A NEW MODEL FOR SPERM CHROMATIN STRUCTURE AND ITS RELATIONSHIP TO FUNCTION

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By
Barbara Sotolongo

Dissertation Committee:
W.S. Ward, Chairperson
Yusuke Marikawa
Helen Turner
Ryuzo Yanagimachi
Tom Huang
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ABSTRACT

Hamster and human spermatozoa is composed of DNA bound by protamines into very condensed toroids. Toroids have an average of 50 kilobases (kb) of DNA. Sperm DNA is also organized into loop domains of about the same size which are attached at their bases to the sperm nuclear matrix. I tested a model of sperm chromatin structure that our lab had proposed, named the Donut-Loop Model. The model predicts that each DNA-loop domain is one protamine-bound toroid. It also proposes that there is a DNase-sensitive toroid-linker site between each toroid and these regions are the sites of DNA-loop-domain attachment to the nuclear matrix. I demonstrated that the first prediction was true, using exogenous DNase I treatment followed by analysis on pulse-field gel electrophoresis (PFGE). I tested the second prediction by examining the loop attachments using the halo assay after DNase I treatment. The PFGE data showed that DNase I released 50 kb fragments of DNA from the nucleus, and the halo assay demonstrated that no DNA remained associated with the sperm nuclear matrix. These combined data supported the Donut-Loop model. One function of these DNase-sensitive toroid linker regions might be to act as a site of endogenous sperm nuclease activity where the nuclease cleaves sperm chromatin at the bases of the DNA
loop domains into large fragments with an average size of 50 kb. I also provided evidence that a previously undescribed sperm nuclease is activated by the presence of calcium and magnesium much more efficiently than with either ion alone resulting in quick DNA degradation in hamster and in human. Furthermore, the human nuclease can be activated by freeze-thawing in non-cryoprotective media. The sperm nucleases in hamster and human act similarly to somatic cell DNases, which also require calcium and magnesium and which digest the chromatin into loop-sized fragments during the process of apoptosis.
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Chapter 1.

A New Model for Sperm Chromatin Structure and its Relationship to Function

Introduction

In this dissertation we demonstrate a new level of sperm chromatin structure, which we have termed the Donut-Loop model, and provide evidence to support its role in sperm DNA degradation. The importance of studying the structure of sperm chromatin as one of the most unique structures of DNA organization in a wide variety of species is for understanding research in many different biological fields. By investigating sperm chromatin, one will be able to answer questions in the areas of embryogenesis, reproduction and infertility, just to name a few areas, as well as basic DNA structure and function, and many others.

Although the level of sperm DNA organization is homologous to nucleosomes in somatic cells, sperm DNA structure differs from somatic with regard to the DNA binding proteins. Sperm cells are unique in binding their DNA to protamines, which are very basic, arginine-rich binding proteins, that bind sperm DNA into the most tightly compacted eukaryotic DNA possible during spermiogenesis (Pogany et al., 1981; Balhorn, 1982). In somatic cells
DNA coils twice around an octomer of histones to form a nucleosome (McGhee and Felsenfeld, 1980; Richmond and Davey, 2003). The DNA loop domains attach at their bases to the nuclear matrix and are associated with DNA replication and RNA transcription (Nelson et al., 1986; Gerdes et al., 1994). The nucleosome is less compact than sperm cells; therefore this structure is more open to DNase digestion in comparison to the protamine-bound sperm DNA. Although sperm DNA also forms loop domains like somatic cells, there is a lack of transcription and translation in mature sperm.

Although sperm structure is not associated with DNA replication and RNA transcription as in somatic cells, the DNA loop domains may have a necessary function for embryogenesis. Klaus et al., (2001) showed that sperm DNA loop domain organization is cell specific and changes during development with regard to the size and number of loops as the sperm progresses towards maturity. We suggested (Sotolongo and Ward, 2000) that intact sperm DNA loop domain is necessary for development in a mammalian system such as the mouse.

In order to fully comprehend the importance of the work discussed in the results section of this dissertation, I must first review the biology of sperm as well as its chromatin structure in this introductory chapter. The importance
of what is known in the somatic cell model and how it might relate to the sperm cell Donut-Loop model will also be discussed.

1.1. Spermatogenesis

Mammalian spermatogenesis is the process by which male germ cells develop, proliferate and differentiate within the seminiferous tubules of the testis throughout a man's lifetime. The first initial stage of development is division by mitosis in order to produce more spermatogonia from primordial germ cells. The next two main stages which complete the process of spermatogenesis are called meiosis and spermiogenesis. Meiosis is described as the transitional process during which spermatogonia differentiate into primary and secondary spermatocytes as the DNA content is increased to 4N and then reduced to 2N and finally to 1N. Spermiogenesis is the differentiation period from the round and elongating spermatid to the mature spermatozoa, which are all haploid (Steger, 2001).

Primordial germ cells (PGC) are formed from the epiblast during gastrulation and migrate to the urogenital ridge (Derooij, 1998). During the formation of the seminiferous tubules, male PGC differentiate into gonocytes and undergo mitotic arrest until after birth. After birth, gonocytes resume mitosis and differentiate into type-A spermatogonia, which are 2N (Brinster,
2002). During the process of mitosis, spermatogonia types A can differentiate into an intermediate form and finally into type B spermatogonia, which are also 2N (Meng, 2000). These type B spermatogonia undergo meiosis and results in the formation of leptotene primary spermatocytes, which are 4N (Hecht, 1995; Krawetz, 1999). The next stages of meiosis among the spermatocytes are zygotene, pachytene and diplotene stages, which are also 4N (Krawetz, 1999). Primary 4N spermatocytes go through meiosis division I and produce secondary 2N spermtocytes. These in turn, go through meiosis division II resulting in primary spermatids, the first haploid cell of spermatogenesis. During meiosis there are various processes occurring such as X-inactivation, homologous chromosome pairing, recombination and synthesis of testis-specific histones (Krawetz, 1999; Holstein, 2003).

Spermiogenesis is a process during which the round, non-motile 1N spermatid undergoes a transformation into an elongating 1N spermatid. The elongating spermatid is further transformed into a mature, motile 1N spermatozoon. The mature spermatozoa undergo complete replacement of histones by protamine proteins. The protamines are responsible for the highly compacted nature of the sperm DNA (Sassone-Corsi, 2002; Mills, 1977). More will be discussed later in the introduction regarding protamines (see figure 1.1).
Figure 1.1. Spermatogenesis in mammalian cells has been shown to be a developmental process by which spermatogonia cells generate into the mature spermatozoon.

The events characterized by modifications in chromatin organization during the two basic processes of meiosis and post-meiotic histone-to-protamine replacement have been diagrammed here. Adapted from Paolo Sassone-Corsi (2002).
Mammalian spermatogenesis has recently been linked to the regulation of cell death by removing abnormal sperm by apoptosis (Print and Loveland, 2000). Blanco-Rodriguez and Martinez-Garcia (1999) have shown in rats that cytoplasmic apoptotic condensates called residual bodies were detached from spermatids before they were released as mature spermatozoa. Their in-vitro studies demonstrated that during the release of spermatozoa from the seminiferous epithelium, partial pinching off of the cytoplasm of the spermatozoa occurs (Blanco-Rodriguez and Martinez-Garcia, 1999). These cytoplasmic stalks broke off and released the spermatozoa from the lumen of the seminiferous tubules. During the next stage, the residual body was phagocytosed by the Sertoli cells. In this case, the apoptotic machinery was not used to destroy and kill sperm cells but to release them as mature spermatozoa while using cytoplasmic apoptotic signaling markers.

Drosophila spermatogenesis has also been found to borrow apoptotic machinery for non-apoptotic functions during sperm differentiation (Arama et al., 2003). Similar to the mammalian cytoplasmic stalk, fly spermatogenesis has occurred within cysts or bundles of 64 spermatids (Noguchi et al., 2003) that retain meiotic cytoplasmic bridges. During the final step in differentiation called individualization, spermatids form a complex close to the nucleus and the spermatid within the cystic bulge (cytoplasmic part) sheds its ‘waste bag’ of
cytoplasm. Then the spermatozoa emerged from this cytoplasmic bulge (Cagan, 2003). This process has been seen as similar to that of mammals in which the apoptotic machinery (caspases and other apoptotic proteins) has been used for non-apoptotic work and in the process of normal spermatozoa development.

1.2. Mammalian Sperm Cell Structure

The four phases of spermiogenesis which contribute to the formation of mature spermatozoa are the golgi, cap, acrosomal and maturation phases. It is during spermiogenesis that the mature spermatozoon is formed and released into the lumen of the seminiferous tubules. There are several different structures that are of importance to a normal sperm cell. These main structures are the tail with the axoneme (axonemal complex), middle piece with the mitochondria, acrosome (acrosomal cap) and the sperm nucleus. Let me first describe the four general phases and then go into more detail on these structures.

During the Golgi phase, the proacrosomal granules of the spermatids accumulate in the Golgi complex and form a single acrosomal granule contained within a membrane-lined acrosomal vesicle. Also during this phase, the centrioles migrate from an area near the nucleus towards the distal part of
the spermatid. The distal centrioles will be involved in the formation of the microtubules forming the flagellar axoneme (Ramalho-Santos et al., 2001; Sinowatz and Wrobel, 1981; Clermont and Leblond, 1955).

During the cap phase, the acrosomal vesicle and granule spread to cover the anterior part of the condensing nucleus to form what is known as the acrosomal cap or acrosome in the sperm head. The acrosome contains enzymes and is a type of lysosome. These acrosomal enzymes are responsible for penetration of the zona pellucida of the ovum during fertilization (Ramalho-Santos et al., 2001; Sinowatz and Wrobel, 1981; Clermont and Leblond, 1955).

During the acrosomal phase, the nucleus becomes more elongated and condensed. The manchette, a cylindrical sheath composed of microtubules, forms at the posterior part of the spermatid. The manchette is a transient structure. The centrioles which began microtubular synthesis during the Golgi phase are now modified to form various parts of the sperm tail. The mitochondria amass toward the proximal part of the flagellum and provide the energy for movement of the tail so that the sperm can be motile after release into the seminiferous tubules (Ramalho-Santos et al., 2001; Sinowatz and Wrobel, 1981; Clermont and Leblond, 1955).

During the maturation phase, cytoplasmic bridges that were formed between sperm cells begin to form residual cytoplasmic bodies or residual
bodies as the process of spermatid differentiation nears completion. The residual cytoplasm is released and phagocytized by Sertoli cells, which are pyramidal-shaped cells adhering to the basal lamina. The spermatids are separated and released into the lumen of the seminiferous tubule during this phase (Ramalho-Santos et al., 2001; Sinowatz and Wrobel, 1981; Clermont and Leblond, 1955).

The flagellum of spermatozoa has the following four segments: the connecting piece next to the head, the middle piece with the mitochondria, the principal piece and the short end piece (Eddy et al., 2003). The flagella are the tails of the sperm that provides the motile complex necessary for motion and penetration of sperm into the egg at fertilization (Inaba, 2003). The connecting piece or the neck region unites the nucleus with the flagellum. As the manchette disappears during late spermiogenesis, the mitochondria form a cylindrically wrapped sheath around coarse fibers in the neck region and distal regions called the middle piece. It is the mitochondria that provide the ATP energy that is used by the sperm tail for its motility. Distal to this middle piece is the principal piece which is composed of nine longitudinal fibers. The end of the flagellum or the fibrous sheath is known as the short end piece.

Another part of the sperm tail that is important is the axonemal complex or axoneme. It is initially formed during the Golgi phase as the centriole makes
the nine peripheral microtubule doublets and two central microtubules that form the axoneme of the sperm tail throughout its flagellar length. The alpha- and beta-tubulin proteins assemble to form linear protofilaments (Mohri, 1968). The axoneme is a cytoskeleton with dynein protein motors (ATPases), molecular chaperones, calcium binding proteins and protein kinases or phosphatases (Inaba, 2003). Gagnon (1996) reported that tubulins can undergo post-translational modifications such as acetylation, glutamylation and glycylation and suggests that these changes may have a role in axonemal motility, stability and interaction with other proteins (Huitorel et al., 1999; Kierszenbaum, 2002).

The axonemes are surrounded by outer dense fibers and mitochondria while a fibrous sheath surrounds the axoneme in the principal piece (Baccetti and Afzelius, 1976; Inaba, 2003). Although immediately after spermiogenesis, newly made sperm cells show no or little motility, prior to fertilization, the axoneme is activated and motility of sperm occurs (Inaba, 2003).

The next important structure in mammalian sperm is the acrosome found in the sperm head. The acrosome is composed of a cap-like structure enclosed with inner and outer acrosomal membranes (Eddy and O’Brien, 1994). The basic structure of the acrosome is conserved in all mammals, however, its shape and size varies between species (Eddy and O’Brien, 1994). The
acrosomal cap contains enzymes such as hyaluronidase, proacrosin (a protease), apexin (a neuraminidase) which are compartmentalized in different domains because different enzymes regulate different aspects of the acrosome reaction and sperm-egg interaction (Kim et al., 2001; Yoshinaga and Toshimori, 2003). The acrosome reaction requires that the spermatozoa undergo epididymal maturation and capacitation within the female genital tract because only capacitated sperm can recognize and bind to the zona pellucida, the egg’s extracellular coat. The sperm-egg interaction is a carbohydrate-mediated act that involves the receptors on the sperm plasma membrane recognizing and binding to glycans on the zona pellucida of the egg. The binding of the sperm receptors to the zona pellucida initiates a calcium-dependent signal cascade which results in the release of the sperm acrosomal enzymes which is called the acrosome reaction (Abou-Haila and Tulsiani, 2000). These acrosomal enzymes and other proteins are necessary for binding to the zona pellucida, activation of the acrosome reaction and penetration through the zona by the sperm in order to fertilize the egg (Toshimori, 2000; Yoshinaga and Toshimori, 2003).

The sperm head is also an important structure that needs to be discussed. It undergoes different re-organizational steps during spermiogenesis. The three types of spermatids found during spermiogenesis are early spermatids with round nuclei (Golgi and cap phases), intermediate
spermatids with elongated nuclei (acrosome phase) and mature spermatids with condensed nuclei during the maturation phase (Roosen-Runge and Holstein, 1978; Dadoune, 1995). The shape and size of the nucleus change in the spermatid as the types of proteins the spermatids are bound to become altered. First, sperm replace the somatic histones with testis-specific histones, then transition proteins in intermediate spermatids and finally, basic proteins called protamines in mature sperm (Hecht, 1990; Hecht, 1998). Compression of mammalian sperm at least six times more condensed than DNA in mitotic chromosomes is involved in making the haploid genome functionally inert and silent which may have an embryonic role in protecting it from DNA breaks during its transit to the egg (Balhorn, 1982; Ward and Zalensky, 1996). It appears that if this sperm chromatin remodeling is impaired, then spermiogenesis and fertility are also impaired (Toshimori and Ho, 2003).

The nucleus has other proteins that may have a role in structural stability and they are called the lamins. The ubiquitous lamins are B1 and B2 and the ones found in differentiated cells are lamins A and C. Alsheimer and colleagues (1999) found that spermatocytes only express transient meiosis-specific splicing variants of lamins A and B2 genes which were the lamins in isoforms C2 and B3, respectively. Collas (2000) found that sperm sea urchin required the assembly of lamin B in vitro for the membranes of the male
pronucleus. The nuclear pore complex of mouse early spermatids, also, like in sea urchin, discovered lamin B (Moss et al., 1993).

The last category of sperm structural regions is the perinuclear theca proteins found surrounding the nucleus, just external to the nuclear envelope (Oko and Maravei, 1995). Among some of the proteins discovered in the perinuclear theca are calmodulin, calicin, spectrin, actin, stat4 cylicin, dystrophins and perf15 (Mujica et al., 2003). One of the perinuclear theca functions is in the activation of the egg during fertilization by SOAF (sperm-born oocyte activating factor) in mammals (Kimura et al., 1998). Another function is in the formation of lipid domains in the sperm plasma membrane (Eddy and O’Brien, 1994). A third function is to maintain the characteristic shape of sperm as in the case of calicin, actin and spectrin proteins (Escalier, 1990). Stat 4 is a transcriptional activator which may be involved in zygotic gene activation (Herrada and Wolgemuth, 1997).

The main structural components of the mammalian sperm cell are the tail with the axonemal complex, the midpiece with the mitochondria, the acrosomal cap in the head and the sperm nucleus. All of these different structural domains as well as other parts of the sperm (perinuclear theca) are of importance during spermiogenesis. These structures regulate a wide variety of
aspects in the sperm including fertility, cytoskeletal shape and stability, motility and the ability to penetrate the egg and fertilize it.

1.3. Somatic cell DNA organization

In somatic cells the negatively charged DNA is packaged with positively charged proteins called histones. In mammalian cells, the histone octamer is composed of two copies, each, of H2A, H2B, H3 and H4 histones wrapped by DNA (Hengartner 2001; Wu et al., 2002). This histone octamer is called a nucleosome. The basic unit of DNA bound to one octamer core of histone proteins is roughly 200 bp-long, as revealed when chromatin is treated with a weak nuclease (Usachenko and Bradbury, 1999). The H1 histone is often referred to as a linker histone because it remains outside of the nucleosome core and just one molecule of H1 binds to the linker DNA at its entry or exit site at the nucleosomal core.

Modification of the histone proteins by phosphorylation, acetylation or ubiquitination can often alter the histones role in the functions of transcription, DNA replication and DNA repair (Usachenko and Bradbury, 1999; Wu et al., 2001). For example, acetylation of lysines in histones destabilize the DNA's negative phosphate charge with the lysines and allows for chromatin
decondensation and open configuration needed in active chromatin sites (Bradbury, 1992; Usachenko and Bradbury, 1999).

Luger (1997) has isolated a crystal structure of the nucleosome core particle which shows alpha helical domains in histone-histone and histone-DNA interactions composed of 146 bp of DNA wrapped 1.8 turns around the histone octamer (figure 1.2). Several octamers of DNA-histones or nucleosomes can be tightly wound and coiled to form a higher order structure called a solenoid (Finch and Klug, 1976). The nucleosomes are arranged in a regular helix with 6 nucleosomes per turn and the linker DNA is in between the nucleosomes in a straight path within the solenoid model. This highly compacted structure forms a 30 nm diameter chromatin fiber (van Holde, 1989; Wedemann and Langowski, 2002). An alternative model was proposed by Woodcock and colleagues (1993) for the 30 nm solenoid called the zig-zag model. The nucleosomes in the zig-zag model are randomly arranged in a zig-zag pattern. In the zig-zag model, the entry and exit points of the linker DNA as well as the tilt angle between two interconnected nucleosomes should be at an angle and random rather than static.

The 30 nm supercoiled solenoid filaments or chromatin is attached at specific sites or regions to the nuclear matrix or scaffold to form independent
Figure 1.2. Approximately equivalent levels of DNA packaging in somatic cells (left) and sperm cells (right).

In somatic cells, DNA is tightly packaged with histone proteins into nucleosomes and then the nucleosomes are further compacted into a solenoid. Active genes tend to be associated with the nuclear matrix at the base and inactive genes are on the outer loop. In the sperm nucleus, protamines bind to the DNA and coil the complex into tightly packaged circles. These circles form a donut-shaped loop structure. Each donut represents one DNA loop attached to the nuclear matrix (From Ward, 1993).
loops (Ward and Coffey, 1991). The nuclear matrix was a residual nuclear protein fraction which has been used to study nuclear proteins as well as the structure of the nucleus (Pederson, 2000). The loop domain attachment sites are estimated to attach to the nuclear matrix at the bases of the loops (Pienta and Coffey, 1984; Cook et al., 1976). The chromatin loop is the both the basic physical and functional unit of chromatin that is involved in DNA replication and gene expressions in DNA since active genes (replication origins) are associated with the base of the nuclear matrix and inactive genes are associated with the unbound part of the loop that faces away from the nuclear matrix and forms what is known as a halo (Pardoll et al., 1980; Vogelstein et al., 1980). DNA loop domains have been shown to be present in somatic interphase nuclei (Cook et al., 1976) as well as in mitotic chromosomes (Earnshaw and Laemmli, 1983). The current size estimated for a loop is in a range of 5 kb to 200 kb (Razin et al., 1995) with an average of 50-60 kbp (Pardoll et al., 1980; Ward 1993) or 60 to 100 kbp (Balhorn, 1982) in length for loop domains. DNA loop domains are loops of DNA attached at their base to the nuclear matrix and first discovered in somatic cells (Berezney and Coffey, 1974; Vogelstein et al., 1980). In somatic cells, the sites of DNA attachment to nuclear matrix regions is sequence specific (Cockerill and Garrard, 1986). The somatic cell matrix
attachment regions at the base of the loops are responsible for replication and transcription (Carri et al., 1986; Nelson et al., 1986; Robinson et al., 1982).

Jackson (1986) proposed that the DNA loop domain attachment sites that are related to gene transcription do exist but are transient and at least one report discusses the possible presence of RNA in the nuclear matrix (Fey et al., 1986). The transcription of RNA by loop domains, unlike DNA replication, has not been well accepted in the scientific field. Iarovaia and colleagues (2004) have shown that a single transcriptional unit may be organized into several loops when actively transcribing a specific gene. Razin and colleagues (2004) have never been able to clearly prove the presence of RNA in the nuclear specific genes like globin on the nuclear matrix, which leaves the question of the source of the RNA still open for experimentation.

At least one type of DNA topoisomerase has been found to be on the nuclear matrix. DNA topoisomerases are enzymes that passing one strand of the DNA through a break (type I) or two strands through a gap in the DNA (type II) in the double helix (Champoux, 2001). DNA topoisomerases are involved in various aspects of DNA replication (initiation of the replication bubble, segregation of newly replicated chromosomes and elongation) as well as transcription of double-helix DNA (Champoux, 2001). Topoisomerase II is known to be localized at the bases of the DNA loop domain (Gromova et al.,
1995). When topoisomerase II is activated in somatic cells during apoptosis, it specifically cleaves DNA attached at the MARs (Li et al., 1999; Gromova et al., 1995).

