al., 1988). These factors involve specific kinases (Balhorn et al., 1991) and reducers (Green et al., 1994) to aid in removal and dissociation of protamines and activation of the male genome. Studies in mammals such as mouse and hamster show that improper proportion of protamine molecules organizing the sperm chromatin can lead to defects in embryonic development or even male infertility, indicating the high specialization of factors in the oocyte environment able to reorganize

and utilize the male genome for proper fertilization events to occur (Cho et al., 2001). Further, these factors appear to be very species specific, since chromatin composition can vary between species, but is tightly regulated within a species (Corzette et al., 2002). Therefore, the sperm chromatin architecture has an important biological function, which is related to the actual survival of species, since it must be ordered and organized in a way which is compatible with the molecular mechanisms particular to the oocyte of the same species.

I.5. Compositional aspects of DNA-associated proteins: effect on chromatin condensation and structure

I.5.1. The nucleosome

A special feature of any chromatin containing histones, be it from somatic or spermatic nuclei, is that these histones are organized into structures called nucleosomes (Fig. I.5.1). The nucleosome is a subunit which has been the subject of study for decades. Electron micrographs provided the first confirmation that the eukaryotic genome is organized into repeating units composed of histones and their associated DNA (Oudet et al., 1975). Early experiments applying nuclease to chromatin showed the typical DNA size released in the digests was 200 base pairs, which could be further digested to particles containing 146 base pairs (Noll and Korngerg 1977). This repeating chromatin subunit called the nucleosome is composed of the histone core octamer around which DNA wraps itself (corresponding to the 146 base pairs observed during extensive chromatin digestion) together with regions of linker DNA of variable length, connecting adjacent nucleosomes (van Holde 1989). The histone octamer, or core particle, is quite evolutionarily conserved, whereas linker DNA associated to histone H1 is much more variable in length (van Holde 1989; Wolffe 1995; van Holde and Zlatanova 1996). There are four histones comprising the histone octamer, which themselves are among the most evolutionarily conserved proteins known. Histone H3 and H4 form a tetramer, and are flanked on either side by [H2A-H2B] dimers (van Holde 1989; Cheung et al., 2000; Khorasanizadeh 2004). Approximately 75% of the core histone protein mass comprises the “histone fold” domains, which form the spool onto which the nucleosomal DNA is wrapped (Arents et al., 1991, 1993; Luger et al., 1997). One molecule of linker H1 associates with the entry and exit sites of DNA which wraps around the nucleosome octamer core, sealing the two turns of DNA (Kamakata and Biggins 2006) (Fig. I.5.1) and further stabilizing the stretches of DNA running between adjacent nulceosomes. The

length of DNA entering and exiting the supercoil, together with the length wrapping around the core octamer, is called the chromatosome (Wolffe 1995; Widom et al., 1998) and is about 168 base pairs.

The high content of basic residues comprising histones provides the major basis for their strong binding to DNA by electrostatic interaction. Linker histone H1 is composed of a conserved globular core region, which is the site of interaction with the entry and exit regions of the DNA wrapping around nucleosome core. Additionally, H1 has highly variable amino and carboxy terminal tail regions whose role is stabilization of the linker stretches of nucleosomal DNA (Wolffe et al., 1997; Lewis et al., 2003b). These tail regions provide this histone with much more evolutionary variability than core histones. The structure of histone H1, which includes a winged helix domain in the globular core region, together with compositional amino acid analysis data suggest an evolutionary relation of histone H1 to protamine-like proteins and protamines (Ausió 1999; Lewis et al., 2004; Eirín-López et al., 2006a; Saperas et al., 2006).

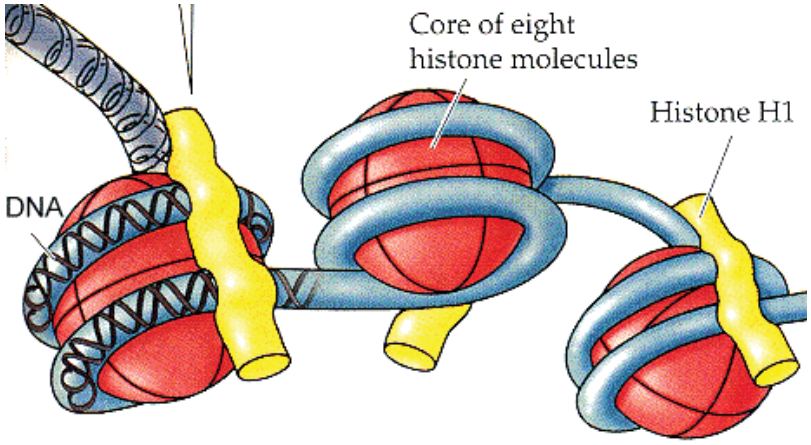

Figure I.5.1. The nucleosomal subunit. The nucleosome consists of 146 base pairs of DNA which wrap around a histone core octamer and the linker DNA connecting adjacent nucleosomes. Histone H1 interacts with the entry and exit sites of DNA around the core particle. The resulting structure is a "beads on a string" configuration. (Life, the Science of Biology, by Purves, Orians, & Heller, 5th ed., 1997).

Nucelosome subunits are further coiled into a fiber of 30 nm (Vaquero et al., 2003) within the somatic nucleus, of which two models have been proposed: a solenoid or a zig-zag arrangement (Fig. I.5.2). This type of DNA packaging into nucleosomes which are organized into solenoid or zig zag fibers results in chromatin with a certain volume,

due to the wrapping of DNA around histones, as well as the added volume of the central core of the solenoid fiber (Pienta and Coffey 1984). This volume is quite relevant when one considers the overall achievement of nuclear reduction during spermiogenesis, and when a comparison is made between the final chromatin compaction of sperm containing histones with sperm containing protamines (compare models in Fig. I.5.6).

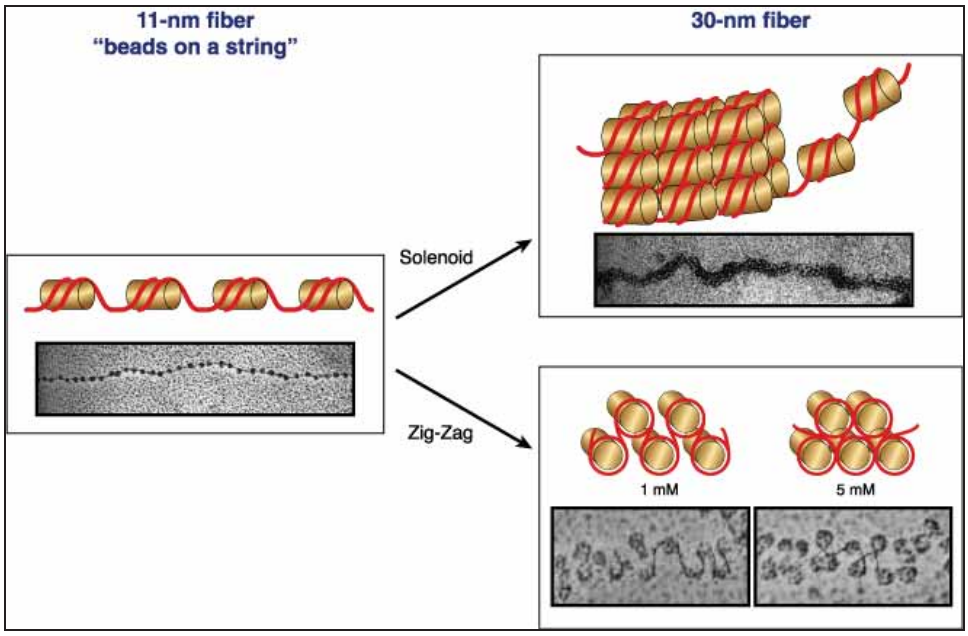

Figure I.5.2. Models for the 30 nm fiber. The figure shows a schematic representation of two models that have been proposed to explain the 30 nm fiber. Within the zig-zag conformation, 1mM and 5mM show two concentrations of TEA-Cl (triethanolamine-chloride) to emphasize the importance of the salt concentration in the compaction of nucleosomal arrays. The electron micrographs of the 11 nm fiber and solenoid were adapted and modified as described in Vaquero et al. (2003).

In addition to stable octamer formation and interaction with one another at interface positions, the histones also have amino-and carboxy-terminal tails that protrude from the nucleosome. These histone tails are 20-35 amino acid residues which play an essential role in controlling the folding of nucleosomal arrays into higher order structures (Woodcock and Dimitrov 2001). This includes their ability to form tail-mediated chromatin folding, as well as inter-nucleosomal and histone-histone interactions (Peterson and Laniel 2004). The tails can fit through DNA gyres through minor grooves and extend beyond the nucleosome surface, forming ideal locations for enzyme-mediated covalent modifications which aid in these types of interactions (Khorasanizadeh 2004).

I.5.2. Histone variants: non-testes specific

Histones with variations in their primary sequences, known as histone variants, are capable of altering chromatin structure. These histone variants are non-allelic isoforms of major core or linker histones, and can replace them within the nucleosome and participate in the functional specialization of nucleosomes and chromatin domains (Malik and Henikoff 2003; Caron et al., 2005); each histone class, except for H4, includes several of these subtypes (Churikov et al., 2004a). Most of these differences occur in the amino and carboxy tail sequences of the major histones.

The main variants pertaining to histone H3 are H3.3 and CENP A. H3.3 has been identified in pre-and post-meiotic cells (Bramladge et al., 1997), and in somatic cells seems to be correlated with actively transcribing chromatin (Ahmad and Henikoff 2002). CENP A is a centromeric variant, harboring an amino-terminal tail which is completely divergent from H3. This variant is deposited on newly duplicated sister centromeres and is required for the recruitment of other proteins to the centromere and kinetochore (Smith 2002).

Histone H2A is the core histone with the largest number of variants. Two of those variants, H2A.X and H2A.Z, are quite evolutionarily conserved, and can be present in sperm. The variant form differs from conventional H2A in the carboxy-terminal sequence region, and their genome distribution. Histone H2A.X has been implicated in the cellular response to double strand breaks in DNA, being phosphorylated at sites of DNA damage (Modesti and Kanaar 2001; Burma et al., 2001; Lewis et al. 2003a). Histone H2A.Z has an acidic patch, which seems to be crucial to its function, which is to alter the folding of a nucleosome array to promote the formation of the 30 nm fiber, and therefore chromatin compaction (Fan et al., 2004). This acidic patch therefore may facilitate the attraction between adjacent nucleosomes by strengthening the bond of H2A with the amino tail of histone H4 from a neighboring nucleosome (Tremethick 2007).

Variant forms of H2B do exist, and tend to be involved in packing DNA in pollen (Ueda et al., 2000), sperm (Gineitis et al., 2000; Zalensky et al., 2002; Churikov et al., 2004b) or in other aspects of sperm development (Aul and Oko 2001). A non-testes specific variant of H2B was identified in the protozoan *Trypanosoma brucei*, (H2B.V) that shares around 38% sequence identity with major H2B, and is essential for viability, along with its dimerization with H2A.Z (Lowell et al., 2005). H2B.V and H2A.Z were found to colocalize throughout the cell cycle and exhibit nearly identical genomic distribution patterns, suggesting that H2B.V and H2A.Z function together within a single

nucleosome, marking the first time an H2A.Z has been shown to associate with a non-canonical histone H2B.

Histone H1 has numerous sequence variants, including sperm and testes-specific variants. The central globular and trypsin-resistant domain of H1 is conserved among H1 subtypes, while both tail domains vary considerably in length and sequence and denote distinctive features of the variant types (Doenecke et al., 1997).

I.5.3. Histone variants: testes specific

A testis-specific linker histone subtype H1t is expressed in mammalian spermatogenic cells. It is highly divergent in its primary sequence from other members of the H1 family. It is found only in the testis, from pachytene spermatocytes to round and elongated spermatids, where it constitutes 55% of the total linker histone (Drabent et al., 1996; Steger et al., 1998). In vitro experiments show that H1t has a lower condensing capacity and is less tightly associated to oligonucleosomes than the other H1 subtypes (De Lucia et al., 1994; Khadake and Rao 1995). This property has been proposed to help maintain chromatin in a relatively open state during meiosis, facilitating meiotic events such as recombination (Oko et al., 1996).

The first testis specific core histone variant corresponding to histone H2B was discovered in rat in 1975 by Shires et al. It was later shown to be expressed in meiotic spermatocytes. At this stage, the nucleosome appears to be in a loosened conformation (Rao and Rao 1987), which could be advantageous for meiotic recombination and to facilitate histone replacement by transition proteins and protamines during in spermiogenesis.

Additionally, a special DNA binding motif, called the SPKK motif was proposed for specialized sperm histones H2B and H1 from sea urchin (Suzuki 1988). This binding motif, composed of sequence repeats of Ser-Pro-Basic-Basic, where the basic residue is either a lysine or an arginine, has been proposed to function allowing the domain of the protein to bind within the minor groove of DNA by folding into a β-turn, creating a concave and flat structure suitable for such binding (Suzuki 1988, 1989). To contribute to the DNA-binding ability of this motif, the side chains of two neighboring basic residues extend upwards and downwards, respectively, and bind two phosphates on opposite strands of the DNA (Kopka et al., 1987). These SPKK motifs are phosphorylated on the serine residues, modulating the interaction of H1 and H2B with DNA, affecting their binding affinities at the tail regions and their chromatin condensing capacities (see section I.5.3b) (Hill et al., 1991).

Several histone H3 variants have also been identified in rat testes. A purified testes-specific histone H3 from rat has both an amino acid composition and a mobility in triton/acid/urea gels which differs from all other H3 subtypes (Trostle-Weige et al., 1984; Witt et al., 1996). This variant is maintained in similar or slightly increased amounts in spermatocytes to round spermatid stages (Trostle-Weige et al., 1984; Meistrich et al., 1985). H4 is the only histone in which no variant form has been observed.

I.5.4. Post -translational modifications (PTMs) of histones: "Histone Code"

Histones are highly susceptible to post-translational modifications at their tail domains, which can affect nucleosomal stability and inter-histone or histone-DNA interactions (Fig. I.5.4). Several functions of post-translational histone modifications are known, and have been proposed to have a number of different functions (reviewed by Workman and Kinston 1998; Li et al., 2007). In addition, a histone code has been hypothesized, proposing that specific histone modifications or combinations of modifications create signals for the docking of specific cellular factors, thus mediating particular chromatin-related functions (Strahl and Allis 2000). They may be involved in gene silencing, transcription activation or silencing, signaling mechanisms, and DNA replication and repair (reviewed by Peterson and Laniel 2004; Groth et al., 2007). Molecular modifications which affect histones include methylation and acetylation of lysine residues, phosphorylation of serines and threonines, ubiquitination, and ADP-ribosylation. With the exception of methylation, these histone modifications result in a change in the net charge of nucleosomes, which could loosen the inter-and/or intra-nucleosomal DNA-histone interactions. In spermiogenesis, possible biochemical mechanisms for removal of histones from DNA include their enzymatic modification (Hnilica 1967; Stellwagen and Cole 1969), and interaction of anionic complexes with histones (Frenster 1965). Some of the main SNBP modifications which may play a significant role in protein transitions occurring during spermiogenesis are primarily acetylation, phosphorylation, and ubiquitination, and will be described in detail. Figure I.5.3 summarizes the different post-translational sites of core histones.

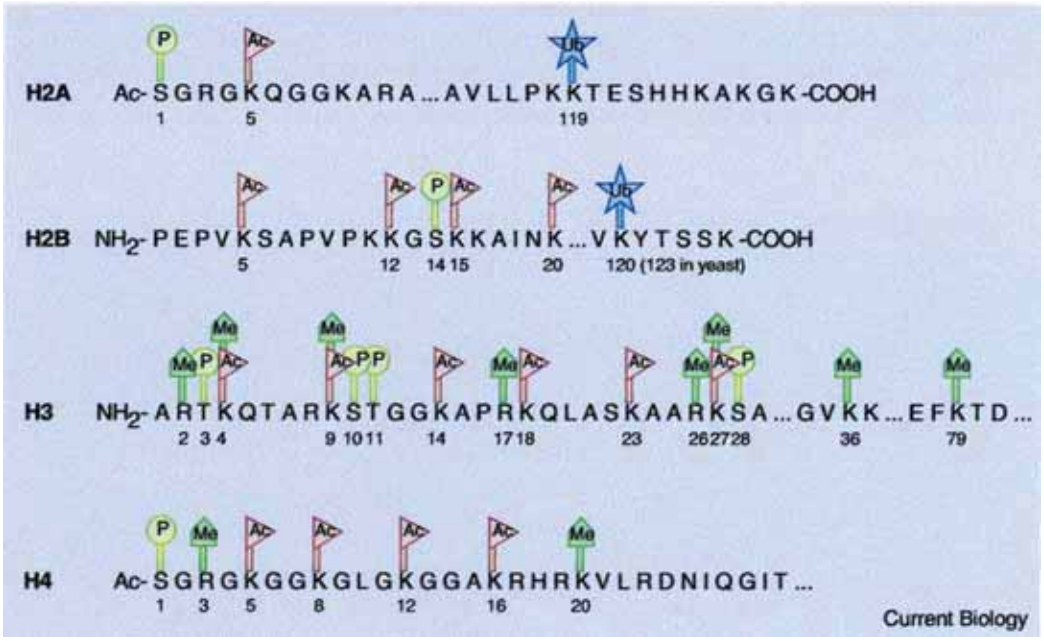

Figure I.5.3. Post-tranlsational modifications of the core histones. The different shapes represent known post-translational modifications of core histones. The histone tails can be methylated at lysine and arginines (pentagons), phosphorylated at serines or threonines (circles), ubiquitylated (stars) and acetylated (triangles) at lysines. From: Peterson and Laniel (2004).

I.5.4.a. Acetylation

Acetylation is one of the most studied post-translational modifications of histones. It was discovered by Allfrey et al. in 1964, and since is thought to be a control mechanism for a variety of biological processes, such as genetic activity, especially transcription. A steady-state balance of proteins with this modification is mediated by enzymes, called histone acetyl-transferases (HATs) and histone deacetylases (HDACs). It was discovered that acetylated histones are easier to displace from DNA, both in vivo and in vitro (reviewed in Oliva and Dixon 1991; reviewed in Li et al., 2007). This modification tends to affect histone H3 and H4; histone H4 is susceptible to acetylation at lysines 5, 8, 12, and 16, while main acetylation sites on histone H3 are lysines 9, 14, 18, and 23.

Histone acetylation has significant implications affecting the substitution of histones by protamine in spermiogenic nuclei. Hyperacetylation of histone H4 has been quite implicated in spermiogenesis of several species, including rainbow trout (Christensen and Dixon 1982; Christensen 1984), rat (Grimes and Henderson 1982), mouse (Hazzouri et al., 2000), rooster (Oliva and Mezquita 1982) and even invertebrate spermiogenesis, such as the mollusk *Sepia officinalis* (Couppez et al., 1987). It is

generally accepted that in spermiogenesis, histone hyperacetylation does not occur in species where histones are retained in spermatozoa (Kennedy and Davies 1980; Govin et al., 2004), and appears to be associated with histone replacement, and subsequent nuclear condensation (Hazzouri et al., 2000). According to the histone code hypothesis (Strahl and Allis 2000), hyperacetylation of histones in elongating spermatids would serve as a signal for recruitment of specific machinery which specifically interacts or binds to acetylated chromatin. Another mechanism of acetylation would be reduction of the affinity of core histones for DNA, permitting easier displacement by protamine (Christensen et al., 1984; Oliva et al., 1987). Aside from the role acetylation could play in facilitating the displacement of histones by protamine, it has been suggested that this modification can relax the nucleosome (Oliva et al., 1987) as well as the chromatin fiber, allowing a more open structure (Calestagne-Moreli and Ausió 2006), and can also inhibit the formation of higher order chromatin structures, such as the 30 nm fiber (Tremethick 2007; see Figs. I.5.2 and I.5.4)

Acetylation of specific lysine residues on the amino terminal of core histones plays an important role in transcriptional regulation. Indeed, histone acetylation has also been associated with transcriptionally-active chromatin, believed to function by decondensing regions of chromatin that are to become transcriptionally active, enabling accessibility to components of the transcription complex (Christensen et al., 1984).

Therefore, two sets of events during spermatogenesis are associated with waves of acetylation. The first is gene transcription which is active in spermatogonia, pachytene spermatocytes, and round spermatids. The cessation of transcription is thought to stop shortly after, in elongating spermatids, possibly accompanied by a deacetylation (Zheng et al., 2008). The second acetylation event takes place during spermatid maturation in some species, when histones are replaced by protamines (Kierszenbaum and Tres 1975, 1978; Hecht 1998). In each case, biological processes are at least partially mediated by a change in binding affinities between histones and DNA and between neighboring nucleosomes. A representation of chromatin structure modulation contributed by acetylation and other chemical modifications can be appreciated in Figure I.5.4.

I.5.4.b. Phosphorylation

Phosphorylation occurs on the hydroxyl groups of serine or threonine residues of the protein it modifies. The addition of this compound brings three negatives charges, diminishing the positive charge of the protein. Sung and Dixon (1970) found that specific sites of testis histones H1, H2B, and H4 from trout were phosphorylated in portions of

the molecule rich in basic residues, and concluded that this alteration in histone binding to DNA could be involved in the substitution of histones for protamine which occurs during trout spermiogenesis. Phosphorylation of H3 has been reported as well, specifically at serine 10, and has been implicated to work in conjunction with acetylated H4 at lysine 16 to enhance transcription (Strahl and Allis 2000). Recently, phosphorylation of serine 1 on the amino terminal tail of histone H4 has been recognized as a conserved histone modification among metazoans, which has been implicated in germ cells to function in modulating chromatin compaction during gametogenesis (Wendt and Shilatifard 2006). Histone H1 and H2B phosphorylation has been described in sea urchin sperm during spermiogenesis, followed by their subsequent dephosphorylation in mature sperm (Hill et al., 1991). This is reminiscent upon protamine phosphorylation during spermiogenesis, one of the most marked post-translational modifications affecting the SNBP interactions with spermiogenic chromatin (see Oliva and Dixon 1991 for review; see also section I.5.8).

I.5.4c. Ubiquitination

All histones except histone H4 have been known to be ubiquitinated in vivo, but H2A and H2B have been the most extensively studied (Jason et al., 2002). In higher eukaryotes, 5 to 15% of H2A and 1 to 2% of H2B are ubiquitinated on a unique target lysine located in the C-terminal part of each histone. Its attachment is achieved by specific ligase enzymes covalently bonding the 8.5 Kd ubiquitin protein to the histone it modifies. Like acetylation, histone ubiquitination is likely to play a role in histone replacement, but the mechanism involved is not known. Ubiquitinated histone H2A has been described in spermiogenesis of mouse (Baarends et al., 1999) and rooster (Agell et al., 1983), and ubiquitination of both H2A.Z and H2B in trout (Nickel et al., 1987), as well as reports of ubiquitinated histone H3 in elongating spermatids of rat (Chen et al., 1998). Since the size of the ubiquitin protein represents 60% of the size of histone H2A, this modification has been suggested to affect the global structure of the nulceosome, facilitating its disruption. It has been proposed that destabilization induced by histone ubiquitination may play a role in facilitating histone-to-protamine replacement (Baarends et al., 1999).

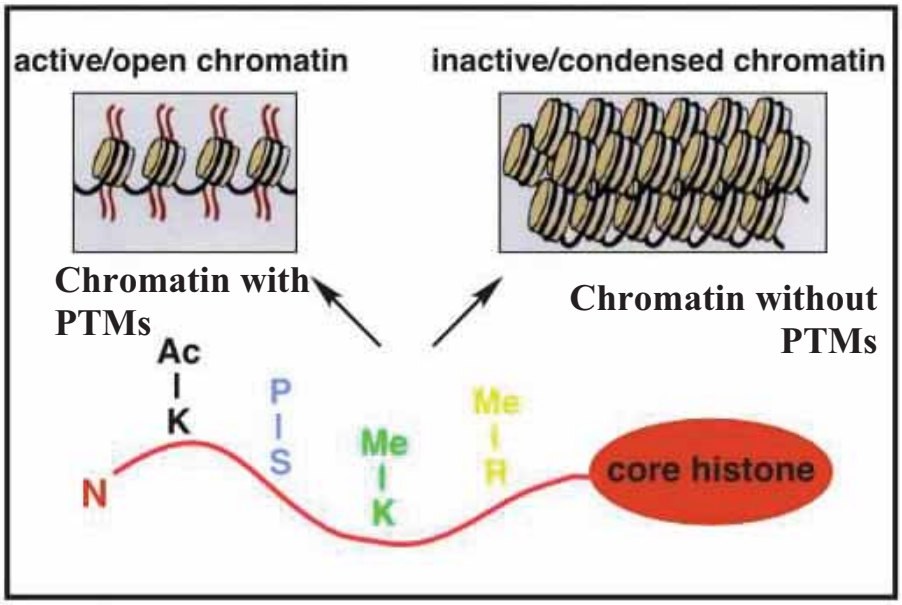

Figure I.5.4. Histone modifications modulate chromatins structure. Covalent post-translational modifications of histones have been shown to play an important role in altering DNA accessibility and chromatin structure. Histones can be reversibly modified in several ways at specific sites on their amino terminal tails (http://www.imt.uni-marburg.de/bauer/research.html).

I.5.5. Transition proteins (TP)

An intermediate step in the histone to protamine exchange occurs in some spermiogenesis, involving transition proteins. These proteins are a part of a dynamic nuclear basic protein transition in spermiogenesis of mammals, and have been well characterized in several species (Balhorn et al., 1984; Zao et al., 2004). During spermatid differentiation in mammals, transition nuclear proteins (TPs) constitute about 90% of the chromatin basic proteins accumulating in cells following histone removal and prior to protamine deposition (Meistrich et al., 2003), and are believed to be involved in such processes. Specifically, Zao et al. (2004) showed that TP replacement triggers proper processing of precursor protamine molecules, and that TP displacement does not occur until proper processing of the precursor protamine into the mature protamine occurs. This suggests an important role for TP in the proper placement of protamine molecules in mature sperm chromatin, indicating as well a specific mechanism of organizing the male gamete genome, likely for its ordered unpacking via machinery found in the oocyte environment upon fertilization (see section I.4.1).

TPs are variable with regard to size and amino acid composition. Generally, TPs are more basic than histones and less basic than protamines. In boar, bull, man, mouse, ram, and rat, the TP family consists of four proteins, TP1-4, of which TP1 and TP2 are

the best characterized. Mammalian TP1s are highly conserved, have a low molecular weight (6.2Kd), and are highly basic, composed of 20% arginine, 19% lysine, and 14% serine. TP2 is much larger, about the size of a core histone (13Kd) with 32% basic residues, including 10% of each arginine and lysine residues, and 22% serine, 13% proline, and 5% cysteine which are capable of forming disulfide bonds (Grimes et al., 1975). Unlike TP1, TP2 is poorly conserved.

Knockout experiments of mice deplete of TP1 or TP2 show abnormal chromatin condensation (Yu et al., 2000). One reason was due to the incomplete precursor protamine processing in the absence of one or both TPs, resulting in lesser compact mature sperm chromatin. TP1 or TP2 are not, however, critical for histone displacement and initiation of chromatin compaction; they seem to be directly related to adequate precursor processing for completion of chromatin condensation (Adham et al., 2001; Zhao et al. 2001).

An interesting study by Brewer et al., (2002) reported condensation rates of free DNA by TP1 and TP2, demonstrating that both molecules are effective in condensing DNA (Fig. I.5.5). This study suggests that, though the level of condensation achieved with TP1/TP2 is not as high as that achieved with protamine, the state of chromatin condensation already begins with the replacement of histones by these molecules, which continues condensing to a further degree with the appearance of protamines.

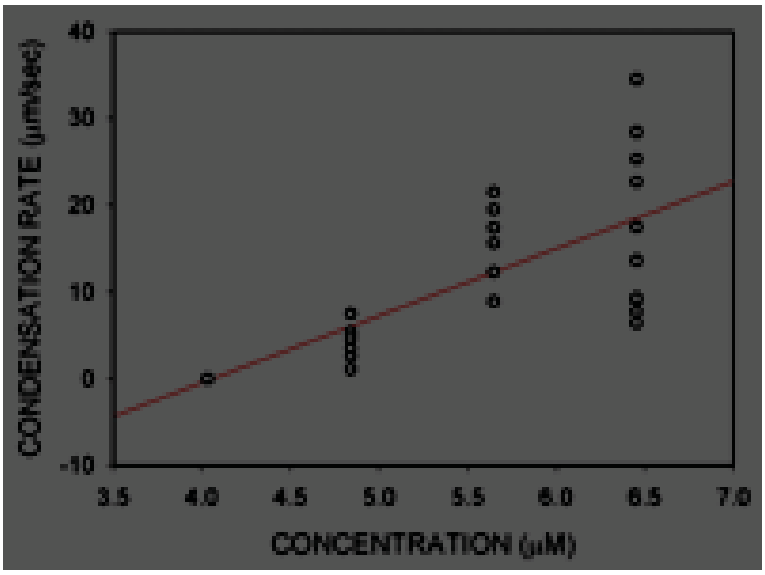

Figure I.5.5. Condensation of DNA by basic proteins. The condensation rate versus peptide concentration for SKRKAVRRRKRTHRAKRRTSGRRYK (carbxy terminus of the TP2 peptide from mouse). The free DNA condensation rate increases with the concentration of the transition protein molecule (Brewer et al., 2002).

I.5.6. Protamine

Cellular aspects that are most directly related to the protamine function are the high degree of sperm chromatin condensation and packing in the sperm nucleus, bringing about a large reduction in final nuclear volume. The result is a protected and inert genome, reduced in volume, which has been proposed to aid streamlining the sperm cell in its plight to gamete fertilization (Braun 2001). Protamine is generally a small protein with some exceptions, but contains a large proportion of positively charged arginine residues that allow it to tightly bind and condense sperm DNA. In fact, the total number of amino acids comprising a protamine, and the positions of the arginine residues, vary considerably among zoological groups. However, the proportion of arginine residues remains nearly the same. This observation, published by Rooney et al. (2000) indicates that evolutionary conservation of a high proportion of arginines in the protamine molecule is maintained at the protein composition level, rather than at the specific amino acid site within the protein.

Aside from the extreme basic nature of the protamine being an important factor in its ability to condense chromatin (Fig. I.5.6), the way in which the molecule binds to DNA also influences the great compaction ability of the protamine. In 1982 Balhorn presented a model of protamine-DNA binding, proposing that protamines bind to the DNA by lying lengthwise inside the minor groove. He also calculated that there were enough positively charged arginine groups on the protamines present in the mouse sperm model to completely neutralize the negatively charged phosphate groups on the DNA. He proposed that the protamine-DNA complex of one strand would fit into the major groove of a neighboring DNA strand, so that the DNA strands in the sperm nucleus would be packaged side by side in a linear array. However, other experiments including X-ray diffraction revealed a correction to this interpretation, showing that the protamine is not situated in the minor groove of the DNA double helix, but in the major groove (Mirzabekow et al., 1977; Fita et al., 1983), which is the accepted model at present. Another feature of mammalian sperm nuclei is that the chromatin is stabilized by intermolecular and intra-molecular covalent disulfide bonds between protamines (Bedford and Calvin 1974; Marushige and Marushige 1975). These important features of protamines allow DNA to be tightly packaged, with high chromatin stability. The maximal volume reduction involved with chromatin packaging by protamine includes a weight reduction of the sperm cell, facilitating the transport of genetic material to the egg for fertilization.

I.5.7. Post-translational modifications (PTMs) of TP and protamine

Phosphorylation is likely a universal modulating element in nucleoprotamine formation, and has been described in several groups of organisms, ranging from mammals (Marushige et al., 1978; Pruslin et al., 1987), to fish (Saperas et al., 1993; Jergil and Dixon 1969). Phosphoryl groups have been found on 75% of the serine residues in early protamine-stage testes, which is diminished to only 5% of the serine residues in protamine of mature sperm (Ingles and Dixon 1967). Therefore, it was concluded that when protamine first binds to spermiogenic chromatin it is in its phosphorylated state, and subsequently undergoes dephosphorylation before the final nucleoprotamine complex is formed (Oliva and Dixon 1991).

Phosphorylation, whether modifying a histone or a protamine, influences the chromatin in a dynamic way during spermiogenesis. The reason has to do with the electrostatic interaction of the protein with DNA. Seryl phosphorylation modifies the affected residues with a negative charge, modulating the interaction of protamines with the DNA, diminishing the interaction (Louie and Dixon 1972). This creates a certain plasticity of the chromatin, letting condensing DNA complexes resolve and reform until the correct chromatin placement and nuclear shape have been accommodated. Once the correct nucleoprotamine complexes have been established, dephosphorylation of protamines would increase the binding affinity of the protamine for DNA and therefore fix the nucleoprotamine complex in a completely compact and rigid state in fully mature sperm chromatin.

Another hypothesis in which phosphorylation could function with the protamine during spermiogenesis is to bring negative charges which could function as cross-linking points to other protamine molecules via their positive arginine residues, forming protein-protein electrostatic interactions (Bode et al., 1983; Bode 1984). It has also been reported that protamine phosphorylation promotes the formation of large ordered macromolecular complexes with DNA. It is thought that the serine phosphates serve to reduce electrostatic attraction between interacting partners, thus permitting a thermodynamically controlled, ordered association that is not possible with unmodified protamines which are more tightly bound to DNA (Bode et al., 1980; 1983; Bode 1984). Phosphorylation of histones H1 and H2B is proposed to have a cross-linking function in sperm chromatin of sea urchin (Hill et al., 1991). Therefore, it seems that phosphorylation is a controlled mechanism which allows both plasticity and flexibility during chromatin condensation, as well as permits the ordered and correct association of the chromatin complexes. The

subsequent dephosphorylation seems to be related to final condensation and fixation of the nucleoprotamine complex in the mature spermatic nucleus.

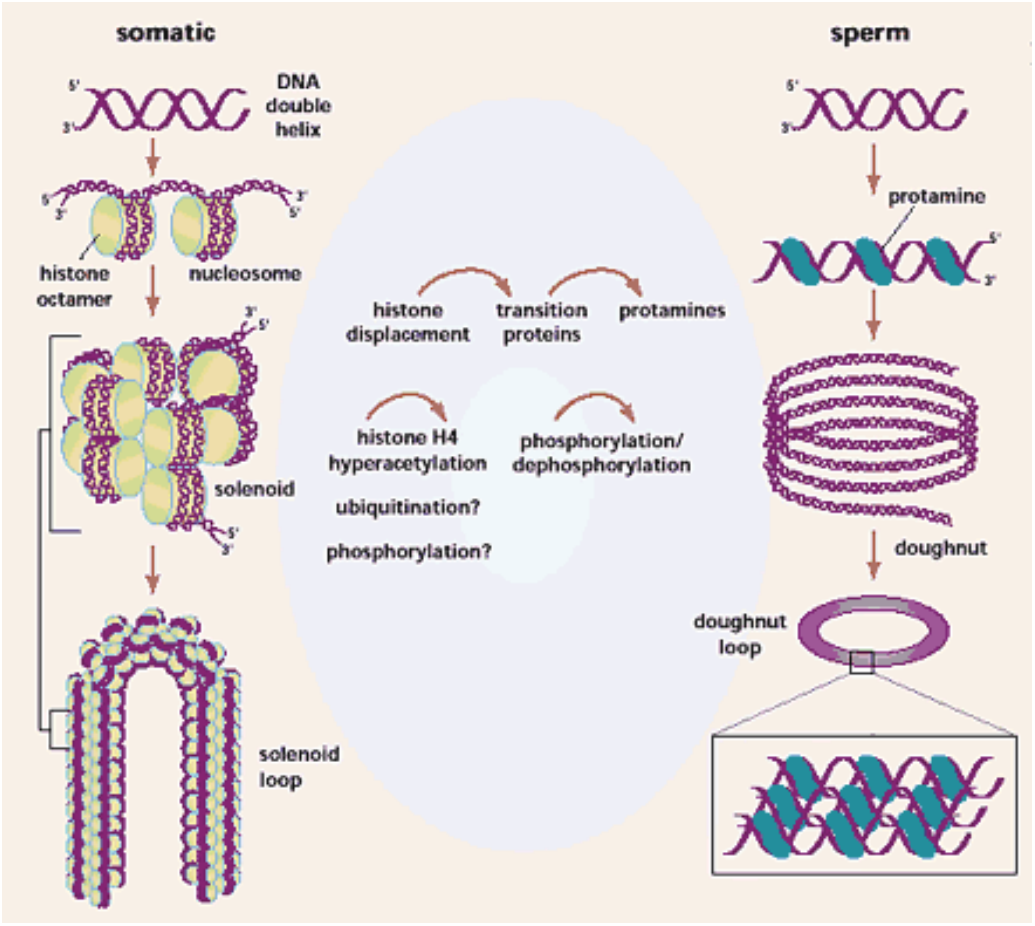

Figure I.5.6. Packaging chromatin. A model of chromatin packaging in somatic cells (left) and mammalian sperm (right). **Left.-** In somatic cells, the DNA is wound twice around histone octamers to form nucleosomes, which are then coiled into solenoids. The solenoids are attached at intervals to the nuclear matrix at their bases and form DNA loop domains. **Right.-** In the sperm nucleus, protamines replace histones and the protamine-DNA complex is coiled into a doughnut shape. Inset shows the tight packaging of protamine-DNA strands, which achieves a maximum reduction in volume, since the protamine fits within the DNA grooves, and does not contribute additional volume to the chromatin. Displacement of the histones is facilitated by post-translational modifications of the proteins, in the form of histone H4 acetylation, ubiquitination, and phosphorylation. Phosphorylation and dephosphorylation of the transition proteins facilitate their displacement before protamines bind. From: Braun (2001).

I.6 Spermiogenesis

In order to understand the sperm cell, a grasp of the biological process leading to the mature sperm cell, called spermiogenesis, is fundamental. One of the most dramatic chromatin remodeling events to ever be observed accompanies male germ cell differentiation, or spermatogenesis, where precursor germinal cells called spermatogonia, differentiate into spermatozoa (Hess 1998). Spermatogonia are mitotically dividing

somatic cells, which eventually enter into meiosis and produce primary spermatocytes. These spermatocytes undergo the preleptotene stage, during which they replicate DNA, followed by passage through leptotene, zygotene, pachytene, and diplotene stages corresponding to the first meiotic division of prophase.

In pachytene spermatocytes, homologous chromosomes are paired up, and exchange segments of DNA through homologous recombination, also known as meiotic crossing over. This process, extremely important for genetic variability, is aided by a number of proteins, localized in recombination sites along the paired chromosomes, in structures called synaptonemal complexes. Identification of these complexes in spermiogenic microscopy observations clearly identifies spermatoctyes in the pachytene stage of meiosis.

The first meiotic division yields secondary spermatocytes, which quickly go through the second meiotic division, generating haploid round spermatids. During the post-meiotic maturation of the spermatid, called spermiogenesis, the spermatid undergoes a dramatic nuclear reorganization. This involves changes in chromatin composition and structure, and changes in nuclear shape and volume. These features of spermiogenesis ensure that the sperm cell achieves proper chromatin structure and organization, nuclear shape, and genome protection.

I.6.1. Changes in nuclear composition

The remodeling of the haploid cell nucleus is a unique process where histone variants, histone post-translational modifications, and many non-histone factors interplay to package the genome in a new way, either by incorporating variant nucleosomes with a tighter organization than in somatic cells, or by obtaining a histone-less molecular composition, which allows increased genome protection (Fig. I.6.1).

I.6.2. Functional changes

Remodeling of the spermatid nucleus is functional, as well. Important nuclear changes occur as the spermatid differentiates into a mature sperm cell. This includes cessation of all transcriptional activity in the advanced stages of the developing spermatid. This change in nuclear function is related to the special chromatin structure acquired by the sperm nucleus during spermiogenesis, inhibiting access of transcriptional machinery to the DNA (Sassone-Corsi 2002). Shutting down transcriptional processes and most metabolic activities by way of altering the nuclear composition and chromatin structure has an important function, which includes protection the male genome, maintaining it in an inert and simplified state. This facilitates the transport and safety of

the male genetic information as it carries itself toward the female gamete, until the proper machinery to reactivate its genome is met upon penetration into the oocyte.

I.6.3. Changes in chromatin structure

Changes in nuclear composition during spermiogenesis are accompanied by changes in chromatin structure. The effect that nuclear compositional changes have on chromatin structure greatly depends on the type of molecule interacting with the spermatic DNA at different points during its differentiation. However, the common tendency is for spermiogenic changes to develop toward a reduction in nuclear volume and a condensed chromatin state. In any sperm cell which retains histones packaging the chromatin, the structure achieved occupies a considerably larger volume than that of non-histone nuclear compositions (see section I.5.7).

Non-histone nuclear compositions of developing sperm cells vary greatly and affect chromatin structure. Acquired chromatin structures can be quite simple, such as the rapid formation of condensed chromatin granules associated with the direct histone replacement by protamine (Saperas et al., 1993). This rapid and direct increase in condensation of chromatin structure has to do with the chemical nature of transition proteins and protamine, and seems to increase proportionally with basic protein concentration (Fig. I.5.5). The way in which the protamine associates with the DNA has an overall effect on nuclear volume reduction as well; the most highly compact chromatin structure possible is achieved in the protamine-DNA nuclear composition (see section I.5.7). Chromatin structures which arise from chemical interactions with intermediate proteins tend to be more dynamic and complex, and depend on the type of transition protein or protamine precursor associated to spermatic DNA. The chemical composition of the intermediate molecule directly influences the type of chromatin condensation, which could also influence the shape.

I.6.4. Interspecific variability in compositional and structural changes

Variability of nuclear shape is the product of interplay between the type of compositional changes in the nuclear biochemistry and structural changes in the chromatin architecture. In the history of sperm study, there has been an evident segregation of morphological/structural spermiogenic studies (achieved with the use of optical and electron microscopy) and biochemical studies of sperm nuclear proteins, protein transitions, gene regulation, and epigenetic chromatin modifications. The relation of these two fields of study is extremely important for understanding the entire process involved in developing a customized male gamete specific for each species. These two

levels of organization, morphological and biochemical, are distinct, but merge toward the same biological principle of creating a sperm cell with the proper nuclear shape and chromatin structure capable of fertilizing the oocyte of its own species, as well as later achieving proper embryonic development.

I.6.5. Proposed relationship between compositional changes (proteins interacting with DNA) and structural changes in spermiogenesis

There appears to be a correlation between the extent and complexity of compositional chromatin changes during spermiogenesis and formed chromatin structures during spermiogenesis. For example, a species whose spermiogenesis involves a great deal of protein complexity and compositional changes during its development is the gastropod mollusk *Murex brandaris* (see ahead section I.7.4) (Cáceres et al., 1994, 1999). The chromatin patterns developed during sperm maturation have been extensively studied (Harrison et al., 2005). However, a direct correlation between composition and structure remains to be elucidated; identification of the chemical composition of the different spermiogenic chromatin structures has not been resolved. In a work done by Oliva and Dixon in 1991, there is mention of the need for experiments which could determine the exact timing and migration of this protein replacement in electron microscopy studies, joining the biochemical and ultrastructural aspect of spermiogenesis.

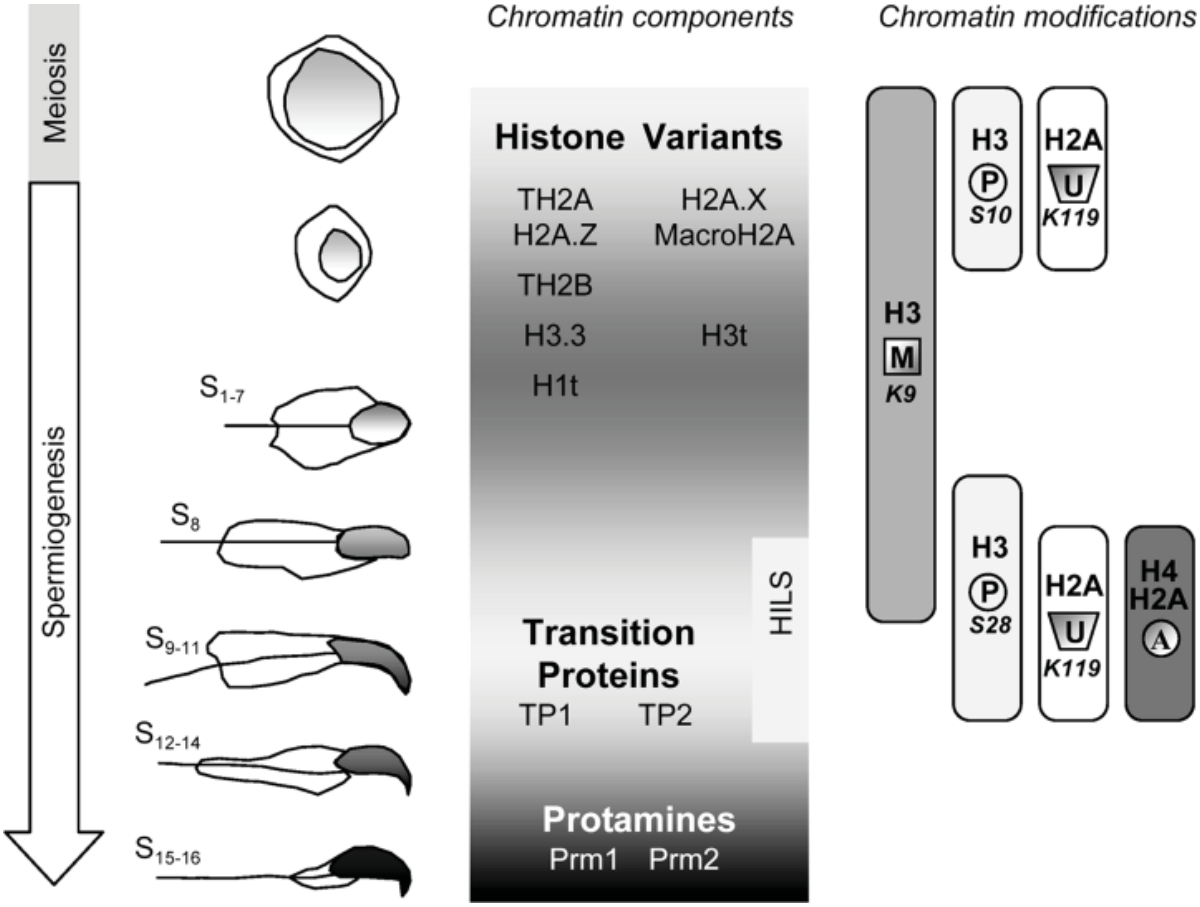

Figure I.6.1. Chromatin components during spermatogenesis in complex systems: the major chromatin components and their post-translational modifications. Histone variants are incorporated during meiosis. Highly basic proteins, transition proteins, and protamines replace histones during late spermiogenesis, depicted in the center. Post-translational histone modifications accompanying meiotic and protein transition phases is depicted on the right: A, acetylation; U, ubiquitination; M, methylation; P, phosphorylation. During postmeiotic maturation of the male germ cell, depicted on the left, a global remodeling of the nucleus occurs, during which it elongates and compacts into the very unique nucleus of the spermatozoa. Govin et al., (2004).

I.7. Spermiogenic Models

An additional way to classify sperm is based on their SNBP interactions during spermiogenesis (Fig. I.7.1). This includes the types of protein exchanges and transitions which occur during the sperm maturation process, and their effect on the patterning of chromatin condensation and final morphology of the sperm nucleus. A summary of the different protein transition models, some of which have been focused on in this study, are as follows:

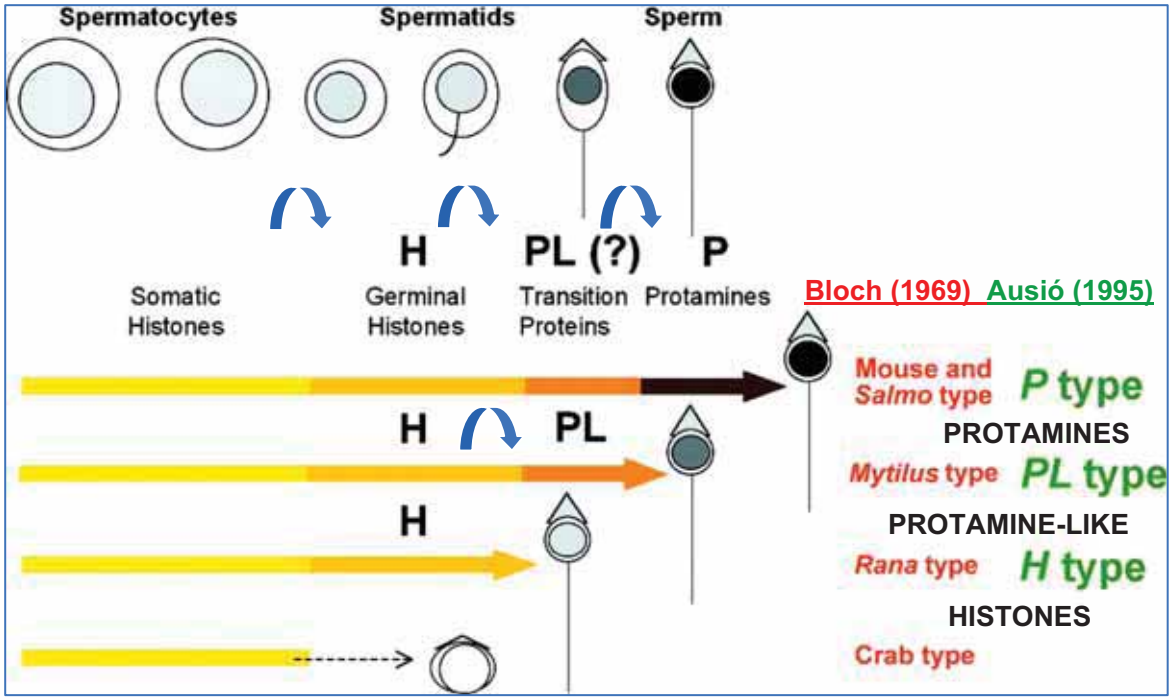

Figure I.7.1. The major SNBP types and SNBP transitions during spermatogenesis in different organizms. The spermatogenic developmental pattern and the associated SNBP transitions are shown on the left. The SNBPs for each transition are shown in black above yellow arrow (H, histones; P, protamines). The classification of the major SNBP types according to Bloch (1969) and the corresponding classification according to Ausio (1995) is shown on the far right. From: Ausio (1999).

I.7.1. Model Histones [H→H]

Species which retain histones in their spermatozoa include invertebrates, such as nemerteans *Cerebratulus californeinsis* and *C. lacteus* (Wang and Ausió 2001), as well as vertebrates, such as several fish species, including toadfish *Opsanus tau* (Casas et al., 1981), lamprey *Petromyzon marinus* (Saperas et al., 1994), and gold fish *Carassius auratus* (Muñoz-Guerra, et al., 1982). In these cases, the histones present in spermatozoa show little if any significant difference from the standard somatic type histone complement. The chromatin of this type of sperm is organized into nucleosomes with a DNA repeat length often longer than that of the typical somatic cell chromatin, which can be correlated to the increase in basicity of the full histone complement of sperm chromatin (Noll 1976; Morris 1976; Zalenskaya et al., 1981). This nucleosomal repeat organization has been studied in detail in *C. auratus*, described by Muñoz-Guerra et al. (1982); in sperm of this fish the nucleosomal repeat length was found to be about 205bp, or 13bp longer than nucleosomes from somatic chromatin of calf thymus. Although the histones were analyzed and found to be identical to somatic type histones, the linker DNA length is longer than that of any somatic cell, with the exception of for chicken

erythrocyte nuclei. These nuclei have the additional lysine rich histone H5, a member of the histone H1 family. Therefore, the increase in linker DNA length in some sperm of the "histones" model is thought to be particularly due to an increased basicity of histone H1 in the spermatic chromatin.

Ultrastructural studies of sperm containing histones show that similarities exist among species who maintain histones in their sperm. During differentiation, the diffuse chromatin of the early spermatid disappears, giving rise to coarse fibro-granular chromatin. This 30 nm fibro-granular structure typical of histone-containing chromatin has been described in *O. tau* and *C. auratus* (Casas et al., 1981; Fribourgh at al. 1970; and Muñoz-Guerra et al., 1982). The mature sperm nuclear morphology has been described as spherical, with a diameter of about 3.2µm (Fribourgh et al., 1970). The spermatozoon does not show the high degree of chromatin packaging achieved in other species which contain protamines in their spermatozoa (Casas et al., 1981).

Sperm of fish species belonging to the Sparidae family, such as *Sparus aurata*, have been described as having an increased proportion of histone H1 (Saperas et al., 1994); spermiogeneses of some members of this family have been studied. The black porgy *Acanthopagurus schlegeli*, a teloest fish pertaining to the order Sparidae, was described by Gwo and Gwo (1993). The round spermatids, of 3.6+/-0.4µm in size, are reduced to 1.9+/-.01µm. The mature sperm diameter is considerably reduced in this model when compared to *C. auratus* sperm which does not contain the additional histone H1 proteins. The chromatin transforms from a mottled, blotched and uneven texture, to homogenous granules in intermediate stages, ending in homogenous tightly packed granules in the spermatozoon.

I.7.2. Histone → Protamine / [H → P] (simple type)

In spermiogenesis of this model, histones are directly replaced by a highly basic, usually very small protamine. Species which conform to this model include amphibians (Kasinsky 1989), some fish (Saperas et al., 1994), and birds (Oliva and Dixon 1991). In the H→P model, the protamine molecule directly substitutes somatic-type histones in the spermiogenic chromatin, causing it to condense and coalesce with the increasing presence of protamine, and disappearance of histones (Saperas et al., 1993).

The model of sperm which directly exchanges histones for the more highly basic protamine molecule has been described in several species. A species responding to this model is the bony fish *Dicentrarchus labrax* (Saperas et al., 1993), whose sperm contains a true protamine, which means it is small (containing only 34 amino acid residues) and

contains arginine clusters (four which contain 4-5 residues and one with 2 residues). The first ultrastructural studies of spermiogenesis of this species (Saperas et al., 1993) show that there is a progression from 25+/-5 nm fibro-granular structures in spermatids which progresses to the formation of coarse granules. The coarse granules evolve from the periphery in areas where the fibro-granular structure becomes depressed and histones are displaced by protamine molecules. In ripe sperm the irregularly shaped coarse granules of 150 +/-50 nm coalesce.

In vitro studies have shown that small protamines such as salmine are only functional in nucleasome disassembly at much higher concentration than is necessary for large protamines, such as galline (Oliva and Mezquita 1986). Additional *in vitro* studies of nulceosome disassembly performed by Oliva et al. (1987) suggest that the size of protamines must be a main factor responsible for differences in the efficiency of nucleosomal disassembly between small fish protamines and large protamines.

I.7.3. Histone → Protamine / [H→P] (special type)

Several species belong to this protein transition model, where histones are displaced by a protamine which is slightly more complex than the previously described type. The protamine molecule of this model is more complex since it is considerably larger in size in some species, or in other species, rich in cysteine residues forming inter- or intra-molecular disulfide bonds in other species. This has become clear since work has also been done on several types of molluskan spermiogeneses and SNBPs (Subirana et al., 1973; Zalensky and Zaleskaya 1980; Cáceres et al., 2000, Giménez-Bonafé et al., 2002 a, b). A pair of related mollusks are ideal specimen of this model, due to their unique protamines which have somewhat deviated from the true protamine paradigm, and their sperm compositions and morphologies will be described here.

Monodonta turbinata is a gastropod mollusk whose sperm and sperm chromatin composition has been extensively described by Daban et al. (1991). Sperm of this species undergo a direct substitution of histones by a single protamine during spermiogenesis, just as in *D. labrax.* In this model, the protamine comprises approximately 95% of the total protein content of mature sperm, along with residual amounts of histone, particularly those migrating similarly to histone H2B. Advanced stages of spermiogenesis involving chromatin condensation have been observed, and the protamine responsible for such chromatin condensation has been characterized (Daban, et al., 1995; Cáceres et al., 2000). The protamine is of this species is the largest known, containing 106 amino acids. Its primary structure shows analogies with some vertebrate protamines, mostly due to the

amino-terminal (RS)n motif and the presence of arginine clusters separated by a few non-basic residues (Daban et al., 1995; Cáceres et al., 2000). From a chemical perspective, this protamine does indeed complete the "true protamine" characteristics; however, being over three times larger than a true protamine, the extreme size of this protein makes the mechanism of histone replacement in this species interesting; it is possible that the large protamine carries out a more efficient nucleosome disassembly for histone replacement.

A final example in the H→P (special) category is *Eledona cirrhosa*, a cephalopod whose spermiogenic chromatin condensation has been related to the unique biochemical nature of the protamine which directly substitutes histones during spermiogenesis (Giménez-Bonafé et al., 2002a,b). The primary structure of this special protamine molecule contains 22.6% cysteine residues, being the most cysteine rich to ever be described. The high level of cysteine residues allows formation of disulfide bonds between SH groups of cysteines. This ultrastructural and biochemical study determined that the protamine enters nuclei in its reactive state, with free SH groups, later forming inter-disulfide bonds in a progressive way during spermiogenic chromatin condensation. In early stages, DNA and protamine are in contact, but with a low degree of chromatin condensation. As the free sulfide groups decrease, inter-protamine disulfide bonds are formed, correlating with the formation of chromatin fibers and their subsequent coalescence. These inter-protamine disulphide bridges were determined to be formed within the fibers, while formation of larger fibers and lamelle is driven by interactions of the sulfide groups between protamines among distinct chromatin fibers. In final stages of chromatin condensation, some free sulfide groups remained, seeming to function in the chromatin plasticity necessary for the unique helical shaping of the nucleus, which is molded by a scaffold of microtubules (Maxwell 1974).

I.74. Histone→Precursor protamine→Protamine / [H→Pp→P]

The replacement of histones by protamines is not direct in all species, and in the most complex cases, such as mammals, occurs in several steps in which histone are replaced by testes specific variants, and then displaced by several transition proteins and/or protamine precursors before their final substitution by protamines (Grimes et al., 1977; Meistrich et al., 2003). Models of this degree of complexity will be discussed in section I.7.4.

The simplest model which demonstrates one increased step in the chemical complexity of protein transitions occurring in spermatic chromatin during spermiogenesis is that of *Sepia officinalis*. In this model the addition of one intermediate protein displaces

histones prior to the final chromatin compaction achieved by the protamine. The precursor molecule undergoes a post-translational cleavage during spermiogenesis (Wouters-Tyrou et al., 1998) leaving the mature protamine to perform the final chromatin compaction. This protein substitution achieves equal chromatin compaction as does a direct substitution of histones by protamine; however, this increase in protein transition complexity allows the formation of chromatin patterns and structures, while permitting changes in nuclear shape which are not observed in models which undergo direct protein substitutions.

Preliminary studies focusing on spermiogenesis of related species were performed by Maxwell, in which he focused on *Eusepia officinalis*, *Loligo forbesi*, and *Alloteuthis subulata* (1975). Later, in a work by Hou and Maxwell (1992), nucleomorphogenesis is described in a cuttlefish species, *Rossia macrosoma*, in which different chromatin and nucleomorphogenic stages of maturation of the spermatid are described. The first biochemical work dedicated to the description of the nuclear protein transitions occurring in cuttlefish spermiogenesis was performed by Rousseaux-Prévost in 1988. In this work, it was established that two nuclear basic protein transitions occur during spermiogenesis of *Sepia officinalis*. However, left to be elucidated were the nuclear morphology and chromatin characteristics identifying each chromatin transition. Additionally, the mechanisms driving the double protein transition were left unclear. This group continued to study *Sepia* proteins, later characterizing two variants of the putative precursor protamine, followed by the characterization of the protamines of this species. Due to their work, a precursor-protamine relation was established between the transition protein replacing histones and the mature protein organizing ripe sperm chromatin; indeed, a deletion of the 21 amino terminal amino acid residues of the precursor molecule converts it into its mature form. Microheterogeneity of the precursor and protamine molecules was discovered (Wouters-Tyrou et al., 1991), as differences in the amino acid sequence of the precursor and mature protamine domains were found, identifying two different forms of the proteins. With a general idea of the morphological changes occuring during spermiogenesis of the cuttlefish, as well as the chromatin patterns formed during sperm maturation, several aspects of spermiogenesis of this species were left unresolved. For example, the relationship between the changes in nuclear shape and chromatin patterns formed during spermiogenesis in *Sepia officinalis* had not established. Also, the biochemical nature of the chromatin related to its condensation pattern at the intermediate

stages of spermiogenesis had not been elucidated, nor had the role of post-translational modifications in this model of spermiogenic nuclear protein transitions.

I.7.5. Model H/ various specific H → Various TP, Pp → Various P

Many examples exist of higher complexity in protein transitions, creating a model in which, in the most elaborate cases, several transition proteins replace histones, and are present in intermediate spermiogenic chromatin stages together with histone variants and precursor forms of several protamines, prior to the final mature sperm chromatin becoming organized by several different protamines, often co-existing with a low proportion of residual histones. In these models, the chromatin condensation pattern also tends to be more complex. For example, the patterning of chromatin condensation is markedly dynamic and elaborate in the model *Murex brandaris* (Amor and Durfort 1990; Caceres et al., 1999). The complex spermiogenic chromatin condensation of this cenogastropod mollusk is accompanied by a series of complex protein transitions involving sequential deletions of a protamine molecule, allowing elaborate lamelle and chromatin patterns to form during spermiogenesis.

Another interesting model of this category of complex protein transitions and condensing chromatin patterns is *Octopus vulgaris*. Mature sperm of this cephalopod is condensed by a set of five different proteins, four of which are protamines, and one which is a putative precursor molecule to one of the protamines (Giménez-Bonafé et al., 2004). In spermiogenesis of this species, chromatin condensation initiates in a pattern of 25 nm fibers, progressively increasing in size to become 40 nm, 60 nm, and 80 nm thick, finally leading to an evenly spaced and compact thread like appearance (Ribes et al., 2004). Though the precise motifs of these complex structural transitions are not clearly understood, they are believed to be correlated with the changes of nuclear proteins during spermiogenesis in *O. vulgaris*, which are much more complex than in many other related species. Each of the four protamines could likely be the product of a separate precursor molecule, and each could be affected by distinct levels of phosphorylation during spermiogenesis. It is likely that dephosphorylation occurs before complete precursor processing, since only a small amount of phosphyrylated proteins are present in mature sperm of this species (Giménez-Bonafé et al., 2004).

Mammals are the most complex model of spermiogenesis, in which up to four transition proteins participate in the repackaging of the spermatid genome in early stages of sperm maturation, coinciding with the removal of histones and the initiation of chromatin condensation (Alfonso et al., 1993; Oko et al., 1996; Meistrich 2003). The

most dramatic chromatin condensation subsequently occurs in late stage spermatids, when precursor forms of the protamine displace transition proteins, followed by one or several cleavages converting precursor molecules into mature protamines in the final stages of spermiogenesis. This causes coiling of the DNA into toroidal subunits of about 50kb of DNA (Balhorn et al., 1999) (Fig. I.5.6).

Several studies have proposed an evolutionary function of having an additional intermediate molecule interacting with spermiogeneic chromatin, since it is known that the maximum compaction state of chromatin is already achieved by the protamine molecule (Subirana et al., 1983), and therefore does not contribute an advantage related to compaction ability. It is proposed that this evolutionary advantage has to do with the way in which the chromatin reaches its end compaction state, involving intermediate structural stages which influence the final shape of the sperm cell itself (Caceres et al., 1994, 2000). It can be understood that a function of these complex and numerous intermediate proteins allows the development of more complex shapes adequate for fertilization of the egg of the same animal species (Fránzen 1970; Daban et al., 1991; Jamieson et al 1991). The involvement of one or several precursor protamines to first displace histones, followed by a post-translational cleavage to convert the molecule into the mature protamine has a morphological and evolutionary importance. Certain plasticity is allowed to condensing chromatin formations and placement within the nucleus, while a species-specific sperm shape is achieved. Through these mechansims, a step by step increase in basicity of the DNA-associated proteins plays an important role in evolution of spermiogenesis

I.7.6. The specialized case of crustacean sperm/ [H→H] (non-condensed chromatin)

This model of sperm DNA void of basic proteins was Bloch's "crustacean model". Published accounts of these sperm support the conclusion that they lack nuclear basic proteins associated to DNA (Chevaillier 1966, 1968; Vaughn 1968; Vaughn and Locy 1968; Langreth 1969; Vaughn and Thomson 1972; Vaughn and Hinsch, 1970, 1972). These conclusions were generally drawn based upon the absence of staining for histones and other basic proteins when cytochemical protocols such as the fast green method of Alfert and Geschwind (1953) and picric acid-bromophenol blue (Bloch and Hew 1960) were applied. Vaugn and Hinsch (1972) drew the same conclusions after isolating sperm chromatin and comparing the melting profile of isolated and deproteinized chromatin.

