Nuclease Present In Mouse Spermatozoa And Surrounding Fluid From The Vas Deferens

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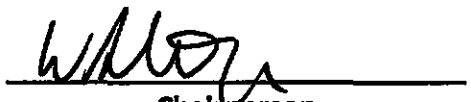

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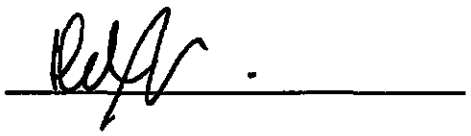

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Abstract

Previous work in our laboratory demonstrated that mouse sperm contain a nuclease that digests chromatin in conjunction with topoisomerase IIB (TOB2B). The focus of this thesis was to develop protocols to enrich this nuclease from sperm preparations and to provide preliminary characterization of its biochemistry. We found that a nuclease that required Mn^{+2} and Ca^{2+} for full activity was present in the sperm cell and in the fluid surrounding the sperm cell in the vas deferens of the mouse. During our fractionation procedures, we discovered that this nuclease has the unique property of requiring incubation first with EGTA, and then with Ca^{+2} to become activated. We term this nuclease EAN, or EGTA activated nuclease. Understanding the biochemical nature of sperm nucleases has important implications for assisted reproductive technologies (ART) because spermatozoa are manipulated and often stored during these processes. Therefore, we examined the role of EGTA in EAN digestion of DNA. We first hypothesized that EAN could become activated through one of the following pathways. (1) EGTA could initiate a complex cell-signaling pathway resulting in the activation of EAN, which then requires Ca^{+2} to digest DNA. (2) EAN could be inhibited by Ca^{+2} binding protein; EGTA releases EAN from this inhibition, and additional Ca^{+2} then binds to EAN enabling it to digest DNA. (3) EGTA chelates some other divalent cation (not Ca^{2+}) that inhibits EAN, and EAN then requires Ca^{+2} to digest DNA. To test these possibilities, we developed a plasmid-based assay, in which a soluble fraction from the vas deferens luminal fluid enriched for

EAN was mixed with plasmid DNA and molded into agarose plugs. The nuclease and its target DNA were sequestered within the agarose plugs, which could then be incubated in different conditions to determine the effects of EGTA and Ca^{+2}. All buffers contained 50 mM Tris, pH 7.5, 100 mM KCl (TKB). When plugs were incubated first in EGTA (10 to 30 mM), then in $CaCl_2$ (1 to 10 mM), the plasmid DNA was digested as expected, and if either EGTA or Ca^{+2} was omitted, the DNA remained intact. These results verified that presence of both EGTA and Ca^{+2} was required for nuclease activity and confirmed that the newly developed plasmid-based assay is suitable for testing EAN. Our next goal was to dissect the combined action of EGTA and Ca^{+2} to determine what role these components play individually. When we incubated plugs in EGTA, then in TKB alone to extract the EGTA, then in Ca^{+2}, we found that the DNA was not digested. When plugs were incubated in EGTA, then TKB alone, then EGTA again, and then Ca^{+2}, EAN became activated and the plasmid DNA was digested. These results were inconsistent with all three possibilities because the TKB incubation between the EGTA and Ca^{+2} treatments should not have reversed either a complex cell signal pathway or reintroduced an extracted cation. They compelled us to test a fourth possibility, that EAN was activated by the presence of EGTA and Ca^{+2} in the same buffer. When plugs were incubated in one buffer with 2.5 mM EGTA and 2 mM Ca^{+2}, DNA was digested. Because under these conditions most of the Ca^{+2} is expected to be

chelated to EGTA, and because our experiments have repeatedly demonstrated that both EGTA and Ca^{+2} are required for EAN to digest DNA, we are currently investigating the possibility that it is actually the EGTA chelated to Ca^{+2} that activates EAN.

Chapter 1. Introduction

1.1. Spermatogenesis

Sperm formation begins with round diploid spermatogonia, which differentiate to condensed haploid spermatozoa. Spermatogenesis is characterized by two major stages: spermatocytogenesis and spermiogenesis.

During spermatocytogenesis progenitor spermatogonia cells are sustained by repeated mitotic divisions in which they proliferate and remain diploid [1]. The path towards spermatozoan production arises when a diploid spermatogonia undergoes a round of mitosis to form the primary spermatocyte, which then duplicates its DNA and undergoes genetic recombination and meiosis I, forming two diploid secondary spermatocytes. These cells then divide to form four haploid spermatids. Spermiogenesis, the last stage of spermatogenesis, begins with the differentiation of spermatids, with the aid of Sertoli cells, resulting in the elongation and condensing of the nuclei [2]. During spermiogenesis the spermatid ejects the majority of its cytoplasm [3] while the histones of the somatic-like nuclear chromatin are replaced with sperm-specific nucleoproteins [2, 4], which organizes the DNA more tightly and further compresses the chromatin. The increase in chromatin compression has been attributed to the replacement of histones with protamines [5], which results in a distinctive spermatozoan chromatin organization [6-8]. Protamine organized chromatin forms toroid shaped "doughnuts" which loop in 50 kb sections [9]. As compression with

protamines is greater than with histones, it is currently thought that transcription of DNA is highly unlikely. Also, since far less DNA is exposed the integrity of the genetic information is better protected than in somatic cells. The extent of genetic protection and integrity provided by the sperm cell has been tested in various ways with viability determined by the ability of the sperm nuclei to decondense and form pronuclei after injection into an oocyte. Hamster, mouse, and human spermatozoa were shown to be moderately heat stable at an upper temperature of 90ºC for 30 minutes while remaining viable. Temperatures higher than 90ºC drastically reduced viability [10]. The viability of mouse and human spermatozoa were also tested against sonication and viability was maintained when injected into the oocyte immediately after sonication [11]. Lastly, viability was also successfully confirmed after freeze-drying spermatozoa and injecting into an oocyte. For sperm to be viable it was thought that all histones were required to be replaced with protamines during the morphological changes that occur during spermiogenesis, as the presence of histones in mature spermatozoa has been linked to infertility and interpreted as evidence suggesting defective chromatin repackaging during spermiogenesis [12]. Alternatively, the presence of histones in the mature spermatozoa has also been interpreted as evidence for the possibility of DNA transcription during early embryonic development [13]. In either case, however, the points where sperm DNA intersects with the nuclear matrix have less protamine bound DNA and therefore is more sensitive to DNA manipulation by

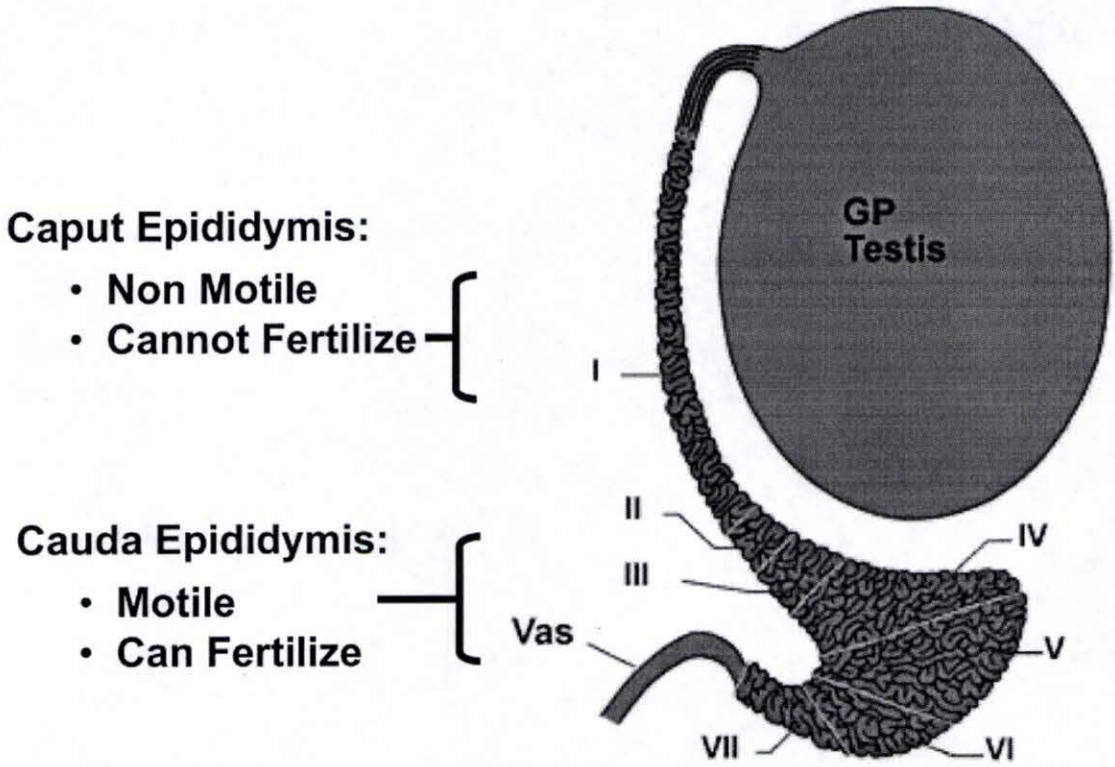
topoisomerase II-beta and nucleases [14] or, in the transcriptionally active spermatozoan model, genetic transcription [15].

1.2. Spermatozoan Maturation.

Spermatozoa exit from the testis fully formed yet immature as they are immotile, cannot locate the oocyte, and cannot undergo an acrosomal reaction in order to penetrate the zona pellucida of the oocyte. It is the migration through the epididymis where the sperm cell undergoes a process, known as maturation, and exits fully capable of fertilization (Fig. 1.1) [16-19].

The maturation [20] of the sperm cell has been the focus of research for many years. Correlations have been made linking sperm cell maturation to interactions of it with various elements found in the luminal fluid of the reproductive tract. Early on it was recognized that epididymal secretions aided sperm cell maturation [16, 17, 21, 22] and aposomes [23-25], apocrine secreted vesicles, are a common method of transferring maturation factors to the sperm cell. Unlike classical merocrine exocytosis, where proteins are processed in the endoplasmic reticulum followed by the Golgi apparatus and finally translocated inside a vesicle to the plasma membrane, during apocrine secretion the protein remains in the cytoplasm and is exported by localizing to apical plasma membrane protrusions, or blebs, which then pinch off into the lumen. Prostasomes, prostrate

Figure 1.1. Sperm Matures in the Epididymis



Gary Hunnicutt, PhD, Population Research Council
http://www.popcouncil.org/projects/BIO_SpermMaturation.html

aposomes secreted [26-29] into the luminal fluid after the vas deferens but prior to ejaculation, were identified in semen using fusogenic abilities to transfer proteins to sperm cells in-vitro [30-36] yet few proteins were actually transferred when measured in-vivo [26, 37, 38]. Prostasomes were also shown to transfer key lipids to the sperm cell [27, 28, 39] with effects on spermatozoan membrane fluidity [26] and have been shown to interact with sperm in seminal fluid aiding in promotion of forward motility and prevention of premature acrosome reaction. They also have antibacterial, antioxidant, and immunosuppressive actions [23, 26, 37, 38, 40-42]. As such, prostasomes have set the precedent for linking sperm maturation with aposomal interactions.

Epididymosomes, another type of aposome, are secreted from the apical epithelial layer of the epididymis and are physically similar to prostasomes but enters the luminal fluid before ejaculation and differs biochemically [43], therefore is more likely to be related to the normal processing of the sperm cell prior to its journey to fertilize the oocyte. During epididymal transit in mice, epididymosomes have been described as exhibiting an increase in the plasma membrane cholesterol:phospholipid ratio and a decrease in polyunsaturated fatty acids (PUFAs), except sphingomyelin which increases, favoring a loss of fluidity [43]. Sperm membranes evolved in the opposite direction during epididymal transit, with an increase in membrane fluidity suggesting the occurrence of a lipid exchange between sperm cells and epididymosomes [43]. The concentration of

sphingomyelin and cholesterol increases during transit through the epididymis. Lipid rafts [44], phospholipid microdomains of the plasma membrane that also exhibit high sphingolipid and cholesterol concentrations, have been found on the surface of epididymosomes [36] and sperm cells. The rafts, on both epididymosomes and sperm cells, present locations where glycosylphosphotidyl-inositol (GPI)-anchored proteins attach and are suggestive of the possibility that epididymosomes may contribute to the formation of lipid rafts on sperm cells [43].

Differences in protein composition contained by epididymosomes from the caput and cauda have been described in the bovine model [45]. Evidence for the gain of zona pellucida binding function has been suggested with the presence of caput derived P25b (zona pellucida binding protein that attaches to the outer sperm membrane with a glycosylphosphotidyl-inositol anchor) [31, 45] and cauda derived HSP90B1 (chaparone for zona pellucida receptor protein) [45]. Protein P25b also has murine and hamster orthologues, SPAM1 (sperm adhesion molecule 1) [46] and P25h [47, 48], respectively. Additionally, epididymosomes from the caput or cauda epididymis have been biotinylated and incubated with sperm from either the caput or cauda epididymis showing that proteins transfer from caudal epididymosomes to both caput and caudal sperm in the same pattern, but biotinylated caput epididymosomes transfer protein differently to caput sperm compared to caudal sperm, further suggesting that protein transfer occurs in a serial manner and has been correlated with sperm maturation during transit through the epididymis [45].

After maturation, sperm cells exit the cauda epididymis and enter the vas deferens, which is thought to act as a general reservoir for mature spermatozoa. Extensive research has yet to be done regarding the occurrence of similar aposome/spermatozoan activity. It has been shown, however, that the vas deferens also has apocrine activity in the production of a protein called MVDP (mouse vas deferens protein), an aldose-reductase present in the cytoplasm of epithelial cells and the luminal fluid [49] (however, it was recently shown that when MVDP was knocked out in mice the protein was not necessary for sperm maturation [50]). The vas deferens also secretes steroids and removes excess water from the luminal fluid [51].

1.3. Apoptosis

Apoptosis, or the genetically planned death of a cell, has been studied for many years [52] and has traditionally been described as morphologically different from necrosis. Apoptosis begins with cell shrinkage due to the fluctuation of osmotically active ions, such as K^+, Na^+, or Cl^-, followed by detachment from the extracellular matrix. With focal adhesion points gone, the cell undergoing apoptosis then assumes a round shape [53]. The detachment of the plasma membrane from the cytoskeleton follows next giving rise to membrane protrusions, or blebbing. The apoptotic cell then undergoes condensation in which multiple invaginations of the plasma membrane pinch off into sealed vesicle compartments. The nucleus is also pinched off into it's own compartment where

nuclear condensation proceeds with the disassembly of the nuclear matrix, nuclear lamina, and the aggregation of chromatin. The DNA is cleaved into 200 bp segments by caspase activated nucleases (CAD/DFF40/CPAN) [54-56] and topoisomerase II. The DNA is also cleaved at internucleosomal regions by topoisomerase II (independent of caspase) [57] as well as nucleases associated with topoisomerase II such as CAD/DFF40 [58, 59]. Finally, the compartmentalized vesicles (apoptotic bodies) are phagocytosed by macrophages and non-professional phagocytes. These characteristics separate apoptosis from necrosis, which does not share the same traits and is visibly distinct from apopotosis, as necrosis involves the swelling of the cell along with the loss of membrane integrity. This ultimately leads to cell lysis and surrounding tissue inflammation.

Even though the events are morphologically distinct, it has been suggested that apoptosis and necrosis are extreme forms of cell death. The signaling involved in apoptosis can be moderated to different strengths giving rise to cells that exhibit both morphologies [60]. As such, it may not be surprising that the machinery used for apoptosis is also used for other purposes as well, such as embryonic development [61], and more specifically, the development of germinal cell lines [62] like spermatozoa [63, 64]. As such, it has been observed that nuclease activity is not restricted strictly to the cellular compartment. Nucleases, like DNASIL3, have been described in extra-cellular seminal fluids [65, 66] outside of the sperm cell.