Razin and colleagues (1991) investigated the sites of DNA that remain bound to the nuclear matrix called MARs or SARs (matrix- or scaffold-attachment regions). Razin found that when DNA containing MARs bound to the nuclear matrix in vitro, it was cleaved after making contact with topoisomerase II. DNA fragments resulted from cleavage at sites of topoisomerase II integration into DNA in vivo were exposed to VM-26 which trapped the fragments in their cleaved state and these fragments were visualized by electrophoresis. The length was 20-300 kb with a peak at 150-200 kb. The lengths of DNA fragments studied were similar to DNA domain or loop sized fragments of less than or equal to 100 kb. When restriction end-labeling with alpha-globin probes were used, a precise localized cluster of three genes, which were not normally expressed in these cells were found within one of the DNA fragments released by cleavage at a topoisomerase II MARs. This experiment suggested that DNA fragments are formed because topoisomerase II specifically cleave at matrix attachment regions and that these fragments rare DNA loops or domains because they correspond to loop-sized fragments of less than 100 kb.
Razin and colleagues (1996) mapped the DNA loops in Drosophila X chromosome by cleaving with high-salt-insoluble topoisomerase II at the nuclear MARs. The loop anchorage sites within a 500 kb region of the chromosome yielded a total of eleven DNA fragments. From these eleven sites, about ten DNA loop-ranged fragments that varied in size from 20 to 90 kb. These DNA fragments were separated by using pulse field gel electrophoresis, which will be discussed in detail in chapter 2. Ten out of a total of eleven anchorage sites colocalized with previously mapped SARs (Surdej et al., 1990). The importance of this experiment was that topoisomerase II cleavage sites at MARs or SARs yields DNA loop-sized domain fragments at specific attachment or anchorage sites. Attachment by loop domains to the MARs or SARs is specific and not random and yields a range of DNA loop sized fragments between 20 to 90 kb in the example of the Drosophila X chromosome.

Pienta and colleagues (1991) suggested that the nuclear matrix condenses, coiling the associated DNA loop domains into a very compact form. Then the loop domains are coiled into 240 nm fibers and further coiled into clusters onto the nuclear matrix. This is the formation of the mitotic chromosome by organized condensation of the DNA loop domains into higher order chromosome bands by stacking 18 loops per radial turn with each loop
containing 60,000 base pairs (Pienta and Coffey, 1984; Nelson et al., 1986). Several researchers (Berezney and Buchholtz, 1981; Pardoll et al., 1980; Ma et al., 1999) have proposed that replicon clusters where DNA replication can occur are approximately 50 kb DNA loop domains attached to the nuclear matrix and can be visualized in living cells. Another hypothesis suggests that DNA loop domains attached to nuclear matrix have both active and inactive genes in their periphery (Cremer and Cremer, 2001). Depending on the location of the genes, there can be three types of chromatin compartments. There can be an open one with loop domains acting as sites of active genes, a closed one with inactive genes and an interchromatin MARs domain where DNA repair, splicing and DNA replication machineries can be found. Gary Stein and colleagues (2000) have also proposed based on their studies with transcription factors that the nuclear matrix may impose regulatory information such as normal or abnormal gene expression based on the discrete sites where the transcription factors are spatially distributed. Also modification of intranuclear targeting signals which direct proteins to specific regions could alter their arrangement with the nuclear matrix and how the proteins are normally regulated to produce a disease state such as cancer in a normal person.
In summary, in somatic cells the DNA is wound around histone octomers to form nucleosomes. Several nucleosomes coil into solenoids. The 30 nm solenoid filament forms the loop domains that are attached to the nuclear matrix at their bases at around 50 kb intervals. Active genes are more closely associated with the nuclear matrix base region and the inactive genes are more closely associated with the site on the loop unbound to the nuclear matrix and far away from the base. The DNA loop domain can coil extensively by forming stacks of radial loops to produce minibands in a chromatid until the desired chromosome length is reached. In somatic cells, DNA replication occurs at the bases of the loop domains on the SARs or MARs, however, it is less clear whether or not transcription of RNA occurs in specific MARs sequences and if these sites are transient. The discovery of topoisomerase II as being associated with the nuclear matrix may offer an explanation of how the DNA helix can unwind, break and rejoin its strands during replication and transcription. Certain studies (Stein 2000) suggest that the specificity of proteins via a nuclear signal onto discrete regions in the nuclear matrix and maintenance of specific DNA loop domain structure is intimately related to proper gene regulation.
1.4. Chromatin structural changes during spermiogenesis

During spermiogenesis protamines bind to DNA lengthwise along the major groove of DNA (Hud et al., 1994), therefore, unlike histone-bound somatic DNA, there is very little supercoiling. The protamines have high amounts of arginine-rich and other basic amino acid residues and are very cysteine-rich (Balhorn, 1982). These cysteine residues form disulfide crosslinks in mature sperm which give sperm chromatin its compact and stable structure (Bedford and Calvin, 1974). Although the majority of sperm is composed of protamines, some studies show that some histones remain (Gatewood et al., 1987). In the case of human sperm which contains 10-15% histones and 85%-90% protamines, the histone to protamine replacement is not complete (Tanphaichitr et al., 1978; Bench et al., 2000). Protamine-bound sperm DNA is resistant to enzymatic digestion, transcriptionally inactive, and binds in a sequence-specific manner to nucleoprotamine components (Gatewood, 1987).

Protamines have been found to coil DNA into donut-shaped toroidal structures containing up to 50-60 kb of DNA (Hud, et al., 1993; Allen et al., 1997; Balhorn et al., 1999; Brewer, et al., 1999; Corzett et al., 2001). An estimated 50,000 toroids can be packaged inside of the nucleus of a typical sperm cell (Brewer et al., 1999). In nature, the organization of DNA into these toroidal structures composed of protamine-complexed DNA, have been
observed only in sperm and bacteriophages (Allen et al., 1997). Although in vivo and in vitro studies have been done to determine how DNA is condensed by protamines and in vitro use of polycations and protamines have produced toroidal subunits, no research knows how the donuts are packaged together (Hud et al., 1995; Allen et al., 1997; Brewer et al., 2002). This dissertation is the first to propose a model of one donut equaling one loop domain which will be discussed further in section 1.10 called the Donut-Loop model. Prior to the Donut-Loop model, no research attempted to integrate the loop domains and the toroidal donut organization of DNA in sperm into a succinct model.

During spermiogenesis, the histone removal and protamine deposition, involves at least 85% basic proteins called transition nuclear proteins (TP or TNP for short) to aid in this process of remodeling of the chromatin (Meistrich et al., 2002). Although some species directly replace histones with protamines, in mammals histones are first replaced by the transition nuclear proteins TP1 and TP2 (Wouters-Tyrou et al., 1998; Zhao et al., 2004). Subsequently, TP1 and TP2 are replaced by protamine 1 and protamine 2 precursor (Chauviere et al., 1992; Zhao et al., 2004). TP1 is very basic due to the fact that 20% of its amino acid residues are arginine and lysine residues (Kleene et al., 1988) but TP2 is slightly less basic with only 10% arginine and lysine residues (Kleene and Flynn, 1987). TP1 and TP2 are localized to the nuclei of elongating and
condensing spermatids (Meistrich, 1989) and can reach levels of up to 90% of the basic chromatin proteins found in condensing spermatids (Yu et al., 2000).

The functions of TP1 and TP2 have various roles during chromatin remodeling during the process of spermiogenesis. TP1 can destabilize nucleosomes and prevent DNA bending in order to help displace histones more easily (Levesque et al., 1998). TP1 is also involved in the repair of DNA stranded breaks (Boissonneault, 2002). TP2 is able to bind to CpG sites and possibly repress RNA synthesis (Kundu and Rao, 1996). Both TP1 and TP2 are capable of condensing DNA (Brewer et al., 2002; Levesque et al., 1998).

A study by Yu and colleagues (2000) showed that targeted disruption of TP1 gene and the production of TP1 null mice in embryonic stem cells does affect spermatogenesis and reduce fertility. The testis weights and sperm production in the TP1 null mice were normal, however the sharp point of the sperm nucleus (apex) was bent more often than among normal mice morphology. Chromatin abnormalities were detected with electron microscopy by the appearance of rod-shaped chromatin condensation units in the nuclei of condensing spermatids of TP1 null mice instead of the normal chromatin fibrils. TP1 null mice also had increased levels of TP2 and protamine 2 precursor. There was a high level of protamine 2 precursor which
remained incompletely processed in the epididymus. There was a severe reduction in sperm motility in TP1 null mice and at least 60% were infertile.

A study by Zhao and colleagues (2001) showed that targeted disruption of TP2 gene affects sperm chromatin structure and reduces fertility in mice. These TP2 null mice were almost normal with no changes in testis weight and epididymal sperm counts as well as normal sperm head morphology, however, TP2 null mice showed increased abnormal sperm tails. The mice were fertile, however, they produced much smaller litters than normal. TP2 mutation also resulted in a deficiency in Protamine 2 precursor processing into the mature form of Protamine 2. Cho and colleagues (2003) have found that protamine 2 deficiency leads to increased sperm DNA damage and embryo deaths in mice.

The results of these studies have shown that either TP1 or TP2 are not essential for production of fertile sperm as long as one is not mutated, since they have redundant functions, although a loss of either one does affect fertility and sperm chromatin structure. When a double mutant TP1 and TP2 mice were produced, the result was complete sterility after the complete absence of thickened chromatin fibers and a loss of uniform condensation of chromatin, therefore at least one of the TPs were required for the production of fertile sperm (Meistrich et al., 2003; Zhao et al., 2004). In the case of the double mutants, over 80% were dead and most live ones were immotile. Also most of
the sperm had abnormal head morphology and the tails were missing mitochondria and had abnormal clumps of cytoplasmic structures. As mentioned before, the few mice that survived (approximately 5 mice) lacking TPs were all sterile (Zhao et al., 2004). A few more recent studies on various TP1 and TP2 null genotype mice have revealed (Shirley et al., 2004; Zhao et al., 2004) a decrease in normal sperm morphology, motility, chromatin condensation, DNA integrity and protamine 2 precursor processing with decreasing levels of transition proteins. Heterozygotes (single knockouts) had fewer abnormalities than homozygotes (double knockouts) for a particular TP null mutation; however, redundancy of TP function was possible among the single knockouts. In the case of TP 1 and TP2 knockout mice, they are required for normal chromatin condensation and functional sperm development. TP1 and TP2 single knockouts tended to partially complement each other (redundancy). In double TP knockouts, however, abnormal DNA condensation, increased DNA breaks and protamine 2 precursor posttranslational processing problems occurred. This resulted in many mature sperms being retained in the testis, abnormal sperm in the epididymus, and severe sterility in these mice even if ICSI (intracytoplasmic sperm injection) was used as a fertilization technique (Zhao et al., 2004).
1.5. Protamines

Protamines are small, basic, arginine-rich nuclear proteins that replace transitional proteins, which themselves temporarily replaced histones, during the maturation of sperm during spermiogenesis. They condense the sperm DNA into the most tightly packaged chromatin known.

Protamines have been found in invertebrates such as mollusks (Subirana et al., 1973), algae (Reynolds and Wolfe, 1984) and some insects (Kasinsky, 1989). Vertebrate protamines were extensively studied in fish such as salmon and were found to contain the shortest protamine molecules but contain no cysteine residues (Lewis, 2003; Retief et al., 1995). The protamines of marsupials such as the marsupial mole and monotremes such as the platypus resemble fish and birds in lacking any cysteine residues (Retief et al., 1993a; Retief et al., 1995). Placental mammalian protamine P1, unlike fish and birds, contain at least six to nine cysteine residues which form stable disulfide bridges in the mature sperm nucleus (Retief et al., 1993b; Retief et al., 1995).

There are two types of protamine molecules in most vertebrates called protamine 1 and protamine 2 which have different amino acid sequences in most mammals (Bench et al., 1996; Vilfan et al., 2004). The protamine 2 sequence is usually larger than that of protamine 1 and has more histidine residues (Bench et al., 1996). Mouse, hamster, stallion, human and other
primates all contain protamine 1 and protamine 2 (Bench et al., 1996). However, other mammalian species such as bull, boar and rat, only have protamine 1 (de Yebra et al., 1993; Maier et al., 1990). Also, the ratio of protamine 1 to protamine 2 was found to be essential in maintaining fertility in humans (Balhorn et al., 1988). In table 1 different mammalian species have various ratios of protamine 1 to protamine 2. For example, hamsters have roughly twice the number of protamine 1 to protamine 2 and mice have that same ratio but in reverse order. Also in table 1 it was listed that the normal ratio of human sperm protamine 1 to protamine 2 is nearly 1:1, however, when abnormal sperm was studied for its protamine content, the ratio was closer to 1.6:1 of protamine 1 to protamine 2 (Balhorn et al., 1988). The delicate balance between the protamine ratios vary among species and have an impact on fertility in humans.

In table 2 the mole % of basic amino acids and cysteines which form the disulfide bridges are compared in salmon, boar and bull. These can be compared to histone proteins in Table 3. The total of basic amino acids as well as cysteines is much higher among the mammalian protamines than the histones. This combination of very basic amino acids and cysteines found in disulfide bonds explains why protamines form stabilizing and strong covalent cross linking. The stable sperm nuclei can only be decondensed and their
Table 1. The percentage of protamine 1 and protamine 2 in various mammalian species.

<table>
<thead>
<tr>
<th>Mammalian Source</th>
<th>Protamine 1 Percentage</th>
<th>Protamine 2 Percentage</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull</td>
<td>100</td>
<td>0</td>
<td>Ref 1</td>
</tr>
<tr>
<td>Boar</td>
<td>100</td>
<td>0</td>
<td>Ref 2</td>
</tr>
<tr>
<td>Rat</td>
<td>100</td>
<td>0</td>
<td>Ref 2</td>
</tr>
<tr>
<td>Mouse</td>
<td>33</td>
<td>67</td>
<td>Ref 3</td>
</tr>
<tr>
<td>Hamster</td>
<td>66</td>
<td>34</td>
<td>Ref 3</td>
</tr>
<tr>
<td>Human (normal)</td>
<td>50</td>
<td>50</td>
<td>Ref 4</td>
</tr>
<tr>
<td>Human (abnormal)</td>
<td>61</td>
<td>39</td>
<td>Ref 4</td>
</tr>
</tbody>
</table>

References for Table 1:


Table 2. The mole percentage of basic amino acids and cysteins in protamines of various species.

<table>
<thead>
<tr>
<th>Protamines</th>
<th>Mole % Basic Amino Acids</th>
<th>Mole % Cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MW(kd)</td>
<td>Arginine (#aa/ Total aa)</td>
</tr>
<tr>
<td>Salmon¹ (P2B)</td>
<td>4.9</td>
<td>62.5 [=20/32]</td>
</tr>
<tr>
<td>Boar² (P1 only)</td>
<td>7.0</td>
<td>50.0 [=25/50]</td>
</tr>
<tr>
<td>Bull³ (P1 only)</td>
<td>7.5</td>
<td>50.9 [=26/51]</td>
</tr>
<tr>
<td>Mouse⁴ (P1)</td>
<td>6.8</td>
<td>54 [=27/50]</td>
</tr>
<tr>
<td>Hamster⁵ (P2)</td>
<td>13.6</td>
<td>55.3</td>
</tr>
<tr>
<td>Human⁶ (P1)</td>
<td>6.7</td>
<td>48 [=24/50]</td>
</tr>
</tbody>
</table>
Table 2 (Continued) References:


Table 3. Mole percentage of basic amino acids and cysteines in histones

<table>
<thead>
<tr>
<th>Human source</th>
<th>Mole% Basic Amino Acids</th>
<th>Mole % Cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arginine</td>
<td>Lysine</td>
</tr>
<tr>
<td>H2A</td>
<td>15.0</td>
<td>9.2</td>
</tr>
<tr>
<td>H2B</td>
<td>13.8</td>
<td>6.3</td>
</tr>
<tr>
<td>H3</td>
<td>15.3</td>
<td>13.2</td>
</tr>
<tr>
<td>H4</td>
<td>11.0</td>
<td>13.6</td>
</tr>
</tbody>
</table>

References for Table 3:

cysteine-containing protamines extracted after treatment with denaturation agents such as 6 M guanidinium chloride and thiols which cleave the disulfide bridges (Oliva and Dixon, 1991). Sperm that lack cysteines, such as salmon sperm in Table 2, are easily decondensed by various treatments such as exposure to mild detergents or metal ions, which are treatments that would be too mild to work on cysteine containing sperm nuclei (Cummins, 1980).

During the process of spermiogenesis, the replacement of somatic histones by protamines occurs in round spermatids (Oliva and Dixon, 1991). In the case of fish and bird species the replacement of histones to protamines is direct with no intermediary stage (Oliva and Dixon, 1991; Zhao et al., 2001). In the case of mammals and some marine vertebrates the replacement involves an intermediary step (Wouters-Tyrou et al., 1998). In these species, first the replacement of somatic histones are done by transition nuclear proteins called TP1 and TP2 in round spermatids and then in the elongating spermatids, the TP1 and TP2 are replaced by protamine P1 and a precursor of protamine 2 which is processed further by proteolysis into P2 (Chauviere et al., 1992; Zhao, 2001).

In the prior section, the role of transition proteins during spermiogenesis was discussed by looking at TP1 and TP2 knockout mice. TP1 and TP2 knockout mice remain fertile, however mice lacking both TPs showed more
DNA breaks, abnormal spermatozoa and reduced numbers of spermatozoa as well as sterility (Zhao, 2001). Also, the process of phosphorylation of serine and threonine residues on the N-terminal of protamines plays an important role in the binding of protamines to the DNA (Hecht, 1989; Lewis et al., 2003). The resulting sperm chromatin which is tightly packaged with protamines is very condensed and believed to be transcriptionally silent (Aoki and Carrell, 2003).

1.6. Sperm DNA Loop Domains

As described above, the chromatin structure of sperm DNA bound to protamines is very different from that of somatic cells bound in nucleosomes to histones. However, there is one level of chromosomal organization that is shared between somatic cells and sperm, and that is the organization of DNA into loop domains.

Ward and colleagues (1989) demonstrated for the first time that DNA loop domains can also be found in sperm cells. Sperm halo structures were made by treating nuclei with high salt (2 M NaCl) and reducing agent, DTT, to remove the protamines. The nuclear matrix, the skeletal part of the sperm nucleus, retained the original shape of the nucleus and the DNA attached to the nuclear matrix at specific regions which formed loops of DNA. The DNA
surrounding the nuclear matrix created a halo which could be examined by staining with ethidium bromide and viewing under fluorescent microscopy is DNA loop was 60% smaller than in somatic cells and was 46 kb versus 76 kb for somatic cells. Sperm DNA was relaxed and not supercoiled as in somatic cells because sperm DNA binds tightly to protamines (Balhorn, 1982). Although the somatic cell DNA loop domains were supercoiled around histones, rather than protamines, and were larger in size than in sperm, both somatic and sperm cells have the same basic structural organization of DNA loop domains.

Ward and Coffey (1990) showed that there was a specific organization of genes in relation to the sperm nuclear matrix. The proximity of six hamster genes (desmin, vimentin, alpha-crystallin, 5S RNA, c-fos, and alpha-adrenergic receptor) to the sperm nuclear matrices were shown by restriction endonuclease treatment of hamster sperm nuclei and southern blot hybridization. Only two out of the six probes were recognized as being associated with the nuclear matrix. One was alpha A-crystallin gene and the other was the 5S RNA gene. The former was a 4 kb sized fragment and the latter was a 12 kb sized fragment. The results showed that DNA loop domains in the mammalian sperm nucleus are organized in a specific, sequence specific manner on the sperm nuclear matrix as in the case of the 5S RNA gene.
Krawetz and colleagues (1998) analyzed the region that defined the MARs for the human sperm-specific expressed protamine gene cluster P1, P2, gene 4 and TP2 domain on human chromosome 16. The sequence of the gene cluster was completed by using computational and biological analysis (screening a chromosome 16 specific cosmid library) of the gene cluster domain. The MARFinder analysis program discovered several sperm-specific MARs were present to mark each of these different protamine gene cluster domains.

This same group (Heng et al., 2004) then used FISH to visualize directly the localization of sperm specific MARs on the nuclear matrix or chromatin loop by examining a 300 kb genomic region from the human chromosome 8. Since on average, one MAR is present at every 60-90 kb of DNA, if the loops were static then loops from different nuclei should display the same configuration when the same sequence (BAC probe) on each loop was painted, however, if the loops were dynamic structures then their ability to use different attachment sites would be displayed as different color configurations for each nucleus. In approximately 20 and 27% of two configurations (red and green colored signals) analyzed by FISH, there were color configuration changes which suggest that endogenous chromatin loop domains are capable of moving with respect to the nuclear matrix attachment sites. Certain sequences such as sperm MAR-containing sequences are tightly anchored on their nuclear matrix
(Schmid et al., 2001) and do not move dynamically. This experiment demonstrated that sperm sequences tend to be fixed on specific nuclear matrix attachment sites, whereas some non germ cell sequences are dynamic.

Klaus et al. (2001), examined loop domain organization of the hamster repetitive gene, 5S rDNA gene cluster, during spermatogenesis and embryogenesis. The hamster 5S rDNA gene cluster was used because it is a single locus which is large enough by its size of 1 megabase to be organized into several loop domains but also small enough that individual loops could be resolved (Hart, 1982). Nuclei from hamsters at the stages of spermatogonia, pachytene spermatocytes, round spermatids and spermatozoa as well as ES cells, brain and liver cells were prepared. Nuclear halos were formed by using high salt solution on all cell types and then FISH, fluorescent in situ hybridization, was performed on the nuclear halos with a double-stranded, biotinylated probes for the 5S rDNA.

Klaus and colleagues (2001) used FISH to directly examine the loop domain organization of the 5S rDNA gene cluster. The results were that the 5S rDNA was organized into three loops or three groups of loops in the sperm nucleus, however, in the somatic liver cell the same gene was organized into a much larger, single loop. When the 5S rDNA gene cluster was examined by FISH during spermatogenesis and embryogenesis, the results were the
following: 1) DNA loop domain structure was cell specific and dynamic, 2) DNA loop domain structure was independent of histone association and changes in nuclear morphology and 3) DNA loop domain structure underwent changes during spermatogenesis and embryogenesis. Let us examine each result in more detail.

The huge difference in size and number of loop domains between large, single loops in somatic cells such as liver and brain versus three loops (or three groups of loops) in the sperm nucleus suggested that the 55 rDNA gene cluster was organized in a cell-specific manner. The DNA loop domains were dynamic because different loop domain numbers and sizes occurred during different developmental stages. In ES cells (2N), the 5S rDNA was organized into two small loop domains which were maintained in the spermatogonia (2N). The pachytene spermatocyte (4N) maintained a similar size to the ES and spermatogonia cells, however, there were 4 small loop domains. The round spermatid (1N) underwent reorganization into three loop domains. This three loop DNA structure was maintained in spermatozoa (1N) but after fertilization, was reorganized again into a two-loop structure like that found in the ES cells.

The DNA loop domain structure was found to be independent of histone association and changes in the nuclear morphology because round spermatids have histones but during spermiogenesis have them replaced by
protamines as is found in hamster sperm nuclei and yet both round spermatids and spermatozoa had the same number of loop domains, three. Therefore, reorganization of the chromatin structure in the sperm from histones to protamines produced no change in loop domain structure.

The DNA loop domain structure underwent changes during spermatogenesis and embryogenesis. There was a change from the pachytene spermatid (4N) with 4 loop domains to the round spermatid (1N) with three much smaller loop domains. Also after fertilization, the three small loops in the spermatozoa were reorganized into two larger loops in embryonic cells as in the example of ES cells. The final, important change occurred when cells such as those found in the inner cell mass of the blastula (future embryonic) cells such as ES cells with two large loops were altered into an even larger single loop in somatic cells. This alteration of a very large, single loop appeared to remain consistent in both differentiated brain and liver tissues studied.