The typical aspect of these sperm containing naked DNA is that they are aflagellated and amotile cells, consisting of an uncondensed nucleus, containing diffuse

chromatin fibers, forming radial arms and surrounding the large and complex central acrosome (Felgenhauer and Abele 1991). The fine structure of this type of sperm is known in various crab species (Yasuzumi 1960; Langreth 1969; Pochon-Masson 1965, 1968; Jamieson 1989a, b, 1990; Medina and Rodríguez 1992). Part of the genetic material is located in the radial arms or processes, the number of which is variable among species, and in some cases even within an individual specimen (Hinsch 1986).

Additionally, brachyuran sperm have a prominent globular acrosome with an internal cylindrical core, which is surrounded by a cup-shaped nucleus extending into the radial projections. This has been described in several species, such as *Eriocheir* (Yasuzumi 1960), *Libinia* (Hinsch 1969), several species of *Oxyhyncha* (Hinsch 1973), *Ovalipes* (Hinsch 1976), *Carcinus* (Chevaillier 1967). The apical portion of the acrosome typically consists of a concave disc, which has an increased electron density than the rest of the acrosome, and may have a function in the initial phases of fertilization in the sperm-egg interaction (Brown 1966; Hinsch 1971; Chiba et al, 1992).

A perforatorium, or acrosomal tubule, creates a hollow space within the center of the acrosome, through which it is believed that the nucleus everts during the acrosome reaction (Hinsch 1986). Microtubules (Hinsch 1986) and actin (Rorandelli et al., 2007) have been described within the acrosomal tubule of several brachyuran crab species (Perez et al., 1986; Hernandez et al., 1989; Tudge and Justine 1994; Tudge et al., 1994), probably functioning in the proper contractile movements needed for transfer of the male genetic material during the acrosome reaction and subsequent fertilization events (Tudge et al., 1994).

The nucleus, contains decondensed chromatin suspended within nucleoplasm, surrounded by a thick cellular membrane. Microtubules present within the nucleus located in the radial projections are considered to provide mechanical support or maintenance of the shape of the arms (Hinsch 1969, 1988). Sperm cells of brachyuran crabs are organized into spermatophores, which surround and protect varying sized populations of sperm cells (Hinsch 1988). These groups of sperm cells are formed by a substance which initially separates the sperm in to discrete clumps prior to the application of the acellular spermatophore wall that eventually surrounds the clumps (Hinsch and Walker 1974). Some view the brachyuran spermatophore as an important structure whose function is to protect the sperm cells during transfer and storage by the female (Krol et al., 1992; Cuartas and Sousa 2007). However, some spermatophores are ruptured during sperm transfer (Sainte Marie and Sainte-Marie 1999; Rorandelli et al., 2008). A proposed

function of the mixed population of free sperm and spermatophores which are transferred to the female during copulation is then thought to either facilitate or delay mobilization of sperm cells so as to favour either immediate use (those sperm free of spermatophore capsule) or long term storage (those stored within the spermatophore protective capsule), and therefore reduce sperm wastage (Sainte-Marie and Sainte-Marie 1999).

I.7.6a Crustacean fertilization and reproductive anatomy

Knowledge of the events leading to gamete contact is helpful in understanding the adaptations that the crustacean sperm cell has acquired. Following transfer of sperm and spermatophores during copulation, female brachyurans store these cells for often lengthy amounts of time in special storage organs called the spermatheca, which have been subdivided into two morphological types: dorsal and ventral (Jensen et al., 1996). Species in the Majidae family pertain to those exhibiting the ventral type of spermathecae. In these animals, the last male to inseminate a female would have the advantage because the sperm are placed nearest the oviduct, physically blocking access of rival sperm to the emerging oocytes. Seminal receptacles in Majids are more developed than in other brachyurans, because they possess greater elasticity and accommodate a larger number of ejaculates; such is the case in the Majid *Inachus phalangium*, whose seminal receptacles can represent more than 10% of female body weight (Deisel 1989). In this species, the male has short setae on the fist gonopod, which burst large the spermatophores during the transfer of sperm from male to female. Only small spermatophores are passed on to the female, along with sperm released from the rupture of large spermatophores. This creates two previously mentioned populations of sperm cells which are passed on to the female: Sperm free of the spermatophores, and sperm which remain within small sized spermatophores. Small males may rely on the transfer of free sperm cells produced by the rupture of large spermatophores to acquire quick fertilization (Rorandalli et al., 2008) since these cells are more readily used for fertilization compared to stored sperm remaining in spermatophores. A disadvantage is that these sperm will not likely survive long in the spermathecae environment, and unless they reach an egg to fertilize fairly soon after copulation, may not be viable long outside the protective spermatophore. Indeed, in *Cancer magister*, sperm found outside of the spermatophore capsule which had been stored for considerable lengths of time were described as misshapen, swelling, shrivelled, or fragmented (Jensen et al., 1996), suggesting that sperm left unprotected by the spermatophore are prone to damage. Interestingly, the spermatophore wall seems to serve a similar protective function of for the crustacean sperm as protamines do for DNA,

packing nuclei of sperm cells to limit access of enzymes, bacteria, and harmful chemical agents to the genetic material.

Candridae females have dorsal-type spermathecae, in which the dorsally opening oviduct and ventrally opening vagina are well separated, and the first male to inseminate the female is thought to have an advantage, because the oocytes encounter this sperm first (Diesel 1991). Within the spermatheca, the dorsal epithelium may serve a protective role, prolonging sperm viability. It has been reported to have a secretory function; propositions include sperm plug dissolution (Spalding 1942), sperm plug formation (Bawab and El-Sherief 1989), dissolution of spermatophore coating to free encapsulated sperm (Ryan 1967; Adiyodi and Anilkumar 1988; Diesel 1989), maintenance of sperm (Johnson 1980), and maintenance of a bacterial population within the spermathecae (Elner and Beninger 1992, Beninger et al., 1993). In *C. magister*, melanin has been described, thought to be secreted by the dorsal epithelium (Jensen et al., 1996), which could function in the spermathecae in two important ways. In many groups, including crustaceans, melanin is involved in immune responses against the presence of foreign substances and diseased or dead host tissue (Salt 1970; Wolke et al., 1985; Soderhall and Smith 1986), due to its bacteriocidic properties. The large amount of melanin present in the spermathecae may be responsible for excluding bacteria and other harmful agents from the spermathecae. On the other hand, within spermatheca of *Chionoecetes opilio* (Majidae), the epithelial secretions are proposed to provide a favourable culture medium for a morphologically homogeneous population of bacteria which, through their metabolic products, may either exclude opportunistic microbes from the spermathecae, or provide substrates for the sperm to metabolize (Elner and Beninger 1992; Beninger et al., 1993). In contrast to *C. opilio*, no bacteria were found in the spermathecae of *C. magister*; the presence of melanin in this case may be to eliminate bacteria from the spermathecae, and in addition, help protect the sperm cells inside by absorbing free radicals produced during metabolism. This type of function for melanin has been reported in cryophilic fish, in which it absorbs free radicals formed during lipid oxidation (Wolke et al., 1985). Sperm in other species, such as the Portunidae *Scylla serrata*, maintain a low level of anaerobic metabolism (Jeyalectumie and Subramoniam, 1991); in brachyuran spermathecae, melanin may promote sperm storage by absorbing free radicals during their metabolism.

I.7.6b. Previous studies on crustacean sperm

An observation was made by Bloch in 1966, which led to his conclusion that formalin fixation may not always be depended upon to completely immobilize protein.

This is an important finding, considering the conclusion that these sperm lack basic proteins was made partially based upon the absence of cytochemical basic protein staining of formalin-fixed cell preparations in several reports (Chavaillier 1966; Langreth 1969). Additionally, it was observed by Reger et al. (1984) that *Carcinas* spermatozoa, following rapid freeze fixation, showed more compact structure compared to chemically fixed samples, suggesting the superiority of cryofixation as opposed to chemical fixation.

These data lead to valid skepticism of previous reports of crustacean sperm deplete of DNA-associated proteins. The possibility remains that the observed absence of nuclear basic proteins associated to the spermatic chromatin of several crustaceans could have been caused by artifactual evidence due to the fixation methods used prior to staining of basic proteins. Further, chromatin of crab sperm was observed to be highly sensitive by Chevaillier (1968), showing that the chromatin fibers clump unless stabilized by heavy metal ions. Due to the possibility that early studies may have erroneously concluded that the sperm nucleus of some crustacean species do not contain any basic proteins associated to the DNA, and considering the sensitive and fragile nucleus of this model, very little is actually known or understood of the biochemical nature of the crustacean sperm nucleus.

Brachyuran crabs are ideal specimen for study of the crustacean sperm model, since they tend to be widely commercially available, and several ultrastructural and biochemical sperm studies have been reported previously for brachyuran crustaceans. Species pertaining to the family Cancridae have been described as simpler brachyuran crabs than those of the Majidae family (Skinner 1967). Langreth (1969) described the spermiogeneic chromatin of *Cancer*, including histochemical descriptions of the protein component of the chromatin. Tudge and colleagues (1994) also described the ultrastructure of the mature sperm cell of *Cancer pagurus*, and described actin in the acrosomal granule, proposed to function in the acrosome reaction. Species from the family Majidae have been quite extensively studied as well, including ultrastructural reports on six majid species by Jamieson et al. (1998), as well as a description of the cytoskeletal and contractile proteins present in sperm of the spider crab *Libinia emarginata* by Perez et al., (1986) and in *Maja squinado* by Tudge and Justine (1994). Other reports include a biochemical description of the mature sperm chromatin of the *L. emarginata* (Vaughn and Hinsch 1972) and ultrastructural descriptions of its mature sperm cell (Hinsch 1969, 1986).

OBJECTIVES

II. OBJECTIVES

In general, sperm studies have focused on the biochemistry of the cell, or morphology of the cell, but few integrate the two. For this work we focused on different sperm models, and characterized the chromatin structurally and biochemically.

Specifically, the experimental design for this study includes the following:

- Relate changes in chromatin structure, changes in protein composition, and post-translational modifications in three models of spermiogenesis using four species:
  - H→H (non-histone proteins are eliminated)
  - H→P (either a protamine or SNBP substitutes histones)
  - H→Pp→P (added chemical complexity of a precursor intermediate)
- Identify common characteristics among these and other spermiogenesis and integrate an evolutionary purpose for maintaining such characteristics in all spermiogenesis throughout evolution
- Try to resolve the longstanding question of how crustacean sperm chromatin is organized by studying the composition and structure of sperm from two families of brachyuran crustacean species

RESULTS

III. RESULTS

CHAPTER 1. SIMPLE MODELS OF SPERMIOGENIC NUCLEAR PROTEIN TRANSITIONS AND CHROMATIN COMPSITION

Spermiogenesis and sperm nuclear basic proteins are quite diverse among species. In order to better understand this important aspect of sperm biology, we analyzed the relationship between spermiogenic chromatin structure and molecular composition. Two simple models of spermiogenic protein transitions were studied in the work entitled "Spermiogenic protein transitions and chromatin condensation. Proposal for an ancestral model of nuclear spermiogenesis" which has been accepted for publication in the *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, in which my participation was complete. The first model is "H→H", in which histones are not replaced during spermiogenesis, and mature sperm chromatin maintains histones and nucleosomal organization (*Sparus aurata*, Fig. 1). The second model, H→P, is slightly more complex. In sperm of this model a protamine molecule directly displaces histones during spermiogenesis (*Dicentrarchus labrax*, Fig. 2, and *Monodonta turbinata*, Fig. 3). The H→P model can be molecularly quite variable, which is why two examples were included for study and analysis.

In this work we show that in all the three species studied, an initial remodeling occurs in the spermiogenic chromatin. This chromatin remodeling includes the transition from a somatic-like nature into fibro-granular structures of 20-25 nm in diameter, uniformly distributed throughout the early/intermediate spermatid nucleus. Additionally, in all cases studied in this work this type of chromatin contains acetylated histones H3 and H4.

From this point, the spermiogenic chromatin condensation pattern diverges in function of the model studied. In the case of *S. aurata* (model H→H) the histones undergo deacetylation and the 20 nm fibro-granular structures join together in partial coalescence to produce a more compact chromatin arrangement in advancing spermatids and mature sperm. In the cases of *D. labrax* and *M. turbinata* (model H→P), acetylated histones are displaced by a protamine (containing 34 amino acids or 106 amino acids, respectively). In both cases, the substitution of histones by the protamine produces a chromatin remodeling, where the 20 nm fibro-granules become large, coarse granules, which are much more irregular in shape and whose size (approximately 80 nm in *D. labrax* and 60 nm in *M. turbinata*) partly depends on the type of protamine interacting

with the spermiogenic chromatin. Finally, these granules undergo coalescence in both species, in order to form the tightly condensed chromatin of the mature sperm nucleus.

In the discussion we analyze the possible evolutionary significance of the 20 nm granular chromatin, and the fact that histones H3 and H4 are found acetylated in this type of chromatin. Subsequent deacetylation of these histones appears to function in final chromatin compaction in the H→H model, likely allowing closer approximation of nucleosomes, and hence, tighter packing of chromatin granules.

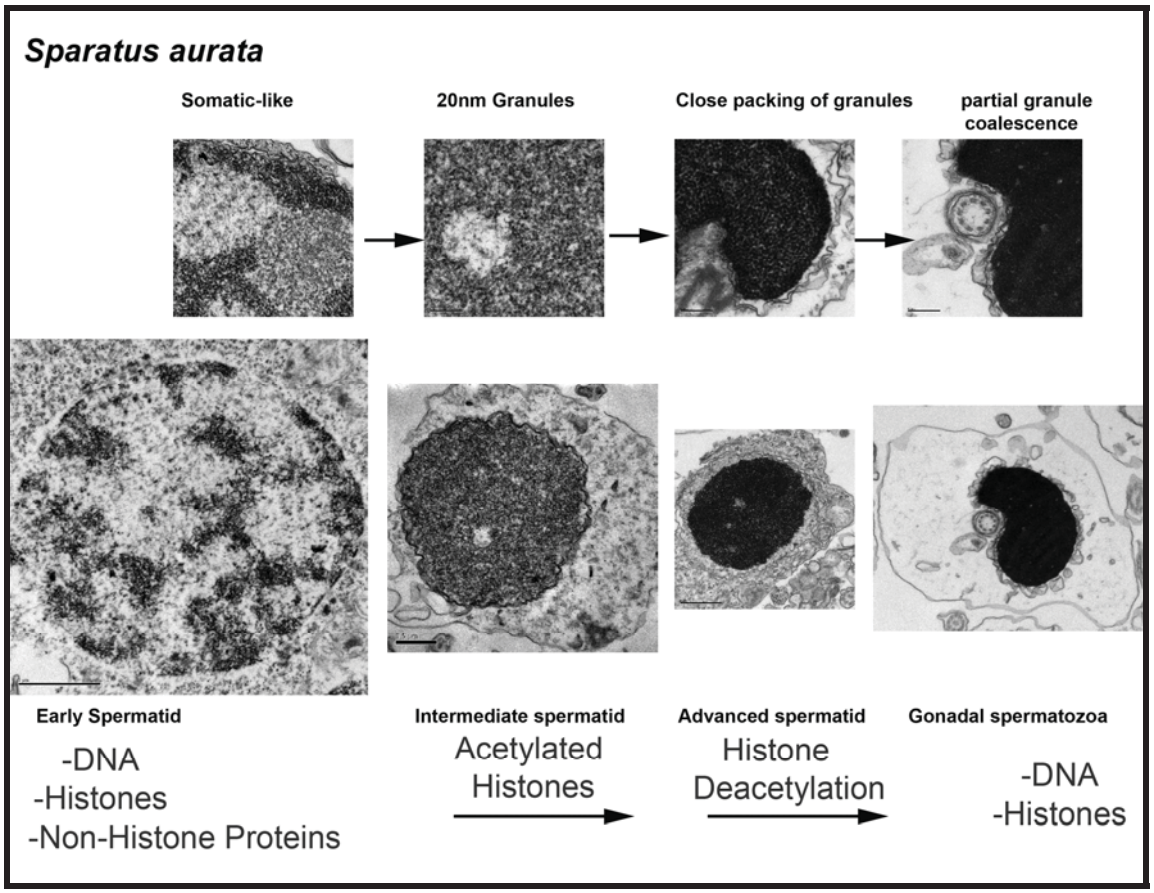

Fig. III.1.1. Spermiogenic chromatin composition and compaction of the H→H model. Beginning from somatic-like organization, the spermiogenic chromatin develops during spermiogenesis into evenly spaced granular structures of 20 nm, simultaneously with a low level of monoacetylation and loss of non-histone proteins. As deacetylation occurs, the granules package closer together and partially coalesce in mature gonadal spermatozoa. The reduction of nuclear volume can be appreciated in the lower images, since all are equally amplified. This volume reduction is reflected in the lower rightmost image of the mature gonadal spermatozoa, where a large space exists between the cellular membrane and the nucleus. An increased amount of histone H1 is likely interacting to achieve this partial coalescence. Bar in above images (all equally amplified): 0.2 μm; Bar early spermatid below: 1 μm; Bar all other images below: 0.5 μm.

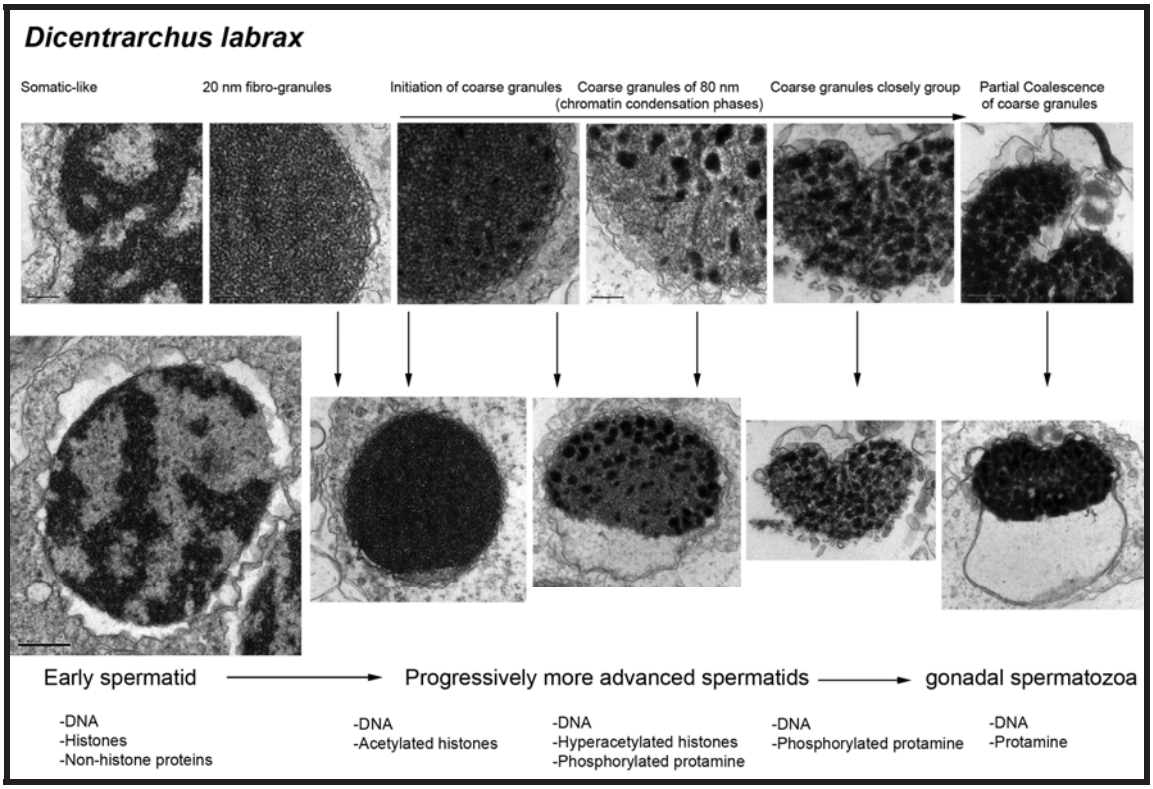

Figure III.1.2. Spermiogenic chromatin composition and condensation of the H→P model (true protamine): From a somatic-like aspect, the chromatin becomes organized first into evenly spaced fibro-granules of 20 nm, and subsequently into coarse granules when the protamine appears to interact with the DNA. These coarse granules progressively group closer together in partial coalescence until the chromatin is fully compact. All top images are equally amplified; bars = 0.2 µm. All bottom images are equally amplified; bar = 0.5 µm.

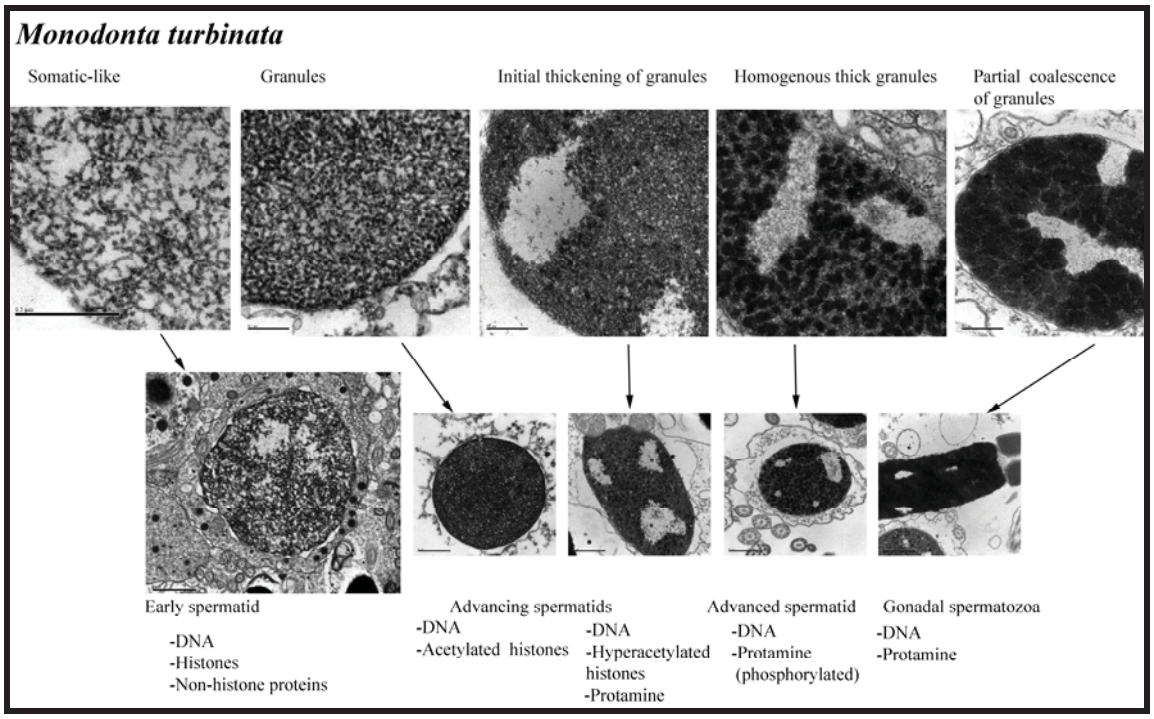

Fig. III.1.3. Spermiogenic chromatin condensation and composition of the H→P model (large protamine). From a somatic-like aspect, where areas of euchromatin and heterochromatin co-exist, the chromatin becomes evenly organized into coarse 20 nm granules comprised of moderately acetylated histones (about 4-5 nucleosomes per granule). When the protamine appears to interact with the DNA, these granules thicken into coarse granules; eventually the protamine achieves full displacement of hyperacetylated histones. At this point, the chromatin is fully composed of 60 nm coarse granules. These granules approximate and gather closely in a partial coalescence, and the chromatin is fully compact, as observed in the gonadal spermatozoa. All above images are equally amplified, and all below images equally amplified. Bars: Above.- Leftmost: 0.5 µm; all others: 0.2 µm. Lower.-Leftmost: 1 µm; all others: 0.5 µm.

Spermiogenic nuclear protein transitions and chromatin condensation. Proposal for an ancestral model of nuclear spermiogenesis

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Spermiogenic nuclear protein transitions and chromatin condensation. Proposal for an ancestral model of nuclear spermiogenesis

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Abbreviations: AU-PAGE: acetic acid/ urea polyacrylamide gel electrophoresis; AUT-PAGE: acetic acid/ urea/ triton X-100 polyacrylamide gel electrophoresis; ECL: electrochemiluminescence; EDTA: ethylenediaminetetraacetic acid; HPLC: high-performance liquid chromatography; HRP: horseradish peroxidase; MNase: micrococal nulcease; PBS: phosphate buffer saline; PCA: perchloric acid; PVDF: polyvinylidene fluoride; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SNBP: sperm nuclear basic protein; Tris: hydroxymethyl aminomethane

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Abstract

We have chosen (*Sparus aurata*, *Dicentrarchus labrax*, and *Monodonta turbinata*) that represent different transition patterns in the composition and structure of spermiogenic nuclei. The transition patterns of these species are representative of spermiogenesis in a large number of animal species. We analyze: a) nuclear protein exchange; b) chromatin condensation pattern; and c) histone acetylation during spermiogenic development. In the simplest spermiogenesis histones and nucleosomes remain in mature sperm. Chromatin of spermatids is organized into 20 nm granules, simultaneous with a nuclear volume reduction. The granules coalesce in the final stage of spermiogenesis. Granular chromatin is correlated with acetylation of histones H3 and H4, while final coalescence is associated with histone deacetylation. We also studied two other spermiogenesis where a basic protein substitutes histones. Each species has a very different substituting protein. One has a typical protamine of 34 amino acids; the other has an SNBP of 106 amino acids. In both, the structural transitions and histone acetylation pattern are similar: in early spermiogenesis chromatin is organized into 20 nm granules, and histones are significantly acetylated, while the nuclear volume decreases. Subsequently, acetylated histones are displaced by the protamine or SNBP. Histone substitution causes chromatin remodeling and additional reduction in nuclear volume. We analyze these three cases together with earlier works and propose that the formation of 20 nm granules containing acetylated H3 and H4 accomplishes the minimum functional requirement to be considered the most evolutionarily ancestral chromatin conformation preceding condensation in animal spermiogenesis.

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Introduction

The composition and structure of mature sperm nuclei is very different than that of somatic cells. Mature sperm nuclei tend to contain basic proteins tightly bound to DNA, and highly condensed chromatin which causes a decreased nuclear volume. These characteristics are acquired during the process of spermiogenesis, during which very important changes occur, not only in sperm chromatin composition and structure, but also in the regulation of genomic activity (Kimmins and Sassone-Corsi, 2005).

The proteins which organize DNA in sperm nuclei (Sperm Nuclear Basic Proteins, or SNBPs) have changed a great deal during evolution, and present an extraordinary interspecific variability (see revision by Kasinsky, '89; Lewis et al., 2003), which will briefly be explained here.

On one hand, mature sperm nuclei may contain histones which are identical to (Muñoz-Guerra et al., '82; Ausió et al., '97), or similar to (if they are more basic) (Vodicka et al., '90) somatic histones. In other examples, the nuclei contain an SNBP which has displaced histones during spermiogenesis. This is the case of typical protamines (reviewed in Oliva and Dixon '91; Saperas et al., '94) and other SNBPs (Daban et al., '90, '91).

There also exist species in which histones of the spermiogenic nuclei are displaced by a precursor protamine, which is later converted into a mature SNBP or protamine (Rousseau-Prévost et al., '88; Martin-Ponthieu et al., '91; Wouters-Tyrou et al., '91). In some cases, the transition of proteins is more complex, in such a way that histones are simultaneously replaced by precursors of various SNBPs, which are processed by a series of multiple deletions (Cáceres et al., '99). In the nucleus of these sperm, various types (or forms) of SNBPs are bound to DNA (Cáceres et al., '99; Giménez-Bonafé et al., 2004).

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The most complex case known occurs in spermiogenesis of mammals (Meistrich, '89; Courtens et al., '95; Meistrich et al., 2003; Zhao et al., 2004). Here, histones of the early spermatid are replaced by testes-specific histones, which are subsequently replaced by transition proteins (TP). The TP proteins are finally substituted by protamines, or by their precursors. The protamines of mammals are basic molecules rich in cysteine, which constitute the main protein component of the sperm nucleus.

The evolutionary changes in composition (and structure) of sperm nuclei represent a puzzle which is difficult to solve. These changes generally have been produced in a divergent way, but can also be found in many examples of evolutionary convergence (see for example the great similarity in the formation and structure of the SNBPs of bivalve mollusks (Ausió and Subirana, '82; Ausió, '92) and of urochordates (Lewis et al., 2004)). Also, many other cases exist in which the spermatic chromatin composition has reverted to a more ancestral state (Saperas et al., '94; Kasinsky et al., '99).

The immediate consequence of this evolutionary variation is that there is no clear correspondence between the taxonomy of animal species and the type of protein (and structure) of its mature sperm nucleus (Kasinsky, '89). In other words, the different types of SNBPs partially show a sporadic distribution throughout the different animal taxa (see the classic classifications of Bloch, '69, '76 and Subirana '75, '83).

For a long time now we defend the point of view that it is necessary to compare spermiogenic processes to be able to understand the diversification of sperm nuclei and their protein components. In the present work we have studied the relation among changes in nuclear proteins, histone acetylaton, and chromatin structure during spermiogenesis in three animal

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species. Two of these (*Sparus aurata* and *Dicentrarchus labrax*) are bony fish, and the other (*Monodonta turbinata*) is an archeogasteropod mollusk.

The species included in this work have not been chosen because of their relation or taxonomic position. They were chosen for two main reasons. First, their types of nuclear protein transitions (from the earliest spermatid to the mature sperm cell) represent a great number of species distrubuted throughout various taxa. Second, they represent the two simplest types of protein transitions which have been found in spermiogenesis. The case of *S. aurata* is representative of many species in which the sperm nucleus contains histones similar to somatic ones (Casas et al., '81; Muñoz-Guerra et al., '82; Saperas et al., '94; Ausió et al., '97). We call this type of transition [H]. Concerning *D. labrax* and *M. turbinata*, they represent the innumerable species in which histones of the early spermatid are directly substituted by an even more basic protein (SNBP, protamine) (reviewed in Oliva and Dixon, '91; Daban et al., '90, '91, '95). In the discussion, the examples included in this work are compared with others which have been previously published by other authors and by ourselves, and we propose a simplified model that possibly represents the most primitive type of nuclear differentiation in animal spermiogenesis.

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Material and Methods

Animals.- Male individuals of the bony fish *Sparus aurata* (gilthead seabream, Family Sparidae), *Dicentrarchus labrax* (European sea bass, Family Percychthydae), and *Trachinus draco* (greater weever, Family Trachinidae) were obtained fresh from the Mediterranean Sea in Barcelona (Spain). Male *Monodonta turbinata* snails (gastropod mollusc) were caught off the northern Mediterranean coast of Catalonia (northeast Spain).

Antibodies.- In this work the following antibodies have been used: anti acetyl lysine (Abcam, cat. # ab 21623), anti H4 specifically acetylated in lysine 12 (anti H4-acK12) (Upstate, catalogue # 07-329) whose antigen is the peptide GKGGA[acK]RHRKC, anti H3 specifically acetylated on lysines 9 and/or18 (anti H3-acK9/18) (Upstate, catalogue # 07-593) whose antigen is the peptide AR[acK]STGGKAPR[acK]QL, and anti histone H4 antiserum. All antibodies were submitted to the same controls mentioned in Kurtz et al. (2007). Briefly, all antibody reactions were compared with *Trachinus draco*, a bony fish which accumulates hyperacetylation of H3 and H4 during spermiogenesis (unpublished results).

Nuclear purification.- Nuclei were obtained as in Saperas et al. (2006) with some modifications. Briefly, male gonads of *S. aurata*, *D. labrax*, and *M. turbinata* were collected during different months of the year, representing various levels of maturity. Mature sperm from *S. aurata* and *D. labrax* was obtained by abdominal massage. Liver from *S. aurata* was used as a control of somatic cells for comparison to germ cells of this species. Tissues were homogenized in ice cold buffer A containing 0.25 M sucrose, 10 mM Tris pH 7.4, 3 mM $MgCl_2$, 5 mM $CaCl_2$, 0.1 mM spermine, 0.25 mM spermidine, with 25 mM benzamidine chloride as an inhibitor of proteolysis. The homogenate was filtered with 4 layers of gauze and cells were collected by centrifugation, washed again in buffer A containing 0.25% Triton X-100 and then washed twice more. The final pellet (purified nuclei) was washed once more in buffer A and used for protein extraction; sperm nuclei from *S. aurata* were also used for micrococcal nuclease digestions.

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Extraction and precipitation of nuclear proteins.- Purified nuclei were washed with ice cold 10 mM Tris pH 7.4 and the basic proteins were extracted with 5 volumes of 0.4 N HCl, cleared by centrifugation, and precipitated with 6 volumes of ice cold acetone, followed by washes with acetone (Saperas et al., 2006).

HPLC chromatography.- Reverse-Phase HPLC was used to fractionate nuclear basic proteins from sperm and liver of *S. aurata* (used as a control of somatic proteins) according to the method described in Ausió ('88).

Amino acid analysis.- Amino acid analyses were carried out after hydrolysis in 6 N HCl at 110ºC for 24 hours.

Electrophoretic analysis of proteins.- One-dimensional acetic acid/urea-PAGE (Saperas et al., 2006) or 15% SDS-PAGE were used to separate nuclear proteins. Two-dimensional gels (AUT/SDS) were performed as in Martínez-Soler et al. (2007a). Proteins were first separated using a variation of the method described in Frehlick et al. (2006), with acetic acid/6 M urea/6 mM triton X-100 (AUT-PAGE), and proteins were separated in the second dimension using 15% SDS gels.

Western blots.- Following one dimensional electrophoresis in AU-PAGE, proteins were transferred to a PVDF membrane and probed for the presence of acetylated histones, as previously described (Kurtz et al., 2007). The membranes were blocked in a solution of PBS with 5% powdered skim milk for 2 hours at room temperature with shaking. All antibodies were

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prepared in this blocking solution; anti acetyl lysine was diluted to 1:500 and anti H4-acK12 and anti H3-acK9/18 were diluted 1:2000, and incubated at 4ºC overnight with shaking. Antibody recognition of all antibodies was detected using an HRP conjugated goat anti rabbit secondary antibody diluted 1:3000 in PBS containing 5% powdered skim milk, and incubated for 1.5 hours at room temperature with agitation. HRP detection was performed using an ECL reagent (Amersham).

Micrococcal nuclease digestion and quantitation of released products.- Purified sperm nuclei of *S. aurata* were digested as described in Kurtz et al. (2008). Briefly, samples of sperm nuclei corresponding to 1 mg/ml of DNA were isolated using the absorbance at 260 nm and the extinction coefficient of A_{260} = 20 $cm^2 \cdot mg^{-1}$ DNA, and suspended in buffer containing 0.25M sucrose, 10 mM Tris pH 8.0, 0.5 mM $CaCl_2$, and 5 mM benzamidine hydrochloride. Chromatin was digested at 37ºC for various times with micrococcal nuclease (Sigma). The enzyme was added to a concentration of 0.6 U/mg DNA. Reactions were halted by the addition EDTA to a final concentration of 10 mM. After centrifugation (10,000 x g for 10 minutes), the supernatants (SI) were collected, and pellets washed and centrifuged again under the same conditions. The resulting pellet (P) was resuspended in 10 mM Tris pH 7.5 with 0.1 mM EDTA. Chromatin fragments from the supernatants (S) and pellets (P) were both DNA and basic protein extracted, as described in Saperas et al. (2006). DNA fragments were analyzed on 1.1% agarose slab gels, while the corresponding basic proteins were analyzed with AU-PAGE. Nucleosome lengths were calculated using the methodology described in Johnson et al. ('76) and used previously in our laboratories (Ribes et al., 2001; Saperas et al., 2006).

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Electron microscopy and immunomicroscopy.- Small sections of testes from *S. aurata*, *D. labrax*, and *M. turbinata* were fixed and embedded in Spur resin for conventional electron microscopy, or in Lowicryl resin for immunomicroscopy experiments (Giménez-Bonafé et al., 2002). For electron immunomicroscopy, the same conditions were applied as those used in Kurtz et al. (2007) to detect antibody labelling by anti acetyl lysine and anti H4-acK12.

Results

I.- Definition of types of nuclear protein transitions during spermiogenesis.

A.- Sparus aurata.- *S. aurata* is a bony fish of the Sparidae family (O. Perciformes). To analyze protein changes during spermiogenesis of this species, nuclei have been separated from immature testes and from mature sperm. In both cases, basic proteins were extracted with 0.4 N HCl and analyzed using two-dimensional electrophoresis (AUT/SDS). In Figure 1 (A,B) the elecrophoretic pattern of these proteins is shown, confirming that mature sperm nuclei contain the complete endowment of histones, and do not contain a significant amount of other non-histone proteins. In both electrophoresis, histone mobility is identical.

We partially studied the most relevant characteristics of sperm chromatin in this species. To begin with, we analyzed histone H1 from ripe sperm nuclei, since this histone is the most variable throughout evolution, and there are many species which contain specific sperm variants of H1 (Ausio, '99; Th'ng et al., 2005). Spermatic histone H1 is resolved by HPLC in two fractions, each with a slightly different electrophoretic mobility (Fig. 1C lanes a,b). The amino acid composition of each one is very similar to that of somatic histone H1 from liver of the same species (also composed of two isoforms), as well as H1 from calf thymus, and does not present any significantly different characteristics (Table 1). Secondly, sperm chromatin was digested

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with micrococal nuclease (MNase) (Fig. 1D), producing a typical pattern of chromatin organized into nucleosomes. Using digestion kinetics the nucleosome length was determined (by extrapolation to time zero), and is 214 base pairs of nucleosomal DNA.

Together these results indicate that during spermiogenesis of *S. aurata* chromatin condensation is carried out by a relatively simple yet global process which is based on the displacement of non-histone nuclear proteins from chromatin, while both histones and nucleosomal organization are conserved in sperm nuclei. In this work, we designate this transition [**H**] (histones). Additional species described in literature with this type of transition include, among others, *Opsanus tau* (Casas et al., '81), *Strongylocentrotus intermedius*, *Aphelasterias japonica* (Zalenskaya et al., '81) and *Carassius auratus* (Muñoz-Guerra et al., '82).

***B.- Dicentrarchus labrax*.-** This bony fish belongs to the Percychthyidae family (O. Perciformes). The nuclear protein transitions during spermiogenesis of this species are shown in a simplified manner in Figure 2A. Nuclei obtained from cells of very immature gonads mostly contain histones as their main nuclear basic protein (Fig. 2A lane a). In nuclei of maturing gonads (which contain more advanced spermiogenic cells) a protein with high electrophoretic mobility is observed (protamine, Fig. 2A lane b) which coexists with histones. In mature spermatic nuclei, the protamine has completely displaced nuclear histones (Fig. 2A lane c). The primary structure of the protamine from *D. labrax* has been previously determined in our laboratory (Saperas et al., '93) and is shown in Figure 2C. The amino acid sequence corresponds to that of a typical protamine and contains 34 amino acids, 21 of which (61.6%) are arginine residues, mainly grouped into four clusters. This type of protein transition in spermiogenic nuclei

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where histones are displaced by a typical protamine is designated here as [**H→P**] (histones to protamine). It is a transition model which has been studied in detail in the past (reviewed in Oliva and Dixon, '91). These studies have established that histone H4 undergoes hyperacetylation during intermediate stages of spermiogenesis. Such acetylation has been proposed to facilitate the proper displacement of nucleosomal histones by the phosphorylated protamine.

C.- ***Monodonta turbinata*****.-** *M. turbinata* is a mollusk belonging to the group Vetigastropods (formerly Archaeogastropods). The nuclear protein transition during spermiogenesis of this species (Fig. 2B) shows a similar general pattern to the transition observed in *D. labrax* and many other bony fishes. The histones of spermiogenic nuclei are progressively substituted by an SNBP of higher electrophoretic mobility (SNBP in Fig. 2B lane b). In the mature sperm nucleus, the main protein associated to DNA is the SNBP (Fig. 2B lane c), which coexists with a small amount of residual histones. The primary structure of this protein was resolved by Daban et al. ('95) and is shown in Figure 2D. This molecule is comprised of 106 amino acid residues, including 61 arginines, (57% of the composition), the majority of which are found in clusters. This SNBP also contains 6 lysine residues (arginine + lysine = 63%), and an N-terminus which has 7 alternating (arginine-serine) residues.

For the purpose of this work, it is important to keep in mind that there is no direct evolutionary relationship between the SNBP of *M. turbinata* and typical vertebrate protamines (such as that of *D. labrax*), since each has appeared independently during evolution (Chiva et al., '92; Saperas et al., '94; Lewis et al., 2004). Nevertheless, these proteins fulfill the very same function. They both participate in the direct and ordered displacement of histones in

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spermiogenic nuclei, and share a similar amino acid composition. For these reasons in the present work we consider that the spermiogenic protein transition of *M. turbinata* pertains to the category [**H→P**]. Other vetigastropods exhibit the same type of protein transition (Daban et al., '90).

II.- Chromatin condensation pattern during spermiogenesis.

Figures 3, 4, and 5 show the decrease in nuclear volume (row I), the chromatin condensation pattern (row II), and histone acetylation state (row III) during spermiogenesis of the three species studied here. In the current section, we will focus only on the changes in volume and chromatin condensation. Acetylation of histones in the various stages will be considered in the section following these results.

A.- *S. aurata*. (Fig. 3).**-** The structural chromatin transition in spermiogenesis of *S. aurata* is relatively simple; this corresponds to the slight change in nuclear protein composition described in the previous section. The earliest spermatid of *S. aurata* possesses a spherical nucleus. Here, the chromatin has not yet begun the condensation process, and has an organization similar to that of somatic cells, with heterochromatin clusters distributed throughout the periphery and in some inner areas of the nucleus (Fig. 3 IA). The initial condensation phase is shown in panels IB and IIB1-B2. Chromatin takes on a granular appearance (or fibro-granular) progressively concentrating toward the center of the nucleus (observe the space of low electrodensity formed between the condensing chromatin and the nuclear membrane in panel IB). The chromatin granules have an average diameter of 20 nm, and can contain 4-6 nucleosomes. In more advanced spermiogenic phases (IC, IIC) the granules pack closer together, leading to an

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important decrease in nuclear volume. The major reduction of sperm nuclear volume takes place in these phases. In the final-most stages of spermiogenesis, granules lose their individual identity, and appear to fuse together, in partial coalescence (IID). Mature sperm chromatin condenses around the centriole, having a relatively homogenous appearance. The volume of the nucleus is finally completely reduced and acquires the shape typical of Sparidae sperm nuclei (Gwo and Gwo, '93; Gwo et al., 2005). The nuclear volume of gonadal spermatozoa is approximately one eighth that of the early spermatid nucleus. It is important to keep in mind that many different factors other than chromatin condensation are involved in the acquisition of the final sperm nuclear shape, such as microtubules and spermatic nuclear matrix, among others (Risley et al., '82; Meistrich, '93; Giménez-Bonafé et al., 2002; Martínez-Soler et al., 2007b). Nevertheless, this work focuses only on those aspects involving chromatin condensation.

B.- ***D. labrax*****.** (Fig. 4). **-** The chromatin condensation pattern of *D. labrax* is more complex than that of *S. aurata*. In *D. labrax*, chromatin condensation is carried out in two distinguishable parts.

In the first part, early spermatid chromatin organizes into granules (fibrogranular pattern) as well, similar to those described for *S. aurata* (IA→B, IIB1-B2). The granules also have a diameter of 20 nm, identical in size to those observed in spermiogenesis of *S. aurata* and *Sepia officinals* (Kurtz et al., 2007; Martínez-Soler et al., 2007a, 2007b; see discussion). In the case of *D. labrax*, the homogenous granular distribution of chromatin in the earliest phases of spermiogenesis also leads to an important reduction in nuclear volume (IA→B).

In the second part of this process, one can observe that the 20 nm granules develop into increasingly larger and more electrodense coarse granules which grow in size up to approximately 80 nm (IC→F; IIC→E). The formation and growth of these granules is due to

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histone substitution by protamine, since anti histone antibodies label fine granular chromatin, but do not react with chromatin organized into 80 nm granules (not shown). The replacement of histones by protamine indicates that the 80 nm coarse granules are not generated by a simple fusion of 20 nm granules, but that the chromatin undergoes a structural remodeling. The formation of coarse granules elicits a further reduction in volume of the spermiogenic nucleus (IC→F). In these final phases of spermiogenesis, the 80 nm granules exhibit a coalescence trend, partially losing their identity and resulting in uniformly packaged chromatin (though not completely uniform; panel IF). The volume reduction of the spermatic nucleus with respect to the early spermatid nucleus is more pronounced than that observed in *S. aurata* and other species of the [**H**] model. This is in agreement with other works (Ward and Coffey, '91; Daban, 2000; Ausió et al., 2007), according to which DNA packaging by protamine (nucleoprotamine) is more compact than the packaging of the DNA-histone complex (nucleohistone).

C.- ***M. turbinata*****.** (Fig. 5).**-** In this case the spermiogenic histones are directly replaced by a protein much larger than the protamine of *D. labrax* (106 and 34 residues, respectively). In spite of this difference and analogous to *D. labrax*, the spermiogenic chromatin condensation pattern is established in two perfectly distinguishable stages. In the first stage, chromatin is newly organized into fine granules of 20 nm in diameter, resulting in the initial nuclear volume reduction (IB→C, IIB→C). From this stage on, the chromatin is remodeled and the 20 nm granules are progressively transformed into larger granules of approximately 60 nm in diameter (IID1, D2). During this structural transition of the chromatin, the nucleus undergoes another decrease in volume (IC→E). The spermiogenic nucleus also experiences a change in morphology due to other phenomena not considered in this work. As in the previous instances, a partial

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coalescence of coarse granules occurs during the final phases of spermiogenesis (IE). It is quite possible that both in *M. turbinata* and *D. labrax*, the final chromatin transformations coincide with dephosphorylation of the SNBP or protamine.

III.- Acetylation of spermiogenic histones

A.- Histone H4.- As has been previously mentioned, histone H4 demonstrates hyperacetylation prior to histone displacement by phosphorylated protamine in spermiogenesis of several bony fish. It has been suggested that hyperacetylation of histone H4 relaxes the DNA-histone interaction, allowing a more efficient displacement of histone by protamines. In a recent work (Kurtz et al., 2007), we observed that two waves of histone H4 acetylation take place during *Sepia officinalis* spermiogenesis. The first mainly involves monoacetylation of lysine 12 on histone H4 (H4-acK12), which is correlated to chromatin restructuring into fine granules of 20 nm. The second wave of acetylation results in hyperacetylation of histone H4, and is correlated with histone displacement by the precursor protamine. It was discussed in this work how histone acetylation plays a functional role in the early phases of chromatin condensation, one which is not directly related to histone displacement. The implications of this observation are that the first wave of histone acetylation that takes place during spermiogenesis of *Sepia* is likely to occur as well in organisms such as *S. aurata*, in which there is no displacement of histones by protamine or any other SNBP.

For this study, we obtained histones from intermediate mature male gonads of the three species described here, and tested for acetylation of lysine 12 on histone H4 using a specific antibody. We chose this antibody because lysine 12 is the residue on H4 that has been previously described as the first lysine to be acetylated in spermiogenesis of other marine species (Coupez

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et al., '87; Kurtz et al., 2007). As shown in Figure 6, acetylation of H4 occurs during spermiogenesis of these three species independent of their subsequent displacement by SNBPs (*D. labrax*, *M. turbinata*) or not (*S. aurata*).

B.- Histone H3.- A great deal of post-translational modifications of histone H3 have been described in different biological systems (Kouzarides, 2007). In spite of this, the specific study of H3 acetylation during spermiogenesis has received little attention until now and has only been sporadically described in mammalian spermiogenesis (Grimes and Henderson, '83; Gatewood et al., '90; Hazzouri et al., 2000). It is possible that H3 acetylation during spermiogenesis could have a similar or complementary function to that of H4 acetylation, and for this reason we have attempted to detect acetylated forms of H3 in gonadal nuclei in the three species studied. To this end, the basic proteins extracted from maturing gonadal nuclei (the same samples as those used in Fig. 6), were analyzed by western blot using the antibody anti H3-acK9/18. The results are shown in Figure 7A. Histone H3 of *S. aurata* is acetylated in gonadal nuclei, but is completely deacetylated in nuclei of mature sperm (Fig. 7A,B lanes SaG, SaS, respectively). Histone H3 in maturing gonadal nuclei of *D. labrax* also demonstrates an intense reaction with anti H3-acK9/18 (Fig. 7A,B lanes Dl), but the labelling of gonadal H3 from *M. turbinata* is very low, practically non-existent (Fig. 7A,B lanes Mt). Since histone H3 can undergo acetylation in positions other than K9 and K18 (K14, 23, and 28), we have repeated the experiment by using the antibody anti acetyl lysine which recognizes any protein with an acetylated lysine residue, independently of its position in the molecule. In Figure 7C (lanes MG) histone H3 of *M. turbinata* shows a considerable reaction with this antibody. The labelling of H3 is similar to that of H4 from the

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same animal, and is equivalent to that of the spermiogenic H3 from the bony fish *Trachinus draco* included as a positive control of H3 and H4 hyperacetylation (see material and methods).

C.- Other histones.- In spermiogenesis of the species studied in this work, no significant evidence of acetylated histones H2A or H2B were found.

IV.-Localization of acetylated histones in the condensing spermiogenic chromatin (row III in Figs. 3, 4, and 5)

We applied antibodies to detect the condensing chromatin phases which contain acetylated histones using electron immunomicroscopy. Anti acetyl lysine antibody was used for *S. aurata* and *M. turbinata*, and anti H4-acK12 was used for *D. labrax* (since background labelling by anti acetyl lysine with this species did not allow nuclear acetylation to be clearly discriminated). The results are shown in Figures 3, 4, and 5 (row III). The acetylation patterns observed both within each species and among the three of them are highly consistent. In all cases, the somatic-like chromatin of the early spermatid appears to have a slight basal level of labelling (III A) which significantly increases in the 20 nm granule stage of intermediate spermatids (III B,C) and disappears completely from chromatin of the most advanced spermatids (gonadal spermatozoa) (III D, E, D2). In *S. aurata*, acetylation disappears in the final stage when fine chromatin granules fuse together (Fig. 3III D), while in the other two species, acetylation disappears in the phases where histones are replaced by protamine/SNBP and forms large size granules (Fig. 4III E, Fig. 5III D2).

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Discussion

In order to clarify the most general conclusions made in our study, it must be kept in mind that among animal species, a sporadic distribution has arisen of the processes involved in spermiogenesis, as well as in the characteristics demonstrated by the mature sperm nucleus. This distribution does not correspond to the general taxonomy or phylogeny of animals. For this reason we have chosen to study three species, each of which represents a model of spermiogenesis which is also exhibited by many other species distributed throughout the various taxa, like a puzzle.

The results of the preceding section describe two types of nuclear protein transitions during spermiogenesis:

The simplest transition is [**H**], in which only non-histone nuclear proteins leave the nucleus during spermiogenesis. The mature sperm of this model retain histones and nucleosomal organization. To study of this model we have chosen spermiogenesis of *S. aurata*, which represents characteristics shared by a great number of species in different taxa (Casas et al., '81; Muñoz-Guerra et al., '82; Vodicka et al., '90; Saperas et al., '94; Ausió et al., '97; see revision by Kasinsky, '89). In fact, any one of these representative species could have been chosen to demonstrate the [**H**] model (compare for example the mature sperm chromatin of *Opsanus tau* (Casas et al., '81) with that of *S. aurata* presented in this work).

The second transition, which involves a higher level of chemical complexity, is [**H→P**]. During this transition, histones are directly replaced by a more basic protein (protamine or SNBP) which ultimately becomes the main protein component of mature sperm nuclei. To study this model we have chosen *D. labrax* and *M. turbinata*, each one which is representative of a

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large number of spermiogenesis of species distributed sporadically in diverse taxa (reviewed in Oliva and Dixon, '91; Daban et al., '91, '95; Saperas et al., '94, '97; Hunt et al., '96).

In previous publications we have described the nuclear protein transitions (and the acetylation pattern) during spermiogenesis of *S. officinalis* (Kurtz et al., 2007; Martínez-Soler et al., 2007a). This spermiogenesis represents still a higher level of complexity. In this instance, histones of early spermatids are displaced by a precursor protamine (Pp) during intermediate spermiogenic stages. Subsequently Pp is transformed into protamine (P) by cleavage and elimination of the N-terminal portion (Martin-Ponthieu et al., '91; Wouters-Tyrou et al., '91). This type of transition was referred to as [**H→Pp→P**] in the paper by Kurtz et al. (2007).

The comparative analysis of these four species (*S. aurata*, *D. labrax*, *M. turbinata*, and *S. officinalis*) allows for a better understanding of some interesting correlations between the nature of the chemical changes and structural/morphological transitions of spermiogenic chromatin. One common feature shared by all four spermiogenesis in their initial stage is the disappearance of the structural differences between euchromatin and heterochromatin. This results in a uniform chromatin distribution throughout the entire nucleus consisting of homogenously organized 20 nm granules. Interestingly, these granules consist of histones in every instance, and based on their size must contain approximately 4-6 nucleosomes (Kurtz et al., 2007; Martínez-Soler et al., 2007a). Additionally, in each species, the histones of this chromatin type are partially acetylated. In *S. officinalis*, we have shown that an important population of histone H4 is monoacetylated on lysine 12 in the 20 nm granules (Kurtz et al., 2007). It is very likely that the partial acetylation of histone H4 (and possibly H3) prevents the formation of higher order chromatin structures (García-Ramírez et al., '95; revised in Zheng and Hayes, 2003; Bulger, 2005; Calestagne-Moreli

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and Ausió, 2006) while favoring the formation of small of nucleosome clusters (from 4-6) which are present in 20 nm fibrogranular structures. A review of the literature on morphological nuclear development during spermiogenesis in different animals indicates that the formation of 20 nm granules in the initial condensation phases of spermiogenesis is a widespread feature in a large number of species, independently of their taxonomic or phylogenetic relationship (Ribes et al., 2001).

The formation of 20 nm granules is associated with an important reduction in the volume of the spermiogenic nucleus (Figs. 3, 4 and 5) albeit the causes directly involved in the process are unknown. The decrease in nuclear volume does not appear to be an intrinsic property of the 20 nm granular chromatin, since in *S. officinalis* and other species such chromatin organization does not occur simultaneously with any reduction in nuclear volume (Martínez-Soler et al., 2007b). The nuclear volume decrease could be the result of the re-organization of other structural components (such as the nuclear matrix) which are related to nucleomorphogenesis; however, this needs further characterization, and is out of the scope this paper.

In the fish *S. aurata* (transition [**H**]) the 20 nm granules partially coalesce in the final stages of spermiogenesis, producing spermatic chromatin which appears nearly homogenous when observed by electron microscopy. The coalescence of these granules occurs simultaneously with the disappearance of acetylated histones (Fig. 3D). This final step is analogous to the protamine dephosphorylation in other traditional types of spermiogenic nuclear protein transitions involving protamines (Lewis et al., 2003). In a similar fashion, histone deacetylation increases the electrostatic interaction between proteins and DNA, allowing for the nucleosomes to redistribute and come into nearer proximity. This results in the dispersal of the 20 nm granules to produce a more compact and homogenous nuclear chromatin organization.

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In *D. labrax* and *M. turbinata* ([**H→P**] transition), the chromatin condensation is extended further. The 20 nm granules disappear forming larger, more electrodense structures of 80 nm and 60 nm, respectively, in which DNA is tightly packed (Figs. 4 and 5). The 20 nm granules disappear simultaneously with an important wave of histone acetylation; these histones are no longer present in the 80 and 60 nm granules (formed by DNA and protamine or SNBP, which are likely phosphorylated at this stage). Although the protamine of *D. labrax* and the SNBP of *M. turbinata* are evolutionarily distant molecules, the general process of chromatin condensation is very similar, differing only in the size of the resulting coarse granules. The fusion of these coarse granules in the final stages of spermiogenesis probably coincides with the SNBP dephosphorylation process, which has been described by several authors (see the reviews by Oliva and Dixon, '91 and Lewis et al., 2003).

Chromatin condensation of *S. officinalis* (Kurtz et al., 2007; Martínez-Soler et al., 2007a, 2007b) ([**H→Pp→P**] type) represents a further step in the extent of complexity. In the early stages of spermiogenesis 20 nm granules appear, composed of DNA and histones (where H4 is monoacetylated). In the stage that follows, the precursor protamine (Pp) enters the nucleus, where it co-exists with histones. The complex formed by DNA, histones, and precursor protamine adopts a fibrillar structure of 35 nm in diameter and later on histone H4 is hyperacetylated and all histones are displaced from chromatin. The remaining complex (DNA-precursor protamine) adopts a 45 nm fiber structure. In the final stages, the Pp is processed to protamine, and the nucleoprotamine fibers undergo a progressive condensation.

Various studies have suggested that histones organize the DNA in the most primitive spermatozoa, and that originating from this model, diverse types of sperm nuclear proteins have evolved, generically called SNBPs (Sperm Nuclear Basic Proteins) (Ausió, '95, '97; Rocchini et

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al., '96). The present work is in agreement with this hypothesis and offers a causal explanation. It is quite possible that both the limited histone acetylation and formation of 20 nm granules are ancestral processes in the evolution of spermiogenesis. The formation and uniform distribution of granules occupying the entire volume of the nucleus results in a structural homogenization that eliminates any differences in chromatin organization in particular areas of the nucleus. This appears to be related to, but not the direct cause of, a subsequent important decrease in volume of the nucleus. Lastly, the 20 nm granules could provide a structure which facilitates the displacement of histones by more specialized SNBPs which have appeared in the course of evolution of the spermiogenesis process.

Our results also show that in addition to histone H4 (Oliva and Dixon, '91), histone H3 is also acetylated during spermiogenesis in some species. Therefore, it appears as if the regulation of electrostatic interactions of the tetramer $[H3\text{-}H4]_2$ with DNA plays a critical role in chromatin restructuring during the earliest stages of spermiogenesis, as well as in histone displacement by SNBPs.

Finally, it must be pointed out that the transitions studied and discussed here are not representative of all animal species. Some organisms have spermiogenesis that exhibit much more complex protein transitions than those described above. They produce even more elaborate patterns of chromatin condensation (Meistrich '89; Cáceres et al., '99; Ribes et al., 2001) and their sperm nuclei can often have very peculiar shapes which likely represent an adaptation to their specific fertilization biology.

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Footnotes

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[2]Amino acid composition (mol %) of histone H1 from sperm (a, b) and liver (L)* of *S. aurata* (Sa) compared to histone H1 from calf thymus histones (Ct).* H1 of liver was only partially purified, and contains some contamination. Fractions Sa a, Sa b, and Sa L, correspond to proteins a, b, and L presented in Figure 1C.

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[2] Table 1. Amino acid composition of histone H1

	Histone H1			
	Ct	Sa L*	Sa a	Sa b
Lys	26.8	20.7	22.7	26.9
His	0.0	1.0	0.0	0.0
Arg	1.8	6.4	4.1	1.6
Asp	2.5	4.6	3.1	2.2
Thr	5.6	3.2	6.8	1.9
Ser	5.6	5.9	5.1	6.7
Glu	3.7	5.4	2.6	2.8
Pro	9.2	8.0	9.5	9.9
Gly	7.2	6.5	7.1	8.1
Ala	24.3	18.7	22.0	27.0
Cys	0.0	0.0	0.0	0.0
Val	5.4	7.4	8.7	7.6
Met	0.0	1.3	0.4	0.3
Ile	1.5	2.4	1.4	0.3
Leu	4.5	5.5	4.6	3.1
Tyr	0.9	1.7	0.9	0.6
Phe	0.9	1.3	0.7	0.5

[2]Amino acid composition (mol %) of histone H1 from sperm (a, b) and liver (L)* of *S. aurata* (Sa) compared to histone H1 from calf thymus histones (Ct).* H1 of liver was only partially purified, and contains some contamination. Fractions Sa a, Sa b, and Sa L, correspond to proteins a, b, and L presented in Figure 1C.

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Figure Legends

Fig. 1.- Protein and spermatogenic chromatin of *S. aurata*. Two-dimensional electrophoresis (AUT/SDS) of proteins obtained from very immature gonads (A) and from mature sperm (B). C.- 15% SDS-PAGE showing: control of H1/H5 from chicken erythrocyte (lane c); H1 extracts from gonadal nuclei of *S. aurata* (lane G); 5% PCA extract from sperm nuclei (lane S); purified HPLC fraction of fast migrating form of H1 (lane a); purified HPLC fraction of slow migrating form of H1 (lane b); 5% PCA extract from liver nuclei of *S. aurata* (Lpca) and HPLC purification of two isoforms of H1 from *S. aurata* liver (L). D.- Agarose gel electrophoresis of DNA fragments obtained from MNase digestion of nuclei purified from *S. aurata* sperm cells. S.- supernatants; P.- pellets; L.- ladder of $(123)_n$ base pairs. From 1 to 6.- 1, 2, 5, 10, 20 and 40 minutes of MNase digestion. Inset.- AU gel electrophoresis of histones contained in chromatin after 20 minutes of digestion (left.- supernatants; right.- pellets).

Fig. 2.- Transition of nuclear proteins during spermiogeneis of *D. labrax* (A) and *M. turbinata* (B). Electrophoresis in acetic acid/ urea/ 15% polyacrylamide of obtained proteins from: lanes a.- immature gonadal nuclei (histones); lanes b.- from gonadal nuclei in maturation; lanes c.- from spermatozoa. H.- histones; P.- protamine. C and D.- Primary structure of *D. labrax* protamine (C) and *M. turbinata* SNBP (D). Clusters of arginines are underlined and a solid line has been placed above alternating $[RS]_n$ residues.

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Fig. 3.- Spermiogenic changes of nucleus (I) and chromatin (II and III) of *S. aurata* observed by electron microscopy.

I: A.- early spermatid nucleus; B, C.- intermediate stages of spermiogenesis; D.- testicular spermatozoon (very advanced spermatid). Each image is presented at the same magnification. Bar: 0.5 μm.

II: B1 and B2.- First stages of chromatin condensation. Chromatin is organized in granules of 20 nm diameter; C.- advanced spermatids. The chromatin granules of 20 nm are closely packed; D.- chromatin from testicular spermatozoon, which appears more uniformly condensed. Each image is presented at the same magnification. Bar: 0.2 μm for all images.

III: Labelling with anti acetyl lysine antibody of *S. aurata* chromatin at different stages of spermiogenesis. A.- early round spermatid; B.- 20 nm granular; C.- 20 nm granular chromatin closely packaged together; D.- Testicular spermatozoon. Each image is presented at the same magnification. Bar: 0.5 μm for all images.

Fig. 4.- Spermiogenic changes of nucleus (I) and chromatin (II, III) of *D. labrax* observed by electron microscopy.

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II: B1, B2.- Stages of 20 nm granular chromatin; C,D.- Remodelling of chromatin from 20 nm granules to coarse granules (80 nm approximately). E.- advanced stages of chromatin condensation, in which the chromatin is organized in coarse granules. Each image is presented at the same magnification. Bar: 0.2 μm for all images.

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III: Anti H4-acK12 labelling of: A.- early spermatidal chromatin; B.- 20 nm granular chromatin; C, D.- chromatin at the beginning of 20 nm→ 80 nm granular remodelling; E.- chromatin organized in coarse granules (80 nm). Each image is presented at the same magnification. Bar: 0.5 μm for all images.

Fig. 5.- Spermiogenic change of nucleus (I), and chromatin (II, III) of *M. turbinata* observed by electron microscopy.

I: A.- spermatogonial nucleus; B.- spermatidal nucleus at the beginning of the first nuclear condensation; C.- nucleus of an intermediate spermatid; D.- more advanced spermatid; E.- testicular spermatozoon. Each image is presented at the same magnification. Bar: 1 μm for all images.

II: B, C.- chromatin organized in granules of 20 nm diameter; D1.- chromatin at the beginning of remodelling from 20 nm granules to coarse (60 nm approximately) granules. Each image is presented at the same magnification. Bar: 0.5 μm for all images.

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Fig. 6.- Left.- Stained PVDF membrane containing transferred proteins from AU-PAGE of histones obtained from nuclei of intermediately mature gonads of *M. turbinata* (lanes Mt), *D. labrax* (Dl) and *S. aurata* (Sa). Right: Immunoblotting analysis of histones using the anti H4-acK12 antibody. Histone H4 is labelled in the three species.