1.4. Role of Topoisomerase II in Apoptosis

The amount of chromosomal DNA that needs to be synthesized and organized during mitosis and meiosis requires topoisomerase II in order to prevent entanglements during the manipulation of DNA. Topoisomerase II operates in this context by creating a temporary covalent bond with DNA and then a temporary double-strand break, allowing for DNA manipulations without entanglements, which is then followed by re-ligation by the enzyme. The size of chromosomal DNA fragments created after a topoisomerase II excision have been repeatedly measured to be between 50 and 100 kb, and the site of the excisions are localized to the internucleosomal regions. The size of the DNA fragments and points of excision in cells induced to apoptosis [67, 68] also coincides with those of cells undergoing cellular division. Further investigations into the nature of topoisomerase II have led to the conclusion that the enzyme is active and functioning during apoptosis [69] as well as neuronal development [70, 71].

1.5. Apoptosis in Sperm

The protein machinery used during apoptosis has been identified in spermiogenic development in the testis. During spermiogenesis topoisomerase II-beta activity has been shown in spermatids undergoing differentiation [72, 73] as well as in fully formed sperm cells taken from the epididymis or vas deferens [14]. As in the early stages of somatic apoptosis, spermatozoan topoisomerase II-beta

attaches to the nuclear matrix at exposed DNA junctions and reversibly cleaves DNA in 50 kb loops. This is followed by the attachment of an as yet unidentified nuclease that proceeds to fully digest DNA. Specifically, caspase activated proteases, ribonucleases, and nucleases are used to digest unnecessary parts of the developing round spermatid leaving an oblong, fully formed sperm cell.

Some sperm, when leaving the testis, have been shown to contain DNA breaks [73-75] however there is little evidence to explain this phenomenon. Theories have been proposed stating that in some species spermiogenesis has evolved to increase sperm production rate at the expense of quality, increasing genetic mutations and which may explain un-religated DNA breaks. Faster spermiogenesis would impart higher rates of success in propagating paternal DNA in sexual selectivity patterns where female to male bonding is not exclusive [76]. Another theory suggests that DNA breaks seen in mature sperm chromatin may be the result of incomplete religation of spermatid chromatin during elongation in spermiogenesis [73]. DNA breaks can be artificially induced in mature spermatozoa by activating endogenous nucleases [77-80]. In species where DNA breaks are found, they have been correlated to infertility in males [81].

1.6. Nuclease Activity

Nuclease activity, which occurs during the end stages of apoptosis, can be activated under various conditions. Typical means include caspase (CAD/DFF40/CPAN) [54-56] as well as ion activation [65]. Ion induced nuclease

activity has been observed in model systems ranging from plant and bacteria to human cells.

Plant systems have been shown to exhibit calcium and magnesium dependent nucleases in apoptotic cells. Extracts from plant cells undergoing apoptosis where able to trigger DNA fragmentation and apoptotic morphology in human cells [82].

Calcium dependent nucleases, commonly present in the nucleus, cytosol, or extra-cellular plasma, have been located on the outer cell membrane of bacteria Mycoplasma hyopneumoniae. As mycoplasmas are not capable of endogenously synthesizing nucleic acid precursors, it is suggestive that exhibiting a cell surface nuclease to digest large chains of DNA serves to provide smaller nucleic acid fragments for cellular consumption [83]. As such, lipid membrane cell surfaces or vesicle surfaces may also provide a method of transport for nucleases in mammalian cells, specifically spermatozoa.

As the initiation of apoptosis relies in part on the fluctuating concentrations of ions, ion channels of the mitochondria help control apoptosis by regulating the ion concentration in the cytosol. This, in turn, regulates the activity of proteases and nucleases. These ion channels also regulate the release of apoptogenic factors from the intermembrane space to the cytosol [84].

In somatic thymocytes calcium dependent nucleases can be inhibited with divalent cation chelators, such as EGTA and EDTA [85].

Rat nuclease DNase gamma, and it's human homologue DNASɪL3 [86], are calcium/magnesium dependent nucleases which have been extensively documented in somatic cells, localized in the nucleus, and associated with reproductive cells. Both enzymes cleave DNA in nucleosome segments. Additionally, the sequence of DNase gamma is 45% similar to DNase I and Dnase X [86], therefore it has been suggested that DNase gamma and DNASɪL3 are part of the DNase I family as the amino acid residues associated with DNA catalytic hydrolysis are conserved. An important difference between DNase gamma and DNase I lies in the location of activity; DNase gamma is found in the nuclear fraction while DNase I is secreted outside the cell. The precursor region of DNase gamma suppresses activity while two sequences localize the protein to the nucleus. On the other hand, the precursor region of DNase I is hydrophobic which is suggestive for extracellular secretion [87, 88]. DNASɪL3, a type of DNase I, has also been associated with sperm development [14, 80, 89, 90].

1.7. Sperm Chromatin Degradation by Topoisomerase II And An As Yet Unidentified Nuclease

The role of topoisomerase II has been well documented in somatic cell division and apoptosis. Its activity has been observed in the sperm cell as well. In spermatozoa topoisomerase II cleaves DNA in similar fashion as in the somatic cell followed by a full degradation of DNA from other nucleases when incubated with

manganese and calcium [14]. The cleavage site taken by topoisomerase II resides on nuclease-sensitive sites of DNA attachment to the nuclear matrix [79, 91]. The spatial location of the other nucleases resides on (or is closely associated to) topoisomerase IIB [92, 93].

A current method for studying sperm/oocyte interactions during fertilization involves placing sperm nuclei, as is or altered according to experimental design, directly into the oocyte, in a process known as ICSI (intracytoplasmic sperm injection) [94]. What follows next (normally decondensation of chromatin DNA followed by replacement of protamines with histones [95], formation of paternal and maternal pronuclei, and finally zygotic cell division [96, 97]) determines whether or not the alterations to the sperm nuclei were critical for fertilization. The very minimum requirements for pronuclear formation under experimental conditions require the nuclear matrix with accessible DNA still attached [98]. Interestingly, if topoisomerase IIB is activated to digest DNA in the sperm nuclei prior to ICSI, paternal pronuclei develop but no DNA synthesis occurs. Alternatively, if topoisomerase IIB is activated, digests and re-ligates the DNA, paternal pronuclei develop and a round of DNA synthesis occurs. As in somatic cells, other nucleases seem to become activated by topoisomerase IIB after excision if the DNA is not re-ligated within a set period of time, resulting in full DNA digestion. When topoisomerase IIB is activated in sperm taken from the epididymis, more time is needed before full digestion occurs in contrast with sperm taken from the vas deferens, where less

time is needed prior to full DNA digestion [99]. As sperm taken from the vas deferens initiates full DNA degradation earlier than from the epididymis suggests that more nucleases are present and perhaps are imported into the cell as it waits in the vas deferens prior to its journey to fertilize the zygote.

Chapter 2. Materials and Methods

2.1. Animals

Male B6D2F1 (C57BL/6J X DBA/2) mice were obtained from National Cancer Institute (Raleigh, NC). Mice were fed ad libitum and kept in standard housing in accordance with the guidelines of the Laboratory Animal Services at the University of Hawaii and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources National Research Council (DHEF publication no. [NIH] 80-23, revised 1985). The protocol for animal handling and the treatment procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Hawaii.

2.2. Extraction of Sperm from Epididymis and Vas Deferens

Mice were sacrificed using carbon dioxide asphyxiation followed by cervical dislocation. The epididymis and/or vas deferens were excised and fluid from the epididymis and/or vas deferens gently extracted, using tweezers, and diluted to a concentration of approximately 10^8 spermatozoa/ml in 25 mM Tris, pH 7.4, 150 mM KCl buffer (TKB) and kept on ice.

2.3. Fractionation of Sperm Fluids and preparation of extracts.

Centrifuging extracted fluid from the epididymis and/or vas deferens at 2,000 x g for 2 minutes at room temperature gives rise to a pellet consisting of sperm cells, which is reconstituted in TKB. The resulting supernatant, Sup A, is comprised of two sub-fractions, Sup B and Pel B, which can be fractionated at 36,000 x g for 10 minutes at 4º C giving rise to it's supernatant, Sup B, and pellet, Pel B. The Pel B, thought to contain vesicles, can be reconstituted in TKB and 0.5% Triton X-100 (lipid membrane solubilization), and fractionated again at 36,000 x g for 10 minutes at 4º C, giving rise to the supernatant Pel B-Sup and pellet Pel B-Pel, which is then reconstituted in TKB.

Various fractions were incubated in 30 mM EGTA and/or 10 mM Manganese, 10 mM Calcium, or 10 mM Magnesium for 15 minutes at room temperature.

2.4. Isolation of Plasmid DNA

CompactPrep Plasmid Midi Kit (Qiagen, Catalog Number 12743) was used to grow eGFP bacteria. *Midi Kit Plasmid* procedure was followed resulting in the harvest of purified plasmid DNA (0.51 μg/μl).

2.5. $CaPO_4$ Precipitation of Sperm Extract

Epididymal and vas deferens fluid was extracted, combined, and diluted in buffer. The solution was divided to two aliquots; one of which was incubated with 10 mM Mn^{2+} and Ca^{2+} at 37º C for one hour, the other aliquot was not incubated (control). Both solutions were then centrifuged at 2,000 x g for 2 minutes at RT. The resulting supernatants were discarded and pellets were resuspended in buffer. Both pellet resuspensions were separately centrifuged with resulting pellets resuspended in buffer two more times with the last resuspension containing 0.25% TX-100. Both solutions were centrifuged a fourth time with resulting supernatants aspirated (S4 and Mn^{2+} and Ca^{2+} incubation derived S4) and pellets discarded.

Aliquots were taken from both S4 solutions and treated with 44 mM $CaCl_2$ and 1 mM PO_4 (PBS), all four solutions were then centrifuged at 36,000 x g for 10 minutes at 4º C. Resulting supernatants (S5) were aspirated to new tubes, pellets were resuspended in buffer (P5). The supernatants and resuspended pellets resulting from S4 treated with $CaPO_4$ were then treated with 15 mM EDTA and 15 mM EGTA.

Nuclease assay scheme: 3 µl of S4 without or with Mn^{2+} and Ca^{2+} pre-incubation (A and B, lanes 1 and 6) were added to separate nuclease assay buffers. A volume of 5 µl of S5 and P5 that were not pre-incubated with Mn^{2+} and Ca^{2+} but treated with $CaPO_4$ (A and B, lanes 2 and 3) or without $CaPO_4$ (A and B, lanes 4 and 5) were added to separate nuclease assay buffers. A volume of 5 µl of S5 and P5 that were pre-incubated with Mn^{2+} and Ca^{2+} and treated with $CaPO_4$ (A and B,

lanes 7 and 8) or without $CaPO_4$ (A and B, lanes 9 and 10) were added to separate nuclease assay buffers. All nuclease assay buffers contained 10 mM Mn^{2+} and 10 mM Ca^{2+}. All samples were analyzed by conventional agarose electrophoresis or denatured in Laemmli's buffer and analyzed by SDS-PAGE.

2.6. Nuclease Assay

Luminal fluid from the epididymis and vas deferens of 9-week-old mice was extracted and collected in 25 mM Tris and 150 mM KCl buffer (TKB) on ice. This was centrifuged with a small tabletop centrifuge (Sigma-Aldrich Micro Centrifuge 100 VAC) for 2 min at 2,000 x g to separate the luminal plasma (supernatant) from the sperm cells (pellet). The supernatant was aspirated and ultra-centrifuged at 36,000 x g (20,000 rpm, in an Allegra 64R tabletop ultracentrifuge from Beckman/Colter using an F2402 rotor) for 30 min at 4ºC. The supernatant (Luminal Plasma Supernatant) and the pellet (Luminal Plasma Pellet) were resuspended in TKB and both used in this assay. The sperm cell pellet was then washed three times with TKB at room temperature and resuspended in TKB. Triton X-100 (TX), a non-ionic detergent, was added to the sperm-TKB solution to a final concentration of 0.25%. This was centrifuged at 700 x g, and the supernatant (Sperm Cell Extract) used for this assay. Each solution - the Luminal Plasma Supernatant, Luminal Plasma Pellet, Luminal Plasma and the Sperm Extract - was pretreated with either: 30 mM EGTA, or 30 mM EDTA for 15 min at

room temperature, or 10 mM MnCa for 1 hour at 37ºC. Then, 3 µl of each solution (with or without pretreatment) was added to 20 µl of TKB, containing 1.25 µg of plasmid DNA, and either no cations, 10 mM $MnCl_2$, 10 mM $CaCl_2$, or 10 mM of each cation for 1 h at 37ºC. The solutions were then run on a 1% agarose gel containing ethidium bromide.

2.7. SDS-PAGE Silver Staining

Analyte samples were denatured by boiling in Lemmli's solution for 10 minutes then electrophoresing on sodium dodecyl sulfate poly-acrylamide gels (SDS-PAGE). Poly-acrylamide gel recipe was prepared according to the BIORAD instruction manual for Mini-PROTEAN II. Protein bands were visualized by silver staining. Silver Stain Plus kit from BioRad (Catalog Number 161-0449) used for protein staining.

2.8. Specific Activity

2.8.1. Total Protein Determination

The protein concentration was determined using by BCA (bicinchoninic acid reagent) Protein Assay Kit (Pierce, Catalogue Number 23227) using a bovine serum albumin standards supplied with the kit.

2.8.2. Determination of Nuclease Units

Specific activity, unit of activity per protein mass, for each fraction was analyzed to discern which fractions were most enriched with nuclease. The plasmid DNA digestion assay was employed to monitor the minimal amount of each fraction required to digest 1 μg of DNA in 1 hour. An activity unit is designated as the full digestion of DNA in 1 hour per volume of fraction. For some specific activity calculations we arbitrarily assigned approximate values (e.g. using 0.9 instead of 1 activity units) to better approximate when a residual amount of DNA was left un-digested. This value was then divided by the total protein concentration of a nuclease-containing fraction, which equals the specific activity:

Specific Activity = (activity unit / ml) / (mg total protein / ml)

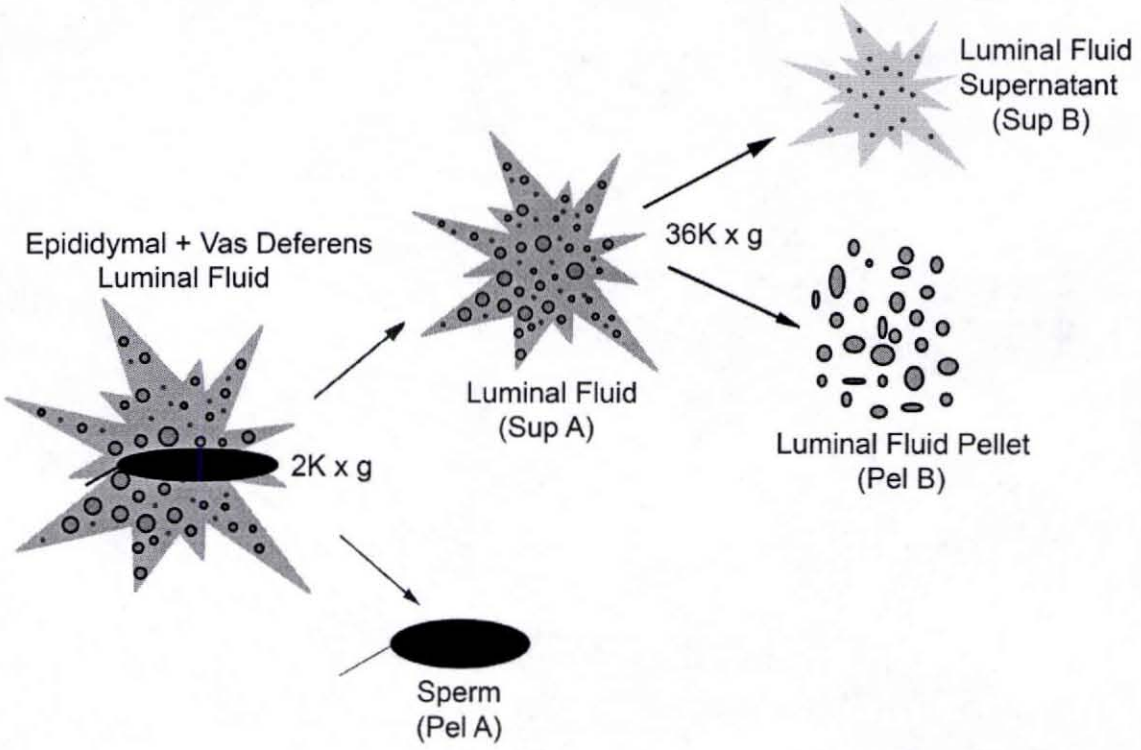
Chapter 3. Results

The focus of this work is the characterization of the nuclease associated with sperm DNA degradation (SDD). As described in the Introduction, this degradation of the sperm chromatin requires the combined efforts of TOP2B and an as yet unidentified nuclease. Previous experiments [14] have shown that combined epididymal and vas deferens luminal fluid could digest plasmids in the presence of manganese plus calcium, or manganese alone, but not calcium alone, or magnesium plus calcium. Because these ion properties fit those of SDD, we hypothesized that this nuclease activity was the same as that found in SDD.