The data presented in these experiments showed that smaller loop domain organization occurred in germline and early embryonic cells than in adult somatic cells. Larger, single loop domain seen only in somatic cells corresponded with the loss of pluripotency as the tissue became differentiated and its fate to become a specific kind of cell was determined. The dynamics of
the different stage-specific loop domains during spermatogenesis and embryogenesis might explain why experiments such as those done by Ward (1999) found that a unstable nuclear matrix which lacked organized loop domains due to the use of DTT and ATAB treated-sperm injected into oocytes, produced no viable embryos and abnormal development occurred. If these loop domains encounter any abnormality as they were being formed, then the loop domain will be disorganized and the embryo will not develop properly, however, if the nuclear matrix remained intact and the loop domains were organized, then normal development proceeded for at least 30% of the case.

1.7. Sperm DNA Loops may be important for Embryogenesis

In section 1.6 the experiments conducted by Klaus and colleagues (2001) demonstrated that changes in DNA loop domain structure occur during both spermatogenesis and embryogenesis in the hamster. These changes in the loop domain structure during embryogenesis may be due to the contribution of paternal DNA. The sperm nucleus may provide the embryo with both the basic DNA sequence and the heritable structural organization of DNA into specific DNA loop domains which govern different developmental stages. Thus, sperm DNA loop structural abnormalities, which disrupt the organization of the loop domain, will also disrupt normal embryogenesis.
In a paper by Sotolongo and Ward (2000), the combination of this heritable structural information in the sperm DNA loops and the DNA sequence were named the “genomic code”. The term “genomic code” implies that DNA sequence in the sperm cell alone does not control how the nuclear matrix functions in embryogenesis, however, the combination of the sperm DNA into loop domains by the nuclear matrix provides heritable information by marking areas along the DNA which attach these areas to the nuclear matrix. These special sites of DNA attachment to the nuclear matrix in the structural form of DNA loop domains could provide the developing embryos with important markers for the origins of DNA replication, mRNA transcriptional start sites, and other unknown functions.

Work by Ward et al. (1999), provided evidence that the structural organization of the sperm nuclear matrix is very necessary for embryogenesis (figure 1.3). The experiment extracted mouse nuclei with either fresh or week-old isotonic nuclear isolation medium (NIM) which was supplemented by ionic detergent, ATAB and either with or without DTT. These treated sperm nuclei were then injected into oocytes using intracytoplasmic sperm injection. Then the embryos were transferred to foster mothers and embryonic development was closely followed up to live births. The measure of the nuclear matrix stability was the ability of the sperm nuclei to form nuclear halos when treated
Figure 1.3. An intact sperm nucleus is necessary for embryogenesis.

This figure showed the results of using ICSI to study the role of sperm DNA organization in embryogenesis. Mouse sperm nuclei were treated first with ATAB and then NaCl and DTT. These nuclei were able to form nuclear halos in vitro and were also able to give viable offspring when injected into oocytes. However, sperm nuclei which were not able to form nuclear halos in vitro, after treatment with ATAB and DTT, then with NaCl and DTT had unstable nuclear matrices. These nuclei were not able to give viable offspring. In both types of sperm nuclei, the DNA is expected to be intact.
with 2M NaCl and DTT. Nuclear halos are extracted nuclei composed of DNA loop domains attached to the nuclear matrix and forming a halo of DNA around the matrix.

The results were that sperm treated with ATAB in the presence of DTT tended to decondense completely when they were extracted with 2M NaCl and 2 mM DTT. These results indicated that these decondensed nuclei had unstable nuclear matrices. Sperm treated with ATAB without DTT did form nuclear halos when extracted with 2M NaCl + 2 mM DTT, which indicated that these sperm had stable nuclear matrices. When the oocytes were injected with these sperm nuclei the results were quite amazing. In the case of the sperm nuclei, previously washed with ATAB and DTT, the sperm nuclei injected into oocytes produced no viable offsprings. In the case of the sperm nuclei, previously washed with ATAB without DTT, the sperm nuclei injected into oocytes produced 30% viable offspring. The DNA in both cases was highly condensed and unlikely to have become damaged, therefore, the only difference between the two nuclei that were injected into oocytes was the stability of the nuclear matrix. This experiment supports the importance of a stable nuclear matrix organization in the sperm nucleus in embryogenesis. The sperm DNA loop domain structure with a stable nuclear matrix (ATAB without DTT) must provide the embryo with crucial information necessary for
its normal development because whenever the nuclear matrix DNA loop
domain organization was disrupted (by adding DTT to the ATAB), there were
no viable offsprings.

The paternal genome requires an intact sperm nuclear matrix, capable of
forming a halo composed of DNA loop domains, in order for heritable
information to reach the embryo and direct normal embryogenesis (figure 1.4). If
the sperm nuclear matrix organization is disrupted as in the experiment with
DTT and ATAB, then viability of the embryo is interrupted and embryonic
development is compromised. The "genomic code" which unites the DNA
sequence and DNA loop domain organization into necessary information for
the embryo to utilize during normal development can only be passed on if the
sperm nuclear matrix is intact and the "genomic code" is preserved.

1.8. Role of Chromatin Structure in Apoptosis in Somatic Cells

Cell death can occur by two general mechanisms called necrosis and
apoptosis. Necrosis is the process by which physical insult such as disruption
of the membranes or chemical damages results in accidental cellular death
(Mastrangelo et al., 1998). Necrotic cells tend to swell and burst open, thus
releasing their lysosomal contents into the extracellular space and causing
inflammation (Wyllie et al., 1980; Bortul et al., 2001).
DNA has genetic information encoded in its sequence of base pairs. The DNA as a linear molecule becomes organized into loop domains by the nuclear matrix. When loop domains are formed, important heritable information is added by marking certain sites along the DNA by attaching these specific points to the nuclear matrix. The combination of the DNA sequence and this three-dimensional organization of DNA is called the "genomic code."

Figure 1.4. The three dimensional structure of DNA contains information.
Apoptosis is a programmed cell death involving a regulated process by which unwanted cells are eliminated during development or in response to various biological stimuli. Apoptosis is controlled by cellular genes, enzymes and signaling cascades (Mastrangelo et al., 1998). An apoptotic cell undergoes cell blebbing or bulging in of the cell membrane due to both condensation of the chromatin and cytoplasmic organelles (Mastrangelo et al., 1998). During apoptosis as the cell shrinks, the nucleus begins to undergo small apoptotic bodies that are phagocytosed in vivo without causing significant inflammation (Bortul et al., 2001; Mastrangelo et al., 1998).

Apoptosis is characterized by the degradation of DNA into specific patterns of high and low molecular weight fragments seen on agarose gels as a distribution of sizes initially between 50 kb and 300 kb, often referred to as “loop-sized fragments” because they are thought to arise from DNA loop domains being cleaved (Walker et al., 1997). Eventually the apoptotic internucleosomal ladder composed of DNA cleaved fragments has ordered multiple bands or ladders of 180-200 bp fragments on gel electrophoresis and this is the typical sized apoptotic somatic cell DNA fragmentation ladder (Gerschenson and Rotello, 1992; Shinoda et al., 1997). Apoptosis can be induced by chemical insults such as etoposides, signaling cascade participants such as cysteine proteases called caspases and biochemical pathways such as
reactive oxygen species and endonucleases such as DNase and topoisomerase II (Mastrangelo et al., 1998).

Razin and colleagues (1995) treated mouse and human cell lines with VM-26, a topoisomerase II poison, which caused topoisomerase II mediated cleavage at matrix attachment sites into chromosomal loop-sized fragments ranging from 50 kb to 600 kb (Gromova et al., 1995). The DNA fragments were visualized on pulse field gel electrophoresis agarose. Another experiment was conducted using tumor necrosis factor (TNF) and serum deprivation, which are two triggers of apoptosis. When TNF was used with either the human or mouse cell lines, excision of 50 kb to 600 kb loop-sized fragments occurred. When serum deprivation was used on human cell lines, excision of 50 kb to 300 kb loop-sized DNA fragments occurred. This study showed that TNF, serum deprivation and topoisomerase II mediated cleavage mechanisms can be involved in high molecular weight DNA fragmentation during apoptotic degradation to produce chromosomal DNA loop-sized fragments.

A study by Li and colleagues (1999) treated live somatic cells with hydrogen peroxide, a reactive oxygen species, and induced oxidative stress which has been known to lead to apoptosis in chromosomal DNA. Both hydrogen peroxide and VM-26 were found to quickly induce excision of loop-sized DNA fragments from chromosomal DNA. The DNA fragments ranged
from 50 kb to 100 kb high molecular weight sizes. The formation of these loop-sized fragments is reversible within 40 minutes to a little under 100 minutes. Hydrogen peroxide induces DNA fragmentation in the presence of DNA topoisomerase II. After this initial and reversible excision of loop-sized fragments, the formation of irreversible apoptotic chromosomal ladders occurs. Since topoisomerase II is located at the base of chromosomal loops (30-100 kb in size), the addition of hydrogen peroxide must covalently modify topoisomerase II specifically at the base of these loops where they form reversible topoisomerase II cleavable complexes.

Koide and colleagues (Yoshihara et al., 1997; Tanaka et al., 1984) had found that purified calcium and magnesium-dependent endonucleases (CAD) such as DNase were located in the nuclei of various somatic cells and bull seminal plasma. They suggested that one role of CAD could be chromatin degradation during apoptosis based on endonuclease activity levels in CAD induced samples being higher than in non-calcium and magnesium added samples (Yoshihara et al., 1997).

Yakovlev and colleagues (1999 and 2000) found that calcium and magnesium-dependent endonucleases (CAD) such as DNAS1L3 have a role in DNA fragmentation during apoptosis. In humans four members of the DNase I family and its homologs DNAS1L1, DNAS1L2 and DNAS1L3 have been
identified. All human enzymes are expressed in tissue-specific manner with DNAS1L3 being expressed in the liver, kidney and thymus (Yakovlev et al., 1999). DNAS1L3 is the only homolog to have a nuclear localization signat at its C-terminal and it was transfected into HeLa S3 cells, then analyzed by agarose gel electrophoresis. The results were that incubation with both calcium and magnesium produced 50 kb loop-sized fragments.

Boulares and colleagues (2001) found that DNA fragmentation factor (DFF), which is a CAD, has also been suggested to be involved in apoptosis along with DNAS1L3. DFF is composed of two subunits called DFF 40 and DFF45. The researchers used DFF 45-/- mouse fibroblast cells and tried to induce apoptosis with TNF but found these cell lines resistant to apoptosis, however DFF +/- fibroblast cells did undergo apoptosis and produced 50 kb DNA fragments.

Gromova and colleagues (1995a and 1995b) used various topoisomerase II poisons (which trap topoisomerase II mediated reactions into a cleavable complex) as well as CAD, calcium-activated DNase (endogenous nuclease), at an amplified c-MYC gene locus in living cells. Not I restriction endonuclease was added because it can only cleave at one site along the 800 kb repeated Myc gene sequence. Finally PFGE and southern blotting was done with a Myc probe. The result was that both CAD and topoisomerase II gave similar
molecular cleavage sites within the Myc Gene at 80 kb, 150 kb and 260 kb, which are loop-sized DNA fragments. Since topoisomerase II is known to be at the base of the loops at the matrix attachment sites, the authors conclude that endogenous nucleases which give similar cleavage fragment patterns must either be at or close to the matrix attachment sites also (Gromova et al., JBC 1995). Therefore, these studies show that both topoisomerase II and CAD endogenous nuclease preferentially cleave DNA at matrix attachment sites and produce DNA loop-sized fragments.

A study conducted by Solovyan and colleagues (2002) used neuroblastoma cells and induced apoptosis by serum deprivation or etoposide (topoisomerase II inhibitor). The results were that high molecular weight DNA cleavage and internucleosomal DNA cleavage may represent separate programs of apoptotic DNA degradation in neuroblastoma cells. The activation of topoisomerase mediated cleavage resulted in 50 kb-100kb DNA fragments suggesting excision of loop domains and was caspase-independent. The CAD pathway was found to be caspase-dependent and also caused apoptotic DNA fragmentation.

The role of CAD nucleases such as DNAS1L3 and DNase as well as topoisomerase II in apoptosis of somatic cells has been well established by these experiments. The location of Topoisomerase at the base of the loops on
the matrix attachment sites and CAD either near or at the matrix attachment sites has also been explored. In the majority of cases the analysis of DNA cleavage into high molecular weight fragments has yielded a range consistent with apoptotic DNA fragmentation and when viewed with gel electrophoresis the loop-sized fragments have an average smear of 50 kb-100 kb (Solovyan et al., 2002; Yakovlev et al., 1999; Li et al., 1999).

1.9. Endogenous nicks during spermiogenesis

There are several different types of endogenous DNA nicks or breaks in sperm which occur during the process of spermiogenesis. One type involves stage-specific endogenous breaks and DNA repair which are directed by DNA topoisomerase II (topo II). The second type involves endogenous nicks in mature sperm that are not healed and the source of this DNA breakage is unknown. The third type of DNA breakage involves an endogenous nuclease, which is present in sperm when it encounters an amount of exogenous DNA which is beyond a threshold level and the nuclease activates apoptotic-like destruction of the exogenous DNA and the sperm itself. All three of these are discussed in greater detail, below.

Recently, Ludovic Marcon and Guylain Boissonneault (2004) described transient DNA stranded breaks in mice and human spermatogenesis. They
used the TUNEL assay to characterize stage-dependent patterns of endogenous DNA stranded breaks that occur during normal spermatogenesis. In the human studies, an increase in DNA nicks occurred during chromatin remodeling and nuclear transition protein to protamine exchange in the spermatids. The role of topo II during chromatin remodeling during spermatogenesis by causing and repairing double-stranded breaks in helping to replace histones with sperm-specific nuclear proteins has been established (Chen and Longo, 1996). The transient DNA breaks are made during the round spermatid stage and appear to be repaired by the time the spermatozoa has become a mature sperm.

In many ICSI procedures, the use of ROSI or round spermatid injections which probably have many transient DNA breaks has been found to be less efficient than using elongated spermatids or mature sperm with little or no transient DNA breaks (Khalili et al., 2002). Therefore, the existence of these transient breaks present in early spermatids may have a role in embryonic developmental failures. In the mouse studies, transient breaks also occurred but in the elongating spermatids and ICSI has been done successfully using round spermatids (Marh et al., 2003; Hayashi et al., 2003). It appears that in mammals, the chromatin remodeling requires transient DNA stranded breaks or nicks and depending on the species, the stages of these nicks will differ,
therefore knowing when the nicks might be at their lowest number or when they might be repaired could increase the use of the best stage of sperm for in vitro procedures and successful births.

Sakkas and colleagues conducted experiments in mice and ejaculated human sperm by using chromomycin A3, a DNA fluorochrome tool for analysis of protamine-deficient, nicked and partially denatured sperm DNA (Bianchi et al., 1993). They found that mature mouse spermatozoa from the cauda epididymis and vas deferens were resistant to in situ nick translation and were chromomycin A3 negative (Bianchi et al., 1993). His results on ejaculated human spermatozoa showed a wide range of results (13%-75%) with regard to positive chromomycin A3 staining (Manicardi et al., 1995). When in-situ nick translation studies were done in correlation with chromomycin A3 staining on ejaculated human spermatozoa, the results were the variable (1% to 38%) with regard to endogenous nick translation but there was a positive correlation between sperm that had stained positive with chromomycin A3 and the percentage of endogenous nick translation (Sakkas et al., 1995). Further studies conducted on maturing (testicular sperm of stages 1-7 and spermatids in stage 15-16) and fertilizing (mature spermatozoa) in epididymidis resulted in testicular spermatids showing a high degree of chromomycin A3 positivity and presence of endogenous nicks, however, during fertilization, endogenous nicks
were not observed in decondensing mature sperm (Sakkas et al., 1995). In summary, the presence of endogenous nicks in sperm as a result of DNA cutting and ligation occurs in sperm. The absence of endogenous nicks during fertilization indicates that decondensing mature sperm lack an endogenous nicking process in the unpackaging of the male chromatin (Sakkas et al., 1995).

Spadafora and his colleagues suggested that spermatozoa from various species have the ability to take up foreign DNA and to internalize them inside of their nucleus where the exogenous DNA is integrated into the sperm genome (Magnano et al., 1998; Spadafora, 1998; Pittoggi et al., 2000). The function of this sperm mediated transfer mechanism is to transfer genetic information into oocytes during the process of fertilization. The invasion of foreign DNA when present above a certain threshold level in sperm cells can activate silent endogenous nucleases in a DNA dose-dependent manner and sequence dependent manner which leads to cell death resembling apoptosis (Maione et al., 1997; Sciamanna et al., 2000). These endogenous sperm nucleases degrade the foreign DNA as well as cause chromosomal DNA damage to the sperm itself.

The three types of endogenous nicks that occur during spermiogenesis appear to work in different manners. One type involves stage-specific nicks that are repaired and are thought to be caused by topo II. The second type
involves DNA breaks in mature sperm that are not healed and the origin is unknown. Finally, the third type involves sperm mediated transfer of exogenous DNA, which under specific dose-dependent and sequence-dependent circumstances, which can release an endogenous nuclease from the sperm to destroy the exogenous DNA as well as the sperm itself. These three mechanisms are examples of how sperm either contain or undergo during spermiogenesis a way to damage DNA.

1.10. Donut-Loop Model for Sperm Chromatin Structure

In this dissertation, we propose a new model that we are calling the Donut-Loop model for sperm chromatin structure that has evolved from the work of various laboratories, and attempts to unite several key pieces of information into an organized manner (figure 1.5). The Donut-Loop model is unique because it unites the concept of toroid structures with DNA loop domain in sperm chromatin. Little is known about how the sperm chromatin is arranged, with the exception of the detailed knowledge of protamine DNA binding provided by the Balhorn laboratory (reviewed in section 1.4).
Figure 1.5. Donut-Loop Model for Sperm Chromatin Structure.

Sperm DNA is formed by protamines into donut-shaped toroids of about 50 kb. We have suggested that each protamine-DNA toroid is one DNA loop domain. This model predicts that linking each toroid is a DNase-sensitive site of sperm chromatin that is attached to the sperm nuclear matrix.
Hud and colleagues have demonstrated that the basic structure of some bacteriophage (lambda) and protamine proteins binding to DNA is the formation of a donut-shaped toroid that has about 50 kb of DNA within a range between 20 kb and 100 kb (Hud et al., 1993). The Ward lab and others have shown that sperm DNA is organized into loop domains that are attached at their bases to the nuclear- (MARs) or scaffold-matrix attachment regions (SARs) and they are attached to MARs or SARs at specific sequences and are about 50 kb in size (Ward and Coffey, 1989; Ward and Coffey, 1990; Choudhary et al., 1995; Kramer et al., 1998). In somatic cells the size of loop domains ranges from 20 to 90 kb and is a topoisomerase II-mediated DNA loop excision cleavage (Iarovaia et al., 1996). According to the research conducted by Schmid et al. (2001), the sperm MAR-containing sequences are tightly attached to the nuclear matrix, whereas in the example of some non-germ-cell DNA sequences this attachment is flexible and dynamic.

Based on the similarity in size of DNA loop domains and toroidal donut-shaped condensates, we proposed a model in which each DNA loop domain in the sperm was a single protamine-bound DNA toroid with an average size of 50 kb although the range could be similar to somatic cells and range between 20 kb and 100 kb (see figure 1.2). This model was named the Donut-Loop model. Our model proposes that between two consecutive
protamine-bound DNA toroids is a MAR linker region which is more sensitive to cleavage by DNase than protamine-bound DNA. Our model predicts that when sperm chromatin is treated with exogenous DNase, DNA loop domains with an average size of 50 kb will be excised. These fragments should be visible by pulse field gel electrophoresis after running and staining with ethidium bromide.

One possible confounding aspect to this prediction may result from the small amount of histones that sperm nuclei contain, as previously described. No one knows to what parts of the sperm chromatin these histones are bound in relation to protamines. Regardless of the arrangement of small percentages of histones with sperm DNA, all sperm DNA is organized into loop domains which are bound to the nuclear matrix. This is the same DNA loop domain structure that is found in somatic cells, discussed in sections 1.5 and 1.6, above. It is possible that the few histones that remain in the sperm nucleus are located at what we predict are the DNase I sensitive regions, the MARs, in our Donut-Loop model, but the placement of the histones will not be tested directly, in this dissertation.

One other aspect of the Donut-Loop model relates to possible function of sperm chromatin. As described above, somatic cells contain both nucleases and topoisomerase II that cleaves the DNA into loop-sized fragments during
apoptosis. Several labs have suggested that apoptosis can occur in mature sperm (Russell et al., 2001; Blanco-Rodriguez, 1999; Hikim et al., 1998). The Donut-Loop model provides a possible mechanism for cleaving sperm DNA into loop-sized fragments. I will provide evidence that the Donut-Loop model is correct, in chapter 3, as well as evidence that such an endogenous nuclease exists, in chapter 4.

1.11. Conclusion

This dissertation explores the structure of sperm chromatin and its unique relationship to function. The main purpose of the work in this dissertation is to test the hypothesis that the organization of sperm DNA into individual loop domains and one loop domain is folded into one protamine toroid. There are toroid linker regions between these toroids that attach the DNA to the nuclear matrix attachment regions. The model predicts that these toroid linker regions are DNase sensitive and when sperm chromatin is treated with DNase, loop-sized fragments of about 50 kb result. Since the 50 kb loop-sized fragments are about the same size as the protamine bound toroids or donuts (Hud et al., 1993; Hud et al., 1995), the cleavage of the toroids must occur at the DNase sensitive sites of the toroid linker regions.
In support of this principal hypothesis, the following specific aims will be examined: 1) I will test whether the Donut-Loop model is correct in mature hamster sperm. 2) I will test the function of the Donut-Loop model by testing for a nuclease that digests the DNA at the sites of attachment to the nuclease. This will be accomplished by using DNase I to verify that an exogenous source of nuclease does cut sperm chromatin into 50 kb loop-sized fragments. Then endogenous mechanisms will be investigated. 3) Finally, both the Donut-Loop model evidence and the testing for a nuclease will be examined in human sperm to see how it correlates to the mammalian hamster system.
Chapter 2.

