The perinuclear theca is a non-ionic detergent-resistant, electron-dense layer surrounding the condensed nucleus of mammalian sperm. The known proteins originating from the perinuclear theca have implicated the structure in a variety of important cellular processes during spermiogenesis and fertilization. Nonetheless, the composition of the perinuclear theca remains largely unexplored. We have isolated a group of low molecular mass (14–19 kDa) perinuclear theca-derived proteins from acrosome-depleted bovine sperm heads by salt (1 M KCl) extraction and have identified them as core somatic histones. N-terminal sequencing and immunoblotting with anti-histone antibodies confirmed the presence of both intact and proteolytically cleaved somatic histones H3, H2B, H2A, and H4. Identical proteins were isolated using 2% SDS or 1 N HCl extractions. Subsequent acid and SDS extractions of intact bovine sperm revealed the presence of all four intact histone subtypes, with minimal proteolysis. Two-dimensional acid/urea/Triton-SDS-PAGE, coupled with immunoblotting analysis, confirmed the somatic nature of these perinuclear theca-derived histones. Estimates of the abundance of perinuclear theca-derived histones showed that up to 0.2 pg per sperm of each histone subtype was present. Immunogold labeling at the ultrastructural level localized all four core somatic histones to the post-acrosomal sheath region of bovine epididymal sperm, when probed with affinity-purified anti-histone antibodies. Little immunoreactivity was detected in residual perinuclear theca structures following the extractions. Taken together, these findings indicate the unprecedented and stable localization of non-nuclear somatic histones in bovine sperm perinuclear theca.

Somatic Histones Are Components of the Perinuclear Theca in Bovine Spermatozoa*

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The perinuclear theca (PT)1 is a specialized cytoskeletal component residing under the acrosome and almost completely surrounding the nucleus of mammalian sperm heads. Based on morphological and protein composition assessments, the PT is divided into two structurally continuous regions, termed the subacrosomal layer, and the post-acrosomal sheath (reviewed in Refs. 1 and 2). Although numerous proteins have been characterized from these PT regions (reviewed in Ref. 3), few of the known PT proteins to date resemble traditional cytoskeletal proteins. Rather, the PT contains a range of proteins of varying function, including Calcin, a basic structural protein that binds actin (4, 5); Stat4, a transcriptional activator hypothesized to contribute to zygotic gene activation (6); SubH2Bv (PT15), a histone H2B-like variant proposed to contribute to acrosome-nuclear docking (3); and PERF 15, a fatty acid-binding protein predominating in the perforatorium of murid sperm (7–9). The PT structure has been implicated in a number of essential cellular processes, such as nuclear shaping during spermiogenesis (2, 10), and egg activation and pronuclear formation during fertilization (11–15), although the PT proteins involved in these processes remain elusive. Their identification and characterization is therefore critical for understanding the mechanisms involved in these, and potentially other, important cellular processes.

To date, all characterized PT proteins share a resistance to non-ionic detergents, such as Triton X-100, and to extraction by high salt (16). High pH buffers (NaOH), or the combination of a reducing agent with a denaturing detergent, are required for their complete removal and/or migration through SDS-PAGE (3). As such, covalent disulfide bonds maintain the protein framework of the PT, consistent with observations in other sperm structures (17). However, the potential for non-covalent, ionic-associated proteins in the PT cannot be excluded (5, 18), as suggested by a recent proposal for classifying PT proteins based on their extractability by ionic detergents, such as SDS (3). Thus far, all known PT proteins are not extractable by SDS alone, whereas salt- and SDS-extractable PT proteins remain largely uncharacterized.

During spermiogenesis, changes in sperm nuclear morphology are concomitant with both the development of the PT (8, 19, 20) and the ultimate replacement of core somatic histones by protamines in sperm chromatin (reviewed in Refs. 21 and 22). Species-specific differences occur both in the subtypes of replacement nucleoproteins and the sequence of their removal, in addition to variation in the persistence of core histones in the mature sperm. Human sperm nuclei retain up to 15% of somatic core histone content (23–25). In contrast, somatic histones from bovine sperm nuclei represent less than 1% of calf thymus nuclei content (26). Somatic histones have also been isolated in the mature sperm of mouse (27, 28) and rat (29), although it remained questionable whether their source was exclusively nuclear (27). In mature bovine sperm, histone-like variants CENP-A and SubH2Bv have been characterized and localized to the nucleus (30) and the subacrosomal layer of the PT (31), respectively. Taken together, these observations demonstrate the potential for both nuclear and non-nuclear localization of histone proteins in mature mammalian sperm.