3.1. Mn/Ca Dependent Nuclease (MCN) is Present in All Components of the Luminal Fluid

Nuclease activity had been shown previously [14] to be present in the contents of epididymal and vas deferens luminal fluid. We first tested whether we could further fractionate the nuclease to the different components of this fluid. Four aspects (Fig. 3.1) of the fluid were considered: (1) the sperm cells, (2) the luminal fluid, (3) the high-speed pellet fraction of the luminal fluid, and (4) the high-speed supernatant fraction of the luminal fluid.

Figure 3.1. Luminal Fluid Separation Scheme.



Fluid extracted from the epididymis and vas deferens was combined and diluted in buffer. The suspension was centrifuged at 2,000 x g for 2 minutes at room temperature (RT). The luminal fluid remained in the supernatant, Sup A, while spermatozoa separated to the pellet, Pel A. The Sup A was then centrifuged at 36,000 x g for 10 minutes at 4ºC. The resulting luminal fluid supernatant, Sup B, was aspirated while the luminal fluid pellet, Pel B, was resuspended in the same volume of buffer.

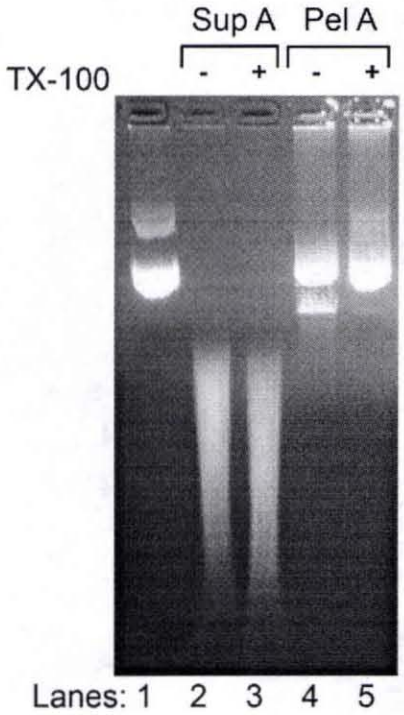
3.1.1. MCN is Present in Two Fractions of the Luminal Fluid.

Centrifugation of combined epididymal and vas deferens fluid at 2,000 x g separates the sperm cells to the pellet (Pel A), leaving non-sperm containing luminal fluid in the supernatant (Sup A). In either of the two fractions we were able to test for the presence of nuclease activity by first incubating a fraction with Mn^{2+} and Ca^{2+} and then aliquoting a small amount to a new tube that contained buffer, an experimental cation combination, and plasmid DNA. The Mn^{2+} and Ca^{2+} incubation is thought to pre-activate the nuclease, while the transfer of pre-activated nuclease solution to the reaction buffer containing a cation fully activates the nuclease. The plasmid DNA was digested if the nuclease was present in the original fraction and fully activated with a proper cation in the reaction buffer. The reaction buffer was then run on an agarose gel containing ethidium bromide and DNA degradation is imaged with UV fluorescence.

We found Mn^{2+} and Ca^{2+} dependent nuclease (Mn^{2+} and Ca^{2+} Activated Nuclease, or MCN) activity present in the luminal fluid (Sup A) (Fig. 3.2, lanes 2 & 3) but initially not in sperm cells (Pel A) (Fig. 3.2, lanes 4 & 5). Plasmid DNA digestion was confirmed with fluorescence by electrophoreses on an ethidium bromide agarose gel. Even though the sperm cell tested negative for MCN activity, it can be influenced to exhibit nuclease activity and the method for doing so will be elucidated later in section 1.

Sup A at this point was further separated with higher speed centrifugation of 36,000 x g into a pellet (Pel B) and supernatant (Sup B). We found DNA

Figure 3.2. Nuclease Activity Localized to TX-100 Treated Luminal Fluid.



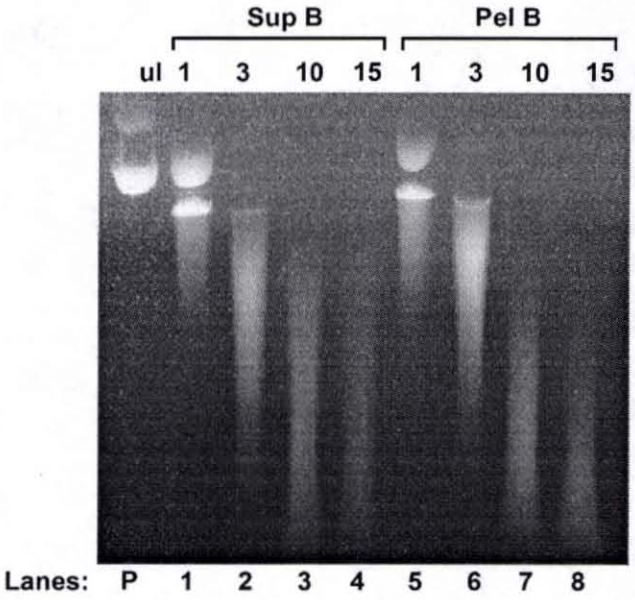
Fluid from the epididymis and vas deferens was combined and diluted in buffer followed by centrifugation at 2,000 x g for 2 minutes at RT. The resulting supernatant, Sup A, was aspirated and the pellet was resuspended in buffer. Both solutions were treated with (lanes 3 and 5) or without (lanes 2 and 4) 0.25% TX-100. One-sixth volume of each fluid was then added to the nuclease assay buffer containing 10 mM Mn^{2+} and 10 mM Ca^{2+}. All samples were analyzed by conventional agarose electrophoresis. Lane 1, plasmid, alone.

nuclease activity to require both manganese and calcium in both high-speed centrifugation fractions under specific conditions (Fig. 3.3). Both Sup B and Pel B from the luminal fluid had nuclease activity that could be activated with Mn^{+2} and Ca^{+2} and 0.25% TX-100. The addition of TX-100 to the individual fractions after separation induces activity levels that are similar between them. It is important to note that addition of TX-100 after separation is necessary in the Pel-B for activity to occur (data not shown). Proteins visualized by running various volumes of Sup B and Pel B on polyacrylamide gels and stained with silver nitrate (Fig. 3.4). Sup B appeared to have a greater total protein concentration than Pel B, yet Pel B retains similar levels of activity (Fig. 3.3). This suggested that Pel B had a higher specific activity for the nuclease, which we confirmed later.

3.1.2. Evidence that MCN is Present in Lipid Vesicles in the Luminal Fluid.

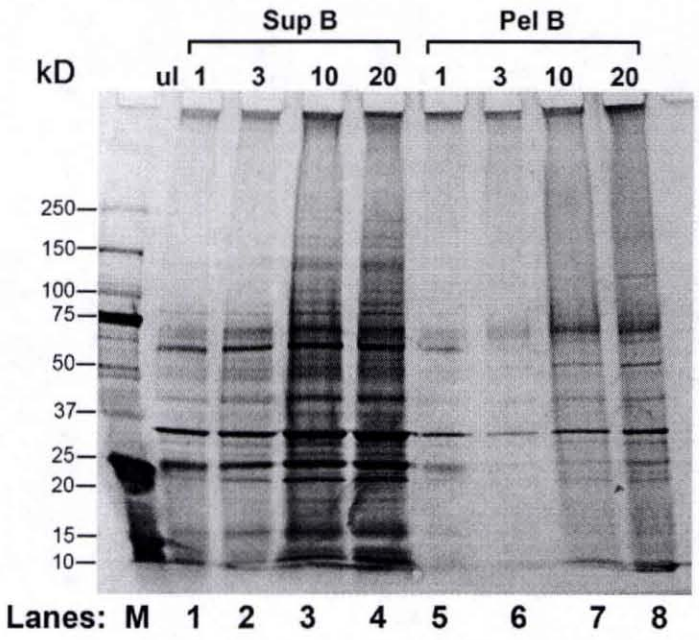
In the experiment depicted in Fig. 3.3, Sup B and Pel B had already been separated prior to the addition of TX-100 used in the nuclease assay. We tested the possibility that the nuclease activity that was present in Pel B pelleted because it was associated with membrane vesicles and was released by treating with TX-100 after high-speed centrifugation. Treating Sup A with TX-100 before centrifuging at 36,000 x g releases nuclease activity from Pel B into Sup B (Figs. 3.5 and 3.6). This supported our hypothesis that the nuclease was associated with lipid vesicles, and that this was the reason for its presence in Pel B.

Figure 3.3. Nuclease Activity Localized to Luminal Fluid Supernatant and Pellet.



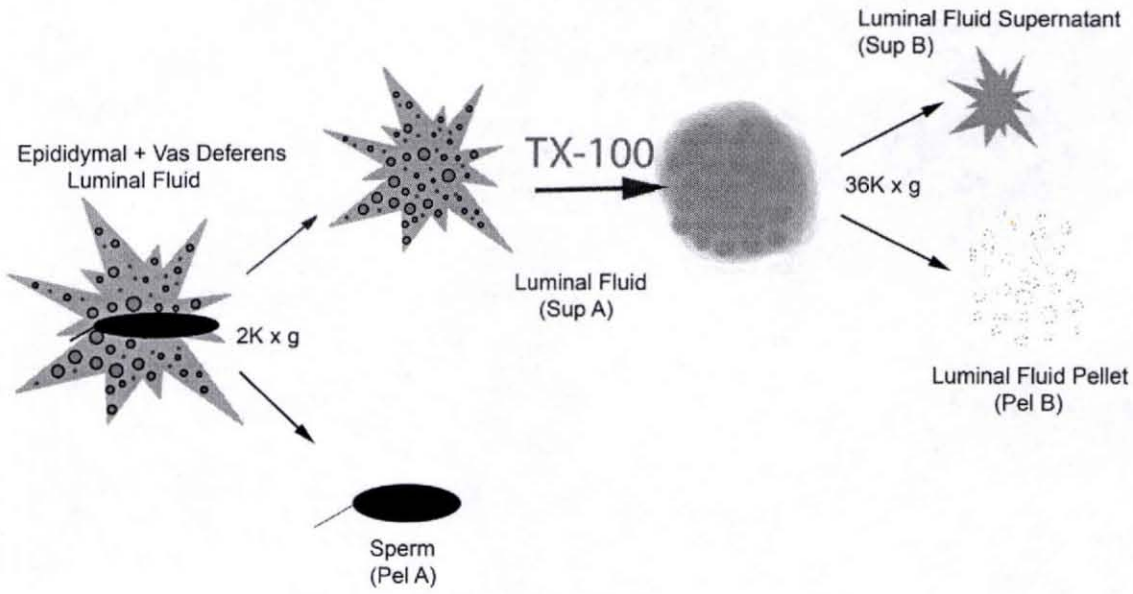
Fluid from the epididymis and vas deferens was combined and diluted in buffer followed by centrifugation at 2,000 x g for 2 minutes at RT. The resulting supernatant, Sup A, was aspirated and centrifuged at 36,000 x g for 10 minutes at 4ºC. The resulting supernatant, Sup B, was aspirated and the pellet, Pel B, was resuspended in buffer. Both solutions were separately treated with 0.25% TX-100. Volumes of 1, 3, 10, or 15 µl of Sup B (lanes 1 – 4) or Pel B (Lanes 5 – 8), respectively, were added to the nuclease assay buffer containing 10 mM Mn^{2+} and 10 mM Ca^{2+}. All samples were analyzed by conventional agarose electrophoresis. Lane P, plasmid alone.

Figure 3.4. Protein Band Visualization in Luminal Fluid Supernatant and Pellet.



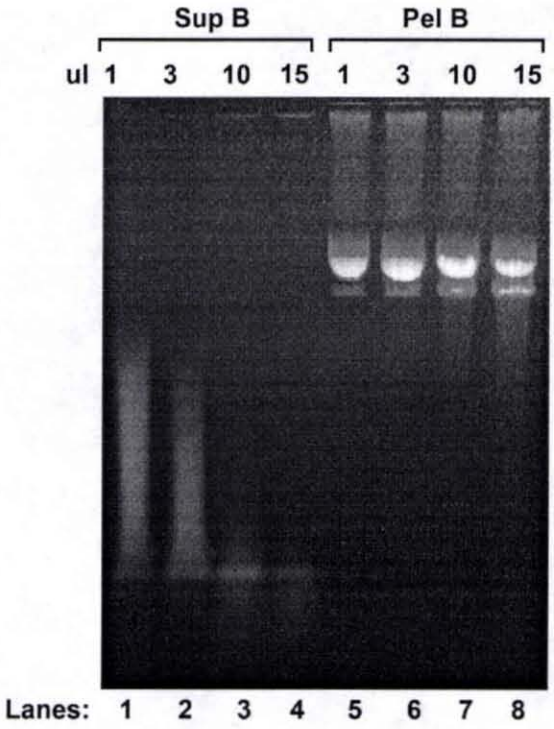
The same solutions of Sup B and Pel B from Fig. 3.3 were denatured in Laemmli's buffer and analyzed by SDS-PAGE. Gel was stained with silver nitrate until protein bands became visible. Lane M, molecular weight markers, as indicated on left. Lanes 1 – 4, Sup B; lanes 5 – 8, Pel B.

Figure 3.5. Luminal Fluid Membrane Solubilization Scheme.



Fluid extracted from epididymis and vas deferens was combined and diluted in buffer. The suspension was centrifuged at 2,000 x g for 2 minutes at RT. The luminal fluid remained in the supernatant, Sup A, while spermatozoa separated to the pellet, Pel A. The Sup A was treated with 0.25% TX-100 followed by centrifugation at 36,000 x g for 10 minutes at 4ºC. The resulting luminal fluid supernatant, Sup B, was aspirated while the luminal fluid pellet, Pel B, was resuspended in buffer.

Figure 3.6. Removal of Nuclease Activity from the TX-100 Treated Luminal Fluid Pellet.



Sup B and Pel B were prepared as described in Fig. 3.5. Volumes of 1, 3, 10, and 15 µl of Sup B (lanes 1 – 4) and Pel B (lanes 5 – 8) were added to the nuclease assay buffer containing 10 mM Mn^{2+} and 10 mM Ca^{2+}. All samples were analyzed by conventional agarose electrophoresis.