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Fig. 7.- AU-PAGE (A), and anti H3-acK9/18 immunoblotting of histones (B) from nuclei belonging to intermediate ripe gonads of *S. aurata* (lanes SaG), *D. labrax* (Dl) and *M. turbinata* (Mt). Lane SaS contains histones from ripe sperm nuclei of *S. aurata*. 3a indicates the acetylated forms of H3, and 3d indicates the dimer of this histone (also reacting with the anti H3-acK9/18 antibody). C.- AU-PAGE (left) and anti acetyl lysine immunoblotting of gonadal histones (right) from the control *Trachinus draco* (TH) and from gonadal histones of *M. turbinata* (MG).

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[2] Table 1. Amino acid composition of histone H1

	Histone H1			
	Ct	Sa L*	Sa a	Sa b
Lys	26.8	20.7	22.7	26.9
His	0.0	1.0	0.0	0.0
Arg	1.8	6.4	4.1	1.6
Asp	2.5	4.6	3.1	2.2
Thr	5.6	3.2	6.8	1.9
Ser	5.6	5.9	5.1	6.7
Glu	3.7	5.4	2.6	2.8
Pro	9.2	8.0	9.5	9.9
Gly	7.2	6.5	7.1	8.1
Ala	24.3	18.7	22.0	27.0
Cys	0.0	0.0	0.0	0.0
Val	5.4	7.4	8.7	7.6
Met	0.0	1.3	0.4	0.3
Ile	1.5	2.4	1.4	0.3
Leu	4.5	5.5	4.6	3.1
Tyr	0.9	1.7	0.9	0.6
Phe	0.9	1.3	0.7	0.5

[2]Amino acid composition (mol %) of histone H1 from sperm (a, b) and liver (L)* of *S. aurata* (Sa) compared to histone H1 from calf thymus histones (Ct).* H1 of liver was only partially purified, and contains some contamination. Fractions Sa a, Sa b, and Sa L, correspond to proteins a, b, and L presented in Figure 1C.

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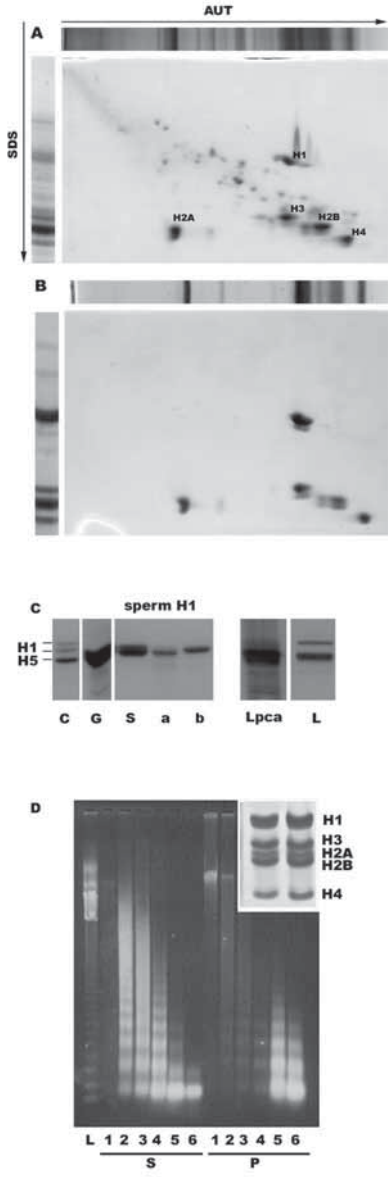
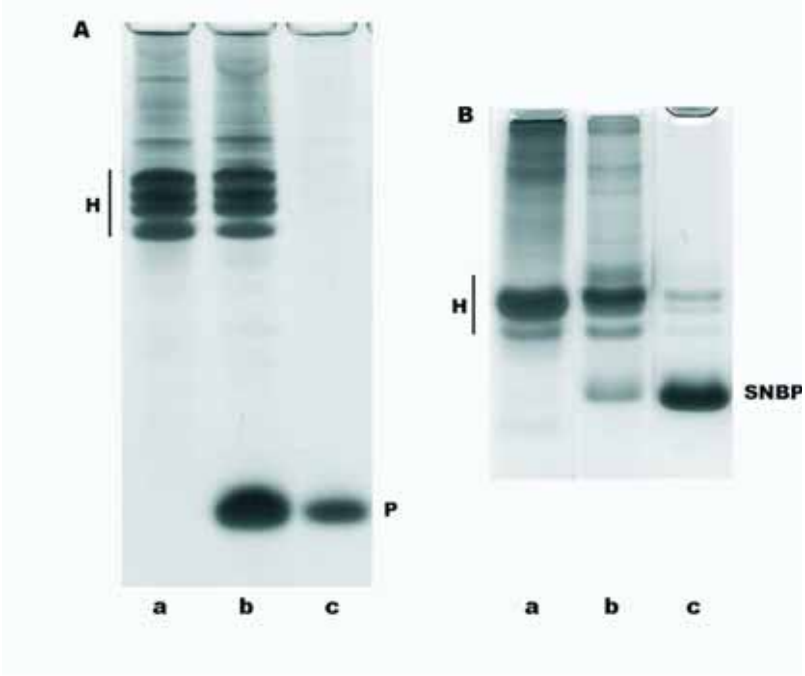


Fig. 1.- Protein and spermatogenic chromatin of *S. aurata*. Two-dimensional electrophoresis (AUT/SDS) of proteins obtained from very immature gonads (A) and from mature sperm (B). C.- 15% SDS-PAGE showing: control of H1/H5 from chicken erythrocyte (lane c); H1 extracts from gonadal nuclei of *S. aurata* (lane G); 5% PCA extract from sperm nuclei (lane S); purified HPLC fraction of fast migrating form of H1 (lane a); purified HPLC fraction of slow migrating form of H1 (lane b); 5% PCA extract from liver nuclei of *S. aurata* (Lpca) and HPLC purification of two isoforms of H1 from *S. aurata* liver (L). D.- Agarose gel electrophoresis of DNA fragments obtained from MNase digestion of nuclei purified from *S. aurata* sperm cells. S.- supernatants; P.- pellets; L.- ladder of (123)n base pairs. From 1 to 6.- 1, 2, 5, 10, 20 and 40 minutes of MNase digestion. Inset.- AU gel electrophoresis of histones contained in chromatin after 20 minutes of digestion (left.- supernatants; right.- pellets). 73x224mm (300 x 300 DPI)



C

PRRRRQASRPVRRRRRTRRSTAERRRRRVVRRRR

D

ARAVRRRRARSRSRSRKSRSRSRSAKRSASRRRSRS

AGRRRRRRTASRRRRRSASRRRSVSRRRRRRSRKSR

GRRRRGRKVRRRRVKRAGRKGRRRTRRRRRRARR

Fig. 2.- Transition of nuclear proteins during spermiogeneis of *D. labrax* (A) and *M. turbinata* (B). Electrophoresis in acetic acid/ urea/ 15% polyacrylamide of obtained proteins from: lanes a.- immature gonadal nuclei (histones); lanes b.- from gonadal nuclei in maturation; lanes c.- from spermatozoa. H.- histones; P.- protamine. C and D.- Primary structure of *D. labrax* protamine (C) and *M. turbinata* SNBP (D). Clusters of arginines are underlined and a solid line has been placed above alternating $[RS]_n$ residues.
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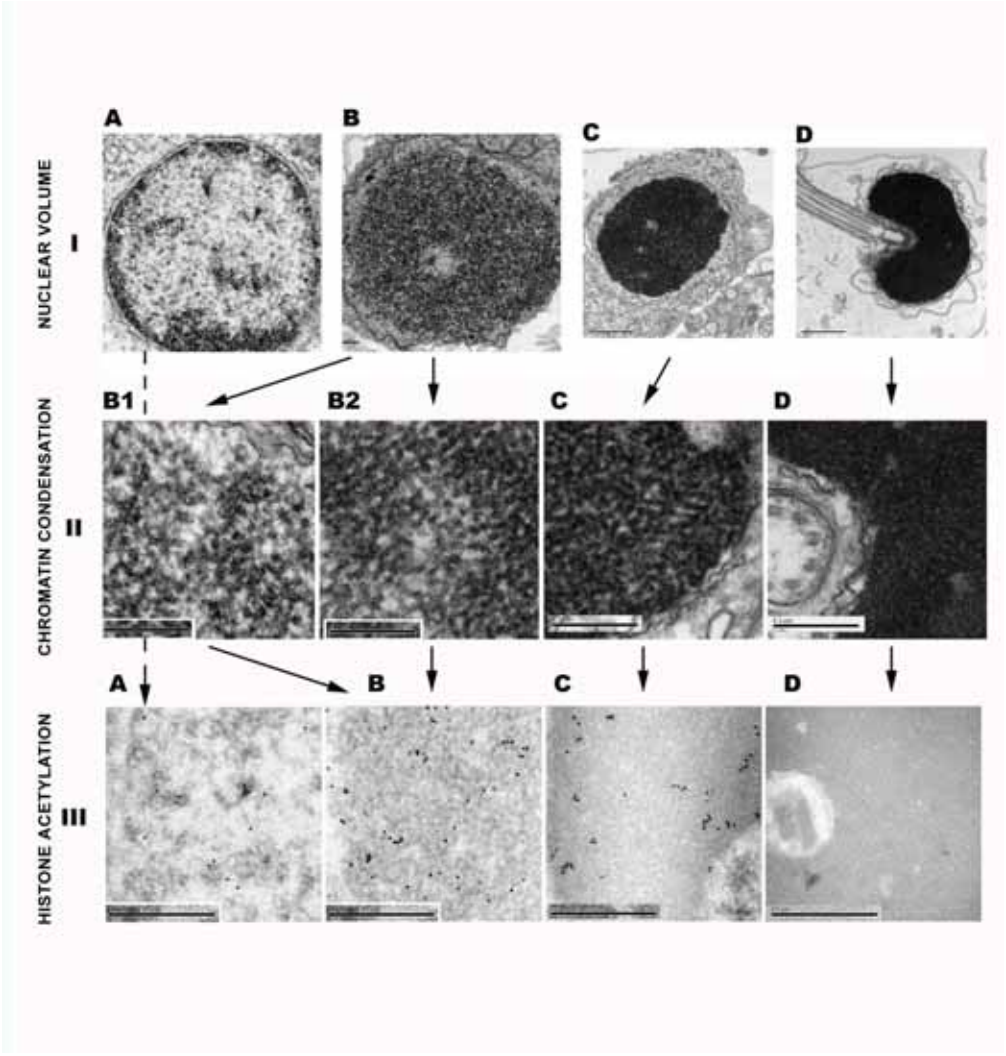

Fig. 3.- Spermiogenic changes of nucleus (I) and chromatin (II and III) of *S. aurata* observed by electron microscopy. I: A.- early spermatid nucleus; B, C.- intermediate stages of spermiogenesis; D.- testicular spermatozoon (very advanced spermatid). Each image is presented at the same magnification. Bar: 0.5 μm II: B1 and B2.- First stages of chromatin condensation. Chromatin is organized in granules of 20 nm diameter; C.- advanced spermatids. The chromatin granules of 20 nm are closely packed; D.- chromatin from testicular spermatozoon, which appears more uniformly condensed. Each image is presented at the same magnification. Bar: 0.2 μm for all images. III: Labelling with anti acetyl lysine antibody of S. aurata chromatin at different stages of spermiogenesis. A.- early round spermatid; B.- 20 nm granular; C.- 20 nm granular chromatin closely packaged together; D.- Testicular spermatozoon. Each image is presented at the same magnification. Bar: 0.5 μm for all images.
161x169mm (600 x 600 DPI)

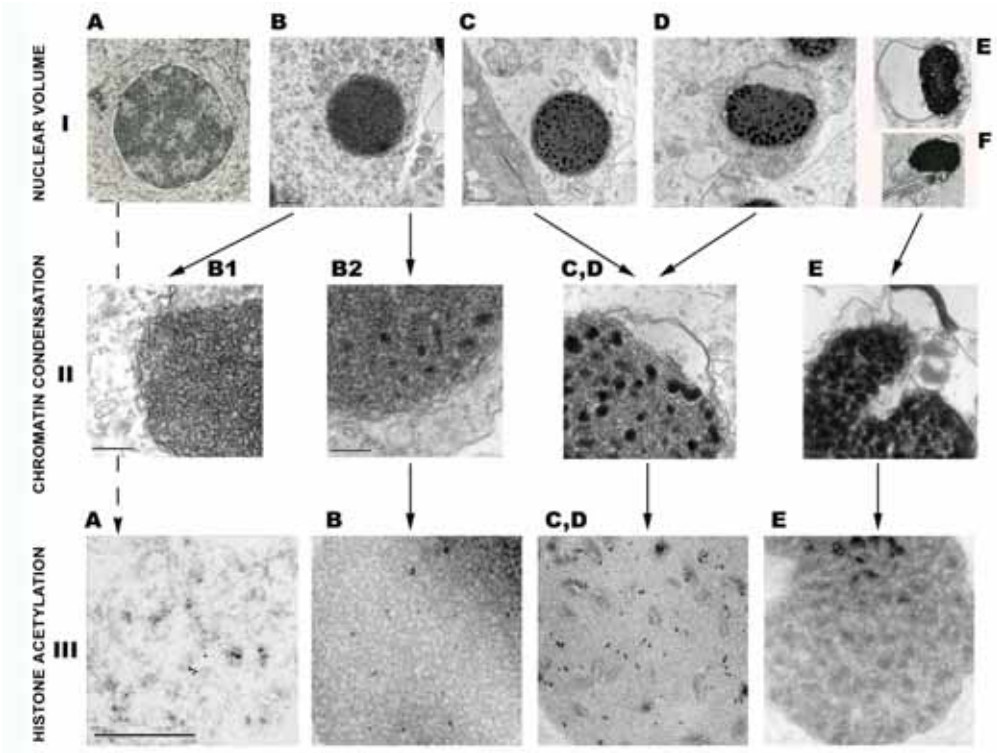

Fig. 4.- Spermiogenic changes of nucleus (I) and chromatin (II, III) of *D. labrax* observed by electron microscopy. I: A.- nucleus belonging to an early spermatid; B.- intermediate; C, D.- advanced; E, F.- very advanced spermatid. Each image is presented at the same magnification. Bar: 0.5 μm for all images. II: B1, B2.- Stages of 20 nm granular chromatin; C,D.- Remodelling of chromatin from 20 nm granules to coarse granules (80 nm approximately). E.- advanced stages of chromatin condensation, in which the chromatin is organized in coarse granules. Each image is presented at the same magnification. Bar: 0.2 μm for all images. III: Anti H4-acK12 labelling of: A.- early spermatidal chromatin; B.- 20 nm granular chromatin; C, D.- chromatin at the beginning of 20 nm→ 80 nm granular remodelling; E.- chromatin organized in coarse granules (80 nm). Each image is presented at the same magnification. Bar: 0.5 μm for all images.
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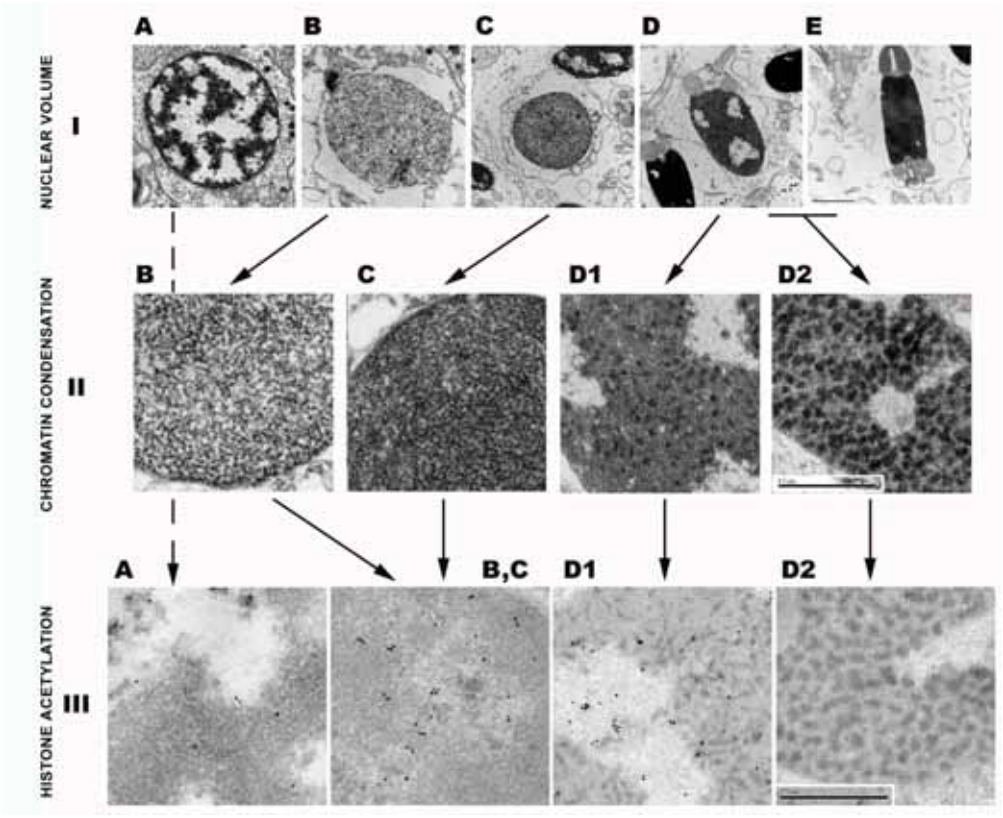

Fig. 5.- Spermiogenic change of nucleus (I), and chromatin (II, III) of *M. turbinata* observed by electron microscopy. I: A.- spermatogonial nucleus; B.- spermatidal nucleus at the beginning of the first nuclear condensation; C.- nucleus of an intermediate spermatid; D.- more advanced spermatid; E.- testicular spermatozoon. Each image is presented at the same magnification. Bar: 1 μm for all images. II: B, C.- chromatin organized in granules of 20 nm diameter; D1.- chromatin at the beginning of remodelling from 20 nm granules to coarse (60 nm approximately) granules. Each image is presented at the same magnification. Bar: 0.5 μm for all images. III: Anti acetyl lysine labelling. A.- earliest spermatidal chromatin; B, C.- chromatin organized in 20 nm granules; D.- chromatin at the beginning of remodelling from granular 20 nm to coars (60nm) granules. Each image is presented at the same magnification. Bar: 0.5 μm for all images.
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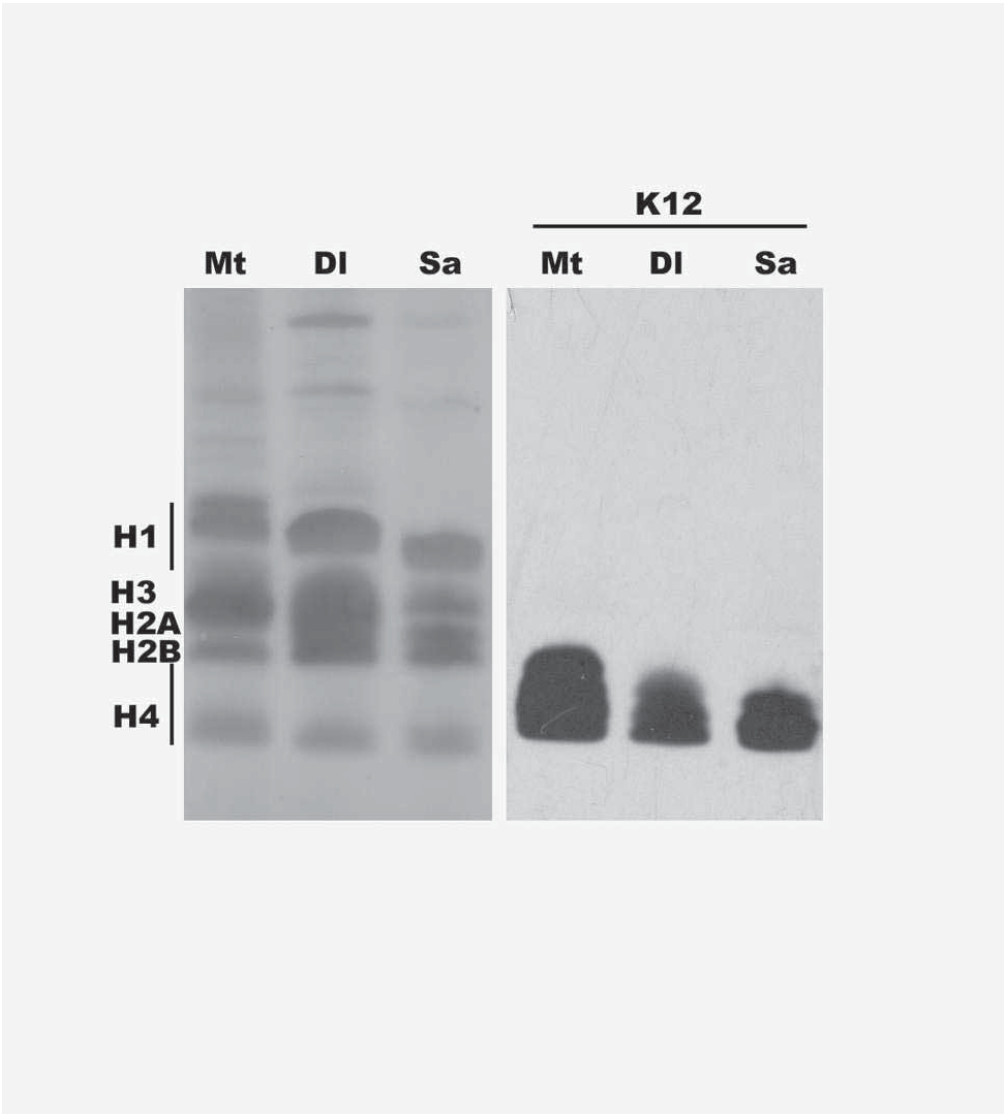

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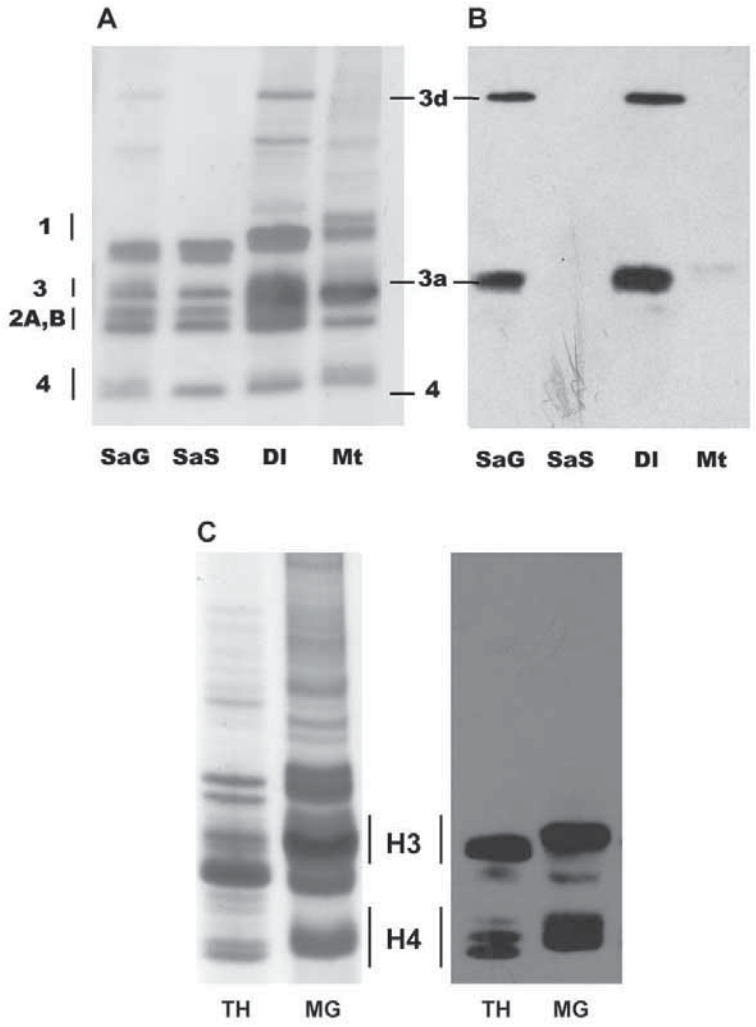
82x91mm (300 x 300 DPI)



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82x119mm (300 x 300 DPI)

III. RESULTS

CHAPTER 2. MODEL INCREASED COMPLEXITY DURING SPERMIOGENCIC PROTEIN TRANSITIONS AND CHROMATIN CONDENSAITON

The cephalopod mollusk *Sepia officinalis* represents a direct increase in complexity in spermiogenic chromatin composition and structure to the model H→P. This model is defined as H→Pp→P, where the precursor protamine displaces histones prior to its conversion into the mature protamine. Early studies (Rousseaux-Prévost et al., 1988; Wouters-Tyrou et al., 1991) confirmed that *S. officinalis* incorporated two protein transitions during spermiogenesis, where a precursor/mature protamine molecule relationship was observed. To be able to relate these protein transitions to chromatin condensation during spermiogenesis, a detailed description of nuclear changes envolved during spermiogenesis was essential. In the publication found in *Tissue and Cell* (2007) entitled "Sperm Nucleomorphogenesis in the cephalopod *Sepia officinalis*" we elaborate on the early descriptions of spermiogenesis of *Sepia* (Longo and Anderson 1970, Maxwell 1975) in order to better understand changes to the developing sperm cell and its condensing chromatin during spermiogenesis. My participation in this article has been significant. I contributed to the characterization of different spermiogenic stages of chromatin condensation and patterning, studying TEM images for fiber measurements. I made observations of changes in nuclear shape and acrosomal and microtubular development which accompany changes in chromatin structure during spermiogenesis.

With a better understanding of the developmental changes to the sperm cell along with spermiogenic structural changes to the chromatin during spermiogenesis, a correlation could be made between these two aspects and the biochemical transitions which were known to occur during spermiogenesis of *S. officinalis*. We took advantage of the fact that the amino acid sequence of the precursor protamine was known (Matin-Ponthieu et al., 1991). This allowed us to isolate the precursor portion of the protein and develop an antiserum specific to this domain. Using this antiserum to treat testicular tissue containing spermiogenic nuclei using immunolocalization and transmission electron microscopy, we were able to identify chromatin patterning stages related to the appearance of the precursor protamine. We also used anti-histone antibodies to identify chromatin structures related to histones alone, and one stage in particular related to the co-presence of residual histones and the appearance of the precursor molecule. In this work, we achieved identification of chromatin changes not related to any loss or gain of histones or other SNBP, chromatin changes related to a loss of some histones and gain of

precursor protamine, and chromatin structures related to conversion of the precursor into the protamine. The study was published in *Molecular Reproduction and Development* (2007) in a work entitled "Transition of Nuclear Proteins and Chromatin Structure in Spermiogenesis *of Sepia officinalis*". For this study, I performed gonadal and epididymal tissue dissections and subsequently obtained pure nuclei for basic protein extraction and electrophoretic analysis. I participated in the purification of histone H2A in order to generate an antiserum for later use in locating the protein in tissue preparations. Prior to use in immunocytochemistry, confirmation of specificity of this antiserum, as well as an anti-protamine antiserum developed by colleagues, was essential. I performed several respective western blots testing for histone H2A and protamine in basic protein extracts from testes of differing grades of maturity. Using these two antisera, I performed immunolocalizations and observed the results using transmission electron microscopy. I participated in analyzing these results to identify spermiogenic stages and chromatin patterns which correlated to histone displacement, as well as condensing chromatin states during which the precursor was likely converted into the mature protamine.

Due to the fact that the simple increase in molecular complexity was shown to bring about such a complex pattern of chromatin condensation during spermiogenesis, we felt it necessary to complete our understanding of the biochemical changes affecting spermiogenic chromatin structure in *S. officinalis*. This was achieved by relating acetylation affecting histone H4 to the patterning changes of spermiogenic chromatin, since histone H4 acetylation had previously been described in this species (Coupez et al., 1987). In the work published in *Journal of Cellular Biology* (2007) entitled "Acetylation of Histone H4 in Complex Structural Transitions of Spermiogenic Chromatin", we related the three structural remodelling events (somatic-like chromatin → 20 nm granules→30-35 nm fibers→40-50 nm fibers) to the acetylation state of histone H4. We were able to conclude that the first chromatin remodelling is associated with a global monoacetylation of histone H4, which (just as in the H→H and H→ P models; see discussion) is speculated to favour gatherings of small groups of nucleosomes causing these types of granules to form. The next remodelling is highly dynamic, including a competition between acetylated histones and precursor protamine for DNA binding. In the third pattern, residual hyperacetylated histone H4 co-exists with the precursor protamine, suggesting that the fibers increase in thickness as the precursor achieves greater histone displacement. In this study, my participation was complete, in which I fully contributed in all methods included in this work.

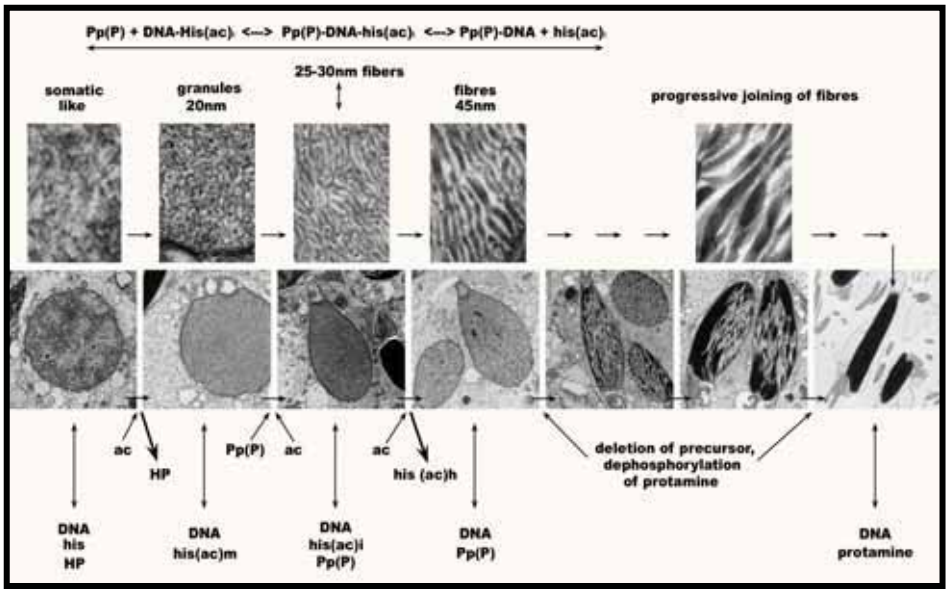

Figure III.2.1. Changes in nuclear morphology, chromatin composition, and chromatin structure during spermiogenesis of *S. officinalis*. In the first transition, non-histone proteins are lost and the chromatin is organized as complexes of DNA-histones (nucleosomes). We have simultaneously observed a *wave* of monoaceylation of histone H4 during this phase. The second transition correlates to the appearance of the bulk of the protamine precursor into the nucleus (part of histone H4 appears hyperacetylated). The next remodeling implies the entrance of the total amount of precursor protamine, while it substitutes the majority of histones. The remaining histones are found in a hyperacetylated form (his (ac)h). The final condensation of chromatin (coalescing of 45 nm fibers) occurs simultaneously with the deletion of the precursor portion and dephosphorylation of the precursor protamine, since in ripe sperm chromatin DNA is associated exclusively with dephosphorylated protamine.

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Sperm nucleomorphogenesis in the cephalopod *Sepia officinalis*

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Abstract

Sperm nucleomorphogenesis in the cephalopod *Sepia officinalis* is the product of the interaction between perinuclear microtubules and condensing chromatin. This interaction occurs during spermiogenesis and is established through the nuclear membrane.

As in other cephalopod species, the perinuclear microtubules are transient structures. In the case of *S. officinalis*, they begin to appear in the basal area of the early spermatid and progress from there, establishing contact with the external nuclear membrane and follow a defined, but not symmetric, geometry. Thus, the microtubules accumulate preferentially in one area of the nuclear membrane which we refer to here as the "dorsal zone". Later, the microtubules will be eliminated before the mature spermatid migrates to the epididymis.

The chromatin is condensed within the nucleus following a complex pattern, beginning as fibro-granular structures until forming fibres of approximately 45 nm diameter (patterning phases). From this stage on, an increase in the chemical basicity of DNA-interacting proteins is produced, and chromatin fibres coalesce together, being recruited to the dorsal zone of the membrane, where there is a higher density of microtubules. This last step (condensation phases) allows the chromatin fibres to be arranged parallel to the axis of the elongating nucleus, and more importantly, is deduced to cause a lateral compression of the nucleus. This lateral compression is in fact a recruitment of the ventral zone toward the dorsal zone, which brings about an important reduction in nuclear volume.

The detailed observations which comprise this work complement previous studies of spermiogenesis of *Sepia* and other cephalopods, and will help to better understand the process of cellular morphology implicated in the evolution of sperm nuclear shape in this taxonomic group.
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Keywords: Cephalopods; *Sepia officinalis*; Spermiogenesis; Nucleomorphogenesis; Chromatin; Microtubules

1. Introduction

The various levels of biological organization related to the reproduction of species have been exposed to an exceptionally fast evolution. This applies at the molecular level (Wyckoff et al., 2000; Swanson and Vacquier, 2002; Lewis et al., 2004), at the cellular level (Jamieson et al., 1995), as well as to the great variety of the reproductive organs and types of fertilization that organisms display.

A particular case on which we have been working is based on the evolutionary changes of sperm nuclear shape of cephalopods, as well as the proteins that condense spermiogenic and spermatic chromatin (Giménez-Bonafé et al., 2002a, 2002b, 2004; Ribes et al., 2004; Martínez-Soler et al., 2007). From studies of conventional optical microscopy, Franzén (1967) defined the evolutionary trend that has occurred in sperm of the phylogenetic group including cephalopod decapods and the octopi *Octopus* and *Eledone*. This trend consists of a nuclear elongation followed by a remarkable spiralization of the nucleus. It is important to mention that this evolution is accompanied by changes in other parts of the cell, mainly in the acrosome and axoneme, which are not taken into account in this work. The nuclear elongation is first produced during the evolutionary divergence: [*decapods-Octopus*], as well as an exclusive spiralization of the acrosome (Galangau and Tuzet, 1968; Longo and Anderson, 1970; Ribes et al., 2002). Later, in some octopi such as *Eledone*, nuclear elongation becomes

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much more severe, and the whole sperm nucleus is spiralized along its elongation axis (Maxwell, 1974; Giménez-Bonafé et al., 2002a).

Despite the fact that the study of variation of shape and components of the mature sperm cell has been extensively used for studies of taxonomy and phylogenetics (see for instance Franzén, 1977; Jamieson, 1991; Jamieson et al., 1999), the studies on the evolutionary changes of the spermiogenic processes are scarcer. In the previously cited works, we have analysed in detail sperm nucleomorphogenesis of *Octopus* and *Eledone*, and the primary structures of proteins responsible for chromatin condensation of spermiogenic cells. In these works, we have given evidence supporting that a limited number of changes in spermiogenic development can lead to important modifications in the nuclear shape of mature sperm. In fact, spermiogenesis is caused by a complex process of progressive activation of a genetic program (Sassone-Corsi, 2002; Kimmins et al., 2004), and therefore of dynamic interactions, ordered in time, between cellular elements. Thus, changes in the appearance of a specific element in this program, as well as changes in the moment of its activation, can produce critical modifications to the final result of spermiogenesis.

The present article shows a detailed analysis of sperm nucleomorphogenesis in *Sepia officinalis*, allowing a better understanding of the processes responsible for the evolutionary changes to the cephalopod sperm cell. The general spermiogeneses of *Sepia* and other cephalopods were first studied by Longo and Anderson (1970), Maxwell (1974, 1975), Fields and Thompson (1976), Arnold and Arnold (1978), Healy (1990a, 1990b, 1993), Selmi (1996) and Giménez-Bonafé et al. (2002a, 2002b).

2. Materials and methods

2.1. Animals

The various specimen of *S. officinalis* studied in this work were collected along the Catalonian coast of the Mediterranean Sea (Northeast of Spain). Gonads were dissected and treated immediately. The species *S. officinalis* (formerly *Eusepia officinalis*), belongs to the class Cephalopoda, order Decapoda, family Sepiidae.

2.2. Electron microscopy and confocal immunofluorescence

Conventional transmission electron microscopy was performed according to the method described in Giménez-Bonafé et al. (2002a). Briefly, portions of male gonads were fixed in 2.5% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.4, and postfixed in osmium tetroxide in cacodylate buffer. Following fixation, the samples were dehydrated and embedded in Spurr's resin. Sections were stained with uranyl acetate and lead citrate and observed on a Hitachi 4-600 transmission electron microscope (Hitachi Ltd., Tokyo, Japan).

Immunomicroscopy was performed as in Martínez-Soler et al. (2007). Specifically, male gonad from *S. officinalis* was fixed in 4% paraformaldehyde with 1% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4, followed by a second fixation in which the glutaraldehyde was omitted. The sample was dehydrated and embedded in Lowicryl K4M resin. Ultrathin sections were applied to nickel grids, and were treated by flotation with anti-α or anti-β tubulin antibodies (Amersham Biosciences) prepared 1:500. An anti-mouse secondary antibody conjugated with 15 nm colloidal gold was used for indirect antibody detection, prepared 1:25. Prior to immunoelectron microscopy observation, the samples were contrasted in the same way as those embedded in Spurr's resin.

Optical immunofluorescence has been performed based on the technique of Zhao et al. (2004) with some variations, and has been used for the inset in Fig. 10. Semi-thin tissue sections (0.75 to 1 μm thick) were blocked with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA)/10% normal goat serum/20 mM Glycine/0.1% Tween-20 for 30 min. The slides were then incubated with anti-α-tubulin antibody (1:25), diluted in the same solution used for blocking, with the glycine omitted, and incubated overnight at 4 °C. Samples were washed several times with PBS/0.1% Tween-20, followed by PBS. They were then incubated for 1.5 h with the secondary antibody, an Alexa Fluor 488 conjugated goat anti-rabbit (Molecular probes) prepared 1:500 in PBS/0.1% Tween-20/1% BSA/1% normal goat serum. Then the nuclei were stained with the far-red fluorochrome TO-PRO-3 (Molecular Probes) prepared 1:6000 in PBS, and washed in PBS before mounting with Immunofluore mounting media (MP Biomedicals).

3. Results

3.1. A. Correlation between the pattern of chromatin condensation and nuclear shape during spermiogenesis of S. officinalis (Fig. 1).

With the objective of determining if nucleomorphogenesis of *S. officinalis* is conditioned by the type of chromatin condensation, we examined the correlation between changes in the chromatin structure and changes in the spermiogenic nuclear shape.

The first change in chromatin structure is produced when somatic-like chromatin of the earliest spermatid nucleus is transformed into homogeneously packaged granules (actually, a fibrogranular structure) approximately 20 nm in diameter (Fig. 1A and B). This first structural remodelling is not correlated with important changes in nuclear shape or volume. In Fig. 1A and B, it is observed that the spermatid nucleus maintains an approximately spherical shape, and that aside from the apparent differences determined by the

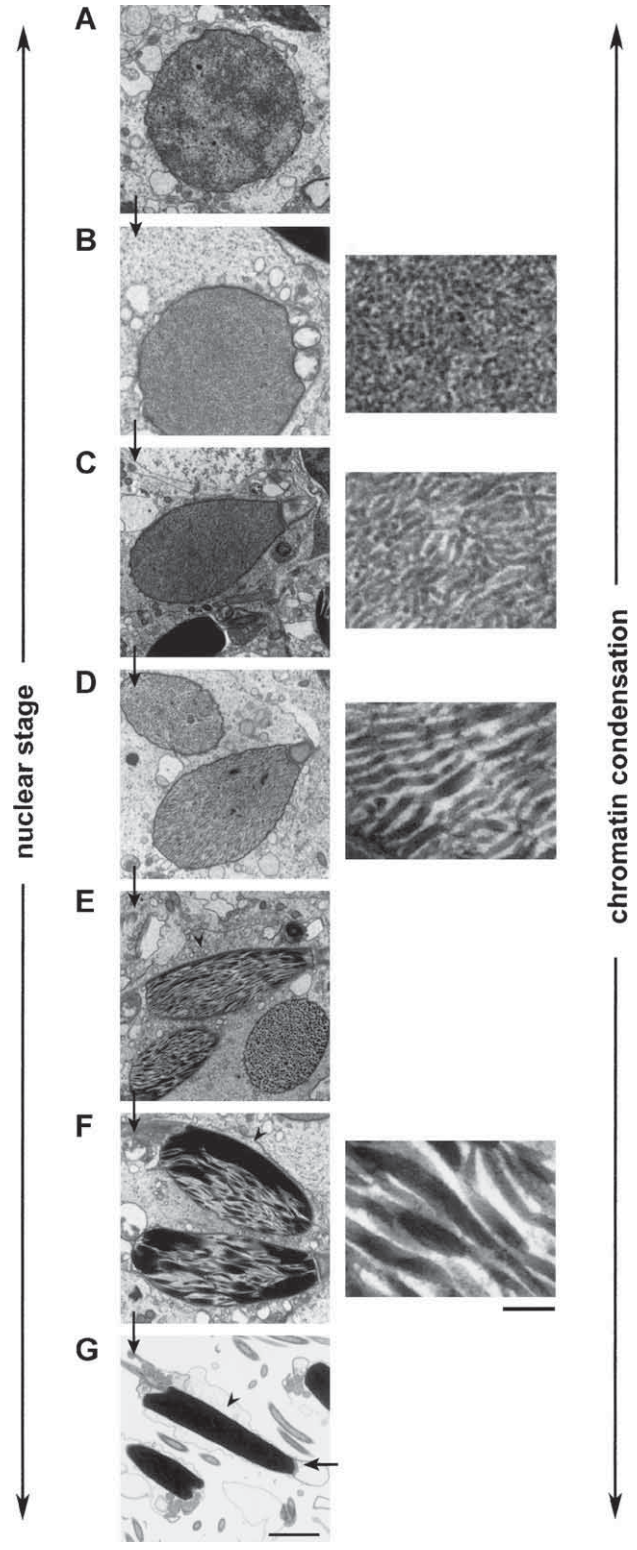


Fig. 1. Spermiogenic nuclei and chromatin in *S. officinalis*—comparison of different stages of spermatid nuclear shape (left) and their correspondence with chromatin structure (right). Phases A–D constitute the "patterning" of chromatin, whereas phases E–G represent the final chromatin condensation process, bringing about a reduction in nuclear volume. Arrowheads in E, F, G indicate the dorsal zone of respective nuclei. Arrow in G: acrosome. Bars—left: 2 μm; right: 200 nm.

sectional plane, its volume does not present any substantial variation.

It is interesting to point out that the acquisition of a homogeneous chromatin distribution before the initiation of its condensation is a phenomenon that is produced in most species observed (Ribes et al., 2001). This could represent a necessary step for proper chromatin remodelling and condensation in the following steps of spermiogenesis.

In the next step (stages B → C in Fig. 1) the granular chromatin is newly remodelled, acquiring a homogeneous organization into 25–30 nm diameter fibres. Nuclei containing this type of chromatin are spindle shaped (Fig. 1C), with the narrow end positioned adjacent to the acrosome, which is in formation.

This chromatin remodelling is not confined to only 25–30 nm fibres. Chromatin is globally transformed into fibres thicker than 40–45 nm in diameter (stages C → D in Fig. 1). This figure also shows that during the transition of 25–30 nm fibres to become 40–45 nm fibres, the nuclear shape and volume do not undergo significant changes. These observations are especially interesting, since it seems to indicate that in *S. officinalis* the internal chromatin remodelling is a process which does not directly affect the shape of the spermiogenic nucleus. An equivalent situation has been described in another gastropod mollusk (Ribes et al., 2001), supporting that changes in chromatin structure exert a permissive effect, instead of a direct effect, over nuclear shaping during the first stages of spermiogenesis.

Conformational changes in the spermiogenic chromatin as understood from stage A to stage D (Fig. 1) are denominated here as "patterning phases", since in each stage the chromatin is uniformly distributed and organized throughout the nucleus. As a consequence, the chromatin is subject to global changes in the DNA interacting proteins (Martínez-Soler et al., 2007). However, from stage D onward, the 40–45 nm diameter fibres are not remodelled into any other structure, but are progressively joined (phases E → G in Fig. 1). We call these last stages "condensation phases" which drive toward a uniform condensation of the spermiogenic nuclear chromatin (Fig. 1G). During condensation phases the electrostatic interaction between DNA and protamine is also increased (Martínez-Soler et al., 2007), provoking the joining of 40–45 nm fibres, which can be observed in the mentioned phases E → G.

During the course of these phases the spermiogenic nucleus is progressively constrained, but this compression is produced by recruitment of the condensing chromatin toward one side of the nucleus, denominated here as the "dorsal zone" (arrowhead in Fig. 1E–G).

The results obtained indicate that during the period of patterning (phases A → D) one type of nuclear shape is compatible with different types of chromatin structures. For instance, round nuclei may contain somatic-like or granular chromatin (A–B), whereas spindle-shaped nuclei may contain different types of chromatin fibres (C–D). We interpret that changes in the chromatin structure produced during this

period do not have any important influence on nucleomorphogenesis. However, during the period of condensation (phases E → G) the union of chromatin fibres determines the shaping that the mature spermatic nucleus will undergo.

3.2. Appearance of the perinuclear microtubules: relation to the nuclear membrane and condensing chromatin

Perinuclear microtubules are one component which appears in spermiogeneses in most animal species. In cephalopods they represent a transitory structure, since in the final stages of spermiogenesis, once the chromatin is completely condensed, they disappear from the cell and are not present in the spermatozoon (Maxwell, 1975; Giménez-Bonafé et al., 2002a). Their function is hence also transitory, and related to nuclear changes, whether acting as substrates of molecular exchange between the nucleus and cytoplasm (Kierszenbaum, 2002), or whether as a physical factor which determines the mature spermatic nuclear shape, as has been demonstrated in *Eledone cirrhosa* (Giménez-Bonafé et al., 2002a, 2002b). In this section we describe the appearance and organization of perinuclear microtubules in the different stages shown in Fig. 1.

3.2.1. Remodelling/patterning phases (stages A → D)

The detailed examination of diverse images corresponding to stage A, as well as immunolocalizations of α and β tubulin, indicate that perinuclear microtubules still have not begun to organize (results not shown). The first observed microtubular structures appear in stage B. The spermatids in this stage (see also Fig. 2, inset) are polarized such that the growing axoneme and mitochondria are located in the basal part, while in the apical portion, the acrosome begins to form. The microtubules have already begun to attach themselves to the nucleus. Their organization is always originated from the basal part, and presents two additional characteristics: (a) it is still incomplete: in stage B the microtubules have not arrived at the acrosome which is in formation (see proacrosomal vesicles in the upper part of inset in Fig. 2), and (b) it is asymmetric.

As may be partially observed in Fig. 2 (showing a detail of the basal zone of a stage B nucleus), one part of the microtubules start from the basal portion following the meridian of the nuclear dorsal zone (arrows), whereas in the opposite part, or ventral zone, the microtubules are obliquely deposited. As a consequence of their slanted position, they do not have a defined appearance, neither in electron microscopy preparations, nor in immunolocalization of tubulin.

The following step of spermiogenic differentiation leads to spermatids of phase C (Fig. 1). This involves a series of simultaneous changes in the cell, namely: (a) complete formation of the proacrosomal vesicle attached to the nucleus (Figs. 3 and 4A); (b) appearance of an electrodense zone which serves as an anchor for the microtubules to the basal portion (arrowhead in Fig. 3; see also Fig. 4B and Fig. 8); (c) complete microtubular growth until arriving at the acrosomal apex (arrowheads in Fig. 4A); and, (d) a chromatin remodelling acquiring a pattern of 30–35 nm fibres (Fig. 1C).

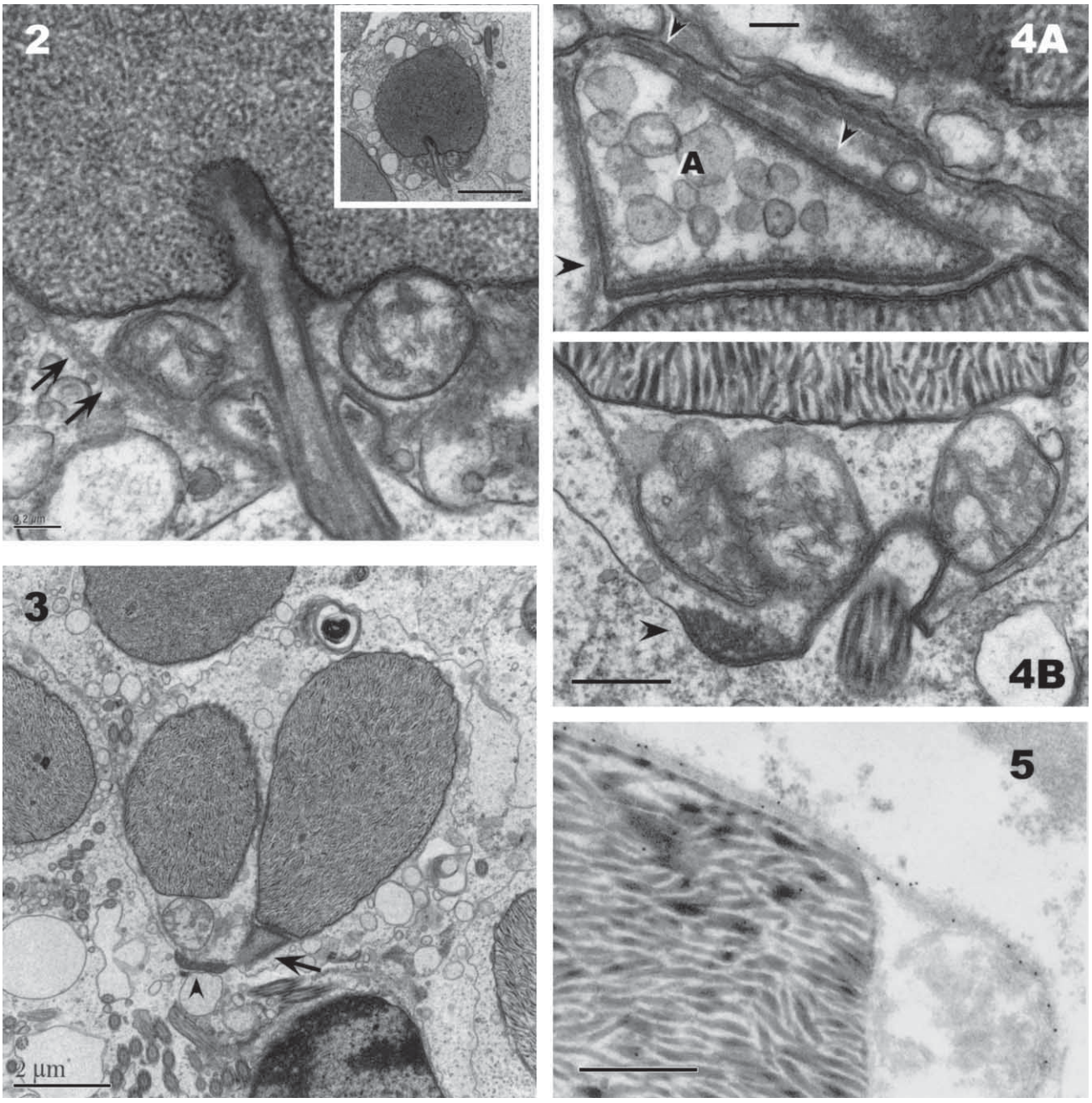
No significant variation in the nuclear shape occurs during the transition stage C → D, nor in the indicated cytoplasmic structures. The only observable morphological change is the third chromatin remodelling driving the 30–35 nm fibres to become thicker fibres of 40–45 nm. For this reason we discuss phases C and D as only one entity.

In these phases, the nucleus has suffered an apical compression and appears slightly spindle shaped. This is due to the growing microtubules adapting to the acrosomal surface, which provokes constriction of the nuclear apex (Fig. 4A). Throughout the basal zone, the microtubules seem to influence the position of the mitochondria (Figs. 4B and 5), and terminate anchored to electrodense basal zones (Fig. 4B). In spite of a clear reaction of microtubules with anti-α and anti-β-tubulin antibodies, we have not found any evidence that electrodense basal zones cross react with these antibodies.

In these phases a significant relationship among chromatin, nuclear membrane, and microtubules begins to be established, which will determine nuclear morphogenesis. Similar to the spermiogenesis of *Octopus vulgaris* (Ribes et al., 2004), chromatin fibres are attached perpendicularly to the apical and basal areas of the nuclear membrane (Fig. 4A and B), but moreover, these fibres establish interactions with other areas of the nuclear membrane. In Figure 6 and those that follow, it is shown that only those chromatin fibres closest to the nuclear dorsal zone (arrow in Fig. 6) run parallel to the axis of nuclear elongation. This disposition is due to the interaction *microtubules ←→ nuclear membrane ←→ chromatin fibre* (see Fig. 7). In this last figure it is shown that microtubules establish a clear association with the nuclear membrane, and that chromatin fibres are found longitudinally attached to these areas. All the observed samples give a strict correspondence between microtubules and chromatin fibres. In the ventral zone the microtubules are found associated in small separated clusters of 3–5 units (see details below in Fig. 12). Microtubules associated with the ventral nuclear zone follow oblique directions with respect to nuclear meridians, and the attachment of chromatin fibres to the nuclear membrane is different from those in the dorsal zone.

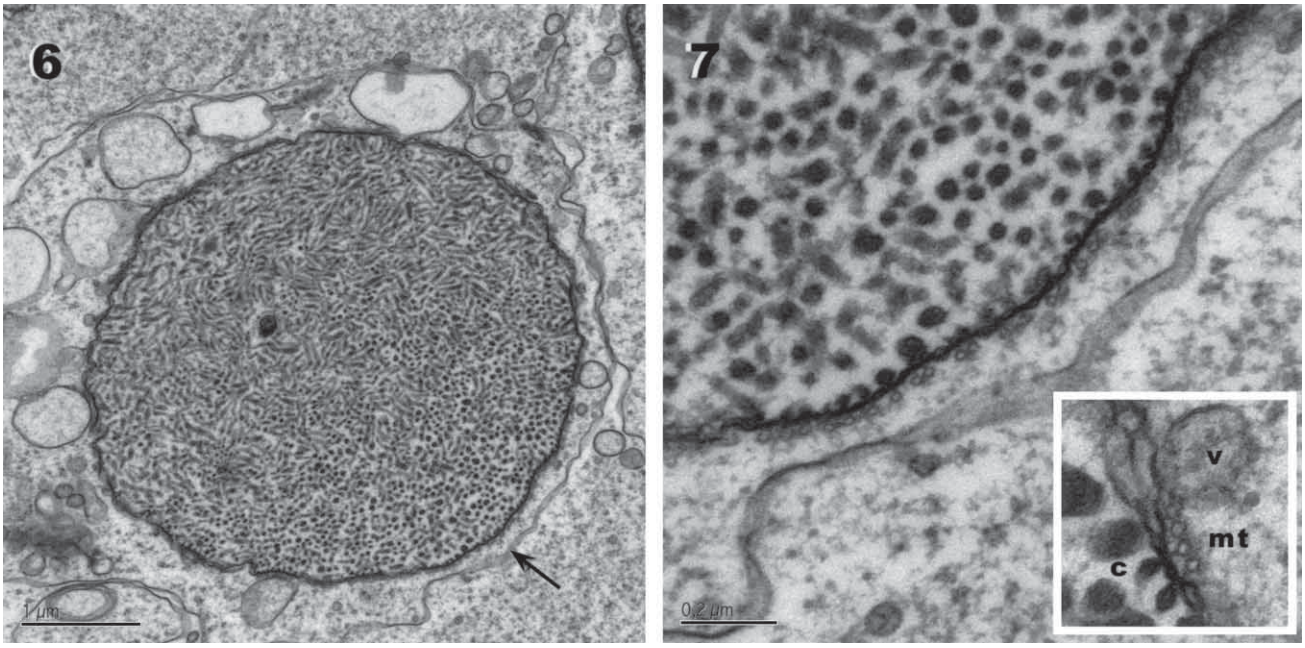
3.2.2. Final phases (E → G)

The starting point of the final phases is represented in Fig. 8. Transverse sections (Fig. 8, left) show the initial tendency of condensing chromatin to concentrate preferentially towards one side of the nucleus. The longitudinal and oblique sections of the same figure show that the area where chromatin is concentrated corresponds to the nuclear dorsal zone and that the microtubules contiguous with the membrane follow the same direction as the chromatin fibres. These chromatin fibres anchor longitudinally along the dorsal membrane, whereas in the basal, apical, and ventral parts



Figs. 2–5. (2) Spermatid in phase B. A global view of the spermatid section is represented in the inset (bar = 2 μm). The arrows indicate growing microtubules in the basal area. These microtubules continue growing from the basal direction toward the apical portion. (3) Spindle-shaped spermatids (phases C and D). Chromatin of these spermatids is now organized into fibres and the microtubules have completed their growth. The arrowhead indicates the electrodense basal area which appears in all spermatids from this phase on. Arrow—Acrosome. (4A) Apical and dorsal details of spindle spermatids. The microtubules (arrowheads) completely surround the acrosome (A) but there is no apparent structure anchoring the microtubules in the apical zone. Chromatin fibres are perpendicularly attached to the nuclear membrane of the apex. Bar—0.2 μm. (4B) Anchoring of microtubules to the electrodense basal areas (arrowhead) can be appreciated in this section. The chromatin fibres are perpendicularly joined to the basal nuclear membrane. Bar—0.5 μm. (5) Labelling with anti-α-tubulin. Microtubules grow permanently associated to the mitochondria, nucleus, and acrosome. Although not analyzed here in detail, the distribution of mitochondria in the middle piece of the spermatozoon (Maxwell, 1975; see also Fig. 4B) seems to be a partial consequence of the microtubule distribution during spermiogenesis.

of the nucleus the chromatin is attached perpendicularly or slantingly to the internal nuclear membrane. In Fig. 8, two details related to this process can be observed. First, an elevated concentration of cytoplasmic vesicles is found in the nuclear ventral zones. These vesicles come from the elimination of part of the nuclear membrane and ventral nucleoplasm. Second, the microtubules of the dorsal zone beneath the nucleus appear always in nearly longitudinal sections (arrows), while in the ventral zone they appear in slanted sections, and therefore are poorly defined. The origin



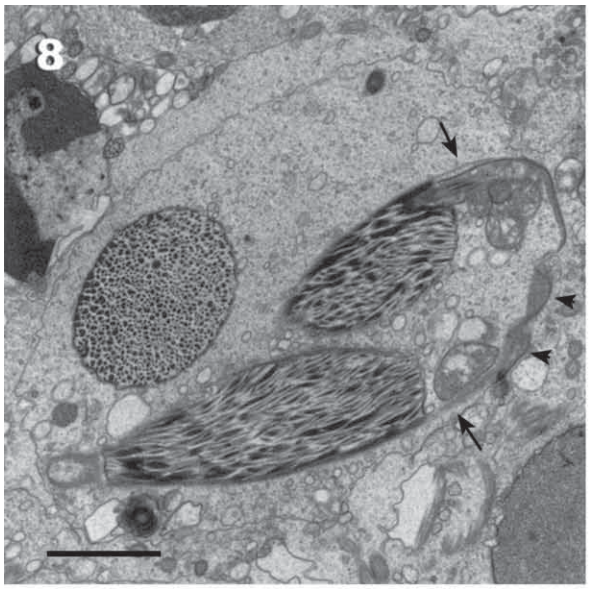
Figs. 6–7. (6) Transversal section of a spindle-shaped spermatid. Only those chromatin fibres closest to the nuclear dorsal membrane (arrow) appear in this transversal section, while in the rest of the nucleus the fibres appear slanted in relation to the plane of the section. (7) Detail of the dorsal zone of Fig. 6. The peripheral chromatin fibres and microtubules follow parallel directions and establish interactions between each other through the nuclear membrane. The longitudinal anchoring of the chromatin to the dorsal membrane is essential for the following processes implicated in nucleomorphogenesis. Inset: Establishment of the association [*microtubule* ←→ *nuclear membrane* ←→ *chromatin fibre*]. mt—microtubules, c—chromatin, v—vesicle formed from part of the nuclear membrane not associated with microtubules/chromatin.

of this distribution can be appreciated in the detail presented in Fig. 9.

In the following stages (Figs. 10–12) chromatin fibres continually fuse and are simultaneously recruited dorsally where they coalesce with other pre-existing fibres. The details (inset in Figs. 10–12) demonstrate once again the different running directions of dorsal and ventral microtubules. An important and noteworthy observation is that in transverse sections of spermatids the ventral microtubules appear in discrete groups slanted in a direction referring to the plane of the section (Fig. 12, arrows). Observed in this figure is an area where chromatin (arrowheads) follows the lines of tension corresponding to recruitment toward the dorsal zone. This chromatin displacement pulls certain areas of the ventral nuclear membrane (sites where the chromatin-membrane-microtubule complex has been established), while the rest of the ventral nuclear membrane is eliminated in the form of vesicles of various sizes. This observation suggests not only that the microtubular "contraction" is the cause responsible for this nuclear compression, but also for the increase in the interaction between chromatin fibres. A representative scheme of this process is expressed in Fig. 14.