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1 The abbreviations used are: PT, perinuclear theca; AP, affinity-purified; AUT-PAGE, acetic acid-urea-Triton X-100 polyacrylamide gel electrophoresis; 2D, two-dimensional; ECL, enhanced chemiluminescence; PVDF, polyvinylidene fluoride; TBS, Tris-buffered saline; TEM, transmission electron microscopy.
Here we demonstrate that the four core somatic histones H3, H2B, H2A, and H4 are present in the PT of the mature bovine sperm head. Unlike other PT proteins characterized to date, PT-derived somatic core histones are extractable by SDS, high salt, or acid. Quantitative analysis of these PT-bound somatic core histones suggests that up to 0.2 pg per sperm of each histone subtype is present, potentially representing 10–30% of a haploid genomic equivalent of somatic histone content.

EXPERIMENTAL PROCEDURES

Isolation of Epididymal Bovine Sperm and Preparation of Sonicated Sperm Heads—Intact whole bovine sperm and sonicated bovine sperm heads from cauda epididymides were obtained and isolated according to methods previously described by Oko and Maravei (18), with some modifications. The isolation buffer, containing protease inhibitors (18), was adjusted to pH 6.4 to minimize histone proteolysis (32, 33). Sperm were counted in a hemacytometer.

Extraction of Salt-, SDS-, and Acid-soluble PT Proteins—Salt-extracted PT proteins were obtained according to previous methods (18), with some modifications. Omitting the initial Triton X-100 incubation, ~1 × 10^15 sonicated sperm heads were gently shaken in 1 ml KCl in an isolation buffer, for 1 h at 4 °C. Following processing of the supernatant (19), the pellet was subjected to a second 2 ml KCl extraction over 16 h to completely remove all salt-soluble PT proteins. SDS-extracted PT proteins were obtained from both whole sperm and sonicated heads by incubating pellets in SDS-PAGE non-reducing sample buffer (2% SDS, 10% glycerol, 0.0125% bromphenol blue, and 62.5 mM Tris-HCl, pH 6.8) for 3 min at 100 °C. The supernatant obtained following centrifugation was analyzed by SDS-PAGE. Acid-soluble PT proteins were obtained using methods for basic nuclear protein extractions described elsewhere (34). However, in place of isolated nuclei fractions, whole sperm or sonicated head fractions were incubated directly with 0.25 M HCl and processed accordingly (34).

The total protein profile of both whole sperm and sonicated heads prior to, and following all extractions, was obtained by incubating pellets in SDS-PAGE sample buffer, containing 2 mM β-mercaptoethanol, for 3 min at 100 °C. To facilitate gel loading, decondensed chromatin was sheared by passing the samples through a 23-gauge needle several times. Total protein concentration in the extracts was determined by using a Bio-Rad protein assay (Bio-Rad, Mississauga, Ontario, Canada) (35).

Gel Electrophoresis, Transfer, and Peptide Sequencing—Three systems of gel electrophoresis were used in this study for analysis of SDS-, salt-, and acid-soluble proteins. SDS-PAGE was conducted under reducing conditions, according to previously described procedures (36). Migration was compared with low molecular weight standards (Amerham Biosciences, Piscataway, NJ) and calf thymus core histones (Roche Applied Science, Laval, Quebec, Canada). 15% acetic acid-urea-Triton X-100 (AUT)-PAGE and two-dimensional (2D) AUT/SDS-PAGE methods (18), except that Fremd’s (14) and 2% SDS extracts from whole sperm.

Transmission Electron Microscopy and Immunogold Labeling—Transmission electron microscopy (TEM) was performed on ultrathin sections of the cauda epididymis and sample pellets obtained from whole sperm and sonicated heads before and after the extractions were fixed in 5% formaldehyde and 0.6% glutaraldehyde and embedded in Epon embedding medium (Ted Pella, Inc.). Sections were stained with uranyl acetate and lead citrate and viewed with a Jeol 100CX microscope (Zeiss, Tokyo).