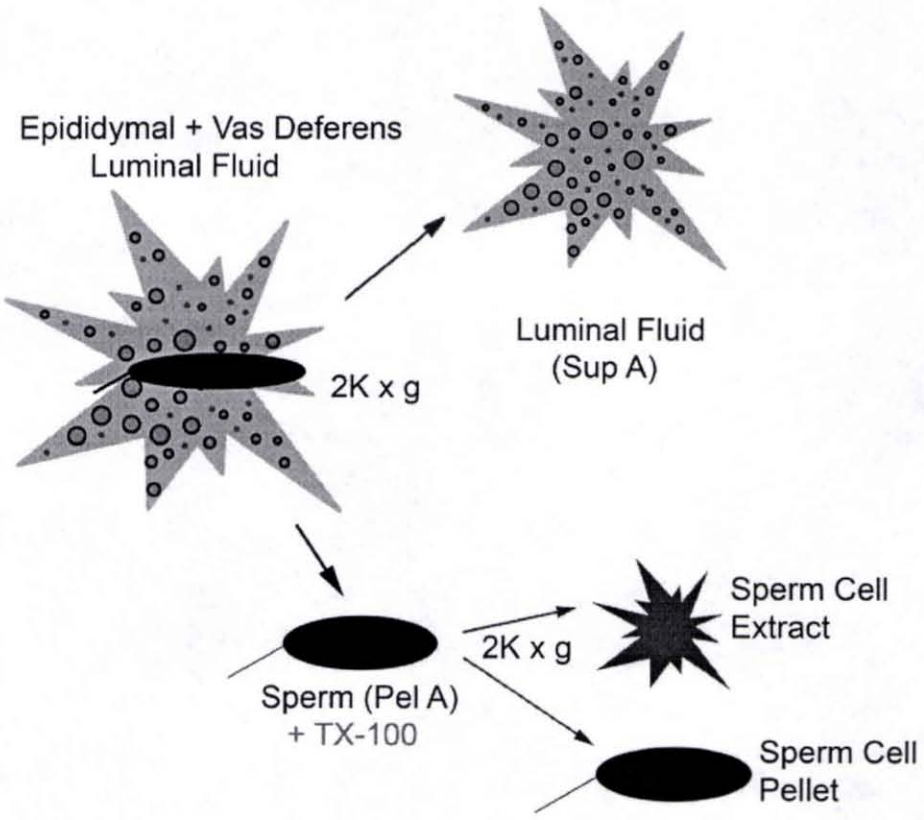
These data suggested that in Sup A, there were two fractions of the nuclease; a soluble form that remained in Sup B and a fraction that was associated with a lipid vesicle, or other TX-100 dissociable organelle, that was pelletable at 36,000 x g and remained in Pel B.

3.1.3. MCN Can be Activated in Spermatozoa with Prior Mn^{+2} and Ca^{+2} Treatment.

In our early experiments when the sperm cell was separated from the luminal fluid and incubated with Mn^{2+} and Ca^{2+} there was no nuclease activity, as shown above (Fig. 3.2). This finding was reproducible and seemed to suggest that the majority of nuclease activity was located outside the sperm cell in the surrounding luminal fluid (Sup A). Yet we know that the sperm cell can be manipulated to digest its own chromatin through nuclease activity [14]. This discrepancy led us to consider the possibility that pretreatment of the sperm cell with Mn^{2+} and Ca^{2+} in the presence of the full luminal fluid either transferred the nuclease to the sperm cell or was required to activate the nuclease in the sperm cell.

To test this hypothesis we incubated the sperm cell (Pel A) along with the rest of the luminal fluid (Sup A) in buffer with Mn^{2+} and Ca^{2+}at 37º C for one hour before centrifugation. This would theoretically allow the transfer of MCN activity from Sup A to the sperm cell or its activation within the sperm cell. The Mn^{2+} and Ca^{2+} incubated solution was centrifuged at 2,000 x g and the pellet Pel A was resuspended in buffer and permeabilized with 0.25% TX-100 (Fig. 3.7).

Figure 3.7. Sperm Cell Extraction Scheme.



Washed sperm cell pellet was resuspended in buffer with 0.25% TX-100 and centrifuged at 2,000 x g for 2 minutes at RT. The resulting supernatant, the sperm cell extract, was aspirated for analysis.

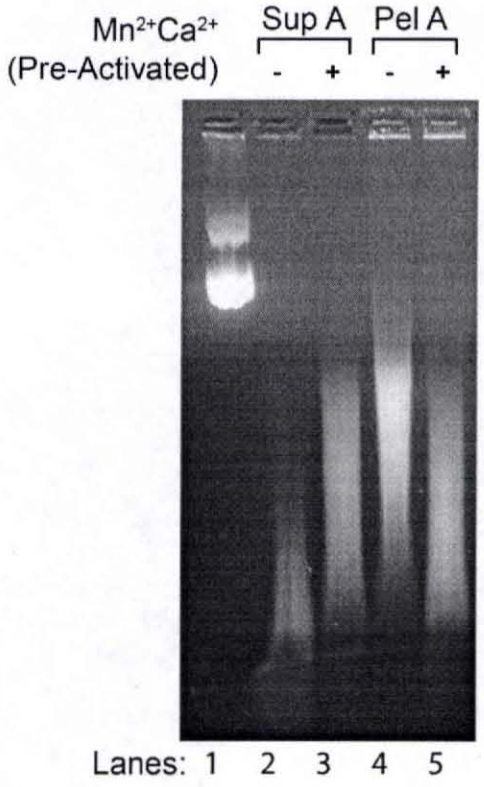
The resuspension was centrifuged again at the same speed. The resulting supernatant consisted of sperm cell extract and exhibited nuclease activity when combined with plasmid DNA in the presence of Mn^{2+} and Ca^{2+} (Fig. 3.8). In this case, however, there was some MCN activity in the sperm cell even without pretreatment with $Mn^{2+}$and Ca^{2+} (Fig. 3.8, lanes 4 and 8). If the incubation proceeded for four hours, activity seemingly moved back into Sup A and disappeared in Pel A (Fig. 3.9). The data suggested that pretreatment with divalent cations could activate MCN in the sperm cell.

3.1.4. Ion Specificity of MCN.

As the nuclease activity requires Mn^{+2} and Ca^{+2}, next we tested other ions with which the nuclease could be expected to react. Also, we examined the concentration ranges of activity.

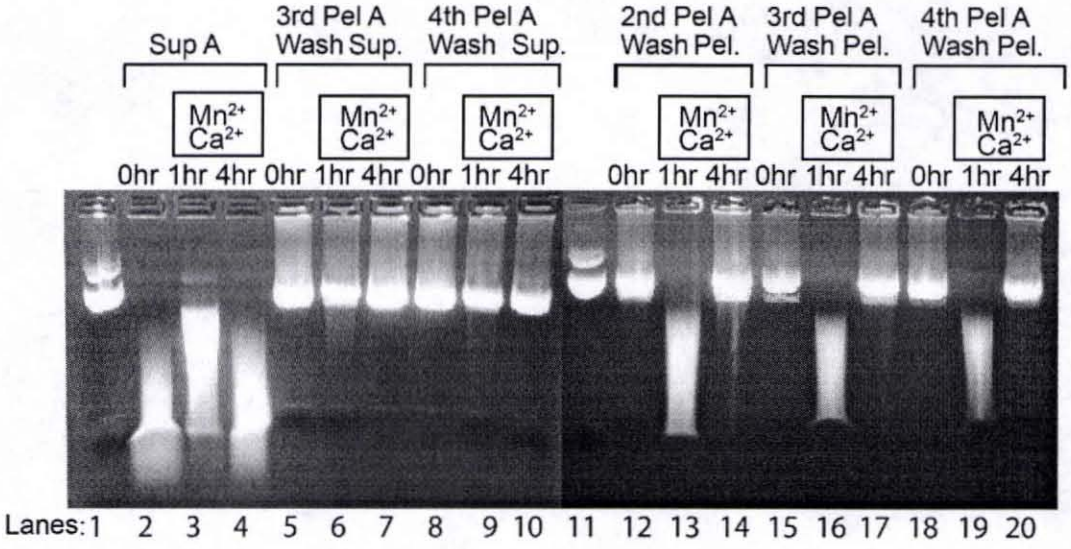
A specific nuclease which requires Mg^{+2} and Ca^{+2}, for activity has been described [2,3]. As such, we tested whether our nuclease was a similar so-called Ca/Mg-Dependent enzyme, and if Mg^{+2} could substitute for Mn^{+2}. We tested for nuclease activity in Sup B and Pel B using all combinations of the three ions: Mn^{+2}, Mg^{+2}, or Ca^{+2} alone, and Mn^{+2} with Ca^{+2}, or Mg^{+2} with Ca^{+2} (Fig. 3.10). We found that Mn^{+2} with Ca^{+2} had the highest activity (Fig. 3.10A, lane 6 & 3.10B, lane 5), and that Mn^{+2} alone had less, but still visible, activity (Fig. 3.10A, lane 2). However, Mg^{+2} alone or Mg^{+2} with Ca^{+2} elicited no nuclease activity in either Sup A or Pel B.

Figure 3.8. Nuclease Activity Localized to the TX-100 Treated Sperm Cell.



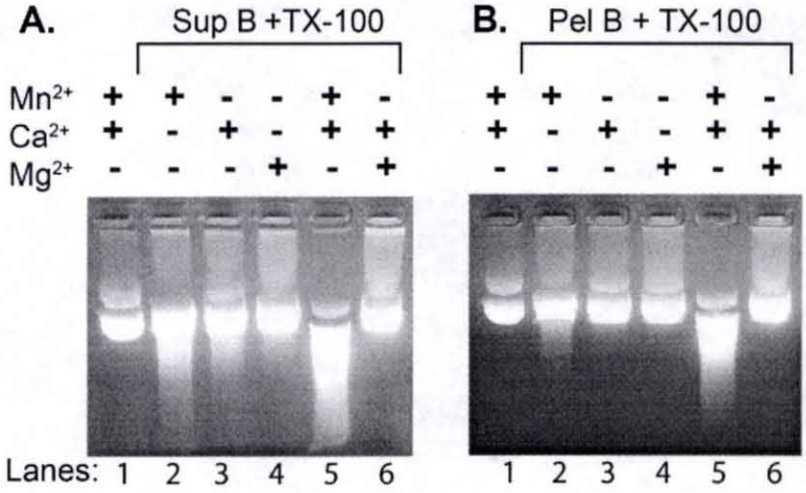
Combined epididymis and vas deferens luminal fluid extracts were incubated with (lanes 3 and 5) or without (lanes 2 and 4) 10 mM Ca^{2+} and 10 mM Mn^{2+} at 37º C for 1 hour. The solution was then centrifuged at 2,000 x g for 2 minutes at RT. The resulting supernatant, Sup A, was aspirated and the pellet, Pel A, was resuspended in buffer. The pellet was cleaned by undergoing two cycles of centrifugation at 2,000 x g, discarding the resulting supernatant and resuspending the pellet in buffer. Both Sup A and Pel A were then treated with 0.25% TX-100 and 3 µl of each were added to the nuclease assay buffer containing 10 mM Mn^{2+} and 10 mM Ca^{2+}. All samples were analyzed by conventional agarose electrophoresis.

Figure 3.9. Nuclease Activity Localized to the Sperm Cell with Incubation Time Restraints.



Combined epididymis and vas deferens luminal fluid extracts were separately incubated without or with 10 mM Ca^{2+} and 10 mM Mn^{2+} at 37ºC for 1 or 4 hours, as indicated in the figure. The suspensions were then centrifuged at 2,000 x g for 2 minutes at RT. The resulting supernatants, Sup A, were aspirated and the pellets, Pel A, were resuspended in buffer. Undergoing two centrifugation cycles cleaned the pellets. Both Sup A and Pel A were then treated with 0.25% TX-100 and 3 µl of each were added to the nuclease assay buffer containing 10 mM Mn^{2+} and 10 mM Ca^{2+}. All samples were analyzed by conventional agarose electrophoresis. Lanes 1 and 11, plasmid, alone. Lanes 2-4, Sup A control. Lanes 5-7, supernatant corresponding to pellet in Lanes 15 – 17. Lanes 8-10, supernatant corresponding to pellet in lanes 18-20. Lanes 12 – 14, pellet of second wash.

Figure 3.10. Nuclease Ion Specificity in TX-100 Treated Sup B and Pel B.



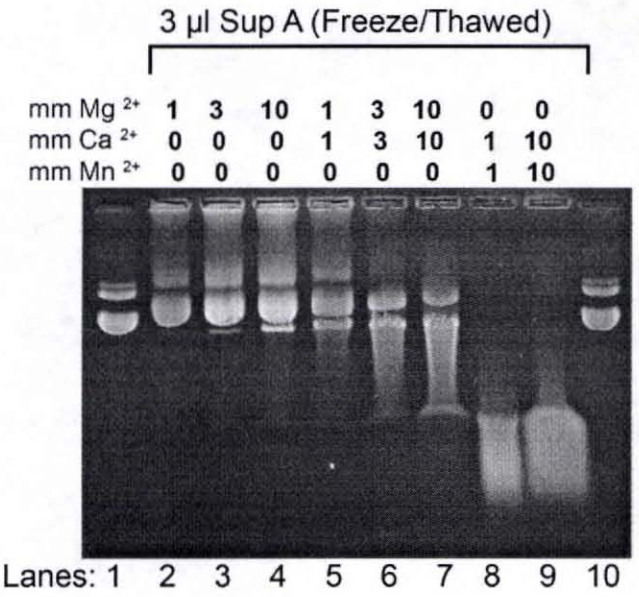
Fluid from the epididymis and vas deferens was combined and diluted in buffer followed by centrifugation at 2,000 x g for 2 minutes at RT. The resulting supernatant, Sup A, was aspirated and centrifuged at 36,000 x g for 10 minutes at 4ºC. The resulting supernatant, Sup B, was aspirated and the pellet, Pel B, was resuspended in buffer. Both solutions were separately treated with 0.25% TX-100. A volume of 3 µl of Sup B (A) or Pel B (B) were added to various nuclease assay buffers containing either 10 mM Mn^{2+} (lane 2), Ca^{2+} (lane 3), or Mg^{2+} (lane 4), alone, or a combination of 10 mM Mn^{2+} and Ca^{2+} (lane 5) or 10 mM Mg^{2+} and Ca^{2+} (lane 6). All samples were analyzed by conventional agarose electrophoresis. Lane 1, plasmid control incubated with 10 mM Mn^{2+} and Ca^{2+} without nuclease, as controls.

As a control, we tested varying concentrations of all three ions with Sup A, before it was fractionated into Sup B and Pel B. In this case, we found some evidence for nuclease activity in the presence of Mg^{+2} and Ca^{+2} (Fig. 3.11, lanes 6 and 7), but this was much lower than the activity of Mn^{2+} and Ca^{2+} (Fig. 3.11, lanes 8 and 9). In fact, there was a much higher nuclease activity with 1 mM Mn^{+2} and Ca^{+2} than with even 10 mM Mg^{+2} and Ca^{+2}, indicating that the nuclease activity is much more highly activated by manganese. Thus, the nuclease is not a classical Ca/Mg-dependent nuclease.

3.2. Spermatozoa Contain a Nuclease that can be Activated by EGTA, (EGTA Activated Nuclease, or EAN)

As was shown in Figure 3.8, MCN activity was found inside the sperm cell when incubated with Mn^{2+}, Ca^{2+}, and Sup A. The sperm cell MCN was extracted into the supernatant at 2,000 x g. Because the fraction obtained from the resulting supernatant was rich in protein we wanted to further isolate an enriched fraction containing MCN activity. We hypothesized that adding Ca^{2+} and PO_4 to the sperm extract prior to centrifugation at 26,000 x g would cause the precipitation of $CaPO_4$ along with extra proteins from suspension. This precipitate could be centrifuged to the pellet leaving fewer proteins in the resulting supernatant. The supernatant, containing less total protein, could then be assayed for nuclease activity and would be enriched for nuclease if it retained activity.

Figure 3.11. Nuclease Ion Specificity in Frozen and Thawed Sup A.