The culmination of this process produces a completely condensed nucleus (Fig. 13) with a circular section of 1 μm in diameter and completely surrounded by microtubules, which later are eliminated from the cell (not shown). The immature spermatozoa found in the first stretches of the epididymis have already irreversibly lost their microtubules. Other aspects of spermatic differentiation which are produced in the epididymis and spermatophores do not directly affect

Figs. 8–13. (8) Nuclei in initial phases of lateral compression (phase E). The chromatin has begun to accumulate toward the nuclear dorsal zone. Observe that the type of anchorage of dorsal and ventral zones is different. There are also abundant vesicles which detach from the ventral nucleus. The dorsal microtubules (arrows) pull longitudinally from the dorsal base, but the ventral microtubules do not clearly appear in these sections. Arrowheads—electrodense basal zones; bar—2 μm. (9) Origin of microtubular distribution. The dorsal microtubules (arrow) start longitudinally along the base, while the ventral ones (arrowhead and inset) initiate in slanted directions associated in small groups of 3–5 units. (10) Recruitment of the chromatin toward the nuclear dorsa. Shown here are two spermatids in transversal and quasi-longitudinal sections. The nucleus is condensed through the union of fibres which are simultaneously pulled toward the nuclear dorsa. The ventral area continually expels nuclear membrane and nucleoplasm. The distribution of microtubules in these phases is represented schematically in Fig. 14. Inset—reaction of anti-α-tubulin observed by fluorescent optical microscopy. The dorsal nuclear zone appears labelled continuously. (11) Dorsal microtubules—they appear adjacently deposited and, together with the chromatin, form a rigid structure which attracts the condensing chromatin. (12) Ventral microtubules—they appear in small groups (arrows) and in sections preferentially slanted. Part of the chromatin follows tension lines (arrowheads) which indicate the direction of recruitment toward the dorsa. Membrane expulsion in form of vesicles can be observed, as well. (13) Fully condensed nucleus (transverse section)—the culmination of both chromatin agglutination and the elimination of nuclear membrane not associated with microtubules or nucleoplasm leads to a nucleus with nearly homogeneous compaction, completely surrounded by microtubules. Arrows indicate the dorsal nuclear zone, while in other areas of the section microtubules are still residually oblique (arrowheads). Inset: detail of the periphery.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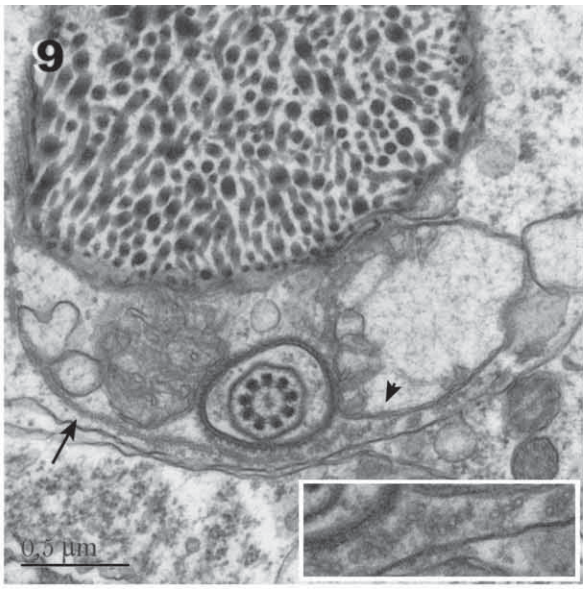
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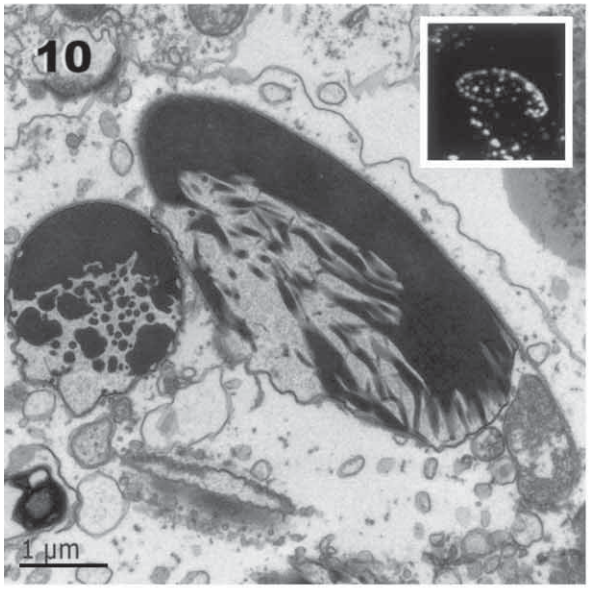
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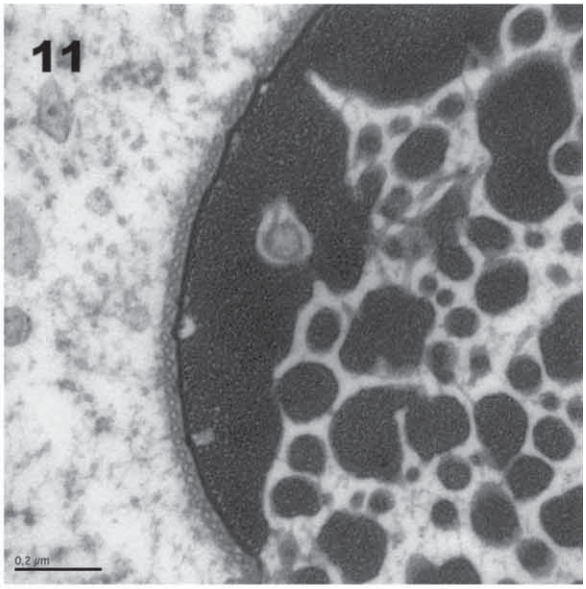
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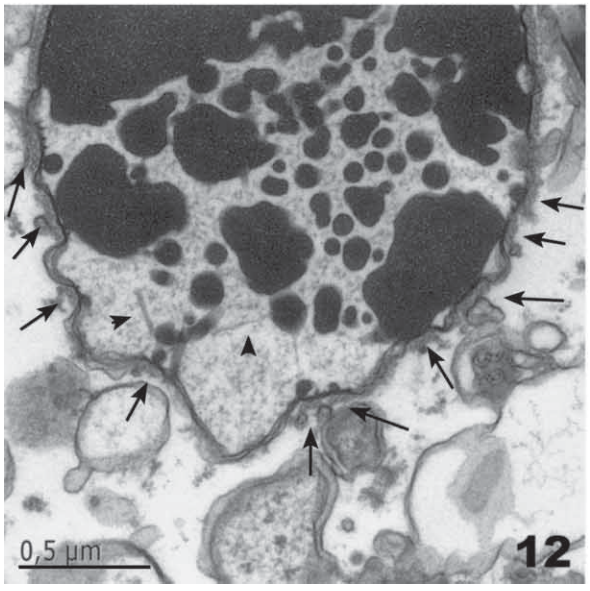
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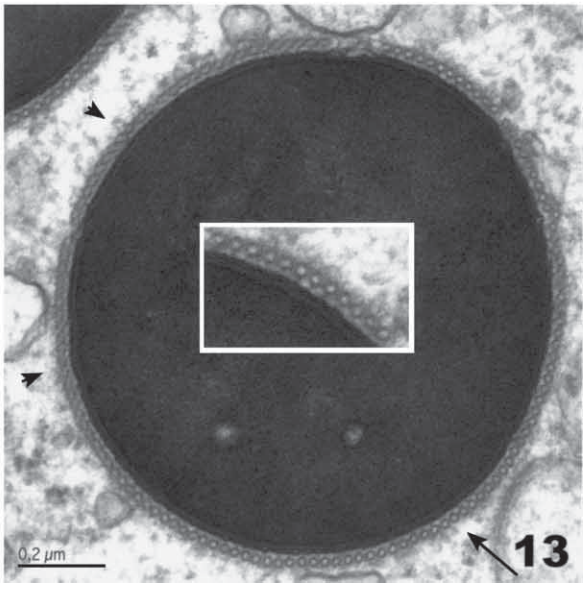
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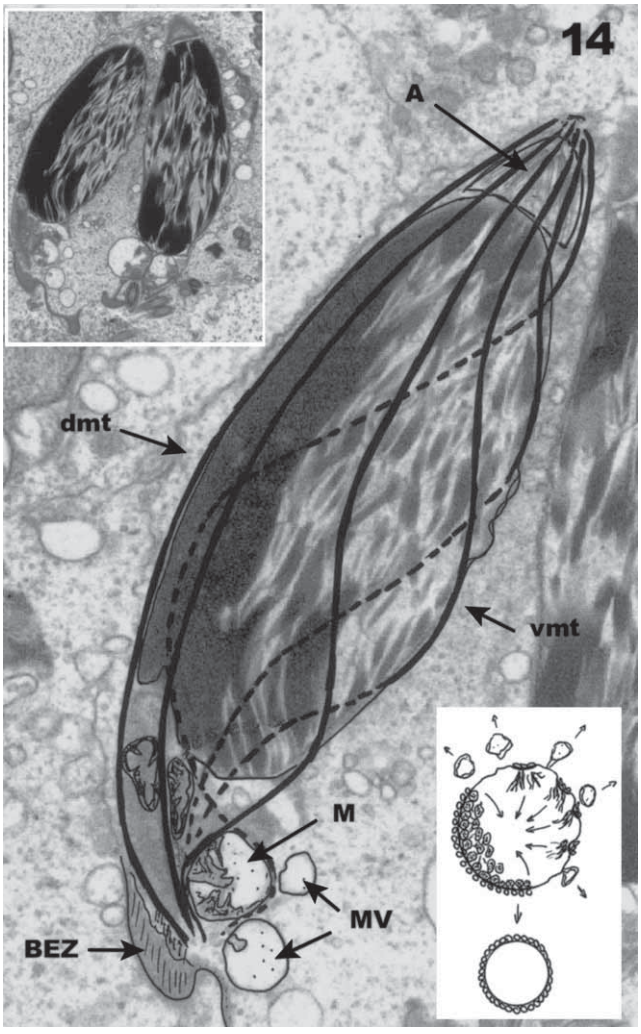

Fig. 14. Diagram idealizing the geometry of microtubules around the nucleus. The spatial distribution of microtubules in the stages of condensation/recruitment of spermiogenic chromatin has been drawn over the image of a spermatid (top, left). In the lower inset, there is an attempt to represent the transversal recruitment of chromatin and reduction of nuclear volume, as well as the elimination of the nuclear membrane and nucleoplasm. A—acrosome; dmt—dorsal microtubules; vmt—ventral microtubules; M—mitochondria in process of reduction; VM—vesicles produced by mitochondrial reduction; BEZ—basal electrodense zone.

the process of nucleomorphogenesis and are not considered in this work.

4. Discussion

The results obtained give evidence that during nucleomorphogenesis of *S. officinalis* there are three processes of predominant importance:

a.- the organization pattern of microtubules around the nucleus,
b.- the condensation pattern of chromatin,
c.- the interaction between microtubules and chromatin through the nuclear membrane.

(a).- The perinuclear microtubules in spermiogenesis are complex structures that develop mechanical functions, cytoplasmic transport, and have a role in the interchange of molecules between the nucleus and the cytoplasm (Kierszenbaum, 2002). The totality of perinuclear microtubules of *S. officinalis* (and other cephalopods) can be considered analogous to the manchette of mammalian spermatids. Both types of spermatogeneses also have in common a very obvious change in the nuclear morphology, as well as some transitions in the DNA interacting proteins.

The perinuclear microtubules of *S. officinalis* originate from electrondense zones located in the base of the spermatid head and grow toward the acrosome, while always attached to cellular organelles (mitochondria → nucleus → acrosome). They are organized in a special geometry (Fig. 14): they preferentially build up on the dorso-nuclear membrane, following the direction of the nuclear meridians, whereas in the ventral part, they grow associated in clusters that are spread out and separated. The microtubules of *S. officinalis* show a cross reaction with anti-α-tubulin (Figs. 10—inset, and 5), as well as with anti-β-tubulin, although the latter with less intensity (not shown). However, we ignore the possibility that the microtubules of cephalopods contain other minor types of tubulin which also constitute the manchette of mammalian spermatids (Smrzka et al., 2000; Oakley, 2000; Kato et al., 2004).

The accumulation of microtubules in the dorsal nuclear zone is an aspect of great importance in sperm nucleomorphogenesis of *S. officinalis*, since it forms a rigid structure that constitutes a recruitment surface for chromatin, and along with it, for the spermatic nucleus. The distribution of microtubules around the nucleus differs from the spermiogeneses of other cephalopods such as *Octopus* and *Eledone* (Giménez-Bonafé et al., 2002a) in which the nucleus does not undergo lateral compression, but rather, an elongation.

(b).- The spermiogenic chromatin condensation pattern of *S. officinalis* is similar to that of *Octopus* and clearly differs from *Eledone*. This is due to the fact that transitions of nuclear spermiogenic proteins and protamines occurring in *Sepia* are quite similar to those which take place in *Octopus*, but completely different from the ones of *Eledone* (compare Giménez-Bonafé et al., 2004 and Martínez-Soler et al., 2007 with Wouters-Tyrou et al., 1991 and Giménez-Bonafé et al., 2002b). It is necessary to point out that the pattern of chromatin condensation does not only depend on the chemical nature of the protamine, but also on the types of transitions and substitutions of nuclear proteins throughout spermiogenesis. Thus, while in *Octopus* and *Sepia* histones are replaced by protamine precursors, which later suffer deletions, in *Eledone* histones seem to be replaced directly by a very special protamine exceptionally rich in cysteine residues.

During the condensation of spermiogenic chromatin of *S. officinalis*, two essentially different parts can be distinguished. First, a period of remodelling, where all areas of nuclear chromatin suffer the same type of change: to become granules and later fibres. It is important to note that chromatin remodelling does not seem to be directly related to nuclear morphogenesis (note that remodelling A → B and C → D occur without a concomitant change in the nuclear shape). Then, a period of condensation (or recruitment), in which the fibres coalesce together. It is only in this second period that lateral compression takes place. A progressive increase in the interaction between chromatin fibres causes both agglutination and recruitment of those fibres fixed on the dorsal nuclear membrane. In these stages it is evident that the origin of general processes which modulate nuclear shape lies on the arrangement of dorsal microtubules.

(c).- As we have pointed out previously, in all images observed during our study, an absolute correlation has been noticed among these three components: [perinuclear microtubules ←→ nuclear membrane ←→ chromatin fibre]. An antecedent of this observation was done by Bergstrom and Arnold (1974) in the squid *Loligo pealei.*

The relation between microtubules and spermiogenic chromatin through the nuclear envelope has been studied at the morphological level in several instances (Bergstrom and Arnold, 1974; Jamieson, 1981), but at the functional level is a poorly known subject that has been reviewed in mammals by Kierszenbaum (2002). The perinuclear microtubule system does not only represent a structural element responsible for rigidity and shape of the spermatic nucleus, but also has a role in nucleocytoplasmic transport. The possible functional role of Ran GTPase is especially interesting. This molecule could participate in the interchange of basic proteins (Kierszenbaum, 2002), and/or could stimulate the direct attachment of chromatin to the nuclear envelope (Bilbao-Cortés et al., 2002). Our morphology results show that the nuclear envelope effectively shows changes in the contact zones with microtubules (Fig. 7), and that these changes are deduced to provoke attachment of condensing chromatin.

The most important general conclusion concerning microtubules is their main functional role in consecutive shaping of the cephalopod sperm nucleus. When spermatic nucleomorphogenesis of *S. officinalis* is compared with *Octopus,* it is observed that chromatin condensation is quite similar in both species, but their nuclei exhibit different shapes due to a different microtubular distribution. These differences determine the way in which condensing chromatin is spatially organized (compare this article with Ribes et al., 2004), and the final morphology of the ripe sperm nucleus.

The point of view explained here for the case of cephalopods does not completely coincide with the opinion of Fawcett et al. (1971). These authors suggest that the chromatin condensation is the main factor in shaping the sperm nucleus, but not the microtubules (manchette). The conclusions of this work are based on the available information in 1971, according to which there is a high degree of order during the packaging of chromatin. Also the chromosomes retain their individuality during spermiogenesis and they become arranged and highly ordered in the condensed sperm nucleus (Inoué and Sato, 1962; Taylor, 1964; Koehler, 1970). Thus, the organized way in which the genetic material becomes ordered during spermiogenesis determines internally the shape of the nucleus. On the other hand, the authors conclude that microtubules are probably not a nucleomorphogenic factor because (among other reasons) the heads of spermatozoa vary greatly in shape without a corresponding diversity in the arrangement of microtubules in different species. In a more recent review, Meistrich (1993) exposes an opposite opinion concerning the effect of chromatin and microtubules on the nuclear shape of spermatozoa. Meistrich (1993) claims that, among other reasons, the specific DNA sequences (including the whole Y chromosome in humans) reveal no obvious specificity with respect to position within the sperm nucleus. Meistrich (1993) also states that a variety of genetic conditions, such as chemical treatments and specific mutations of the β2-tubulin gene, cause nuclear abnormalities as a consequence of manchette irregularities in spermatids and spermatozoa.

Another possibility is that in some types of spermiogenesis, microtubules may be the morphogenic factor of the sperm nucleus, whereas chromatin condensation may be the main factor in other types of spermiogenesis. It is necessary to continue the study of this possibility.

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Transition of Nuclear Proteins and Chromatin Structure in Spermiogenesis of *Sepia officinalis*

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ABSTRACT During spermiogenesis of *Sepia officinalis* histones are directly substituted by a molecule of precursor protamine, which is later transformed into the protamine through a deletion of the amino terminal end. In the present work, it is shown that the pattern of spermiogenic chromatin condensation consists of a phase of "patterning" and a phase of "condensation." In the phase of patterning, three structural remodelings are produced in the chromatin structure: [*somatic-like chromatin → 18 nm granules → 25 nm fibers → 44 nm fibers*]. The first remodeling of the chromatin into granules of 18 nm takes place without the entrance of specific proteins in the spermiogenic nuclei. The second remodeling [*granules of 18 nm → fibers of 25 nm*] is due to the entrance of the precursor protamine and its interaction with the DNA–histone complex. The third remodeling [*fibers of 25 nm → fibers of 44 nm*] occurs simultaneously with the disappearance of histones from the chromatin. In the phase of condensation, the fibers of 44 nm coalesce among themselves to form progressively larger aggregates of chromatin. In this phase there are no substantial variations in the nuclear proteins, so that the condensation of the chromatin must respond to posttranscriptional changes of the precursor protamine (dephosphorylation, deletion of the amino-terminal end). *Mol. Reprod. Dev. 74: 360–370, 2007.* © 2006 Wiley-Liss, Inc.

Key Words: cephalopod; decapod; sperm; nucleus; DNA-interacting proteins

INTRODUCTION

The genetic material of animals condenses throughout spermiogenesis following a temporal order in its morphology. This process, which implies very important structural changes, denominates the "pattern of condensation" of the spermiogenic chromatin.

The pattern of condensation of the chromatin in spermiogenesis is related to certain functions which develop within two different levels of biological organization. Firstly, referring to the molecular level, the pattern of condensation organizes the genome into an extraordinarily compact form, easily reversible during fertilization. It is also very probable that this condensed genome is not distributed randomly within the mature spermatic nucleus (Wykes and Krawetz, 2003) since some specific parts occupy concrete or preferential positions (Zalensky et al., 1993; Joffe et al., 1998; Hazzouri et al., 2000a). The nonrandom distribution of the genome within the sperm nucleus may also have importance in the first steps of embryonic development (Ward et al., 1999). All of this implies that the spatial organization of the genome within the spermatic nucleus and its consequences depend at least partially on the route the chromatin has followed in its condensation during spermiogenesis.

Next, within the cellular level, the pattern of condensation of the spermiogenic chromatin exerts an important permissive effect on the shape that the mature spermatic nucleus adopts (Ribes et al., 2001). The spermatic nuclear form is quite variable among distinct animal species (see for instance Jamieson, 1991; Jamieson et al., 1995, 1999) and is found highly adapted to both physicochemical characteristics of the environment that surrounds the oocyte, as well as to particular properties of the membrane and the size of the oocytes (Bedford, 2004).

Due to the immense importance on the viability of the nucleus and the spermatic genome for the biology of fertilization, and hence for the survival of biological species, the pattern of condensation of the spermiogenic chromatin must be a process affected by strong cellular and evolutionary controls.

If the reduction of the nuclear volume is a practically universal phenomenon in spermiogenesis, among each distinct animal species the spermiogenic chromatin condensation pattern is evolutionarily highly variable. This pattern depends upon the temporal organization of the changes in the interactions between DNA and nuclear proteins; in other words, the type of interaction

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between nuclear proteins and DNA, as well as the progressive change of these interactions during spermiogenesis. Until now, a great amount of the DNA-interacting proteins have been studied in many diverse spermiogeneses (Kasinsky, 1989; Chiva et al., 1995), but the way in which these interactions determine the different patterns of condensation has been left practically unanalyzed. In the first attempt to theoretically approach this problem, we have recently proposed that during the first part of spermiogenesis the dynamic aspects of the interaction between proteins and DNA could have importance equal to or greater than the type of protein itself (Harrison et al., 2005).

For the majority of species, the types of nuclear histones are substituted by specific proteins during spermiogenesis, while in some other species, histones can be found (and therefore nucleosomic organization) in the mature sperm nuclei. In the spermiogeneses in which histones are substituted by specific proteins, a reduction of spermatic nuclear volume is achieved, much greater than those in which the exchange of proteins is not carried out (Ward and Coffey, 1991; Daban, 2000). In the rest of this introduction, we will only refer to those cases in which a substitution of nuclear proteins is produced.

The simplest pattern of condensation of spermiogenic chromatin is found in the species in which histones of the early spermatid are substituted directly by protamine (a protein exclusive of the sperm nucleus). Many examples of this type of substitution (in which other chemical processes also participate, such as acetylation of histones and phosphorylation/dephosphorylation of the protamine (Oliva and Dixon, 1991)) may be found among fish, amphibians, birds, and mollusks. In all of these cases, the DNA is condensed in granules which grow progressively until adopting a size, depending on the type of protamine. In these species, the mature spermatic nuclei have a relatively simple form: they could be round, oval, or elongated, but never present very elaborate forms, such as elongated nuclei furrowed by the axoneme, etc.

Nevertheless, from the relative simplicity that the transition [*DNA-histones* → *DNA-protamine*], evolution has produced a large number of modifications that increase the complexity of the reaction of the spermiogenic exchange of DNA-associated protiens. Simultaneously, these changes also present an increase in the morphological complexity of the condensation patterns of the spermiogenic chromatin, and in certain cases, they can be found associated to very elaborate shapes of mature spermatic nuclei. The case of mammals illustrates the most complex transition of proteins known. Without taking into consideration the posttranscriptional modifications (acetylation, polyubiquitination, phosphorylation, etc.), the histones of the early spermatids are partially replaced by testicular specific histones (or preferential ones), and these are replaced progressively by several transition proteins; afterwards the transition proteins are substituted by protamines, some of which appear in precursor form to be transformed later on into the mature protamine in the final stages of spermiogenesis (Balhorn et al., 1984; Meistrich, 1989; Churikov et al., 2004; Govin et al., 2004).

In this work we present a detailed study of the condensation of the spermiogenic chromatin of the decapod cephalopod *Sepia officinalis*, including a re-examination of the types of gonadal histones, as well as an attempt to correlate the condensation pattern of the chromatin with the concrete exchange of nuclear proteins. There are two main reasons why we have chosen the model *S. officinalis*. Firstly, if we consider that the simplest transition of DNA-interacting proteins is the reaction [*DNA-histones* → *DNA-protamine*], the case of *S. officinalis* exhibits the substitution reaction representing the next order of complexity, meaning:

$$[DNA - histones \rightarrow DNA - precursor\ protamine \rightarrow - protamine]$$

(since here histones are replaced by a precursor to the protamine which posteriorly is transformed into the protamine; see Martin-Ponthieu et al., 1991; Wouters-Tyrou et al., 1991; Fig. 1 and Discussion for more details). As a consequence, this case may illustrate an added complexity to the relatively simple patterns of chromatin condensation which appear in fish, amphibians, birds, etc. Secondly, the model of *S. officinalis* allows us to complete a series of studies which we previously began, concerning the evolutionary changes in nuclear proteins, chromatin condensation pattern, and the spermatic nuclear form in cephalopods (Giménez-Bonafé et al., 2002a,b, 2004; Ribes et al., 2002, 2004. See also Martínez–Soler et al. accompanying article).

MATERIALS AND METHODS

Animals

Various specimen of the cuttlefish *S. officinalis* were used for this experiment. The animals were captured in the Mediterranean Sea (off the northeast coast of Spain) and immediately transported to the laboratory for further processing.

Nuclei and Chromatin

With the purpose to study the proteins associated with the chromatin, populations of total nuclei have been obtained from the gonads, as well as spermatic nuclei of the deferential conduct and epididymis of *S. officinalis*. In order to make a comparison with this experiment, nuclei were also obtained from the prostate gland of the same species. The collection of nuclei has been carried out by the method described previously in Giménez-Bonafé et al. (2004). Before extracting the proteins with 0.4 N HCl, the nuclei were first homogenized with 10 mM Tris pH 7.4, 10 mM EDTA, and centrifuged (5,000*g* for 10 min). This process was later repeated with 1 mM Tris pH 7.4. These washes are hypotonic solutions which partially swell the nuclei and facilitate the solubilization of nuclear proteins. From the gonadal nuclei a fraction resistant to sonication was obtained (Rousseaux-Prévost et al., 1988) which in our

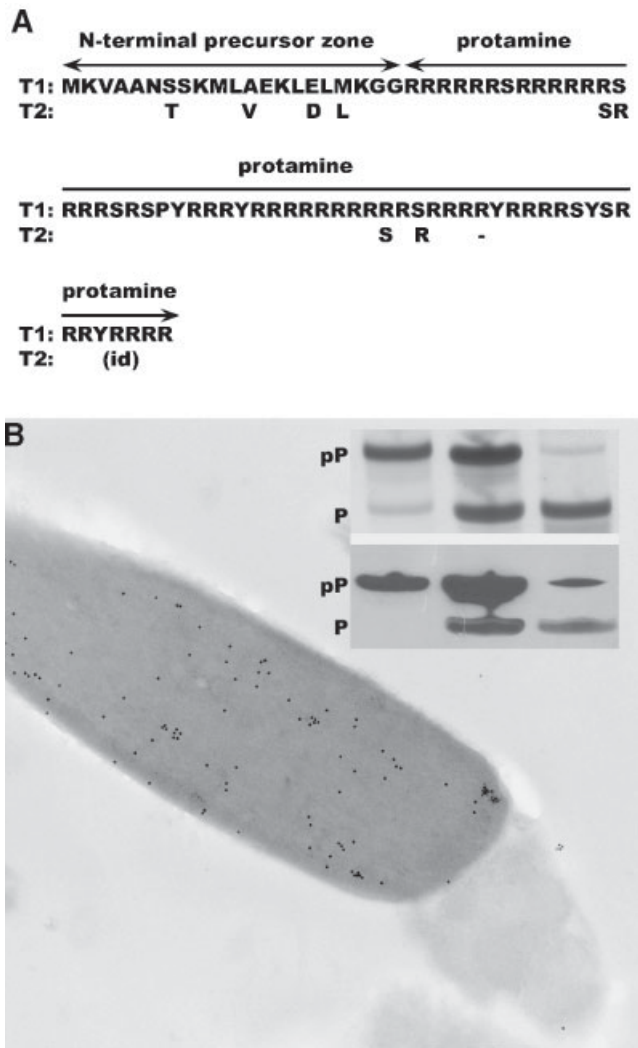

Fig. 1. Sequence of the precursor and the protamine and obtaining of antiserum. **A**: Amino acid sequence of the two microheterogenous forms T1 and T2 of the protamine precursor (from Wouters-Tyrou et al., 1991). The residues not noted for T2 are identical to those of T1. The first 21 residues of the N-terminal portion compose the strictly precursor part of the molecule, which will be eliminated in the most advanced stages of spermiogenesis. **B**: Control of antiserum: The right part of the figure shows an electrophoresis of distinct concentrations of the precursor (pP) and the protamine (P) (top inset), and the reaction that these proteins present with the obtained antiserum in Western blot (bottom inset). In spite of the fact that the antiserum reacts with much more intensity to the precursor, this molecule elicits an important cross-reaction with the protamine. In the left part of the figure, immunolabeling of the antiserum obtained may be observed in an epididymal spermatozoal nucleus of *S. officinalis*. The nuclei of the epididymal spermatozoa only contain the protamine, due to a deletion of the precursor part in the gonad (see text). In spite of this, the antiserum exhibits a clear signal in these nuclei. (Conditions of labeling are described in Material and Methods.)

case consists of advanced spermatids and testicular spermatozoa.

Analysis of Proteins

The gonadal histones of *S. officinalis* and the protamine precursor have been purified by HPLC following the method described in Ausió (1988).

For the one-dimensional electrophoresis polyacrylamide/acetic acid/urea gels (PAGE) were used (Hurley, 1977), while the two-dimensional electrophoresis were developed in gels of polyacrylamide/acetic acid/urea/triton-X 100 (AUT) (for the first dimension) and SDS gels for the second dimension.

Amino acid analyses were carried out after hydrolysis in 6 N HCl at 110°C for 24 hr. No corrections were made for hydrolytic losses.

To identify the subfractions of histone H1 of the gonad and of somatic tissue of *Sepia*, we carried out electrophoresis followed by Western blot and immunodetection with the antiserum raised against the cuttlefish H1 immunogen (see below).

Antisera

Antiserum against the protamine precursor. The precursor molecule (see Fig. 1) was purified by HPLC, and was hydrolyzed with chymotrypsin. The resulting peptides were separated by HPLC, and have been analyzed by mass spectrophotometry, and the corresponding peptide at the amino terminal end of the molecule was isolated (this part of the molecule corresponds to the precursor portion which is deleted and eliminated in the last phases of spermiogenesis). From these fragments an antiserum was produced against the precursor following protocols which have been previously utilized (Cáceres et al., 1999). It is very important to point out here that in the control experiments which were carried out, the antiserum obtained does not react with histones, but presents a cross-reaction with the mature protamine molecule (Fig. 1B, Western blot). Evidence of this is also observed in electron immunomicroscopy controls: the epididymal spermatozoal nuclei contain the protamine molecule exclusively, but in spite of this are labeled with the obtained antiserum (Fig. 1B image). Consequently, the antiserum used should be considered to effectively label both the precursor form as well as the protamine form.

Antiserum against histone H2A. Histone H2A was extracted from calf thymus by the method of Johns (1967) and later was repurified by carboxymethylcellulose CM52 chromatography, as in Cáceres et al. (1999), but using a gradient of NaCl from 0.2 to 0.8 M. Later it was used to generate antiserum in the same way that the anti-precursor protamine antiserum was produced. The controls for antiserum activity that were carried out by Western blot (not shown) have demonstrated that the antiserum obtained is absolutely specific for histone H2A. Further, in the immunolabeling experiments, no cross-reaction of the antiserum is observed with very mature spermatids or with spermatozoal nuclei (see Results).

Antiserum against linker histone H1. An anti-histone H1 antiserum was obtained by selective solubilization of gonads with 5% PCA. The protein was later used to generate an antiserum, the same way in which the precursor protamine antiserum was produced.

Electron microscopy and immunomicroscopy. Small sections of gonad have been fixed and embedded in Spur resin for conventional electron microscopy, or in

Lowicryl resin for immunomicroscopy experiments (Giménez-Bonafé et al., 2002a).

For electron immunomicroscopy examination, we have tried out a variety of blocking conditions and dilutions of both antiserum and secondary antibody. The following working conditions are those which have been selected.

Anti-precursor antiserum. The nickle grids containing the ultrafine sections of gonadal or epididymal tissue were blocked for 30 min in 0.1 M PBS containing 4% BSA, 1% normal goat serum, 0.05% Tween-20 (w/v), and 20 mM glycine. The antiserum was diluted 1:600 and 1:1,000 in the same blocking solution, with the glycine omitted and incubated for 2 hr. The secondary antibody used was a 15 nm colloidal gold conjugated goat anti-rabbit antibody, diluted 1:25 in the same solution which was used to dilute the antiserum, and applied for 1 hr. All treatments were performed at room temperature. The tissue samples treated with anti-histone H2A antiserum were blocked in the same conditions as those treated with anti-precursor antiserum, and diluted 1:500. The same secondary antibody and dilution was used to detect H2A in these samples.

Under these circumstances the controls treated exclusively with secondary antibody produced minimal or no labeling in spermatic nuclei, and the general background conditions were the lowest observed. These are the only variations of the general method described previously (Giménez-Bonafé et al., 2002a) for electron immunomicroscopy.

RESULTS

Analysis of Nuclear Proteins in the Gonad of *S. officinalis*

The works previously cited (Martin-Ponthieu et al., 1991; Wouters-Tyrou et al., 1991) describe the general composition of the nuclear proteins in spermatogenesis and in spermiogenesis of *S. officinalis*, as well as go into more depth concerning the study of the primary structures of the precursor and protamine molecules. In this section we re-examine the protein transitions in the gonadal nuclei and apply a more detailed analysis of histones which have not been previously studied.

In Figure 2A the extracted proteins from total nuclei of an immature gonad (lane 1), mature (2), and very mature (3) can be observed. The rest of the electrophoresis shows the proteins obtained in nuclear fractions of the gonad resistant to sonication (advanced spermatids and immature testicular spermatozoa) (lane 4), and the spermatozoal nuclei obtained from the deferential duct (a short conduct which leaves the gonad and leads to the epididymis) (lane 5), and finally from the mature spermatozoa of the epididymis (lane 6). The figure shows that the histones are progressively substituted by the precursor protamine in the gonadal nuclei, and that in the last phases of spermiogenesis the molecule of the precursor is transformed into the mature protamine.

The relation between the amounts of protamine compared to its precursor is always very low in all the mature gonads which we have examined. With the objective of understanding this situation, we used the tissue sections examined by conventional optical microscopy, and have quantified the different types of spermiogenic cells in function of the level of maturity of the gonad. The results have verified that the proportion of elongated spermatids (equivalent to mature spermatozoa) in the testicle is always low in comparison to the global quantity of spermiogenic cells (2%, 5%, and 12%, respectively, in the three gonads in Fig. 2A, lanes 1, 2, 3). If we consider that the quantity of precursor with respect to the protamine is very elevated, we must conclude that the most mature spermatozoa rapidly abandon the gonad in order to enter the deferential conduct. This observation is interesting for the support of some of the results obtained later which indicate the large majority of the gonadal protamine is found in its precursor form.

The gonadal histones have been compared to the somatic histones in the same species through their two-dimensional electrophoretic behavior (Fig. 2C,D). They have been posteriorly purified by HPLC and their amino acid composition has been analyzed (Fig. 2E and Table 1). The results indicate that the four nucleosomic core histones (H2A, H2B, H3, H4) do not present significant differences with respect to their somatic counterparts. This fact, in contrast with spermiogenesis of mammals (Churikov et al., 2004; Govin et al., 2004), indicates that the model of substitution of nuclear proteins in spermiogenesis of *S. officinalis* is relatively simple, since the precursor protamine displaces histones directly, without the involvement of other histone variants or transition proteins.

On behalf of histone H1, it exhibits a remarkable heterogeneity, both in its electrophoretic behavior and in HPLC (Fig. 2C–E). Similar to other cephalopods (Mennella et al., 2002), histone H1 of *S. officinalis* is resolved into three main subfractions in one dimensional AU electrophoresis (not shown), but from bidimensional electrophoresis and HPLC analysis, a minimum of five subfractions of histone H1 can be recognized, none of which is exclusive to gonadal nuclei (see asterisks in Fig. 2D,E). The amino acid compositions of the fractions of H1 obtained by HPLC also demonstrate that, in effect, the gonadal H1 histones of *S. officinalis* do not present any major differences to the H1 histones of somatic tissues (Table 1).

The phosphorylation state of the *S. officinalis* protamine is another aspect that has not been analyzed to date. Considering that the protamine and precursor molecules contain, respectively, 7 and 9 serines, we have applied an anti-phospho-serine commercial antibody (Sigma) to detect the phosphorylation in Western blot (Fig. 2B), of these molecules from protein extracts of immature gonads (lane 1), mature gonads (lane 3), spermatozoa of the deferential conduct (lane 5), and epididymis (lane 6). The figure shows that the protamine is found in a highly phosphorylated state in the gonadal

nuclei, but that the phosphorylation has almost totally disappeared when it is found in the deferential conduct and epididymis. One unforeseen result of these experiments was the notably weak labeling of the protamine precursor molecule. Even though the ratio between the precursor and the protamine is highly elevated in the gonadal nuclei (see Fig. 2A, lanes 1, 3), the labeling with anti-phosphoserine is always much more intense in the protamine molecule (Fig. 2b, lanes 1, 3). This preliminary result suggests that the phosphorylation of the protamine occurs within the cellular nucleus, nearly simultaneously along with the deletion of the precursor portion, as well as possibly the phosphorylation of the precursor protamine molecule. The phosphorylation of precursor protamine would be the signal that initiates its rapid transformation into the protamine. Evidently this preliminary result deserves more experimental work and is presented here only as a suggestion.

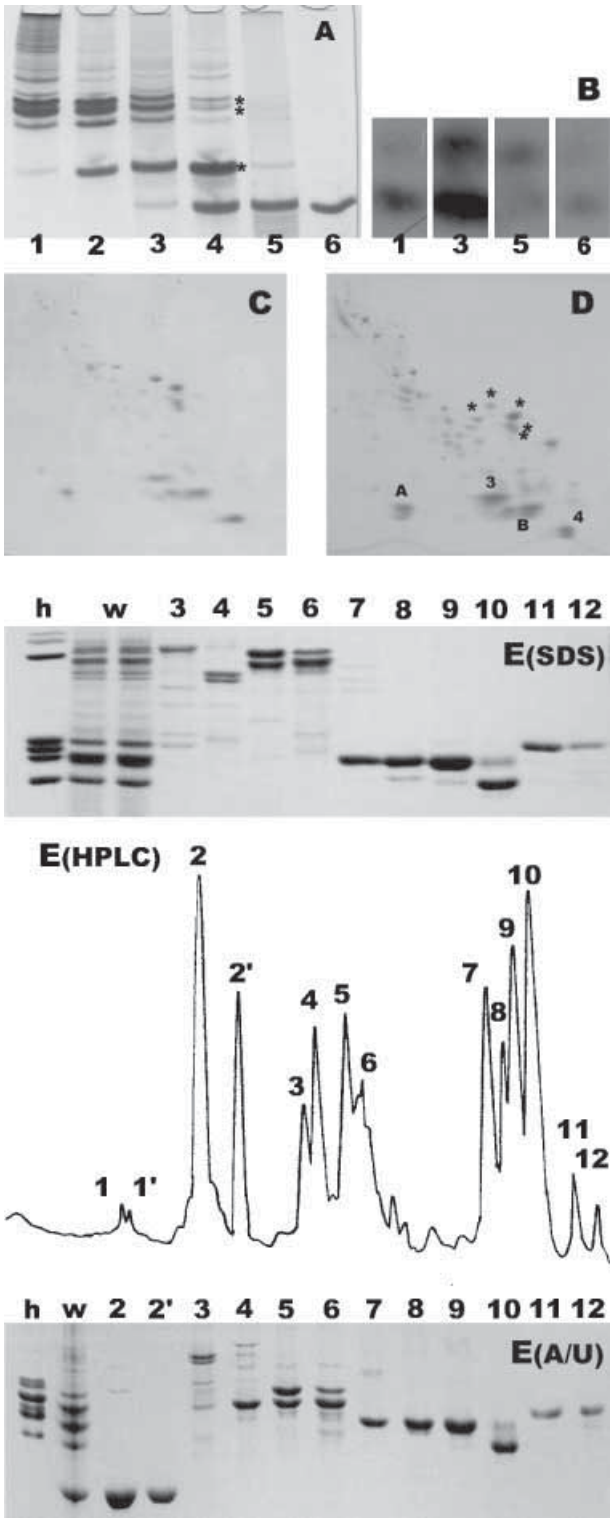

Fig. 2. Nuclear proteins in the gonad of *S. officinalis*. **A**: AU-PAGE of extracted proteins in the population of total gonadal nuclei of an immature testicle (**lane 1**), mature (**lane 2**), and very mature (**lane 3**); **lane 4**: proteins of advanced spermatic testicular nuclei (fractions resistant to sonication); **lane 5**: proteins of spermatozoal nuclei obtained from the deferential conduct; **lane 6**: protein from spermatozoa of the epididymis (protamine). **, histones; *, protamine precursor. **B**: Immunoblot treated with anti-phosphoserine antibody, showing a clear reaction with the protamine (lower band) and the precursor (upper band). Lane 1—Immature gonad (corresponding to A, lane 1); lane 3—very mature gonad (corresponding to A, lane 3); lane 5—spermatozoa from the deferential duct (corresponding to A, lane 5); lane 6—epididymal spermatozoa (corresponding to A, lane 6). In spite of the superior quantity of precursor in 1 and 3 in relation to the quantity of protamine, the labeling of the protamine in these gonadal nuclei is much more intense. **C** and **D**: Comparison by two-dimensional electrophoresis of the behavior of histones from a mature gonad of *S. officinalis* (**C**) and the histones proceeding from the nuclei of the prostate gland of the same animal (**D**). The asterisks signal the different isoforms of histone H1 identified by reaction with the antiserum generated against H1 from the gonad of *S. officinalis* (see Material and Methods). The vertical duplication of histone bands observed in D is an artefact of electrophoresis. The first dimension has been developed in the AUT system and the second in SDS slab gels. **E**: Behavior by HPLC of the basic proteins extracted from the nucleus of the mature gonad. The peaks 1–1′ and 2–2′ correspond, respectively, to the microheterogenous forms of the protamine (not loaded in the gel) and the precursor. The group of peaks 3–6 correspond to histone H1 and peaks 7–12 correspond to the core-histones. The electrophoretic control of the fractions obtained appears in SDS (upper gel) and AU (lower gel). Lanes 3, 4, 5, 6—H1 isoforms. Lanes 7, 8—H2A. Lane 9—H2B. Lane 10—H4. Lanes 11, 12—H3. See also the amino acid composition of the gonadal histones of *S. officinalis* in Table 1.

Pattern of Spermiogenic Chromatin Condensation of *S. officinalis*

This section will be restricted to the analysis of the morphology of the pattern of condensation. The participation of the condensation of the chromatin in the more general process of nucleomorphogenesis is analyzed together with other details in the accompanying article (Martínez-Soler et al., this issue).

In Figure 3 are presented six images (of equal magnification) which summarize the process of chromatin condensation. The aspect of the chromatin in the nuclei of early spermatids is a somatic type ("somatic-like chromatin") (Fig. 3A). In these nuclei, regions of euchromatin and regions of heterochromatin are found clearly differentiated, which means zonal differences in the proteins which interact with the DNA. This point is the initiation of the first structural transition of

TABLE 1. Amino Acid Composition (mol%) of the Gonadal Histones of *S. officinalis* (So) in Comparison to Somatic Histones of Calf Thymus (CT) Mayes and Johns (1982)

	H1					H2A		H2B		H3		H4	
	So_1	So_2	So_3	So_4	CT	So	CT	So	CT	So	CT	So	CT
Lys	16.5	20.1	22.5	22.0	26.8	11.2	10.2	14.0	14.1	10.2	10.0	10.9	11.4
His	1.3	1.5	1.2	1.4	—	2.6	3.1	2.6	2.3	1.6	1.7	2.3	2.2
Arg	7.5	6.5	4.2	4.1	1.8	9.0	9.4	7.1	6.9	12.2	13.0	12.7	12.8
Asx	4.2	1.4	2.7	4.3	2.5	5.2	6.2	4.8	5.0	4.1	4.2	4.8	5.2
Thr	3.7	6.3	5.6	5.3	5.6	5.9	3.9	5.6	6.4	7.1	6.8	5.4	6.3
Ser	6.8	6.1	7.7	7.1	5.6	4.5	3.4	10.7	10.4	4.2	3.6	2.3	2.2
Glx	7.8	3.5	3.5	4.0	3.7	8.6	9.8	8.4	8.7	11.5	11.6	7.0	6.9
Pro	5.0	7.5	8.5	7.5	9.2	4.5	4.1	3.6	4.9	4.7	4.6	2.2	1.5
Gly	7.4	5.4	7.0	7.9	7.2	9.0	10.8	6.6	5.4	6.1	5.4	15.3	14.9
Ala	23.0	20.9	20.3	21.7	24.3	10.5	12.9	11.2	10.8	12.9	13.3	8.3	7.7
Cys	—	—	—	—	—	—	—	—	—	—	1.0	—	—
Val	5.1	8.7	5.7	5.4	5.4	7.5	6.3	8.0	7.5	5.0	4.4	8.4	8.2
Met	—	1.6	0.9	—	—	1.5	—	2.0	1.5	1.3	1.1	1.0	1.0
Ile	2.1	3.0	2.8	1.0	1.5	4.0	3.9	4.9	5.1	5.0	5.3	5.4	5.7
Leu	3.5	3.3	4.0	5.0	4.5	12.8	12.4	4.6	4.9	8.9	9.1	8.8	8.2
Tyr	3.5	2.4	1.7	1.8	0.9	2.5	2.2	3.7	4.0	2.3	2.2	3.2	3.8
Phe	2.6	2.2	1.7	1.6	0.9	1.0	0.9	2.0	1.6	2.9	3.1	1.9	2.1

spermiogenic chromatin (Fig. 3A–C). The nuclear chromatin is left absolutely uniform so that the differences between eu- and heterochomatin disappear (Fig. 3B), and it immediately appears organized in granules (or fibro-granular structures) with a diameter of approximately 18 ± 2 nm (Fig. 3C). This remodeling of the chromatin is simultaneous and global within the whole nucleus, that is, no significant zonal differences are detected in any of the studied sections which we have been able to observe; in any concrete moment the whole nuclear structure presents an identical morphology. Therefore, the remodeling reaction is produced as a

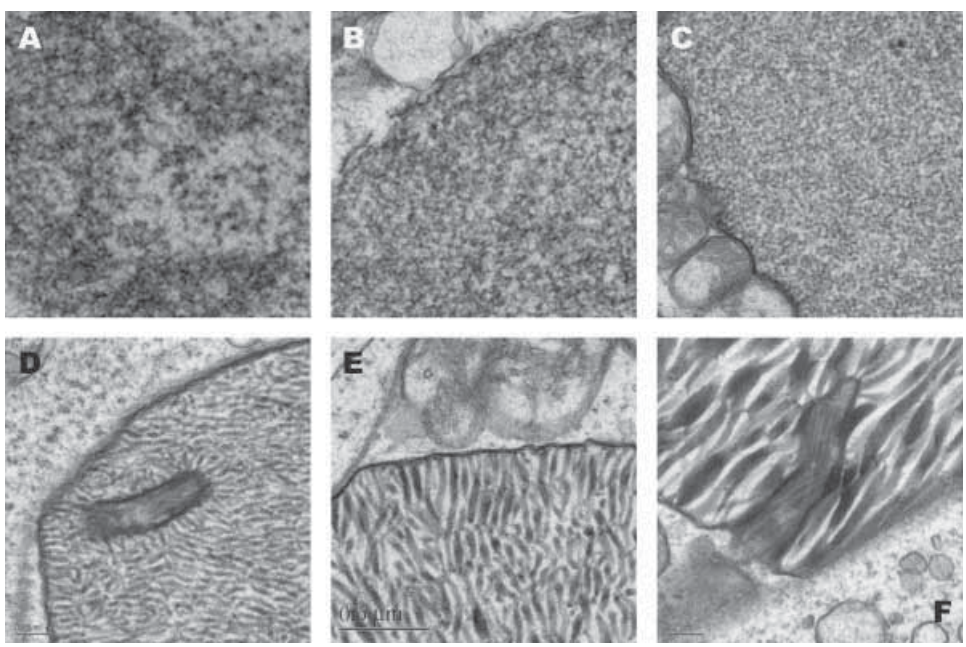

Fig. 3. Pattern of spermiogenic chromatin condensation of *S. officinalis*. **A**: The early spermatids contain a chromatin which is distributed in areas of greater (heterochromatin) and lesser (euchromatin) condensation. The regions of eu and heterochromatin acquire a homogeneous aspect (**B**), passing into a granular aspect (18 ± 2 nm) distributed throughout the whole spermatic nucleus (**C**). The granules are remodeled in fine fibers (25 ± 4 nm) (**D**) and later form fibers of 44 ± 5 nm (**E**). None of these fibers appears formed by a simple accumulation of granules which implies a profound conformational change in the chromatin (remodeling); (**F**) The larger fibers continue to unite together to form fibrous aggregates in the beginning (and later completely condensed chromatin). The chromatin corresponding to D can be found in round or very slightly elongated nuclei; those corresponding to E are slightly elongating; in contrast, the process of condensation of the chromatin (from F onward) is produced in spermatids which are clearly elongated. The stages understood between A and E are denominated in this text as "phase of patterning;" those stages from F and onward are referred to as "phase of condensation." The magnification of each image is identical (see bars in D–F).

unit within the nucleus and hence all nuclear areas find themselves in the same conditions.

From the stages depicted in Figure 3C the structure of granular chromatin undergoes a new remodeling. The granules lose their physical identity, and the chromatin adopts a fibriliar structure which in the beginning exhibits a diameter of 25 ± 4 nm (Fig. 3D) and later is transformed into fibers of 44 ± 5 nm in diameter (Fig. 3E). Similar to the first remodeling, in all of these transformations the nucleus always shows one uniform distribution of the chromatin (which is homogeneous), so that all of the nuclear areas simultaneously change in structure.

In the later stages, the fibers of 44 nm keep joining among themselves and form aggregates of chromatin which progressively grow larger in size (Fig. 3F shows an initial phase of the coalescence of these fibers). This process implies an increase in the interaction between fibers, but not a true remodeling since the fibers of 44 nm do not disassemble to reorganize themselves into another type of chromatin structure.

Following the points of view expressed in a previous work (Harrison et al., 2005), we denominate "patterning phase" to be understood as the remodeling of chromatin in early spermatids (Fig. 3A) up until the formation of fibriliar chromatin of 44 nm (Fig. 3E), and "phase of condensation" as the final stages of the chromatin compaction (Fig. 3F and following phases).

Appearance of the Precursor Protamine and the Disappearance of the Histones in the Spermiogenic Chromatin of *S. officinalis*

In the Material and Methods section, we have explained the process of obtaining the antiserum against the precursor part of the protamine. In the controls performed it was observed that the antiserum strongly reacts with the precursor molecule, but also produces a signal with the mature protamine (Fig. 1B). These controls imply that immunolocalizations of proteins using electron microscopy allow us to be sure of the moment in which the protamine precursor appears, but that we cannot know exactly when the precursor is transformed into the protamine (see below).

Figure 4 shows by electron microscopy the labeling of the spermiogenic chromatin of *S. officinalis* with the antiserum obtained against the protamine precursor. The immunoreactivity of this antiserum is very elevated and can be observed with a certain intensity of background in the "somatic-like" chromatin and in granular chromatin. Nevertheless, Figure 4 shows very clearly that the first structural remodeling ["*somatic-like*" *chromatin* → *granular chromatin*] is produced without the precursor protamine having entered in the spermiogenic nuclei (Fig. 4A,B), while the transition of granules to fibers of 25 nm is carried out simultaneously with the massive entrance of the precursor protamine (Fig. 4C). The labeling of the chromatin with the antiserum against the precursor protamine is found constantly elevated in all the following gonadal stages [*44 nm fibers* → *coalescence of the fibers* → *uniformly compacted chromatin*] (Fig. 4D–F).

To study the disappearance of the histones in the spermiogenic chromatin, we have utilized an antiserum recognizing the gonadal histone H2A from *S. officinalis* and we have carried out the same controls as those performed with the antiserum against the precursor protamine. We interpret that the immunolabeling of histone H2A is representative of the nucleosomic organization, at least in the first phases of spermiogenesis. In Figure 5 appears a summary of the results obtained in this experiment. The image represented in Figure 5A corresponds to part of a secondary spermatocyte nucleus, which we use as a positive control. The labeling of histone H2A is significantly high and is found localized preferentially in heterochromatin where an elevated concentration of nucleosomes is produced (a result similar to this is described in Manochantr et al., 2005 and Suphamungmee et al., 2005). In the "somatic-like" chromatin of the early spermatids (Fig. 5B) the labeling is also strong and predominantly accumulated in the areas occupied by heterochromatin. For its part, Figure 5C (which shows the entire nucleus with granular chromatin) verifies that these phases maintain the labeling for histone H2A, and therefore the nucleosomal organization of the chromatin.

Within the concrete stage of organization into fibers of 25 nm we have obtained relatively variable results. In some cases (Fig. 5D) the concentration of the labeling is comparable to the somatic-like chromatin of the early spermatid, while in more extreme cases (Fig. 5E) the labeling diminishes to approximately 65%. We cannot disregard a slight decrease in the presence of the histones in this phase, but it is very possible that the differences in the union with the antiserum form part of the quantitative variability in immunolabeling experiments. As a consequence, we assume that, for the most part, the fibriliar chromatin of 25 nm conserves its consistency in nucleosomal histones. On the other hand, the signal obtained in the chromatin organized in fibers of 44 nm (Fig. 5F) and in the following stages is practically nonexistent.

The quantitative comparison to the labeling between different phases of chromatin condensation is very difficult to perform for various reasons. In the first place, the grade of immunolabeling presents an inevitable experimental variability, and in the second place, both the form and the volume of the nucleus and chromatin keep changing throughout spermiogenesis. Nevertheless, the results shown in Figures 4 and 5 (represented qualitatively in Table 2) indicate two very important correlations: (a) the structural transition [*granular chromatin* → *25 nm fibers*] is produced simultaneously to the entrance of the molecule of precursor protamine, while (b) the transition [*fibers of 25 nm* → *44 nm fibers*] takes place while the nucleosomic histones disappear from the chromatin.

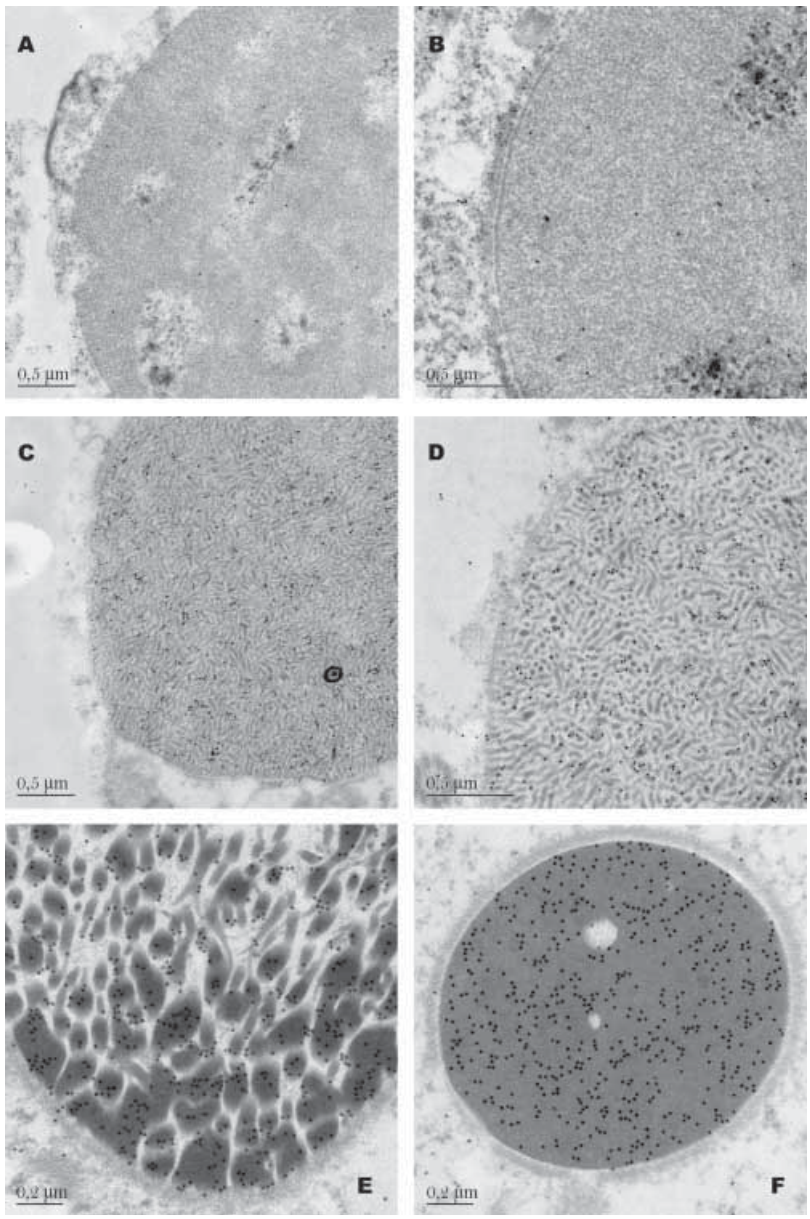

Fig. 4. Anti-precursor labeling of the spermiogenic chromatin of *S. officinalis*. The "somatic-like" chromatin and the granular chromatin do not show significant labeling (**A**, **B**). The remodeling of the granular chromatin into fibers of 25 nm correlates with the entrance of the precursor (**C**). The labeling of these nuclei (round or slightly oblong) is very intense. The precursor is maintained in fibers of 44 nm (**D**). The precursor, or the product of it as the mature protamine, is found present in all the following stages of condensation (**E**, **F**). *Note*: Due to a great electrodensity of the chromatin in compaction, the level of brightness intensity has been conveniently adjusted. The intensity of the intranuclear labeling is the sum of the intensity of the colloidal gold particle and the chromatin. For this reason, the extranuclear background (mainly in E and F) appears with lower intensity.

DISCUSSION

The most important previous studies about spermiogenic composition and protein changes in cuttlefish come from the group of Dr. Sautiére (Couppez et al., 1987; Rousseaux-Prévost et al., 1988; Martin-Ponthieu et al., 1991; Schindler et al., 1991; Wouters-Tyrou et al., 1991; Martin-Ponthieu et al., 1994). These works put together demonstrate that in the spermiogenic nuclei of *Sepia* the histones are substituted by two microheterogenous forms of the molecule called T, which turns out to be the precursor protamine (Fig. 1A). Later, by a deletion of the amino-terminal residues of the precursor, they are transformed into the mature protamine, which is also constituted by two microheterogenous forms. In one of these studies (Rousseaux-Prévost et al., 1988) the authors obtain an antiserum against the precursor molecule of the protamine, and show through optical immunomicroscopy that the antiserum begins to mark the round nuclei of young spermatids, and that it produces a maximal labeling in spermatids with nuclei in elongation, and finally no reaction with the elongated

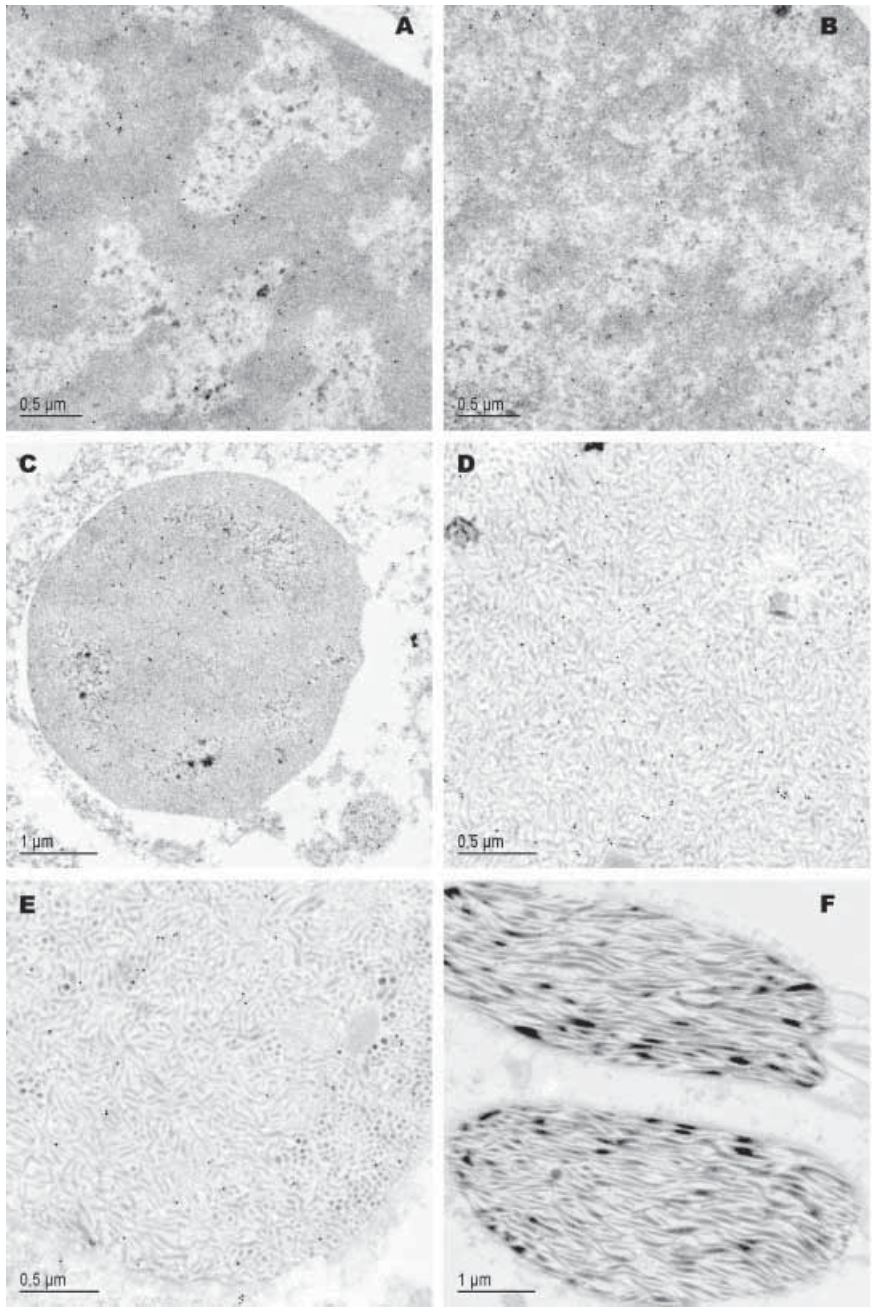

Fig. 5. Immunolabeling of the spermiogenic chromatin of *S. officinalis* with the antiserum against histone H2A. **A**: Chromatin of a secondary spermatocyte used as a control of the maximum labeling observed in these experiments; (**B**) "Somatic-like" chromatin of very early spermatids; (**C**) general view of a nucleus with granular chromatin; (**D**) and (**E**) labeling of 25 nm fibers; (**F**) labeling of 44 nm fibers and in the following stages practically null.

nuclei of testicular spermatozoa (the most advanced phases that can be found in the testicle). In another interesting article, (Couppez et al., 1987) the authors describe an important proportion of gonadal histone H4 which is found acetylated, showing that some molecules even reach the level of tetracetylation; however, in the work they did not identify which spermiogenic phases the acetylation of the histones is produced.

From the morphological point of view, the only antecedent dealing with cuttlefish spermiogenesis is found in a study by Maxwell (1975) in which the general features of spermiogenesis in three decapod species is described through electron microscopy.

From these foregoing studies and the considerations explained in the Introduction section, we have broadened our understanding of conformational transitions

TABLE 2. Semi-qualitative Appearance of Histones and Protamine Precursor During the Different Stages of Chromatin Condensation Patterns in Spermiogenesis of *S. officinalis*

Chromatin type	Somatic like	Granules 18 nm	Fibers 25 nm	Fibers 44 nm	Advanced steps
Presence of core hisontes	+++	+++	++(+)	–	–
Presence of precursor/protamine	–	–	+++	+++	+++

of the spermiogenic chromatin, and we have studied the appearance of the precursor to the protamine and the disappearance of histones.

The pattern of condensation we have found is quite more complex than that which appears in those species in which the histones are directly substituted by the protamine (compare for instance Saperas et al., 1993 with this work). In the pattern of *S. officinalis* we distinguish a complex stage of "patterning" and a phase of "condensation". In the "patterning" stage are produced three sequential remodelings of the chromatin: 1. —[*somatic-like chromatin* (*eu and heterochromtin*) → *granules of 18 nm*]; 2. —[*granules of 18 nm* → *25 nm fibers*]; 3. —[*25 nm fibers* → *44 nm fibers*]. The remodeling simultaneously affects the whole nuclear volume and implies a loss of physiological identity of one structure in order to reform itself into the next (e.g., the fibers of 25 nm are not a simple accumulation of the 18 nm granules).

In the first chromatin remodeling, granules are left organized in a quite homogenous size which fluctuates around 18 nm. The fact that neither a loss of the core-histones nor the entrance of the precursor occurs here suggests that these granules are formed due to small but quite regular accumulations nucleosomes (approximately 4–6 nucleosomes per granule). In this work we do not differentiate the initial reaction which produces this transition, and it will be necessary to study the variation of posttranslational modifications of histones and the absence of heterochromatin proteins in the nuclei with granular chromatin. It is interesting to point out that in mammals a massive deacetylation of the histones is produced in the first stages of spermiogenesis, which is recuperated by an acetylated stage in spermatids in elongation (Hazzouri et al., 2000b).

In the second transition the granules disappear and the chromatin deviates toward being organized in fine fibers of 25 nm. This change is related with the union of histones in a way that the conformation of 25 nm fibers must respond with a stage of equilibration in the following reaction:

$$Prec + DNA - HistPrec - DNA - Hist\,\mathrm{Pr}ec - DNA + Hist$$

The following remodeling (formation of fibers of 44 nm) represents a displacement toward the right of the above reaction, now that the histones have separated from the chromatin, leaving the precursor molecule exclusively bound to the DNA. This point is worthy of some considerations. First, it is unlikely that this displacement of histones from the chromatin might be due to an increase in the concentration of the precursor, since the quantity of the precursor is practically at a maximum in chromatin fibers of 25 nm (see Fig. 5). Secondly, concerning histone replacement, it is highly unlikely that the histone displacement should be due to the conversion of the precursor into the protamine. In fact, nuclei with 44 nm fibers are found in elongation and Rousseaux-Prévost et al. (1988) also demonstrated that in nuclei in elongation the labeling of the precursor (but not the protamine) is at a maximum. Additionally, we must take note that in the gonad the majority of the protamine is found in its precursor form, and the nuclei with 44 nm fibers represent very early phases in elongation (see also Martínez-Soler et al. this issue). On the contrary, it is more likely that the transition toward fibers of 44 nm lies on an increase in the electrostatic interaction between the precursor and DNA, or on a decreased in the electrostatic interaction of histones with DNA. However, just as it has been stated before, this and other points must be studied more extensively.

From the fibers of 44 nm initiates a second phase, or "condensation" of the chromatin. The fibers enter progressively in coalescence with each other, and end up producing uniformly compact chromatin, which is strongly condensed. The causes that explain the union of these fibers must deal with the conversion of the precursor into the protamine and/or complete dephosphorylation of these molecules, now that in the completely mature spermatic nuclei the precursor has been transformed into the protamine, which appears not to be phosphorylated.

In summary, the present work represents a first approximation to analyze the effect of the exchange of nuclear proteins on the structure of spermiogenic chromatin. The simple evolutionary substitution of the protamine by a precursor molecule which is less basic brings a remarkable increase to the complexity of protein transitions and the chromatin's structural remodeling during spermiogenesis (compare for instance Saperas et al., 1993 with this article). Over time, chromatin remodeling allows for a larger evolutionary plasticity in the mature sperm nuclear form. This final point is analyzed, together with other processes, in the accompanying article.