Immunoblotting—1D- and 2D-resolved salt-, acid-, and/or SDS-extractable PT proteins, from both whole sperm and sonicated heads, were immobilized on PVDF and probed with affinity-purified anti-H3, anti-H2B, and anti-H4 antibodies (1:20–1:500 of unconcentrated stock), and the following commercially acquired antibodies: C-terminal-specific anti-H2B (against human H2B residues 118–126, 1:2000, Upstate Biotechnology), C-terminal-specific anti-H3 (human H3 residues 120–135, 1:1000, Santa Cruz Biotechnologies, Santa Cruz, CA), anti-H4 (1:1000, Upstate Biotechnology), and anti-H2A (against acidic patch, 1:1000, Santa Cruz Biotechnologies). Non-specific sites were blocked with 5% skim milk in 25 mm Tris-buffered saline, pH 7.8, and 0.1% Tween (TBS-TW) for 1 h at room temperature. Diluted primary antibodies in 2% skim milk/TBS-TW were incubated overnight at 4 °C. Anti-rabbit IgG (1:20,000, Vector Laboratories, Burlingame, CA) or anti-goat IgG (1:20,000, Sigma) peroxidase-conjugated antibodies were used for 2-h secondary incubations at room temperature. Immuneoreactivity was detected using a Super Signal West Pico Enhanced Chemiluminescence (ECL) kit (Pierce, Rockford, IL).

Transmission Electron Microscopy and Immunogold Labeling—Transmission electron microscopy (TEM) was performed on ultrathin sections of the cauda epididymis and sample pellets obtained from whole sperm and sonicated heads before and after the extractions were fixed in 5% formaldehyde and 0.6% glutaraldehyde and embedded in Epon embedding medium (Ted Pella, Inc.). Sections were stained with uranyl acetate and lead citrate and viewed with a Jeol 100CX microscope (Zeiss, Tokyo).

Figure 1. Protein extraction profiles from whole sperm and sonicated heads resolved by 15% SDS-PAGE. A, proteins extracted from sonicated heads by 1 M KCl (lane 2), with corresponding profiles of fully solubilized sonicated heads before (lane 1) and after (lane 3) salt extraction. B, comparison of migration of calf thymus core histones (H) with 1 M KCl extracts (K), 2% SDS extracts (S), and 1 N HCl extract (A) from sonicated heads. C, comparison of migration of calf thymus core histones (H) with 1 N HCl extracts (A) and 2% SDS extracts from whole sperm.

Figure 2. N-terminal sequence of the major protein bands from sonicated sperm head salt extracts aligned with histone sequences. A, alignment of the N-terminal sequences from the 15.7- and 17-kDa bands with protein sequences from bovine somatic H2B (bH2B), testis-specific H2B variants from rat (rH2B) and human (hH2B), and H2B-like SubH2Bv (bSubH2Bv). Gaps (-) are indicated for proper alignment of sequences. Note: A second minor sequence (PEPAKSAPA) was detected in the 17-kDa band, representing contaminating sequence from the 15.7-kDa band. B, alignment of the N-terminal sequences from the 14.5-kDa band with protein sequences from bovine somatic H3 (bH3) and human H3-like centromere-specific histone variant CENP-A (hCENP-A).
Histones Are Present in Perinuclear Theca Extracts from Bovine Sperm—Protein extractions from sonicated heads using 1 M KCl were analyzed by one-dimensional 15% SDS-PAGE, revealing three prominent polypeptide bands of 14.5, 17, and 18 kDa (Fig. 1A). A comparison of the protein profile of reduced sonicated heads before (lane 1) and after (lane 3) extraction showed both the presence and absence of these bands, respectively (Fig. 1A), indicating their complete extraction. The N-terminal sequence of the 18-kDa band (PEPAPKSAPKKGS) exactly matched the N-terminal sequence of bovine somatic histone H2B, H2A, or H4 and sperm-equivalent volumetric loads of whole sperm SDS extracts were probed with affinity-purified anti-histone anti-sera. Densitometry of reactive bands, resolved by ECL, was assessed using the Scion Image Beta 4.02 Acquisition and Analysis software (Scion Corp., Frederick, MA). Regression analysis of recombinant histone standards and final estimation of sperm equivalence was conducted using Microsoft Excel Data Analysis software. Three separate whole sperm SDS extractions were analyzed for quantification of each histone protein from the same bull.