Materials and Methods

A. Cell Isolation

2.1. Hamster Spermatozoa Isolation

In a typical experiment, three retired breeder male Syrian golden hamsters (Charles River Laboratories, Wilmington, MA) were sacrificed by asphyxiation with 80-100% concentration of carbon dioxide gas in 10-15 minutes and the six caudae epididymis were dissected. Spermatozoa were obtained from the isolated caudae epididymis by making two vertical slits with scissors in each epididymis while each cauda was held in place at one end with a sharp-edged forcep. One forcep held the cauda in place as motile sperm were teased out with a second pair of fine forceps from the distal (larger) part of each epididymis into 5 mls of ice-cold 1X Phosphate buffered saline [1X PBS has 1.7 mM KH₂PO₄, 5.2 mM Na₂HPO₄, and 150 mM NaCl at pH 7.4] (Biowhittaker, Inc., Walkersville, MD, or Cambrex BioScience, Baltimore, MD, #51225).
2.2. Hamster Sperm Nuclei Isolation

Five hamsters were sacrificed and their cauda epididymides were dissected, incised and spermatozoa were extruded with fine forceps as previously described under hamster sperm isolation methods. The spermatozoa were put into 40 mls of ice cold 50 mM Tris-HCl, pH 7.4. The sperm were briefly sonicated with the mini-probe sonicator (Branson Sonifier cell disruptor and converter, Danbury CT) at a setting of microtip output control of 7, timer for 4 minutes, continuous pulsing and 50% duty cycler setting in an autoclaved glass beaker. The suspension was diluted with an additional 45 mls of ice cold 50 mM Tris, pH 7.4 and 45 mls of cold 2 M sucrose, 50 mM Tris, pH 7.4 and 5 mM MgCl₂. A triple step centrifugation gradient was constructed as follows: 10 mls of ice cold 2M sucrose, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, was placed into each of six Sorvall ultracentrifuge tubes (Sorvall Surespin 630 rotor, Kendro Lab Products, Philadelphia, PA). Twenty five mls of the sonicated sperm suspension was slowly and carefully layered onto each of the 10 ml sucrose cushions with a 25 ml pipette held tightly against the side of the tube. Finally 5 mls shaken three times of 0.45 g/ml CsCl, 25 mM Tris, pH 7.4 , 0.25% NP-40 (v/v) (bottom-most layer) was underlaid with a syringe attached to a small piece of tubing (approximately 10 cm long). The triple step centrifugation gradient was centrifuged at 20,500 rpm in a Sorvall
ultracentrifuge using a Surespin 630 rotor for 45 minutes at 4°C. After centrifugation, the various gradients were carefully aspirated with a pipette, leaving the intact pelleted nuclei in the shape of a white ring at the bottom of the tube. The pelleted nuclei were resuspended in 1X PBS. The reference for this triple step centrifugation gradient was developed in Dr. Ward’s lab.

2.3. Preparation of Spleen Nuclei

Hamster spleens were dissected out and cut into sections of approximately 1 mm. Then the spleens were ground up in a 90 mm ceramic mortar and pestle in the presence of liquid nitrogen and then mixed with 1X PBS. The spleen cells in 1X PBS were then plugged with equivalent amounts of pretempered (53°C) 1% pulse field gel electrophoresis agarose. This method was used for experiments conducted in chapters 3-5.

If there was no liquid nitrogen available, then the spleen cells were homogenized on ice in 1X PBS with a 15 ml Dounce tissue homogenizer tube with pestle (Bellco Glass Co., Inc, Vineland, NJ, #198410015). The homogenized spleen cells were plugged with equivalent amounts of pretempered (53°C) 1% pulse field gel electrophoresis agarose.
2.4. Incubation of Live Hamster Spermatozoa

Spermatozoa were obtained from the surgically isolated cauda epididymis of one fully mature retired breeder golden Syrian hamster male (Charles River Laboratories, Wilmington, MA). A small piece of dense sperm mass (bolus) from the tubules was cut out and placed with the forceps into the bottom of a sterile 5 ml polypropylene transport tube (Perfector Scientific Inc., Atascadero, CA, #2000S). Three mls of m-TALP-3 media [m-TALP-3 had the following composition: 101.02 mM NaCl, 2.68 mM KCl, 1.8 mM CaCl2, 0.49 mM MgCl2·6H2O, 0.36 mM NaH2 PO4·H2O, 35.7 mM NaHCO3, 4.50 mM D-glucose, 1.0 mM Na-pyruvate, 9.0 mM Na-lactate (60% syrup), 0.5 mM hypotaurine, 0.05 mM (-) epinephrine, 50 µg/ml gentamycin sulfate and 15 mg/ml bovine serum albumin] (Cummins and Yanagimachi, 1986) were added to the transport tube with the bolus of sperm mass. Each tube with the bolus in m-Talp3 media was incubated in an incubator with 5% CO2 [Heraeus, Kendro Lab Products, Philadelphia, PA or Asheville, NC, # BB 6220] at 37° Celsius for 10 minutes. After 10 minutes, the top 1.5 mls of the sperm swim-up suspension from each tube was pooled with a sterile disposable graduated transfer pipet (Fisher Scientific, Pittsburgh, PA, 13-711-9B) into several 35 X 10 mm polystyrene Petri dish [Fisher Scientific, Pittsburgh, PA, Falcon, 351007]. The pooled sperm suspension was aliquoted into several 35 X 10 mm plastic
Petri dishes containing approximately 2-3 milliliters each. The sperm suspension was looked at with a Spencer Phasestar phase contrast microscope [American Optical Corp, Buffalo, NY, # 105GA] with a 37° Celsius stage warmer [Air Curtain Incubator, Sage Instruments, Inc., Freedom, CA, # 277] at low magnification objective (20X) and motility was visually assessed, as described in the following section.

2.5. Assessment of Live Hamster Sperm Motility

After 10 minutes of incubation in a Petri dish, the sperm suspension was taken out of the 5% CO₂ incubator. The sperm suspension was observed with a phase contrast microscope with a 37° Celsius stage warmer at low magnification objective and motility was assessed. At the start of the assessment and up to the 10-15 minute time period, the vast majority of spermatozoa should be randomly motile and within the field of view of the microscope one should see at least 90-100% spermatozoon motility at the beginning. If motility was not initially at least at 90%, then the samples were discarded and the experiment was repeated from the beginning. If at the start, motility was approximately 100%, then after 2-3 hours, the spermatozoa were no longer aggregating, capacitation began and they were ready to fertilize eggs. The sperm motility, on average, decreased to between 70-80% motility in 2-3
hours. By the 5-6th hour of incubation, the acrosome reaction had occurred and capacitation was completed. The motility rate after 5-6 hours showed a decrease to a level of 60-70% motility. After overnight incubation, approximately 10-20% of sperm were motile, therefore there was still some motility and survival of spermatozoa, albeit markedly reduced, even after capacitation and overnight incubation.

2.6. Isolation and Treatment of Human Spermatozoa

Ejaculated human spermatozoa samples were obtained from the Kapiolani Medical Center, Pacific IVF Institute, Reproductive Biology Laboratory, Honolulu, Hawaii under the directorship of Thomas Huang, Ph.D. Every sample and semen analysis report was given an anonymous case number and no patient names were revealed to me. Standard semen analysis was performed within 30 minutes of collection and only those samples having normal motility, concentration and morphology using WHO criteria (WHO, 1999) were used for this study. Each semen analysis report had the following information: semen volume (in ml), concentration (in million/ml), motility (% motile), grade of activity, forward progression, liquefied within (in minutes), viscosity, WBC’s (in million/ml), sperm agglutination, semen fructose (mg/ml) and sperm morphologies (% normal). The information that was used for the
experiments was concentration and motility because similar samples were sometimes compared. Each sample came in a 15 ml falcon tube with the case number, motility and concentration of sperm/ml, as well as information on whether the samples had been treated with a Puresperm (standard percoll gradient) wash or a 2X wash (HEPES HTF Sperm washing medium, Irvine Scientific, Santa Ana, CA., #9983).

After the standard semen analysis was completed, spermatozoa were purified by centrifugation for 15 minutes at 1900 rpm over a two step gradient of PureCeption (40%:80%, Cooper Surgical, Trumbull, CT). Then the spermatozoa were rinsed in HEPES-buffered Human tubal fluid (HTF) medium (for the formula, see [Quinn et al.] supplemented with 15% synthetic serum substitute (Irvine Scientific, Irvine, CA). The spermatozoa from these preparations were 90-95% motile, free of seminal fluid components, and had > 90% normal morphology. Some spermatozoa samples were frozen within one minute at this stage without cryoprotectant and stored. Prior to this quick freezing, the sperm samples had normal morphology and motility.

Four samples were used for the experiments which I numbered 1, 2, 3 and 4. Whole semen samples were washed twice in HTF media buffer (Irvine Scientific, Santa Ana, CA, #9922), then either used fresh (sample #1) or frozen at -20° Celsius (sample #2). Some samples were isolated on Percoll gradients
and after isolation, were either used fresh (sample #3) or frozen at -20° Celsius (sample #4). Approximately 1 ml of samples was in each tube.

After these samples were transported to our University of Hawaii at Manoa laboratory, they were incubated with DNase I or Triton X-100 treatment (section 2.3) in a manner identical to the hamster spermatozoa protocol, except that the media used was not 1X PBS but HTF (human tubal fluid) media which Dr. Huang’s lab had previously used to store the sperm in.

2.7. Counting Spermatozoa

Spermatozoa, isolated sperm nuclei, and spleen nuclei were counted using a method developed in our lab to more easily visualize nuclei to increase accuracy. Cells were counted by pipetting the suspensions in 1X PBS with 1 µl of 0.5 µg/ml of ethidium bromide stain mixed with 9 µls of sperm suspension into the V-shaped notch of the hemocytometer (Hausser phase contrast hemocytometer, Fisher Scientific, Pittsburgh, PA, 02-671-54) and covering it with a microscope cover glass for less than 5 minutes. The sperm cells were counted in the counting grid under 40X (4X objective) microscopic magnification with a fluorescent microscope (Nikon fluorescence microscope Eclipse E600). The technique to use the phase contrast hemocytometer with ethidium bromide staining to look at the sperm cell outer structure for the
purpose of counting was developed by the Ward lab. The cells were counted
with the ethidium bromide filter in place so that the sperm heads were much
more easily visible, with enough transluminescent light to have seen the
hemocytometer grid.

The formula used to get the final count in cells/ml was:

\[
\text{Final cells/ml} = n \times 10^4 \times \text{dilution factor}
\]

where \( n \) = the number of cells in one mm square on the hemocytometer. The
concentration range used in the experiments after counting was approximately
\( 2 \times 10^7 \) cells/ml.

The human sperm samples were counted by the laboratory that
provided the samples using a Coulter Counter.

2.8. Treatment of Spermatozoa and Sperm Nuclei with DNase I or Divalent
Cations

For treatment with DNase I, hamster spermatozoa, hamster sperm
nuclei, hamster spleen nuclei, or human spermatozoa isolated and counted as
described above were diluted to 0.25% Triton X-100 (v/v) and 20 mM MgCl₂,
(Sigma Aldrich, St. Louis, MO, #M4880) and aliquoting into 2 ml eppendorf
tubes. The sperm cells were incubated in some cases with DNase I (Sigma
Aldrich, St. Louis, MO, #D4527) with various concentrations of 0, 10, 30, 100,
and 300 µg/ml. The eppendorf tubes were incubated at 37° C in a water bath for between 1-20 hours.

For treatment of cells with divalent cations (used for spermatozoa or sperm nuclei, only) sperm suspensions were made up to 0.25% Triton X100 and then to various concentrations of MgCl₂, CaCl₂, or both and incubated for various times points. The exact concentrations and times points were provided in the Results sections when the experiments were described.

2.9. Test for Release of DNA from the Sperm Nuclear Matrix by Centrifugation

One ml each of sperm with a concentration of 4 x 10⁷ cells/ml was subdivided into two groups. Group 1 was treated with 0.25% Triton X-100 an 10 mM of MgCl₂ and incubated in a water bath overnight. The next day, half was diluted to 2 x 10⁷ cells/ml with 0.5 ml of 1XPBS and then plugs were made. This sample was considered a control with total contents. Total contents were defined as all DNA in the sperm cell. The other half was used treated with 4 M NaCl, 10 mM DTT and halos formed (see section 2.13). The samples were centrifuged for 10 minutes at 5 rpm and the pellets and supernatants were collected and plugged separately. The plugs were analyzed by PFGE. The PFGE agarose gel was stained with 0.5 µg/ml ethidium bromide and analyzed by the Kodak EDAS 290 system.
In group 2, the samples were treated with 0.25% Triton X-100, 10 mM magnesium chloride and 100 µg/ml of DNase I and incubated overnight. From this 1 ml, half was diluted with 1X PBS and then plugs were made. This sample was considered a control with total contents. The other half was treated with 4 M NaCl and 10 mM DTT to form nuclear matrix, then spun for 10 minutes at 5 rpm. The supernatant and pellet were separated and plugs were formed from each.

B. Pulse Field Gel Electrophoresis

2.10. Embedding the Samples in Agarose Plugs

Pulse Field Gel Electrophoresis has been used to separate very large fragments of DNA (up to 2 mb of DNA), but such large fragments could not be easily manipulated without causing DNA breaks. The samples must be embedded in agarose before further manipulations can be made. After treatment and incubation, cell suspensions were mixed with an equivalent volume of 1% pulse field gel agarose (which had been tempered in a 53° C water bath for 15 minutes). This cell suspension-agarose mixture was pipetted into either disposable plug molds (Biorad, 1703713) or reusable plug molds (Biorad, 1703622). Fifteen minutes elapsed at room temperature in order for the plugs to set and then the plugs were removed from the molds.
2.11. Protease Digestion of Samples

Before the DNA can be electrophoresed, the proteins had to be extracted completely. This was done by treating the agarose embedded samples with SDS and proteinase K overnight. The PFGE plugs were incubated overnight in approximately 2 ml of lysis buffer solution per plug (adapted from Korzik, 1998) [10 mM Tris-HCl, pH 8.0, 10 mM Ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 20 mM dithiothreitol (DTT), 2% SDS, 20 μg/ml of proteinase K. The DTT and proteinase K were purchased from Sigma Aldrich, St. Louis, MO and the other chemicals were purchased from Fisher Scientific, Pittsburgh, PA. The plugs were incubated in the lysis buffer overnight in a 53° C water bath (Isotemp, Fisher Scientific, Pittsburgh, PA, #3013H).

The following day, the lysis buffer treated plugs were washed three times for a minimum of 20 minutes each in 25 mls of autoclaved sterilized TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer mixed with 25 mls of 1 M glycine, which had been previously heated in the 53° C water bath for 15 minutes in a 50 ml polypropylene Falcon tube (Fisher Scientific, Pittsburgh, PA, 1443222) for each sample treatment. The Falcon tubes containing the samples being washed were placed horizontally on a slow speed rotomix orbital mixer at a speed of 40 rpm at continuous speed (Thermolyne, Barnstead International,
Dubuque, Iowa, #M71735) for the duration of the washes. The plugs were washed with 50 mls of TE buffer alone two additional times for 10 minutes each with rotation on the shaker. Finally the samples were stored at 4°C in ~ 2 ml of TE buffer in 2 ml eppendorf tubes.

2.12. Preparation of the PFGE Gel

Approximately 100 ml of a 1% PFGE agarose gel (Pulsed Field Certified Agarose, Bio-Rad Laboratories, Hercules, CA,162-0137) in sterilized autoclaved 0.5X TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0, diluted from sterile autoclaved 10X TBE buffer, Biowhittaker, Inc., Walkersville, MD, or Cambrex BioScience, Baltimore, MD, BW16-012Y) was tempered in a water bath at 55°C temperature for 10 minutes and then poured into the level Chef Mapper XA PFGE 14 cm X 13 cm casting stand (Bio-Rad Laboratories, Hercules, CA, 170-3659). The 1% PFGE agarose gel was allowed to harden at room temperature for 30 minutes.

Usually two different sized ladders were also added to the 1% PFGE agarose in the first two lanes. Approximately 0.1 -0.2 mm width of marker was sliced off with a 22 mm microscope cover glass (Fisher Scientific, Pittsburgh, PA, 12-542b) from the syringe containing the PFGE markers and one sample from each of the following two markers were placed with two spatulas into the
first two wells, respectively. First, one molecular weight marker was a high molecular weight ladder composed of polymers of the 48.5 kb lambda phage genome with size ranges from 48.5 kb-1018.5 kb (lambda ladder PFG marker, New England Biolabs, Beverly, MA, N0340S). The other molecular weight marker was a low molecular weight ladder with Hind III digested lambda phage genome with size ranges of 2 kb-23 kb (low range PFG marker, New England Biolabs, Beverly, MA, N0350S).

2.13. Electrophoresis of plugs

The samples of DNA were loaded onto the 1% PFGE wells after treatment, lysis buffer incubation and TE/Glycine buffer washes. The wells were sealed with 3 μls of 1% PFGE agarose. Once the samples were loaded, they were ready to be electrophoresed. First, 2.2 liters of autoclaved 0.5X TBE was poured into the Chef Mapper chamber. The conditions for electrophoresis were chosen by using the automatic program on the Chef Mapper. The cooling module was set to 14 degrees and the temperature was allowed to equilibrate for approximately 15 minutes. The flow pump was set to 70 (flows 1 liter/minute). The Chef Mapper was programmed to run under the following additional conditions: linear ramping, 27 hours, 12 minutes total run time, 4 v/cm voltage, 120 degree angle, range 50 kb to 1 mb, 6.75 seconds of initial
switch time and 33.69 seconds of final switch time. After the run was completed, the 1% PFGE agarose was stained with 0.5 μg/ml ethidium bromide for 20 minutes in 250 mls of 0.5X TBE. The digital photographs were taken with the Kodak EDAS 290 gel documentation and ID Image analysis systems discussed in section 2.12.

C. Pulse Field Gel Electrophoresis Analysis

2.14. Image Analysis of PFGE DNA Patterns

The 1% PFGE agarose gel was stained for 20 minutes with 0.5 μg/ml ethidium bromide and image analysis was performed directly on the gel that was illuminated by an ultraviolet light box. A digital camera was attached to the Kodak EDAS 290 (electrophoresis documentation and analysis system) gel imaging system for taking pictures. The 1% PFGE agarose gels were analyzed by the Kodak 1D image analysis software, version 3.4 (Eastman Kodak, Rochester, NY). Each PFGE lane was densitometrically scanned and the molecular size in base pairs of the peaks of intensity was calculated by the software using the two molecular weight markers (low and high molecular weight PFGE ladders) as standards.
D. Halo Preparation

2.15. Halo Assay

Hamster spermatozoa were isolated in 1X PBS (10X PBS buffer, Biowhittaker, Inc., Walkersville, MD, or Cambrex BioScience, Baltimore, MD, BW16-006Y). Spermatozoa were treated with either 0.25% Triton X-100, 20 mM MgCl₂ and 100 µg/ml DNase I or 0.25% Triton X-100 and 20 mM MgCl₂ alone. Finally, the suspension was incubated at 37° C for 1-20 hours. After incubation, the DNase I (Sigma Aldrich, St. Louis, MO, #D4527) treated samples had their further nuclease activity deactivated by the addition of 0.5M EDTA (EDTA, Fisher Scientific, Pittsburgh, PA, AC118432500). Approximately 100-250 µl (5 X 10⁶ cells/ml) of the hamster spermatozoa was incubated in a 2 ml eppendorf tube with 0.5% Sodium dodecyl sulfate (10% SDS solution, Fisher Scientific, Pittsburgh, PA, BW16007B), 50 mM Tris-HCl, pH 7.5 (Fisher Scientific, Pittsburgh, PA, BP152-11) and spun at 2000 rpm for 10 minutes in a Sorvall Biofuge pico microcentrifuge (Kendro Lab Products, Asheville, NC). The pellets were resuspended in 2 ml of 50 mM Tris-HCl, pH 7.5, and then spun at 2000 rpm for 10 minutes in a Sorvall Biofuge pico microcentrifuge. The pellets were resuspended in eppendorf tubes with 200 µl of 2 M NaCl (Fisher Scientific, Pittsburgh, PA, S2713), 50 mM Tris and then 10 mM DTT (Sigma Aldrich, St. Louis, MO, #457779) was added. The eppendorf tubes were
incubated in water at 37° C for 10 minutes and the samples were placed on a microscope slide and stained with 2 μl of 200 μg/ml ethidium bromide for examination under a digital microscope with ultraviolet light (300 nm). Digital photographs of the halos on the slides were taken at 40X magnification (Nikon fluorescence microscope Eclipse E600 used with Olympus Magnafire software program connected to a Sony computer G 200 series). The digital photographs were imported as tiff 24 files onto a CD and were later opened up, viewed, and cropped as tiff file 24 in Adobe Photoshop 5. The picture and figures were labeled with Arial font under the computer program Powerpoint 2003.
Chapter 3.

The Donut-Loop Model for Hamster Sperm Chromatin Structure

3.1. Experimental Model Tested with DNase I

In these series of experiments the main focus was to test the experimental model of sperm chromatin structure called the Donut-Loop model, which was described in detail in the Introduction (see figure 3.1.). Balhorn and colleagues demonstrated that sperm protamines bound DNA into a tightly compacted toroid that contains about 50 kb of DNA (Hud et al., 1993 and Brewer et al., 1999). Our model predicted that each DNA-loop domain is one protamine-bound toroid. It also predicted that in between each toroid was a DNase hypersensitive region of toroid linker areas. Furthermore, these toroid linker regions were the sites of DNA-loop-domain attachment at their bases to the nuclear matrix of the sperm the matrix attachment sites (MARs).

From prior experiments conducted in our lab, this DNA-loop domain attachment to MARs was cell specific by using 5S-rRNA gene, a repetitive DNA segment, which organized the sperm loop domains into several, smaller compact loops versus a single, large loop domain in somatic liver cells of the same species (Nadel et al., 1995). Also, actively transcribed genes were associated with the nuclear matrix, while inactive genes were localized to DNA
The Donut-Loop Model Predicts 50 kb Digestion by DNase I

DNAse I

SDS, DTT, and Proteinase K

20 to 100 kb DNA Fragments
(average loop size = 50 kb)

Figure 3.1. The Donut-Loop Model for Sperm Chromatin Structure Predicts DNase I Treatment will result in 50 kb, Loop-Sized Fragments.

The Donut-Loop model predicts that DNase I sensitive toroid linker regions exist between each protamine toroid, and that the sperm DNA that is tightly bound to protamines within the toroid are protected from DNase I digestion in the loop domain itself (Nadel et al, 1995 and Gerdes et. al, 1994). The functional role predicted for the interaction between the sperm DNA and its nuclear matrix was the compaction during spermiogenesis and organization of information during embryogenesis (Ward, 1994, Sotolongo and Ward, 2000).
In designing our test of the Donut-Loop model, we took advantage of two predictions. First, the protamine bound toroids were less sensitive than most other types of chromatin that were histone-bound, and second, the toroid linker regions were more sensitive to DNase than the rest of the chromatin. If both predictions were true, then the treatment of membrane permeabilized sperm nuclei with DNase I should have digested only the toroid linker regions. Also, if the predictions were true, then the protamine bound toroid DNA would have remained intact.