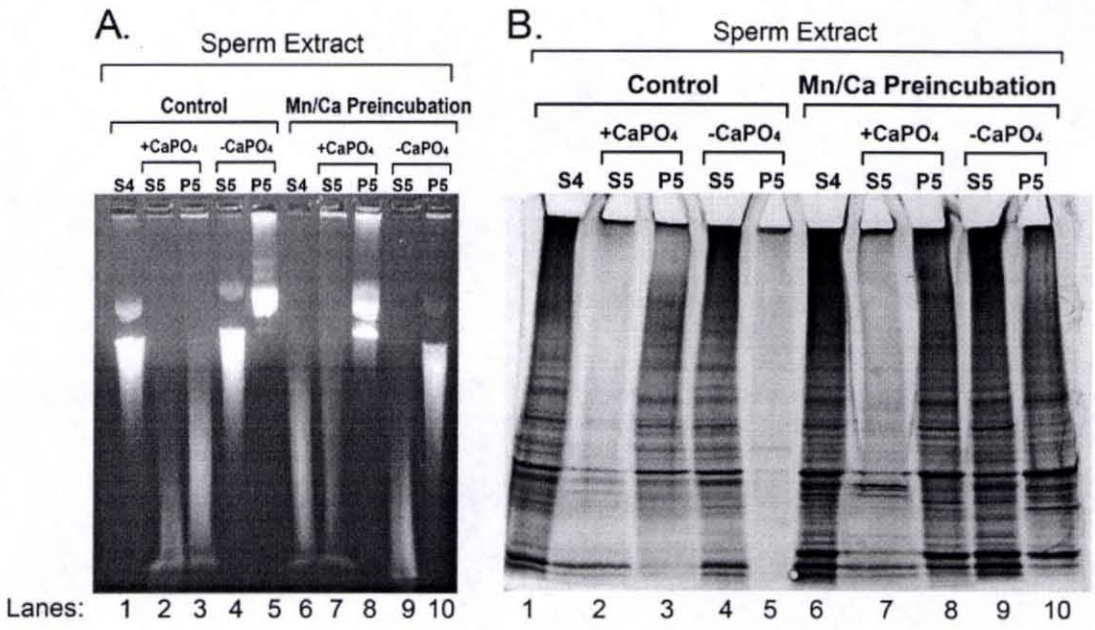
Epididymal and vas deferens fluid was extracted, combined, and diluted in buffer followed by centrifugation at 2,000 x g for 2 minutes at RT. The resulting supernatant, Sup A, was frozen overnight at -80ºC and thawed. A volume of 3 µl was added to separate nuclease assay buffers containing: 1, 3, or 10 mM Mg^{2+} (lanes 2 – 4), alone, or combinations of 1 mM Mg^{2+} and Ca^{2+} (lane 5), 3 mM Mg^{2+} and Ca^{2+} (lane 6), 10 mM Mg^{2+} and Ca^{2+} (lane 7), 1 mM Mn^{2+} and Ca^{2+} (lane 8), or 10 mM Mn^{2+} and Ca^{2+} (lane 9). Lanes 1 and10, plasmid only controls. All samples were analyzed by conventional agarose electrophoresis.

3.2.1. EDTA and EGTA Activate MCN.

For our test we acquired the sperm extract from epididymal and vas deferens luminal fluid in the same manner as described for Fig. 3.8 except a control group was not incubated with Mn^{2+} and Ca^{2+} for pre-activation (Fig. 3.12). $CaCl_2$ and PBS were added to the experimental samples and all were centrifuged at 26,000 x g for 10 minutes at 4º C. The supernatants were aspirated to new tubes while the pellets were resuspended in buffer. EDTA and EGTA were added to samples containing $CaPO_4$ with the intention of chelating the Ca^{2+} in the pellet. In doing so the precipitate would become solubilized releasing any proteins that may have come down during centrifugation. EDTA/EGTA was also added to samples not treated with $CaPO_4$ as a control. All samples were then tested with our MCN activity assay. However, an unexpected outcome resulted from the assay. Normally, without Mn^{2+} and Ca^{2+} pre-incubation of the sperm cell (Pel A), the resulting sperm extract would not exhibit nuclease activity. However lanes 1-4 of Figure 3.12 all exhibited activity even though they were control samples that are derived from non-Mn^{2+} and Ca^{2+} incubated sperm cells. They should not have had any activity according to our historical trends.

A follow up experiment was designed to further understand how sperm cell extracted nuclease could become activated in the absence of Mn^{2+} Ca^{2+} pre-incubation. For this the sperm cell was not pre-incubated with Mn^{2+} and Ca^{2+} prior to extraction. Sperm extract was then mixed with buffer alone, Ca^{2+} alone,

Figure 3.12. Ca PO_4 Precipitation of Sperm Extract.



(A) Sperm extract (S4, indicating the spermatozoa were washed four times by centrifugation) was prepared as described in Methods from combined epididymal and vas deferens fluid that was either not treated, or pretreated with 10 mM Mn^{2+} and Ca^{2+} at 37º C for one hour. Sperm extracts from control and pretreated samples were taken from both S4 solutions and treated with 44 mM $CaCl_2$ and 1 mM PO_4 (PBS) (lanes 2, 3, 7 and 8), or without $CaPO_4$ (lanes 4, 5, 9 and 10). All four solutions were then centrifuged at 36,000 x g for 10 minutes at 4º C. Resulting supernatants (S5) were aspirated to new tubes; pellets were resuspended in buffer (P5). The supernatants and resuspended pellets resulting from S4 treated with $CaPO_4$ were then treated with 15 mM EDTA and 15 mM EGTA. Three µl of each S4 control (lanes 1 and 6), and 5 µl of each S5 and P5 suspension were added to separate nuclease assay buffers, incubated for 1 hr at 37º C, then plasmid digestion was assayed by gel electrophoresis. All nuclease assay buffers contained 10 mM Mn^{2+} and 10 mM Ca^{2+}.

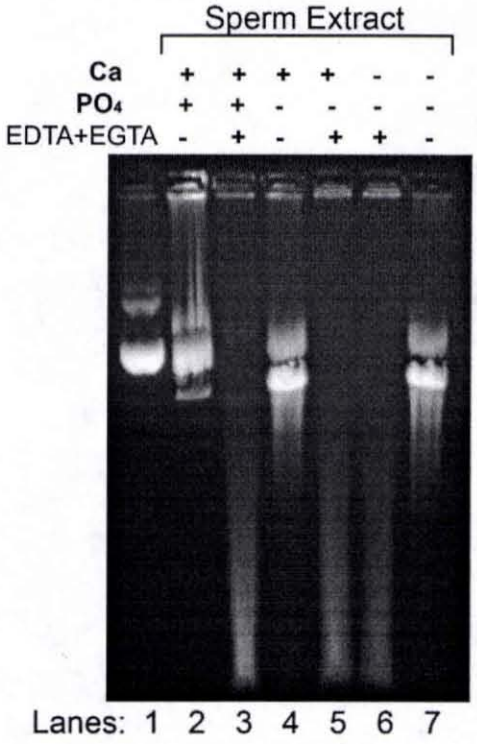
(B) Equivalent volumes of each sample in (A) were assayed by SDS-PAGE.

PBS alone, or Ca^{2+} and PBS. The extract and a quantity of the two mixtures were separately mixed with EDTA and EGTA for a total of six samples that were assayed with Mn^{2+} and Ca^{2+} and plasmid DNA. It was clear that all EDTA/EGTA containing samples, even those that were pre-incubated with only EDTA/EGTA had strong activity (Fig. 3.13, lanes 3, 5, and 6). This suggested that the increased activity we saw in Fig. 3.12 was not due to the $CaPO_4$ precipitation concentrating MCN, as we had hoped. Rather, it seemed that EDTA and EGTA alone activated the nuclease.

3.2.2. *EGTA Activated MCN Can Digest DNA in the Presence of Ca^{2+}.*

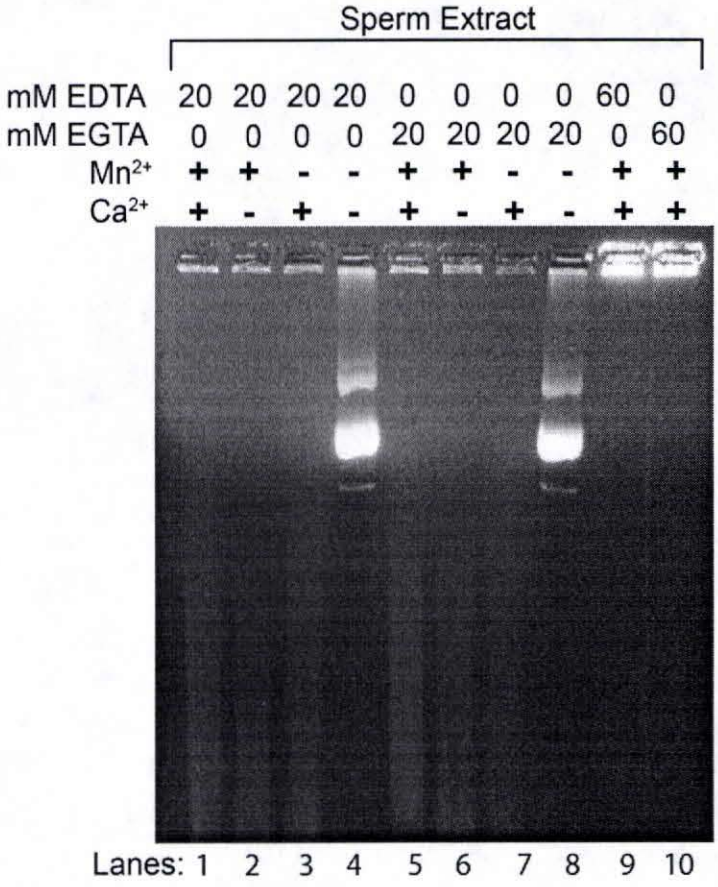
We next tested whether both EGTA and EDTA were necessary to activate MCN, and retested the cation requirements for EGTA/EDTA activated MCN, as a control. Sperm cell extract that was not pre-incubated with Mn^{2+} and $Ca^{2+}$was prepared with 20 mM EDTA, 20 mM EGTA, or both and incubated for 15 minutes at room temperature. An aliquot of the mixture was diluted to volume with buffer, 10 mM Mn^{2+} and Ca^{2+}, or with each ion, individually, and 1.25 µg DNA to assay for the nuclease. Our results showed that both EDTA and EGTA are capable of pre-activating the nuclease with nucleolytic activity following exposure to cations (Fig. 3.14). Surprisingly, we also found, for the first time, that Ca^{2+} alone was just as active in stimulating the nuclease to digest DNA as Mn^{2+}, or Mn^{2+} and Ca^{2+}. We must emphasize that the cation specificity for MCN activity never included Ca^{2+} alone. EAN activity, as shown in Fig. 3.14, clearly shows Ca^{2+} alone (Fig. 3.14, lanes 3 & 7) is sufficient to activate the nuclease.

Figure 3.13. EGTA and EDTA Activates the Nuclease.



Sperm extract without any pretreatment was prepared as described in Methods. Aliquots of the sperm extract were separately added to buffers containing 44 mM Ca^{2+} and 1 mM PO_4 (lane 2), 44 mM Ca^{2+}, 1 mM PO_4, 10 mM EDTA, and 10 mM EGTA (lane 3), 44 mM Ca^{2+} (lane 4), 44 mM Ca^{2+}, 10 mM EDTA, and 10 mM EGTA (lane 5), 10 mM EDTA and 10 mM EGTA (lane 6). A volume of 3 µl of each was then added to separate nuclease assay buffers containing 10 mM Mn^{2+} and 10 mM Ca^{2+}. All samples were analyzed by conventional agarose electrophoresis.

Figure 3.14. EGTA Followed by Ca^{2+} Alone Activates the Nuclease.



Sperm extract was derived from the epididymis and vas deferen, as in Fig. 3.13. Separate aliquots of the sperm extract were treated with 20 mM EDTA, 20 mM EGTA, 60 mM EDTA, and 60 mM EGTA. Three µl aliquots of 20 mM EDTA treated extract were added separately to nuclease assay buffers containing plasmid DNA with either: 10 mM Mn^{2+} and 10 mM Ca^{2+} (lane 1); 10 mM Mn^{2+} (lane 2); 10 mM Ca^{2+} (lane 3); or buffer containing no cations (lane 4). Lanes 5-8, as in lanes 1 – 4, except that EGTA was used in place of EDTA. Lanes 9 and 10, as in lanes 1 and 5, respectively, except that 60 mM EGTA or EDTA was used.

These results were so unique, that we changed our standard assay parameters to be pretreatment with EGTA then nuclease digestion with Ca^{2+}, alone. Furthermore, from this point on, we term the nuclease pre-activated by EGTA/EDTA "EAN" (EGTA/EDTA Pre-Activated Nuclease).

An interesting artifact did occur in Fig. 3.14 that is reproducible when using higher concentrations of chelators EGTA or EDTA. When the sperm cell extract was pre-activated with 60mM EDTA or 60 mM EGTA followed by the addition of Mn^{2+} and Ca^{2+} and plasmid DNA, it resulted in DNA being retained in the electrophoresis well (Fig. 3.14, lanes 9 & 10).

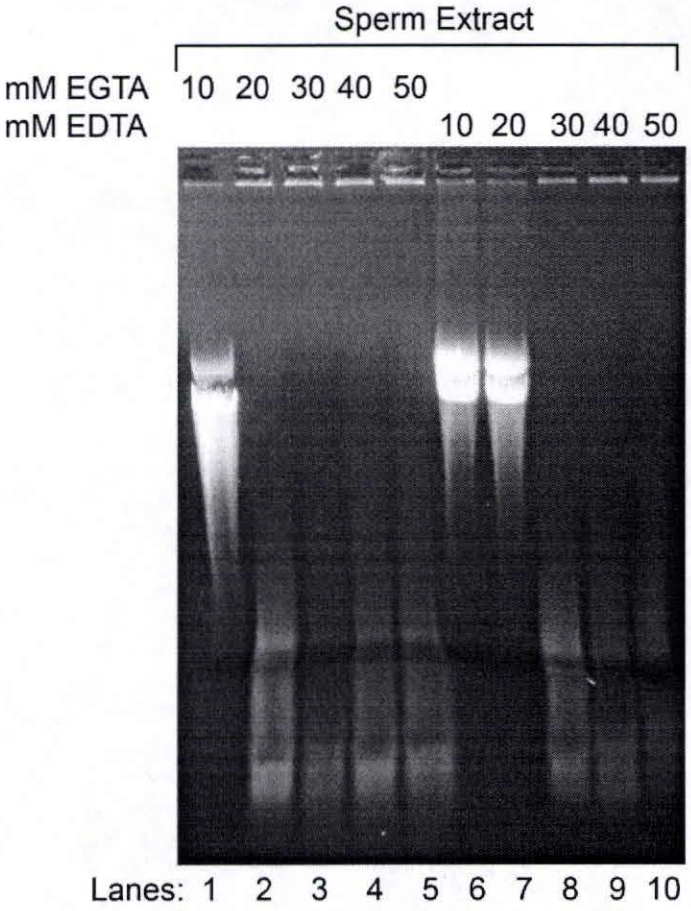
3.2.3. *EGTA can Activate EAN at Lower Concentrations than EDTA.*

We next tested for the minimum concentration of EGTA and EDTA necessary for EAN activity (Fig. 3.15). We extracted sperm cell contents and pre-activated them with increasing concentrations of either EGTA or EDTA. EGTA elicited a stronger nuclease response (Fig. 3.15, lane 2 versus lane 7) with activity occurring between 10 and 20 mM while 20 to 30mM EDTA was needed for equivalent activity.

3.2.4. *Spermatozoa Digest their DNA with EGTA Pretreatment Followed by Ca^{2+} Treatment: Evidence that EAN is a Sperm Nuclease.*

Other colleagues in the laboratory had already established by this point that (A) Mn^{2+} and Ca^{2+} nuclease pre-activation occurred inside the sperm cell resulting

Figure 3.15. EGTA is a Stronger Activator of the EAN than EDTA.