RESULTS

Histone subtype identities of the 14- to 19-kDa bands from whole sperm and sonicated heads and characterization of affinity-purified anti-histone antibodies. A, blots containing 15% SDS-PAGE resolved calf thymus core histones (H) and SDS extracts from whole sperm (WS) and sonicated head (SH) probed with C-terminal-specific anti-H3, C-terminal-specific anti-H2B, anti-H2A, and anti-H4 antibodies. B, single blot from the same gel containing calf thymus histone (H) and SDS extracts from whole sperm (WS) and sonicated head (SH) probed with N- and C-terminal-specific anti-H3 antibodies. Salt and acid extracts from whole sperm and sonicated heads showed identical immunoreactivity when probed with antibodies shown in A and B (data not shown). C, preparative strips containing calf thymus core somatic histones probed with affinity-purified antibodies against recombinant H3 (APH3), H2B (APH2B), H2A (APH2A), and H4 (APH4). Also shown is a Coomassie Blue-stained strip demonstrating histone migration level (CT-His) and a strip probed with the unpurified serum (NP-anti His).

Migration of the 18-, 17-, and 14-kDa bands extracted from sonicated head by salt, acid, and SDS treatment matched identically to control calf thymus histones H2B, H2A, and H4 (Fig. 1B). No matching band corresponding to H3 was detected in any of the Coomassie Blue-stained sonicated head extracts, nor did the 14.5-kDa band match with any of the core histone bands. In contrast, crude acid and SDS extracts from whole sperm revealed the presence of the same 14- to 18-kDa bands, together with a 19-kDa band, corresponding to the migration of calf thymus H3 (Fig. 1C). Furthermore, the presence of the 14.5-kDa band was also inconsistent in both whole sperm extracts. Although additional proteins were present in these extracts above 20 kDa, the 14- to 19-kDa bands were fully depleted from whole sperm pellets following acid or SDS extraction (data not shown).

Histone subtype identities of the 14- to 19-kDa bands from whole sperm extracts were further elucidated by immunoblots probed with anti-histone antibodies (Fig. 3). N- and C-terminal-specific anti-H3 antibodies labeled the 19-kDa band from whole sperm SDS (Fig. 3, A and B) and acid extracts (data not shown). Similarly, the 14-, 17-, and 18-kDa bands were specifically immunoreactive to respective anti-H4, anti-H2A, and C-terminal-specific anti-H2B antibodies, respectively (Fig. 3A).
Whole sperm pellets after extractions were non-immunoreactive to these antibodies (data not shown). Quantification of PT-derived somatic histones indicated that 0.2, 0.1, 0.03, and 0.02 pg per sperm of H3, H2B, H2A, and H4, respectively, were present in the whole sperm SDS extracts.

H3, H2B, and H4, but not H2A, were sensitive to proteolytic cleavage as a result of the sperm head isolation process, because no such cleavage products were detected in immunoblots of whole sperm extracts (Fig. 3, A and B). Furthermore, anti-histone antibodies confirmed the presence of histone proteolytic cleavage products in sonicated head extracts. For example, in sonicated head extracts, but not whole sperm extracts, C-terminal-specific anti-H3 antibodies were strongly immunoreactive with the 14.5-kDa band (Fig. 3B). Labeling was abolished when these extracts were probed with N-terminal-specific anti-H3 antibodies. This corroborates the N-terminal sequencing data, suggesting the 14.5-kDa band contains an H3 cleavage product lacking the N-terminal region, which was otherwise detectable by the latter probe. The 18-, 17-, and 14.5-kDa bands from sonicated head extracts were all immunoreactive to C-terminal-specific anti-H2B antibodies (Fig. 1A). This confirmed the presence of both intact and proteolytically cleaved H2B that were initially detected in the 18- and 17-kDa bands, respectively, by N-terminal sequencing. Anti-H2A and anti-H4 antibodies labeled the 17- and 14-kDa bands, respectively, from sonicated head extracts, demonstrating the presence of these histones despite the lack of N-terminal sequence data. An additional band migrating below 14 kDa was detected by anti-H4 antibodies and is indicative of a potential H4 cleavage product.

