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Comparative structure of vertebrate sperm chromatin

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ABSTRACT

A consistent feature of sperm nuclei is its exceptionally compact state in comparison with somatic nuclei. Here, we have examined the structural organization of sperm chromatin from representatives of three vertebrate lineages, bony fish (*Danio rerio*), birds (*Gallus gallus domesticus*) and mammals (*Mus musculus*) using light and transmission electron microscopy (TEM). Although the three sperm nuclei are all highly compact, they differ in morphology and in the complement of compaction-inducing proteins. Whereas zebrafish sperm retain somatic histones and a nucleosomal organization, in the rooster and mouse, histones are largely replaced by small, arginine-rich protamines. In contrast to the mouse, the rooster protamine contains no cysteine residues and lacks the potential stabilizing effects of S–S bonds. Protamine driven chromatin compaction results in a stable, highly condensed chromatin, markedly different from the somatic nucleosome-based beads-on-a-string architecture, but its structure remains poorly understood. When prepared gently for whole mount TEM, the rooster and mouse sperm chromatin reveal striking rod-like units 40–50 nm in width. Also present in the mouse, which has very flattened sperm nuclei, but not rooster, where nuclei take the form of elongated cylinders, are toroidal shaped structures, with an external diameter of about 90 nm. In contrast, similarly prepared zebrafish sperm exhibit nucleosomal chromatin. We also examined the early stages in the binding of salmine (the salmon protamine) to defined sequence DNA. These images suggest an initial side-by-side binding of linear DNA–protamine complexes leading to the nucleation of thin, flexible rods with the potential to bend, allowing the ends to come into contact and fuse to form toroidal structures. We discuss the relationship between these *in vitro* observations and the rods and toroids seen in nuclei, and suggest an explanation for the apparent absence of these structures in TEM images of fully condensed sperm nuclei.

1. Introduction

Deciphering the underlying architecture of compact DNA–protein complexes is often challenging, requiring a range of microscopical and other techniques. For compact nuclear chromatin consisting of arrays of nucleosomes, decades of work appear to be approaching a consensus concerning its structural organization (reviewed in Bian and Belmont, 2012; Grigoryev and Woodcock, 2012; Maeshima et al., 2010). Sperm chromatin is even more compact, and the details of its structural organization are still poorly understood.

Within the vertebrate lineage, there is a wide variation both in sperm morphology (Baccetti and Afzelius, 1976; Jamieson et al., 1995), and in the sperm nuclear basic proteins (SNBPs) that are associated with DNA in the mature sperm (Ausió, 1999; Bloch, 1969, 1976; Kasinsky, 1989). Evolutionary aspects of this diversity have been discussed extensively elsewhere (Ausió, 1999; Eirin-Lopez and Ausió, 2009). In brief, a reversible transition in SNBPs from histones (H) to protamines (P) through a protamine-like intermediate (PL) has recurred in many metazoan groups in the course of evolution resulting in a sporadic yet non-random distribution where SNBPs of the P type are always present at the tips of the phylogenetic tree. Fish provide an excellent example of this (Ausió et al., 2011a,b; Saperas et al., 1994,) where the three types of SNBPs are present in different species. Species with H-type SNBPs occur in agnathans (Saperas et al., 1994) and in the order Cypriniformes which includes *Danio rerio* (Muñoz-Guerra et al.,

Abbreviations: TEM, transmission electron microscopy; AFM, atomic force microscopy; SNBP, sperm nuclear basic protein; Hepes, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DIC, differential interference contrast; DTT, dithiothreitol; CCD, charge coupled device; CTAB, cetyl trimethyl ammonium bromide (Cetrimide); Cryo-EM, cryo-electron microscopy; S.D., standard deviation; S.E., standard error.

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1982). Salmonids and their protamine (salmine) (Oliva and Dixon, 1991) are representative of the P-type.

Differing sperm DNA compaction modalities have been recognized associated with the different SNBPs (Ausio, 2007; Eirin-Lopez and Ausio, 2009). In one type, exemplified here by the zebrafish (*D. rerio*), the full complement of somatic histones is retained, and the somatic nucleosomal organization persists. What exactly promotes sperm compaction in these cases remains a matter of debate, although as discussed below, the higher ratio of H1 linker histone to core histones, and changes in the levels of some post-synthetic histone modifications are likely major contributors. A second modality seen in the majority of vertebrates involves the replacement of the majority of somatic histones with small, highly basic protamines that may contain substantial amounts of cysteine. We selected the rooster (*Gallus gallus domesticus*) as an example of sperm compaction based on protamines that lack cysteine. In mammals, exemplified here by the mouse (*Mus musculus*), the protamines contain multiple cysteines, which, in the mature sperm, may undergo S–S bond formation (Balhorn, 2007), enhancing chromatin stability. Additionally, cysteine-bearing protamines may interact with the zinc ions present in these cells (Bjorndahl and Kvist, 2014). Another feature reported in some eutherian mammals, is the retention in mature sperm of some (5–15%) somatic histones which are associated with genes that become activated immediately after fertilization (Hammond et al., 2009; Hisano et al., 2013; Wu et al., 2011). These regions retain a nucleosomal organization in mature sperm.