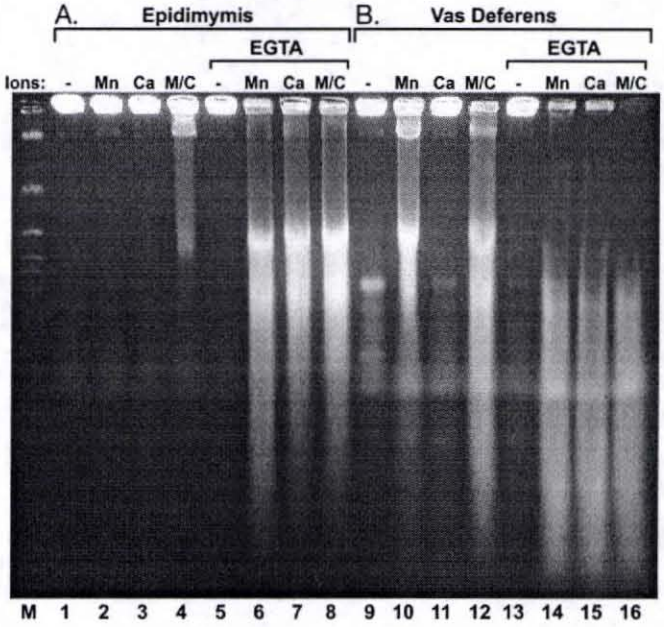


Sperm extract was derived from the epididymis and vas deferens, as in 3.13. Separate aliquots of the sperm extract were treated with increasing concentrations (10, 20, 30, 40, and 50 mM) of EGTA (lanes 1 – 5) or EDTA (lanes 6 - 10). A volume of 3 µl from each was separately added to nuclease assay buffers containing 10 mM Mn^{2+} and 10 mM Ca^{2+}. All samples were analyzed by conventional agarose electrophoresis.

in the cell's own chromatin degradation and (B) that the activity was stronger in spermatozoa from the vas deferens than from the epididymis. We felt it was important to test for EAN activity inside the sperm cell that targeted the sperm's own chromatin before pursuing the isolation and characterization of EAN. Because EAN pre-activation had been demonstrated to work well in vitro using plasmid DNA as the substrate suggested that the same mechanism may exist inside the sperm cell using chromatin as the substrate. For these experiments the sperm cells from the epididymis or vas deferens were separately mixed with agarose and cast in plugs followed by incubation with 2 M $NaCl_2$ and 2 mM DTT to remove protamines. The agarose plugs where then incubated with or without EGTA, then in Mn^{2+} alone, Ca^{2+} alone, or Mn^{2+} and Ca^{2+} for 1 hour at 37º C, then analyzed by field inversion gel electrophoresis (FIGE).

The results demonstrated several points. First, as mentioned above, spermatozoa from the vas deferens had much stronger activity than those from the epididymis (lanes 4 and 12, Figs. 3.16A versus 3.16B). Secondly, spermatozoa contain EAN activity as defined by us: they could digest their DNA when treated with EGTA then with Ca^{2+}, alone (Figs. 3.16a and 3.16b, lanes 7 and 15). This was the first demonstration that sperm chromatin could be digested in the presence of Ca^{2+}, alone. Third, they demonstrated that EAN activity was much greater than

Figure 3.16. EGTA Activated Nuclease (EAN) Activity Degrades Sperm Chromatin.



EGTA stimulates sperm DNA degradation after salt treatment. Total fluid from (A) the epididymis (lanes 1 – 8) or (B) vas deferens (lanes 9 – 16) was embedded in agarose plugs and treated with 2 M $NaCl_2$, 2 mM DTT for 1 hr at 37º C, then treated with or without 30 mM EGTA, as indicated. All plugs were then treated with 10 mM $MnCl_2$, alone (lanes 2, 6, 10 and 14), or with 10 mM $CaCl_2$, alone (lanes 3, 7, 11, and 15), or with both (lanes 4, 8, 12 and 16) as indicated. The reactions were stopped with SDS, and the DNA analyzed by field inversion gel electrophoresis (FIGE).

MCN activity in salt extracted spermatozoa (compare lanes 4 and 7, and 12 and 15, Fig. 3.16). This suggested to us that it was possible that EAN and MCN was the same enzyme, but that it was activated much more strongly by EGTA and Ca^{2+}. These results were published [89].

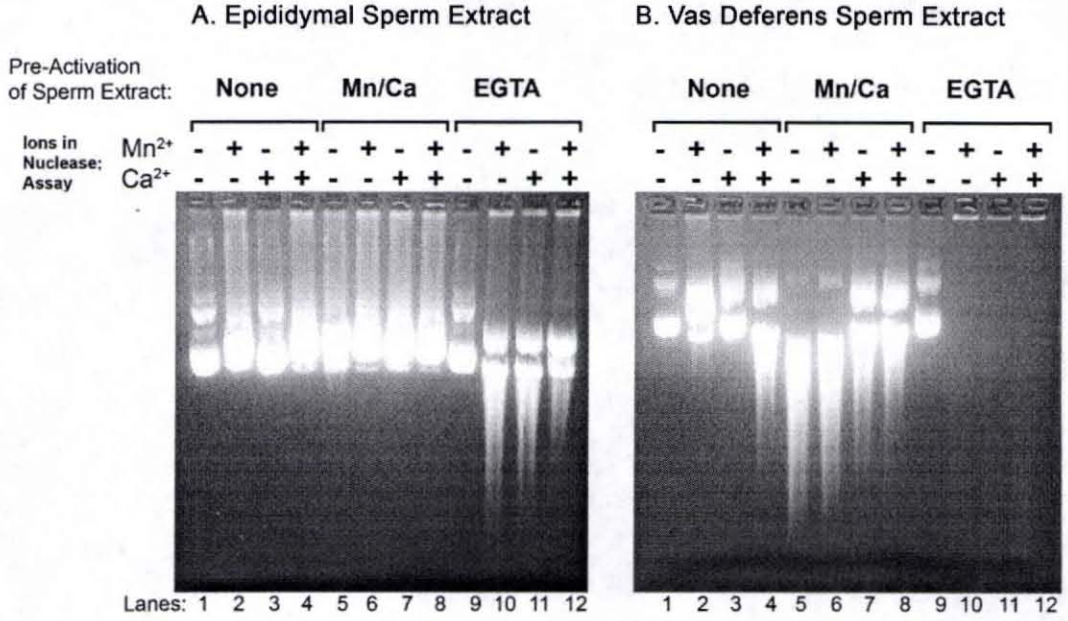
3.2.5. EAN Activity is Stronger in the Vas Deferens Luminal Fluid than in the Epdidiymis.

Because EAN activity in sperm chromatin was shown to be stronger when the sperm were derived from the vas deferens rather than from the epididymis, we tested to see if EAN activity on plasmid DNA was also stronger when the sperm extract was extracted from sperm cells derived from the vas deferens rather than from the epididymis (Fig. 3.17). As with the sperm chromatin digestion study (Fig. 3.16), vas deferens derived sperm had stronger EAN activity than the epididymis (Fig. 3.17, lanes 22-24 versus lanes 10-12). Again, Ca^{2+} alone was sufficient for nuclease activity (Fig. 3.17, lane 11 & lane 23). These results were also published [89].

3.3. EAN is Present in all Luminal Fluid Fractionsbut Appears to be Most Highly Concentrated in Lipid Vesicles

Since Mn^{2+} and Ca^{2+} activated nuclease is present in all fractions of the luminal fluid (Pel A, Sup A, Sup B, and Pel B) we were curious as to whether EAN was present in the same fractions. For our test parameters we analyzed Sup B and Pel B for EAN in place of Sup A because these two fractions compose Sup A. We

Figure 3.17. EAN Activity is Greater in Vas Deferens Sperm Extract than that from the Epididymis.



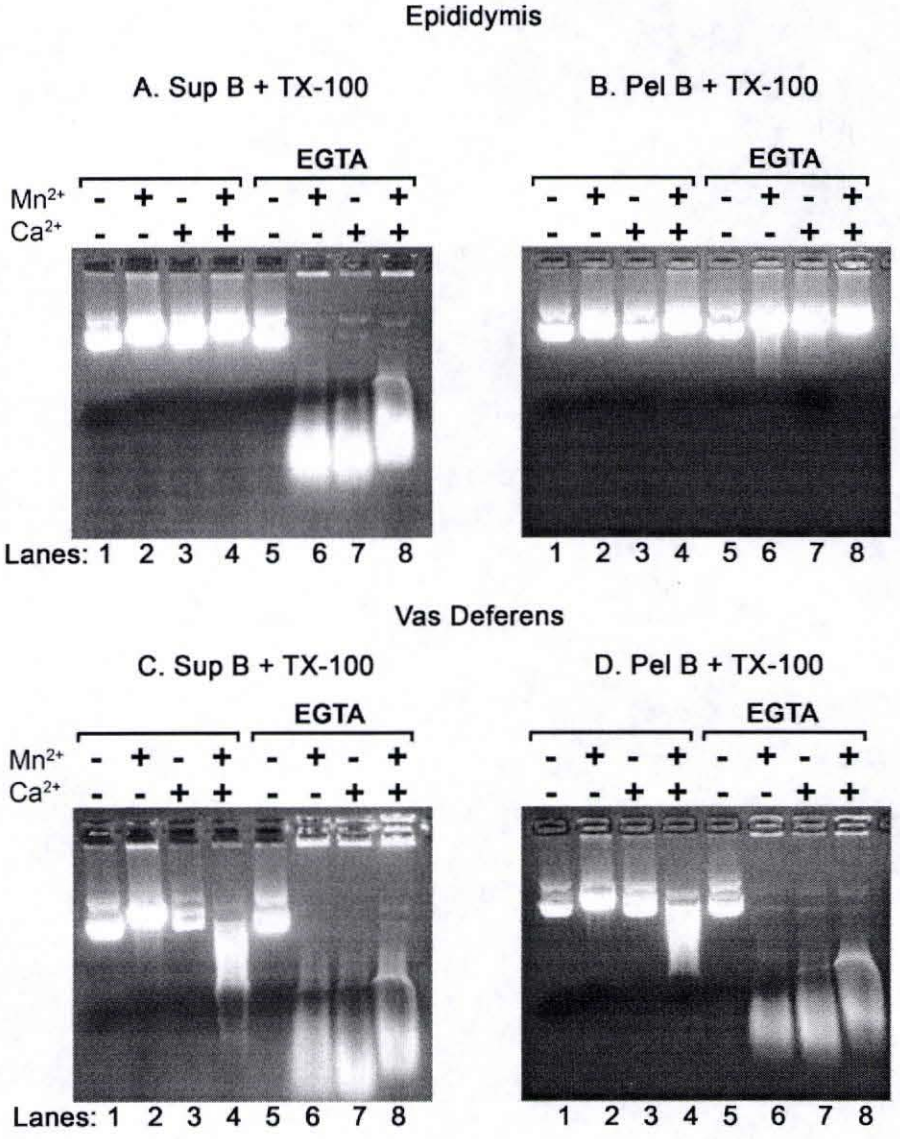
Sperm extract was derived separately from the epididymis or vas deferens using the same methods as in Fig. 3.13. Portions of both extracts were either left untreated or treated with 10 mM Mn^{2+} and 10 mM Ca^{2+} or 20 mM EGTA. A volume of 3 µL of untreated extract from both the epidydimis and vas deferens was assayed for nuclease activity with no cations present as a control (A and B, lane 1), with 10 mM Mn^{2+} alone (A and B, lane 2), 10 mM Ca^{2+} alone (A and B, lane 3), and 10 mM Mn^{2+} and 10 mM Ca^{2+} combined (A and B, lane 4). Lanes 5 – 8 (A and B) as in lanes 1 – 4, except that the sperm extract was pretreated with Mn^{2+} and Ca^{2+}. Lanes 9 – 12 (A and B), as in lanes 1 – 4, except that sperm extract was pretreated with 30 mM EGTA. All samples were analyzed by conventional agarose electrophoresis.

did not analyze Pel A for EAN as it was already described above. Secondly, we also compared EAN activity in Sup B and Pel B from the vas deferens against the same fractions in the epididymis as these two organs contained different levels of MCN activity (Fig. 3.17). We will now describe EAN activity as specifying a distinct nuclease that requires EGTA for pre-activation, and is fully activated with Ca^{2+}. EAN can be fully activated with other cations like Mn^{2+}, and Mn^{2+} and Ca^{2+}, however, we have found no other nuclease in sperm that can be activated with Ca^{2+} alone, thus we operationally define EAN as pre-activatable with EGTA then activatable with Ca^{2+}.

3.3.1. EAN Activity is Present in Vas Deferens Lipid Vesicles.

For our test (Fig. 3.18) the luminal fluid from the epididymis and vas deferens were extracted separately and centrifuged at 2,000 x g for 2 minutes. The resultant Sup A from the epididymis and vas deferens were separately aspirated to new tubes and centrifuged at 26,000 x g for 10 minutes at 4º C. The supernatants (Sup B) were aspirated to new tubes and the pellets (Pel B) were resuspended in buffer. Aliquots of each sample were pre-activated with 30 mM EGTA for 10 minutes at RT. All samples were treated with 0.25% TX-100 and 3 µL of each were added to 17 µl of nuclease assay buffer containing 10 mM of various cations, and 1.25 µg of plasmid DNA. Epididymal Sup B and Pel B did not contain any MCN activity (Figs. 3.18A and B, lanes 1-4). There was EAN activity present in the Sup B (Fig. 3.18A, lanes 6-8), however none present in Pel B. Vas deferens Sup B and Pel B exhibited

Figure 3.18. EAN and MCN Activity are both in the Luminal Fluid Supernatant and Pellet of the Vas Deferens



Fluid was collected separately from the epididymis and vas deferens. Sup B and Pel B, from either the epididymis or vas deferens, were prepared as described in Figure 3.10. Three µl of Sup B or Pel B from the epididymis were assayed for nuclease activity alone, with Mn^{2+}, with Ca^{2+}, or with Mn^{2+} and Ca^{2+} (A and B, lanes 1 – 4). Sup B and Pel B from the epididymis were also treated with 30 mM EGTA then 3 µl of each were assayed for nuclease activity alone, with Mn^{2+}, with Ca^{2+}, or with Mn^{2+} and Ca^{2+} (A and B, lanes 5 – 8). The same conditions used to test for nuclease activity were also performed on Sup B and Pel B from the vas deferens (C and D). All samples were analyzed by conventional agarose electrophoresis.

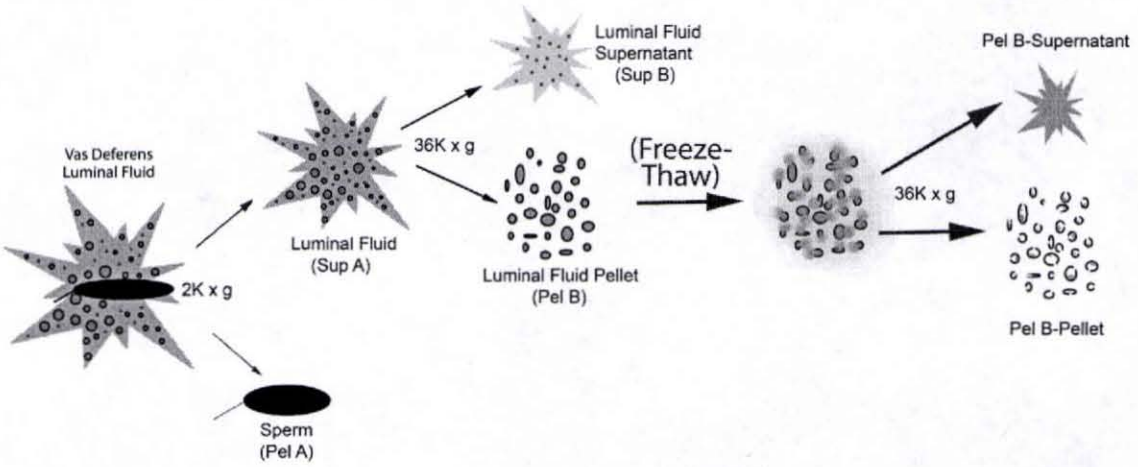
similar MCN activity (Figs. 3.18C and D, lanes 1-4). EAN activity was present in both Sup B and Pel B (Figs. 3.18C and D, lanes 6-8). It was clearly noticeable that the epididymis contained less overall nuclease activity in the Sup A than the vas deferens. Another observation was noted; an increase in EAN activity as luminal fluid travels from the epididymis to the vas deferens may suggest that nuclease activity transfers into the Pel B fraction, as the Pel B consists of lipid vesicles.