The somatic nature of the histones isolated from whole sperm acid extracts was further established by AUT- and 2D AUT/SDS-PAGE analysis. Numerous proteins in crude whole sperm acid extracts were resolved in Coomassie-stained gels by AUT-PAGE (Fig. 4A) and 2D AUT/SDS-PAGE (Fig. 4B). Nonetheless, bands and spots corresponding to calf thymus core somatic histones were detected. Subsequent probing of 2D-resolved whole sperm acid extracts by anti-histone-specific antibodies revealed strong monospecific reactivity of spots corresponding to respective histones subtypes. These labeled spots matched 2D-resolved and immunoprobed calf thymus somatic histones (Fig. 4C).

**Somatic Histone Are Localized to the Post-acrosomal Sheath Region in Bovine Sperm Perinuclear Theca—**Ultrastructural assessment of sonicated sperm heads prior to, and following, salt, SDS, or acid extractions by transmission electron microscopy indicated that neither the isolation, nor the extraction procedures produced any observable perturbation of the condensed nucleus (Fig. 5A). These findings were consistent with previous assessments of sonicated sperm head morphology following Triton X-100 and NaCl extractions (2, 18, 43).

Attempts at labeling sperm samples with commercially available anti-histone antibodies were marred by high background and nonspecific labeling (data not shown). Therefore,
antiserum raised against sonicated head KCl extracts was affinity-purified against each histone subtype and used to probe the sperm samples. The characterization of the antisera and subsequent affinity-purified anti-H3, -H2B, -H2A, and -H4 antibodies are presented in Fig. 3C. Prior to the extractions, immunogold labeling with each affinity-purified anti-histone antisera revealed the presence of somatic histones in the exposed post-acrosomal sheath of sonicated heads (Fig. 5A). There was negligible nuclear labeling with all anti-histone antibodies (Fig. 5A, inset). In contrast, little to no labeling was evident over the post-acrosomal sheath in sonicated heads following extractions (Fig. 5B and C). No immunogold labeling was detected in the PAS of sperm head sections probed with either preimmune serum or gold-conjugated secondary antibodies alone (not shown). Bar = 0.2 μm. Gold particles = 10 nm.

**DISCUSSION**

We report unequivocal biochemical and ultrastructural evidence for the stable, non-nuclear localization of somatic-type core histones in mature bovine sperm. Their localization appears to be confined to the post-acrosomal sheath region of the PT, a specialized cytosolic component unique to mammalian sperm. Moreover, our estimates show that up to 0.2 pg per sperm of each PT-derived somatic histone subtype are present.

**Somatic Histones Are SDS-/Salt-soluble Components of Bovine Sperm PT**—We sought to extend the compositional analysis of the bovine sperm PT by characterizing salt-extractable PT proteins previously observed (18). Most PT proteins characterized to date share a high resistance to Triton X-100 and high salt treatments and require much harsher chemical conditions for their extraction (3, 5, 6, 8, 18, 31, 44). Using sperm heads obtained by sonication, we were able to extract histones from the PT with 1 M KCl. However, immunoblotting and N-terminal sequencing analysis revealed the presence of histone proteolytic cleavage products in this extract. This obser-
vation is consistent with the previously reported susceptibility of histones to proteolysis during isolation, from both somatic (45) and spermatogenic nuclei (46, 47), despite the use of protease inhibitors. Histone cleavage products were also observed in sonicated head SDS and acid extracts, indicating that they were not the result of the salt extraction procedure alone. SDS-PAGE and immunoblotting analyses of the crude acid and SDS extracts from whole bovine sperm revealed a remarkable reduction in histone proteolysis, demonstrating that the histone cleavage products were likely the indirect result of the sonication procedure. More importantly, these latter findings indicate that all four core somatic histones are present and intact in bovine epididymal sperm.