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Acetylation of Histone H4 in Complex Structural Transitions of Spermiogenic Chromatin

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Abstract In spermiogenic nuclei of the cephalopod mollusc *Sepia officinalis* histones are replaced by a precursor-protamine molecule, which is later converted into protamine. Simultaneously, spermiogenic chromatin undergoes a complex structural change. Somatic-like chromatin belonging to the earliest spermatid is progressively reorganized into: (a) granules of 20 nm diameter, (b) fibres of 30–35 nm, and (c) fibres of 40–50 nm. In the final phases of spermiogenesis these fibres of 40–50 nm join to form larger structures of condensed chromatin, and lastly, the uniformly packed chromatin in the sperm nucleus. Using specific antibodies for mono- and hyperacetylated forms of histone H4, in this work we show that the first structural remodelling of chromatin (from somatic-like organization into 20 nm granules) is given concomitantly with a massive mono-acetylation of H4 (acetylation in lysine 12), whereas the structural remodelling from 30–35 to 40–50 nm fibres is produced simultaneously with hyperacetylation of H4 and the nuclear removal of histones. J. Cell. Biochem. 102: 1432–1441, 2007. © 2007 Wiley-Liss, Inc.

Key words: H4 acetylation; spermiogenesis; chromatin structure

During the development of spermiogenesis in the major part of animal species, important changes occur in type and chemical modifications of nuclear proteins. This fact implies a series of progressive changes in the interaction between DNA and proteins, which bring about a continuous remodelling of chromatin structure, reaching to highly condensed chromatin in the ripe sperm nucleus.

In the most simple cases (Model: H → P), histones of the spermatidyl nucleus are directly replaced by a small and very basic protein designated as "protamine" which is the sole protein associated to DNA in the sperm nucleus. Spermiogenic protein transitions belonging to the H → P model are found among bony fish and birds, and they have been studied in the past by several authors (see Oliva and Dixon, 1991 for a review).

Nevertheless, evolution has generated enormous diversity in the spermiogenic processes of nuclear protein transitions and consequently, in the interaction of DNA and proteins, and also in the patterns of spermiogenic chromatin condensation [Chiva et al., 1995; Lewis et al., 2003, 2004; Harrison et al., 2005]. In the course of spermatogenesis of the cephalopod *Sepia officinalis*, nuclear histones are replaced by a basic protein of 77–78 amino acid residues during intermediate steps of spermiogenesis. This molecule (Protamine-precursor: Pp) is afterwards converted into mature protamine by an enzymatic deletion of its first 21 N-terminal residues [Martin-Ponthieu et al., 1991; Wouters-Tyrou et al., 1991]. We are studying the spermiogenesis of *S. officinalis* (Model: H → Pp → P) because it constitutes the level of complexity immediately superior to the model H → P.

In previous studies [Martínez-Soler et al., 2007a,b] we have analysed the progressive changes in the structure of *S. officinalis* spermiogenic chromatin, as well as the basic proteins associated with each particular type of chromatin. As can be observed in Figure 8, the

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chromatin of the earliest spermatid displays a somatic-like appearance, where histones are the main proteins interacting with DNA. In the next stages of spermiogenesis, the chromatin structure suffers a re-organization into granules of 20 nm diameter but continues containing histones as the main nuclear protein component. Afterwards, the chromatin is remodelled again into 30–35 nm diameter fibres that contain both histones and protamine-precursor. In more advanced steps, spermiogenic chromatin is reorganized into 40–50 nm fibres constituted by DNA associated with protamine-precursor molecules, and a very small amount of histones. In the final stages of spermiogenesis, the 40–50 nm fibres join to form aggregates of condensing chromatin displaying irregular sizes. These aggregates contain protamine.

The aim of this work is to study how the acetylation of histone H4 participates in the process of remodelling of *S. officinalis* spermiogenic chromatin by means to localize mono- and hyperacetylated forms of H4 in each particular chromatin structure.

In a previous work, Couppez et al. [1987] demonstrated that in the testis of cuttlefish, histone H4 is found partially in the non-acetylated form (41.4% of the whole gonadal H4), and partially in mono-, di-, tri- and tetra-acetylated forms (32.3, 18.0, 6.4 and 2%, respectively). The same study also established that in the male gonad of cuttlefish, acetylation of histone H4 is accomplished sequentially, in such a way that lysine 12 (K12) is the main site of acetylation in the monoacetylated form, whereas lysine 12 and 5 are found acetylated in diacetylated H4, and lysine 12, 5 and 16 are found acetylated in triacetylated H4.

Considering these previous results from Couppez et al. [1987], in the present work we have used a specific antibody against H4 acetylated on lysine 12 (anti-H4-acK12) in order to detect monoacetylation of histone H4, whereas hyperacetylation of H4 has been studied using the specific antibody against H4 acetylated on lysine 16 (anti-H4-acK16). We have analysed by electron immunomicroscopy the labelling of spermiogenic chromatin by each one of these two antibodies. The combination of results obtained shows that the remodelling of chromatin from somatic-like to 20 nm granular structures develops simultaneously with a massive monoacetylation of histone H4 (without removal of histones from the nucleus), whereas the remodelling from 30–35 nm into 40–50 nm fibres initiates with a hyperacetylation of H4 and finalizes with the removal of histones from the cell nucleus. These results complement the study of the main processes responsible for the structural transitions of chromatin in a complex model of spermiogenic nuclear protein changes ($H \rightarrow Pp \rightarrow P$) [Martínez-Soler et al., 2007a].

MATERIALS AND METHODS

Animals

Specimen of *S. officinalis* (a cephalopod mollusc) and *Trachinus draco* (an osteichthyan fish) were caught at the Mediterranean coast and identified by their morphological characters.

Antibodies

In this work four antibodies have been used: anti-acetyl lysine (Abcam, cat. # ab 21623), anti-H4 specifically acetylated in lysine 12 (anti-H4-acK12) (Upstate, catalogue # 07-329), whose antigen is the peptide GKGGA[acK]RHRKC, anti-H4 specifically acetylated in lysine 16 (Upstate, catalogue # 07-595), whose antigen is the peptide GKGLG[acK]GGAKRC, and an antibody against histone H2A (anti-H2A). The antibody anti-H4-acK16 presents low reactivity when used for electron microscopy immunolabelling, but displays a normal reactivity when used in Western blots obtained from denaturing electrophoretic gels. A possible explanation for this behaviour is that lysine 16 of histone H4 occupies a position very close to the globular core of molecule, and it may be only slightly accessible to the antibody when H4 protein is not completely denatured.

Whereas the three first antibodies are commercial (anti-H4-acK12 and anti-H4-acK16 from Upstate, and anti-acetyl lysine from Abcam), anti-H2A antibody was generated from H2A histone purified from calf thymus according to the method of Johns [1967], and submitted to controls explained in Martínez-Soler et al. [2007a].

Nuclear Proteins

Nuclei were obtained from testis of *S. officinalis* as in Saperas et al. [2006]. Briefly: Gonads of *S. officinalis* and *T. draco* were homogenized in buffer A (0.25 M sucrose, 5 mM $CaCl_2$, 10 mM Tris/HCl pH7.4, 10 mM benzamidine chloride),

filtered and centrifuged. Pellets were homogenized and centrifuged again with buffer A containing 0.25% Triton X-100 and the complete procedure was repeated two more times. The last sediment (purified nuclei) was washed with 10 mM Tris pH 7.4, and basic proteins extracted by 5 volumes of 0.4 N HCl, cleared by centrifugation, precipitated and washed with acetone [Saperas et al., 2006].

Electrophoretic Analysis of Proteins

Two-dimensional electrophoresis was performed according to the procedure described by Dimitrov and Wolffe [1997] with minor variations. In the first dimension, the histones were separated in 6 M urea, 6 mM Triton X-100 in 15% acrylamide. A single lane of electrophoresed proteins was excised from the gel of the first dimension, and incubated briefly in 8 M urea containing methylene green as a running marker. This first dimension was then laid on top of a second dimension gel, which contained two portions: a running gel, overlain with a concentrating gel. The running gel contained 6 M urea, 0.9 M acetic acid, and 0.6 mM Triton X-100. The overlaying concentrating gel was about 2 cm thick, containing the same components of the running gel, but with the Triton X-100 detergent omitted. The gel was run at 100 V until the methylene green marker ran off the bottom of the gel.

Protein Acetylation Analysis by Western Blots

Following electrophoresis of the second dimension, the gel was transferred to a PVDF membrane and probed for the presence of acetylated histones, using anti-acetyl lysine (Abcam), anti-H4-acK12 (Upstate), or anti-H4-acK16 (Upstate). The antibodies recognizing specific acetylated lysine residues on H4 were diluted 1:2,000 in PBS containing 5% powdered skim milk, and anti-acetyl lysine was prepared in PBS containing 2.5% foetal calf serum and 2.5% powdered skim, at a dilution of 1:250, and incubated at 4°C overnight with shaking. Antibody recognition of acetylated residues by all antibodies mentioned was detected using an HRP conjugated goat anti-rabbit secondary antibody diluted 1:3,000 in PBS containing 5% powdered skim milk, and incubated for 1.5 h at room temperature with agitation. HRP detection was performed using an ECL reagent (Amersham). PVDF membranes were stained with Ponceau-S (Sigma) and protein spots or bands on the film were unambiguously identified by superposition, and quantified in an automatic system (Phoretix 1D).

Electron Microscopy and Immunomicroscopy

Small sections of gonad have been fixed and embedded in Spur resin for conventional electron microscopy, or in Lowicryl resin for immunomicroscopy experiments [Giménez-Bonafé et al., 2002].

For electron immunomicroscopy examination, we have tried out a variety of blocking conditions and dilutions of both primary and secondary antibodies. The following working conditions are those which have been selected.

Anti-acetyl lysine (Abcam). The nickel grids containing the ultrafine sections of gonadal or epididymal tissue were blocked for 30 min in 0.1 M PBS pH 8.5 containing 2% powdered skim milk, 2% normal goat serum, and 20 mM glycine. The antibody was diluted 1:25 and 1:100 in the same blocking solution, with the glycine omitted. Tissue samples treated with anti-H4-acK16 were blocked for 30 min in 0.1 M PBS pH 8.5 containing 1.5% of each powdered skim milk and normal goat serum with 20 mM glycine. The primary antibody was diluted 1:100 in this same solution, with the glycine omitted. Anti-H4-acK12 and anti-H2A were treated with the same conditions as those used for anti-H4-acK16. All primary antibody incubations lasted for 2 h. The secondary antibody used for all treated samples was a 15 nm colloidal gold conjugated goat anti-rabbit antibody, diluted 1:25 in the same solution which was used to dilute the respective primary antibody, and applied for 1 h. All treatments were performed at room temperature.

Under these circumstances the controls treated exclusively with secondary antibody produced minimal or no labelling in spermatic nuclei, and the general background conditions were the lowest observed. These are the only variations of the general method described previously [Giménez-Bonafé et al., 2002] for electron immunomicroscopy.

RESULTS

General Acetylation of Histone H4 in Testis of *S. officinalis*

To study the behaviour of antibodies anti-H4-acK12/-acK16 by electron microscopy, and in order to verify the agreement of our results with

those described by Couppez et al. [1987], we have re-examined the appearance and the proportions of H4 acetylated populations in testis of *S. officinalis*.

It is necessary to point out that the relative ratios among different stages of spermiogenic cells change depending on the degree of maturity of *S. officinalis* testis. Advanced steps of spermiogenic cells (which may contain a higher degree of histone acetylation) are more frequent in ripe gonads than in those that are immature [Martínez-Soler et al., 2007b]. For these reasons, the relative proportion of H4 acetylated forms described by Couppez et al. [1987] should be considered only as an approximate, but not absolute, value.

Figure 1A displays a two-dimensional electrophoretic development of proteins extracted from whole gonadal nuclei prepared from a mature testis of *S. officinalis*. The electrophoresis has been performed in 6 mM Triton/6 M urea (first dimension) and 0.6 mM Triton/6 M urea for the second dimension, and has been stained with Coomassie blue. Under these conditions, histone H4 and its acetylated forms show a good resolution without any contamination of other protein spots, because they separate from the diagonal formed by the major part of proteins [Dimitrov and Wolffe, 1997]. The zone corresponding to histone H4 and acetylated H4 appears magnified in Figure 1B. Stained by Coomassie blue, the non-acetylated (0 in Fig. 1B), mono-acetylated (1 in Fig. 1B) and di-acetylated H4 (2 in Fig. 1B) appear clearly in the gel with a decreasing intensity, but tri- and tetra-acetylated are minor fractions of histone H4 and are not detected by Coomassie blue at the protein concentration used for this electrophoresis. The proportion between non-, mono- and di-acetylated H4 estimated from Figure 1B is respectively 5/2/1. This value is roughly coincident with ratio given by Couppez et al. [1987].

General acetylated forms of H4 histone have been revealed by Western blot of two-dimensional electrophoresis using the antibody anti-acetyl lysine, which unespecifically recognizes acetylated proteins. In Figure 1C one can observe that the intensity of labelling for H4 acetylated forms decreases from mono- to tetra-acetyl H4. However, the relative intensity of each population cannot be measured in a reliable way in these blots, due to overlapping of spots.

Detection of H4 Acetylation by Western Blot Using the Antibodies Anti-H4-acK12 and Anti-H4-acK16

Figure 2 shows the cross-reaction between antibody anti-H4-acK12 and the whole testicular proteins of *S. officinalis* (s1, s2 in Fig. 2), compared with a control (t1, t2) obtained from *T. draco*, a bony fish which accumulates hyperacetylation of H4 during spermiogenesis (unpublished results). Mono-acetylated H4 of *S. officinalis* clearly appears as the major acetylated form, while the intensity of the next acetylated levels of H4 decrease progressively. The approximate ratio between acetylated populations of H4 has been evaluated from the intensity of bands appearing in lane s1 (Fig. 2). The value obtained is 4/2.5/1 for mono-, di- and tri-acetylated H4, respectively. Tetra-acetylated H4 appears always as a very minor fraction in *S. officinalis* testis (see Discussion)

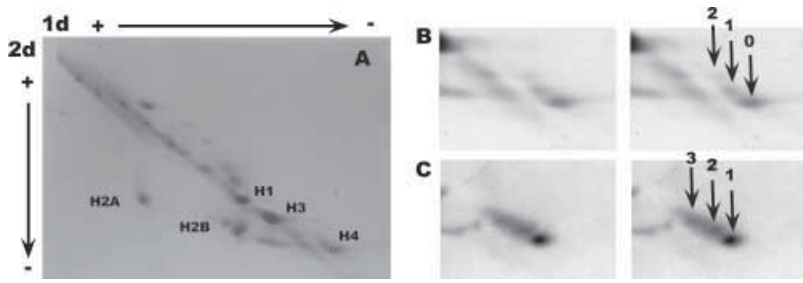

Fig. 1. Immunodetection of acetylated forms of H4 by anti-acetyl lysine antibody in *S. officinalis* male gonad. **A**: Two-dimensional electrophoresis of whole proteins extracted from testicular nuclei of *S. officinalis* by 0.4 N HCl. First dimension: 6 mM Triton/6 M urea; second dimension 0.6 mM Triton/6 M urea. **B**: Magnification of zone corresponding to H4 (0) and acetylated forms (1: mono-acetylated H4; 2: di-acetylated H4). **C**: Western blot and immunodetection of acetylated H4 by the antibody anti-acetyl lysine (1: mono-; 2: di-, 3: tri-acetylated H4).

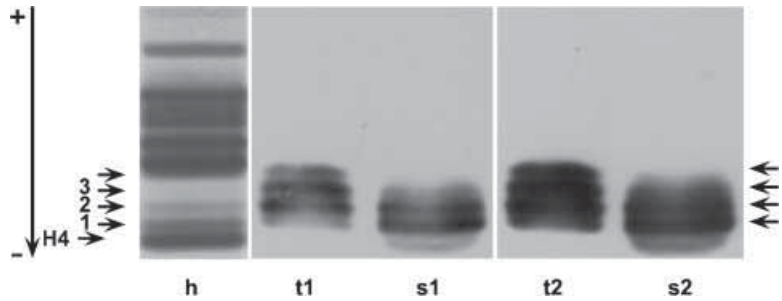

Fig. 2. Immunodetection of acetylated forms of H4 by anti-H4-acK12 in testis of *S. officinalis*. **Lane h**: Acetic acid/urea electrophoresis of testicular histones of *S. officinalis*. **Lanes s1** and **s2**: Immunodetection of acetylated H4 by anti-H4-acK12 (s2 identical to s1 with developing increased). **Lanes t1** and **t2**: Forms from mono- to tetra-acetylated H4 obtained from intermediate spermatidyl nuclei of *Trachinus draco*. In this species of bony fish all the gonadal H4 accumulates hyperacetylation before to be replacement by protamine (1, 2, 3: mono-, di-, and tri-acetylated H4).

and does not appear clearly in the blots performed in this work.

The values obtained here are in agreement with those obtained by Couppez et al. [1987] and with the ratios evaluated from Figure 1B. A more important point is that this coincidence in results corroborates that lysine K12 is the first lysine to be acetylated in gonadal H4 molecules. If this were not the case, the proportion found using anti-H4-acK12 would differ from the general proportion of acetylated forms.

The cross-reaction of antibody anti-H4-acK16 with acetylated H4 gonadal forms is displayed in Figure 3. In this case the intensity of labelling of mono-, di- and tri-acetylated forms is similar (1/1/1, respectively) (Fig. 3, lane s). This ratio is not a complete contradiction to the results of Couppez et al. [1987], according to which lysine 16 is the third lysine to be acetylated in gonadal H4, but that a small proportion of monoacetylated H4 also exists whose site of acetylation is lysine 16. Our results also show that a proportion of mono- and di-acetylated H4 is acetylated on lysine 16. Nevertheless, if we take into consideration that the absolute level of tri-acetylated H4 is very low compared to mono- and di-acetylated H4 (Figs. 1B and 2), we should conclude that antibody anti-H4-acK16 reacts preferentially (not exclusively) with tri-acetylated H4.

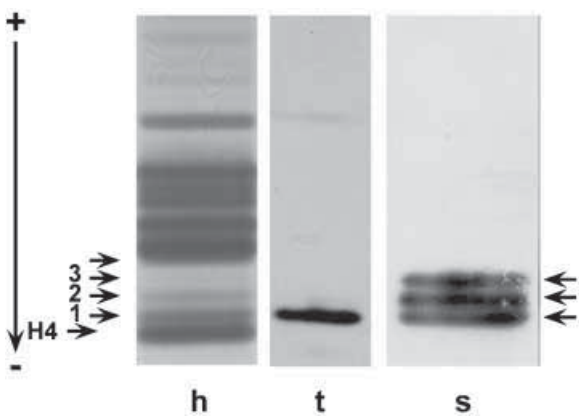

Fig. 3. Immunodetection of acetylated forms of H4 by anti-H4-acK16 in testis of *S. officinalis*. **Lane h**: Acetic acid/urea electrophoresis of testicular histones of *S. officinalis*. **Lane s**: Immunodetection of acetylated H4 by the antibody anti-H4-acK16. **Lane t**: Control of mono-acetylated H4 obtained from *T. draco* (1, 2, 3: mono-, di-, and tri-acetylated H4).

Immunolabelling of *S. officinalis* Spermiogenic Chromatin

We have assayed a series of working conditions to observe by electron microscopy the antibody labelling of *S. officinalis* spermiogenic chromatin. We have chosen the specific conditions in which the nuclear labelling is maximum, whereas extranuclear background is maintained in a practically null level. All the following results are presented at the same conditions (described in Materials and Methods).

The labelling of different phases of *S. officinalis* spermiogenic chromatin by the antibody anti-H4-acK12 is shown in Figure 4. Earliest spermatids contain somatic-like chromatin that shows a very low degree of cross-reaction with the antibody (Fig. 4A). The first structural remodelling of chromatin drives to granular structures of 20 nm which are homogeneously distributed in the cell nucleus. This chromatin strongly reacts with anti-H4-acK12 (Fig. 4B). Chromatin organized in 30–35 nm fibres (next structure in spermiogenesis) reacts also intensely with this antibody (Fig. 4C), where the

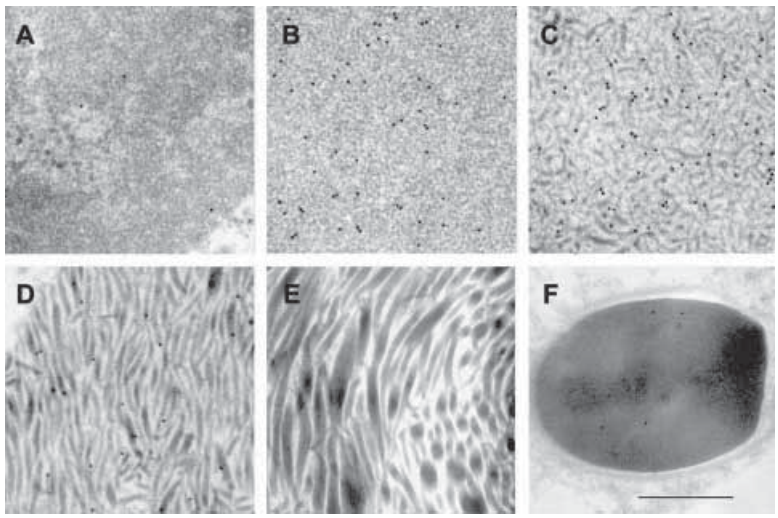

Fig. 4. Immunogold labelling of *S. officinalis* spermiogenic chromatin structures by anti-H4-acK12. **A**: Somatic-like chromatin; **B**: 20 nm granular chromatin; **C**: 30–35 nm fibrillar chromatin; **D**: 40–50 nm fibrillar chromatin; **E**: Advanced step of chromatin condensation; **F**: Chromatin fully condensed belonging to a testicular spermatozoon. Bar: 500 nm for (A)–(E), and 1,000 nm for (F).

labelling is even slightly superior than in 20 nm granular chromatin. The reaction with anti-H4-acK12 appears remarkably diminished in the next spermiogenic chromatin structure (40–50 nm fibres) (Fig. 4D), and at the final phases of condensation (Fig. 4E,F) the reaction with the antibody completely disappears.

We have performed the same observations using the antibody anti-acetyl lysine. The results (not shown) are qualitatively identical, but the level of labelling is superior, since in this case all acetylated proteins participated in the reaction.

The chromatin labelling by antibody anti-H4-acK16 is always weak when compared with the former (see Materials and Methods). In Figure 5A,B one can observe that both somatic-like chromatin from earliest spermatids, as well as granular chromatin do not react significantly with anti-H4-acK16. Nuclei with chromatin organized in 30–35 nm fibres exhibit the highest degree of antibody labelling (Fig. 5C,D). In the following spermiogenic stage, the labelling of 40–50 nm chromatin fibres diminishes with respect 30–35 nm fibres (Fig. 5D), and in the next stages (Fig. 5E,F) the labelling is null.

To understand correctly the preceding results observed, using the same conditions we studied the cross-reaction of spermiogenic chromatin of *S. officinalis* with an anti-H2A antibody, elicited from histone H2A purified from calf thymus. This part serves here as a control of the presence of histones in each one of the particular structures of spermiogenic chromatin. The observations (Fig. 6) show that in somatic-like chromatin of earliest spermatids, the reaction with anti-H2A is given mainly in the zone occupied by heterochromatin, were density of nucleosomes is high (Fig. 6A). The signal found in granular chromatin is similar or slightly superior (Fig. 6B), whereas in 30–35 nm fibres, labelling returns to the initial values (Fig. 6C). Labelling remarkably diminishes during the remodelling from 30–35 to 40–50 nm fibres (Fig. 6D), and disappears finally in the last steps of condensation. A semiquantitative comparison of immunogold labelling by the different antibodies used is represented in Figure 7.

DISCUSSION

In spermiogenesis belonging to the model $H \rightarrow P$, the transition of nuclear proteins is relatively simple. Histones of early spermatids become hyperacetylated in the intermediate stages of spermiogenesis and are replaced by phosphorylated protamine. Afterwards, in the last phases of spermiogenesis, protamine suffers dephosphorylation and remains in the sperm nuclei maintaining DNA in a highly packed state [Subirana, 1983; Christensen et al., 1984]. The changes of DNA-interacting proteins induce also relatively simple transitions in chromatin structure (condensation pattern). The pattern of condensation starts from somatic-like chromatin (first spermiogenic

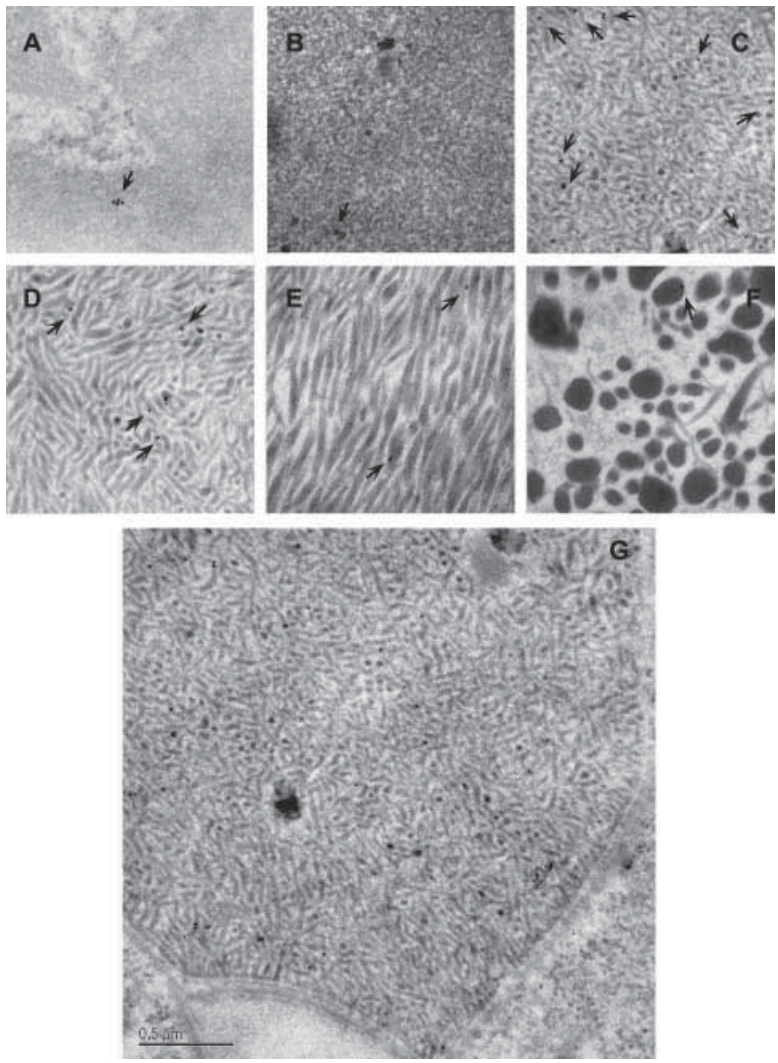

Fig. 5. Immunogold labelling of *S. officinalis* spermiogenic chromatin structures by anti-H4-acK16. **A**: Somatic-like chromatin; **B**: 20 nm granular chromatin; **C**,**G**: 30–35 nm fibrillar chromatin; **D**: 40–50 nm fibrillar chromatin; **E**,**F**: Advanced steps of chromatin condensation. Bar: 500 nm for all images.

stages) evolving to granular chromatin (intermediate stages), and finally to condensed chromatin by protamine [Saperas et al., 1993].

Spermiogenesis of *S. officinalis* belongs to a more complex model of nuclear protein transitions (H → Pp → P). In these cases an additional protein appears between histones and mature protamine. This protein is a precursor of the protamine molecule [Wouters-Tyrou et al., 1991]. This fact (relatively simple at the molecular level) produces a remarkable increase in complexity in the spermiogenic pattern of chromatin condensation; that is, the number of different structures adopted by chromatin during spermiogenesis is higher than would be expected. In Figure 8 appears a selection of main steps in the pattern of condensation of *S. officinalis* spermiogenic chromatin. This pattern comprises three structural remodellings (Fig. 8A–D: somatic-like chromatin → 20 nm granules → 30–35 nm fibres → 40–50 nm fibres), a final period where the 40–50 nm fibres are joining to form larger structures, and lastly, uniformly packed chromatin (Fig. 8E,F). In a previous work [Martínez-Soler et al. 2007a] we showed that the endowment of histones associated to chromatin remains complete until 30–35 nm fibres, and drastically diminishes in fibres of 40–50 nm. Simultaneously, protamine-precursor (Pp) appears

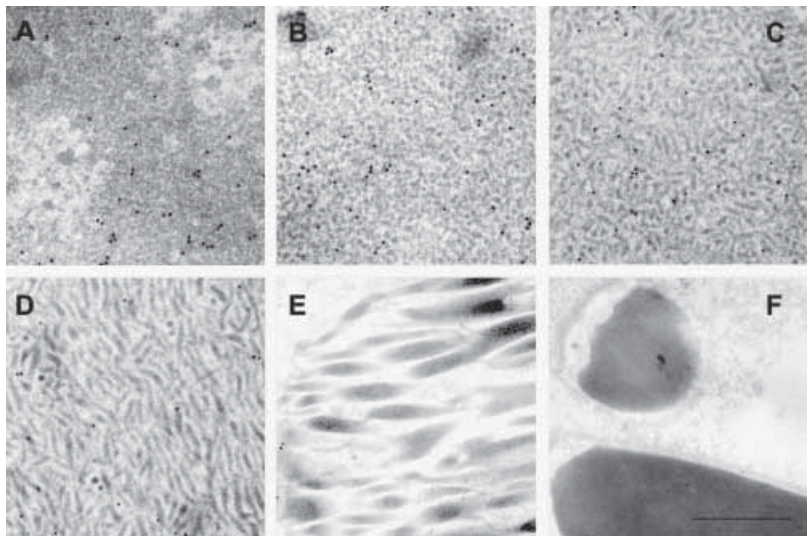

Fig. 6. Immunogold labelling of *S. officinalis* spermiogenic chromatin structures by anti-H2A. **A**: Somatic-like chromatin; **B**: 20 nm granular chromatin; **C**: 30–35 nm fibrillar chromatin; **D**: 40–50 nm fibrillar chromatin; **E**: Advanced step of chromatin condensation; **F**: Chromatin fully condensed belonging to a testicular spermatozoon. Bar: 500 nm for (A)–(E), and 1,000 nm for (F).

swiftly in chromatin organized into 30–35 nm fibres and is converted into the phosphorylated protamine in the final phases of spermiogenesis.

In the present work we analyse the presence of acetylated forms of histone H4 in the pattern of *S. officinalis* chromatin condensation using specific antibodies for acetyl-H4 lysine12, and acetyl-H4 lysine 16. The results (discussed next) have been incorporated in Figure 8.

During the first remodelling (somatic-like → 20 nm granules) the labelling with antibody anti-H4-acK12 suffers an important increase, but we have not detected any significant labelling with anti-H4-acK16. If we consider that these antibodies preferably react with mono-acetylated H4 (anti-H4-acK12), and tri-acetylated H4 (anti-H4-acK16), we should conclude that first step of chromatin remodelling develops simultaneously with a massive mono-acetylation of histone H4 at lysine 12. To our knowledge, this is the first report of global histone H4 acetylation [Calestagne-Morelli and Ausió, 2006] affecting only this residue. The size of granules (20 nm in diameter) allows a packing

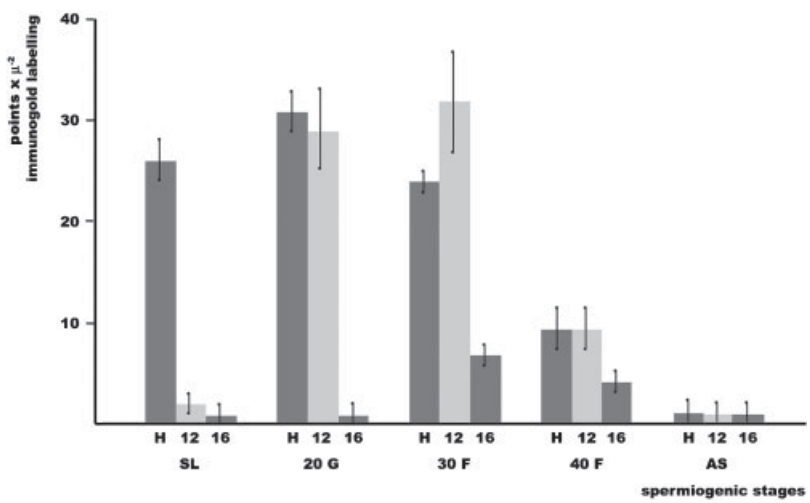

Fig. 7. Immunogold labelling (particles/μm^2) by antibodies anti-H2A (H), anti-H4-acK12 (12), and anti-H4-acK16 (16) at different stages of spermiogenic chromatin of *S. officinalis*. SL: Somatic-like chromatin. 20G: 20 nm granular chromatin. 30F: 30–35 nm fibres. 40F: 40–50 nm fibres. AS: advanced stages.

of 4–6 nucleosomes. We speculate that monoacetylation of H4 at lysine 12 (and other histones not considered here) may favour an ordered gathering of small groups of nucleosomes, although this point deserves to be studied with more detail. The next remodelling drives to 30–35 nm fibres that contain an interesting composition of proteins. First, the level of histones in this chromatin (detected by antibody anti-H2A independently in this work, and in Martínez-Soler et al. [2007b]) seems to be practically the same as that which is found in the somatic chromatin. Second, the level of acetylation/hyperacetylation of histone H4 is the largest found among all spermiogenic chromatin stages. Third, this chromatin also contains the complete endowment of protamine-precursor molecules [see Martínez-Soler et al., 2007b]. The structure of 30–35 nm fibres should be related to a dynamical equilibrium of the reaction

$$\mathrm{Pp} + \mathrm{DNA} - \mathrm{acHist} \leftrightarrow \mathrm{Pp} - \mathrm{DNA} - \mathrm{acHist} \leftrightarrow \mathrm{Pp} - \mathrm{DNA} + \mathrm{acHist}$$

in which, nucleosomes will leave DNA when histone acetylation reaches a threshold. The threshold value seems to be tetra-acetylated status since tetra-acetylated form of H4 practically cannot be detected in *S. officinalis* testicular nuclei (see Figs. 1–3). Displacement of the reaction to the right is related to structural chromatin remodelling. In 40–50 nm fibres there are residues of hyperacetylated histones (compare labelling of anti-H4-acK12 and anti-H4-acK16 with anti-H2A in Figs. 4–6), but the main DNA-interacting protein is largely the protamine-precursor [Martínez-Soler et al., 2007b]).

In the last phases of spermiogenesis (condensation phases, Fig. 7D–F) histones have completely disappeared and chromatin associated proteins are protamine-precursor, protamine and their dephosphorylated forms [Martínez-Soler et al., 2007b]. Therefore, the final condensation of chromatin is given by the

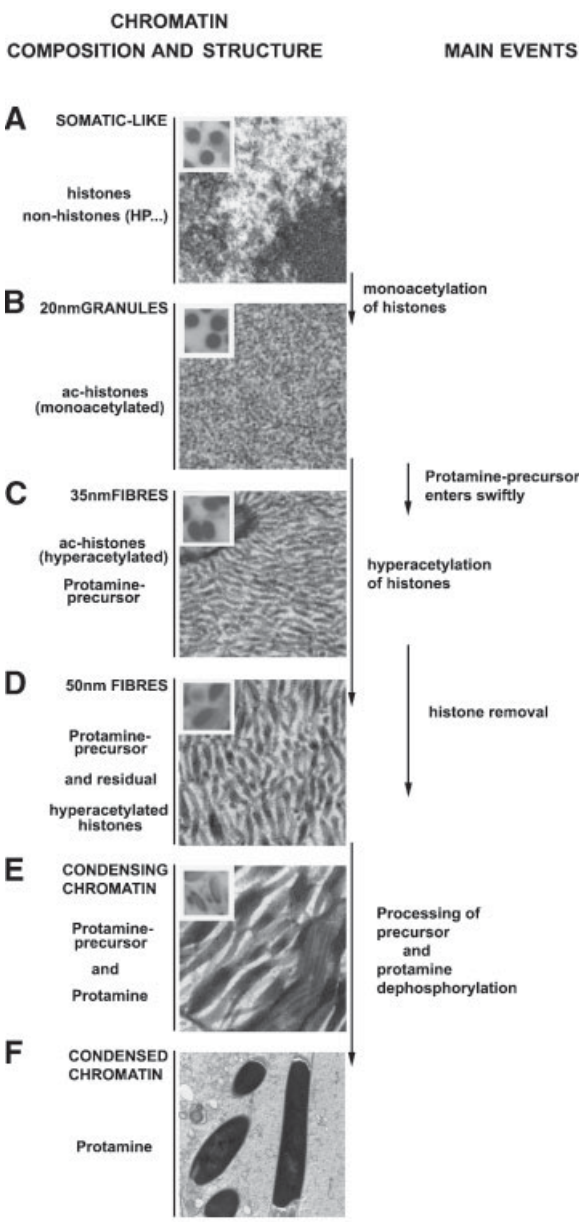

Fig. 8. Changes in chromatin condensation and in DNA-interacting proteins during *S. officinalis* spermiogenesis (from this work and from Martínez-Soler et al. [2007b]). Nuclei belonging to earliest spermatids (**A**) are spherical and contain somatic-like chromatin (eu- and heterochromatin). Histones from these spermatids display only basal levels of acetylation. First structural remodelling produces a chromatin organized into granules of 20 nm diameter, which are uniformly distributed in each round nucleus (**B**). Histone H4 is found mono-acetylated and the precursor-protamine has still not entered inside nuclei. This structural remodelling carries out without changes in the nuclear shape (inset). The second remodelling into fibres of 30–35 nm (**C**) develops simultaneously with the entrance of protamine-precursor to nuclei and with hyperacetylation of H4. This type of chromatin is found in nuclei that begin the elongation (inset). The third remodelling drives the formation of fibres of 40–50 nm in diameter and coincides with nuclear removal of histones. Remnants of H4 histone in this chromatin contain a high level of acetylation. Cell nuclear shape is elongated (inset). Condensation of chromatin in final phases (**E**,**F**) is given from progressive joining of 40–50 nm fibres. The mature sperm nucleus (F) is nearly cylindrical, 1 μm in diameter, with a length of 6 μm. Chromatin obtained from ripe sperm nuclei only contain non-phosphorylated protamine.

complete processing of the precursor molecule and protamine dephosphorylation.

The levels of histone acetylation in spermiogenesis depend on the equilibrium between activities of acetyl transferases and deacetylases [Hazzouri et al., 2000; Lahn et al., 2002], and has been found in all the cases studied. Nevertheless, until now the influence of histone acetylation over structural changes of spermiogenic chromatin has not been established. In this work we have found that during spermiogenesis of *S. officinalis* (model $H \rightarrow Pp \rightarrow P$), there are two structural changes of chromatin closely related with histone H4 acetylation: transition into 20 nm granules (shared by the majority of spermiogenesis) mainly correlated with a massive mono-acetylation of H4 K12, and transitions from 30–35 to 40–50 nm fibres, correlated with hyperacetylation and removal of nuclear histones.

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III. RESULTS

CHAPTER 3. SPECIALIZED SPERM CHROMATIN MODEL: CRUSTACEAN SPERM

In the past, several studies have been carried out which indicate that DNA of crustacean of sperm is not organized by any type of basic proteins (Chevaillier 1966, 1967, 1968; Vaughn and Locy 1968; Vaughn and Thomson 1972; Vaughn and Hinsch 1972). Crustacean sperm nuclei became one of Bloch's categories of sperm types (1969) yet still remains the most poorly understood. Since among all metazoan cells there exist no known cases where DNA is not interacting with some type of basic protein (Sassone-Corsi 2002; Sanman and Reeve 2005), and due to great interest in understanding which type of counter ions are responsible for organizing the DNA in these sperm, we re-examined the composition and partially the structure of the mature sperm chromatin of *Cancer pagurus* in the work published in the *Journal of Cellular Biochemistry* entitled "Histones and nucleosomes in *Cancer* sperm (Decapod: Crustacean) previously described as lacking basic DNA-associated proteins. A new model of sperm chromatin". My participation in this work was complete.

Our results show that: a) the sperm DNA of *C. pagurus* is bound by histones forming small nucleosomes; b) the ratio [histones/DNA] in sperm of two *Cancer* species is much lower than in other sperm types; and c) histone H4 is highly acetylated in mature sperm chromatin of *C. pagurus*. The low ratio of histones to DNA, their high level of acetylation, especially hyperacetylated H4, and the rapid chromatin digestion kinetics which suggest the existence of large genomic regions which are not stabilized by basic proteins, all help to explain the non-compact, decondensed state of the peculiar chromatin of crustacean sperm. It is likely that the lax chromatin of the nucleus is a gamete adaptation to the process of fertilization of these species.

We also used a similar approach to examine the mature sperm chromatin of a brachyuran crab from another family, the Majid *Maja brachydactyla* in an initial study called "Preliminary study of sperm chromatin characteristics of the brachyuran crab *Maja brachydactyla*. Histones and nucleosomes in crustacean sperm previously described without SNBPs." This work is currently submitted and under consideration for publication in the journal *Tissue and Cell*. In this study we verify that, despite previous reports that fellow Majids do not have basic proteins associated to the sperm DNA, histones are present in sperm of *Maja brachydactyla*, and indeed are associated to chromatin. However, electrophoretic analysis in various gel systems demonstrates that the

stoichiometries of some of these histones are unusual, because of the important decrease found in the proportion of histone H3, while histone H2B appears to be present in a higher proportion than in somatic chromatin.

Additionally, we describe that histone H3 is hyperacetylated in mature sperm of this species. Due to the preliminary nature of the work on *M. brachydactyla* sperm chromatin, several aspects remain elusive. The ratio of histones/DNA has not been determined due to the apparent disproportion of some core histones, mainly a lower amount of H3. Histone H1 appears to be in lower stoichiometric amounts than typical nucleosomal chromatin; just how much lower is still unknown. Also, several experiments suggest a nucleosome arrangement of the histones with DNA; however, another co-existing organization of only some core histones and possibly additional proteins with DNA was also evidenced in several experiments. This work provides a strong initial basis for characterization of sperm chromatin of *Maja brachycactla* and supports the suggestion that crustacean sperm deserves further attention and classification.

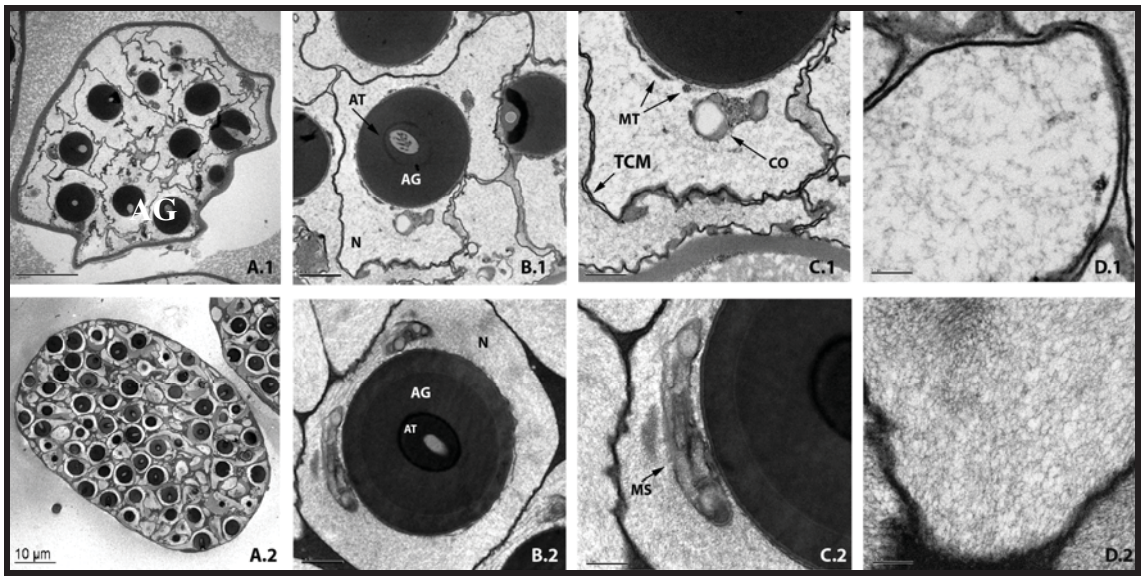

Figure III.3.1. Specialized sperm chromatin model: *C. pagurus* vs. *M. brachydactyla* sperm. A.1-D.1 correspond to sperm of *C. pagurus*; A.2-D.2 correspond to sperm of *M. brachydactyla*.; A.- sperm cells grouped into spermatophores; B.- sperm cell; C.- amplification of radial processes of the sperm cell in which organelles and cytoplasmic structures are immersed within the nucleoscytoplasm; D. - comparison of the different fibrilar chromatin arrangements of the decondensed sperm chromatin model. Bars: A.1: 2 µm; A.2: 10 µm; B.1 and B.2: 1 µm; C.1 and C.2: 0.5 µm; D.1 and D.2: 0.5 µm. MT: microtubules; CO: cytoplasmic organelle; TCM: triple cellular membrane; MS: membrane system; N: nucleus; AG: acrosomal granule, AT: acrosomal tubule.

Table III.1. Comparison of sperm chromatin from *C. pagurus* and *M. brachydactyla*

	Cancer pagurus	*Maja brachydactyla*
Nucleosomes	Yes	Yes
Acetylated histones	Hyperacetlated H4, slighly H3	Hyperacetylated H3, slightly H4
Histone/DNA ratio	0.6	Not determined
Arrangement of chromatin	loose fibrilliar arrangement	Close fibrilliar arrangement
Typical brachyuran sperm cell morphology	Yes	Yes
Possibly lower than normal amount of histone H1	Yes	Yes

Comparison of characteristics found in sperm of the two brachyuran crab species used for biochemical and ultrastructural studies for this work.

ARTICLE
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Histones and Nucleosomes in *Cancer* Sperm (Decapod: *Crustacea*) Previously Described as Lacking Basic DNA-Associated Proteins: A New Model of Sperm Chromatin

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ABSTRACT

To date several studies have been carried out which indicate that DNA of crustacean sperm is neither bound nor organized by basic proteins and, contrary to the rest of spermatozoa, do not contain highly packaged chromatin. Since this is the only known case of this type among metazoan cells, we have re-examined the composition, and partially the structure, of the mature sperm chromatin of *Cancer pagurus*, which has previously been described as lacking basic DNA-associated proteins. The results we present here show that: (a) sperm DNA of *C. pagurus* is bound by histones forming nucleosomes of 170 base pairs, (b) the ratio [histones/DNA] in sperm of two *Cancer* species is 0.5 and 0.6 (w/w). This ratio is quite lower than the proportion [proteins/DNA] that we found in other sperm nuclei with histones or protamines, whose value is from 1.0 to 1.2 (w/w), (c) histone H4 is highly acetylated in mature sperm chromatin of *C. pagurus*. Other histones (H3 and H2B) are also acetylated, though the level is much lower than that of histone H4. The low ratio of histones to DNA, along with the high level of acetylation of these proteins, explains the non-compact, decondensed state of the peculiar chromatin in the sperm studied here. In the final section we offer an explanation for the necessity of such decondensed chromatin during gamete fertilization of this species. J. Cell. Biochem. 105: 574–584, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: CHROMATIN; ACETYLATION; CRUSTACEA; SPERM

The study of proteins associated to DNA in sperm nuclei is a subject which began over 130 years ago [Miescher, 1874]. The first classification of sperm nuclei according to the type of molecule associated to its DNA was performed by Bloch [1969]. In this work, Bloch established five main categories of sperm nuclei based on the counter ions which are associated to their DNA. The first four categories of nuclei (those containing histones, monoprotamines, diprotamines, and keratinous protamines) have been followed up with additional studies, largely increasing our understanding of the chemical structure of molecules organizing mature sperm DNA [Ausió, 1999]. Furthermore, this molecular characterization helps to explain the vast evolutionary variability they demonstrate [Subirana, 1983; Subirana and Colom, 1987; Daban et al., 1991a,b; Rooney et al., 2000; Lewis et al., 2004; Eirin-Lopez et al., 2006a,b]. Still, the fifth category established in the original work of Bloch remains poorly understood. This category includes sperm nuclei of diverse crustacean species, however representing a great portion of all animal species, in which no basic proteins were found associated to DNA. Nevertheless, the alternative counter ion to basic DNA-interacting proteins is not mentioned in the original work by Bloch.

Around the time period of Bloch's classification of sperm nuclei, diverse species of crustacean sperm were being studied, in an attempt to describe possible proteins associated to the DNA [see for example Chevaillier, 1966, 1967, 1968; Vaughn and Locy, 1968; Vaughn and Hinsch, 1972; Vaughn and Thomson, 1972]. These studies were consistent in describing the peculiarities which are particular to sperm of this taxonomic group, and agree that, for the most part, their cellular components are not homologous to other types of sperm cells [see Langreth, 1969].

Due to the great diversity of crustacean species, in the rest of the introduction we will focus exclusively on sperm of brachyuran decapod crustaceans. Sperm nuclei of these species are positioned in

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the periphery of the cell, surrounding an acrosomal granule approximately spherical in shape [Langreth, 1969; Vaughn and Hinsch, 1972; Hinsch, 1988; Chiba et al., 1992; Medina and Rodríguez, 1992; see also Fig. 1]. In addition, these nuclei exhibit two particular characteristics which differentiate them from other mature sperm nuclei. First, they are not entirely separated from the cytoplasm by a membrane or envelope [Langreth, 1969; Hinsch, 1986], and therefore the cytoplasmic structures which are reminiscent of sperm cells are found immersed within the chromatin itself. (The sperm nuclei of the crustaceans studied here have been designated by several authors under the term *nucleocytoplasm*.) A second characteristic is the apparent decondensed, non-compact chromatin (see Fig. 1). This situation is not at all shared by sperm nuclei of other animal species which contain highly condensed chromatin.

The peripheral position of the nucleus, as well as its decondensed nature, has made difficult the purification of these organelles, and consequently, the analysis of specific counter ions of the DNA. The experiments designed to study the nuclear contents were mainly carried out by specific histochemical staining methods [Alftert and Geschwind, 1953; Bloch and Hew, 1960], even though in some cases an attempt was made to solubilize the chromatin by drastic mechanical methods to analyze the proteins released in the supernatants [Vaughn and Hinsch, 1972]. Specifically in species of the genus *Cancer*, Langreth [1969] observed that the histones apparently leave the nucleus in the most advanced phases of spermiogenesis. Also, Chevaillier [1966, 1967] described that in *Eupagurus lambardus* and *Carcinus maenas* the histones migrate from the nucleus to the acrosomal granule in the latest steps of spermiogenesis. On the other hand, Vaughn and Hinsch [1972] describe only acidic proteins associated to sperm DNA of *Libinia emarginata*.

Due to great interest in understanding which type of counter ions are responsible for organizing the DNA in mature sperm of these animals, we have re-examined the composition and (partially) the structure of mature sperm chromatin of *Cancer pagurus*, since sperm of this species has been described as void of basic DNA-interacting proteins.

MATERIALS AND METHODS

ANIMALS

Male individuals of *C. pagurus* (a decapod brachyuran crab) were purchased live from markets in Barcelona, Spain following proper identification according to their morphological characteristics. Male *Cancer magister* crabs were purchased live from markets in Victoria, British Columbia, Canada, also identified by morphological characteristics. Male individuals of the echinoderm *Holothuria tubulosa* were obtained off the Mediterranean coast of Catalunya, Spain, and the sperm isolated. Both *Patella aspera* and *Murex brandaris* (molluscs) were caught and treated as described elsewhere [Daban et al., 1991a,b; Cáceres et al., 1999, respectively]. The deferential duct was removed from *C. pagurus* and *C. magister*. Fresh sperm and spermatophore suspensions were used for further experimentation. Sperm from *H. tubulosa* were dounce homogenized with ice cold buffer A (0.25 M sucrose, 10 mM Tris pH 7.4,

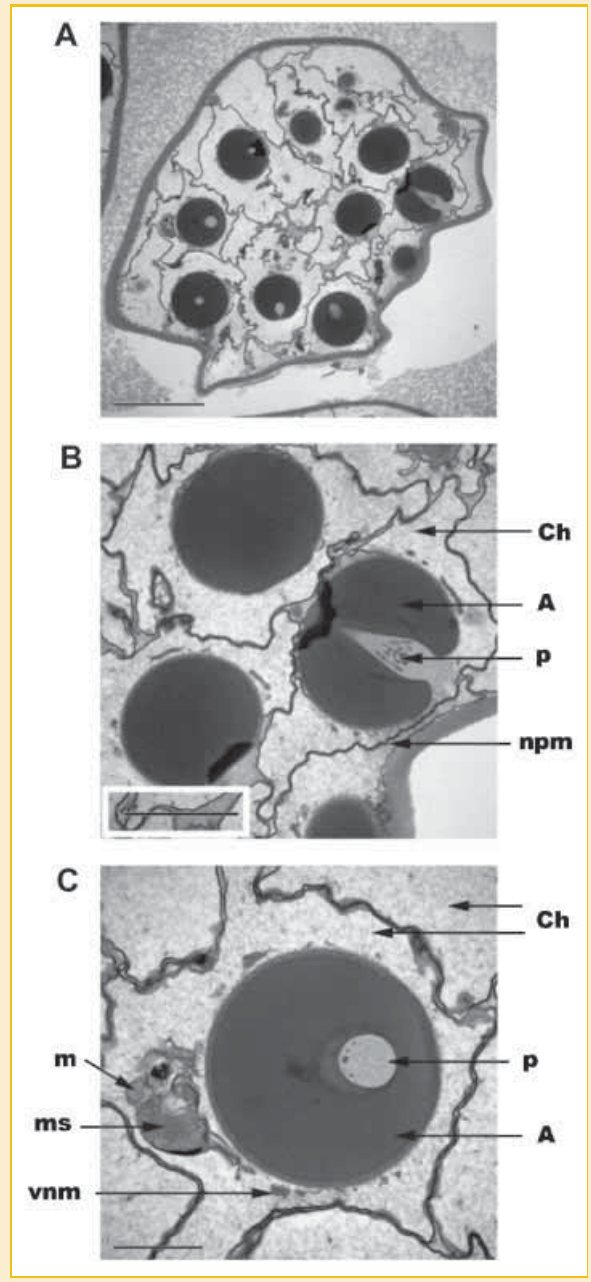

Fig. 1. Sperm structure of *C. pagurus* observed by transmission electron microscopy. A: Spermatophore obtained from the deferential duct of a specimen of *C. pagurus*, consisting of a capsule surrounding several sperm cells contouring around one another to achieve a tightly packed sperm bundle. B: Section cut along the meridian of a mature sperm cell. C: Section cut along the central equator of a mature sperm cell, in which organelles of cytoplasmic origin can be observed, immersed within the chromatin. A, acrosome; Ch, chromatin; m, mitochondrion; ms, membrane system; npm, nucleocytoplasmic membrane; p, perforatorial column; vnm, vessiculate nucleocytoplasmic membrane (nomenclature of organelles is based on that which is used by Tudge et al. [1994] and by Jamieson [1994]). Bars—A: 5 μm; B: 2 μm; C: 1 μm.

3 mM $MgCl_2$, 5 mM $CaCl_2$, 0.1 mM spermine, 0.25 mM spermidine, with 25 mM benzamidine chloride as an inhibitor of proteolysis) and made to 50% glycerol, followed by storage at −20°C until later use.

ELECTRON MICROSCOPY

Small sections of deferential duct have been fixed and embedded in Spur resin for conventional electron microscopy [Giménez-Bonafé et al., 2002].

OBTAINING FREE SPERM CELL POPULATION OF *C. pagurus* AND *C. magister*

The deferential ducts of *C. pagurus* and *C. magister* were gently homogenized with a dounce homogenizer in buffer A, and filtered through four layers of gauze. The filtrate (containing spermatophores and free sperm cells) was stirred during 15 min at 4°C to free sperm cells from spermatophores. Free sperm cells were separated from spermatophore capsules and full spermatophores by sedimentation at unit gravity through a discontinual sucrose gradient of 0.25 M sucrose, 1 M sucrose, and 2.2 M sucrose. The denser material, being full spermatophores and spermatophore capsules, sunk to the interphase of 1 M sucrose and 2.2 M sucrose. After allowing full separation of free sperm from spermatophores in the sperm cell suspension, the top layers containing free sperm suspended in 0.25 M/1 M sucrose were collected and washed in buffer A. All procedures were performed at 4°C. From this point, the sperm cells freed from spermatophores were used for micrococcal nuclease digestions or extraction of basic proteins. An aliquot of these sperm cells can be seen in Figure 7A,B.

FLUORESCENCE MICROSCOPY

Sperm cell nuclei of *C. pagurus* were stained for 10 min on ice with either Draq 5 (1:1,000 dilution in buffer A) or Topro-3 (1:3,000 dilution in buffer A), and viewed on a Leica optical microscope. The fluorescence resulting from DNA intercalating agents was observed by excitation with a 633 nm red helium neon laser. Alternatively, nuclei of these sperm cells were stained with 0.1 mg/ml Hoesch 33258 in distilled water and observed using an Olympus DP-11 fluorescent microscope, exciting the flourochrome with 295 nm spectra.

EXTRACTION OF NUCLEAR PROTEINS

Free sperm cells were pelleted and extracted with 5 volumes of 0.4 N HCl, or alternatively, with 5% perchloric acid (PCA), and cleared by centrifugation, precipitated and washed with acetone [Saperas et al., 2006].

ANALYSIS OF PROTEINS

Two-dimensional electrophoresis was performed according to the procedure described by Dimitrov and Wolffe [1997] with minor variations, described in Kurtz et al. [2007].

Electrophoresis of native oligonucleosomal fragments was first performed on a 6% acrylamide native gel in TAE (40 mM Tris pH 7.2, 20 mM sodium acetate, 1.5 mM EDTA) buffer. For native two-dimensional analysis, a lane was excised from the first dimension and equilibrated for 5 minutes in buffer containing 2% SDS, 10 mM β-mercaptoethanol, 10 mM Tris pH 7.5, and then placed atop the stacking layer of a 15% SDS gel. The proteins associated to the oligonucleosomal fragments were allowed to resolve at 80 V until loading dye reached the bottom of the gel.

One-dimensional SDS–PAGE and AU-PAGE gels were performed as in Martinez-Soler et al. [2007], and AUT-page gels were performed as in Frehlick et al. [2006].

Amino Acid analyses were carried out after hydrolysis in 6 N HCl at 110°C for 24 h.

Reverse-Phase HPLC was performed following the method described in Ausió [1988].

PROTEIN AND ACETYLATION ANALYSIS BY WESTERN BLOTS

Following electrophoresis, proteins were transferred to a PVDF membrane and probed for the presence of histones H4 or H2A using antisera raised in rabbits as described in Martinez-Soler et al. [2007]. The presence of acetylated histones was detected using the commercial antibodies anti-acetyl lysine (Abcam), anti H4 specifically acetylated in lysine 12 (anti-H4-acK12, Upstate), and anti H4 specifically acetylated in lysine 16 (anti-H4-acK16, Upstate).

The anti-H2A and anti-H4 antisera were prepared 1:1,000 in PBS containing 5% powdered milk and the membrane probed overnight at 4°C with shaking. Commercial antibodies specific for acetylated lysines were prepared in PBS containing 2.5% fetal calf serum and 2.5% powdered skim, at a dilution of 1:250, and incubated at 4°C overnight with shaking. Antibody recognition of all antibodies mentioned was detected using an HRP conjugated goat anti-rabbit secondary antibody diluted 1:3,000 in PBS containing 5% powdered skim milk, and incubated for 1.5 h at room temperature with agitation. HRP detection was performed using an ECL reagent (Amersham).

MICROCOCCAL NUCLEASE DIGESTION AND QUANTITATION OF RELEASED PRODUCTS

Digestions were performed as in Saperas et al. [2006]. Sperm chromatin digestions of *H. tubulosa* were performed in parallel with those of *C. pagurus*, since the nucleosome of this species has been previously described in detail [Cornudella and Rocha, 1979]. Briefly, samples of sperm cells or sperm nuclei corresponding to 1 mg/ml of DNA in buffer containing 0.25 M sucrose, 10 mM Tris pH 8.0, 0.5 mM $CaCl_2$, and 5 mM benzamidine hydrochloride were digested at 37°C for various times with micrococcal nuclease (Sigma). The enzyme was added to a concentration of 0.33 U/mg DNA for *H. tubulosa*, which was diminished to 0.11 U/mg DNA for *C. pagurus* to avoid over digestion of the DNA. Reactions were halted by the addition EDTA to a final concentration of 10 mM. After centrifugation (10,000*g* for 10 min), the supernatants (SI) were collected, and pellets washed and centrifuged again under the same conditions. The resulting pellet (P) was resuspended in 10 mM Tris pH 7.5 with 0.1 mM EDTA. Chromatin fragments from the supernatants (SI) and pellets (P) were both DNA and basic protein extracted, as described in Saperas et al. [2006]. DNA fragments were analyzed on 1.1% agarose slab gels, while the corresponding basic proteins were analyzed with AU-PAGE. Nucleosome lengths were

calculated for *H. tubulosa* and *C. pagurus* using the methodology described in Johnson et al. [1976] which was also applied in the study done by Cornudella and Rocha [1979] and used previously in our laboratories [Ribes et al., 2001; Saperas et al., 2006]. Briefly, a standard curve of molecular weight versus migration distance was produced using a molecular weight marker which was included in the DNA electrophoresis along with digested DNA fragments, and used to extrapolate the molecular weight of these fragments in each reaction. The total nucleosome length (linker region + core region) was determined by extrapolating the theoretical mononucleosomal fragment size at time = 0 minutes of the SI fraction. The core size was determined by the limiting fragment size, resistant to further digestion, and the linker length was determined by subtracting the core length from the total nucleosomal length.

For four different species (*C. pagurus*, *H. tubulosa*, *M. brandaris*, and *P. aspera*) aliquots of prepared sperm nuclei, each containing 0.5 mg DNA, were digested in parallel as described above. Following digestion times, EDTA was added to 2 mM and NaCl and PCA were added to final concentrations of 1 M each. The digested chromatin was sedimented and the soluble DNA was quantified by the absorbance at 260 nm using the extinction coefficient of $A_{260} = 20\ cm^2\,mg^{-1}$ DNA. The amount of soluble DNA in each sample was calculated and presented as the percentage of total starting DNA.

PROTEIN/DNA NORMALIZATION

Fresh sperm obtained from spermatophores released from deferential ducts of *C. pagurus* and *C. magister*, or purified nuclei of control species *H. tubulosa*, *P. aspera*, and *M. brandaris* were gently homogenized in ice cold buffer A. Suspensions of sperm cells (*Cp*, *Cm*) or sperm nuclei (*Ht*, *Pa*, *Mb*) were separated into equal aliquots for either DNA or basic protein quantitation, and several replicates were performed for each. Aliquots for DNA quantitation were incubated at 37°C in 10 mM Tris pH 8.0, 0.1 M EDTA, 40 μg/ml RNase, and 0.5% SDS with later addition of proteinase K to 0.1 mg/ml at 50°C until cells or nuclei were fully digested. Following incubation, DNA was isolated with the addition of phenol:cholorform:isoamyl alcohol (25:24:1), and precipitated in ethanol, dried, and solubilized in distilled water. The absorbance of each aliquot was measured at 260 nm, the DNA quantified using the extinction coefficient of $A_{260} = 20\ cm^2\,mg^{-1}$, and the values obtained for each aliquot were averaged. In parallel, basic proteins from sperm aliquots were obtained with 0.4 N HCl solubilization, and precipitated with cold acetone (6 volumes). Sperm basic proteins were analyzed on a 15% SDS gel which included known amounts of chicken erythrocyte histones as a standard which had been previously quantified. *Photoretix 1D* densitometry program was used to analyze the resulting SDS gel, where the band intensities obtained from the sperm samples were compared to those of the quantification standard. The amount of protein present in the sperm samples was extrapolated and averaged. Values were consistent among aliquots for both DNA and basic protein quantification. The relation of protein/DNA was calculated as the dividend of the average protein weight and average DNA weight extracted from sperm aliquots.

RESULTS

DESCRIPTION OF THE NUCLEOCYTOPLASM OF *C. pagurus*

The mature sperm of *C. pagurus* is found tightly grouped into capsules called spermatophores (Fig. 1A). These spermatophores protect the sperm in the spermatheca of the female and allow them to remain during a prolonged period of time without any loss of their functional ability. Longitudinal and transversal sections of sperm can be observed in Figure 1B,C. The chromatin coexists, without complete physical separation from laminar membrane systems, with mitochondria, microtubules, and other components which originally are formed in the cytoplasm of developing spermatids [Hinsch, 1969, 1986; Langreth, 1969; Tudge et al., 1994; Rorandelli et al., 2008]. (Note that these elements are a source of contamination when the chromatin is studied or isolated.) The nuclear membrane has been fused with the plasma membrane, forming a dense envelope that wraps around the majority of the cell. [For more detailed studies on the complete nucleocytoplasmic structures and the substructure of the acrosomal granule one can consult Langreth, 1969; El-Sherief, 1991; Chiba et al., 1992; Jamieson, 1989, 1994.]