3.3.2.. Fractionation of the Vas Deferens Lipid Vesicles to Enrich for EAN Activity.

Since our data suggested that the nuclease in the vas deferens Pel B is contained within lipid vesicles (Section 1) that seemingly act to naturally encapsulate the nuclease we next tested the hypothesis that the lipid membrane of the vesicles could be ruptured allowing the nuclease contained within to escape. Because the focus of this work was to isolate and characterize the sperm nuclease associated with SDD, and because our data so far suggested that EAN was this nuclease, we focused on the vas deferens lipid vesicles as the most likely fraction from which we could identify and characterize EAN. Additionally it was thought that the total protein concentration found in the contents of the vesicles in Pel B would be lower yet still maintain nuclease activity and would be, in effect, an enzyme enriched fraction.

To test this idea we ruptured the vas deferens lipid vesicles by freeze/thawing, according to the method diagrammed in Fig. 3.19. We first extracted the luminal contents of the vas deferens and centrifuged it at 2,000 x g

Figure 3.19. Luminal Fluid Pellet Fraction Separation Scheme

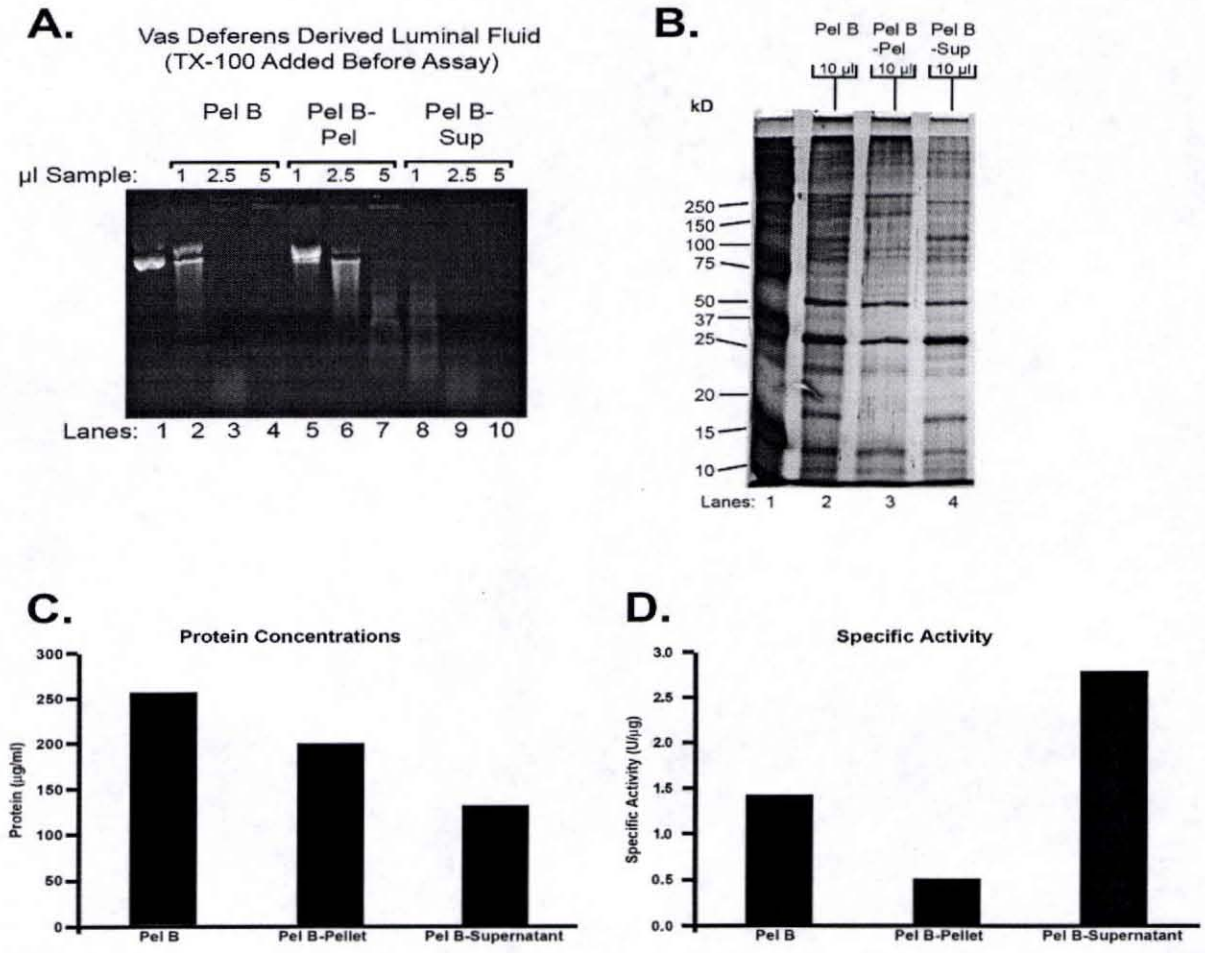


Fluid extracted from the vas deferens was diluted in buffer. The suspension was centrifuged at 2,000 x g for 2 minutes at room temperature (RT). The luminal fluid remained in the supernatant, Sup A, while spermatozoa separated to the pellet, Pel A. The Sup A was then centrifuged at 36,000 x g for 10 minutes at 4ºC. The resulting luminal fluid supernatant, Sup B, was aspirated while the luminal fluid pellet, Pel B, was resuspended in the same volume of buffer and was kept frozen at -80º C overnight. Pel B was then thawed and centrifuged at 36,000 x g for 10 minutes at 4º C. The resulting supernatant, Pel B-Supernatant, was aspirated while the resulting pellet, Pel B-Pellet, was resuspended in the same volume of buffer.

for 2 minutes. The resulting supernatant, Sup A, was then centrifuged at 26,000 x g for 15 minutes at 4º C. The resulting pellet, Pel B, was resuspended in buffer and frozen at -80º C overnight. The next day Pel B was thawed and centrifuged at 26,000 x g for 15 minutes at 4º C. The resulting supernatant, Pel B-Sup, was aspirated to a new tube and the pellet, Pel B-Pel, was resuspended in buffer. Next we pre-activated aliquots of thawed Pel B, Pel B-Pel, and Pel B-Sup with 30 mM EGTA for 15 minutes at RT. Aliquots of 1, 2.5, and 5 µL of the pre-activated samples were brought up to assay volume with buffer, 10 mM Ca^{2+}, and 1.25 µg of plasmid DNA. At this point the cation Ca^{2+} was used as it specifically activates EAN. Pel B (Fig. 3.20A, lanes 2-4) exhibited the strongest EAN activity with only 2.5 µL needed to digest nearly all the DNA. Pel B-Pel (Fig. 3.20A, lanes 5-7) exhibited the least activity of the three and Pel B-Sup (Fig. 3.20A, lanes 8-10) exhibiting higher activity than Pel B-Pel but not as high as Pel B. It is interesting to note that Pel B-Sup seems to be twice as strong in nuclease activity as Pel B-Pel as only 2.5 µL of Pel B-Sup was needed to digest the same amount of DNA as 5 µL of Pel B-Pel.

We next examined the protein profiles of Pel B, Pel B-Pel, and Pel B-Sup by denaturing aliquots of each in Laemmli's buffer, running them through SDS-PAGE, and staining the gel with silver nitrate (Fig. 3.20B). For each lane, equivalent volumes of the suspensions were electrophoresed, so that we could compare the relative amounts of protein. It is clear that Pel B (Fig. 3.20B, Lane1) has overall darker protein staining and more bands. Pel B-Pel and Pel B-Sup (Fig. 3.20B, lanes 3 & 4) differ in the amount of protein staining at the top of the lanes (heavier

Figure 3.20. Luminal Fluid Pellet Derived Fractions: Comparative EAN Activity and Protein Analysis



(A) The fractions Pel B, Pel B-Pellet, and Pel B-Supernatant were prepared from the vas deferens as described in 3.19 and treated with 30 mM EGTA for 10 minutes at RT. Aliquots of 1, 2.5, and 5 µl of Pel B (A, lanes 2 – 4), Pel B-Pellet (A, lanes 5 – 7), and Pel B-Supernatant (A, lanes 8 – 10) were each were assayed for nuclease activity in 10 mM Ca. All samples were analyzed by conventional agarose electrophoresis.

(B) Aliquots of the same solutions of Pel B, Pel B-Pellet, and Pel B-Supernatant were denatured in Laemmli's buffer and analyzed by SDS-PAGE. Gel was stained with silver nitrate until protein bands became visible. Lane M, molecular weight markers, as indicated on left. Lanes 1 – 3: Pel B, Pel B-Pellet, and Pel B-Supernatnat, respectively.

(C) BCA protein assay performed on triplicate aliquots of Pel B, Pel B-Pellet, and Pel B-Supernatant resulting 258, 203, and 130 µg protein / µl, respectively.

(D) Results from figures 3.20a (minimal volume needed to digest 1 µg DNA in 1 hour) and 3.20c (protein concentration) were used to calculate the respective specific activities of the Pel B fractions, Pel B, Pel B-Pellet, and Pel B-Supernatant as 1.40, 0.49, and 2.76, respectively, with equation:
Specific Activity = (activity unit / ml) / (mg total protein / ml).

proteins in Pel B-Pel) and have differences in the pattern of protein banding.

An analysis of the individual total protein concentrations of Pel B, Pel B-Pel, and Pel-B Sup was performed next. Using a BCA assay kit (serum albumin protein standards and bicinchoninic acid reagent) to determine individual protein concentrations (Fig. 3.20C); Pel B, Pel B-Pel, and Pel B-Sup contained 258, 203, and 130 μg protein / μL, respectively.

Lastly we analyzed the individual specific activities (Fig. 3.20D), units of activity per protein mass, of each fraction in order to discern which fractions were most enriched with nuclease. We used the plasmid DNA digestion assay (Fig. 3.20A) to monitor the minimal amount of each fraction required to digest 1 μg of DNA in 1 hour. Volumes of 1, 2.5, and 5 μL of each fraction were assayed. We defined an activity unit as the full digestion of DNA in 1 hour per volume of fraction. For some specific activity calculations we arbitrarily assigned approximate values (e.g. using 0.9 instead of 1 activity units to better approximate when a residual amount of DNA was left un-digested). This value was divided by the total protein concentration of a nuclease-containing fraction to give us each specific activity:

Specific Activity = (activity unit / ml) / (mg total protein / ml)

We were able to calculate the estimated specific activities of Pel B, Pel B-Pel, and Pel B-Sup as 1.40, 0.49, and 2.76, respectively.

3.4. EGTA Activates EAN only in the Presence of Ca^{2+}: Evidence that EAN is Activated by Ca^{2+}-Chelated EGTA

The experiments described above suggested that vesicles were present in the luminal fraction Pel B that contained the nuclease, and that these could be ruptured by freeze thawing to release some of the nuclease. Centrifugation of ruptured vesicles at high speed separated the contents held in the vesicle from the lipid membranes that constitute the vesicle itself. The pellet of freeze/thawed Pel B would contain ruptured vesicles that may contain attached EGTA activatable nuclease with or without its activating factors. Based on the presence of activity in both Sup B and Pel B (Fig. 3.18, C & D, lanes 6-8) we hypothesized that some of the nuclease was attached to lipid membranes of ruptured vesicles and remained associated with the vesicle membrane in Pel B, while some of the nuclease was released into Sup A. We therefore attempted to clean the Pel B with several cycles of centrifuging and resuspended the pellet. The pellet would retain EAN activity while subsequent supernatants would lack EAN activity. Additionally we tested to see if EGTA pre-activated nuclease would still be activatable with Ca^{2+} following removal of EGTA. Lastly, we tested if activity could be restored if EGTA was reintroduced.

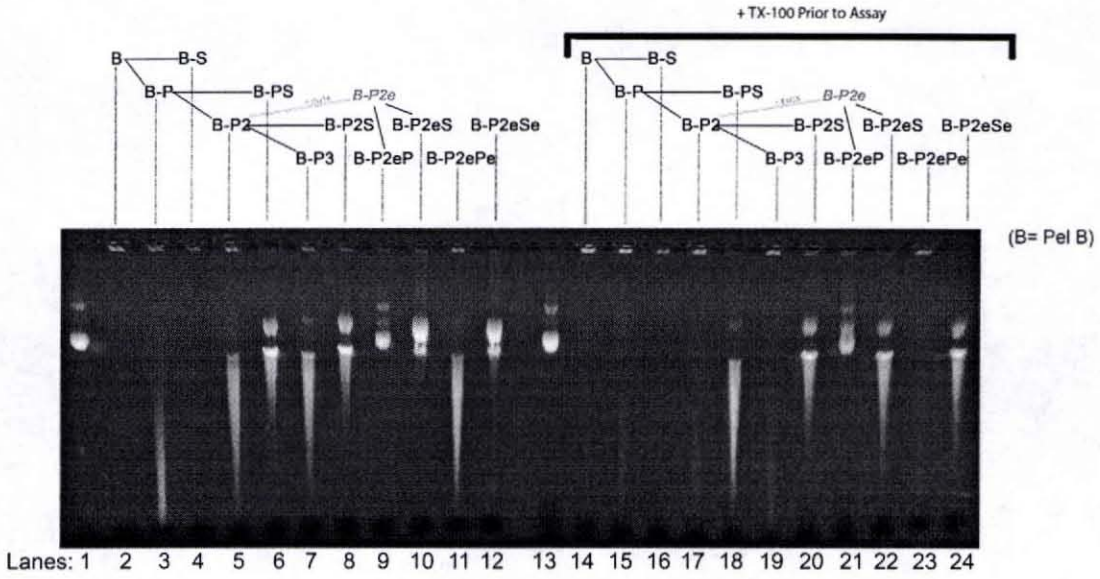
3.4.1. EAN Appears to be Bound to Vesicle Membranes.

In order to test if nuclease remained attached to the putative vesicle membranes we began with Pel B (obtained from the vas deferens) frozen at -80ºC

which was thawed and centrifuged at 26,000 x g for 10 minutes at 4º C; giving rise to the first pellet (Pel B-P) and supernatant (Pel B-S, Fig. 3.19). Pel B-P was resuspended in buffer and centrifuged again at 26,000 x g, giving rise to the second pellet (Pel B-P2) and supernatant (Pel B-PS). Pel B-P2 was resuspended and an aliquot of it and Pel B-PS was transferred separately to new tubes. The remaining Pel B-P2 was centrifuged at 26,000 x g, giving rise to the third pellet (Pel-B-P3) and respective supernatant (Pel B-P2S). All samples were then pre-activated with 30 mM EGTA for 15 minutes at RT. Lastly, a 3 µL aliquot of each sample was brought up to assay volume with buffer, 10 mM Ca^{2+}, plasmid DNA and separately with buffer, 10 mM Ca^{2+}, 0.25% TX-100, plasmid DNA and 0.25% TX-100. We used TX-100 on the second set of samples to ensure solubilization of any enduring vesicle membranes thereby releasing remaining nuclease. As shown in Fig. 3.21, these fractions mark a stage were each successive pellet (lanes 5 and 7) afterward continued to retain EAN activity while their respective supernatants (lanes 6 and 8) continued to maintain residual amount of EAN activity. By the third wash, there was very little EAN activity in the supernatant, while there was still activity in the pellets.