Subsequent TEM immunogold labeling localized all four somatic histones to the post-acrosomal sheath region of bovine sperm, immunogold labeled with an affinity-purified anti-H2B antibody. B, oblique section through the PAS region of a double-tailed sperm, immunogold labeled with an affinity-purified anti-H3 antibody. C, transverse section through post-acrosomal sheath region of bovine sperm, immunogold labeled with an anti-H2B antibody. D, example of heterochromatic labeling in somatic nuclei (Sertoli cell), labeled with an affinity-purified anti-H2B antibody. No immunogold labeling was detected in somatic nuclei and bovine sperm PAS when probed with either preimmune serum or gold-conjugated secondary alone (not shown). Ac, acrosome; ES, equatorial segment; N, nucleus; OPL, outer periacrosomal layer; PAS, post-acrosomal sheath; T, tail. Bar = 0.2 μm (except in B inset, where the bar = 1.0 μm; gold particles = 10 nm).

PT-derived Histones Are Not Testis-specific Variants—Several lines of evidence in our study confirm that the isolated PT-derived histones are somatic in nature. N-terminal sequencing determined that PT-derived histones are an exact match to bovine somatic histone sequences. Failure of H2A and H4 detection by N-terminal sequencing can be attributed to N-terminal acetylation reported in these latter histones (48–50), which blocks the N-terminal sequencing procedure. PT-derived histone sequences do not share any homology to mammalian testis-specific histone variants (51, 52), although no bovine testis-specific histone variant has been characterized to date (53). Furthermore, 2D AUT/SDS-PAGE, and subsequent immunoblot analysis confirmed that these PT-derived histones are somatic-type, based on their identical characteristics to calf thymus core somatic histones.
PT-derived histones also share no N-terminal sequence homology to reported bovine sperm histone variants, such as CENP-A (26) and SubH2Bv (31). In addition, the localization of these latter two variants to the nucleus and the subacrosomal region of mature bovine sperm, respectively, clearly differ from the post-acrosomal sheath localization we observe in PT-derived somatic histones.

Quantification of PT-derived Somatic Histones—Estimates of the abundance of PT-derived somatic histones were obtained from SDS extracts of intact and freshly collected bovine sperm. The rationale for making estimates on crude extracts was to minimize the occurrence of histone cleavage products observed in this study. Values obtained by densitometry of immunoblots ranged from 0.2 to 0.02 pg per sperm, whereby the order of abundance of each subtype was H3>H2B>H4>H2A. The confidence levels for this potential non-stoichiometric representation are uncertain, and it should be noted that Coomassie Blue-stained acid extracts on SDS-PAGE indicate a more equivalent representation, particularly with H2A and H4 (Fig. 1C). The possible underestimation of PT-derived H2A and H4 may be due to limitations in the quantification technique, such as epitope accessibility in the crude extracts. Cumulative estimates of PT-derived H3 (0.2 pg per sperm) and H2B (0.1 pg per sperm) alone represent at least 10% haploid genomic equivalence of histone content by weight (based on 1:1 total histone to DNA ratio per nucleosome by weight, where one sperm nuclei contains ~3 pg of DNA (54)). These estimates far exceed the reported 1% somatic histone content reported in bovine sperm nuclei (26), further supporting the notion that the larger population of somatic histones we report must arise from a non-nuclear source.

Insights into the Structure of Bovine PT—Consistent with other PT proteins characterized to date, PT-derived somatic histones are highly basic in nature, supporting earlier observations that the PT is a lysine-rich structure (55). Interestingly, the observed proteolytic cleavage in some of the isolated histone subtypes in sonicated head extracts suggests that the N-terminal tails of PT-derived histones are located along the periphery of the post-acrosomal sheath. Cleavage of H3, H2B, and possibly H4 occurred near the N terminus, and showed a consistent pattern in all sonicated head extracts, as seen by N-terminal sequencing and immunoblotting analysis. H3, H2B, and H4 have relatively long N-terminal tail domains, containing 44, 32, and 26 amino acids, respectively (56). Moreover, the N-terminal tail domains that are rich in basic lysine and arginine residues tend to be unstructured, providing suitable target sites for a number of proteases. Given this susceptibility and sensitivity to proteolysis, it is possible that, despite the use of protease inhibitors, acrosome proteases may readily act upon PT-bound proteins, due to their proximity and ensuing release upon sonication (1, 57). The fact that minimal H2A cleavage was observed in sonicated head extracts further substantiates this view. In contrast to the other histones, the N-terminal tail domain of H2A is relatively short, at 16 amino acids (56), and might minimize exposure to released proteases.