BASIC PROTEINS IN THE WHOLE SPERM CELL: PRESENCE OF HISTONES

Due to the absence of tight chromatin packaging and apparent decondensed nature, the sperm nuclei of *C. pagurus* rupture when conventional nuclear purification methods are applied. Additionally, the presence of organelles of cytoplasmic origin which are immersed in the chromatin increase the possibility of proteolysis during experimental handling (see Discussion Section). Since the various *Cancer* species which have been described prior to the present study were concluded to have no histones bound to mature sperm DNA, we carried out an exhaustive examination using diverse electrophretic methods of the basic proteins that were extracted from freshly obtained sperm from the deferential ducts.

Figure 2 (top) shows the total proteins solubilized with 0.4 N HCl from sperm as resolved in SDS–PAGE, acetic acid/urea AU-PAGE, and acetic acid/urea/triton AUT-PAGE. In these different electophoretic systems some extracted basic proteins demonstrate a migration similar to that of histones used as a control. Analysis in polyacrylamide/SDS indicates that two of these proteins (asterisks in Fig. 2) have identical mobilities as histones H3 and H4 from chicken erythrocyte, while another two proteins present slight variations in mobility with respect to histones H2A and H2B. This result is congruent with the fact that histones H3 and H4 are the most evolutionarily conserved [Baxevanis and Landsman, 1996; Piontkivska et al., 2002]. The putative H1 exhibits mobility in SDS–PAGE similar to histones H5 and H1 of chicken erythrocyte. When the proteins are analyzed in high resolution gels of AU-PAGE (Fig. 2: AU), or in AUT gels (Fig. 2: AUT), the electrophoretic development appears more complex since these systems resolve diverse types of histone posttranscriptional modifications [Zweidler, 1978; Dimitrov and Wolffe, 1997]. It is especially interesting to observe that the possible core histones of *C. pagurus* are transformed into more than nine bands in the AU gels, and into an even greater number of bands in AUT gels. Also, we have used AU gels of low resolution to observe the possible presence of a protamine type protein. However, those

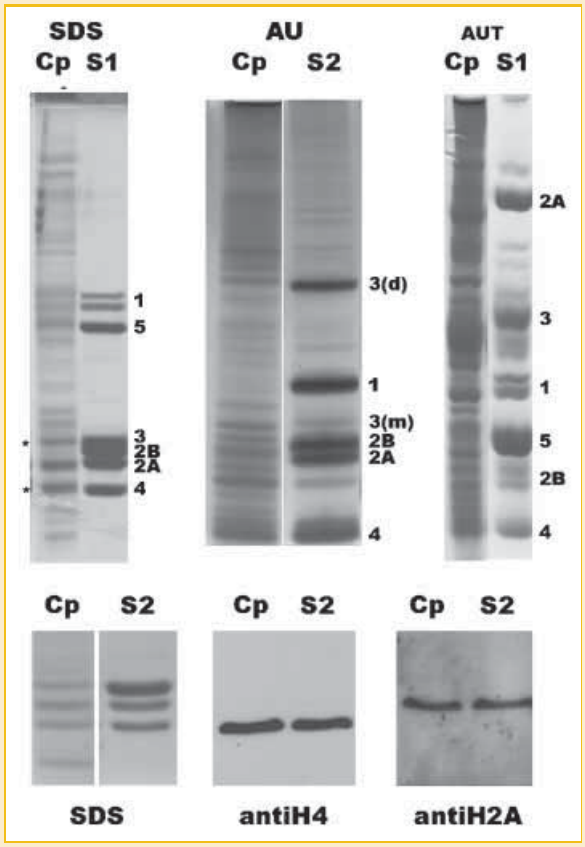

Fig. 2. Basic proteins in the sperm of *C. pagurus*. Electrophoretic analysis of solubilized proteins with 0.4 N HCl from sperm of *C. pagurus*. Top (left to right). Polyacrylamide–SDS gels (SDS), polyacrylamide–acetic acid–urea (AU) and polyacrylamide–acetic acid–urea–triton (AUT). From 1 to 5: Histones H1, H2A, H2B, H3, H4, and H5; 3(d) Dimer of histone H3; 3(m) monomer of histone H3. Asterisks mark histones H3 and H4 of *C. pagurus* sperm. Bottom: Immunodetection of basic proteins from *Cancer* sperm with anti-H4 and anti-H2A antisera. Cp, *C. pagurus*, S1, Standard of histones from chicken erythrocyte; S2, Standard of histones from lamprey sperm [Saperas et al., 1994].

results (not shown) demonstrate that sperm of *C. pagurus* do not contain protamine.

In order to unmistakably verify the identity of the histones in this sperm, we carried out Western blot analyses of the proteins resolved in SDS gels, using an antiserum specific for histone H4 (indicator of the tetramer $[H3\text{-}H4]_2$) and one specific for histone H2A (indicator of the dimer [H2A-H2B]). The results of these Western blots, which are shown in Figure 2 (bottom) assures that the extracted proteins of the sperm are indeed nucleosomal core histones. Histone H1 will be described later in this work.

THE HISTONES OF *C. pagurus* SPERM ARE BOUND TO DNA

Currently, it is known that in practically all cells of eukaryotic organisms, except for some protozoa such as dinoflagelates [Bodansky et al., 1979; Herzog and Soyer, 1981], the DNA is organized via its interaction with basic proteins, mainly histones (and/or SNBPs in the sperm nucleus). However, evidence contradicting this biological principle has been shown in sperm nuclei of crustaceans, and consequently it is imperative to this study whether the histones we describe here are interacting with DNA in the nucleocytoplasm, or if they have left their position in the nucleus.

Due to the impossibility of obtaining pure nuclei, we used sperm released from spermatophores and permeabilized with 0.25% Triton X-100/0.1% Nonidet NP-40. The permeabilized cells were incubated with micrococcal nuclease (MNase) at different times. Since the nucleocytoplasm occupies a peripheral position in the sperm cell (see Fig. 1), the chromatin is accessible to nuclease digestion, and the enzyme acts to release chromatin fragments to the supernatant during the reaction. The digestion product of the MNase digestion has been examined in a two-dimensional gel system, in which the first dimension separates chromatin fragments (protein–DNA complexes), and the second dimension resolves the protein content of these chromatin fragments. In Figure 3A it is shown that the chromatin fragments are organized in sizes corresponding to the multiple nucleosome subunits (M, D, T in Fig. 3A), which clearly contain the complete endowment of core histones.

To study in more detail the nucleosome size of sperm chromatin of *C. pagurus*, we have analyzed the DNA fragments released at different incubation times when treated with MNase. As a comparative control, we used sperm nuclei from *H. tubulosa*, which contain histones forming nucleosomes of a known size [Cornudella and Rocha, 1979; Azorin et al., 1985]. The result (which can be appreciated in Fig. 3B and in Table I) indicates that the nucleosome size in mature sperm of *C. pagurus* is composed of 170 base pairs of DNA (core: 145 bp; linker: 25 bp, see Table I). This small nucleosomal size can easily be observed in Figure 3B (top) where oligonucleosomal fragments of *C. pagurus* have a faster migration than those of *H. tubulosa* (compare fragment mobilities in S portion of *C. pagurus* to those in lane H). Figure 3B also shows that in *C. pagurus* sperm, all DNA is readily MNase digestible, since after just a few minutes of incubation with the enzyme no large DNA fragments were left undigested in the supernatants or pellets. This result indicates that there are no existing regions of DNA protected by a protamine or any other type of special protein. Although the kinetics of the MNase digestion do not allow the proportion of DNA free from histones to be known with exact precision, the analysis of the soluble DNA fraction in cold 1M PCA offers an estimation of the proportion of DNA readily accessible to digestion, and therefore with a high probability of being unbound to any histones. The fraction of cold-acid soluble DNA (1M PCA) obtained at various times for sperm of *C. pagurus* is compared in Figure 4 with the equivalent digestion kinetics of sperm nuclei containing histones, protamines, or protamine-like proteins. In this figure, it can be observed that the sperm chromatin of *C. pagurus* is digested at a faster rate and in greater proportion to the other shown chromatin types, with nearly 50% of the genome accessible to enzyme digestion.

CHARACTERISTICS OF HISTONES FROM THE CHROMATIN OF *C. pagurus*

Amino acid composition. The histones of *C. pagurus* have been purified by reverse phase HPLC (see Materials and Methods Section) and the results shown in Figure 5. The behavior in HPLC of the core

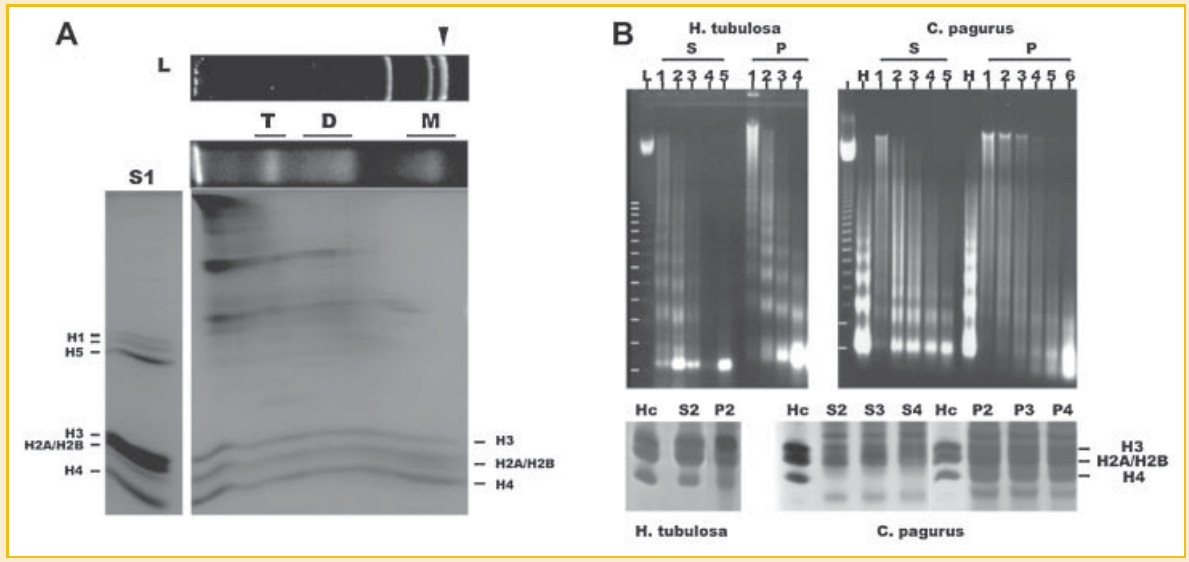

Fig. 3. Sperm chromatin of *Cancer*. A: Two-dimensional gel resolving the protein fraction of chromatin fragments. The first dimension (from left to right) resolves the trimer (T), dimer (D), and monomer (M), of the nucleosome subunit. The arrow on the ladder (L) indicates the core particle size. The second dimension corresponds to electrophoresis in SDS which allows identification of core histones bound to the chromatin fragments (H1 isoforms are not identified in this gel and will be analyzed in Fig. 5). S1, standard of chicken erythrocyte histones. B: (top) Kinetics of *Holothuria* and *Cancer* MNase digestion of sperm nuclei. L, ladder indicating multiples of 123 base pairs. S, supernatants; P, pellets; from 1 to 6: incubation times corresponding, respectively, to 2, 5, 10, 20, 40, and 120 min. H, pellet obtained from *H. tubulosa* sperm chromatin digestion at 20 minutes serving as a nucleosomal repeat marker to compare with nucleosomes observed in *C. pagurus* sperm digests. Bottom: Proteins in supernatants (S) and in pellets (P) at different times. Hc: Histone core standard.

histones studied in *Cancer* (Fig. 5A,C) is practically identical to other histones of sperm or somatic origin studied in our laboratories [Saperas et al., 1994; Roccini et al., 1996]. However, HPLC chromatography of the fraction of histones soluble in 5% PCA (histone H1 and other closely related proteins, Fig. 5B) only partially purified some histone H1 forms and other additional or contaminating proteins (Fig. 5D).

The core histones and the main H1 fraction which were purified have amino acid compositions which are very similar to somatic histones of calf thymus (Table II). Sperm nuclei of *C. pagurus* contain at least three isoforms of histone H1 (a, d, e in Fig. 5B,D and in Table II). These H1 isoforms are identified as such based on their amino acid compositions. Two of these (d, e) exhibit mobilities in SDS which are nearly the same as H1/H5 of chicken erythrocyte. One isoform (a, Fig. 5B) has a much slower electrophoretic mobility than H1/H5 despite its amino acid composition identifying this fraction as an H1 isoform. Some proteins obtained in the 5% PCA soluble fraction also have mobilities in the H1/H5 region, but are too acidic in nature to be considered H1 proteins, and therefore are designated as H1-like (b, c in Fig. 5B,D and in Table II).

TABLE I. Nucleosome Size of Crustacean Sperm Chromatin From Genus *Cancer*, Compared to That of *H. tubulosa*, Used as a Control

	Core (bp)	Linker (bp)	Nucleosome (bp)
Holothuria (1)	145	82	227
Holothuria (2)	145	80	225
Cancer (2)	145	20–25	165–170

(1) From Cornudella and Rocha [1979]; the rest (2) are from the present work.

Histone acetylation. In Figure 2 the examined histones can be observed in AU and AUT gels, which have been resolved into several bands. This behavior corresponds to the different posttranslational modifications of these proteins. In this section we analyze the acetylation state of *C. pagurus* sperm histones. On one hand, the proteins extracted with 0.4 N HCl have been separated by two-dimensional electrophoresis according to a variation of the method described by Dimitrov and Wolffe [1997] and used previously in our

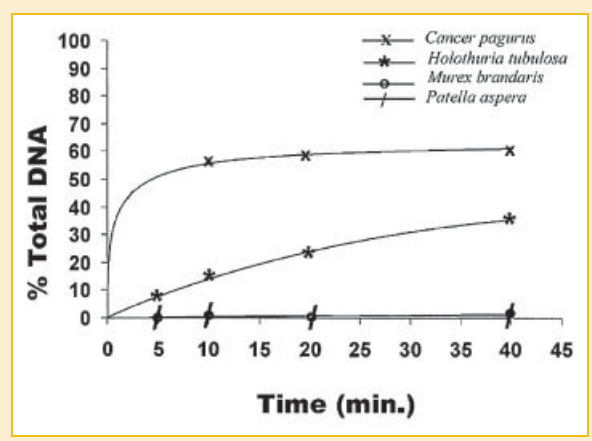

Fig. 4. Kinetics of MNase digestion in sperm of various chromatin compositions. Percent of sperm DNA which is soluble in the presence of cold 1 M PCA from various species presenting different sperm chromatin types. *C. pagurus* (X) (decondensed sperm chromatin); *H. tubulosa* (*) (sperm chromatin containing histones); *P. aspera* (/) (sperm chromatin containing protamine-like proteins); *M. brandaris* (o) (sperm chromatin containing protamine).

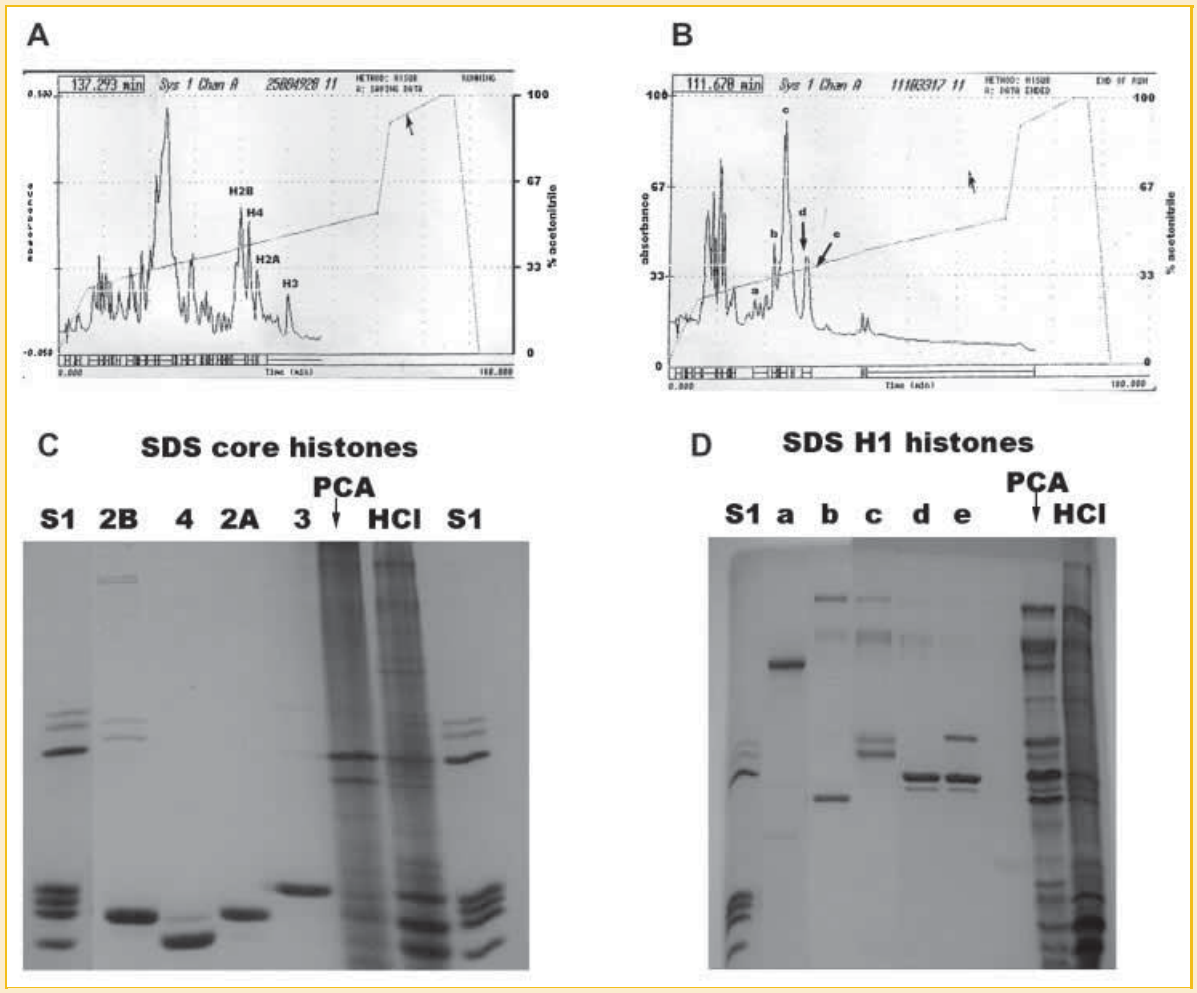

Fig. 5. Purification of histones from *Cancer* sperm. A: HPLC chromatogram and (C) Electrophoresis in SDS, of core histones purified by reverse-phase HPLC. B: HPLC chromatogram and (D) Electrophoresis in SDS, of proteins soluble in 5% PCA, separated by reverse-phase HPLC. S1, histone standard from chicken erythrocyte; HCl, total basic sperm proteins solubilized with 0.4 N HCl; PCA, sperm proteins solubilized with 5% perchloric acid (H1 and family).

TABLE II. Amino Acid Composition (mol%) of the Histones of *C. pagurus* (Cp) Compared to the Amino Acid Composition of Histones From Calf Thymus (Ct)

	H2A		H2B		H3		H4		H1				H1 Like	
	Ct	Cp	Ct	Cp	Ct	Cp	Ct	Cp	Ct	Cp (a)	Cp (d)	Cp (e)	Cp (b)	Cp (c)
Lys	10.2	10.1	14.1	13.8	9.7	9.0	11.4	8.5	26.8	18.3	16.1	20.3	13.9	7.0
His	3.1	1.8	2.3	2.3	1.4	1.5	2.2	2.3	0.0	1.1	0.6	0.5	0.8	1.6
Arg	9.4	8.6	6.9	6.4	13.0	12.8	12.8	12.2	1.8	4.3	2.1	4.7	2.4	2.9
Asp	6.2	7.5	5.0	6.9	4.2	4.3	5.2	6.0	2.5	5.8	6.2	5.9	8.4	11.3
Thr	3.9	3.0	6.4	5.2	6.8	6.3	6.3	6.4	5.6	10.3	6.0	7.5	7.6	8.3
Ser	3.4	5.1	10.4	8.4	3.6	3.7	2.2	3.2	5.6	6.7	4.8	6.1	6.0	7.1
Glu	9.8	9.3	8.7	8.5	11.6	11.8	6.9	7.5	3.7	4.6	8.2	1.6	14.1	13.0
Pro	4.1	4.7	4.9	3.3	4.6	4.8	1.5	2.3	9.2	9.4	10.6	10.6	9.1	8.4
Gly	10.8	11.1	5.4	5.5	5.4	6.2	14.9	14.7	7.2	5.3	5.0	5.1	5.0	4.9
Ala	12.9	9.7	10.8	11.5	13.3	12.0	7.7	7.4	24.3	9.5	21.2	19.3	9.9	4.5
Cys	0.0	0.0	0.0	0.0	1.0	0.3	0.0	0.0	0.0	0	0.0	0	0.0	0.0
Val	6.3	6.8	7.5	6.4	4.4	5.3	8.2	7.7	5.4	9.2	8.2	8.4	11.2	10.3
Met	0.0	1.5	1.5	2.2	1.1	1.8	1.0	1.5	0.0	0.2	1.0	0	0.9	2.4
Ile	3.9	5.1	5.1	6.4	5.3	5.3	5.7	5.7	1.5	4.8	3.1	3.5	4.6	6.1
Leu	12.4	10.8	4.9	6.0	9.1	9.0	8.2	7.9	4.5	6.8	4.5	4.2	4.3	6.8
Tyr	2.2	2.9	4.0	4.5	2.2	2.7	3.8	4.4	0.9	0.9	1.5	1.1	1.0	1.9
Phe	0.9	1.8	1.6	2.5	3.3	3.3	2.1	2.4	0.9	2.7	0.9	1.2	0.8	1.7

H1 and H1 related proteins a, d, e and b, c (respectively) correspond to protiens a–e of Figure 5.

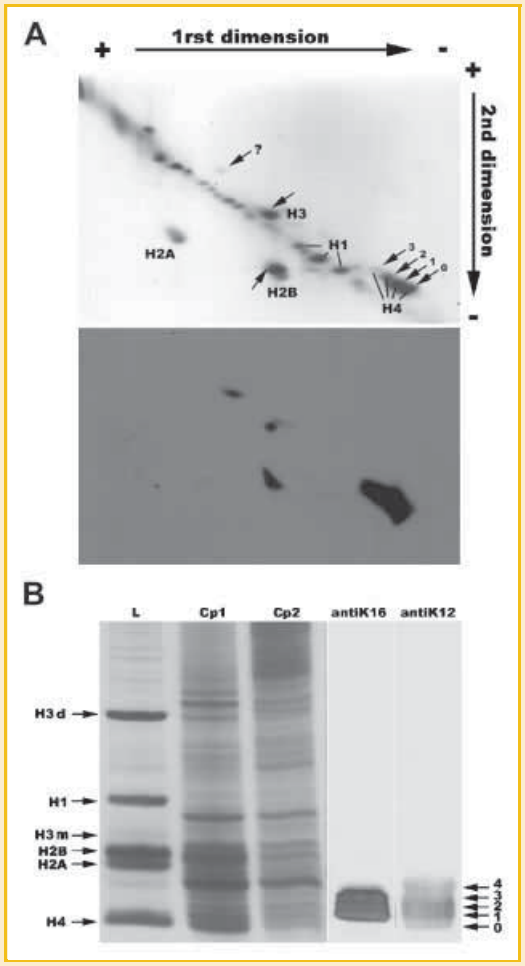

Fig. 6. Acetylation of histones from *Cancer* sperm. A: Two-dimensional electrophoresis [a variation of the method used in Dimitrov and Wolffe, 1997, described in Kurtz et al., 2007] of proteins extracted from sperm of *C. pagurus* with 0.4 N HCl (top) and Western blotted with anti-acetyl lysine antibody (bottom). In the 2D gel, the histones have been identified, along with their acetylated forms. B: Mono-, di-, and triacetylated isoforms of histone H4 are demonstrated by Western blotting using antibodies specific for acetylated residues on the amino-terminal tail of H4 (anti-H4-acK12 and anti-H4-acK16). The proteins were resolved AU-PAGE. L, sperm histones of lamprey; Cp 1 and 2, histones of *C. pagurus*: Cp1 corresponds to the immunoblot for anti-H4-acK16 and Cp2 corresponds to the immunoblot for anti-H4-acK12; 0, 1, 2, 3, 4: non, mono, di, tri, and tetra acetylated forms of histone H4.

lab to identify acetylated forms of spermatic histones [Kurtz et al., 2007]. Then, the histones were analyzed by Western blot with an anti-acetyl lysine antibody, which detects any protein possessing acetylated lysine residues. The results of these experiments (Fig. 6A) show that histone H4 has a high level of acetylation in mature sperm, while histones H2B and H3, along with an unidentified protein, appear to have low levels of acetylation. Histone H4 acetylation (mono-, di-, and triacetylated forms) is unquestionably proven using antibodies recognizing specific acetylated lysine residues on the amino terminal tail of histone H4 (anti-H4-acK12 and anti-H4-acK16, Fig. 6B).

Proportion of histones/DNA. To complete the analysis of *Cancer* sperm chromatin, we estimated the histones/DNA (w/w) ratio as well as the ratios of basic protein/DNA from other sperm types; we describe the method used for this estimation in Materials and Methods Section. In these experiments, we quantified the relation of histones/DNA (w/w) in sperm from two species of the genus *Cancer* (*C. pagurus* and *C. magister*), and the basic protein/DNA ratio in sperm nuclei of the following three control species: (a) *H. tubulosa* whose sperm contain histones; (b) *P. aspera*, whose sperm contain specific proteins of protamine-like type (PL); and (c) *M. brandaris*, whose sperm contain protamine. The obtained ratios are compared in Table III. It is interesting to observe that while all three control species (*Ht*, *Pa*, *Mb*) have a basic protein/DNA ratio of approximately 1/1 (w/w) (despite all having different types of basic proteins), the amount of histone in sperm of two *Cancer* species is only 0.5 and 0.6 per unit weight of DNA.

DISCUSSION

A DECONDENSED CHROMATIN STRUCTURE IN THE SPERM OF *Cancer* IN THE PRESENCE OF A LOW HISTONE/DNA RATIO

In contrast to what is observed in sperm nuclei of most organisms studied to date, the nucleocytoplasm of the decapod crab *Cancer* exhibits a decondensed organization [Langreth, 1969; Tudge et al., 1994]. This is despite the association of DNA with histones, forming nucleosome structures with a sort linker DNA (see Table I), as has been described in the previous section.

In spite the fact that sperm histones of *Cancer* possess a composition practically indistinguishable from somatic histones of calf thymus, our results provide an explanation for the decondensed chromatin state. First is the low proportion of histones per DNA content. While other sperm nuclei have a proportion of DNA-interacting proteins/DNA of approximately 1/1 (w/w) sperm chromatin of *Cancer* has a much lower value of approximately 0.5–0.6 histone/DNA (w/w). The histone–DNA association results in a nucleosome organization with a short linker DNA size (25 bp) which is much shorter than the average linker found in somatic cells [van Holde, 1988] and in other sperm cells (Table I). This suggests that in *Cancer* sperm, chromatin is organized into short oligonucleosomal arrays, consisting of closely packed nucleosomes interspersed in the midst of non-nucleosomally organized DNA, accounting for

TABLE III. Comparison of the Ratio of DNA Interacting Proteins/DNA (w/w) in Sperm With Different Sperm Nuclear Basic Proteins Types

	Cancer pagurus	*Cancer magister*	*Holothuria tubulosa* (H)	*Patella aspera* (P-L)	*Murex brandaris* (P)
Nuclear proteins/DNA (w/w)	0.5	0.6	1.0	1.0	1.2

Sperm of both *Cancer* species contains chromatin with histones, sperm of *H. tubulosa* contains histones, sperm of *P. aspera* contains protamine-like proteins, and sperm of *M. brandaris* contains protamines.

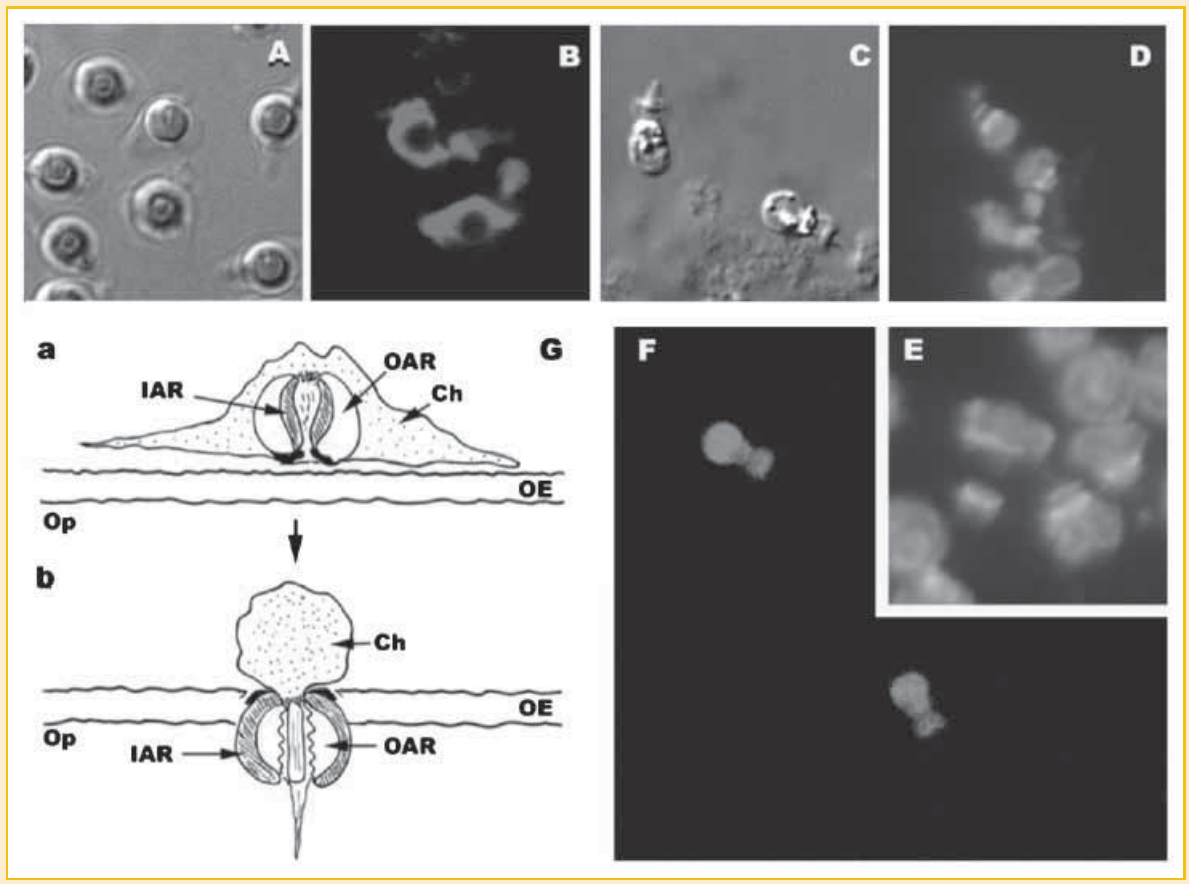

Fig. 7. Change in sperm shape during the activation of *C. pagurus* spermatozoa. Light micrograph of *C. pagurus* sperm cells (A), showing Draq 5 epifluorescence of the cell nuclei in the peripheral position (B). When the sperm cells are activated (spontaneously in buffer A, or by adding calcium) the acrosome everts, and the nucleus changes shape (light micrograph in C, and epifluorescent staining by Topro-3 in F, or by Hoechst reagent in D, E). Image G shows a simplified schematic representation of the activation process of the *C. pagurus* spermatozoon (a) before activation; (b) after activation. IAR.– inner acrosomal region; OAR, outer acrosomal region; Ch, chromatin; OE, oocyte envelope; Op, oocyte cytoplasm.

approximately 50% of the genomic DNA (see Fig. 4). This type of chromatin organization seems evident as well, considering that in a canonical nucleosome organization of 200 bp with one histone H1 per nucleosome the (w/w) histone/DNA ratio is equal to one. The histone-free regions appear to be stabilized mainly by metal ions.

The HPLC analysis of a 5% PCA extract from *Cancer* sperm (Fig. 5B,D) allowed us to identify three fractions (a, d, and e in Fig. 5B,D) that have an amino acid composition rich in alanine, lysine, and proline which is characteristic of members of the histone H1 family (Table II). However, the results presented here suggest that these histones are present in the oligonucleosomal chromatin fraction in sub-stoichiometric amounts (i.e., less or no more than 1 H1 per nucleosome). Also, though we have properly used proteolytic inhibitors, the possibility of degradation during chromatin isolation cannot be discarded, since the bands corresponding to fractions a, d, and e from Figure 5D and Table II are very faint in Figure 3A. The seemingly low proportion of H1 in this sperm chromatin is not surprising, considering the result that the nucleosomal repeat length in *Cancer* (170 bp) is very close to that of the chromatosome (160–168 bp) [Simpson, 1978; van Holde, 1988].

However, even in the presence of histone H1 it is very unlikely that the oligonucleosomal structures just described adopt a higher order folded structure such as that exhibited by the chromatin of organisms that contain histones with a canonical histone/DNA ratio in their mature sperm [Williams and Langmore, 1991]. Furthermore, preliminary results from our lab suggest that the linker histone fraction associated with *Cancer* chromatin is phosphorylated (results not shown). Linker histone phosphorylation has also been shown to lead to unfolding of the chromatin fiber [Horn et al., 2002].

In addition, the histones of *Cancer* sperm chromatin exhibit a high extent of acetylation of H4 (together with a minor acetylation of H2B and H3) (see Fig. 6). Histone acetylation leads to unfolding of nucleosome arrays [Garcia-Ramirez et al., 1995] and its role in spermiogenesis in other species has been linked to chromatin relaxation prior to displacement of histones by protamines (or other SNBP) [Oliva and Dixon, 1991; Kurtz et al., 2007]. However, acetylation of histones in organisms that retain histones in their spermatozoa has never been described before. Thus, the highly unfolded chromatin characteristic of *Cancer* sperm is unique and is not shared by any other species with condensed sperm nuclei.

BIOLOGICAL IMPLICATIONS OF A DECONDENSED CHROMATIN ORGANIZATION FOR FERTILIZATION

All the structural characteristics of *Cancer* chromatin described above suggest a decondensed chromatin organization, which is most likely quite flexible. This ''most likely'' has important biological implications that reflect the very peculiar mode of fertilization of this organism. In contrast to most other spermatozoa, the sperm of crustaceans lack flagella. Therefore, the mechanism of oocyte penetration by the sperm appears to involve an eversion of the acrosomal granule that takes place when it contacts the oocyte coating [Hinsch, 1971; Brown, 1976; Goudeau, 1982; Medina and Rodríguez, 1992]. This process results in a modification of the relative position of cellular components, as the sperm nucleus is pulled forward passing through the relatively narrow internal canal created for its own passage through the acrosome (see Fig. 7). In spite of many aspects of this process which still are not fully understood (or have not been studied), the sperm nucleus must undergo important changes in shape during fertilization. A condensed nucleus in which chromatin is highly compact would lack the flexibility necessary to allow it to pass through the narrow acrosomal canal. This type of sperm chromatin organization would be at the opposite end of the highly rigid organization resulting from the interaction of DNA with cysteine-rich SNBPs [see, e.g., Giménez-Bonafé et al., 2002].

CONSIDERATIONS OF PREVIOUS STUDIES

One final aspect to consider has to do with the discrepancy between the results reported by us and previous work which reported a complete lack of histones or any other SNBPs in the sperm of brachyuran crabs, including *Cancer*. Most of the earlier works were carried out using histochemical staining methods and electrophoretic analysis. The relatively low histone/DNA ratio and the occurrence of histone acetylation possibly hampered the resolution of the staining histochemical analysis. Also, the electrophoretic analysis of the SNBPs of this organism is not trivial. SDS electrophoresis of total *Cancer* sperm protein extracts (not shown) reveals a highly complex electrophoretic pattern in which histones are almost undetectable in the background of a multiplicity of proteins likely arising from the large acrosomal granule. Only when basic proteins from sperm were extracted with 0.4 N HCl (Fig. 2 SDS) or when chromatin was highly purified (Fig. 3) could histones be clearly visualized. Furthermore, we have noticed that release of mature sperm from spermatophores during purification elicits a proteolytic activity that results in important histone degradation unless careful manipulation and adequate use of protease inhibitors are used.

The main conclusion of the present study is that *Cancer* sperm nuclei which had previously been considered deplete of SNBPs, do indeed contain nucleosomes, albeit with an unusually unfolded and unique chromatin organization. This should be considered as a chromatin adaptation to the peculiar fertilization process of the gametes involved.

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Abstract: An interesting characteristic of crustacean sperm nuclei is that they do not contain highly packaged chromatin. In the present study we re-examine the presence of DNA-interacting proteins in sperm nuclei of the crustacean Maja brachydactyla. Although previous reports have indicated that, unlike the majority of sperm cells, DNA of crustacean sperm is not organized by basic proteins, in this work we show that: 1) histones are present in sperm of Maja brachydactyla; 2) histones are associated to sperm DNA; 3) histone H3 appears in lower proportions than the other core histones, while histone H2B appears in higher proportions; and 4) histone H3 in sperm nuclei is acetylated. This work complements a previous study of sperm histones of Cancer pagurus and supports the suggestion that crustacean sperm chromatin deserves further attention.

Barcelona, September 22, 2008

Dear Dr. Dallai

Please find enclosed the manuscript "Preliminary study of sperm chromatin characteristics of the brachyuran crab *Maja brachydactyla*. Histones and nucleosomes in crustacean sperm previously described without SNBPs." by K. Kurtz et al., to be considered for publication in Tissue and Cell.

This work is original, and has not been published (nor is under consideration for publication) elsewhere. There is no conflict of interest which influenced this work in any way.

Thank you for your consideration.

Sincerely yours,

Kathryn Kurtz
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Preliminary study of sperm chromatin characteristics of the brachyuran crab *Maja brachydactyla*. Histones and nucleosomes in crustacean sperm nuclei previously described without SNBPs.

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Running title
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Key words
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Abstract

An interesting characteristic of crustacean sperm nuclei is that they do not contain highly packaged chromatin. In the present study we re-examine the presence of DNA-interacting proteins in sperm nuclei of the crustacean *Maja brachydactyla*. Although previous reports have indicated that, unlike the majority of sperm cells, DNA of crustacean sperm is not organized by basic proteins, in this work we show that: 1) histones are present in sperm of *Maja brachydactyla*; 2) histones are associated to sperm DNA; 3) histone H3 appears in lower proportions than the other core histones, while histone H2B appears in higher proportions; and 4) histone H3 in sperm nuclei is acetylated. This work complements a previous study of sperm histones of *Cancer pagurus* and supports the suggestion that crustacean sperm chromatin deserves further attention.

Introduction

During spermiogenesis, differentiating spermatids are converted into mature sperm. In most species, this process involves a great reduction of nuclear volume and change in chromatin architecture, producing extreme packaging of the genome. The proper interaction of histones or other sperm nuclear basic proteins (SNBPs) with DNA provokes the progressive condensation of spermiogenic chromatin. The final sperm nucleus possesses chromatin which is very compact, reduced in volume, and with a simplified protein composition. However, the transformation of the spermatid nucleus into the mature sperm nucleus is a complex process which varies in each taxonomic group. This process is particularly interesting in crustaceans, a group of organisms which is the fourth most species diverse (Martin and Davis, 2001). Mature sperm nuclei from several crustacean species are very different from sperm nuclei of other studied taxonomic groups (Yasuzumi, 1960; Moses, 1961; Shigekawa and Clark, 1986; Medina, 1994). Sperm chromatin of some crustaceans is not at all electron dense, and appears in a non-compact organization, loosely arranged and suspended in the nucleoplasm (Hinsch, 1969, 1986; Tudge et al., 2001; Medina et al., 2006). Due to the fact that spermiogenesis in many crustacean species involves a decondensation of the spermiogenic chromatin (Langreth, 1969; Shigekawa and Clark, 1986), in the past much focus has been placed on describing the nuclear protein transitions and/or basic proteins associated to spermiogenic and mature sperm cells of these organisms, specifically in brachyuran crabs.

The crustacean type of sperm nuclei was first designated in 1969 when Bloch published a classification of sperm nuclei according to the sperm nuclear basic proteins (SNBPs) interacting with the DNA. Despite several studies dedicated to characterizing this type of sperm (Vaughn and Locy, 1968; Langreth, 1969; Vaughn et al., 1969), the crustacean category of sperm nuclei still remains poorly understood. Around the time of Bloch's sperm classification, sperm nuclei of diverse crustacean species were reported to have no basic proteins associated to DNA, though the alternative stabilizing component of the DNA was never determined. Recently, a study on *Cancer* crabs provided some answers to the longstanding question of how chromatin of crustacean sperm is organized (Kurtz et al., 2008). In this work, micrococcal nuclease digestions of mature sperm chromatin led to the discovery that chromatin is organized by histones into small size nucleosomes; some of these histones appeared to be acetylated or hyperacetylated, in particular, a large portion of histone H4. Additionally, these mature sperm were found to have a much lower basic protein/DNA ratio than other sperm types. In *Cancer*, the low histone/DNA ratio, short linker region of the nucleosomes, and rapid kinetics of the chromatin digestion collectively suggest that among regions of sperm chromatin organized by nucleosomes there exist large regions of sperm DNA which are not stabilized by any basic protein. It is supposed that divalent cations are present to stabilize areas without basic proteins.

Several ultrastructural studies have described in detail the morphology of sperm from a variety of crustacean species. The nucleus generally is cup-shaped and extends into radial arms, is positioned in the periphery of the cell, and surrounds a globular, roughly round acrosomal granule (Langreth, 1969; Hinsch, 1988; Chiba et al., 1992; Medina and Rodriguez, 1992; Jamieson 1994; Jamieson et al., 1998; see also figure 2). Additionally, these nuclei exhibit a few particular characteristics which differentiate them from other sperm nuclei. To begin with, these organelles are not entirely separated from the cytoplasm by a membrane or envelope (Langreth, 1969; Hinsch, 1986, Tudge et al., 2001); some cytoplasmic structures reminiscent of spermiogenic cells are found

immersed within the chromatin itself, together forming *nucleocytoplasm*. Another characteristic of brachyuran crustacean sperm is its uncondensed, fibrous chromatin (Langreth, 1969; Jamieson et al., 1998; see figure 2). This characteristic is quite different than sperm nuclei of other animal species with highly condensed chromatin, a feature which has been interpreted, among other reasons, as protection of the DNA against mutagenic factors (Subirana, 1975; Braun et al., 2001).

The peripheral location of the nucleus, as well as its decondensed nature, has made it complicated to purify these nuclei, and therefore, accurately perform biochemical analysis of the chromatin. Previous experiments designed to study the molecular contents of the nucleus of several brachyuran crabs were mostly carried out by specific histochemical staining methods (Alfert and Geschwind, 1953; Bloch and Hew, 1960), though an attempt was made to solubilize the chromatin using harsh mechanical methods to analyze the solublized proteins (Vaughn and Hinsch, 1972). Species of the genus *Cancer* were studied by Langreth (1969), who concluded that all histones apparently leave the nucleus in the most advanced steps of spermiogenesis; a discrepancy to these findings was described in the recent re-examination of sperm chromatin of *Cancer* crabs (Kurtz et al., 2008). Also, Chevaillier (1966, 1967, 1968) described that in *Eupagurus lombardus* and *Carcinus maenas* the histones migrate from the nucleus to the acrosomal granule in the latest steps of spermiogenesis, while Vaughn and Hinsch (1972) describe only acidic proteins associated to sperm DNA of the Majid *Libinia emarginata*.

Due to great interest in understanding the biochemical nature and organization of the chromatin in crustacean sperm, we have re-examined the basic protein composition of sperm chromatin of the brachyuran crab *Maja brachydactyla*. This species is an interesting model, considering sperm of several Majid species have been used in earlier ultrastructural studies (Hinsch, 1969, 1971; Chiba et al., 1992; Tudge and Justine, 1994; Jamieson et al., 1998), and have also been portrayed as void of basic DNA-interacting proteins (Vaughn and Hinsch, 1972). Since Majids are more specialized crabs than Cancrids (Skinner 1967), the subjects of a previous study with a similar focus (Kurtz et al., 2008), we recognize that differences in sperm chromatin composition between sperm of these two families may contribute to a better overall understanding of crustacean sperm, and makes *Maja brachydactyla* an interesting comparative model.

Material and Methods

Animals.- Male individuals of *Maja brachydactyla* and *Cancer pagurus* (both decapod brachyuran crabs) were purchased live from markets in Barcelona, Spain following proper identification according to their morphological characteristics and the deferential duct containing mature sperm within spermatophores was removed. Male individuals of the bony fish *Sparus aurata* were purchased live from fishermen in Barcelona, Spain, and male echinoderms *Holothuria tubulosa* were obtained off the Mediterranean coast of Catalonia, Spain, and the sperm isolated from each species. Sperm were washed with ice cold buffer A (0.25 M sucrose, 10 mM Tris pH 7.4, 3 mM $MgCl_2$, 5 mM $CaCl_2$, 0.1 mM spermine, 0.25 mM spermidine), containing either 25 mM benzamidine chloride or proteolytic inhibitor cocktail tablets (Roche Diagnostics) to inhibit proteolysis.

Transmission electron microscopy (TEM).- Small sections of testes and deferential duct were dissected from live male individuals of *M. brachydactyla*, and were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 24 hours at 4º C. Samples were rinsed in cacodylate buffer (3 times for 10

minutes and 3 times for 30 minutes) and post-fixed for one and a half hours at 4º C in 1% osmium tetraoxide in cacodylate buffer. The samples were then rinsed in cacodylate buffer twice for 5 minutes and once for 30 minutes. TEM samples were then dehydrated using an increasing acetone series (30% for 15 minutes, 50% for 15 minutes, 70% for 15 minutes, 70% overnight at 4º C, 90% for 60 minutes, 95% for 75 minutes, and twice 100% for 30 minutes) and embedded in Spurr's resin. Ultra-thin sections were made using a Leica UCT ultramicrotome, and counterstained with uranyl acetate and lead citrate. Observations were made on a Jeol EM-1010 transmission electron microscope at 80 kV.

Obtaining purified sperm nuclei or free sperm cell populations.- The purification of sperm cell nuclei from *S. aurata* and *H. tubulosa* was performed as previously described in Giménez-Bonafé et al. (2004). To obtain free sperm cells from *M. brachydactyla*, the deferential ducts were gently homogenized with a dounce homogenizer in buffer A, and filtered through 4 layers of gauze. The filtrate (containing spermatophores and free sperm cells released from spermatophore capsules) was stirred during 15 minutes at 4º C to free sperm cells from spermatophores. Free sperm cells were separated from spermatophore capsules and full spermatophores by sedimentation at unit gravity through a discontinual sucrose gradient of 0.25 M sucrose, 1 M sucrose, and 2.2 M sucrose. The denser material, being full spermatophores and spermatophore capsules, sunk to the interphase of 1 M sucrose and 2.2 M sucrose. After allowing full separation of free sperm from spermatophores in the sperm cell suspension, the top layers containing free sperm suspended in 0.25 M/1 M sucrose were collected and washed in 6 volumes of buffer A. All procedures were performed at 4º C. From this point, the sperm cells freed from spermatophores were used for micrococcal nuclease digestions or extraction of basic proteins.

Extraction of nuclear proteins.- Several types of protein extractions were attempted, using sperm obtained from various individuals on independent occasions. Total protein extraction was performed with 2% SDS, 60 mM Tris, 0.7 M β-mercaptoethanol, and 5 mM EDTA; separate extractions in buffer containing 6 M urea, 20 mM Tris pH 8.0, and 8% β-mercaptoethanol, or with 0.4 N H_2SO_4, were applied as well (results not shown). In all cases the relative amount of soluble histone proteins was the same; however, additional proteins were solubilized in some buffers which overpowered and masked the histone bands by comparison. Ultimately, the following form of protein extraction was determined to be the most appropriate for protein analysis: Free sperm cells were pelleted and total basic proteins were extracted with 5 volumes of 0.4 N HCl; histone H1 and related proteins were extracted with 5% perchloric acid (PCA). Solubilised proteins were cleared by centrifugation, precipitated with 6 volumes cold acetone, and washed with acetone before being vacuum dried (Saperas et al., 2006). For comparative and proteolytic control purposes, fresh and untreated spermatophores were extracted directly with 0.4 N HCl. The type and relative amounts of histones observed in the electrophoretic pattern obtained using this method was identical to that obtained from free sperm cells.

Analysis of proteins.- One-dimensional 15% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and acetic acid/ urea polyacrylamide gel electrophoresis (AU-PAGE) were performed as in Martinez-Soler et al. (2007).

Two-dimensional AUT/AUT electrophoresis was performed according to the procedure described by Dimitrov and Wolffe (1997) with minor alterations, described in

Kurtz et al. (2007). Briefly, the first dimension resolved basic proteins using acetic acid/ 6 M urea/ 6 mM Triton-X 100/ 15% polyacrylamide gel electrophoresis. The second dimension was similar, except the Triton-X 100 concentration was lowered to 0.6 mM.

Reverse-Phase HPLC was performed following the method described in Ausió (1988).

Amino Acid analyses were carried out after hydrolysis in 6N HCl at 110ºC for 24 hours.

Protein and acetylation analysis by western blots.- Equal amounts of total histones from *M. brachydactyla* sperm and standard histones from chicken erythrocyte were loaded for SDS-PAGE used for western blots. Following SDS-PAGE, AU-PAGE, or AUT/AUT two-dimensional PAGE, proteins were transferred to a PVDF membrane and probed for the presence of histones or modified histones as described in Martínez-Soler et al. (2007). Histones H4 or H2A were detected using antisera raised in rabbits as described in Martinez-Soler et al. (2007). Histones H3 and H2B were detected using the commercial antibodies anti-H3 (cat no. ab18521, Abcam) and anti-H2B (cat no. ab 47190, Abcam). The presence of acetylated histones was detected using the following antibodies used in Kurtz et al. (2007; 2008): anti-acetyl lysine (Abcam), anti-H4 specifically acetylated in lysine 12 (anti-H4-acK12, Upstate), anti-H4 specifically acetylated in lysine 16 (anti-H4-acK16, Upstate). Anti-H3 specifically acetylated on lysines 9 and/or 18 (anti-H3-acK9/18, cat no. 07-593, Upstate) was used, as well.

The following antibody dilutions were applied: anti-H2A and anti-H4 antisera at 1:1000; anti-acetyl lysine and anti-H3 at 1:500; anti-H4-acK12, anti-H4-acK16, and anti-H3-acK 9/18 at 1:2000, and anti-H2B at 1:250. All antibodies were prepared in PBS containing 5% powdered skim milk and the membrane incubated overnight at 4º C with shaking. Antibody recognition of all antibodies mentioned was detected using an HRP conjugated goat anti-rabbit secondary antibody diluted 1:3000 in PBS containing 5% powdered skim milk, and incubated for 1.5 hours at room temperature with agitation. HRP detection was performed using an ECL reagent (Amersham).

Micrococcal nuclease digestion and analysis of released products.- Digestions were carried out as mentioned in Kurtz et al. (2008). Control sperm chromatin digestions of *H. tubulosa* were performed parallel to those of *M. brachydactyla*, since the nucleosome of this species has been previously described (Cornudella and Rocha, 1979). Briefly, samples of free sperm cells (*M. brancydactyla*) or sperm nuclei (*H. tubulosa*) were washed in buffer A containing 0.25% Triton-X 100/ 0.1% Nonidet-P40 and incubated on ice for 10 minutes. Then, sperm cells or sperm nuclei corresponding to 1 mg/ml of DNA in buffer containing 0.25 M sucrose, 10 mM Tris pH 8.0, 0.5 mM $CaCl_2$, and 5 mM benzamidine hydrochloride were digested at 37º C for various times with micrococcal nuclease (Sigma). The enzyme was added to a concentration of 0.33 U/mg DNA for *H. tubulosa*, which was diminished to 0.11 U/mg DNA for *M. brachydactyla* to avoid over digestion of the DNA. Reactions were halted by the addition EDTA to a final concentration of 10mM. After centrifugation (10,000 x g for 10 minutes) the supernatants (S) were collected. Chromatin fragments from the supernatants (S) were DNA extracted, as described in Saperas et al. (2006). DNA fragments were analyzed on a 1.1% agarose slab gel. Separately, *M. brachydactyla* sperm chromatin was digested for 15 minutes, and the solubilized native chromatin fragments were separated as in Kurtz et al. (2008) using electrophoresis on a 6% acrylamide native gel in TAE (40 mM Tris pH 7.2, 20 mM sodium acetate, 1.5 mM EDTA) buffer. For native two-dimensional analysis, a lane was excised from the first dimension and equilibrated for 5 minutes in

buffer containing 2% SDS, 10 mM β-mercaptoethanol, 10 mM Tris pH 7.5, and then placed atop the stacking layer of a 15% SDS gel. The proteins associated to the chromatin fragments were allowed to resolve at 80 volts until loading dye reached the bottom of the gel.

Results

I.-Chromatin decondensation during spermiogeneisis and the sperm nucleus of M. brachydactyla

Chromatin organization of the early spermatid of *M. brachydactyla* does not differentiate in the same way as spermatids of other types of spermatogenesis. It presents several clusters of heterochromatin scattered throughout the nuclear interior and periphery (figure 1A), but in the stage immediately following (figure 1B), the heterochromatin begins to disappear, so that the chromatin of intermediate spermatids appears lesser condensed (figure 1C). Nevertheless, dense areas of chromatin appear in sections of these nuclei, some of which are formed by clearly defined granules (figure 1D). In more advanced phases of spermiogenesis, the nuclear shape is transformed, due to the growth of the proacrosomal vesicle, adopting a cup shape surrounding it (figure 2A, B, C), and finally extends into several expansions, or nuclear arms (visible in the section shown in figure 2D).

Although chromatin in the mature sperm nucleus is not in a condensed state, and has a fibrilar appearance (estimated to measure 10-12 nm in diameter), there are areas of the chromatin in which the fibers appear to aggregate together (figure 2E, F). This fact suggests the presence of a nuclear matrix to which the sperm DNA could anchor, providing a certain consistency to the nucleus. Notably, in several other Majids the presence of a nuclear matrix has been suggested. Perez et al. (1986) observed labeling in the nucleocytoplasm of mature sperm of *Libinia emarginata* using antibodies specific for actin, myosin, and tubulin; Tudge and Justine (1994) observed antibody labeling with anti-actin and anti-tubulin in the same region of mature sperm of *Maja squinado*, although some studies mention inability to observe intact microtubules (or other structures) using conventional electron microscopy (Perez et al., 1986). It is still unknown if there could be a relation between the denser areas of chromatin in the transforming spermatid nucleus and the fiber aggregates in the mature sperm nucleus.

To summarize, beginning from the loss of condensed areas of chromatin observed in intermediate spermatids, the nucleus develops a great capacity to transform in shape. This capability to adapt its nuclear shape to its surroundings is a characteristic which is maintained in the mature sperm nucleus, as it is observed even in the spermatophore, where the nuclear arms contour around one another in order to closely pack together (Kurtz et al., in preparation).

II.- Extraction and electrophoretic pattern of M. brachydactyla sperm histones

Just as was described in a previous study (Kurtz et al., 2008), sperm nuclei of many decapod crustacean species cannot be purified using standard methods, since these nuclei rupture when mechanical homogenization procedures are used. Consequently, the histones must be obtained from entire sperm cells, taking careful measures to prevent proteolysis. After having tested various types of extractions (see Material and Methods), we chose to extract fresh sperm cells directly with cold 0.4 N HCl, since a lower proportion of contaminating proteins accompanying solubilized histones is observed, and proteolysis is minimized. The electrophoretic pattern of basic proteins extracted in this way and separated in SDS gels (figure 3A, lane Mb) is similar

(though not identical) to the histone pattern of chicken erythrocyte histones used as a control (figure 3A, lane S1). Proteins with a similar migration to H1/H5 are distinguished, as well as proteins which migrate as those comprising the nucleosome core particle (H2A, H2B, H3, H4). Putative histone H4 demonstrates a similar migration to H4 from chicken, while putative histones H2A and H2B migrate faster than those from chicken. The supposed histone H3 migrates identical to H3 from chicken, but demonstrates a much lower proportional intensity compared to the other core histones (figure 3A).

The electrophoretic pattern described for core histones (which have identical migrations to histones H3 and H4, but slight differences in the migration pattern of H2A and H2B) correlates with the fact that histones H3 and H4 are evolutionarily more widely conserved than histones H2A and H2B (Baxevanis et al., 1996; Piontkivska et al., 2002). Nevertheless, the low proportion of histone H3 is a peculiar characteristic of the sperm chromatin of *M. brachydactyla* which has been reproduced in all experiments, and with all individual specimen included in this study. When sperm are extracted with buffer containing SDS, much more protein is solubilized than with 0.4 N HCl, but the amount of H3 and other histones remains the same (explained in the Material and Methods section).

With the purpose of unmistakably proving that the extracted proteins of sperm from *M. brachydactyla* are histones, we performed a study applying specific antibodies for each of the nucleosomal core histones.

III.- Identification of M. brachydactyla sperm histones

Sperm of a distinct individual of *M. brachydactyla* were separately extracted with 0.4 N HCl and the solubilized proteins were loaded onto a 15% SDS-PAGE, alongside equal amounts of chicken erythrocyte histones, included as a histone standard and positive control. These two lanes were repeated four times in the electrophoresis, and the separated proteins transferred to a PVDF membrane. The core histones were individually detected using specific antibodies for each one, according to the conditions described in the Material and Methods section. Figure 3B shows electrophoresis in SDS of histones from sperm of *M. brachydactyla* (Mb) and chicken erythrocyte (S1), and the reaction of each of the specific histone antibodies (anti- H3, -H2B, -H2A, and -H4, respectively) with these proteins. This figure shows that the basic protein bands extracted from *M. brachydactyla* sperm react with the anti-histone antibodies in a similar way as the histones from chicken erythrocyte. Interestingly, the antibody labeling produced with histone H3 again indicates an observably lower amount than the other histones. Also, histone H2B appears to resolve into more than one isohistone.

This experiment indicates that sperm of *M. brachydactyla* contain histones, although the (deficient) amount of H3 and the (excess) amount of H2B apparently do not respond to the stoichiometrics of typical somatic chromatin, in which all the core histones appear in equimolar proportions.

The quantitative differences in histones H3 and H2B have additionally been verified with the use of antibodies in western blots produced from two-dimensional AUT/AUT electrophoresis (according to Kurtz et al., 2007). In figure 3C (left) the electrophoretic development is shown in two-dimensional gels that contain 6 mM Triton-X 100/ 6 M urea/ 15% polyacrylamide in the first dimension, and 0.6 mM Triton- X 100/ 6 M urea /15% polyacrylamide in the second dimension. In this gel system histones have been identified according to the patterns described in Dimitrov and Wolffe (1997) and Kurtz et al. (2007, 2008). The labeling of these proteins with the anti-H2B antibody is shown in figure 3C (middle), and labeling with anti-H3 antibody

is shown in figure 3C (right). This figure demonstrates again that the amount of histone H3 appears to be diminished both in chromatic staining with Coomassie blue, as well as in its reaction with the anti-H3 antibody. On the other hand, histone H2B resolves into 4 isohistones which together represent a higher amount of H2B than any other histone.

Linker histone H1 is the least evolutionarily conserved histone (Wolffe et al., 1997; Lewis et al., 2003), and hence exhibits great interspecies variability. For this reason it is not suitable to use a commercial antibody, raised to recognize histone H1 from a mammal, in order to identify histone H1 of a brachyuran crustacean species such as *M. brachydactyla*. Histone H1 of these mature sperm appears clearly resolved in the two-dimensional gels of figure 3C and will be analyzed in the following sections of this work.

IV. Histones of M. brachydactyla are bound to sperm DNA

In a great deal of previous studies on spermiogenesis of decapod crustaceans, it was concluded that the nucleus loses histones in the most advanced stages of spermiogenesis, leaving the mature sperm DNA without associated basic proteins (Langreth, 1969; Chevaillier, 1966; 1967). The studies which refer to this subject were mainly carried out in the late 1960's and early '70s, and are based mostly on histochemical staining methods specific for basic protein detection. Despite the intrigue of chromatin apparently lacking in basic proteins, these studies have not been re-examined nor continued until recently, in a work done by Kurtz et al. (2008) in which we demonstrated that sperm DNA of *Cancer pagurus* is bound to histones, some of which have special characteristics.

In the present work we have shown that sperm of *M. brachydactyla* contains histones, but we still have not established that these histones are found in the nucleus of the cell, interacting with DNA. For this purpose, we have taken fresh sperm, solubilized their cellular membrane with Triton-X 100 and Nonidet-P40, and incubated the permeabilized sperm with micrococcal nuclease at a concentration of 0.11 U/mg DNA. (Note that the sperm nucleus of this species is located in the periphery of the cell, and is susceptible to the effects of the nuclease enzyme without necessity of prior purification). The products released from the nuclei by micrococcal nuclease were examined in a two-dimensional gel, in which the first dimension (acrylamide) separates the chromatin fragments (DNA bound by proteins), while the second dimension (SDS) resolves the protein component of the fragments. Figure 4A (first dimension) shows the analysis of the fragments released from the sperm nuclei after 15 minutes of incubation with the micrococcal nuclease enzyme. According to this figure, the nuclease generates a series of discrete chromatin particles (A, B in figure 4A) that are undoubtedly composed of DNA and histones, although again, histone H3 appears in a much lower proportion.

In order to identify the DNA fragment that constitutes the main chromatin particle (A in figure 4A) we performed an identical experiment, in which permeabilized sperm cells of *M. brachydactyla* were incubated from 2 to 15 minutes with the same low concentration of micrococcal nuclease, and the DNA released into the supernatant was deproteinized and analyzed (figure 4B). The released DNA formed discretely small fragments (figure 4B); by incubation times of 5 and 15 minutes a small limiting DNA fragment is clearly observed. This limiting fragment produced in the digestion corresponds to the DNA constituting fragment A in the first dimension of figure 4A. It was estimated to contain about 100 base pairs of DNA, and its protein component (core histones with a low proportion of histone H3) appears beneath the limiting chromatin fragment (A) in the SDS gel of figure 4A. Thus, this limiting chromatin fragment can be

interpreted as analogous to the nucleosome core particle. However, this fragment is not at all identical to the nucleosome core particle, since it is much shorter in length, and apparently is not composed of two molecules of each core histone, evidenced by the low amount of H3 observed in the SDS electrophoresis of figure 4A.

Due to the unusual composition of histones in these chromatin particles, in the description of these results we avoid referring to the chromatin fragments as nucleosomes. It appears that the sperm chromatin of *M. brachydactyla* is organized into units which are similar to, but not the same as, nucleosomes, due to an alteration in their protein composition and DNA length. This is a very important point which deserves further and in-depth examination.

V.- Histone characteristics

Va.-Amino acid composition

Histones obtained by extraction with 0.4 N HCl were later fractionated into the portion soluble in 5% PCA (H1 and family) and the fraction insoluble in PCA (core histones and contaminating proteins). The proteins of each fraction were separated by reverse phase HPLC. The chromatogram of the fraction corresponding to the core histones is complex due to the large proportion of additional minority proteins that are extracted with hydrochloric acid (figure 5A: m, n, o, p, q). However, the nucleosome core histones are eluted at acetonitrile concentrations of 39.9, 42.0, 42.1 and 46.1% (figure 5A), which is similar to the elution profile of other somatic histones (Roccini et al., 1995; Ausió et al., 1997) and have electrophoretic mobilities (figure 5A') and amino acid compositions practically identical to somatic counterparts, as well (Table 1).

Histone H1 (PCA soluble fraction) is resolved into three main bands in the reverse phase HPLC chromatogram (Figure 5B, B'). The amino acid composition of these fractions is included in Table 1, compared to the H1 fractions found in *C. pagurus* (Cp), as well as somatic H1 from calf thymus(Ct). The mobility of these fractions in SDS is similar to H1/H5 of chicken erythrocyte. Aside from their solubility in 5% PCA, the amino acid compositions and mobilities of these fractions designate them as H1 isoforms. It is important to point out that proper identification of these H1 fractions in global extracts is quite difficult, due to the large amount of contaminating proteins, likely from the acrosome, which migrate in the H1/H5 region of SDS gels.