In Figure 3.1., this experimental protocol was diagrammed. The hamster spermatozoa were treated with the non-ionic detergent TX-100 to allow access to the nucleus, then with DNase I which cleaved the toroid linker regions. Next, the sperm proteins were extracted with SDS detergent and proteinase K destroyed the proteins when they were added simultaneously. After this treatment, only DNA loop-sized fragments with an average median size of 50 kb and a wide range of 20 to 100 kb were expected based on the Donut-Loop model.

3.2. Somatic Cell Chromatin is rapidly degraded by DNase I

The first of the two predictions of the Donut-Loop model suggested that the highly compact protamine bound DNA was much less sensitive to DNase I digestion than histone bound chromatin found in most other cell types. In
order to be able to compare the protamine-bound sperm DNA with histone-bound DNA, spleen nuclei were used as a control and as an example of somatic cell chromatin structure in mammals. The spleen nuclei from the same hamsters, whose spermatozoa were used in these experiments, were digested with DNase I under the same conditions as spermatozoa.

The spleen nuclei were treated for 1 hour, 3 and 14 hours with increasing concentrations of DNase I at 37°C. Increasing concentrations of DNase I from 0 to 300 μg/ml were used, as shown in Figure 3.2. Then plugs were made and the PFGE agarose was electrophoresed. The PFGE agarose was stained with 0.5 μg/ml of ethidium bromide for 20 minutes and Kodak EDAS 290 digital photographs were taken after UV illumination.

For the purpose of simplicity and to avoid redundancy, only the one hour condition was shown in Figure 3.2, since by 3 and 20 hours, all of the lanes looked identical to lane 6 and the DNA was completely degraded. The results of the DNase I treated spleen nuclei was that incubation with no DNase I for 1 hour showed a typical somatic cell high molecular weight compression.
Figure 3.2. Somatic Cell chromatin was degraded by DNase I.

Spleen nuclei were treated with DNase I. Spleen was treated with 0.25% Triton X-100, 20 mM of MgCl₂ and then increasing concentrations of DNase I for 1 hour and then electrophoresed on PFGE. For the gel the DNase I concentrations were: lane 1, 0 µg/ml; lane 2, 3 µg/ml; lane 3, 10 µg/ml; lane 4, 30 µg/ml; lane 5, 100 µg/ml; lane 6, 300 µg/ml. λ, Lambda phage molecular weight marker in 48.5-kb increments; MW, low molecular weight marker. Figure previously published in Sotolongo et al., (2003).
zone. In studies conducted by Leroy Liu and others (Li et al., 1999), when PFGE was used with somatic DNA, the largest DNA molecules did not resolve at all and migrated in a compression zone just slightly below the well. The compression zone in lane 1, which was spleen without DNase, yielded large molecular weight fragments that remained in the typical compression zone as well as some nonspecific degradation. In lane 2 of the PFGE agarose, the spleen nuclei were digested into visibly large fragments after treatment of the spleen chromatin with 3 μg/ml of DNase I. In lanes 4 and 5, which used 30 μg/ml and 100 μg/ml of DNase I, respectively, the DNA fragmentation yielded lower molecular weight fragments. Lane 6, which utilized 300 μg/ml of DNase I, however, completely digested all of the spleen DNA. Somatic cell nuclei, such as the spleen samples exemplified here, were highly sensitive to DNase I digestion. This was consistent with the prediction that histone-bound DNA was less compact than sperm DNA, therefore, more easily degraded. This experiment showed that histone bound chromatin is completely digested with as little as 10 μg/ml of DNase I, in 1 hr at 37°C.

3.3. Hamster sperm chromatin is digested to loop sized fragments by DNase I

We next tested the second prediction of the Donut-Loop model, that the toroid linker regions were more sensitive to DNase I digestion than the rest of
the protamine bound sperm chromatin. Hamster sperm were treated with
0.25% Triton X-100, 20 mM MgCl₂ and the same concentration range of DNase I
that was used in the spleen nuclei treatments (see section 3.2). The treatment
times were 1 hour, 3 and 20 hours. For figures 3.3 A-C shown below the DNase I
concentrations were from 0 to 300 µg/ml.

Unlike the spleen nuclei or other somatic cell types which show a high
molecular weight compression zone in PFGE agarose, the sperm DNA in lane
1, when no DNase I was used in figures A through C, remained in the well.
This was probably due to the greater degree of compactness found in
protamine-bound DNA versus histone-bound DNA (Ward and Coffey, 1991;

Figures 3.3 A - C, lanes 2 through 6 showed that exposure to DNase I caused
digestion of sperm chromatin. The sizes of the digestion products centered
around the 48.5 kb molecular weight marker. These data were consistent with
the model where DNase I digestion has resulted in the excision of loop sized 50
kb fragments of DNA (Li et al., 1999).

There was a remarkable consistency of these loop-sized DNA fragments
after 1 hour, 3 hours and overnight (20 hours) treatment. In contrast to spleen
nuclei, the sperm nuclei never demonstrated complete degradation of all DNA
Figure 3.3. A-C. Sperm nuclei treated with DNase I released loop-sized fragments.

Sperm nuclei (A-C) were treated with 0.25% Triton X-100 and 20 mM MgCl₂ and increasing concentrations of DNase I for 1, 3 or 20 h, as indicated, then electrophoresed on PFGE. For all panels, the DNase I concentrations were: lane 1, 0 µg/ml; lane 2, 3 µg/ml; lane 3, 10 µg/ml; lane 4, 30 µg/ml; lane 5, 100 µg/ml; lane 6; 300 µg/ml. λ, Lambda phage molecular weight marker in 48.5-kb increments; MW, low molecular weight marker. Figure previously published in Sotolongo et al. (2003), as figure 2 B-D.
as was seen in lane 6 of figure 3.2 in spleen nuclei. DNase I treatment of sperm nuclei even after overnight incubation and increasing DNase I concentrations, yielded loop-sized DNA fragments around an average range of 50 kb as shown in figures 3.3 A through C.

3.4. Calculation of the Average Size of the Digested DNA

The PFGE gels were quantitated using the Kodak ID Image Analysis software to determine the average size of the digested DNA fragments. Each PFGE lane in figure 3.3 A was scanned into the software and the molecular size in base pairs of the peaks of DNA degradation was calculated using the lambda molecular weight markers as standards. The densitometric scans of PFGE gels that had been treated with various DNase I concentrations for the 1 hour time range was shown here in figure 3.4 as an example of the range of DNA fragmentation. Each DNase I digestion profile had a readily identifiable peak, suggesting that the DNA was digested to a clearly identifiable average size. The peaks of DNA fragmentation with the different concentrations of DNase I for the one hour treatment of sperm nuclei ranged from 28.1 kb at the second highest concentration of DNase I (100 \( \mu \)g/ml) to 77.6 kb with the lowest concentration of DNase I used (3 \( \mu \)g/ml).

The same densitometric analysis was performed for all DNase I treatment conditions shown in figures 3.2 and 3.3. To present the data in a
Each lane in the gel in Figure 3.3A was scanned using the Kodak 1D Image Analysis software, as described, and shown here as an example. Arrows point to the peak of the intensity, and the calculated molecular sizes for each peak are indicated. The concentration range of DNase I was from 0 μg/ml to 300 μg/ml. The peak range was from 28.1 kb when treated with 100 μg/ml of DNase up to 77.6 kb when treated with 10 μg/ml of DNase. Sperm DNA was digested to sizes that ranged from an average mean of 43.1 ± 20 kb for all DNase concentrations averaged together. Figure previously published as figure 3 in Sotolongo et al., 2003.
manner that would be easily comprehensible, the molecular sizes of the peak of each scan was plotted for each of the four conditions tested, as shown in figure 3.5. Two important points stood out from this graph. The first was that the difference between DNase I digestion of the histone-bound spleen chromatin and that of the protamine-bound sperm chromatin was clearly demonstrated.

The solid line on the graph correlated with the data shown in the PFGE gels in which the spleen began at high molecular weights of over 140 kb at around 3 μg/ml of DNase I and by the one hour mark had steadily and progressively degraded all of its DNA when treated with the highest concentration used in this study of 300 μg/ml of DNase I. As was shown by the PFGE figure in 3.2, spleen (somatic) cells when treated with DNase I were completely degraded from large molecular sized fragments of 436 kb to lower sized fragments (below 48.5 kb) and at high DNase I concentrations of 300 μg/ml within one hour, were completely degraded. The graph confirmed that spleen DNA was degraded much more rapidly and completely than the sperm DNA by the one hour time point, thus supported by the data from figure 3.2 at the quantitative level.

In figure 3.5, the lines on the graph that were labeled sperm treatment with DNase I for 1, 3 and 20 hours showed a relatively consistent size
Figure 3.5. A comparison of DNA fragmentation peaks after DNase I treatment between spleen and spermatozoa DNA in figures 3.2 and 3.3 A, B, and C.

Each lane of Figures 3.2 and 3.3 A-C was scanned. Then the molecular sizes of the various peaks were calculated, as in figure 3.4. Each line on the graph represents one gel in figures 3.2 and 3.3. The spleen chromatin was digested to very large molecular sized DNA (145 kb) at the lowest DNase concentrations, then to roughly sizes in the range of 20-30 kb, then digested completely at the highest DNase concentration of 300 μg/ml. The sperm DNA was digested at the same 1 hour time point to an average mean range of 23 to 63 kb and at the 20 hour time point to an average mean range of 34-38 kb. This figure previously published as figure 4 in Sotolongo et al., 2003.
minimum for the average size of sperm DNA that resulted from DNase I digestion, in all times and concentrations, that was 30 to 50 kb. The graph confirmed the PFGE results that sperm nuclei, when treated with DNase I, were unable to completely degrade their DNA as was demonstrated by spleen DNA. Sperm nuclei remained within a consistent fragmentation range of around 50 kb loop-sized DNA fragments. As both the concentration of DNase I and the time of treatment were increased the graphs for all three time points for sperm nuclei leveled to this range. This data has suggested that there was a structural limitation to the amount of DNase I digestion that could occur in sperm chromatin, just as the Donut-Loop model had predicted. The average size of the peak size of the digested sperm DNA was $43.1 \pm 20$ kb. These ranges fall well within the estimated 20 to 100 kb that were predicted in the model based on somatic cell DNA fragmentation patterns (Balhorn, 1982; Ward and Coffey, 1989).

Supporting the data from figure 3.4 at the quantitative level, the range of the sizes centered around 50 kb loop-sized fragments. The appearance of some fragments, which were much larger (77.6 kb for the 10 µg/ml DNase I treatment) and much smaller (28.1 kb for the 100 µg/ml DNase I treatment) could be explained as follows. The Donut-Loop model for sperm chromatin structure proposed that each protamine-toroid was a DNA loop domain and
the linker regions between each toroid were nuclear matrix attachment sites. This explanation for the model was strongly supported by the PFGE data in figures 3.2 and 3.3 and the somatic cell literature which estimated the average size of the somatic cell loop domain to be an average mean of 50 kb (Balhorn, 1982; Vogelstein, 1980; Ward and Coffey 1989). An alternative explanation regarding the narrow size range of smaller (28 kb) or larger (77 kb) DNA fragmentation must be mentioned and could be easily explained, if one accepted the concept that toroids might be partially digested by the DNase I at the ends and yield different sized fragments. Another possible explanation would be that the toroids, which up to now had been estimated at 50 kb sizes, were in actuality smaller than 50 kb and closer to 20-40 kb and therefore two toroids linked together may be yielding the larger fragments if their linker regions were not completely and efficiently digested by the DNase I at the DNase I hypersensitive sites.

3.5 DNase I treatment for 5 days did not increase DNA digestion

The results of the overnight DNase I treated spermatozoa revealed the consistency of 50 kb loop-sized DNA fragments during a time period of 1 hour, 3 hours and overnight. The question that remained to be answered was would DNase I treatment completely degrade the DNA fragments, if given a much longer time course of treatment or would it never completely degrade beyond a
minimum size regardless of the time course. To test whether DNase I could ever digest the protamine-bound DNA to sizes smaller than the 50 kb average size which we saw after overnight digestion (figure 3.3), I treated spermatozoa with DNase I for much longer times.

For the experiment the following control and experimental samples were prepared. 1) Spleen was used as a control without any treatment (lane 1). (2) Spleen was subjected to lysis treatment for 1 hour and overnight (lanes 2-3). (3) Spermatozoa were treated with Triton X-100 and MgCl₂ alone (lane 14). (4) Spermatozoa were treated with DNase I, Triton X-100, MgCl₂ (lanes 5-13). Both the spleen and spermatozoa samples were each plugged with 1% PFGE agarose, electrophoresed, then stained with 0.5 μg/ml of ethidium bromide, viewed with UV illumination and photographed with the Kodak digital camera.

Spleen-derived DNA (lane 1) that was not exposed to DNase I showed no degradation at all (figure 3.6). Spleen treated for at least 1 hour (lane 2) showed low molecular weight DNA fragmentation. When spleen was treated overnight with DNase, no DNA remained and complete degradation occurred as in prior experiments.

The PFGE results for the DNase I treated sperm plugs (lanes 6-13) showed a consistent degradation of DNA fragmentation of loop sized fragments even
after 5 days. There were some fragments that ran into the lower molecular weight areas after overnight treatment and well into day 5 (figure 3.6, lanes 10-13). The results answer the question that remained to be answered regarding rate versus maximum velocity of DNA fragmentation. DNase I treatment did not completely degrade the DNA fragments as it did in the case of spleen chromatin. When the DNase I treated sperm cells were given a much longer time course of treatment, it never completely degrade beyond the minimum size of the 50 kb average, regardless of the time of incubation. In sperm, degradation was limited to loop-sized fragments regardless of how much degradation time was allowed in the experiment. These results supported two predictions of the Donut-Loop model. The first, that protamine bound DNA was less sensitive to DNase I than histone-bound chromatin, was shown by this experiment. Secondly, that the linker regions were DNase I sensitive were also supported by this experiment. These results did not exclude the possibility that the DNase I could be cutting at one place inside of the toroid.

Unexpectedly, treatment with TX-100 alone (lane 14) did result in a similar pattern of sperm DNA degradation to that of DNase I treatment, but only after overnight incubation. This suggested that an endogenous sperm
3.6. **DNase I treatment of Hamster spermatozoa for up to 5 days.**

The spleen samples were in lanes 1, 2 and 3. The treatment times were 0 minutes, 1 hour, and overnight, respectively. The DNase I spermatozoa samples were in lanes 5-13 treated for the following time points: (lane 5) 0 minutes, (lane 6) 15 minutes, (lane 7) 1 hour, (lane 8) 3 hours, (lane 9) 1 day, (lane 10) 2 days, (lane 11) 3 days, (lane 12) 4 days, (lane 13) 5 days. The overnight Triton X-100 treated spermatozoa sample was in lane 14. λ, Lambda phage molecular weight marker in 48.5-kb increments were in lanes 4 and 15.
nuclease does exist but that it is not activated quickly. This endogenous nuclease was the subject of the next series of experiments of this dissertation, described in Chapter 4.

3.6. Test of the Donut-Loop Model by the Halo assay

The Donut-Loop model predicted that each protamine toroid was one loop domain. The model also predicted that the toroid linkers were also the sites of attachment of the loops to the sperm nuclear matrix at the MARs. Organization of DNA into loops could be visualized in preparations that were called the nuclear halo assay or halo assay, for short. Nuclear halos were prepared by extracting nuclei with 2 M NaCl (as well as DTT in the case of spermatozoa) to remove the histones and/or protamines bound to the DNA (Vogelstein et al., 1980; Ward et al., 1989). These nuclear halos were made up of the nuclear matrix, the skeletal component of the nucleus, with a halo of DNA composed of loops surrounding it. The extracted nuclei were then stained with ethidium bromide and visualized under fluorescent microscopy. The DNA surrounding the nuclear matrix appeared as a halo of fluorescence made up of DNA loop domains attached at their bases to the nuclear matrix.

The Donut-Loop model predicted that exogenous DNase I was digested only at the bases of the loop domains. Thus, it would also have predicted that
sperm nuclei digested by DNase I would release all of the DNA loops into solution, if extracted by 2M NaCl, because the attachment sites would be digested by the DNase I.

The halo assay was used for DNase I treated spermatozoa (figure 3.7). It was also used for TX-100 treated spermatozoa, however, Triton X-100 treated halo assays will be discussed in chapter 4. The halo assay allowed direct visualization of DNA loop domains by treating the sperm with high salt (2M NaCl) and 10 mM DTT to remove the proteins called protamines. Then the sperm were stained with ethidium bromide and their DNA was directly viewed with the microscope at 40 X magnification.

The control sperm nuclei, which were not treated with DNase I or Triton X-100, revealed strong fluorescent halos surrounding the sperm nucleus. The DNA loop domains attached at the bases of the nuclear matrix were visualized as halos. Only the DNase I treated sperm at low concentrations (3 μg/ml) of DNase I or high concentrations (300 μg/ml) of DNase I showed no fluorescent halos. These DNase I treated sperm with no fluorescent halos surrounding the sperm nucleus were interpreted as having released their DNA into the solution.
Figure 3.7. Halo Assay for DNase and TritonX-100-treated spermatozoa.

Spermatozoa were extracted with high salt and DTT to remove the protamines, and then stained with ethidium bromide to reveal the DNA structure under a microscope. (A) In control sperm nuclei, this treatment resulted in a halo of fluorescence surrounding the sperm nucleus made of DNA loop domains attached at their bases to the nuclear matrix. Spermatozoa treated with either 3 µg/ml (B) or 300 µg/ml DNase (C) released their entire DNA into solution. Figure 10 A-C previously published in Sotolongo et al., 2003.
3.7. Conclusions

In somatic cells, DNA is wound loosely around histone proteins to form nucleosomes, which when coiled form loop domains bound to the nuclear matrix (Finch et al., 1977). The sites where DNA was bound to the nuclear matrix is the site of DNA replication and RNA transcription and the along the loop were inactive regions (Gerdes et al., 1994; Vogelstein et al., 1980). In contrast to the somatic model of chromatin packaging, sperm DNA is bound to protamine proteins in very compact donut-loop structured toroids. These toroids contained on average 50 kb of DNA (Balhorn, 1982; Hud et al., 1995). As in somatic cells, sperm DNA was organized into loop domains. In marked contrast to somatic chromatin, mature sperm DNA does not undergo replication or transcription, despite having the same basic loop domain structure. Sperm DNA loop domain organization was determined to be cell-specific and changed during development, therefore, its functions might be involved in both spermatogenesis and embryogenesis (Klaus et al., 2001). Although these pieces of information were known about sperm DNA, few models existed of how sperm DNA structure was organized and its functions until the Donut-Loop model was proposed. Our model called the Donut-Loop model proposed that each protamine-toroid was a DNA loop domain (figure 3.8). It also predicted that the links between each toroid are toroid linker
regions that connect to the nuclear matrix attachment sites. Since a small percentage of sperm DNA was found to be bound to histones, not protamines (Gatewood, 1987; Ausio, 1999), we suggested that the DNA closest to the nuclear matrix attachment sites were associated with histones, rather than protamines, thus more easily cleaved than compacted DNA-protamine sites. Our model predicted that these histone bound sites would be relatively accessible to cleavage by various exogenous enzymes. DNase I was selected for testing our experimental model due to the sensitivity of histone-bound DNA to DNase I digestion in comparison to DNA bound to protamines in the toroids. This model has suggested that intact sperm DNA loop domain might be necessary for both normal mammalian spermatogenesis and embryogenesis (Klaus et al., 2001; Sotolongo and Ward, 2000).

Both DNase I digestion and the halo assay experiments supported our Donut-Loop model. The DNase I digestion of somatic cells resulted in quick loop sized DNA fragmentation and complete degradation in the histone-bound DNA organization of somatic cells such as spleen. When sperm cells were treated with DNase I digestion, a few differences were witnessed. First, protamine-bound DNA in sperm made the organization resistant to complete degradation as was seen in the somatic cells.
Figure 3.8 Model for sperm DNA degradation.

The data suggested that DNase I digested the nuclease sensitive protamine linker region at the MARs, cleaving DNA loops. After DNase I treatment these loops were treated with the halo assay 2M NaCl and 20 mM DTT treatment and the loop sized fragments were released. This figure was modified from figure 11 one previously published in Sotolongo et al., (2003).
Second, DNase I digestion, also produced loop-sized fragments, however they remained consistently within an average mean of 50 kb. Both somatic and sperm cells shared similar loop-sized domain structures, however, protamine-bound DNA in sperm in comparison to histone-bound DNA in somatic cells, made the loop-domain structure in sperm relatively impermeable to complete degradation as had been observed in somatic cells. These conclusions suggested that the protamine-bound DNA was more compact than the histone-bound DNA. Another conclusion suggested by the data was that DNase I cleaved in histone-sensitive regions and not protamine-sensitive toroid regions since it gave consistent 50 kb fragments which corresponded to the size of what we believe a toroid to be (Balhorn, 1982; Hud et al., 1995). In short, the DNase I treated samples electrophoresed on PFGE agarose, alone, said that the fragments were loop-sized.

The halo assay results showed that in control conditions, the halos remained intact and attached to the nuclear matrix, thus showing the classical halo of fluorescence around the nucleus. When DNase I treatment was added to the halo assay, the nuclear matrix of the sperm remained but no halos of DNA loops were seen. The halo assay, alone, said that the DNA was released from the nuclear matrix.
The first set of data with the PFGE agarose said that fragments were loop-sized and were on average of 50 kb. The second set of data with the halo assay said that the DNA was cleaved and released from the nuclear matrix. The only manner in which to explain both sets of data was that DNase I was cutting the DNA at the bases of the loops and probably at no other places and the size of these fragments were 50 kb loop-sized.

There could be alternative explanations regarding the range of DNA fragments with DNase I treatment between 28 kb to 77 kb; however, the average mean was 46 kb which was very close to our expected average of 50 kb. Also, somatic cells have been found to be within a range of 20 kb to 100 kb with an average size, which has shown that loop-sized domains are dynamic and flexible, although within an average range of around 50 kb. The alternative explanations for the variety in loop-sized domains would be that the DNase I was cutting into the ends of the toroids and therefore producing smaller than expected fragments. Another possible explanation would be that the toroid size would be closer to 30-40 kb and in some cases the DNase I has cut one toroid and in cases of incomplete digestion, the DNase I has cut two toroids.

Although these alternative explanations have appeared possible, the reason that our model has remained as the most accurate explanation has been
the results themselves. We predicted an average size of 50 kb with DNase I digestion and the results were consistent with that. We also predicted that like somatic cells, there could be a range of as much of 80 kb between the loop domains and once again the results have shown a variety within a narrow range of 50 kb. We predicted that protamine-bound toroids would be more resistant to DNase I digestion than the histone-bound toroid linker regions and the results have shown a strong resistance to complete digestion in sperm (in the toroid regions) versus in somatic cells, which are histone-bound DNA or in the linker region of the sperm. Finally, the halo assay and the DNase I digestion followed by PFGE results verify one another, the DNA was cut into loop sized fragments with an average of 50 kb and the loop sized fragments could not be seen when a halo assay was conducted because they had been cleaved away from the nuclear matrix at the matrix attachment region.