De Novo Synthesis of Non-nuclear Somatic Histones during Spermiogenesis—In the context of the sperm nucleus, nucleoprotein transitions during spermatogenesis have been well established in numerous mammalian species, whereby the variation in somatic histone fate is species-specific. We have preliminary evidence for PT-localized histones in rat epididymal sperm,4 indicating that non-nuclear histones in mature sperm are not a species-specific occurrence. In light of this finding, the question arises whether these histones are recycled after displacement by transition proteins and protamines during earlier stages of spermatogenesis, or if they are synthesized de novo. A variety of studies both directly and indirectly support the latter notion. Proteolytic breakdown of nuclear somatic histones has been reported in rat and mouse spermatids (46, 47). Moreover, post-translational modifications, such as acetylation (58–61) and ubiquitination (32, 62), of somatic histones have been associated with their removal and subsequent degradation from mammalian spermatid nuclei. Within the confines of our analysis, we detected no post-translational modifications of PT-derived somatic histones. Thus, somatic histones of nuclear origin are likely degraded, rendering the notion of histone recycling improbable.

More direct lines of evidence for de novo synthesis stem from autoradiographic studies demonstrating histone synthesis occurring in late spermatids in mouse (28). Histones synthesized in this period are retained in epididymal sperm, although their source in the sperm was not ascertained (28). In this latter study, epididymal sperm nuclei purification for histone extraction employed the use of Triton X-100, which would not have solubilized any of the PT proteins characterized to date, including the PT-derived somatic histones reported here. Interestingly, the timing of late spermatid histone synthesis reported by Goldberg et al. (28) coincides temporally with the synthesis and formation of PT structures (2). Preliminary developmental studies have also localized somatic histones to manchette microtubules in bovine testis,2 a structure associated with post-acrosomal sheath formation and nuclear-cytoplasmic transport (10, 63, 64). Taken together, there is considerable evidence suggesting de novo synthesis of PT-derived somatic histones. However, more species-specific studies are required to fully elucidate this notion.

Functions for PT-derived Somatic Histones—Provided their arrangement along the PT can support DNA binding, the localization of PT-derived somatic histones mechanistically favors a potential role in sperm-mediated gene transfer (65). In this process, binding of exogenous DNA is thought to involve sperm submembrane compartment proteins and occurs in demembranated, Triton X-100-treated sperm heads (66). Specific localization of exogenous DNA binding to sperm appears confined to PT structures, such as the subacrosomal layer and the post-acrosomal sheath, and may involve proteins of less than 20 kDa in sperm of various species (67). Given the localization of PT-derived somatic histones characterized in this study, and the inherent capabilities of somatic histones for DNA binding, it is likely that they are potential candidates for this process.

Although the levels of PT-derived histones are apparently too low for a general role in genomic nucleosomal organization, the possibility remains that PT-derived histones are present in sufficient amounts to exert nuclear stability during male pronuclear formation. Cumulative quantitative estimates of PT-derived somatic histones are comparable to HMG 2 protein levels calculated in rat spermatids (47 μg/mg DNA), which play a role in chromosomal reorganization (68). Since mammalian sperm PT proteins have been implicated in the process of fertilization (12), the potential for PT-derived somatic histones to contribute to male pronuclear development exists. Further studies are clearly required to substantiate this claim. Although the functional roles for non-nuclear somatic histones in sperm are indeed speculative, this unprecedented finding of PT-derived somatic histones may provide insight into numerous ill-defined sperm functions.

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4 P. R. Tovich and R. J. Oko, unpublished observations.
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REFERENCES
1. Eddy, E. M., and O'Brien, D. A. (1994) in The Physiology of Reproduction (Kobl, E., and Nell, J. D., eds) pp. 29–77, Raven Press Ltd., New York.
2. Oko, R., J. (1995) Reprod. Fert. Dev., 7, 777–797.
3. Oko, R., Aul, R. B., Wu, A., and Sutovsky, P. (2001) in Andrology in the 21st Century: Proceedings of the VIIth International Congress of Andrology (Robaire, B., Chem, H. E., and Morales, C. R., eds) pp. 37–51, Midemont Publishing Co., Inc., Englewood, NJ.
4. van Bulow, M., Heid, H., Hess, H., and Franke, W. W. (1995) Exp. Cell Res., 316, 297–316.

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