Vb.- Histone acetylation

Sperm histones of *M. brachydactyla* were separated in two-dimensional gels (Figure 6A), and analyzed by western blot with an anti-acetyl lysine antibody. This antibody marks any protein which has an acetylated lysine residue, independently of where that acetylated lysine happens to appear in the molecule. In contrast to the fellow brachyuran crab *C. pagurus* (Kurtz et al., 2008), histone H4 of *M. brachydactyla* does not highly react with the antibody (Figure 6B); in fact, nearly no reaction was observed at all, unless the antibody reaction was overexposed. To confirm that H4 is not one of the mainly acetylated histones, antibodies specific for acetylated forms of H4 (H4-acK12 and H4-acK16) were used to detect any acetylation on histone H4. In this experiment sperm basic protein extracts from *C. pagurus* were included alongside sperm extracts from *M. brachydactyla* as a positive control (Kurtz et al., 2008), and developed in AU-PAGE, a system which resolves acetylated fractions of histone H4. Indeed, H4 showed very low labeling with both of the two antibodies, yet highly reacted with *C. pagurus* H4 (Figure 6C). The main protein which is highly acetylated in this chromatin according to the reaction with anti-acetyl lysine antibody is histone H3 (figure 6B). We used an antibody specific for acetylated histone H3 (anti-H3-acK9/18) to confirm that the reaction with anti-acetyl lysine is indeed histone H3, and not a

contaminating protein migrating similar to H3. In this experiment, basic protein extracts from spermiogenic nuclei of the bony fish *Sparus aurata* were included as a positive control of H3 acetylation, and basic proteins from mature sperm of the same animal were included as a negative control (unpublished results). Indeed, the antibody highly reacted with histone H3 in basic extracts of mature sperm of *M. brachydactyla* and gonadal tissue of *S. aurata* (Mb and Sg in figure 6D), but showed no reaction with the negative control (Ss in Figure 6D). The strong antibody reaction suggests that a very high portion of H3 is acetylated in sperm of *M. brachydactyla*.

Discussion

Considerations on obtained results

Contrary to spermiogenesis in most species, the developing sperm nucleus of *M. brachydactyla* undergoes chromatin decondensation. Spermiogenic chromatin of this species transforms in shape according to external forces. This characteristic is maintained in the mature sperm nucleus, in which certain flexibility allows it to change shape in reaction to stimuli, a characteristic which appears to be an important adaptation during the acrosome reaction (Fasten, 1921; Nanshan and Luzheng, 1987; Hinsch, 1971; Goudeau, 1982; Medina and Rodríguez, 1992). The main proteins associated to sperm DNA and how they organize the chromatin in a mature sperm nucleus with such an unusually malleable nature has been the focus of this study.

The entire histone complement found in sperm of *M. brachydactyla* constitutes an atypical pattern when compared to chromatin of somatic cells. On one hand, the presence of all nucleosomal histones (H2A, H2B, H3, H4, and H1) have been identified, using their electrophoretic migrations, amino acid compositions, and (in the case of the four core histones) their reactions with respective specific antibodies. Nevertheless, the proportion of core histone H3 seems to be diminished with respect to its canonical proportion, while the relative proportion of histone H2B appears increased. The low amount of H3 which was found does not appear to be an experimental artifact, such as proteolysis, since several methods of extractions were performed, using many different individual animals (see Material and Methods), and this same result was reproduced each time. Despite the fact that stoichiometric disproportion of histones is a highly infrequent occurrence, it is not exclusive to the sperm of *M. brachydactya*. Indeed, the sperm nucleus of the amphibian *Xenopus laevis* contains an endowment of histones H3 and H4 equal to somatic cells, but has reduced amounts of histones H2A and H2B, and lacks histone H1 entirely (Bols and Kasinsky, 1973; Risely and Eckhardt, 1981; Wolffe, 1989). In this case, the sperm nucleus is found in a state of condensation due to other basic proteins (Sp1 to Sp6) which substitute the [H2A-H2B] dimers (Mann et al., 1982; Abé, 1987).

Another significant result is the demonstration that these histones are bound to the sperm DNA. This result is verified in figure 4A, in which histone H3 again appears in a very low proportion compared to the other histones. The analysis of the micrococcal nuclease digestions also show that the DNA-histone complexes form structural units that may be analogous to nucleosomes (A in figure 4A). Nonetheless, these fragments cannot be identical to nucleosomes, since they contain a low proportion of H3, and nucleosomes have equimolecular amounts of core histones (van Holde, 1988).

Finally, our results show that histone H3, in spite of its low proportion, appears in a considerable state of acetylation. Acetylation of histones diminishes their capacity for electrostatic interaction with DNA and impedes the formation of superorder

chromatin structures (García-Ramírez et al., 1995; reviewed in Zheng and Hayes, 2003; Calestagne-Moreli and Ausió, 2006). This fact would be related to the decondensed state of *M. brachydactyla* sperm chromatin. Another possibility which does not exclude the previous one, is that during spermiogenesis, histone H3 could become hyperacetylated and possibly depart from the chromatin, leaving only residual amounts of acetylated H3 remaining in mature sperm chromatin. In support of this possibility, it is an established fact in various spermiogenesis that expulsion of histones from the nucleus is preceded by an important wave of acetylation (Oliva and Dixon, 1991; Hazzouri et al., 2000; Lahn et al., 2002). In addition, it is interesting to point out that in mature sperm nuclei of other brachyuran crustaceans studied (*Cancer pagurus*) histone H4 also appears to have an elevated level of acetylation. However, aside from a small portion of acetylated histones which remain together with protamine in mature human sperm (Gusse et al., 1986; Gatewood et al., 1990), in sperm with condensed chromatin (representing the majority of all species), acetylation of histones has never been found.

Our work presented here leaves many important questions open and unanswered concerning the structure of sperm chromatin of brachyuran decapod crustaceans. Despite this, the results obtained (mainly the low proportion of H3, its high degree of acetylation, and the unusual proportion of H2B) broaden our understanding of the non-condensed sperm nucleus of *M. brachydactyla*.

Considerations concerning the biology of fertilization in brachyuran decapod crustaceans

The sperm nucleus of any animal species completes a primordial function, which is the preservation of its genetic material in optimal conditions, so as to guarantee fertilization and continuity of the species. Additionally, it is adapted to the concrete type of fertilization produced within its own species (Franzén, 1970, 1977).

In the majority of animal species, their sperm nuclei are highly condensed, with a greatly reduced volume. Condensation of the genetic material is related to (among other aspects) the preservation of DNA against mutagenic changes often produced by physicochemical agents of the environment (Subirana and Puigjaner, 1973; Subirana, 1975). In the case of brachyuran crustaceans, the mature sperm which leave the male for fertilization are found grouped together within spermatophores (Hinsch, 1986, 1988; El-Sherief, 1991; Chiba et al., 1992; Jensen et al., 1996) which may have a similar protective function. In this way, sperm nuclei of *M. brachydactyla* are able to exist in a non-compact state without their DNA being susceptible to the often harsh effects of their environment.

The non-condensed and flocculent characteristic of the sperm chromatin in these crustaceans is undoubtedly related to the process of their nuclear penetration of the oocyte cytoplasm. When the sperm enters in contact with the oocyte envelope, the acrosome goes through an eversion (Fasten, 1921; Hinsch, 1971; Goudeau, 1982; Nanshan and Luzheng, 1987; Medina and Rodríguez, 1992) and the nucleus is dragged toward the oocyte cytoplasm though a narrow acrosomal canal (formed by the sperm's own eversion). This type of acrosomal reaction could not occur if the sperm chromatin were compact and rigid. On the contrary; in sperm nuclei of crustaceans, a relaxation of the DNA-protein interactions occurs, which permits deformation of the cell nucleus, and allows it to enter into the oocyte cytoplasm.

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Figure Legends

Figure 1.- Early changes in the spermiogenic chromatin of *M. brachydactyla*.
A.- Corresponds to the nucleus of the earliest spermatid stage found in our study. The nucleus (N) is organized into clusters of heterochromatin and lesser dense areas of euchromatin.

B.- In the stages immediately following, the heterochromatin begins to disappear from the spermiogenic nucleus.

C. and D.- The nucleus of the intermediate spermatid contains homogenous chromatin, apparently decondensed, except for some small areas (visible in D) which conserve an electrodense and granular appearance. Observe that in spite of the chromatin decondensation and homogenation, no significant increase in volume of the cell nucleus has occurred (verified in various sections not shown).

Bars: A- D: 2 μm.

Figure 2.- Final changes in spermiogenic chromatin of *M. brachydactyla*.
A.- In the most advances stages, a proacrosomal complex begins to form (PA), which provokes a displacement of the nucleus (N) toward one cellular pole, as well as a change in its shape.

B.- As the acrosome continues to grow and becomes organized into the proacrosomal vesicle (PA), the nucleus (N) continues to adopt a cup shape and begins to surround the acrosome.

C. and D.- In the final stages of spermiogenesis the nucleus (N) nearly completely surrounds the acrosome (PA in C and A in D) and extends into radial expansions (nuclear arms). The nuclear chromatin is not perfectly uniform; areas with aggregates of chromatin fibers are observed. NA.- Transversal section of a nuclear arm.

E. and F.- Details of nuclear areas in which large aggregates of chromatin fibers are observed (marked in F with arrows). Using these images (and others not shown) we have estimated the diameter of the chromatin fibers, which have an approximate value of 10-12 nm.

Bars: A.-C: 2 μm; D: 1 μm; E: 0.5 μm; F: 0.2 μm

Figure 3.- Identification of the core histones in sperm of *M. brachydactyla*.
A.- Electrophoreses in SDS of proteins extracted from sperm of *M. brachydactyla* (lane Mb) compared to control histones of chicken erythrocyte (lane S1). The basic proteins of *M. brachydactyla* have the same migrations as nucleosomal histones (H2A, H2B, H3, and H4). Observe that the histone band corresponding to H3 in *M. brachydactyla* appears with a lower intensity than the rest of the core histones. Also, one can observe in this same extract a complex series of electrophoretic bands that migrate in the H1/H5 region.

B.- Electrophoresis in SDS of the core nucleosome proteins of *M. brachydactyla* (Mb) and chicken erythrocyte (S1), and corresponding western blot of these proteins with antibodies specific for each one of the four histones comprising the nucleosome core (anti- H3, -H2B, -H2A, and -H4, respectively). The intensity of labeling of each core histone of *M. brachydactyla* is equivalent to its homologue in chicken erythrocyte, except for histone H3, in which the intensity is lower. Note that in the SDS gel pattern the intensity of histone H3 *of M. brachydactyla* is also lower than its corresponding control. Notice as well that histone H2B resolves into two bands which together exhibit intensity greater than the H2B control.

C.- Two-dimensional electrophoresis in AU with 6 mM Trion/ AU with 0.6 mM Triton (according to Kurtz et al., 2007) of the extracted proteins from sperm cells of *M. brachydactyla* with 0.4 N HCl (left), and western blot treated with anti-H2B (center) and anti-H3 (right). These gels resolve histone H2B into four subfractions (left and center) and again demonstrate that the amount of extracted of histone H3 is very low (left and right). C blue st.- coomassie blue staining.

Figure 4.- Analysis of *M. brachydactyla* sperm chromatin using micrococcal nuclease (MNase) digestions.
A.- (left and center).- Two-dimensional gel which resolves the chromatin fragments and associated proteins, following an incubation of 15 minutes with MNase. The first dimension (from left to right) separates the main chromatin fragment (A) and its putative dimer (B). Fragment A could be similar, but not identical, to the core particle of the nucleosome. The second dimension (from top to bottom) corresponds to electrophoresis in SDS which separates the proteins associated to DNA in each particle. The proteins of fragments A and B correspond to core histones, in which histone H3 appears in a low proportion, once again. In the left portion of this figure a control of sperm histones from *M. brachydactyla* developed in SDS-PAGE has been included.

B.- (right)-Analysis of DNA solubilized by MNase at different incubation times (2, 5, and 15 minutes, respectively). As the incubation time progresses, the DNA is digested toward a limiting fragments size (visible at 15 minutes of incubation) of approximately 100 base pairs. The DNA digested at 15 minutes correlates to DNA of chromatin fragment A in figure 4A. h.- nucleosome core particle of *H. tubulosa* obtained in parallel with the chromatin digestion of *M. brachydactyla*. The size of the nucleosome core is 146 base pairs, L.- DNA ladder showing multiples of 123 base pairs.

Figure 5.- Purification of the sperm histones of *M. brachydactyla*.
A.- Reverse phase HPLC chromatogram of the fraction of proteins soluble in 0.4 N HCl and insoluble in 5% PCA (perchloric acid). Proteins eluted in the major peaks co-migrate in SDS gels with core histones (A') and display and amino acid composition very similar to other known core-histones (Table 1). Control lane in A': Mb.-Fraction of *M. brachydactyla* sperm proteins insoluble in PCA.

B.- Reverse phase HPLC chromatogram of the protein fraction from *M. brachydactyla* sperm soluble in 5% PCA. Peaks a, b, and c correspond to three proteins which migrate in SDS gels in the electrophoretic area of histones H1/H5 (B'), and have an amino acid composition typical of histone H1 (Table 1). Control lanes in B':S1.- H1/H5 from chicken erythrocyte. Mb.- Fraction of *M. brachydactyla* sperm proteins soluble in PCA.

Figure 6.- Acetylation of histones from *M. brachydactyla* sperm.
A.- Two-dimensional electrophoresis, also shown in figure 3C, which corresponds to the gel transferred for western blot and treated with anti-acetyl lysine antibody (B), which recognizes any protein with an acetylated lysine residue.

C.- The low acetylation level of histone H4 is demonstrated by western blot using antibodies specific for acetylated residues on the amino terminal tail of H4 (anti-H4-acK16, center, and anti-H4-acK12, right). The proteins were resolved in an AU-PAGE gel (left). Cp.- sperm histones of *C. pagurus* used as a positive control; Mb.- histones of *M. brachydactyla*.

D.- Western blot of proteins resolved using AU-PAGE (left) and treated for the presence of acetylated H3 at lysines 9 and/or 18 (right). Sg.- histones from *S. aurata* gonadal nuclei, included as a positive control of H3 acetylation; Ss.- histones from mature sperm of *S. aurata*, included as a negative control of H3 acetylation; Mb.- *M. brachydactyla* sperm proteins.

Figure

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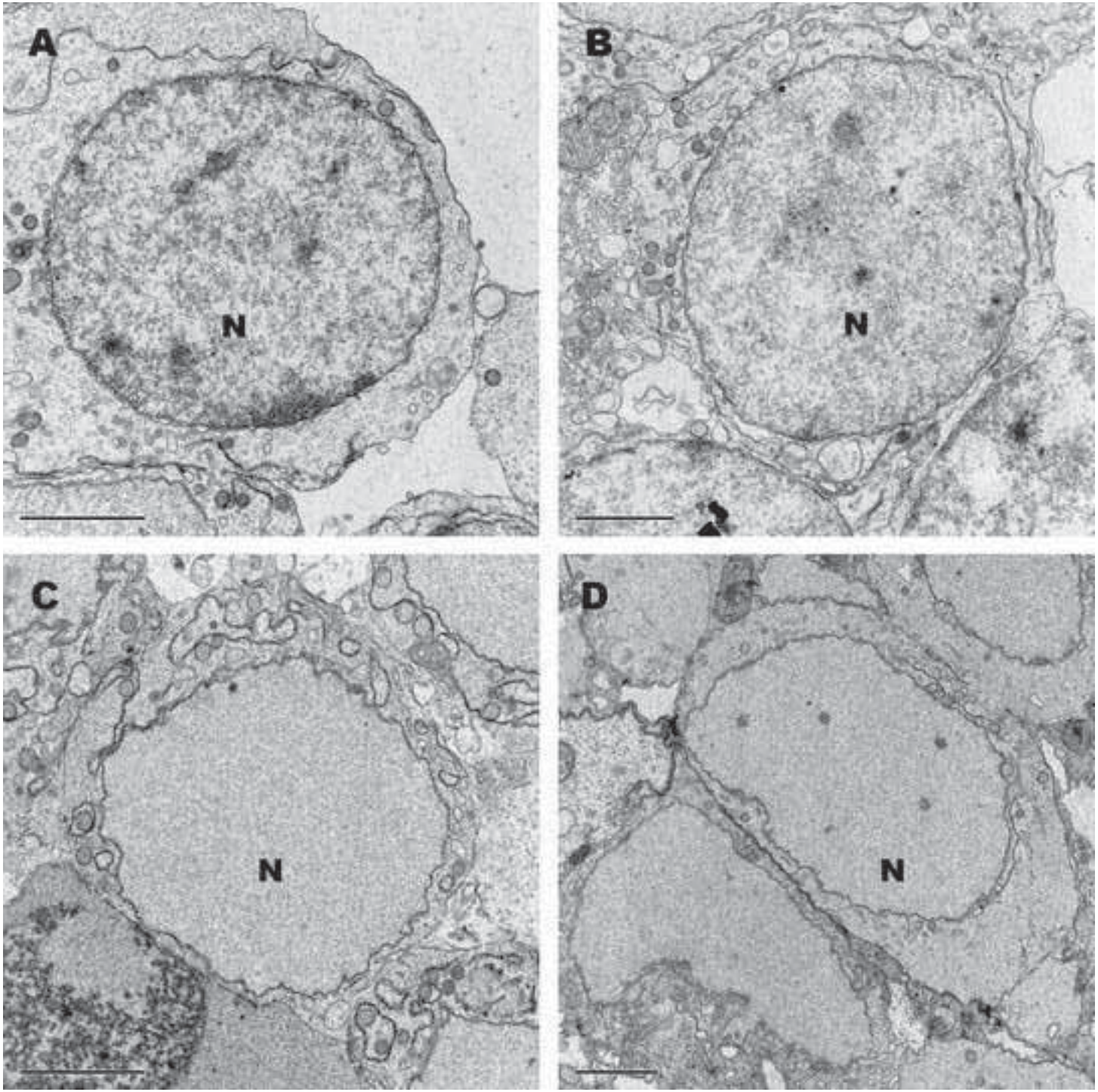

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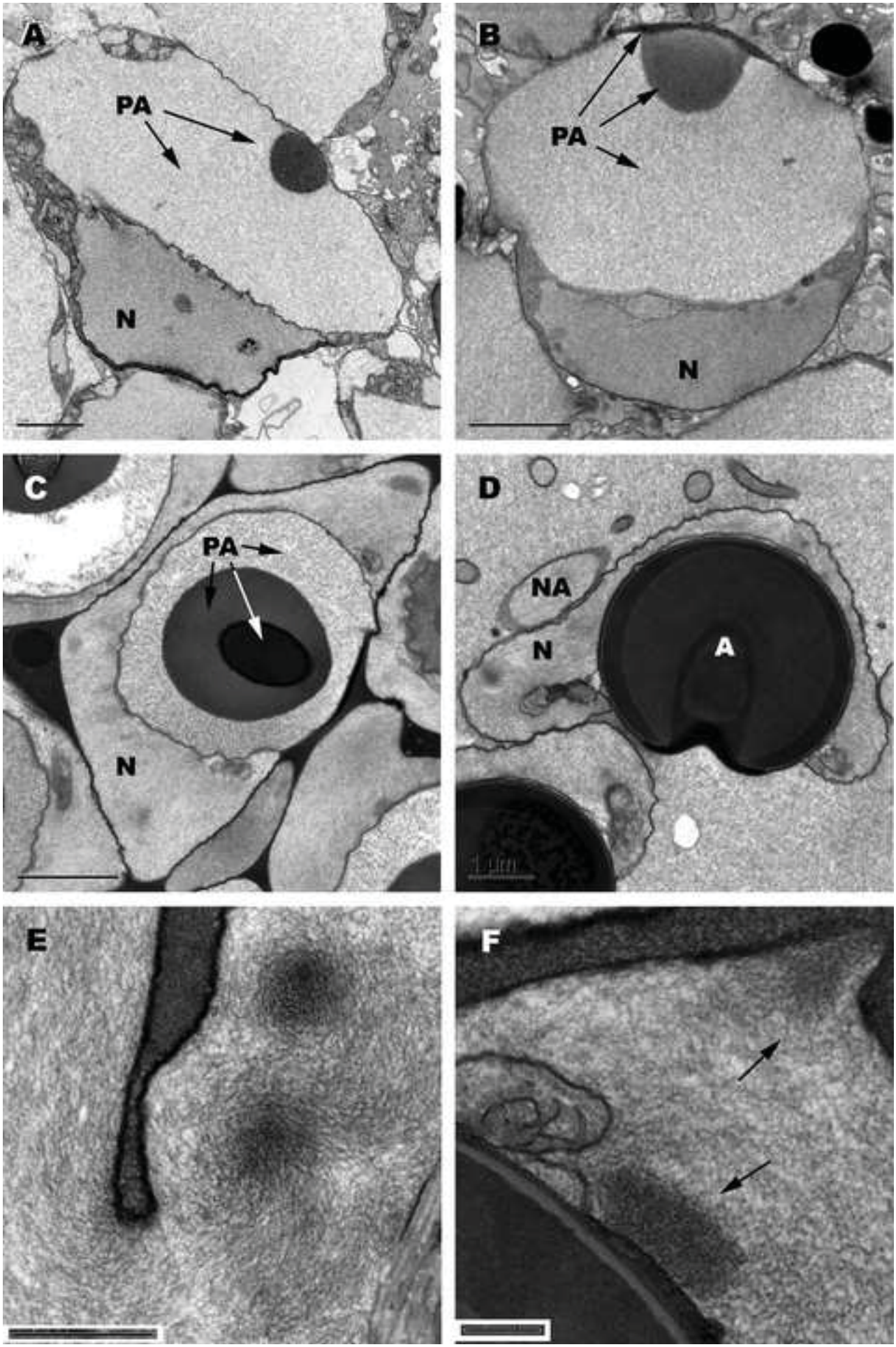

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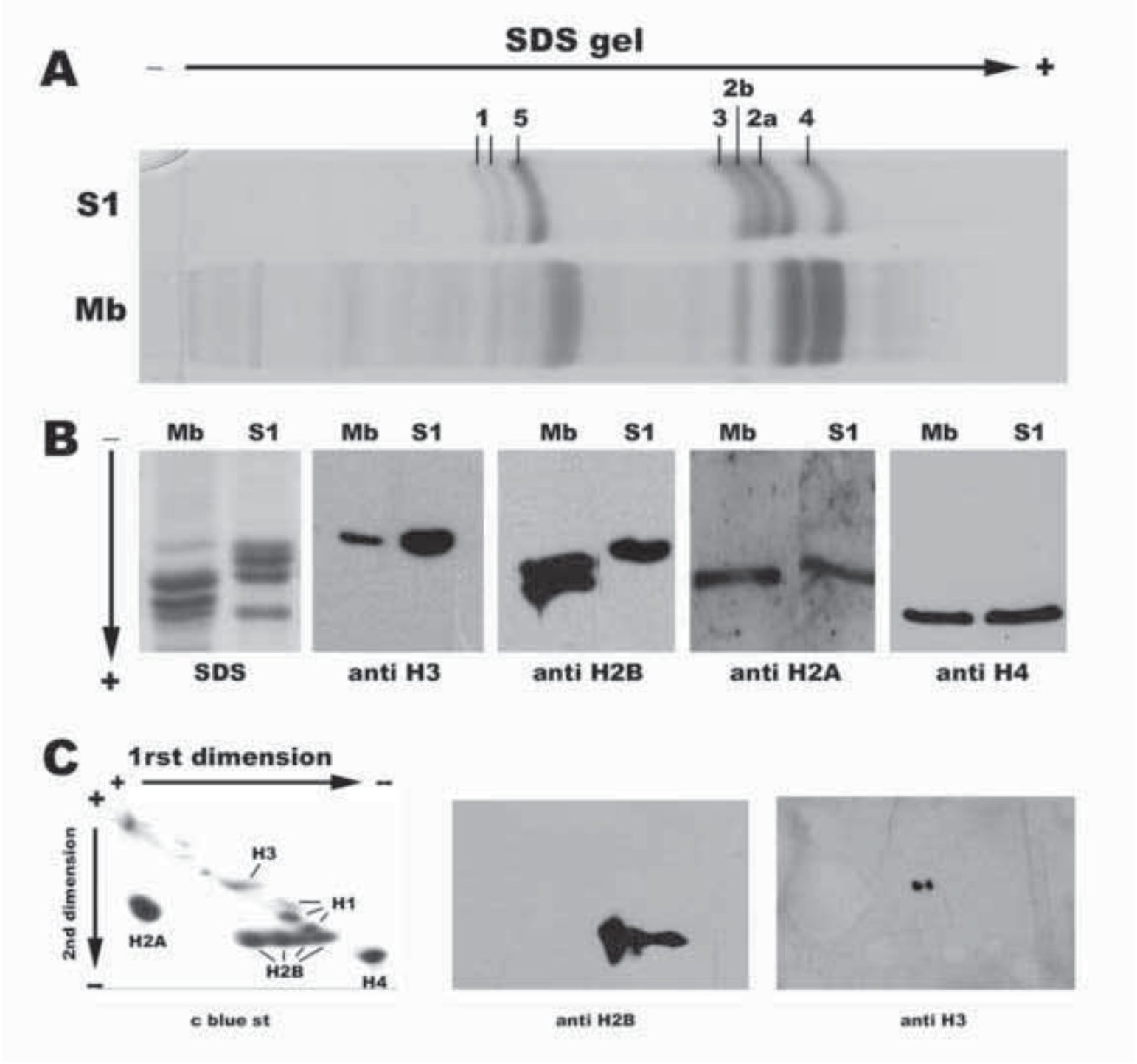

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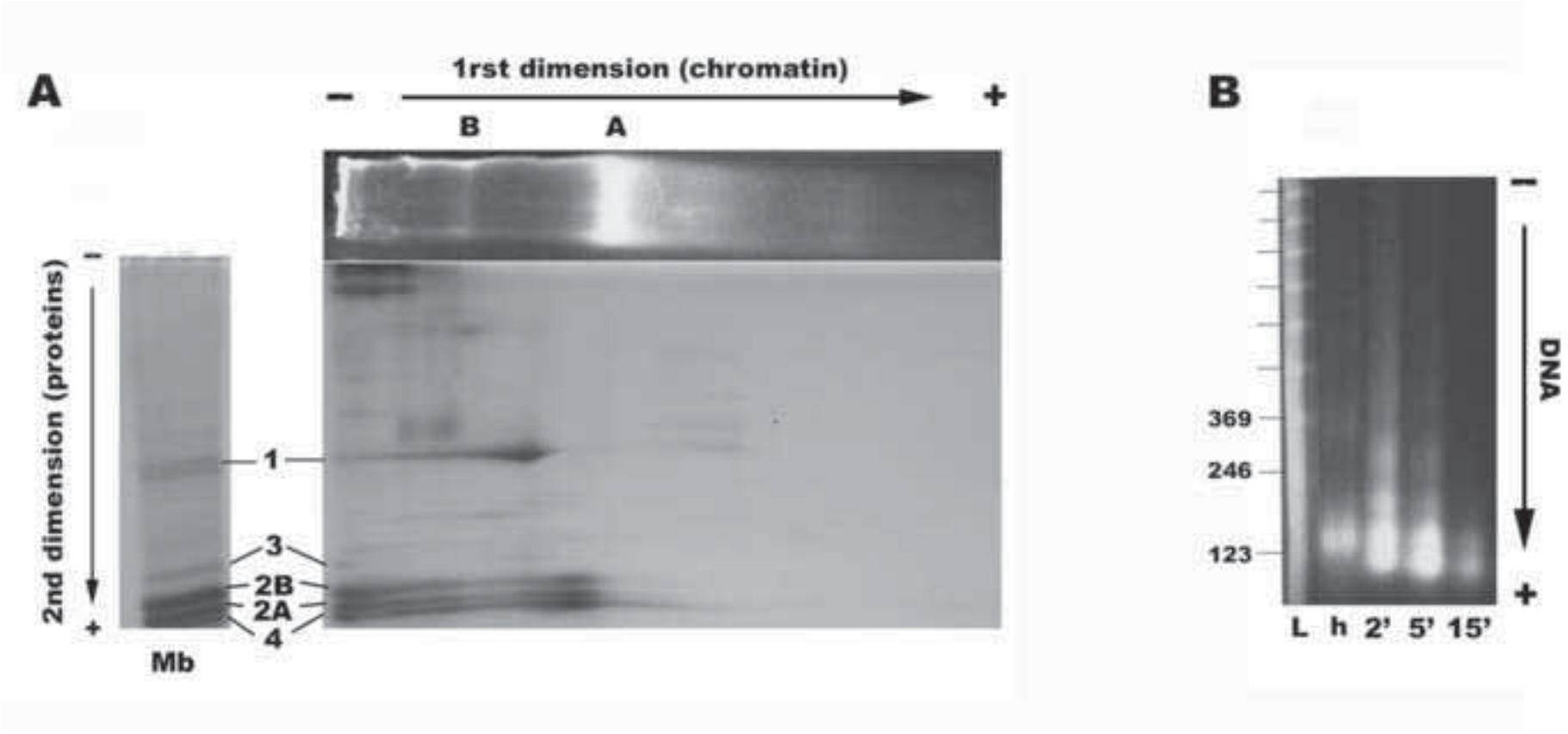

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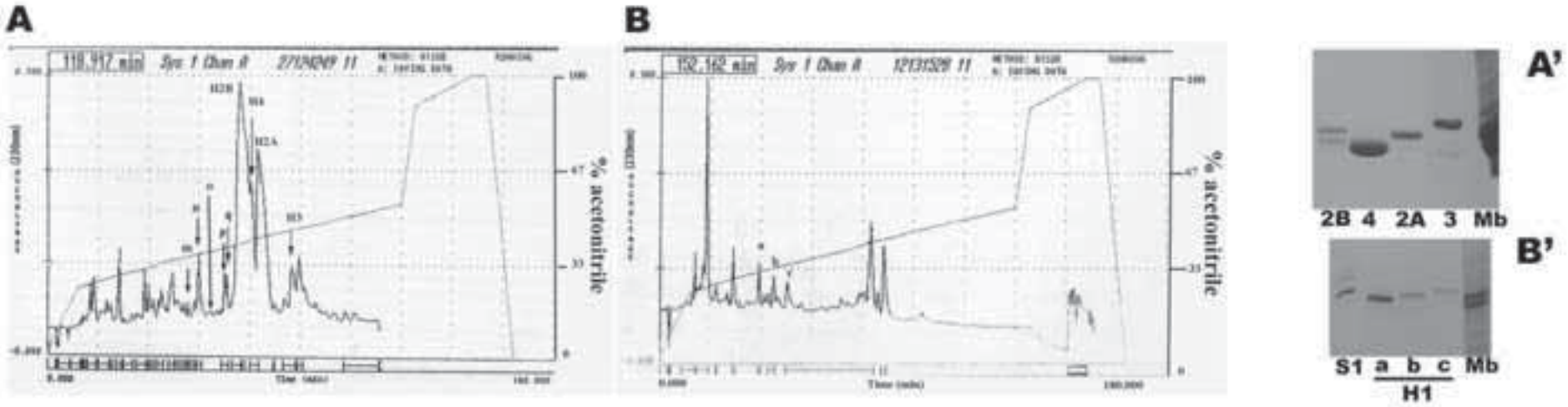

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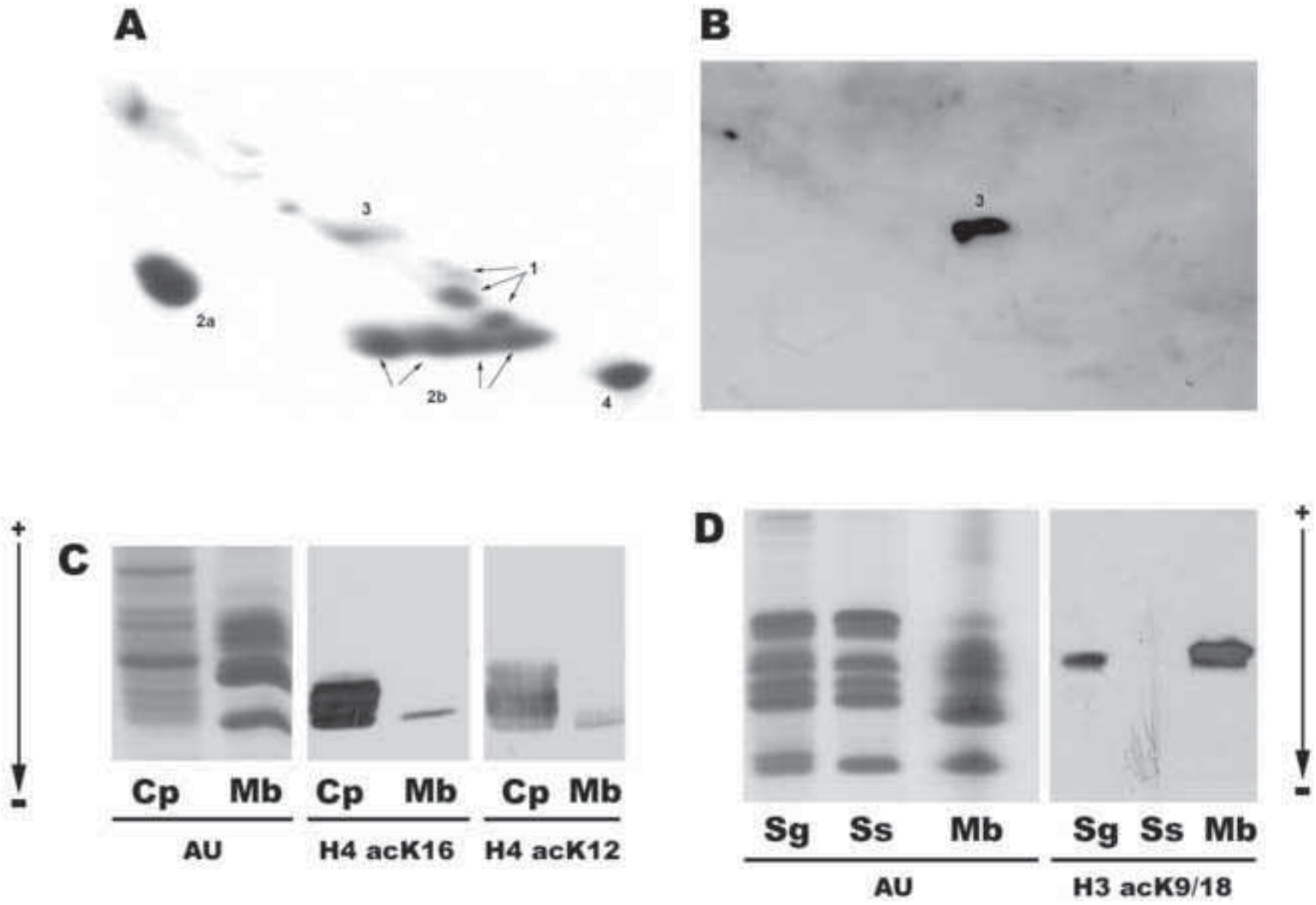

Table

	H2A			H2B			H3			H4			H1						
	Ct	Cp	Mb	Ct	Cp	Mb	Ct	Cp	Mb	Ct	Cp	Mb	Ct	Cp (a)	Cp (d)	Cp (e)	Mb (a)	Mb (b)	Mb (c)
Lys	10.2	10.1	10.5	14.1	13.8	13.1	10.0	9.0	7.6	11.4	8.5	10.5	26.8	18.3	16.1	20.3	24.8	17.5	16.9
His	3.1	1.8	1.8	2.3	2.3	2.3	1.4	1.5	2.1	2.2	2.3	2.2	0.0	1.1	0.6	0.5	1.0	1.7	2.2
Arg	9.4	8.6	9.9	6.9	6.4	6.9	13.0	12.8	12.2	12.8	12.2	12.7	1.8	4.3	2.1	4.7	2.6	3.1	3.9
Asp	6.2	7.5	6.3	5.0	6.9	6.6	4.2	4.3	4.7	5.2	6.0	5.7	2.5	5.8	6.2	5.9	4.4	5.4	4.9
Thr	3.9	3.0	3.0	6.4	5.2	6.3	6.8	6.3	6.8	6.3	6.4	6.1	5.6	10.3	6.0	7.5	7.1	5.2	8
Ser	3.4	5.1	5.0	10.4	8.4	9.2	3.6	3.7	3.7	2.2	3.2	2.7	5.6	6.7	4.8	6.1	5.5	7.9	12.7
Glu	9.8	9.3	9.1	8.7	8.5	10.2	11.6	11.8	11.1	6.9	7.5	6.9	3.7	4.6	8.2	1.6	5.8	8.4	3
Pro	4.1	4.7	4.7	4.9	3.3	4.4	4.6	4.8	4.8	1.5	2.3	1.9	9.2	9.4	10.0	10.6	7.7	9.5	8.7
Gly	10.8	11.1	11.0	5.4	5.5	6.1	5.4	6.2	5.7	14.9	14.7	14.8	7.2	5.3	5.0	5.1	6.2	6.1	9.4
Ala	12.9	9.7	10.6	10.8	11.5	9.9	13.3	12.0	11.3	7.7	7.4	7.5	24.3	9.5	21.2	19.3	20.7	18.7	14.5
Cys	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Val	6.3	6.8	6.9	7.5	6.4	5.4	4.4	5.3	6.0	8.2	7.7	8.0	5.4	9.2	8.2	8.4	3.4	4.8	5.7
Met	0.0	1.5	1.1	1.5	2.2	2.3	1.1	1.8	1.6	1.0	1.5	1.1	0.0	0.2	1.0	0	0.9	1.3	0
Ile	3.9	5.1	4.7	5.1	6.4	5.8	5.3	5.3	5.4	5.7	5.7	5.8	1.5	4.8	3.1	3.5	3.4	3.9	3.6
Leu	12.4	10.8	11.6	4.9	6.0	5.3	9.1	9.0	9.6	8.2	7.9	7.9	4.5	6.8	4.5	4.2	3.9	4.0	3.7
Tyr	2.2	2.9	2.6	4.0	4.5	3.9	2.2	2.7	2.4	3.8	4.4	4.3	0.9	0.9	1.5	1.1	1.6	1.8	1.4
Phe	0.9	1.8	1.1	1.6	2.5	2.2	3.3	3.3	2.9	2.1	2.4	2.0	0.9	2.7	0.9	1.2	1.1	1.4	1.3

Table 1. Amino acid composition (mol %) of histones of *M. brachydactyla* (Mb) compared to the amino acid composition of histones from *C. pagurus* (Cp) and calf thymus (Ct). H1 proteins a, b, and c correspond to proteins a, b, and c of figure 5B, B'. All histone fractions from *Cancer pagurus* (Cp) are from Kurtz et al., (2008).

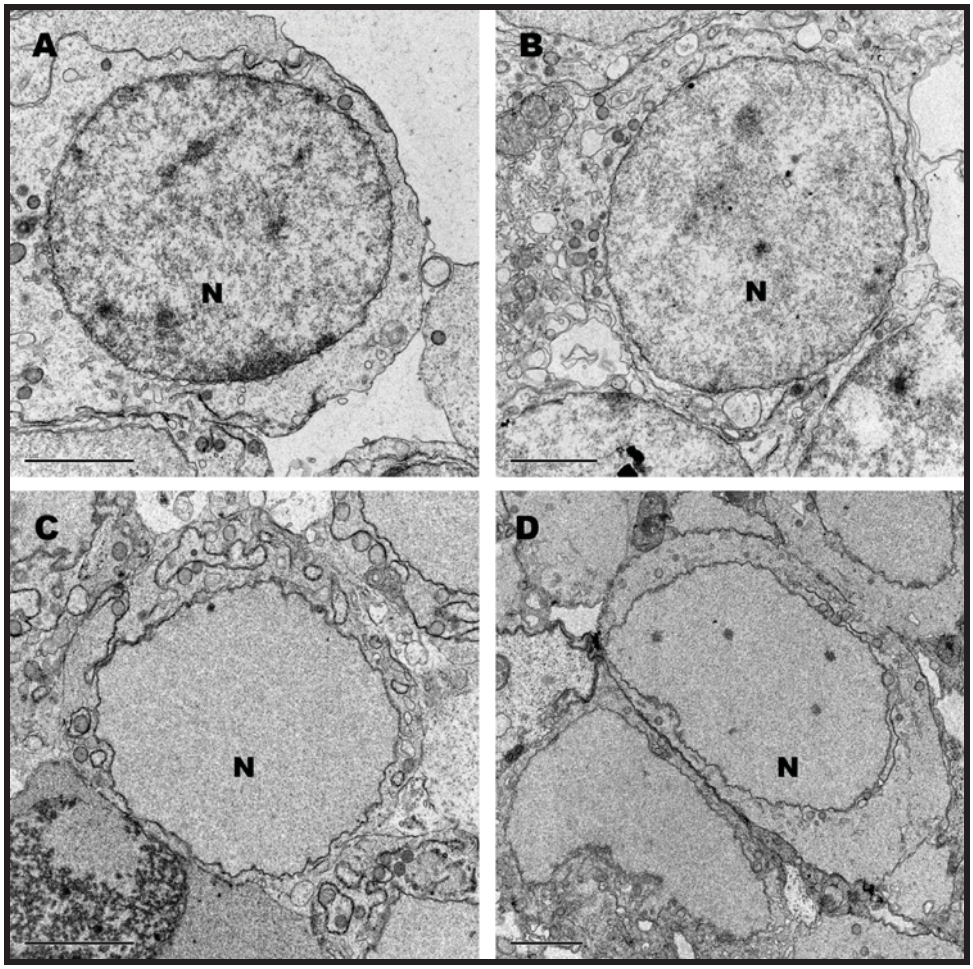

FIGURE 1

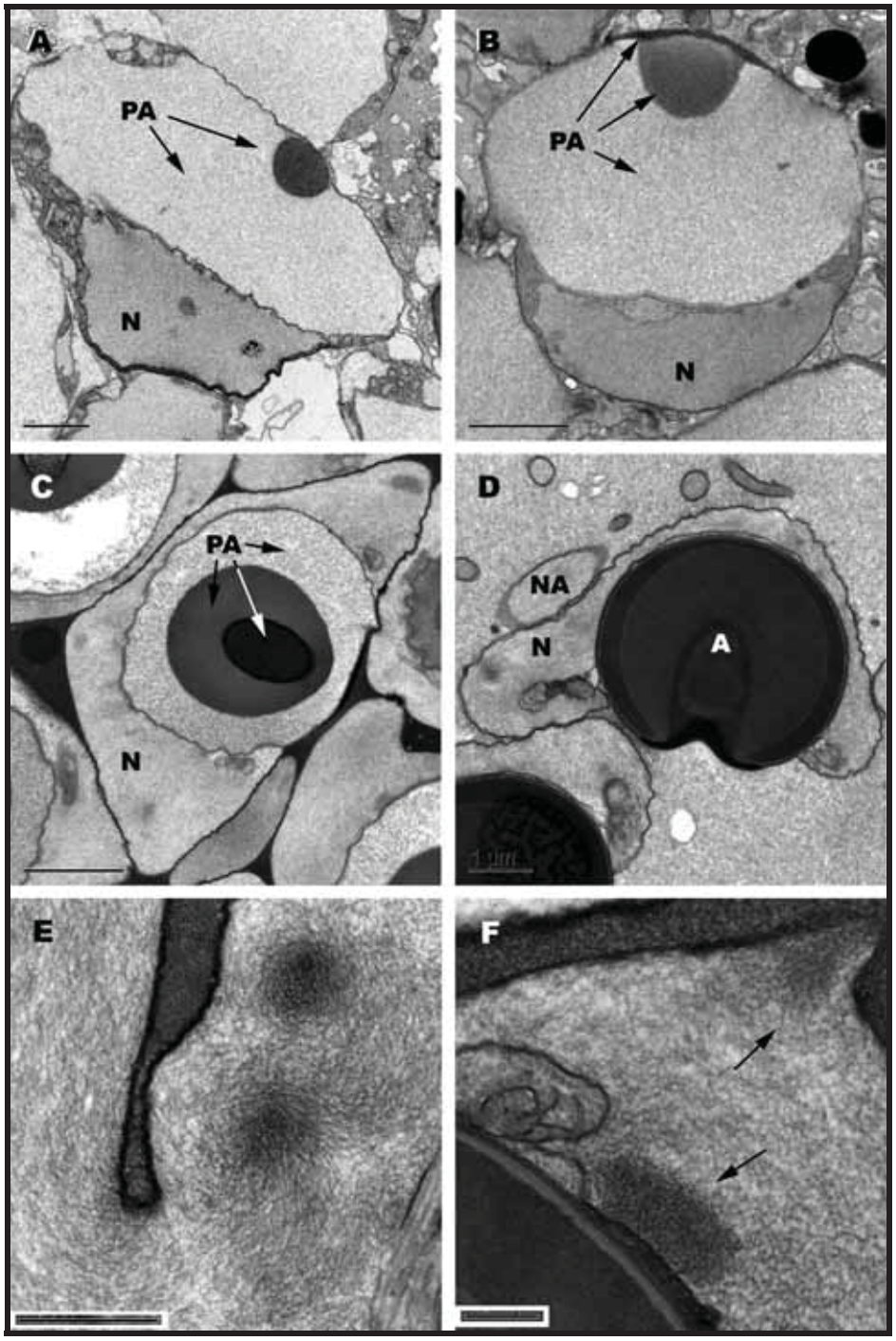

FIGURE 2

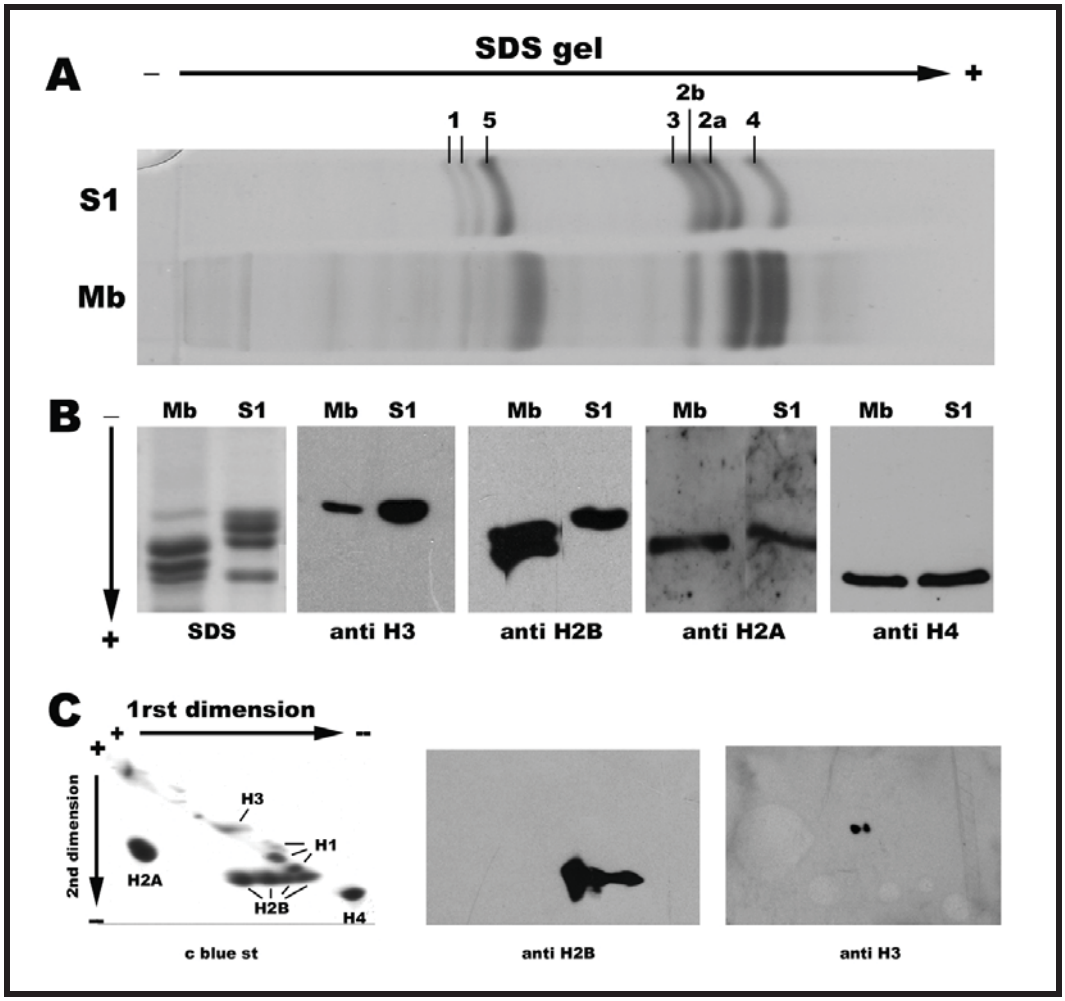
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FIGURE 3

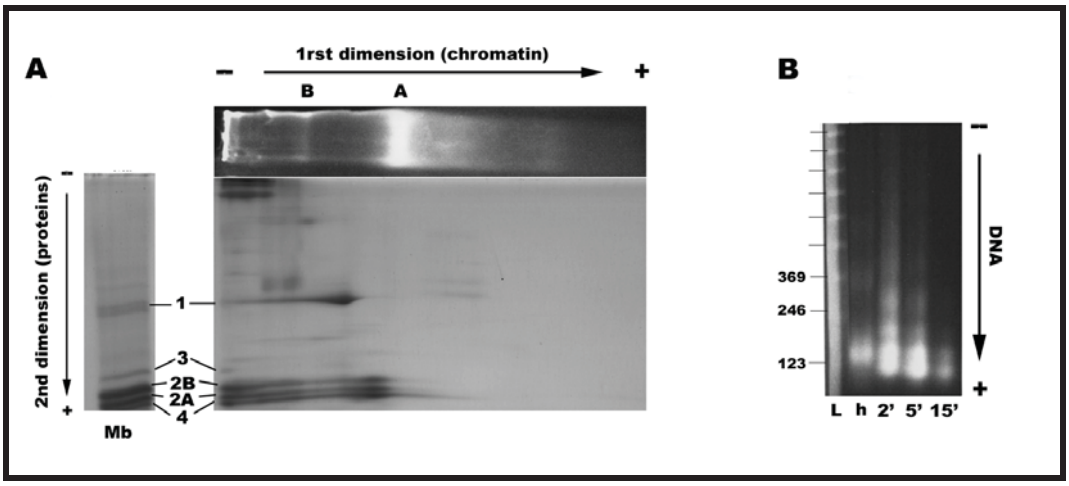
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FIGURE 4

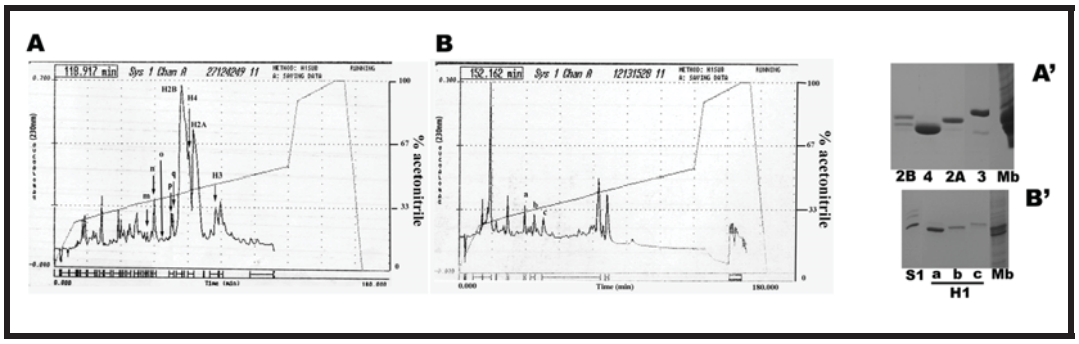
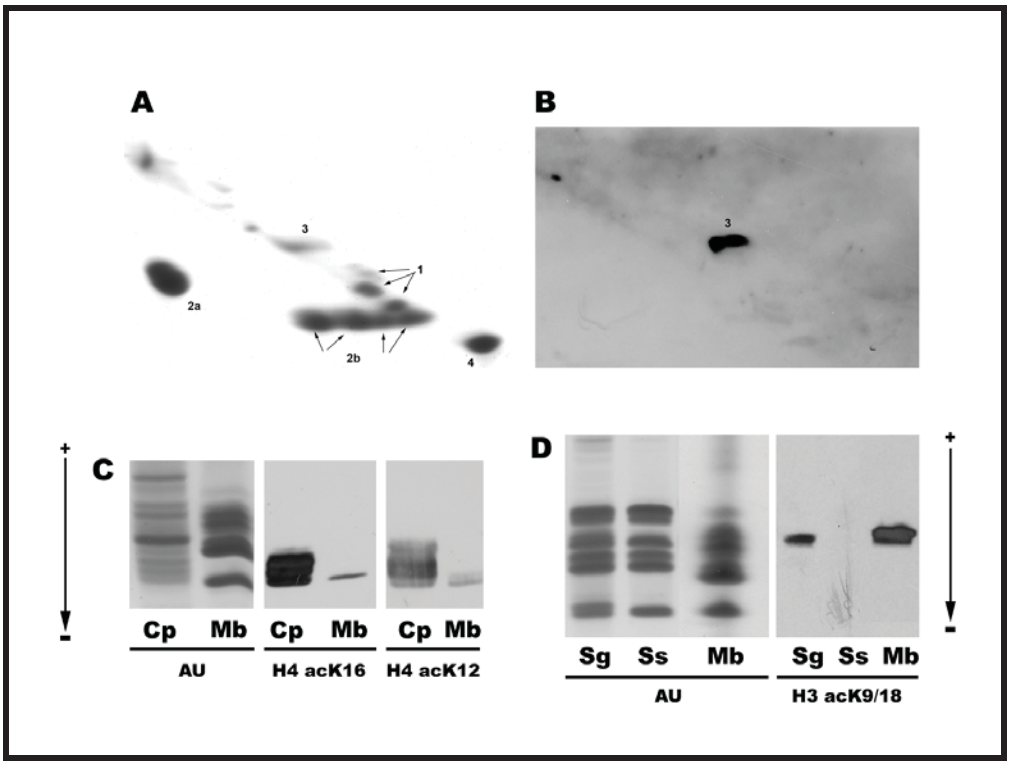


FIGURE 5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H4 acK16
H4 acK12
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Ss
Mb
AU
H3 acK9/18

FIGURE 6

DISCUSSION

IV. DISCUSSION: SIGNIFICANCE OF SPERM CHROMATIN MODELS

IV.1. MODEL "HISTONES"

In this work, four simple sperm models were studied, and the sperm nuclear basic protein transitions, chromatin structures, and histone modifications were analyzed. In the most primitive sperm model, H→H or "histones", histones are maintained in the mature sperm chromatin. This model, represented in this study by the bony fish *Sparus aurata*, is considered the most primitive since the histones are not replaced by any other protein during spermiogenesis. Without any change in the basic protein component of the spermiogenic chromatin in the H→H model, other changes which affect the maturing sperm cell during spermiogenesis are also quite simple. This includes chromatin compaction, reduction in nuclear volume, and alteration in nuclear shape, none of which are as prominent in the H→H model as in other sperm models which involve the exchange of sperm nuclear basic proteins during spermiogenesis.

Though no change in the sperm nuclear basic proteins exists in the "histones" model, structural transitions are observed during spermiogenesis. The somatic-like chromatin of the early spermatid contains areas of heterochromatin which are dispersed among the euchromatin areas in the nucleus. Next, this chromatin is transformed into evenly spaced 20 nm fibro-granular structures in intermediate spermatids. During advanced phases of spermiogenesis, the chromatin granules aggregate closer together, until they achieve closely packed chromatin, in which the granules are nearly fused together in partial coalescence. This tight packaging of the 20 nm fibro-granular structures involves a slight reduction in nuclear volume; however, this reduction is not as great as that which occurs in other models which do not retain histones and nucleosomes in mature sperm chromatin.

Despite the absence of protein transitions in this model, changes in the overall nuclear protein component of the sperm chromatin do occur. These changes simply include the loss of non-nucleosomal proteins (such as heterochromatin proteins and proteins involved in transcription), as well as the addition and subsequent removal of post-translational modifications, such as acetylation. The loss of these non-histone nuclear proteins and histone modifications produces mature sperm chromatin solely composed of unmodified histones, which are organized into nucleosomes.

The pattern of histone acetylation found to occur in the spermiogenic chromatin of the "histones" model has contributed a great deal to our understanding of the structural changes occurring in spermiogenic chromatin. With antibodies specific for acetylation,

we were able to detect the presence of acetylated histones H3 and H4 in intermediately mature male gonad extracts with western blots. Using immunolocalizations observed by transmission electron microscopy, this acetylation was correlated to the 20 nm fibro-granular chromatin structures produced prior to tight chromatin packing. The moderate acetylation observed with this chromatin structure suggests that the acetylation plays an important role in maintaining the evenly spaced 20 nm granules to uniformly organize the genome properly, prior to subsequent tight chromatin packing. The granules are able to aggregate closer together once deacetylation of the tetramer histones has occurred; indeed, from thereon, no histone acetylation was observed, which is correlated to the closer packaging and subsequent partial coalescence of the 20 nm fibro-granular chromatin. Acetylation of the tetramer histones H3 and H4 in 20 nm fibro-granular chromatin is an important and novel result, since acetylation of histones has never before described in spermiogenesis of species which do not exchange nuclear proteins, due to its prior association with histone replacement. Here, another function for histone acetylation has been identified, which is a mechanism influencing the organization and even spacing of 20 nm chromatin granules into groups of 4-6 nucleosomes prior to sperm packaging, and helps to achieve a uniform and ordered sperm chromatin compaction.

The H→H sperm model is the simplest observed in our study of sperm models. In association, representative species of this model are also considered more primitive organisms. Therefore, it is quite possible that spermiogenesis of the earliest existing species were representatives of this model, since species of the H→H model tend to be evolutionarily less complex. Though there is no strict phylogenetic or taxonomic order of these species, animals considered to be the most primitive, such as cnidarians and ctenophores (Roccini et al., 1995, 1996), sponges (Ausió et al., 1997), echinoderms (Subirana 1970), and agnathan fish (Saperas et al., 1994) also happen to be members of the H→H model. Including this primitive model in our study has provided valuable insight concerning the minimum requirements of a developing spermatid to achieve ordered genome packaging and condensation. The H→H model also represents a primordial model, which could serve as a foundation for developing other sperm types, with more specialized chromatin organizations and structures, throughout evolution.

IV.2. MODEL "HISTONES TO PROTAMINE"

The model H→P, or "histones to protamine" is next in order of spermiogenic complexity. To represent this model, two species were used, each which contain very different protamines. The protamine of the bony fish *Dicentrarchus labrax* is considered

a “true” protamine due to its small size and high amount of arginine organized into clusters. The protamine of the gastropod mollusk *Monodonta turbinata* has several chemical and structural characteristics in common with the protamine of *D. labrax*, yet is unusual due to its extremely large size. However, in the H→P model the structural transitions occurring in both representative species are similar. They include the transformation of somatic-like chromatin of the early spermatid into homogenous 20 nm fibro-granular structures in the subsequent spermiogenic stage. This event is reminiscent of the structural transition mentioned in the first stages of spermiogenesis in the H→H model. These 20 nm chromatin granules of the intermediate spermatid become larger, coarse chromatin granules, which measure around 80-90 nm in the “true protamine” example, and around 60 nm in the “large protamine” example. The final stages of spermiogenesis are identical as well, which involve rapid condensation and tight packing of the coarse granules of chromatin.

The protein transitions which occur during spermiogenesis of this model coincide with these structural changes. The first formations of large, coarse chromatin granules in advancing spermatids indicate the beginning of protamine interaction with the spermatic chromatin. As the coarse granules increase in number and in size, fewer histones remain in the chromatin, until full histone displacement is achieved in advanced spermatids. Once the full protamine complement is interacting with the spermatic chromatin, its compaction and condensation is rapid and complete. Acetylation plays an important role in the protein transitions in the H→P model, and occurs in two acetylation events. The first event is the moderate acetylation of the tetramer histones involved in the formation of the evenly spaced 20 nm granules formed in intermediate spermatids. The second event involves hyperacetylation of histone H4, which appears to be related to histone displacement by protamine and nucleosomal disassembly. Similar accounts of hyperacetylation have been observed in spermiogenesis where histones are displaced, to facilitate their removal, but the monoacetylation of intermediate spermatids is a new and interesting result.

Based on the description of structural changes, protein transitions, and pattern of acetylation during spermiogenesis, it is appears that the H→P model represents a continuation of the simpler H→H model. This is evident due to the early events which occur in spermiogenesis of both models which are identical, mainly the formation of evenly spaced 20 nm granules which contain histones, of which H3 and H4 are moderately acetylated. Beyond the point of 20 nm fibro-granular chromatin, the H→P

model continues to develop further changes which do not occur in the H→H model. This is due to the replacement of histones by the protamine, which interacts with spermiogenic chromatin to cause further chromatin compaction and nuclear volume reduction, both of which extend beyond what is observed in the H→H model.

The H→P model of spermiogenesis is represented by many types of species (birds, reptiles, molluks, fish: reviewd in Oliva and Dixon 1991; Balhorn 2007) and does not encompass one class of organisms. This increase in complexity from the H→H model to the H→P model could have originated independently many times throughout evolution. Species of the H→P model have spermiogenesis which are more complex due to the addition of a specialized protein, the protamine, which replaces histones and nucleosomal organization in sperm. Interaction of the protamine with DNA rather than histones with DNA brings about a more specialized sperm cell, with a maximally decreased nuclear volume and more compact chromatin, better able to withstand harsh physiochemical factors of its environment. This slightly more complex sperm cell is, therefore, better equipped for its function to pass on genetic material in the creation of viable offspring.

IV.3. MODEL "HISTONES → PROTAMINE PRECURSOR → PROTAMINE"

The next model in order of complexity is H→Pp→P. Spermiogenesis of this model, represented by *Sepia officinalis*, involves more structural chromatin transitions than would have been imagined in spermiogenesis with simply one additional intermediate protein transition. These structural transitions include the somatic-like chromatin of the early spermatid becoming 20 nm fibro-granular chromatin in the first stages of spermiogenesis. These chromatin granules become fine 30-35 nm chromatin fibers as spermiogenesis advances, which subsequently thicken and elongate to become 45 nm fibers in advancing spermatids. These structural changes mentioned thus far constitute the "patterning" phases of spermiogenesis in this model. From these phases onward, the "condensing" stages involve the thick 45 nm chromatin fibers merging and coalescing together, as the chromatin condenses laterally to become one fully condensed mass of compact chromatin in the final stage of spermiogenesis.

The protein transitions and waves of acetylation which occur in this type of spermiogenesis explain well the structural chromatin transitions which occur in this model. The loss on non-histone proteins and moderate histone acetylation cause the somatic like chromatin to become 20 nm fibro-granular chromatin. At this point, the precursor protamine molecule appears to transform the chromatin into 30-35 nm fine

chromatin fibers; histone H4 becomes hyperacetylated at this stage, and a dynamic competition for DNA binding occurs between acetylated histones (now with a reduced DNA affinity) and the precursor protamine (with a slightly higher DNA binding affinity than histones, but lower than would have the protamine). As the precursor protamine achieves greater DNA binding with histone removal (facilitated by histone hyperacetylation), the fibers thicken into 45 nm fibers. This chromatin structure is correlated with only residual hyperacetyleated histones and an increased proportion of precursor protamine. Once the precursor protamine is converted into the mature protamine form via an amino-terminal cleavage, the condensing phases of spermiogenesis begin, and rapid coalescence and condensation of the spermiogenic chromatin directly produce fully compact, highly packaged mature sperm chromatin.

Due to one simple additional intermediate step in protein substitutions, this model is considered next in terms of complexity. Interestingly, the single increase in protein transitions involves several intermediate stages of chromatin structures. This makes the H→Pp→P model ideal in order to analyze the overall effect and purpose of intermediate steps involved in achieving condensed sperm chromatin. Indeed, the chromatin at the end of spermiogenesis is just as compact in this model as in the H→P model, but there are more intermediate chromatin structures that are formed during spermiogenesis due to the intermediate protein is involved.

Many diverse species fit into the model H→Pp→P. However, during spermiogenesis of most of representatives, histones are replaced by several protein intermediates prior to the involvement of one or more protamines; the complexity in protein transitions affecting spermiogenic chromatin structure is much more elaborate than the relatively simple one chosen to represent the H→Pp→P model in this study. Due to greater complexity in protein transitions and chromatin patterns produced during spermiogenesis, occurring in species such as *Murex brandaris* (Amor and Durfort 1990; Càceres et al., 1999) and *Octopus vulgaris* (Giménez et al., 2004; Ribes et al., 2004), other members of this model have been studied in much less detail. However, an important aspect of the H→Pp→P model of spermiogenesis is that complex chromatin patterns can be formed (fibers and/or laminae), which are not observed in spermiogenesis of the H→H or H→P models. This added chemical complexity in spermiogenic protein transitions causes elaborate condensing chromatin patterns, and permits these sperm cells to adopt often sophisticated and specialized sperm nuclear shapes, unique and adequate for fertilization within their own species.