The results of using exogenous nuclease, DNase I, with sperm and having produced loop-sized fragments left unanswered questions to be explored. First, if an exogenous nuclease revealed a system of sperm degradation into loop-sized 50 kb fragments, could the sperm have an endogenous system that could be revealed that would also produce the 50 kb fragments when activated? If so, what would the most likely candidates have
been for this endogenous nuclease? Would the endogenous nuclease work in an identical manner to the exogenous nuclease, DNase I, in sperm?
Chapter 4.

The presence of an endogenous hamster sperm nuclease that cleaves chromatin into loop-sized fragments

Some of the data described in Chapter 3 suggested the idea that sperm nuclei have an endogenous nuclease that requires Triton X-100 permeabilization of the sperm membranes in order for the DNA to be degraded into loop-sized fragments. Several labs had reported that calcium-magnesium dependent endonucleases existed in somatic cells (Boulares et al., 2002; Yakovlev et al., 1999; Yakovlev et al., 2000) as well as bull seminal plasma and were involved in cleavage of DNA during the process of apoptosis into loop-sized fragments, similar to those we observed in Chapter 3, figure 3.6 (Hashida et al., 1982; Yoshihara et al., 1997). Topoisomerase II has also been shown to cleave DNA into loop-sized fragments during apoptosis of somatic cells (Solovyan et al., 2002; Li et al., 1999; Lagarkova et al., 1995). In this Chapter, I explored the possibility that spermatozoa contain an endogenous nuclease capable of cleave its DNA into 50 kb fragments.
4.1. Controls of the DNase I experiments suggested a possible endogenous nuclease in sperm.

During our work on the DNase I treatment of hamster spermatozoa described in Chapter 3, we found that control experiments in which spermatozoa were treated with TX-100 and MgCl₂ but without DNase I also digested their DNA into loop sized fragments (figure 3.6, lane 15). This suggested that sperm contain an endogenous nuclease to digest their own DNA into loop-sized fragments that was distinctly different from that of the DNase I induced DNA fragmentation. We tested this by incubating spermatozoa for various time points without DNase I (figure 4.1). We also compared the digestion of the endogenous nuclease with that of DNase I that we had previously performed (figure 4.1).

When the samples of hamster sperm were prepared as previously described in the materials and methods section and run on a 1% pulse field gel agarose in figure 4.1, the results for the DNase I, MgCl₂ and Triton X-100 treated (lanes 9-14) versus Triton X-100 and MgCl₂ (lanes 2-7) samples results were similar. The solubilization of the sperm membranes by Triton X-100 treatment did cleave the DNA into 50 kb loop-sized fragments as did the DNase I treatment, however, there were some important differences.
Figure 4.1. Spermatozoa treated with Triton X-100 without DNase I Digest DNA into Loop-Sized Fragments.

Spermatozoa were treated with either Triton X-100 or DNase I for the following times: lanes 1 and 8 for 0 minutes, lanes 2 and 9 for 15 minutes, lanes 3 and 10 for 1 hour, lanes 4 and 11 for 2 hours, lanes 5 and 12 for 4 hours and lanes 6 and 13 overnight. The samples were then electrophoresed on PFG agarose, and stained with ethidium bromide. Lanes 7 contained the lambda DNA ladder on the PFGE gel. DNase I digested sperm chromatin quickly within 15 minutes (lane 7), but Triton X-100 treated sperm did not completely digest sperm chromatin until overnight incubation (lane 4).
In figure 4.1, the DNase I treated samples in lanes 9-14 showed 50 kb loop-sized fragments at various time intervals from 0 minutes up to overnight treatment. The DNase I treated hamster sperm samples all reached the same loop-sized fragments over time. The Triton X-100 treated samples in lanes 2-5 showed no 50 kb loop-sized fragments at the time intervals from 0 minutes to 2 hours. The sperm DNA remained stable during this time point and remained in the well. The Triton X-100 treated samples in lane 6 showed some digestion visible in the larger molecular weight range with the 4 hour incubation. In lane 7, the digestion of Triton X-100 treated sperm into 50 kb loop-sized fragments was completed by overnight incubation.

The results showed that the DNase I treated hamster spermatozoa degraded sperm chromatin more quickly than the endogenous Triton X-100 induced nuclease. The DNase I treated sperm resulted in quick digestions within 15 minutes and remained at a steady state even overnight. The Triton X-100 treatment of sperm resulted in a slower induction of an endogenous nuclease begun around the 4 hour mark and completed by overnight treatment. These experimental conclusions suggested that there was an endogenous nuclease.
4.2. Spermatozoa contain an endogenous nuclease activity that cleaved DNA into loop-sized DNA fragments.

The controls for the DNase I experiments shown in the PFGE gel in figure 4.1 suggested the existence of an endogenous nuclease. We next tested whether TX permeabilization was necessary for this endogenous nuclease to be activated. The prediction was that if an endogenous nuclease existed in the absence of DNase I but under the influence of Triton X-100 treatment, the spermatozoa would be induced to cleave DNA into loop-sized DNA fragments only after TX treatment had induced the process of endogenous cleavage in the sperm DNA. If the spermatozoa were left intact without DNase I or Triton X-100 treatment, then I expected that the endogenous nuclease might not be activated and either no or very little DNA fragmentation might occur, if it required membrane solubilization during a short period of time such as a few minutes or hours. It was unknown what might occur over a longer time course such as several days.

First, controls of spermatozoa in 1X PBS were incubated at 37°C for various time points ranging from zero minutes, 15 minutes, and finally one through 5 days (lanes 2-8). These controls were not washed with anything further and were simply embedded in PFGE agarose after incubation. This set was labeled intact spermatozoa. A second set of spermatozoa in 1X PBS were
washed with 0.25% Triton X-100 detergent and 20 mM MgCl₂ only (lanes 9-15).
This second set was also incubated for the time points as the intact spermatozoa and were labeled Triton X-100 treated.

When the control spermatozoa were left intact without Triton X-100 or MgCl₂ treatment, the sperm DNA remained stable for up to 5 days and did not cleave into loop-sized DNA fragments as seen in lanes 2-8 of figure 4.2. The sperm that were treated with Triton X-100 and MgCl₂ degraded their DNA into the approximate loop-sized fragments seen with DNase I treatment as seen in lanes 11-15 of figure 4.2. This result indicated the presence of an endogenous nuclease activity in spermatozoa existed that released loop-sized DNA fragments. Since intact sperm did not degrade after a long time course of 5 days, we can conclude that the membranes were required to be broken in order for DNA digestion to occur.

There were similarities between the DNase I treated samples in figure 3.6 and the Triton X-100 treated samples in figure 4.2. First, DNase I and the Triton X-100 treatment both caused DNA fragmentation to occur in the 48.5 kb loop-sized fragment size. In the case of DNase I treatment the DNA fragmentation was quick and occurred within 15 minutes. Complete DNA fragmentation occurred in the Triton X-100 and MgCl₂ treated experiments only
Figure 4.2 Membrane Permeability is required for Endogenous Nuclease Activation.

Lane 1 contained the lambda DNA ladder on the PFGE gel. Treatment samples were either intact spermatozoa which were treated with PBS alone (lanes 2-8), or with 0.5% Triton X-100 and 10 mM MgCl₂ (lanes 9-15) for a time period from 0 to 5 days, then electrophoresed on PFGE gels, and stained with ethidium bromide. Endogenous nuclease activity exists in spermatozoa that cleaved DNA into loop-sized fragments in the Triton X-100 treated sperm. Figure previously published in Sotolongo et al., 2003.
after overnight incubation. The results of these experiments showed that the 
endogenous nuclease treatment worked at a much slower pace than the DNase 
I (exogenous nuclease) treatment, however, they both produced similar DNA 
loop-sized fragments. Another result was that the DNA did not degrade any 
further in the Triton X-100 treated spermatozoa beyond the 48.5 kb loop-sized 
fragments as seen in lanes 11-15 of figure 4.2. This result mimicked the DNase 
I treatments which also did not degrade any further beyond the loop-sized 
fragments. Image analysis was done on the Triton X-100 samples versus the 
DNase I treated ones and the results will be discussed in section 4.4

4.3. Comparison of Triton X-100 versus DNase I treatment in releasing loop- 
sized fragments.

I next performed side-by-side digestions of the sperm DNA with the 
endogenous nuclease and exogenous DNase I with the intent of examining the 
digestion patterns by image analysis to compare the average sizes of the DNA 
fragments released by each method. The DNA cleavage of hamster 
spermatozoa treated with DNase I under various time points began loop-sized 
fragmentation as early as 15 minutes (although some fragmentation was seen 
in the zero incubation condition), which indicated that just addition of DNase I, 
Triton X-100 and MgCl₂ was enough to induce fragmentation and the loop
sized fragments were not further degraded by days 1 through 5 (figure 4.3). In comparison, the Triton X-100 and MgCl₂ treated sperm began to show partial fragmentation into loop-sized fragments around 4 hours and complete by overnight treatment. The result with the same sample pool was that the DNase I treated samples degraded sperm chromatin at a faster rate than the endogenous system induced by the Triton X-100 treatment.

The data shown in PFGE in figures 4.3 and 4.4 were compared by image analysis of the gels. The results indicated that DNase I treatment released very high molecular weight fragments initially at the 15 minute mark of around 140 kb but after several days, the fragmentation equilibrated to an average peak size of around 55 kb. The Triton X-100 treated spermatozoa released slightly higher fragments of around 70 kb averages. Thus, the average sized fragments for the endogenous nuclease Triton X-100 treated sperm were slightly larger than those of DNase I treated sperm, but both average sizes of the peaks were within the loop-sized range and do not digest any further over the course of several days of treatment.
4.3. DNase I released loop sized fragments faster than endogenous nuclease.

Spermatozoa were treated with TX or DNase I for various times, as indicated, then electrophoresed on PFGE, as indicated. The first lane contains the lambda 48.5 kb ladder. DNase I (100μg/ml) digests sperm chromatin within 15 minutes, but Triton X-100 treatment does not induce complete sperm DNA fragmentation until overnight incubation. This figure was previously published as Figure 7 in Sotolongo et al., 2003.
Figure 4.4. Comparison of DNase I - and Triton X-100-mediated sperm DNA fragmentation.

The data in Figures 4.2 and 4.3 were compared by image analysis of the gels. This figure was previously published as Figure 8 in Sotolongo et al., 2003.
4.4. Assessment of live hamster sperm motility

I next tested whether the experiments described above could be repeated in live hamster spermatozoa, or whether they were an artifact of isolating spermatozoa and placing them immediately in cold PBS. In Chapter 2 a complete description was given of how to incubate live hamster spermatozoa and assess its motility (number alive). In figure 4.5 below live hamster sperm was incubated in mTalp-3 media and assessed for motility for four different time periods in two different experiments. The time periods were 5-15 minutes, 2-3 hours, 5-6 hours and overnight incubation. During the 5-15 minute time period, the best swim-up sperm are isolated from the upper 1.5 mls from a total of 3 mls. This time period is the best time to find approximately 100% uncapacitated motile sperm because if one waits too long then the sperm will begin to die (as per Dr. Yanagimachi communication November 1, 2003). Also at around the 3 hour period, the sperm should be free and vigorous and capacitation should have begun. At the 6 hour time period, the sperm should still be freely swimming sperm, however, the acrosome reaction should have occurred by this time period.

In figure 4.5, the uncapacitated sperm were at 100% motility for experiment 1 and at 90% motility for experiment 2 after 5-15 minutes of incubation with mTalp3 under the conditions described in chapter 2. A similar
Hamster Spermatozoa Viability in mTALP Media at 37°C

Figure 4.5 Hamster spermatozoa viability in mTalp-3 media.

Assessment of live hamster sperm motility in mTalp-3 for 5-15 minutes, 2-3 hours, 5-6 hours postcapacitation and overnight in CO₂ incubator showed a steady decrease in viability in two separate experiments.
experiment conducted by Cummins and Yanagimachi (1986) found similar results between 2-5 minutes of incubation with roughly 85%-95% motile sperm population. Between the 2-3 hour time period, the motility was between 70% (for experiment 2) and 80% (for experiment 1) as the sperm began to become capacitated. At the 5-6 hour time period during which the acrosome reaction should have begun, the motility was between 60% (experiment 2) and 70% (experiment 1). Finally, when the hamster sperm were looked at after overnight incubation, the results were between 10% (experiment 2) and 20% (experiment 1) were still motile. These experimental results served as a baseline for normal live hamster sperm motility in mTalp-3 during different time periods. Although the results of a control with 0.25% Triton X-100 and 20 mM MgCl₂ in mTalp-3 for 1 hour were not included in figure 4.5, this detergent treatment which disrupted the plasma membrane of the sperm, resulted in death to all of the sperm.

Figure 4.6 shows a second experiment conducted with live hamster media mTalp-3 in a (37° C, carbon dioxide) incubator which required measuring motility under different treatment controls. Initial motility before any treatment
Figure 4.6 PFGE of live hamster sperm in mTalp-3 media with control and 0.25% TX-100, 20 mM MgCl₂ lanes.

Lane 1 is 48.5 kb lambda ladder. Lane 2 is a low range ladder. Lane 3 is control for fast swimming sperm. Lane 4 is TX-100, MgCl₂ treated fast-swimming sperm. Lane 5 is a control for slower swimming sperm. Lane 6 is TX-100, MgCl₂ treated slower swimming sperm.
modality was instituted was 90% live hamster motility at 10 minutes for the uppermost layer of swim-up sperm which have the highest initial motility. These sperm were considered high motility sperm (see lanes 3 and 4). A second group was used from the next layer of swim up sperm which have many dead and dying sperm and these had a motility of 80% live hamster motility. This second group was considered the low motility sperm (see lanes 5 and 6).

The control condition (figure 4.6, lanes 3 and 5) were incubated for 5 hours, motility was assessed and then the samples were plugged immediately with 1% PFGE agarose. For sperm incubated with TX-100, MgCl₂ for 1 hour (see lanes 4 and 6), then motility was assessed and further incubation for 4 hours was conducted for a final incubation of 5 hours, then motility was again assessed and then the samples were plugged immediately with 1% PFGE agarose. The reason that 5 hours was chosen for incubation was that by the 5-6 hour time period, capacitation and the acrosome reaction should have begun to occur. Sperm must have completed the acrosome reaction before penetrating the oocyte's zona pellucida during the process of fertilization; therefore we wanted to assess intact sperm (control) versus (Triton X-100) plasma membrane disrupted sperm after capacitation had begun.
The results for the fast swimming (lane 3) and the slower swimming control (lane 5) were similar in that most of the DNA did not degrade and the assessed motility of live sperm after 5 hours of incubation was between 50-60% live. The results for the fast swimming (lane 4) and the slower swimming Triton X-100 treated sperm (lane 6), both showed 50 kb loop sized DNA degradation patterns. The assessed motility for the Triton X-100 treated sperm was 100% dead sperm after 1 hour incubation as well as at the 5 hour time period.

The results of the live hamster sperm motility assay and the PFGE agarose provide further support to the results found in figure 4.3 for Triton X-100. The control condition which showed normal motility levels comparable to figure 4.4 of 90% motility after 10 minutes and 50-60% after 5 hours produced no 50 kb DNA degradation pattern on the PFGE agarose (lanes 3 and 5). The Triton X-100 treated sperm, which had disrupted plasma membranes, showed 0% motility and showed a 50 kb DNA degradation pattern on the PFGE agarose (lanes 4 and 6). This corresponds with the results of figure 4.3 for 0 hour (control) and 4 hour Triton X-100 treated sperm which also showed no DNA fragmentation for nontreated sperm and loop sized DNA fragments for Triton X-100 treated sperm.
Assessment of live hamster sperm motility can be successfully combined with PFGE in order to verify DNA fragmentation patterns being indicative of dying or dead sperm in Triton X-100 treated conditions as well as motile sperm showing intact PFGE DNA patterns for control conditions.

4.5. Halo assay used to analyze DNase I versus Triton X-100 treated spermatozoa

In Chapter 3, I demonstrated that exogenous DNase I cleaved DNA at the bases of the loop domains using the halo assay (figures 3.7 and 3.8). In this section, I used the same halo assay to test whether the endogenous nuclease also released the DNA loop domains, thereby indicating that it also cleaved the DNA at the bases of the loop domains. As in the DNase I treatment, Triton X-100 halo assays were prepared in order to test the hypothesis that sperm nuclei might be digested at the MARs (matrix attachment regions) and to visualize it under a 40X microscopic objective and digital pictures of the microscope pictures were taken.

The prediction was that one would see a visible halo around the sperm nucleus if the DNA loop domains were still attached at their bases to the nuclear matrix. I expected to only see halos in the case of the control which
showed no DNA fragmentation pattern when the samples were intact as in figure 4.2 (lanes 2-8). I did not expect to see any halos in the case of Triton X-100 treated spermatozoa since DNA was cleaved to loop-sized fragments as in figure 4.3 where Triton X-100 treatment resulted in loop-sized fragments on PFGE agarose.

The results of the experiment are shown in figure 4.7. The first three panels of this figure are reproduced from figure 3-7 in the previous chapter because the experiments were the same. The results of the halo assay for the control sperm nuclei was the predicted halo fluorescence around the hooked shaped hamster sperm nuclei which were made of DNA-loop domains attached at their bases to the nuclear matrix at MARs as seen in figure 4.7A. The Triton X-100-treated spermatozoa appeared to retain most of their DNA loop domains and had the characteristic halo around the nuclei (figure 4.7D).

The halo assay data does not support the model's claims that Triton X-100 treatment will cut the DNA from the sperm matrix since most of the DNA appeared to remain associated with the nuclear matrix. One possible explanation for this was that the DNA has been cleaved in the Triton X-100 treated condition, but it was not able to get released from the DNA-loop domain attachment regions. Another possible explanation is that only a few of the TX treated spermatozoa digested their DNA, but those that did release their
DNA loop domains into solution. We tested this hypothesis in the next section.

4.6. A Modified Halo Assay shows TX-100 Treated Spermatozoa do Appear to Release DNA Loops into Solution

I conducted an experiment to try and determine why both DNase I and Triton X-100 treated spermatozoa when run on 1% PFGE after overnight treatment demonstrated DNA loop-sized 48.5 kb fragments, however, loops were only visible in the Triton X-100 treated samples and not in the DNase I treated samples when the original halo assay was conducted in section 4.6.

As described in the methods, spermatozoa were treated with Triton X-100 or DNase I, and then extracted with 2 M NaCl and DTT to remove the protamines. These samples were then centrifuged, and the pellets and supernatants collected, and analyzed independently by PFGE. If the 48.5 kb sperm DNA fragments were released from the nuclear matrices, they should be present in the supernatant. However, if the DNA remained associated with the nuclear matrices, then the DNA would be found in the pellets.

Spermatozoa were treated in various ways and were extracted with high salt and DTT to remove the protamines, then stained with ethidium bromide to reveal DNA. A) In control sperm nuclei, this treatment results in a halo of fluorescence surrounding the sperm nucleus, made of DNA-loop domains attached at their bases to the nuclear matrix. Spermatozoa treated with either 3 µg/ml (B) or 300 µg/ml DNase (C) released all of their DNA into solution. D) Spermatozoa treated with 0.25% TX overnight retained most of their DNA-loop domains. This figure was previously published as Figure 10 in Sotolongo et al., 2003.
The prediction was that the Triton X-100 treated total and pellet should show the smear on pulse field, but not the supernatant since the model was that the DNA loop domains remained attached to the matrix (pellet) and did not float away into the supernatant when halo assays were conducted since the halo was visible with ethidium bromide staining under a microscope. The prediction for the DNase I treated samples was that the total and supernatant would show smears of DNA since the nuclear matrix showed no halos when viewed under a microscope after doing a halo assay, therefore the loop domains were predicted to go into the supernatant and not remain attached to the pellet (matrix portion) after DNase I treatment.

The results for the Triton X-100 treated samples were that the total gave a smear in the DNA loop-sized range. The pellet gave a very small smear also in this range and the supernatant gave the strongest lower range smear. These results did not confirm those of the halo assay described in the previous section. Some of the loop domains remain attached to the nuclear matrix and form halos (pellet) in the Triton X-100 condition but most of the DNA went into the supernatant. We suggest that this experiment more accurately reflects the true condition, since this experiment tests the entire population of sperm within the experiment.
The results for the DNase I treated samples was that the total and supernatant gave a very light smear; however, the pellet gave no smear at all. This part of the donut loop model verified the loop domains going into the supernatant and not remaining attached to the nuclear matrix as visualized by the halo assay by no halo visibility at all. In these assays, the amount of DNA in the total and in the supernatant fractions was less than in the TX treated cells. We believe this to be the result of residual DNase I activity that digests the more vulnerable, protamine extracted DNA after the salt extraction (figure 4.8).

In conclusion, this modified halo assay is more sensitive because it examines all of the sperm attached to the nuclear matrices. This modified halo assay shows that Triton X-100 is actually releasing loops from the attachment sites after all.
The Donut-Loop Model for DNase I and Triton X-100 experiments indicated that in both models the cleaved DNA loop-sized fragments were capable of leaving the nuclear matrix. The control total sample was treated with 0.25% Triton X-100 and 10 mM MgCl₂, then incubated overnight at 37 degrees and plugged. A second sample was treated first like the total and then was further treated with 4M NaCl, 10 mM DTT and halos were formed. Then samples were centrifuged for 10 minutes at 5 rpm and the pellets and supernatants were collected and plugged separately. The plugs were electrophoresed in PFG agarose and stained with ethidium bromide.

Figure 4.8. The donut loop model for DNase I and Triton X-100 treatments.
4.7. Dithiothreitol did not increase Triton X-100 Degradation in hamster sperm

Experiments conducted by Szcygiel et al., (2002) in mice spermatozoa found that treatment with both nonionic detergent Triton X-100 and dithiothreitol (DTT) caused chromosomal breaks when these spermatozoa were used for (intracytoplasmic sperm injection (ICSI). The use of both reagents together resulted in severe chromosomal breaks in the paternal pronuclei, while neither reagent, alone, caused them. Most endogenous nucleases were calcium and magnesium dependent and the addition of chelators such as EGTA and EDTA should have prevented the chromosomal breakage being caused by a nuclease. When EDTA or EDTA and EGTA together were added after Triton X-100 and DTT treatment, the chromosomal breaks were still evident, although reduced. This suggested the presence of an endogenous nuclease after disruption of the plasma membrane by detergent and disruption of the protamines by DTT.