Sperm quality is known to be important for successful fertilization, and defects in compaction are often associated with infertility (Oliva, 2006). Thus, understanding the structural principles of sperm chromatin formation and compaction has both intrinsic interest and clinical implications. Achieving this understanding has proven to be difficult – the extreme chromatin compaction hampers structural studies of native sperm, which appears uniformly dense in thin sections imaged by TEM (e.g. Dooher and Bennett, 1973, 1977; Fawcett and Bedford, 1979). Substructure in the form of sheets has been reported in freeze-fracture images of the highly flattened sperm of rat and rabbit (Koehler, 1970; Koehler et al., 1983), but not in human sperm (Koehler, 1972). An *in vitro* approach that has provided important insights into the structural consequences of DNA–SNBP interactions involves imaging the products obtained by mixing DNA, often of defined length and sequence, with pure protamines or small polycations. These studies have revealed two prominent DNA–protamine motifs, rod-like and toroidal, and much effort has been devoted to their characterization. Cryo-EM of toroids prepared from DNA and hexammine cobalt chloride, $Co(NH_3)_6Cl_3$ III, has suggested an organization in which extended linear complexes of DNA and polycations associate side-by-side in a close-packed hexagonal arrangement (Hud and Downing, 2001; reviewed in Hud and Vilfan, 2005), consistent with X-ray (Roque et al., 2011) and molecular modeling studies (Balhorn, 1982). These suggest that protamines wrap around DNA, interacting with the minor groove, creating linear complexes that pack side-by-side in hexagonal arrays while retaining the B-form of DNA. AFM imaging has also been applied both to *in vitro* complexes and native sperm *in situ*, providing general support for a toroidal-based organization (Allen et al., 1993, 1997; reviewed in Balhorn, 2007). An extension of the laminar sheet model of Koehler has the sheets formed by planar arrays of toroids (Balhorn et al., 1999). However, this arrangement has not been experimentally verified. Moreover, it is still not known how toroidal structures are formed *in vivo*, how they are arranged within the nucleus, and the path of DNA within and between them.

Here, we report the first comparative study of the structural organization of native sperm nuclei from zebrafish, rooster and mouse, focusing on the features revealed by TEM imaging of partially decondensed sperm nuclei. The work highlights the striking differences between the three systems, especially in terms of chromatin structure, stability and resistance to decondensation. As expected from its protein composition, zebrafish sperm chromatin has a nucleosomal organization, whereas the protamine-bearing rooster and mouse sperm have quite different architectures. The images reveal the presence of a striking rod-like motif in partially decondensed chicken and mouse sperm nuclei. In the mouse, toroids are also present, intermixed with rods. To aid in understanding the process of DNA–protamine interaction, we also investigated the structures formed *in vitro* at the earliest stages after mixing defined sequence DNA with salmine, the salmon protamine. This work provides novel insights into the structural motifs present in sperm chromatin and the processes involved in their formation.

2. Materials and methods

2.1. DNA–salmine complexes

Defined sequence DNA, consisting of 4 tandem repeats of a 207 bp unit was prepared as described (Ghosh et al., 2010), and salmon sperm protamine (salmine) was obtained from Sigma Corp., (St. Louis, MO, USA). Complexes were formed by mixing DNA and salmine in HEN buffer (10 mM Hepes, 0.1 mM EDTA, 2.5 mM NaCl, pH 7.5), for 10 min at room temperature.

2.2. D. rerio sperm nuclei

Testes were dissected from euthanized adult male zebrafish, placed on a drop of Ginzberg's fish Ringer's solution (150 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 2 mM $NaHCO_3$) on a glass slide, and covered with a cover glass. The cover glass was tapped gently to release sperm, then removed, and the suspension harvested with a micropipette. Sperm from ~5 fish were combined and made to 1.0 ml with buffer, and held on ice for 10 min to allow large particles to sediment. Supernatants were centrifuged in a swinging bucket centrifuge at 200*g* for 5 min to gently pellet sperm, resuspended in buffer, and inspected for swimming sperm using phase contrast or differential interference contrast microscopy (DIC). For long-term storage, 100 µl aliquots were flash frozen in liquid nitrogen, and stored at −80 °C. Typically, samples stored for several months appeared completely normal when rapidly thawed at 37 °C, with some even regaining motility. To release nuclei, sperm were pelleted at 200*g*, and resuspended on ice in 10 mM HEPES pH 7.5, 2 mM EDTA, 150 mM NaCl. Non-idet P40 detergent was added to 2%, and the sample vigorously vortexed. Phase microscopy was used to determine when the majority of nuclei were released, at which time the nuclei were pelleted at 2000*g* for 10 min, and resuspended in detergent-free buffer. Long-term storage of nuclei at −80 °C was as described above.

2.3. G. gallus domesticus sperm nuclei

Epididymi were obtained from sexually mature roosters and placed in Whitten's sperm isolation medium (Whitten and Biggers, 1968) without pyruvate. They were then cut into 2–3 mm segments, gently teased to release sperm, and treated to generate demembranated nuclei as described for *M. musculus* except that sonication was not needed, and dithiothreitol (DTT) was omitted.