The first steps of spermiogenesis are shared in common among the H→H model, H→P model, and H→Pp→P model. The transformation of somatic-like chromatin into uniformly distributed 20 nm fibro-granular structures which contain moderately acetylated histones has been a part of all spermiogenesis mentioned thus far, which supports the idea that these events are necessary in all spermiogenesis, likely preparing the genome for subsequent events in the process of spermiogenesis, regardless of the type of protein or structural transitions which occur later on. This point implies the significance of the homogenously distributed, moderately acetylated 20 nm fibro-granular chromatin in subsequent chromatin packaging, despite how this packing will eventually be achieved in spermiogenesis. Therefore, the organization of sperm chromatin in intermediate spermatids during spermiogenesis of all species universally appears to occur the same way, but later continues to develop in its own independent way, depending on the sperm model the species represents.

IV.4. MODEL "SPECIALIZED CHROMATIN": CRUSTACEAN SPERM

There exist many species which do not have sperm with condensed chromatin, as well as many groups of organisms with aflagellated sperm cells (Bowen 1925; Dallai and Afzelius 1995; Jamieson 1998; Morrow 2004). However, the chromatin in these cases has not been studied in enough detail to fully understand its structure or composition. In the examples of crustacean sperm included in this work, the structural transition of the chromatin is very simple. The transition from early to intermediate spermatid involves the chromatin becoming homogenously dispersed throughout the nucleus. From this evenly distributed chromatin, no structural changes take place in the spermatic chromatin; no further condensation occurs as spermiogenesis advances. The chromatin remains uncondensed which allows the nucleus to transform in shape around the developing acrosomal granule, susceptible to changes in its shape according to external forces.

The protein transitions occurring during spermiogenesis are unknown, though it is quite probable that these sperm no not undergo any transition of sperm nuclear basic proteins. Indeed, though the transitions are unknown, the proteins organizing the mature sperm chromatin have been identified. Histones with characteristics nearly undistinguishable from somatic histones are present in the sperm chromatin of crustacean sperm. Depending on the species, they organize the chromatin into nucleosomal fragments (or nucleosome-like fragments). In *Cancer*, it is known that the ratio of histones/DNA is much lower than in other sperm types containing histones or other basic proteins, and that a large portion of the genome is not stabilized by basic proteins. An

interesting possibility is that these genomic regions in *Cancer* sperm which are not organized by nucleosomes could be the AT rich satellite areas of DNA which were described in *Cancer pagurus*. This characteristic was described for DNA isolated from germ cells of this species, found to be composed of 24% light satellite (AT rich) regions (Pochon et al., 1966; Skinner 1967). In *Maja*, the basic protein/DNA ratio remains unknown, though it does appear that histone H3 is in lower stoichiometric proportions than what appears in somatic cells, while histone H2B is in higher proportions.

Having found acetylation in this specialized type of sperm chromatin correlates well with what is known about the effect that histone acetylation has on histone-DNA binding, inter-histone interactions, and inter-nucleosomal interactions. Finding hyperacetylated histone H4 in mature sperm of *Cancer*, along with the low proportion of H3 retained in *Maja* sperm which is highly acetylated, is not surprising considering the non-condensed nature of this chromatin. The fact that acetylated histones in mature sperm chromatin has never been described before makes sense as well, due to the fact that other known species which contain histones in the mature sperm also have sperm with condensed chromatin. This suggests that acetylation could be one of the mechanisms utilized to achieve the non-condensed sperm chromatin of this model, which appears to be an adaptation of the sperm cell to accomplish oocyte fertilization. Additionally, the presence of histone H3 and H4 acetylation in this fibrilliar type of chromatin challenges the reassessment of the hypothesis that 20 nm granular chromatin with acetylated tetrameric histones is an ancestral model. Instead, non-condensed mature sperm chromatin could be the ancestral model, where acetylated tetrameric histones found in the crustacean sperm model were maintained in chromatin of other sperm types, except in these cases they are subsequently deacetylated during spermiogenesis. The step of histone deacetylation could be an evolutionary adaptation for achieving a higher degree of chromatin packaging in other sperm types, which is particularly relevant to the H→H model.

The presence non-condensed mature sperm chromatin is surprising considering the known importance and purpose of tightly packaged and condensed chromatin for sperm cell function and survival (Subirana 1975; Braun et al., 2001). However, in brachyuran crabs, the organization of mature sperm into spermatophores has a similar function, which includes protection of the genetic material, and packing the sperm nuclei into a tight, enclosed area. In this way the fragile sperm nucleus may be non-condensed

and flexible in nature, while avoiding harsh environmental conditions, remaining protected and enclosed within the spematophore.

An interesting extension of this “specialized sperm chromatin” model would be to study the sperm chromatin of other aflagellated sperm types. Indeed, it is a possibility that the non-condensed nature of the sperm chromatin is not only an adaptation of the sperm cell to the mode of fertilization of a species, but also to achieve flexibility for sperm contractile movement in sperm types which do not have flagella to provide motility. It is likely that other aflatellated sperm would also demonstrate chromatin with a non-condensed and fibrilliar nature, and it would be interesting to find out if similar mechanisms, such as acetylation of the tetrameric histones, are associated characteristics in sperm of other species with a similar sperm chromatin or morphology.

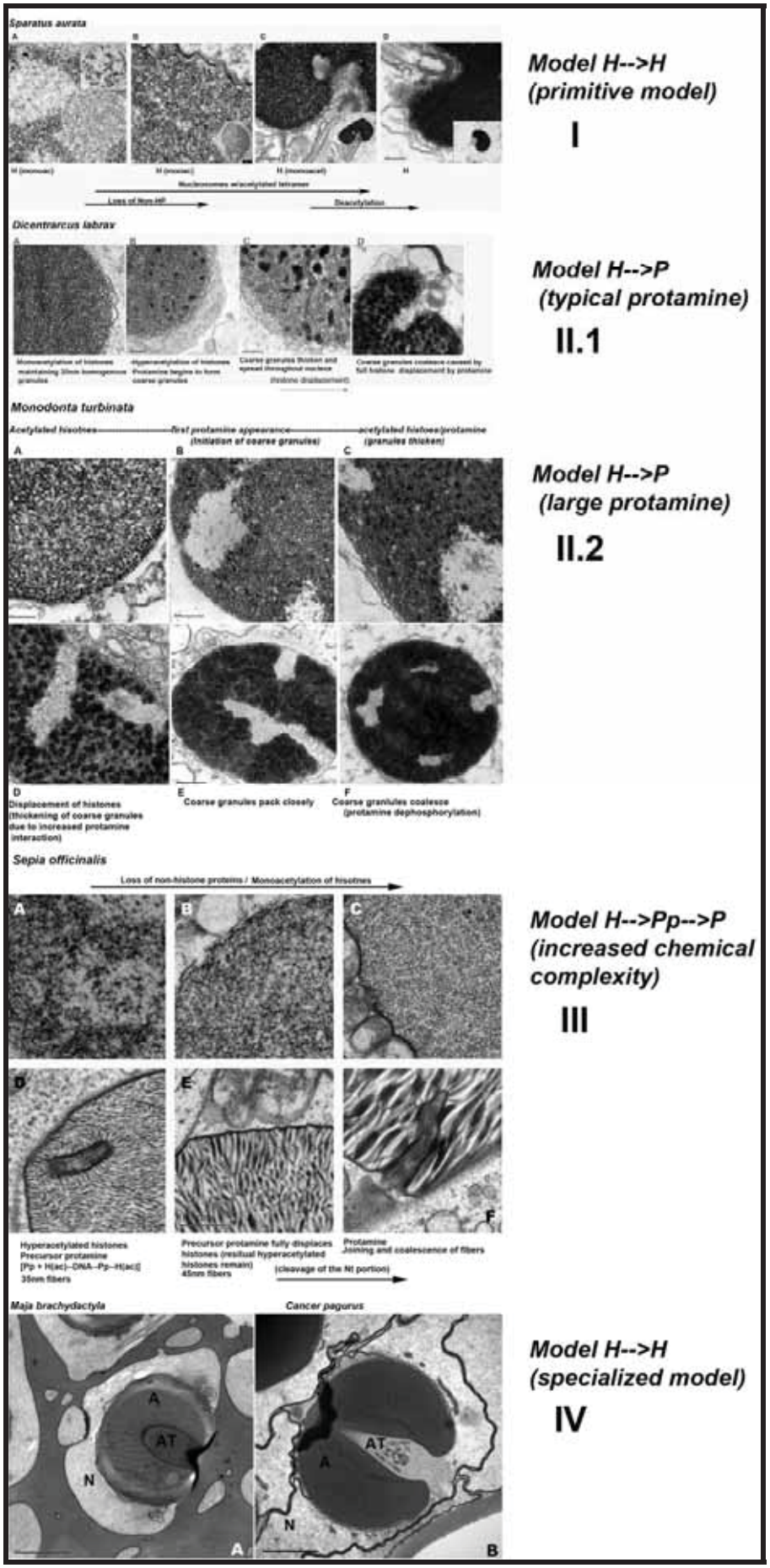
Sparatus aurata
Model H-->H
(primitive model)
I
Dicentrarcus labrax
Model H-->P
(typical protamine)
II.1
Monodonta turbinata
Acetylated histones
first protamine appearance
(initiation of coarse granules)
acetylated histones/protamine
(granules thicken)
Model H-->P
(large protamine)
II.2
Displacement of histones
(thickening of coarse granules
due to increased protamine
interaction)
Coarse granules pack closely
Coarse granules coalesce
(protamine dephosphorylation)
Sepia officinalis
Loss of non-histone proteins / Monoacetylation of histones
Model H-->Pp-->P
(increased chemical
complexity)
III
Hyperacetylated histones
Precursor protamine
[Pp + H(ac)--DNA--Pp--H(ac)]
35nm fibers
Precursor protamine fully displaces
histones (residual hyperacetylated
histones remain)
45nm fibers
(cleavage of the Nt portion)
Protamine
Joining and coalescence of fibers
Maja brachydactyla
Cancer pagurus
Model H-->H
(specialized model)
IV
A
AT
N
B

Figure IV.1.1. Summary of sperm models. I-III.-Spermiogenic protein transitions and chromatin compaction in various models. **I.-** *S. aurata*. A.- Somatic like ealy spermatid; B.- spermatid with homogenous 20 nm granules; C.- advanced spermatid shows homogenous granules beginning to package closer together; D.-Mature gonadal spermatozoa with partially coalesced condensed chromatin. Insets A-D: view of entire sperm nucleus in the equivalent spermiogenic stage, showing how the nuclear volume reduces, creating a space between the nuclear membrane and condensing chromatin. Bar: A-D.- 0.2 μm. Insets A, B: 1 μm; C, D: 0.5 μm. **II.1.**- *D. labrax*. A.-Homogenous 20 nm granules are formed from slight monoacetylation of nucleosomes. B.- Hyperacetylated histones are easily displaced by a typical protamine, evidence of which is shown by the initiation of coaser chroamtin granules. C.- The granules thicken as the protamine achieves histone displacement, forming thicker 50 nm granules. D.- 80 nm granules are the product of full protamine-DNA interaction in the most advanced spermatid. Bar A-D: 0.2 μm. **II.2.-** *M. turbinata*. A.- Monoacetylated histones maintain chromatin of the early spermatid in a 20 nm granular strucutre. B.- In intermediate spermatids, histones become hyperacetylated, and the protamine apppears forming granules of progressively larger diameter (C). D.- In advanced spermatids, the full endowment of protamine achieves near total histone displacement, as 60 nm coarse granules form. E.- The coarse granules package closely together, and F.- coalesce, corresponding to a likely dephosphorylation of the protamine in the most mature gonadal spermatozoa. Bars: 0.2 μm. All images are equally amplified. **III.-** *S. officinalis*. A: Somatic-like chromatin from an early spermatid. A-C: First remodeling: the chromatin acquires a granular aspect similar to model A and B (20 nm granules.). C-D: Second chromatin remodeling: granules convert into fibers of 25 nm-30 nm, and histone H4 monoacetylation occurs. D-E: Third remodeling: in more advanced stages the chromatin appears organized in 45 nm fibers, along with a residual level of hyperacetylated H4, before the precursor protamine displaces hisontes. E-F.- Conversion of the precursor into the protamine causes coalescence of fibers to become fully compact chromatin. Bar E: 0.5 μm; Bar D, F: 0.2 μm. All images are equally amplified. **IV.-** Transversal view of mature sperm cell found within spermatophore of A.- *Maja brachydactyla*; B. *Cancer pagurus*. A.- acrosome; N.- Nucleus; AT.- Acrosomal tubule. Bar: A.-2 μm; B: 1 μm.

CONCLUSIONS

V. CONCLUSIONS

1.- The complexity of the spermiogenic protein transition affects the chromatin condensation pattern, as well as the sperm nuclear shape and volume, which has developed during evolution to aid in customizing gametes specific for fertilization of a species.

2.- A common feature shared among many spermiogenesis is the disappearance of euchromatin and heterochromatin regions into a uniform chromatin distribution, consisting of homogenously organized 20 nm granules in early/intermediate spermatids. These homogenously dispersed chromatin granules should contain 4-6 nucleosomes with moderately acetylated tetrameric histones. This post-translational modification is correlated to the even spacing of chromatin granules. A reduction of nuclear volume occurs in some species along with this structural transformation, but is not an intrinsic property. This type of chromatin structural transition occurs regardless of the type of SNBP substitution which occurs later on during spermiogenesis, and from this stage on, each spermiogenesis follows its own specific structural transition pattern. These 20 nm chromatin fibro-granules could represent a primitive, ancestral model of spermiogenesis.

3.- A second wave of acetylation occurs in spermiogenesis in which histones are displaced. The degree of acetylation is greater than the first wave, and has a separate and distinct function which is correlated with chromatin compaction due to the beginning of protamine binding and histone removal.

4.- The slight increase in chemical complexity due to the added intermediate of a precursor protamine, together with histone acetylation, involves a much more complex chromatin condensation pattern in spermiogenesis in *Sepia officinalis* compared to other models in which histones are directly substituted by a protamine or other SNBP.

5.- Crustacean sperm is organized by histones, forming small size nucleosomes or nucleosome-like particles. Histone acetylation (including hyperacetylation of H4 in *Cancer* and H3 in *Maja*), the low ratio of basic proteins/ DNA in sperm of *Cancer*, and the unusual stoichiometrics of histones H3 and H2B found in sperm chromatin of *Maja* probably contribute to their extremely non-condensed and fibrilliar sperm chromatin. In the past, cytochemical staining methods likely failed to detect histones due to their highly acetylated nature and low proportion compared to other histone containing nuclei.

6.- Nucleosomes must not be the only structural component of sperm DNA of crustaceans, since the genome is susceptible to rapid digestion with low amounts of micrococcal nuclease. Large regions of the genome are likely stabilized by some other component, possibly divalent cations. The nucleosomes that do exist are expected to be situated close together, due to the short linker region found.

ADDENDUM

TRANSICIÓ DE PROTEÏNES NUCLEARS I CONDENSACIÓ DE LA CROMATINA ESPERMIOGÈNICA. PROPOSTA D'UN MODEL ANCESTRAL DE L'ESPERMIOGÈNESI NUCLEAR

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RESUM

En aquest capítol revisem la correlació entre: a) el recanvi de proteïnes en els nuclis espermiogènics, b) el patró de condensació de la cromatina, i c) l'estat d'acetilació de les histones.

L'estudi s'aplica a l'espermiogènesi de quatre especies que presenten transicions de proteïnes nuclears de complexitat creixent: *Sparus aurata* que reté les histones en els nuclis espermàtics madurs (transició **[H → H]**); *Dicentrarchus labrax* i *Monodonta turbinata*, en les quals les histones de les espermàtides son substituïdes per una molècula de proteïna molt més bàsica (protamina o SNBP respectivament) (transició **[H → P]**); i *Sepia officinalis*, en l'espermiogènesi de la qual, les histones son desplaçades per una proteïna precursora de la protamina que es transforma en protamina en les últimes fases de l'espermiogènesi (transició **[H → P_p → P]**).

Els resultats indiquen que en la primera fase de l'espermiogènesi de totes les quatre especies, es dona un procés idèntic, en el qual: a) la cromatina perd la organització de tipus somàtic (desaparició de les diferencies entre eucromatina i heterocromatina), b) la cromatina queda homogèniament organitzada en grànuls de 20 nm de diàmetre que contenen de 4 a 6 nucleosomes, i c) les histones del tetràmer experimenten una acetilació moderada.

A partir de l'estructura granular de 20 nm, cada tipus d'espermiogènesi segueix camins diferents. En les espermàtides de *S. aurata* (**[H → H]**) les histones es desacetilen, i la cromatina es condensa definitivament per a donar la cromatina espermàtica madura. En canvi en les altres espermiogènesis es produeixen transicions estructurals més complexes que son analitzades en el text.

La consideració d'aquests casos, conjuntament amb altres que ens ofereix la literatura, ens permeten proposar que la formació de grànuls de 20 nm (contenint les histones H3 i H4 moderadament acetilades) compleix els requisits funcionals mínims per a poder ser considerada com un estat ancestral en l'evolució de la cromatina espermiogènica.

ABSTRACT

In this work we study the correlation between: a) nuclear protein exchange in spermiogenic nuclei, b) the chromatin condensation pattern, and c) the acetylation state of histones.

The study in applied to spermiogenesis of four species which demonstrate an increasing complexity in nuclear protein transitions. In *Sparus aurata* histones remain in

sperm nuclei (transition **[H → H]**). In *Dicentrarchus labrax* and *Monodonta turbinata* the histones in spermatids are substituted by a much more basic molecules (protamine and SNBP, respectively) (transition **[H → P]).** In spermiogenesis of *Sepia officinalis* histones are replaced by a precursor protamine, which is transformed into the protamine in the last phases of spermiogenesis (transition **[H → P_p → P]**).

The results indicate that in the first phase of spermiogenesis of all four species an identical process is involved, in which: a) the chromatin looses typical somatic organization (loss of differences between euchromatin and heterochromatin), b) the chromatin ends up homogenously organized into granules measuring 20 nm in diameter, which contain 4 to 6 nucleosomes, and c) the histones of the tetramer undergo a moderate acetylation.

From this 20 nm granular structure, each type of chromatin goes its own way. In spermatids of *S. aurata* **[H → H]** the histones are deacetylated and the chromatin definitively condenses, producing mature sperm chromatin. On the other hand, in the other species, much more complex structural transitions develop which are analyzed in the text.

Considering these cases, together with others taken from the literature, we propose that the formation of 20 nm granules (containing moderately acetylated H3 and H4) completes the minimum structural and functional requirements to be considered as an ancestral state during spermiogenic chromatin evolution.

I**NTRODUCCIÓ**

En les espermiogènesis dels animals, el nucli de les espermàtides experimenta una important transformació que el converteix en el nucli de l'espermatozoide. El procés de diferenciació nuclear es produeix en molts casos a través de l'expressió seqüencial de proteïnes específiques (anomenades genèricament, *Sperm Nuclear Basic Proteins*, però en alguns casos concrets s'anomenen protamines). Aquestes proteïnes penetren en el nucli i van desplaçant a les histones i altres proteïnes nuclears. La substitució de les histones per les SNBP provoca la condensació progressiva de la cromatina. Així, el nucli final de l'espermatozoide conté una cromatina molt compactada, un volum reduït i una composició en proteïnes molt simplificada.

La transformació del nucli de l'espermàtida en nucli espermàtic és un procés complex que varia segons el grup taxonòmic estudiat. En alguns peixos ossis i aus, la histona H4 de les espermàtides presenta hiperacetilació en les etapes intermèdies de l'espermiogènesi, i a partir d'aquí les histones son reemplaçades per una proteïna molt bàsica (protamina) que es troba fosforilada (Sung i Dixon, 1970; Christensen i Dixon, 1982; Christensen *et al*., 1984; revisat en Oliva i Dixon, 1991). La substitució de les histones per la protamina produeix un desmuntatge dels nucleosomes (Oliva *et al*., 1987) i organitza el DNA en una estructura molt més compacte (nucleoprotamina). La substitució de proteïnes esmentada es dona en els estadis intermedis de l'espermiogènesi, els quals coincideixen amb la progressió de la condensació de la cromatina. En els estadis finals de l'espermiogènesi té lloc la desfosforilació de la protamina i l'últim pas de la condensació de la cromatina. Una altra de les espermiogènesis més estudiades és la dels mamífers. Aquí es dona una seqüència més complexa en les substitucions de les proteïnes que interaccionen amb el DNA. Les histones es veuen parcialment reemplaçades per altres histones específiques del testicle, les quals son desplaçades més endavant per proteïnes de transició. Les proteïnes de transició seran reemplaçades posteriorment per protamines (una de les quals es troba en forma de precursor) (Meistrich, 1989; Courtens *et al*., 1995; Meistrich *et al*., 2003; Zhao *et al*., 2004; Churikov *et al*., 2004). En el decurs d'aquests canvis del tipus de proteïnes que s'uneixen al DNA també es produeixen una sèrie de modificacions postraduccionals que permeten que el recanvi de proteïnes s'efectuï organitzadament. Entre aquestes modificacions cal destacar la hiperacetilació de la H4 i altres histones específiques del testicle i la ubiqüitinització de H3, H2B i TH3 (Grimes i Henderson, 1983; Chen *et al*., 1998; Hazzouri *et al*., 2000; Lahn *et al*., 2002). En el nucli espermàtic madur s'hi troben protamines i també una certa proporció d'histones acetilades, les quals en l'espermatozoide humà organitzen un 15% del genoma (Gusse *et al*., 1986; Gatewood *et al*., 1987; 1990). Evidentment aquests canvis de proteïnes comporten canvis en l'estructura i condensació de la cromatina que es poden evidenciar parcialment a través de la observació per microscòpia electrònica.

Les molècules relacionades amb la biologia reproductiva (així com els altres nivells de complexitat biològica relacionats amb la reproducció) han experimentat una evolució excepcionalment ràpida (Swanson i Vacquier, 2002). Això comporta que les característiques bioquímiques i morfològiques de l'espermiogènesis siguin molt variades entre les especies. Des de fa temps, s'han fet classificacions molt interessants i acurades de les cèl·lules espermàtiques i de la seva possible evolució (vegeu per exemple Franzén, 1977), però encara no es coneix com pot haver evolucionat el procés de l'espermiogènesi per a produir tota la varietat d'espermatozoides que existeixen. En el present treball enfoquem aquest subjecte centrant-nos en el nucli espermàtic. Analitzem diferents tipus de canvis nuclears en quatre espermiogènesis escollides i intentem proposar un model ancestral del procés que segueix el nucli cel·lular en el decurs de la diferenciació espermàtica.

MODELS DE CANVIS NUCLEARS EN LES ESPERMIOGÈNESIS

A.- ***Sparus aurata*** (figura 1), (model: **[H → H]**)

La figura 1 mostra en primer lloc (part superior) el canvi, o transició de proteïnes nuclears en l'espermiogènesi d'aquesta espècie. És una transició molt simple: les espermàtides més joves contenen histones (marcades de H1 a H4 a la figura) en el seu nucli. En el decurs de l'espermiogènesi, desapareixen les proteïnes *no histones* (minoritàries a la figura1), però en els nuclis espermàtics madurs hi romanen les histones. Les digestions dels nuclis dels espermatozoides amb nucleasa micrococcal produeixen el patró típic de les cromatines organitzades en forma de nucleosomes (Saperas, 1992; Kurtz *et al*., en preparació). Per tant, l'estructura nucleosòmica no es perd en els nuclis espermiogènics.

En la segona part de la figura 1 apareixen les imatges, obtingudes per microscòpia electrònica, de la compactació dels nuclis espermiogènics (fila I), així com del patró de la condensació de la cromatina (fila II) i del marcatge amb anticòs anti[acetil-lisina] (fila III) en cada un dels estadis de la condensació. El marcatge amb anti[acetil-lisina] dona informació de l'acetilació de les histones H3 i H4, tal i com ha estat comprovat per *western-blot* (Kurtz *et al*., en preparació). L'espermàtida primerenca conté una cromatina organitzada de manera similar als nuclis somàtics, formats per eucromatina i per grumolls d'heterocromatina acumulades a la perifèria i en algunes zones interiors dels nuclis (IA a la figura 1). En aquests nuclis (els quals contenen histones i una certa proporció d'altres proteïnes) apareix un nivell basal relativament baix d'acetilació (IIIA). En els següents estadis de l'espermiogènesi, la cromatina de l'espermàtida s'organitza homogèniament en grànuls. El diàmetre dels grànuls és de 20 nm la qual cosa suggereix que poden estar composats per 4-6 nucleosomes cada un d'ells (un nucleosoma és un cilindre d'aproximadament 5,5nm de radi i 5,5 nm d'alçada). Els grànuls de 20nm contenen histones en un estat significatiu d'acetilació (IIIB). De fet l'acetilació en aquests estadis és la més intensa de tot el procés espermiogènic d'aquesta espècie. A mesura que progressa l'espermiogènesi de *S. aurata*, els grànuls de 20 nm es van concentrant en direcció al centre nuclear (IB → C) sense que es modifiqui la seva mida (IIC). Aquest pas comporta una reducció del volum nuclear, i possiblement una certa pèrdua de l'acetilació de les histones (IIIC). En els estadis finals, la cromatina adopta un aspecte més homogèniament compactat (ID), la organització en grànuls de 20 nm desapareix. És interessant observar que aquesta fase final coincideix amb la completa desacetilació de les histones (IIID).

B.- ***Dicentrarchus labrax*** (figura 2), (model: **[H → P]**)

La figura 2 és un recull de les transicions composicionals i estructurals del nucli i cromatina del peix ossi *D. labrax* durant la seva espermiogènesi. L'anomenem model [H → P] perquè les histones de les espermàtides son substituïdes per una (o més) protamines en els nuclis espermàtics madurs. A la part superior de la figura 2 es presenta el canvi en proteïnes així com l'estructura primària de la protamina de *D. labrax* (Saperas *et al*., 1993). Es tracta d'una molècula molt petita (34 residus aminoacídics) formada en un 61,8% per residus arginina. Similarment al cas anterior, l'espermàtida més primerenca presenta un nucli amb una organització de tipus somàtic (a la figura 2, IA apareix un nucli d'una espermàtida no totalment primerenca on la cromatina de tipus somàtic comença a transformar-se). La cromatina d'aquests nuclis, a més de contenir DNA conté histones com a principals proteïnes bàsiques associades (i *no-histones* en menor proporció) (figura 2, part superior). El marcatge amb anti[acetil-lisina] dona una reacció basal relativament dèbil (IIIA).

El pas següent és la reorganització de la cromatina en grànuls de 20 nm de diàmetre (IIB1, B2) idèntics als que presenta *S. aurata*. En aquesta transició també s'ha produït una acumulació dels grànuls en direcció al centre nuclear, la qual cosa ha provocat una important reducció del volum nuclear (IB). En els grànuls de 20 nm les histones (concretament les H3 i H4, quan és comprovat per *western-blot*) manifesten un grau significatiu d'acetilació (IIIB). En els estadis següents, la protamina penetra en els nuclis (electroforesi intermèdia, part superior de la figura 2). Això provoca una remodelació de l'estructura de la cromatina: els grànuls de 20 nm passen a formar grànuls majors i més electrodensos que creixen fins adoptar un diàmetre aproximat de 80 nm (IC, D; IIC,D, E). La formació dels grànuls de 80 nm no s'efectua per simple fusió dels grànuls de 20 nm ja que els grànuls de 80 nm no contenen histones, només DNA i protamina, la qual empaqueta el DNA d'una manera diferent de la nucleosòmica (Ward i Coffey, 1991; Daban, 2000). Les histones que apareixen en aquest estadi es troben en el seu grau màxim d'acetilació, la qual cosa està d'acord amb treballs anteriors en els que es mostra que quan la protamina desplaça les histones aquestes estan hiperacetilades. Aquesta transició o remodelació estructural provoca una segona reducció del volum del nucli espermàtic (IC → F). En les fases finals, el nucli ha reduït considerablement el seu volum (IF), i la cromatina apareix organitzada en grànuls de 80 nm (IIE). No hi ha histones en aquesta cromatina, només protamina (electroforèsi, figura 2)

C.- ***Monodonta turbinata*** (figura 3), (model: **[H → P]**)

En aquesta espècie de mol·lusc, les histones que es troben en les espermàtides primerenques son substituïdes per una proteïna de 106 residus aminoacídics que formarà part del nucli espermàtic (figura 3, electroforèsi). Aquesta proteïna no és una protamina típica, ni té un origen comú amb les protamines típiques (Chiva *et al*., 1992; Lewis *et al*., 2004). No obstant, la seva estructura primària (resolta per Daban *et al*., 1995) comparteix característiques força semblants amb les protamines típiques (entre elles una gran proporció de residus bàsics i la disposició de les arginines en grups de 4 a 6 residus, vegeu la figura 3).

En els primers estadis de la diferenciació espermiogènica, la cromatina de les espermàtides s'organitza de nou en grànuls de 20 nm (IB; IIB, C), molt homogenis en el valor del seu diàmetre i que contenen histones en un estat significatiu d'acetilació (IIIB, C). En aquesta primera etapa de la diferenciació espermiogènica, el nucli també disminueix el seu volum sense que la cromatina perdi la seva estructura granular (IB → C; IIB, C). A partir d'aquest estadi, els canvis en la cromatina segueixen també un patró similar al que hem trobat en *D. labrax*. Els grànuls de 20 nm es remodelen en grànuls de 60 nm quan la "protamina" de *M. turbinata* substitueix les histones (IC, D; IID1). En els estadis en que la protamina comença a substituir les histones, aquestes es troben en el seu màxim estat d'acetilació (IIID1). Posteriorment, la cromatina completa la seva remodelació i els complexes DNA-"protamina" adopten la forma de grànuls densos d'aproximadament 60 nm de diàmetre (IID2; IIID2). En el pas final, els grànuls de 60 nm es fusionen parcialment i la cromatina del nucli espermàtic presenta un estat relativament homogeni (IE)

D.- ***Sepia officinalis*** (figures 4 i 5), (model: **[H → P_p → P]**)

La transició de proteïnes en aquesta espècie és un grau més complexa que en els casos anteriors. A les figures 4 i 5 es presenta un resum dels estudis efectuats en aquesta espermiogènesi. Aquí, les histones son substituïdes per una molècula precursora de la protamina (figura 4, electroforesi) la qual es transforma en protamina per escissió del seu extrem amino-terminal (Martin-Ponthieu *et al*., 1991; Wouters-Tyrou *et al*., 1991; figura 6). En aquest cas doncs, les histones no son substituïdes directament per la protamina que trobarem al nucli espermàtic madur, sinó per una proteïna menys bàsica (el precursor). La

transició estructural de la cromatina és més complicada del que esperaríem basant-nos en la transició de proteïnes (vegeu figura 4, modificada de Martínez-Soler *et al*., 2007b). Es produeixen quatre transformacions (remodelacions) en l'estructura de la cromatina espermiogènica. El nucli de l'espermàtida primerenca conté cromatina de tipus somàtic que es reconverteix (com en els casos anteriors) en cromatina granular de 20 nm (figura 4B). A diferència del que passava en les altres espermiogènesis, el nucli no pateix un decrement de volum (Figura 4 A→ B). En estadis posteriors, la cromatina es remodela a una configuració fibril·lar (30-35 nm de diàmetre), i simultàniament el nucli adopta una forma fusiforme (figura 4C). En un estadi següent, tota la cromatina es reconverteix en fibres de 40-50 nm sense que es modifiqui la forma nuclear (figura 4D). En els últims estadis d'aquesta espermiogènesi, les fibres de 40-50 nm es van ajuntant progressivament per a formar fibres majors i finalment la cromatina uniforme i compactada del nucli espermàtic (figura 4E → G). Els canvis de forma del nucli espermiogènic, i les seves principals causes, varen ser descrits en un treball anterior (Martínez-Soler *et al*., 2007b), però no es consideren aquí en detall.

Donada la complexitat de les transicions estructurals varem estudiar la composició de proteïnes (Martínez-Soler *et al*., 2007a), i l'estat d'acetilació de la histona H4 (Kurtz *et al*., 2007) en cada tipus de cromatina. Els resultats globals d'aquests estudis es presenten resumidament en la figura 5: la cromatina de tipus somàtic de l'espermàtida més jove conté histones (més altres proteïnes minoritàries) que presenten un estat d'acetilació baix. En la primera remodelació, els gràmuls de 20 nm es troben formats per histones de les quals la histona H4 està en forma monoacetilada. En el pas immediatament posterior, la molècula precursora de la protamina penetra en el nucli i coexisteix amb les histones i el DNA. Aquest complex [histones-DNA-precursor] adopta la forma de fibres de 30-35 nm. En aquestes estructures la histona H4 es troba hiperacetilada (fins a tres acetilacions per molècula d'H4). En l'estadi següent, les histones han abandonat el nucli i només el precursor de la protamina es troba interaccionant amb el DNA. La cromatina s'organitza ara en fibres de 40-50 nm (seguiu la figura 5). Finalment, el processament del precursor (i possiblement la desfosforilació de la protamina) provoquen que les fibres de 40-50 nm es vagin fusionant entre elles, fins a formar el nucli espermàtic de *Sepia*, que conté només DNA i protamina i que presenta la cromatina molt electrodensa.

Discussió

En aquest treball definim tres tipus de transicions de proteïnes nuclears en l'espermiogènesi. En el tipus més simple de transició (**[H → H]**) només les proteïnes *no histones* abandonen els nuclis espermiogènics. El nucli espermàtic conserva les histones i l'organització nucleosòmica. En el següent nivell de complexitat (transició **[H → P]**) es produeix un desplaçament directe de les histones per una proteïna molt bàsica, que posteriorment serà la principal (o la única) proteïna present en els nuclis de les cèl·lules espermàtiques. L'altra transició espermiogènica presenta una complexitat superior. Les histones de les espermàtides son desplaçades per una molècula precursora de la protamina, que posteriorment es transformarà en protamina a través d'una escissió i eliminació de la seva part amino-terminal (Martin-Ponthieu *et al*., 1991; Wouters-Tyrou *et al*., 1991). Aquest tipus de transició fou anomenat **[H → P_p → P]** per Kurtz *et al*., (2007).

Una anàlisi comparativa de l'espermiogènesi de les quatre espècies (*S. aurata*, *D. labrax*, *M. turbinata* i *S. officinalis*) ens permet establir algunes interessants correlacions entre canvis químics i canvis estructurals/morfològics de la cromatina espermiogènica. Apareix una característica que és compartida per totes aquestes espermiogènesis: En les primeres etapes del procés, les diferencies estructurals entre eucromatina i

heterocromatina desapareixen i tot el material nuclear s'organitza homogèniament en grànuls de 20 nm. La mida d'aquests grànuls és idèntica en totes les espècies. Els grànuls de 20 nm contenen en tots els casos histones i en conseqüència han d'estar constituïts per 4-6 nucleosomes. També en tots els casos les histones de la cromatina es troben parcialment acetilades. En *S. officinalis* hem demostrat que la histona H4 es troba monoacetilada en la lisina 12 en els grànuls de 20 nm (Kurtz *et al*., 2007). Sembla molt probable que l'acetilació parcial de la histona H4 (i possiblement la histona H3) impedeixi la formació d'estructures d'ordre elevat d'empaquetament dels nucleosomes (García-Ramírez *et al*., 1995; revisat per Bulger, 2005; Calestagne-Morelli i Ausió, 2006) però que per altra banda afavoreixi l'agregació de petits grups de nucleosomes per a formar els grànuls de 20 nm. De fet, un repàs de la literatura sobre el desenvolupament morfològic de l'espermiogènesi de diferents animals, indica que la formació dels grànuls de 20 nm en les fases inicials de la condensació de la cromatina és un fenomen que es produeix en la majoria de les espècies (no en totes) (Ribes *et al*., 2001).

Per altre cantó, la formació de grànuls de 20 nm també permet que el nucli espermiogènic experimenti una reducció important en el seu volum (figures 1, 2 i 3). No sembla que això sigui una propietat intrínseca de la cromatina de 20nm doncs en *S. officinalis* (i en altres espècies) la reorganització de la cromatina en grànuls de 20 nm no es produeix simultàniament a una reducció del volum nuclear (figures 4 i 5). Aquesta disminució hauria de ser deguda a altres components (com per exemple les seves interaccions amb la matriu nuclear, etc.) relacionats amb la nucleomorfogènesi, però que han de ser estudiats encara en la major part de les espècies.

En el cas de *S. aurata* (transició **[H → H]**), els grànuls de 20 nm experimenten una coalescència parcial en les darreres fases de l'espermiogènesi, produint una cromatina espermàtica, d'aspecte quasi homogeni quan és observada per microscòpia electrònica. La coalescència d'aquests grànuls es produeix conjuntament amb la desaparició de l'acetilació de les histones (figura 1). Aquest pas final és anàleg a la desfosforilació de la protamina en els altres tipus de transicions proteiques. La desacetilació de les histones augmenta la interacció electrostàtica entre proteïnes i DNA, permet una major aproximació i redistribució dels nucleosomes. Això provoca la "disgregació" dels grànuls de 20 nm.

En els restants casos analitzats en aquest treball, la cromatina prossegueix el procés de compactació. En *D. labrax* i *M. turbinata* els grànuls desapareixen per a donar lloc a estructures més grans i més electrodenses de 80 i 60 nm de diàmetre respectivament (figures 2 i 3). La desaparició dels grànuls de 20 nm és precedit per una hiperacetilació de les histones, les quals ja no es trobaran presents en els grànuls de 80 i 60 nm (formats exclusivament per DNA i protamina probablement fosforilada). Malgrat que les protamines de *D. labrax* i *M. turbinata* son diferents, el procés general de la condensació és molt similar i només es diferencia en la mida dels grànuls majors. La fusió d'aquests grànuls majors al final de les espermiogènesis coincideix amb la desfosforilació de la protamina descrita per altres autors.

La condensació de la cromatina en *S. officinalis* és més complicada. En una primera fase es formen grànuls de 20 nm composats per DNA i histones (amb l'H4 monoacetilada). En una etapa immediatament posterior la molècula precursora de la protamina entra en el nucli on coexisteix amb les histones (fibres de 35 nm). En etapes més avançades, la histona H4 és hiperacetilada i totes les histones son desplaçades de la cromatina (fibres de 40-50 nm). En les etapes finals, el precursor es converteix en protamina i les fibres es van ajuntant entre elles.

En diversos estudis ha estat suggerit que el tipus més primitiu d'espermatozoide conté histones en el seu nucli, i que a partir d'aquest model poden haver evolucionat

diversos tipus de proteïnes nuclears espermàtiques anomenades genèricament SNBP (Ausió, 1995; Roccini *et al*., 1996; Ausió *et al*., 1997). El treball que presentem aquí està d'acord amb aquesta hipòtesi, i n'ofereix una explicació causal. És molt possible que l'acetilació limitada de les histones i la formació de gràmuls de 20 nm siguin processos ancestrals en l'evolució de l'espermiogènesi. D'una banda la formació del gràmuls en tot el volum nuclear provoca una homogeneïtat estructural, anul·lant les diferencies zonals en la organització (i possiblement en l'estat funcional) de la cromatina; d'altra banda permet que el nucli espermàtic experimenti una important disminució en el seu volum. Finalment, representa una estructura que permet fàcilment el desplaçament de les histones per part de les SNBP que puguin haver sorgit en l'evolució. Aquestes SNBP (entre les quals hi ha les protamines típiques) poden produir una condensació addicional del nucli espermàtic.

Hem de manifestar que les transicions estudiades no representen a totes les espècies animals. En altres espermiogènesis es produeixen transicions molt més complexes amb patrons de condensació de la cromatina més elaborats (Meistrich, 1989; Cáceres *et al*., 1999; Ribes *et al*., 2001). Els nuclis d'aquests espermatozoides poden presentar morfologies molt peculiars adaptades als detalls de la biologia de la fertilització de cada espècie.

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PEUS DE FIGURES

Figura 1.- Característiques dels nuclis espermiogènics de *S. aurata*.

Part superior: Electroforesi bidimensional (àcid acètic/urea/tritó i SDS) de les proteïnes extretes dels nuclis d'una gònada molt immadura (A), o dels nuclis d'espermatozoides (D).

Fila I: Nucli d'una espermàtida molt jove (A), i d'espermàtides en diferenciació (B, C). Nucli d'un espermatozoide testicular (espermàtida molt avançada) (D). Barra: 0,5 μ per a totes les imatges de la fila.

Fila II: Patró de condensació de la cromatina espermiogènica. Cromatina en grànuls de 20 nm (B1, B2, C). Cromatina condensada en les espermàtides molt avançades (D). Barra: 0,2 μ per a totes les imatges

Fila III: Marcatge amb anticòs anti[acetil-lisina] en cada estadi de la condensació de la cromatina. Barra 0,5 μ per a totes les imatges.

Figura 2.- Característiques dels nuclis espermiogènics de *D. Labrax*.

Part superior: Electroforesi en àcid acètic/urea del canvi de proteïnes nuclears en l'espermiogènesi de *D. Labrax* (H: histones; P: protamina). També apareix la seqüència aminoacídica de la protamina d'aquesta espècie.

Fila I: Estadis de la diferenciació nuclear, des d'una espermàtida jove (A) a un espermatozoide testicular (F). Barra 0.5 μ per a totes les imatges.

Fila II: Patró de condensació de la cromatina . B1 i B2: grànuls de 20 nm; C, D: remodelació dels grànuls de 20 a 80 nm; E: cromatina d'una espermàtida molt avançada. Barra 0,2 μ per a totes les imatges.

Fila III: Marcatge amb anti[acetil-lisina] de cada tipus de cromatina. Barra 0,5 μ per a totes les imatges.

Figura 3.- Característiques dels nuclis espermiogènics de *M. Turbinata*.

Part superior: Electroforesi en àcid acètic/urea de la transició de proteïnes nuclears espermiogèniques (H: histones; P: protamina). També apareix l'estructura primària de la molècula de "protamina" de *M. Turbinata*.

Fila I: Diferenciació del nucli en l'espermatogènesi (el nucli en A correspon a un espermatogoni representant l'organització de tipus somàtic de la cromatina). Barra 1 μ per a totes les imatges de la fila.

Fila II: Patró de condensació de la cromatina espermiogènica. B, C: grànuls de 20 nm; D1: remodelació dels grànuls de 20 a 60 nm. D2: cromatina d'una espermàtida avançada. Barra per la fila, 0,5 μ

Fila II: Marcatge amb anti[acetil-lisina] de la cromatina en les diferents fases de la condensació. Barra 0.5 μ per a tota la fila.

Figura 4.- Nucli i cromatina espermiogènica de *S. officinalis*.

Columna esquerra: Modificació de la forma nuclear al llarg de l'espermiogènesi. Barra 2 μ per a tota la columna.

Columna central: Patró de condensació. Cromatina granular de 20 nm (B); Cromatina en fibres de 30-35 nm (C); En fibres de 40-50 nm (D); Unió progressiva de les fibres de 40-50 nm (E → F). Barra 200 nm per a tota la columna

Columna dreta: Composició proteínica de cada tipus de cromatina. (H: histones; P_p: precursor de la protamina; P.- protamina)

Figura 5.- Resum del patró de condensació, composició i acetilació de proteïnes en l'espermiogènesi de *S. officinalis*.

Columna dreta: Composició i estat d'acetilació de les histones.

Columna central: Patró complet de condensació de la cromatina espermiogènica. Barra 300 nm per a totes les figures excepte per la última (3 μ)

Columna dreta: Principals esdeveniments causants dels canvis en la condensació de la cromatina.

Figura 6.- Precursor i protamina de *S. officinalis.*

La protamina de *S. officinalis* és una proteïna microheterogènia que prové d'una proteïna major, o precursor. En aquesta figura es mostren les seqüències de les dues formes de la protamina de *S. officinalis* i dels seus precursors (T1 i T2 en la qual només apareixen els residus diferents). Els 21 residus de l'extrem amino, son eliminats de la molècula (i del nucli) en els estadis avançats de l'espermiogènesi. Les seqüències provenen de Martin-Ponthieu *et al*. (1991) i de Woters-Tyrou *et al*. (1991).

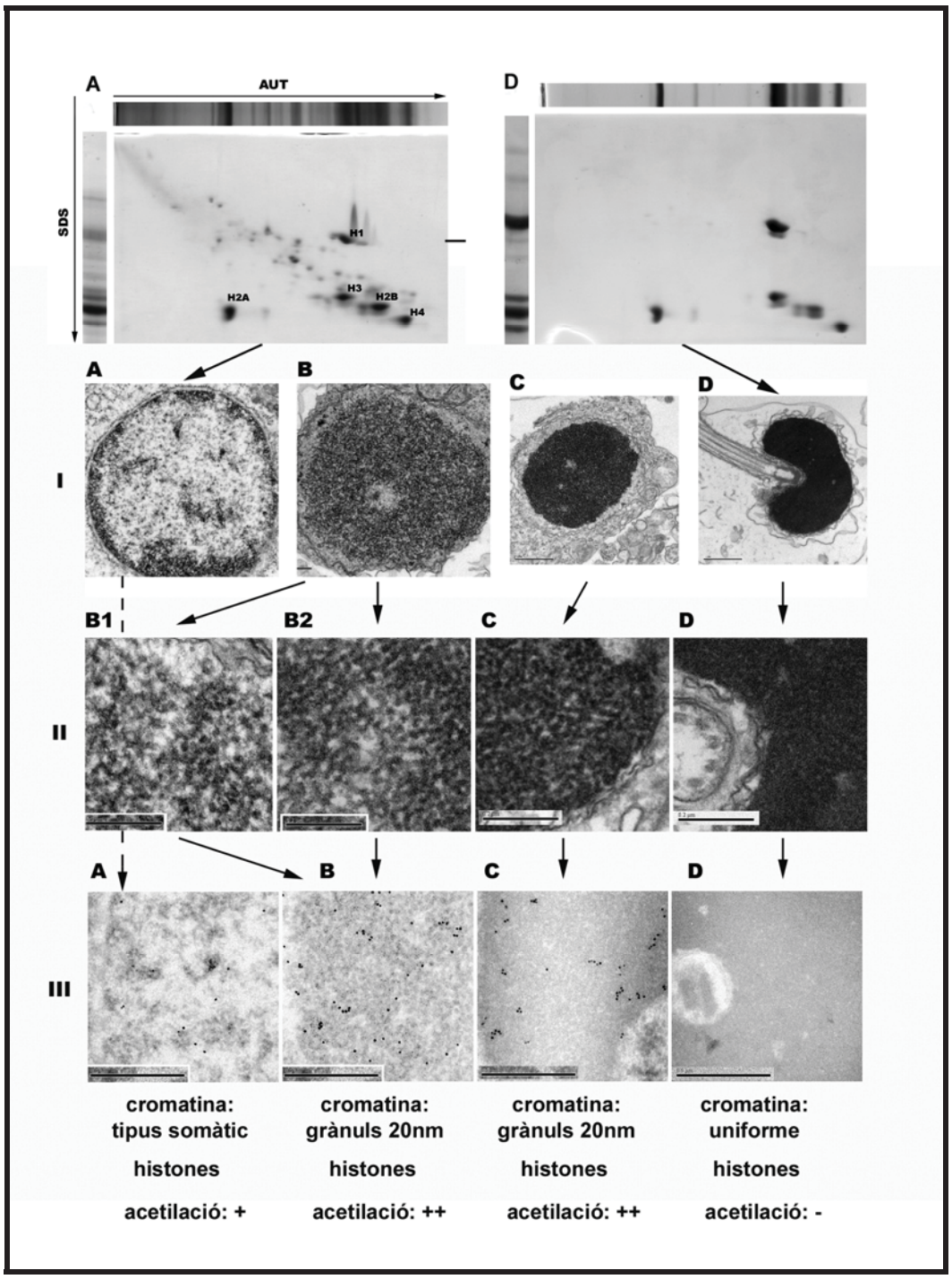
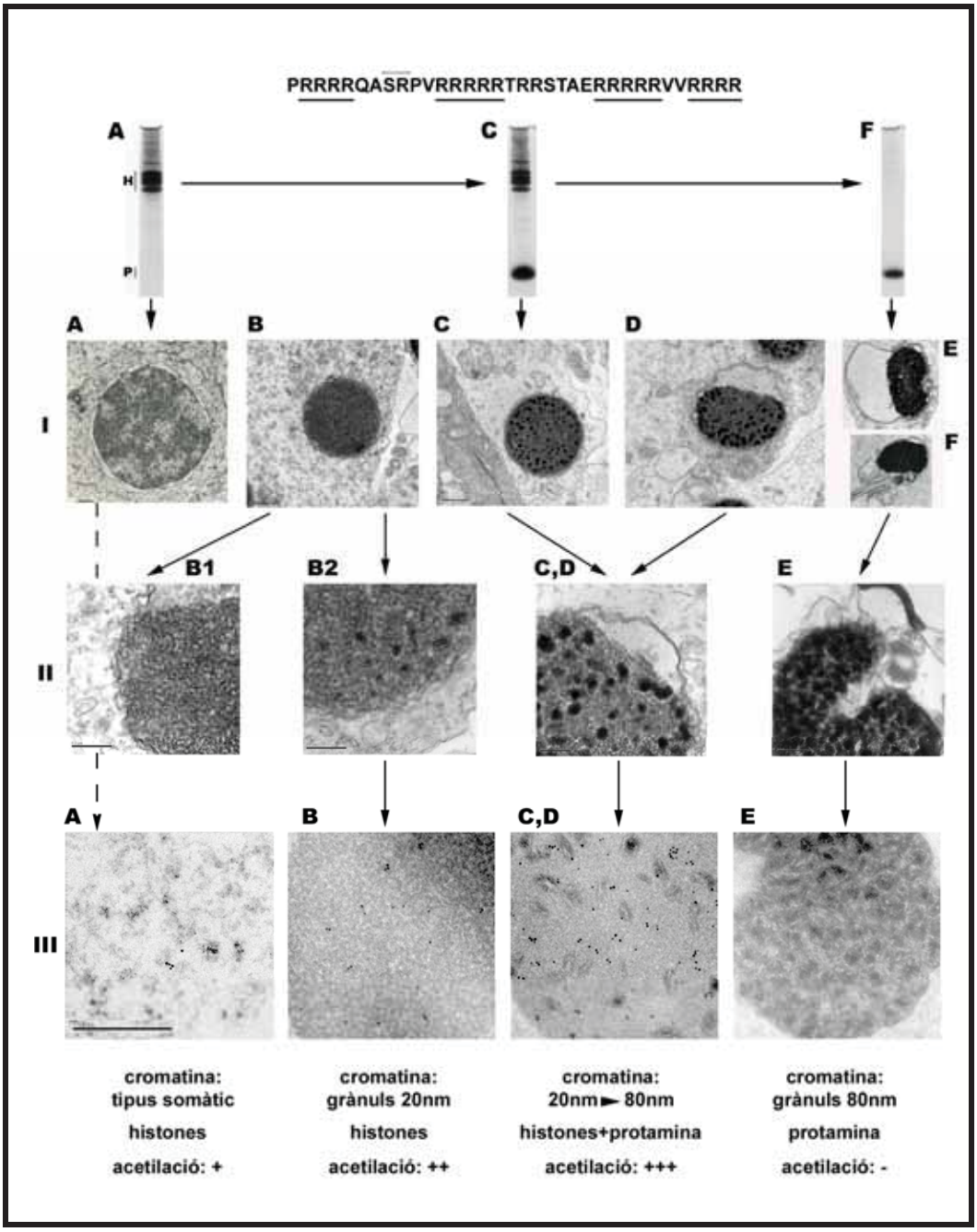

A
AUT
SDS
H1
H2A
H3
H2B
H4
D
I
A
B
C
D
II
B1
B2
C
D
III
A
B
C
D
cromatina: tipus somàtic
histones
acetilació: +
cromatina: grànuls 20nm
histones
acetilació: ++
cromatina: grànuls 20nm
histones
acetilació: ++
cromatina: uniforme
histones
acetilació: -

FIGURE 1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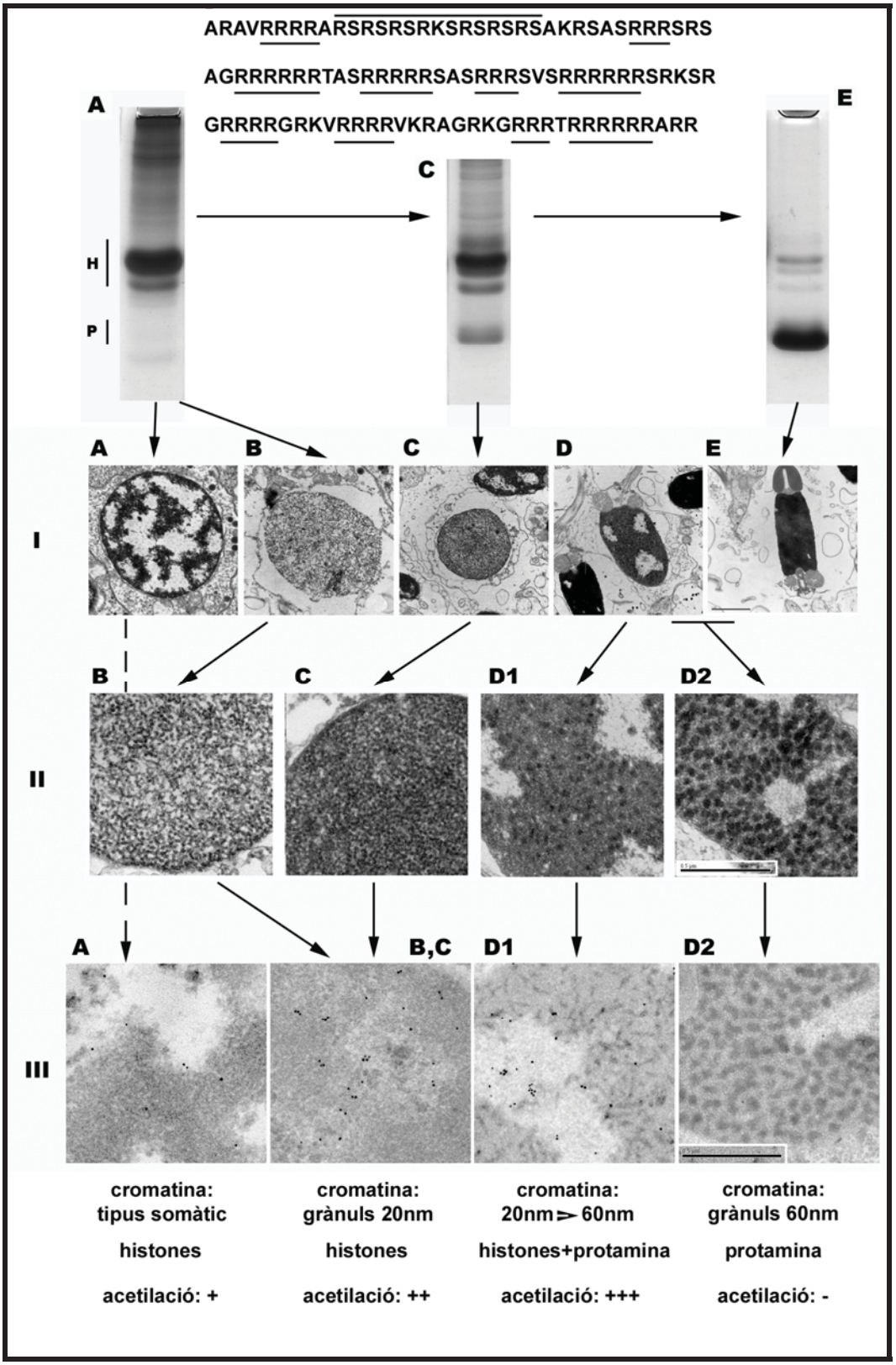
A
H
P
C
F
A
B
C
D
E
F
I
B1
B2
C,D
E
II
A
B
C,D
E
III
cromatina:
tipus somàtic
histones
acetilació: +
cromatina:
grànuls 20nm
histones
acetilació: ++
cromatina:
20nm► 80nm
histones+protamina
acetilació: +++
cromatina:
grànuls 80nm
protamina
acetilació: -

FIGURE 2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E
C
H
P
A
B
C
D
E
I
B
C
D1
D2
II
A
B,C
D1
D2
III
cromatina:
tipus somàtic
histones
acetilació: +
cromatina:
gránuls 20nm
histones
acetilació: ++
cromatina:
20nm > 60nm
histones+protamina
acetilació: +++
cromatina:
gránuls 60nm
protamina
acetilació: -

FIGURE 3

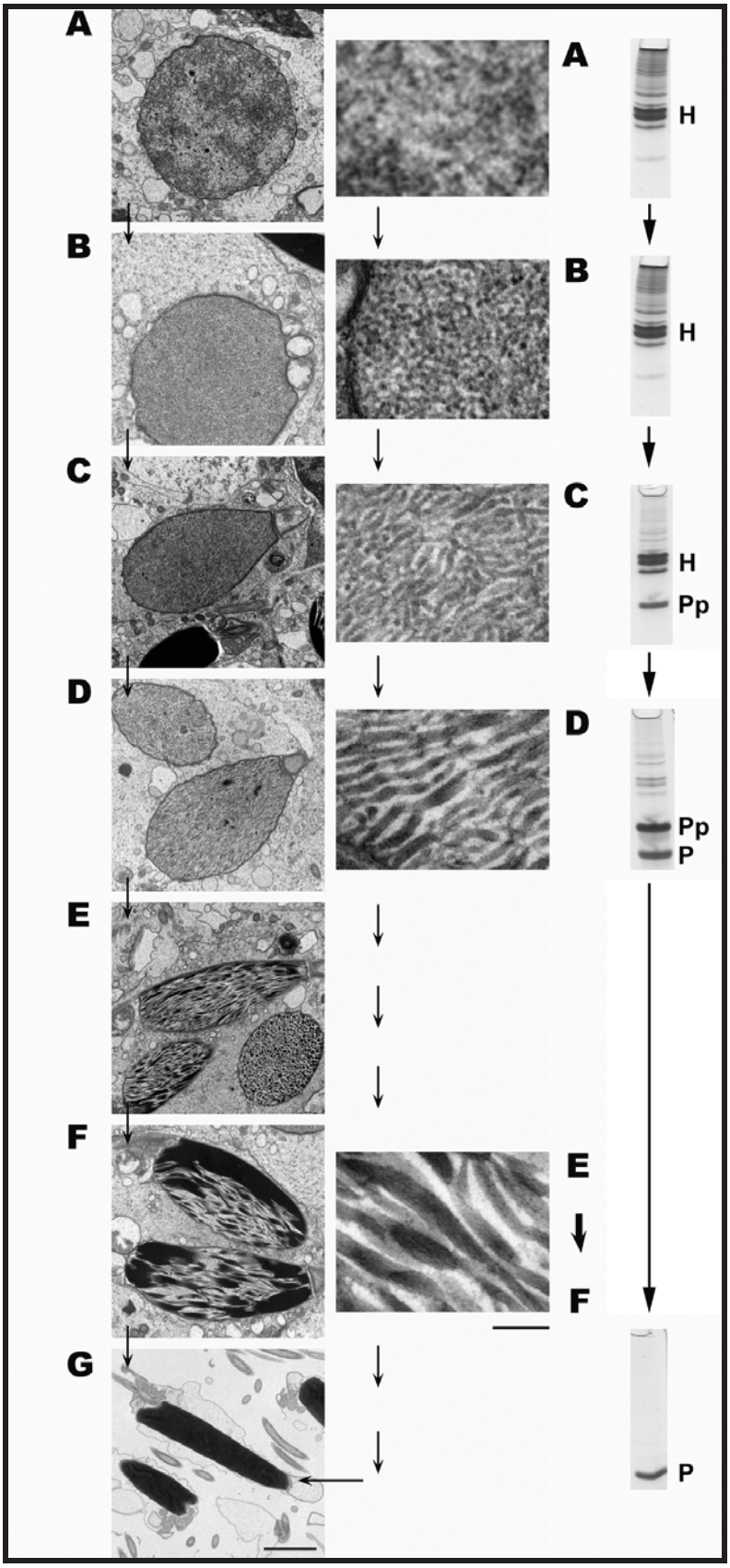

FIGURE 4

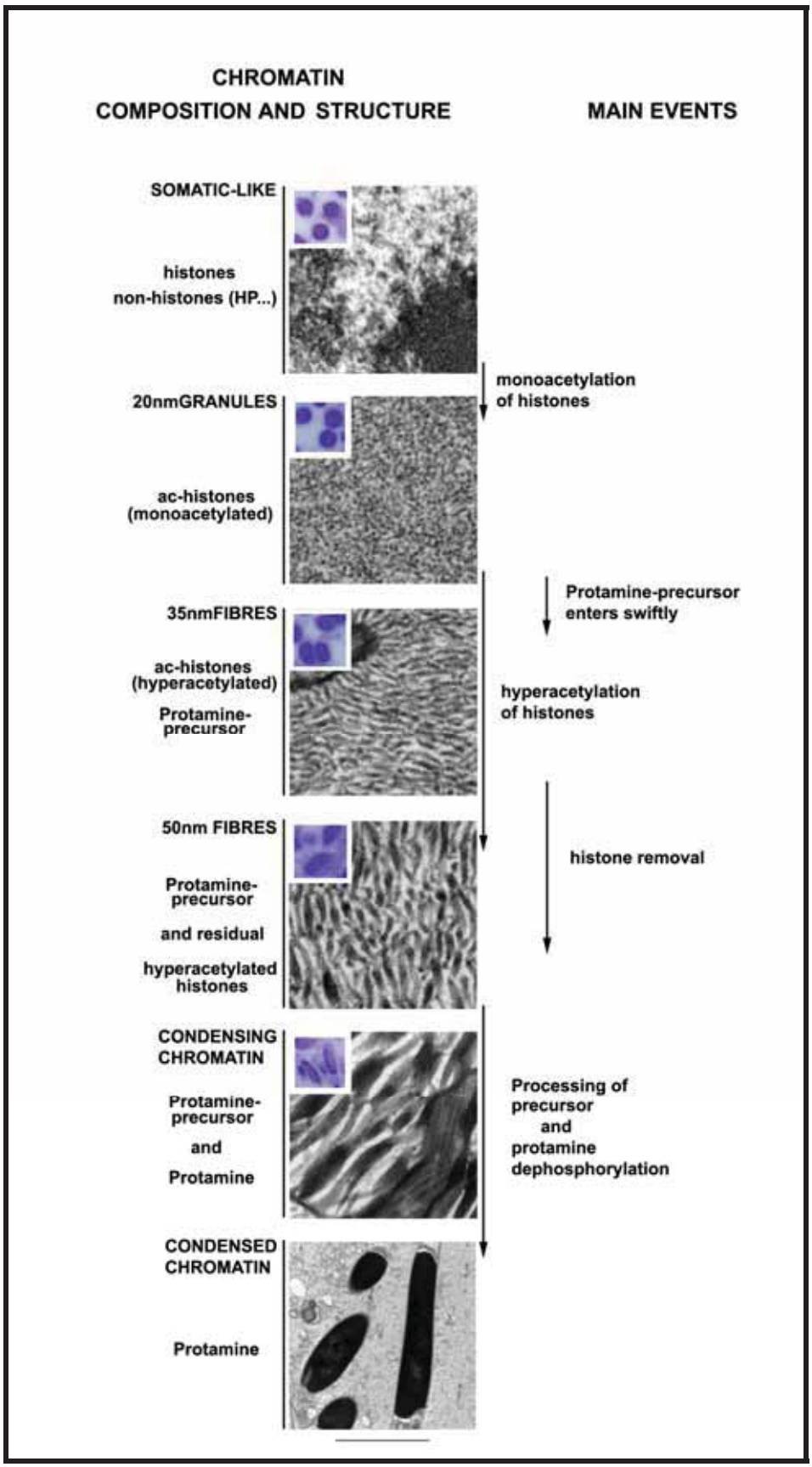
CHROMATIN
COMPOSITION AND STRUCTURE
MAIN EVENTS
SOMATIC-LIKE
histones
non-histones (HP...)
monoacetylation
of histones
20nmGRANULES
ac-histones
(monoacetylated)
Protamine-precursor
enters swiftly
35nmFIBRES
ac-histones
(hyperacetylated)
Protamine-
precursor
hyperacetylation
of histones
50nm FIBRES
Protamine-
precursor
and residual
hyperacetylated
histones
histone removal
CONDENSING
CHROMATIN
Protamine-
precursor
and
Protamine
Processing of
precursor
and
protamine
dephosphorylation
CONDENSED
CHROMATIN
Protamine

FIGURE 5

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