Since these results with the synergistic damaging effects of DTT and Triton X-100 were done in mice, I decided to see if another mammalian species such as hamster spermatozoa would give similar results of DNA fragmentation on PFGE agarose. A time course study was done using 0.25% Triton X-100 and 20 mM of MgCl₂ alone in hamster sperm in 1X PBS or with 10 mM DTT
added. The samples were electrophoresed on 1% PFGE agarose and stained with ethidium bromide for visualization.

In figure 4.9, compares the sperm DNA degradation pathway between spermatozoa treated with TX alone or with TX + DTT, from 0 to 20 hours. The 50 kb digestion pattern was only evident after overnight (20 hr) treatment in both treatments. These results suggest that the chromosomal breakage seen by Szczygiel et al., (2002) do not correspond to the PFGE fragmentation pattern that I have described in this dissertation. I would predict that different mechanisms direct these two activities.

4.8. Calcium and magnesium activated hamster sperm nuclease more than magnesium alone.

Several studies indicated that endonucleases depended on calcium and/or magnesium (Yakovlev et.al., 1999; Yakovlev et al., 2000; Lagarkova et al., 1995) existed in somatic cells that cleaved DNA into large loop-sized fragments ranging between 20-100 kb. Other studies had suggested that the absence of calcium and magnesium from media for the mouse sperm head improved chromosomal stability (Kuretake et al., 1996; Tateno et al., 2000). Based on the role of calcium and/or magnesium in prior somatic cell research as well as the mouse sperm head research, the existence of a nuclease that
4.9. Dithiothreitol treatment did not enhance the release of loop-sized fragments in Triton X-100 treated hamster spermatozoa.

Spermatozoa were treated with TX-Mg or TX-Mg + DTT for the following times: various times: lanes 2 and 9 for 0 minutes, lanes 3 and 10 for 15 minutes, lanes 4 and 11 for 1 hr., lanes 5 and 12 for 2 hrs., lanes 6 and 13 for 3 hrs., lanes 7 and 14 for 4 hrs., lanes 8 and 15 overnight. The samples were treated for the times indicated, then electrophoresed on PFGE, as indicated. The first lane contains the lambda 48.5 kb ladder. Lanes 2-8 were Triton X-100 treated and Lanes 9-15 were Triton X-100 and DTT treated sperm. Lanes 8 and 15 showed sperm DNA fragmentation did not occur until overnight incubation. This figure was previously published as Figure 7 in Sotolongo et al., 2003).
would depend on either calcium, magnesium or both synergistically hamster sperm was detected in the following experiments.

Hamster spermatozoa were suspended in PBS and 0.25% Triton X-100 and treated with either 10 mM CaCl$_2$ or 10 mM MgCl$_2$. The samples were incubated for various time points ranging from immediate (0 minutes) up to overnight incubation. After the incubation, the sperm were embedded in PFGE agarose, electrophoresed, and stained with ethidium bromide. The results suggested that the nuclease was slightly more sensitive to calcium than to magnesium. Magnesium induced DNA fragmentation around the 4 hour mark (figure 4-10, lane 14), while calcium induced DNA fragmentation within 1 hour (figure 4-10, lane 4).

I next tested whether calcium and magnesium, together, would activate the nuclease more efficiently than either cation alone. Spermatozoa were treated with 0.25% Triton X-100 and various concentrations of both CaCl$_2$ and MgCl$_2$ for various time points. After incubation the plugs were embedded in PFGE agarose, electrophoresed, and stained with ethidium bromide and were shown in figure 4.11. The results showed no DNA digestion for control lane 3 which was plugged immediately. In the case of the 5 mM, 10mM and 20 mM treatments of calcium and magnesium, DNA digestion began at 15 minutes in all three cases (lanes 4, 8, and 12). In all three cases, overnight treatment led to
4.10. Hamster sperm nuclease was activated by calcium and magnesium alone.

Hamster sperm were isolated and treated with 0.25\% Triton X-100, then incubated with 10 mM calcium alone or 10 mM magnesium alone at different time points. The spermatozoa were embedded in agarose, incubated with lysis buffer and electrophoresed on a pulse-field gel. Sperm DNA was digested into loop sized fragments within 1 hour with calcium (lane 4) and 4 hours for magnesium (lane 14).
4.11. Hamster sperm nuclease was activated by calcium and magnesium together.

Hamster sperm were isolated and treated with 0.25% Triton X-100, then incubated with various concentrations of calcium and magnesium together at the following time points: lanes 4, 8, and 12 for 15 minutes, lanes 5, 9, and 13 for 1 hour, lanes 6, 10, and 14 for 4 hours, and lanes 7, 11, and 15 overnight. The spermatozoa were embedded in agarose, incubated with lysis buffer and electrophoresed on a 1% pulse-field gel. Sperm DNA was digested to loop-sized fragments within 15 minutes of treatment with calcium and magnesium together. The control in lane 3 was plugged immediately without treatment.
complete digestion of sperm DNA (lanes 7, 11, and 15). The results suggested that the endogenous nuclease did respond more efficiently to both cations.

4.9. Sperm nuclease is localized in the nucleus.

The identity of the specific sperm nuclease that I described in these experiments has not been completed thus far. In somatic cells, several enzymes had been discovered that cleave DNA into loop-sized fragments. These enzymes might be related to the sperm nuclease. One was called DNAS1L3 or DNase gamma (Yakovlev, 1999). DNAS1L3 was isolated from bull semen and has been found to be a nuclease associated with chromatin. Another somatic cell nuclease that was identified was DFF40, which was activated after proteolytic cleavage of a special binding protein called DFF45 (Zhang et al., 1998). Topoisomerase II in somatic cells was the third possibility for the sperm nuclease. Topoisomerase II (Topo II) has created double-stranded breaks in DNA and was found during apoptosis to cleave at the MARs (Li et al., 1999; Solovyan et al., 2002). At least one of the enzymes described above digest chromatin into loop-sized fragments and was active at the bases of the DN loop domains (Gromova et al., 1995).

Since the hamster sperm used for these experiments were always obtained from the epididymus, I needed to rule out that the nuclease might be
located in the epididymal fluid rather than the sperm nucleus. In order to localize the sperm nuclease, I isolated hamster sperm nuclei using sucrose step gradients described in Chapter 2 in the methods. I tested for the presence of the sperm nuclease as previously described in figure 4.10 with one set incubated with 5 mM CaCl$_2$ and 5 mM MgCl$_2$, another with 10 mM CaCl$_2$ and 10 mM MgCl$_2$, and a third set with 20 mM CaCl$_2$ and 20 mM MgCl$_2$ for various time points. The samples were embedded in 1% PFGE agarose, electrophoresed and stained with ethidium bromide.

The data are shown in figure 4.12. Low concentrations of CaCl$_2$ and MgCl$_2$, 5 or 10 mM each, sperm DNA were digested to loop sized fragments. However when 20 mM of both cations was used, there was no digestion. This is similar to what has been reported for other Ca/Mg nucleases which are inhibited by high concentrations of divalent cation. This is explored more fully in the next section. Finally, the sperm nuclease was shown by these experiments not to be in the epididymal fluid but rather in the sperm nuclei themselves since calcium and magnesium activation at low concentrations cleaved DNA into loop-sized fragments.
Hamster sperm nuclei were isolated by sucrose density gradients. The sperm nuclei were tested for their nuclease activity by their ability to digest DNA into loop-sized fragments after treatment with calcium and magnesium, which often activate nucleases. Lane 1 showed the 48.5 kb lambda DNA ladder and in lane 2 was the low range PFGE ladder. Lanes 3, 6, and 9 were treated for 1 hour. Lanes 4, 7 and 10 were treated for 4 hours. Lanes 5, 8 and 11 were treated overnight. The treatments were for the 5 mM, 10 mM and 20 mM concentrations of magnesium and calcium treatments shown for the respective lanes above. The 5 mM and 10 mM treatments, respectively, showed DNA cleavage into loop-sized fragments. High 20 mM concentrations of calcium and magnesium (lanes 9-11) showed no fragmentation at all.

Figure 4.12. The hamster sperm nuclease is present in the nucleus and not the epididymal fluid.
4.10. High concentration of calcium and magnesium may inhibit sperm nuclease

The experiments in the previous section suggested the possibility that high concentrations of divalent cation would inhibit the sperm endogenous nuclease. I, therefore, designed an experiment to test this hypothesis. In figure 4.12, the 20 mM concentrations of magnesium and calcium did not show digestion of DNA into loop-sized fragments, therefore, more experiments were conducted with the hamster nuclei to see if higher concentrations of divalent cations might inhibit sperm nuclease activity.

Hamster sperm nuclei that had been purified with sucrose density gradients were incubated for 15 minutes, 4 hours and overnight with calcium chloride and magnesium chloride. The first set was incubated with 5 mM CaCl$_2$ and 5 mM MgCl$_2$, the next set with 10 mM CaCl$_2$ and 10 mM MgCl$_2$, and the final set with 20 mM CaCl$_2$ and 20 mM MgCl$_2$. As shown in figure 4.13, the higher concentrations calcium and magnesium did inhibit the nuclease reaction.
Figure 4.13. High concentrations of calcium and magnesium might inhibit hamster sperm nuclease in the sperm nuclei.

Hamster sperm nuclei were isolated using sucrose density gradients and then incubated with various concentrations of magnesium and calcium at various time points. At low concentrations of calcium and magnesium, DNA is cleaved into loop-sized fragments quickly (lanes 3-5) but at higher concentrations of 10 mM or 20 mM (lanes 6-8 or 9-11 respectively) cleavage occurred at the 15 minute time point (lane 6 and 9) and longer time points of 4 hours and overnight showed no cleavage of DNA fragments. The lambda ladder was placed in lanes 1 and 13 and the low range ladder was placed in lanes 2 and 14. Low concentrations of 5 mM calcium and 5 mM magnesium cleaved DNA into fragments as quickly as the 15 minute mark and continue in the 4 hour and overnight conditions (lanes 3-5). Slightly higher concentrations of 10 mM calcium and 10 mM magnesium cleaved DNA into fragments quickly at the 15 minute mark but inhibited at the 4 hour and overnight time points (lanes 6-8). The highest concentration of 20 mM each of magnesium and calcium also gave DNA fragments at the 15 minute mark but the smear was far weaker than at the lower concentrations and inhibition occurred at the higher time points of 4 hours and overnight (lanes 9-11). The control was sperm nuclei in buffer only without calcium or magnesium as seen in lane 12.
4.11. EDTA and EGTA inhibit endogenous nuclease

Prior experiments involving mouse sperm used for ICSI, chelating agents EGTA and EDTA when added to isolated sperm media before treatment with detergent, Triton X-100 and DTT, preserved sperm chromosomal integrity when compared to conditions when the chelators were not present (Szycygiel et al., 2002). Since the prior experiments conducted by me indicated that the endogenous nuclease was activated by calcium and magnesium, the prediction would be that chelators such as EGTA and EDTA would interfere or slow down the activity of an endogenous nuclease.

The hamster sperm were treated with 0.25% Triton X-100 to solubilize the plasma membrane and then 10 mM of MgCl₂ and/or 10 mM of CaCl₂ were added or not to the samples. In conjunction with the calcium and magnesium treatments to induce endogenous nuclease activity, 2 mM of EDTA and/or 2 mM of EGTA were added or omitted from the samples. All samples were incubated overnight, embedded in agarose, incubated with lysis buffer, electrophoresed and the 1% PFGE agarose was stained with ethidium bromide.

Figure 4.14 shows that EDTA and EGTA prevented the degradation of sperm DNA. The presence of EDTA with MgCl₂ also prevented the degradation, but EGTA with CaCl₂ could not. This may be because the nuclease was more sensitive to calcium than to magnesium (figure 4.12). These
Inhibition of prior nuclease activity activated by TX-100 and magnesium were seen in lanes 5, 6, 7, and 10 in this 1% pulse-field gel agarose stained with ethidium bromide. Lanes 1 and 12 had the lambda DNA ladder and lane 13 had the low range PFGE ladder. In lane 2, overnight incubation with Triton X-100 alone induced DNA loop-sized cleavage. In lane 3, magnesium alone was added and also induced DNA loop-sized cleavage. In lane 4, calcium alone was added and induced DNA loop-sized cleavage. In lane 5, EDTA was added and inhibition of nuclease activity occurred. In lane 6, magnesium treatment followed by EDTA resulted in an inhibition of the DNA loop-sized cleavage by the chelator. In lane 7, EGTA alone was added and inhibition of nuclease activity occurred. In lane 8, calcium treatment was followed by EGTA but EGTA failed to prevent the inhibition of the nuclease activity when calcium was involved. In lane 9, magnesium and calcium alone were added and they induced DNA loop-sized cleavage. In lane 10, both EDTA and EGTA were added and they both can inhibit nuclease activity by complete inhibition. In lane 11, magnesium and calcium treatments were followed by EDTA and EGTA.
results showed that both EDTA and EGTA together or separately can easily prevent DNA loop-sized cleavage in Triton X-100 treated sperm as well as in magnesium-treated sperm. Both chelators EDTA and EGTA inhibit the endogenous nuclease activity induced by Triton X-100 and magnesium treated samples. EGTA and EDTA both inhibited the nuclease.

4.12. Conclusions

The presence of an endogenous nuclease after treatment with Triton X-100 in sperm DNA into loop-sized fragments was confirmed with the Triton X-100 versus DNase I treatments. The use of DTT and Triton X-100 in chromosomal studies for ICSI demonstrated damage, however, in the hamster sperm experiments conducted with PFGE, DTT had no effect on increasing Triton X-100 induced degradation. Calcium and magnesium had a synergistic effect in activating the hamster sperm endonuclease in a similar manner to calcium and magnesium activated nucleases in somatic cells. The nuclease was found to be localized in the sperm nucleus and to be inhibited by high concentrations of calcium and magnesium. EDTA and EGTA, chelators, were found to inhibit endogenous nuclease activation by Triton X-100 as well as calcium with magnesium activated nuclease activity. The halo analysis of Triton X-100 sperm indicated that all of the DNA loop domains remained
attached to the nuclear matrix, since a halo was visible under the microscope after treatment with salt and DTT. A modified halo experiment was done that looked at separating the pellet, which should have the nuclear matrix portion, from the supernatant, revealed that the Triton X-100 treated sperm did go into the supernatant.
Chapter 5

Endogenous Nuclease in Human Spermatozoa

In Chapter 3 the experimental model for chromatin organization in mammalian sperm in animal models such as mouse, hamster and human was proposed. This model was called the Donut-Loop model because it proposed that each protamine bound toroid (donut) was a single DNA loop domain. In Chapter 4 it was demonstrated that hamster spermatozoa have an endogenous nuclease that cleaves relatively open chromatin areas predicted by the Donut-Loop model. In this chapter, I examined how the principles of sperm chromatin structure and function that I found in the hamster applied to human spermatozoa.

5.1 Human sperm has a Donut-Loop Model organization for its chromatin.

Ejaculated human spermatozoa were treated with exogenous DNase I. These experiments were conducted to test whether human sperm chromatin was also organized into Donut-Loops in a manner similar to that of the hamster (and mouse, unpublished data). As in my previous experiments in Chapter 3, human spermatozoa were treated with 0.25% TX-100, and then incubated with
DNase I in the presence of 20 mM MgCl₂ as was done previously with the hamster experiments. Every experiment was done at least three times for accuracy. The results of the experiments were that various concentrations of DNase I treatment of human sperm can cause the 50 kb digestion pattern (figure 5.1). However, in contrast to hamster sperm chromatin which required at least an hour to obtain this pattern, human sperm chromatin was digested within of 15 minutes. This result shows that human sperm is much more sensitive to DNase I than hamster sperm. It also supports the concept that the Donut-Loop model organization exists not just in hamster sperm but also in human sperm since we saw the expected 50 kb loop-sized fragments on the PFGE agarose. Much longer incubation times with DNase I caused more complete digestion in human sperm than in comparison with the hamster sperm. These results support our prediction that the Donut-Loop model is a basic feature of mammalian sperm chromatin.

Initially all of the ejaculated human sperm samples used in my experiments were stored frozen at -20°C. This was because the clinic that provided the samples was not originally geared up to provide fresh samples. However, at least one study conducted by Chatterjee and Gagnon (2001) provided strong evidence that oxygen free radicals are produced during
Fresh and frozen Percoll-purified human sperm were treated with 0.25% Triton X-100 and 20 mM MgCl₂ and then increasing concentrations of DNase I for 15 minutes at 37 degrees Celsius. The samples were loaded onto a 1% PFGE and electrophoresed. The Fresh samples were in lanes 3 to 8 and the frozen in lanes 9 to 14. For the gel, the DNase I concentrations were: lanes 3 and 9, 0 μg/ml; lanes 4 and 10, 0.01 μg/ml, lanes 5 and 11, 0.1 μg/ml, lanes 6 and 12, 1.0 μg/ml and lanes 7 and 13, 10 μg/ml. The control lanes which were plugged with no treatment at all were lanes 8 and 14. Lanes 1 and 15 has λ, Lambda phage molecular weight marker in 48.5–kb increments; lane 2, MW, low molecular weight marker.
freezing and thawing of bovine spermatozoa and that these reactive oxygen species may be a cause for damaging plasma membranes, reducing motility and reducing fertilizing ability of spermatozoa. Other studies have shown strong support for data that links oxidative DNA damage with sperm function defects and male infertility (Kodoma et al., 1997; Shen et al., 1999). Although these studies do not prove that in human sperm freezing and thawing could directly cause the activation of a nuclease which in turn causes the 50 kb DNA fragmentation pattern, these studies prompted me to investigate whether freeze-thawing had any influence on digestion of DNA into loop sized fragments in human sperm. This was not an issue in the hamster experiments because these studies were done with fresh hamster sperm. The results of fresh versus frozen percoll treated epididymal sperm were shown and summarized in Figure 5.1.

In Figure 5.1 fresh percoll treated epididymal sperm (lanes 3-8) were compared to -20° Celsius frozen percoll treated epididymal sperm (lanes 9-14) from the same donor. Both sets were treated with 0.25% Triton X-100, 20 mM MgCl₂ and various concentrations of DNase I for fifteen minutes at 37° Celsius. In all lanes except for the ones that were controls (lanes 8 and 14) and no DNase I, neither Triton X-100, nor additional MgCl₂ was added to the sperm in HTF, there were loop-sized fragments released. Therefore, freezing and
thawing had no effect on DNA fragmentation versus fresh. The treatment lasted for a shorter interval than in the hamster (15 minutes) and yet released loop-sized fragments; therefore the mechanism in the human sperm was activated at a much faster rate than in the hamster model. Finally, it appeared that for the DNase I sensitivity at these concentrations, there were no major differences between fresh and frozen Percoll purified human sperm.

5.2 Human sperm has an endogenous nuclease.

I next tested whether human spermatozoa, like those of hamster, contained an endogenous nuclease. Two different experiments, that were controls for the DNase I experiments described above, suggested that human sperm do have an endogenous nuclease similar to hamster. First, in figure 5.1, in lanes 3 and 9, which had no DNase, but did have 0.25% Triton X-100 and 20 mM MgCl₂, DNA fragmentation into loop-sized 50 kb fragments was evident, which supports the hypothesis that an endogenous nuclease exists in sperm. In a preliminary experiment without DNase I, which only used Triton X-100 and magnesium chloride, DNA was also cleaved into loop-sized fragments of approximately 50 kb after 4 and 24 hrs (figure 5.2). These experiments strongly indicate the presence of an endogenous sperm nuclease, as in the hamster.
5.2 Human sperm has an endogenous nuclease when treated with TX-100.

Frozen percoll-purified human sperm were treated with 0.25% Triton X-100 and 20 mM MgCl₂ at various time points at 37 degrees Celsius. The samples were loaded onto a 1% PFGE agarose and electrophoresed. In lane 1 was λ, Lambda phage molecular weight marker in 48.5-kb increments. In lane 2, the time course was 0 minutes, in lane 3, 4 hours and in lane 4, 24 hours.
In my attempt to focus on what possible pathways could be involved in this endogenous sperm nuclease activation, several experiments (Razin, 1995; Smulson, 1999) pointed towards calcium and magnesium dependent endonucleases that cleaved DNA into fragments similar to loop-size. In several experiments that I conducted with hamster sperm, described in Chapter 4, the addition of calcium and magnesium cleaved sperm DNA very quickly just as would be expected by a calcium and magnesium dependent endonuclease since all of the human sperm were provided to us in HTF (human tubal fluid) which contains calcium already, these data are consistent with a nuclease in human spermatozoa that resembled that of the hamster in its requirement for both cations.

Human epididymal sperm samples that were frozen and had undergone a standard wash were treated with 0.25% Triton X-100 and MgCl₂ for 0 minutes, 4 hours and overnight (lanes 2, 3 and 4 respectively). In figure 5.2., lanes 3 and 4 which were incubated for at least 4 hours and overnight with detergent and magnesium in HTF diluted sperm, showed loop sized fragments. The control in lane 2 showed no fragmentation at all. The presence of loop-sized 50 kb fragments in lanes 3 and 4 without DNase I and only Triton X-100 and MgCl₂ treatment provided support for the existence of an endogenous nuclease in human sperm. In the prior figure 5.1, there was a fifteen minute treatment with
Triton X-100 and MgCl₂ treatment that resulted in loop-sized fragments, however, the sperm in that condition were percoll gradient isolated and in this case, they were simply washed twice in HTF buffer. Human sperm samples that have undergone standard washing behave similarly to hamster sperm and its endogenous nuclease was activated at a slower rate than those that were isolated by percoll gradient.

5.3 Sensitivity of Human Sperm to DNase I and of the Endogenous Nuclease to Percoll Purification.

The work described above suggested that human sperm chromatin had two similarities with hamster sperm chromatin; it was organized into Donut-Loops and it had an endogenous nuclease. However, there were differences when working with human sperm, also. First, the chromatin appeared to be more sensitive to exogenous DNase I than hamster, and second, I had four different preparations of human sperm from which to choose, frozen or fresh and total ejaculated sperm (washed) vs. Percoll-purified sperm (Percoll). In this section, we tested the sensitivity of human sperm chromatin to exogenous DNase I by using very low concentrations of DNase I, and compared two of the four preparations of human spermatozoa.
Washed and Percoll-purified human sperm were treated with 0, 0.01, 0.10 and 3 μg/ml DNase I for 15 minutes at 37°C with Triton X-100 and MgCl₂ (figure 5.3). In lanes 1 and 5 the human sperm controls with lacked DNase I, with DNase I, loop size fragments resulted, however, in the case of Percoll isolated sperm treated with DNase I, there was complete digestion of the DNA fragments. The endogenous nuclease worked more completely and faster in the case of Percoll treated sperm than total sperm since even small concentrations of DNase were enough to activate the complete digestion in the case of Percoll treated in lanes 6 through 8 versus total (lanes 2-4). In the case of hamster sperm experiments described in Chapter 3, longer periods of incubation with DNase I gave strong loop-sized fragments, which supported the hypothesis of the existence of an endogenous nuclease. In the case of total human sperm treated for periods as long as 5 days did not cause complete digestion of the 50 kb DNA fragments. The Percoll treated human sperm in comparison to the hamster sperm has a nuclease that was more easily turned on than that of the hamster sperm.

5.4 Frozen Percoll purified and total washed human sperm comparison.

Since the prior experiments conducted were done with percoll treated sperm samples (see Figure 5.1), I wanted to see if there would be a difference
between the purified percoll and washed treated human sperm samples when both were frozen. The experiment described above in which frozen, washed sperm was compared with frozen Percoll sperm (figure 5.3) indicated that some differences might exist between these two preparations. The purified percoll gradient treated human sperm, which is free of seminal fluid components, and the washed or total human sperm, which still retained seminal fluid components, were compared in order to see if the nuclease might be found more in the sperm itself (percoll) or in seminal fluid and sperm (washed). In this experiment I also controlled for artifacts of different donors by using matched sets of washed and Percoll purified sperm from the same donors.

Frozen Percoll purified and total washed human sperm were incubated with nothing (figure 5.4, control in lanes 2, 5, 9, and 12), no DNase I but 0.25% Triton X-100, 5 mM MgCl2 (lanes 3, 6, 10 and 13) or 0.01 µg/ml of DNase I treatment (figure 5.4, lanes 4, 7, 11, 14). In this case, the samples were taken from the same donor to rule out the effects of donor variability. In all conditions, loop-sized fragments occurred in the case of the frozen percoll and total washed human sperm. These results indicated that an endogenous nuclease existed that was activated by DNase I or by the very process of collection in HTF since the control also showed degradation. The reason these
Total and percoll-purified human sperm were treated with 0.25% Triton X-100 and 20 mM MgCl₂, and then increasing concentrations of DNase I for 15 minutes at 37 degrees Celsius. The samples were loaded onto a 1% PFGE and electrophoresed. The total samples were in lanes 1 to 4 and the percoll samples were in lanes 5 to 8. For the gel, the DNase I concentrations were: lanes 1 and 5, 0 μg/ml; lanes 2 and 6, 0.01 μg/ml, lanes 3 and 7, 0.1 μg/ml, lanes 4 and 8, 3.0 μg/ml. λ, Lambda phage molecular weight marker in 48.5-kb increments; MW, low molecular weight marker.

Figure 5.3. Sensitivity of Human Sperm to DNase I and of the Endogenous Nuclease to Percoll Purification
Two separate matched sets of frozen percoll purified (lanes 4-6 and 11-13) were matched with total washed human sperm (lanes 1-3 and 8-10), respectively. Frozen percoll purified and total washed human sperm were treated with 0.25% Triton X-100 and 5 mM MgCl₂ and then either no DNase I (lanes 2, 5, 9, and 12) or 0.01 μg/ml of DNase I for 15 minutes at 37 degrees Celsius (lanes 3, 6, 10 and 13). The control samples were plugged immediately without treatment and were found in lanes 1, 4, 8 and 11). The samples were loaded onto a 1% PFGE and electrophoresed. The λ, Lambda phage molecular weight marker in 48.5-kb increments; lane 7, LR, low molecular weight marker.
results could not confirm or deny the sensitivity of human chromatin to DNase I. This is not very surprising, since the amount used was 0.01 μg/ml, a concentration of DNase I that will not digest chromatin in somatic cells within 15 minutes.

These results also suggested that the endogenous nuclease in human spermatozoa was very easily activated, even at the zero time point in this case. The endogenous nuclease in frozen sperm, whether it is the total ejaculate, or Percoll purified (figure 5.5) has been activated, possibly by membrane permeabilization. We tested this more thoroughly in the following section.

5.5 The Endogenous Human Sperm Nuclease is Not Activated in Fresh, Percoll Purified Sperm.

The experiments conducted with percoll and washed frozen human sperm did not show a difference with regard to DNA fragmentation pattern. The human sperm nuclease was activated by freezing in both conditions. The nuclease activation was therefore not dependent upon the addition (washed) or exclusion (percoll) of seminal fluid components, therefore the difference in activation of the nuclease might be found by comparing fresh versus frozen percoll treated human sperm. I wanted to see if the endogenous nuclease would be activated more in fresh or frozen human sperm. The results appear
Figure 5.5 Human percoll and total human sperm matched set have an endogenous nuclease.

Lanes 1 through 3 had total washed human sperm and lanes 4-6 had percoll purified human sperm. The total washed human sperm control sample was plugged immediately without treatment and was found in lane 1 and the percoll control sample was in lane 4. The total washed human sperm that were treated with 0.25% Triton X-100 and 5 mM MgCl₂ and then either no DNase I for 15 minutes at 37 degrees Celsius was in lane 2 and the percoll treated equivalent sample was in lane 5. The total washed human sperm sample treated with 0.25% Triton X-100 and 5 mM MgCl₂ and 0.01 μg/ml of DNase I for 15 minutes at 37 degrees Celsius was in lane 3 and the percoll treated equivalent sample was in lane 6. The samples were loaded onto a 1% PFGE and electrophoresed. The λ, Lambda phage molecular weight marker in 48.5-kb increments; LR, low molecular weight marker.
to be that the human sperm have endogenous nuclease that are activated more in frozen sperm as shown in figure 5.6.

Two pooled samples that were fresh or frozen percoll isolated purified human sperm were treated with or without magnesium, Triton X-100 and EDTA. The samples that had EDTA resisted DNA degradation. The samples that had magnesium and Triton X-100 but lacked EDTA in the fresh condition degraded rapidly. The samples that were the control, magnesium treated or Triton X-100 treated samples without EDTA in the frozen condition degraded rapidly. Frozen samples had the nuclease activation occur in the presence of either detergent or the magnesium cation; however, fresh samples lacking membrane permeabilization due to the absence of Triton X-100 did not digest their DNA. EDTA definitely offered protection from DNA degradation.

5.6 Conclusions

Human sperm chromatin is organized into Donut-Loops and has a similar nuclease as that found in hamster spermatozoa, which can also cleave its DNA into loop-sized fragments. Human sperm can be induced to digest its DNA within 15 minutes of incubation with DNase I, therefore it is more
Figure 5.6. Human sperm have endogenous nuclease that is activated more in frozen sperm (one gel, matched set).

Samples from two donors were Percoll purified and separated into two aliquots. One aliquot (Fresh) was further separated into five samples, and treated with Magnesium, TX100, or EDTA alone or Triton X-100 with Magnesium or control as indicated. The other sample was frozen overnight at -20°C, then thawed and treated in the same way. Spermatozoa were then embedded in agarose, treated with SDS and Proteinase K, and electrophoresed on pulse-field gel electrophoresis.
sensitive to DNase I treatment than hamster sperm chromatin which usually requires longer incubation times. Longer incubation times with the exogenous DNase I caused more digestion than in the hamster nuclei. Human sperm has an endogenous nuclease that can be activated by freezing overnight at -20°C and thawing as well as in fresh samples. The presence of a cryoprotectant such as EDTA protected the human sperm from digestion into loop-sized fragments. These results show that human spermatozoa behave very similarly to hamster spermatozoa and also organize their chromatin as in the Donut-Loop model.
Chapter 6.

Discussion.

The dissertation has proposed that the Donut-Loop Model for sperm chromatin structure is found in both hamsters and humans as 50 kb loop-sized fragments. Treatment with an exogenous nuclease, DNase I, was used to show that DNA fragmentation is faster in human (15 minutes) than in hamster sperm (1 hour-overnight). An endogenous nuclease was activated after TX100 treatment in both species. The combination of calcium and magnesium activated endonuclease induce cleavage of sperm DNA better than just magnesium alone. EDTA/EGTA, as well as high concentrations of calcium and magnesium, inhibits the endogenous nucleases. The halo assay, the modified halo assay, sperm motility assay and the nuclease localization experiments in chapters 3 and 4 showed that the nuclease is in the nucleus, DNase I cleaves and releases the loop-sized fragments from the nuclear matrix. Like DNase I treated spermatozoa, TX-100 treated spermatozoa become immotile and releases loop-sized fragments.

Human sperm is similar to hamster sperm in that it also has an endogenous nuclease but is much more sensitive to DNase I treatment than hamster sperm in showing DNA degradation. The endogenous nuclease is not
activated in fresh percoll purified sperm, however, frozen percoll purified and total washed is activated to form loop-sized DNA fragments. The frozen human sperm appear to activate their nuclease by membrane permeabilization as well as via calcium and magnesium-dependent nuclease activation. The sperm media HTF in which the sperm are frozen happens to have calcium already in it, therefore activation of the nuclease may begin quickly. Also freezing and thawing allows for oxygen free radicals to damage the plasma membranes and decrease both motility and fertility (Chatterjee and Gagnon, 2001). EDTA also protects human sperm DNA from degradation like in hamster sperm.

The experiments conducted have helped to test the proposed Donut-Loop model and to show that it is correct. One of the functions of the Donut-Loop model by testing for a nuclease that digests the DNA at the sites of attachment to the nuclease has also been examined. These experiments, however, have opened up several important questions and possible explanations that will be discussed in this section regarding the purpose and functions for the sperm chromatin Donut-Loop Model and the nuclease(s) involved within the general scope of science as well as within the more defined scope of reproductive biology.
6.1 Sperm Chromatin Donut-Loop Model and its Role in Human Embryogenesis, Apoptosis, Fertility and Cancer

Although chromosomal anomalies are well documented in pregnancy failures (Lamond et al., 2003), not much is known about the paternal contribution to human failed pregnancies. The role of the Donut-Loop Model and how sperm undergoes DNA fragmentation can, therefore, provide great insight into issues of fertility, embryogenesis, apoptosis and diseases such as cancer. A study by Lamond et al., (2003) found that chromatin damage (DNA strand breaks) were apparent in sperm intended for use with the ICSI technique that had been kept in IVF media. These sperm exhibited not just DNA strand breaks but also decreased motility, mitochondrial damage (antioxidants that blocked mitochondrial damage blocked chromatin damage as well), reduced sperm membrane permeability and hypersensitivity of specific genes such as tumor suppressor (VHL), neurotransmitter (NOS1), oncogene (BRCA1) to hybridize with DNA fragments from the human sperm and perform endogenous cleavage within these genes.

I found that I had similar results in my work to those conducted by Lamond et al., (2003) in his study. The experiments conducted with live hamster sperm media in Chapter 4 mimic some of his results on human sperm. In both cases decreased sperm motility and DNA fragmentation in IVF media
or live sperm media occur. In chapter 4 I discussed the use of TX-100 in my experiments with the live hamster sperm media and as a result all motility ceased, severe damage to the sperm membrane occurred and DNA fragmentation was found to occur in loop-sized fragments that supported the Donut-Loop Model. The human sperm experiments that I conducted and described in chapter 5 also show that frozen percoll and total washed sperm can release loop-sized fragments when their nuclease is activated by either DNase I or possibly the calcium in the HTF media. Even the control human sperm degraded into loop-sized fragments when it was in HTF or treated with calcium and magnesium. Based on my experiments, it is logical to suggest that sperm have a mechanism to destroy its DNA if the mitochondria, plasma membrane, or chromatin structure are damaged or if some signal activates the nuclease due to DNA loop domain breaks. A disrupted loop domain could produce an interruption in the proper stage-specific formation of loops and DNA sequence which is important and will be inherited by the embryo.

We hypothesized (Sotolongo and Ward, 2001) that the organization of the DNA into loop domains provided sperm with a level of heritable information encoded within its DNA sequenced that could be passed on to future generations. Klaus et al., (2001), found that changes in the DNA loop domain structure during spermatogenesis and embryogenesis in hamsters
were specific to the developmental stage. The Donut Loop Model states that each toroid is a DNA-loop domain in sperm; therefore, together with prior work from our lab (Klaus et al., 2001) we can speculate that the structure of the loop domain is essential for conveying proper embryogenesis in the next generation. The role of the Donut-Loop domain in embryogenesis, diseases and apoptosis can be understood clearly, however, what could the significance and function be for sperm DNA fragments to hybridize to specific cancer and neurodegenerative genes? Before we discuss the possible functions of sperm DNA fragments activating endogenous cleavage in oncogenic and neurodegenerative genes, let me first discuss the functions of apoptosis, fertility and embryogenesis in more detail.

Magnano et al., (1998) found that foreign DNA such as those found in a virus could activate silent endogenous nucleases to cause cellular death in an apoptotic manner in sperm. This implies that if normal sperm are attacked by any kind of foreign DNA then they may when a certain threshold is met, undergo apoptotic-like self-destruction and destroy their DNA with loop-sized fragments, plasma membrane and mitochondria, rather than allow the defective sperm to be passed on to future generations. This may be a normal programmed mechanism that may eliminate mature sperm that may have DNA stranded breaks but survived spermiogenesis. I found while treating
sperm with TX100, an endogenous nuclease existed, which could degrade the DNA into loop-sized fragments; therefore, my experiments support the existence of an endogenous nuclease in sperm.

Another possibility is that this apoptotic-like mechanism is during the process of fertilization. One mechanism in which fertility may evoke this apoptotic-like process is for normal sperm that have entered the female tract but will not be able to fertilize the egg to get targeted for destruction. A possible mechanism would be for an oxygen free radical signal to target the sperm, the nuclease becomes activated, then DNA fragmentation occurs along with mitochondrial and plasma membrane damage. The fact that antioxidants prevented the DNA fragmentation (Lamond et al., 2003) in much the same way that cryopreservatives such as EDTA and EGTA in my experiments prevented DNA fragmentation suggests the intriguing possibility that oxygen radical signal pathways might be activated either directly or indirectly by the nuclease activation that releases loop-sized DNA fragments.

Defective sperm DNA may also be involved in failed pregnancies and infertility. Various studies (McVicar et al., 2004; Evenson et al., 2002; Sakkas et al., 1999) in human sperm have found that DNA stranded breaks in ejaculated sperm correspond to higher levels of abnormality in the ejaculate. One of the signals to destroy abnormal sperm may be the destructive targeting
of mitochondria which provide the sperm with motility and energy, the plasma membrane which protects the nucleus and other cellular structures and the DNA stranded breaks which destroy the DNA message and loop domain organization. The main goal of this mechanism is to destroy defective sperm before it reaches the oocyte and could be one of the main reasons of male infertility.

Inheritance of an interrupted DNA loop domain or DNA fragments by the embryo could be an underlying cause of early genetic diseases and cancers in both newborns and young children. A secondary mechanism besides the ones just discussed could be to target genes. The specificity of the damaged sperm DNA fragments with these specific genes implies that some childhood cancers, developmental defects, and oncogenic diseases may be directly influenced by the paternal genome. Lamond et al., (2003), reported microarray hybridization experiments with sperm DNA fragments that suggested that there is a strong paternally-derived genetic message that in this case binds to and may activate oncogenic and neurodegenerative genes.

The activation of these specific genes as a result of DNA fragmentation in sperm could be a cause of inherited newborn cancers such as pediatric brain tumors involving either tumor suppressor genes or oncogenes as in the case of retinoblastoma (RB1 gene) and meningioma (NF2 gene) in many cases (Rice,
Another result of the activation of these genes could be the failure of certain developmental processes to cease at the right time (cell cycle dysfunctions) that could lead to genetic defects at birth. Many developmentally related illnesses such as autism, which appear to have no specific genetic cause, however, have been found to be much more prevalent in males than in females and has a sexual bias (Stone et al., 2004). Developmentally related diseases could be the result of abnormal activation of genes by the fragmented sperm DNA such as in the case of normal neurotransmitter, NOS1 (involved in antioxidant signaling pathway), such that either the timing of the gene activation is wrong or the genetic message is mutated (either too much product, too little product or the wrong product is made) in these individuals. For example, in mammals there are three NOS genes that make NOS1, NOS2 and NOS3 enzymes and these enzymes are involved in many critical processes in the central nervous, reproductive, skeletal, gastro intestinal, etc. system (Mungrue et al, 2003).

Other non-destructive roles may be performed by this mechanism of sperm chromatin fragmentation. The embryo can be viewed at certain period of time during its development as an uncontrolled cellular growth machine. Among the activated genes, tumor suppressor and oncogenic genes, which control off and on switches to cellular growth, appear to be involved according
to the study by Lamond et al, (2003). These genes might be involved in rapid, uncontrolled growth during developmental circumstances which may be initially activated by the paternal DNA fragmentation. DNA fragmentation might be a necessity in the process of normal growth and survival in the embryo after the male message of “uncontrolled growth” has been inherited.

6.2. Sperm nuclease. What are possible mechanisms of the endogenous nuclease? Why does a nuclease exist in sperm?

In all of my experiments the size of the sperm DNA loop-sized fragments was always similar to the somatic apoptotic fragments of 50 kb. Regardless of whether I used the exogenous DNase I or activated the endogenous nuclease with calcium, calcium and magnesium, freeze-thawing or Triton X-100, the sperm DNA loop-sized fragments were on average 50 kb, although the range was sometimes 20 kb above or below this average. The results of my experiments provide a clue into the nature of the endogenous nuclease, although no specific one was identified. It can be activated by calcium or calcium with magnesium, however, the calcium-magnesium activated nuclease has a better activation than just calcium alone. This suggests that the nuclease may be a calcium-magnesium dependent nuclease such as DNase I. The fact that using an exogenous DNase I provided an identical
result of DNA loop-sized fragments as using calcium and magnesium to activate the endogenous nuclease suggest that a nuclease similar to or identical to DNase I is a likely one. The nuclease, according to my experiments, was much more sensitive and degraded the DNA in human sperm faster than in hamster sperm. Although both are mammalian systems, human sperm and hamster sperm (see chapter 1, tables 1, 2, and 3) have different compositions of protamines binding to the DNA as well as basic amino acids and therefore the human sperm appears to be more susceptible to nuclease attack. Freeze thawing which has been known to activate oxygen free radicals (Chatterjee and Gagnon, 2001) and permeabilize the plasma membrane of sperm was also found to activate the nuclease in human sperm (chapter 5). The experiments conducted in chapter 4 localized the sperm nuclease in the nucleus (see figure 4.12). According to my experiments in chapter 4 (see figure 4.14), both EDTA and EGTA, metal-dependent proteases (such as calcium), inhibit the endogenous nuclease.

There are several possible mechanisms for the endogenous nuclease in sperm according to the literature. As discussed in chapter 1, section 1.8, and the two most likely mechanisms discussed were topoisomerase II and CAD endonucleases such as DNAS1L3 or DFF. According to the study by Razin and colleagues (1995), in the case of topoisomerase II, he had the same loop-sized
DNA fragments with topoisomerase II-mediated cleavage as with the endogenous nuclease activated-apoptotic cleavage by serum deprivation. The CAD endonucleases such as DNAS1L3 were involved in apoptotic DNA fragmentation into loop-sized fragments when incubated with calcium and magnesium. Gromova and colleagues (1995a, 1995b) showed that both topoisomerase and CAD endogenous nuclease preferentially cut the DNA at the matrix attachment sites and produce DNA loop-sized fragments. Both of these two systems could be equally involved in the Donut-Loop model since the loop-sized fragments in sperm are similar to those seen under apoptotic conditions with either enzyme. My data can only collaborate that the fragments are loop-sized and appear to be calcium-magnesium dependent which correlates more with the CAD endonucleases, however, I was unable to either disprove or prove the role of topoisomerase II with my experiments.

Even more elusive than the finding that there is a nuclease in sperm, the question of why sperm would have a nuclease is more surprising. One reason could be that there is a necessity for a "self-destruct" mechanism among sperm if they are in the female tract and will never have an opportunity to fertilize the oocyte. A study by Chantler and colleagues (1989) found that the female cervical mucus changes in humans to form either a sperm-receptive or a sperm-hostile environment. Antisperm antibodies were found in the cervical mucus
of immunologically infertile patients. Sutovsky and colleagues (2001) found that an ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa existed in the mammalian epididymis. This suggests that the female tract has mechanisms to prevent certain sperm from reaching the oocyte and sperm have mechanisms for destroying defective sperm that wouldn’t be successful even if they could reach the oocyte. In the first case with the female tract attempts to make the environment hostile to the sperm, the sperm probably activate their endonuclease mechanism and destroy these sperm that have been blocked from reaching the egg successfully. In the second case, ubiquitin-conjugating enzyme E2 in sperm and prohibitin in sperm mitochondrial membranes appear to control the elimination of defective sperm in the epididymis.

Another reason could be that the mature sperm have endogenous nicks which target it for further DNA fragmentation without being in the female tract. Marcon and Boissonneault (2004) have found that sperm have transient DNA stranded breaks during mice and human spermatogenesis as part of their normal development. The breaks appear to be repaired by the time the sperm have become mature in most cases (Sakkas and colleagues, 1995). Further studies will reveal whether or not mature sperm may have persistent endogenous nicks that were not repaired during spermatogenesis and could
not be detected by current techniques, but which cause the sperm to be targeted for future endonuclease cleavage and DNA fragmentation.

Another reason for DNA fragmentation into loop-sized fragments could be the result of exposure to an environmental problem such as oxidative stress. Barroso and colleagues (2000) found a strong correlation between oxidative stress and impaired sperm function and increased DNA fragmentation. According to Donnelly and colleagues (1999), sperm DNA is very vulnerable to damage induced by oxygen species especially damage to its membrane. Although his group found that seminal plasma offers some protection from oxidative species by having antioxidant enzymes such as SOD and catalase which remove peroxides and other harmful oxygen free radicals, oxygen radicals may still enter the sperm and activate DNA fragmentation. Lamond and colleagues (2003) found that antioxidants that specifically target the mitochondria could prevent chromatin damage in human sperm. Li and colleagues (1999) induced topoisomerase-mediated DNA cleavage in somatic cells by inducing apoptosis with oxygen free radicals. All of these studies support the existence of a DNA fragmentation pathway in sperm which can be induced by the presence of oxygen radicals and can be prevented by antioxidants. Although oxygen radicals may damage the plasma membrane and harm the mitochondria, this does not exclude the simultaneous or further
activation of DNA fragmentation by an endonuclease as this process is occurring due to an environmental attack.

The Donut-Loop Model of sperm chromatin structure and its relationship to the various functions of the sperm has been discussed in this dissertation. The sperm DNA loop domain organization on the nuclear matrix and the similarity in sperm loop-sized domains with somatic apoptotic loop domains was discussed. The Donut-Loop model, unlike other models, connected the importance of one donut equaling one loop. The existence of an endogenous endonuclease which can cleave in hamster and human sperm to release on average, 50 kb sized loop-sized domains was shown in the experiments in chapters 3-5. The various reasons for the existence of such a DNA fragmentation with regard to fertility, embryogenesis, apoptosis and cancer were also discussed. Finally, the possible mechanism of the endonuclease and its possible functions was also discussed. The Donut-Loop Model has importance in the area of reproduction with regard to fertility issues and sperm DNA fragmentation, however, it also has consequences in the general area of science because it provides a unique model for the study of DNA that appears to be transcriptionally silent and is therefore, the most basic type of DNA available for research.
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