The results, demonstrated that from the first spin, Pel B (the starting source of EAN) and Pel B-S (the supernatant of Pel B) contained the highest amounts of EAN activity when assayed with or without TX-100 (Fig. 3.21, lane 2-4, and 14-16). This is consistent with our previous results that showed that most of the EAN is released from Pel B by freeze/thawing. High activity levels were expected as the

Figure 3.21. Localization of EAN Activity Within Luminal Fluid Pellet Fractions



The fractions Pel B (lanes 1 and 14), Pel B-Pellet (Pel B-P, lanes 2 and 15), and Pel B-Supernatant (Pel B-S, lanes 3 and 16) were prepared from the vas deferens as described in 3.19. The Pel B-P was then centrifuged at 36,000 x g for 10 minutes at 4º C resulting in the supernatant Pel B-PS (lanes 6 and 18) and pellet Pel B-P2 (lanes 5 and 17). Pel B-P2 was then resuspended in buffer and centrifuged at 36,000 x g for 10 minutes at 4º C resulting in the supernatant Pel B-P2S (lanes 8 and 20) and pellet Pel B-P3 (lanes 7 and 19). Pel B-P3 was then resuspended in buffer. An aliquot of Pel B-P2 was then treated with 30 mM EGTA for 15 minutes at RT and then centrifuged at 36,000 x g for 10 minutes at 4º C resulting in the supernatant Pel B-P2eS (lanes 10 and 22) and pellet Pel B-P2eP (lanes 9 and 21). The pellet was then resuspended in buffer. Separate aliquots of Pel B-P2eS and Pel B-P2eP were then treated EGTA for 15 minutes at 4º C and renamed Pel B-P2eSe (lanes 12 and 24) and Pel B-P2ePe (lanes 11 and 23). An aliquot of each sample was then separately treated with 0.25% TX-100 after which 3 µl of each was assayed for nuclease activity in 10 mM Ca. All samples were analyzed by conventional agarose electrophoresis.

contents of Pel-B have been shown to be EAN rich and soluble, therefore would remain in the supernatant. The 1st resuspended pellet of Pel B, Pel B-P, exhibited reduced activity in the absence of TX-100 (Fig. 3.21, lane 2) and strong activity in the presence of TX-100 (Fig. 3.21, lane 15). The difference in activity suggests that solubilizing the lipid membrane using TX-100 releases embedded nuclease from vesicle membranes and may allow for more interaction between Ca^{2+} activated nuclease and its substrate, plasmid DNA.

Without TX-100 the EAN activity levels of the 2nd and 3rd resuspended pellets of Pel B, Pel B-P2 and Pel B-P3, were moderate and equal to each other yet slightly weaker than Pel B-P (compare lane 3 with lanes 5 and 7, Fig. 3.21).

The difference in activity between Pel B-P and subsequent pellets may be due to residual Pel B-Pel with TX-100 (Fig. 3.21, lanes 15, 17, and 19, respectively). This suggested that there was much more EAN activity retained in the washed pellets than was apparent when EAN was assayed without TX-100.

3.4.2. EAN in Supernatants May also be Membrane Bound.

The supernatants of centrifuging resuspended Pel B-P and Pel B-P2, Pel B-PS and Pel B-P2S, exhibited similar nuclease activity levels suggesting the amount of unattached nuclease released from Pel B-P and Pel B-P2 was equal and minor (Fig. 3.21, lanes 6 and 8). However TX-100 treated Pel B-PS exhibited stronger activity than TX-100 treated Pel B-P2S suggesting the amount of unattached nuclease decreases with each successive centrifugation cycle. The difference

between the two TX-100 treated and untreated supernatants may suggest that centrifugation at 26,000 x g is not enough to pellet small nuclease embedded membrane fragments, or that small, lipid bound fragments are released from the vesicles that contain EAN bound to them. The resulting supernatant, when treated with TX-100, would have a higher concentration of available nuclease interacting with DNA.

3.4.3. *EGTA Activation is Not Permanent.*

We next began to test the mechanism of EGTA activation. At this point, two broad scenarios were possible. One was that EGTA could activate a complicated signaling pathway that was essentially irreversible once it was initiated. This might have included caspase or caspase like proteolytic cleavage, for example. The second possibility was that EGTA performed a much simpler function, such as removing Ca^{2+} from a calmodulin or calmodulin-like protein that inhibited EAN, and that the additional Ca^{2+} required for EAN activity bound directly to the nuclease. In this case, EGTA activation might be reversed with the addition of Ca^{2+} before DNA was present. We tested for the reversibility of EGTA activation by treating Pel B-P2 with EGTA and centrifuging a third time. The aliquot of Pel B-P2 was pre-activated with 30 mM EGTA at RT for 15 minutes followed by centrifugation at 26,000 x g for 15 minutes at 4º C giving rise to its pellet (Pel B-P2eP) and supernatant (Pel B-P2eS). It should be noted that the centrifugation of EGTA activated Pel B-P2 would leave its pellet, Pel B-P2eP, with

no EGTA as it would have remained in the supernatant during centrifugation. Pel B-P2eP was next resuspended in buffer and aliquots of it and Pel B-P2eS were set aside and not treated at all or pre-activated again with EGTA.

The supernatant of this reaction, Pel B-P2eS, was aspirated and the pellet, Pel B-P2eP, was resuspended in buffer. Pel B-P2eP did not digest any DNA in the presence of Ca^{2+}, only, without additional EGTA, whether or not TX-100 was included (Fig. 3.21, lanes 9 and 21). As noted above, EGTA was part of the supernatant and was removed during aspiration after centrifugation. This suggested that whatever effect EGTA had on EAN, it was either not permanent, or EGTA has no effect until the other components of the nuclease assay are added. When EGTA was added again to this resuspended pellet, there was substantial EAN activity (Fig. 3.21, lane 23). This indicated that EAN was present in the Pel B-P2eP, but that the EGTA pre-activated nuclease in Pel B-P2 still required the presence of EGTA in Pel B-P2eP with the addition of Ca^{2+} to fully activate the nuclease.

When the supernatant of EGTA pre-activated Pel B-P2, Pel B-P2eS, was treated with TX-100 and activated in the presence of Ca^{2+} the nuclease digested a moderate amount of plasmid DNA. This suggested that unattached nuclease in the presence of both EGTA and Ca^{2+} was activated and accessible to plasmid DNA.

3.4.4. EGTA Activation of Soluble EAN is Also Not Permanent.

The reversible nature of membrane bound EAN activation contained in the pellet of freeze/thawed PelB, as described in lanes 9 and 21 of Fig. 3.21 was another unique characteristic of EAN since as far as we know no other nuclease has been described with this activity. We therefore tested if similar activity patterns could be found in released vesicular contents found in the supernatant of freeze/thawed Pel B. If so, this would further support our hypothesis that the EAN activity in the Pel B pellet and supernatant were the same enzyme. As Pel B contained pelletable nuclease bound to vesicle membranes, which acted as scaffolding for the nuclease, the membranes easily allowed us to change the resuspension solution and likewise the environment of the enzyme. However, the supernatant of freeze/thawed Pel B did not naturally provide such a scaffold for the nuclease, so we devised another technique through which we could remove the EGTA. We embedded the supernatant, along with plasmid DNA, in agarose gel plugs. The agarose plug, containing the nuclease and DNA, could then be sequentially incubated in solutions containing EGTA or Ca^{2+}. As the two molecules easily diffuse through an agarose plug we were able to change the incubation conditions of the enzyme.

For our test we began with Pel B (obtained from the vas deferens) frozen at -80º C which was thawed and centrifuged at 26,000 x g for 10 minutes at 4º C; giving rise to the first pellet (Pel B-P) and supernatant (Pel B-S). Pel B-S was aspirated and mixed with buffer, DNA and agarose. The solution was then cast into 5 plugs. Because not all plugs were being incubated concurrently, the inactive

ones were storedat4º C until use. Each plug was incubated sequentially in EGTA or buffer for 15 minutes intervals at room temperature (RT). For the last incubation, all plugs except for the negative control were incubated at 37º C for 1 hour. The negative control was left at RT for 1 hour. The order for each incubation is shown in Table 1. The expected outcomes were based on the results of the Pel B-P experiment, shown in Fig. 3.21.

Table 1. Protocol for Pel B-S Incubations in Agarose Plugs.

		15" RT	15" RT	15" RT	15" RT	60" 37º C	Expected Activity
1	Plug	Ice	Ice	Ice	Ice	RT	No
2	Plug	Ice	Ice	Ice	Ice	EGTA	No
3	Plug	Ice	Ice	Ice	EGTA	Ca	Yes
4	Plug	Ice	EGTA	TKB	TKB	Ca	No
5	Plug	EGTA	TKB	TKB	EGTA	Ca	Yes

For our negative control (plug 1, Table 1) no activity was initiated by leaving DNA and nuclease together for 1 hour at RT without EGTA or Ca^{2+} (Fig. 3.22, lane 1). As expected, EGTA in the absence of Ca^{2+} did not activate the nuclease when incubated at 37º C for 1 hour (Fig. 3.22, lane 2). For a positive control, we activated EAN activity by incubating the plug in 30 mM EGTA followed by incubation in 10 mM Ca^{2+} (Fig. 3.22, lane 3).

Figure 3.22. Luminal Fluid Pellet Derived Supernatant: EAN Activation Conditions

Step 1: 15" RT	ice	ice	ice	ice	EGTA
Step 2: 15" RT	ice	ice	ice	EGTA	TKB
Step 3: 15" RT	ice	ice	ice	TKB	TKB
Step 4: 15" RT	ice	ice	EGTA	TKB	EGTA
Step 5: 60" 37°C	RT	EGTA	Ca^{2+}	Ca^{2+}	Ca^{2+}

Lanes: 1 2 3 4 5

The fraction Pel B-S was prepared from the vas deferens as described in Figure 3.19, mixed with plasmid DNA, cast into agarose plugs, and then kept near 0º C . Individual plugs were then incubated for 15 minutes at RT in various solutions in specific order: EGTA, buffer, buffer, EGTA, and Ca (lane 5); EGTA, buffer, buffer, Ca^{2+} (lane 4); EGTA and Ca^{2+} (lane 3); EGTA only (lane 2). The last incubation step for each plug was designed as a nuclease assay and was kept for one hour at 37º C except for the control (lane 1) which was at RT for one hour. All EGTA and Ca^{2+} solutions were 30 mM and 10 mM, respectively. All samples were then analyzed by conventional agarose electrophoresis.

Our next finding, interestingly, shows that incubating the plug in 30 mM EGTA followed by two buffer incubations and finally in 10 mM Ca^{2+} does not initiate activity (Fig. 3.22, lane 4). By incubating the plug in two successive buffer solutions the concentration of EGTA in the plug was extracted, suggesting that EGTA along with Ca^{2+} must be present at the same time in order for activity to occur. This result is in agreement with Fig. 3.21, lanes 9 and 21. Both experiments provide examples of EGTA added to and then removed from the nuclease followed by a lack of activity in the presence of Ca^{2+}. When the nuclease is incubated in 30 mM EGTA, twice in buffer, than 30 mM EGTA again before 10 mM Ca^{2+}, EAN activity is restored (Fig. 3.22, lane 5). This result also agrees with lanes 11 and 23 of Fig. 3.21, as in each situation the nuclease has EGTA added, removed, and added again along with Ca^{2+} resulting in activity.

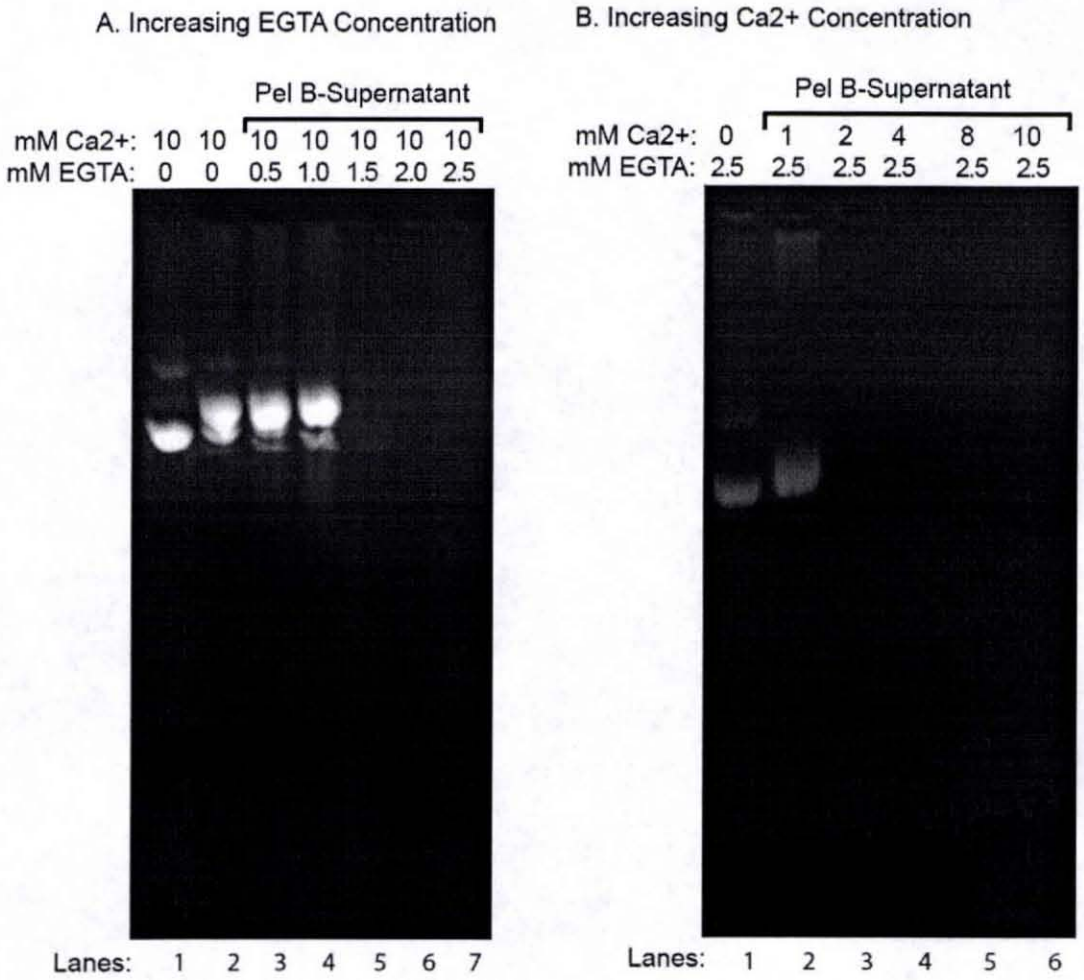
3.4.5. EGTA Chelated to Ca^{2+} Activates EAN.

The results shown in Figures 3.21 and 3.22 suggested a novel possibility for the mechanism of EGTA activation of EAN. In the first set of experiments in which EGTA was removed from membrane bound EAN in Pel B-P, the only way to activate EAN was to add 30 mM EGTA back to the suspension. Then, 3 µl of this suspension was added to a final volume of 20 µl of reaction buffer containing 10 mM $CaCl_2$. In this assay, the EGTA was effectively diluted to 7.5 mM, and the DNA was being digested in the presence of both EGTA and Ca^{2+}. In the second experiment, in which agarose plugged EAN was serially incubated first in EGTA

and then in Ca^{2+}, there is a point in the final incubation when EGTA is being extracted at the same time that Ca^{2+} is diffusing into the plug. Thus, it was possible that EAN required the presence of both EGTA and Ca^{2+}, simultaneously.

To test this, EAN activity was examined in a series of different concentrations of EGTA and Ca^{2+} incubated together, rather than sequentially. We first tested the lowest EGTA concentration needed to initiate EAN activity in conjunction with greater and constant Ca^{2+} concentrations. The goal of this experiment was to saturate EGTA with Ca^{2+} to determine if EAN was still able to digest DNA in the absence of any free EGTA. If all available molecules of EGTA were bound with Ca^{2+} at lower EGTA concentrations, resulting activity would further suggest that *free* EGTA is not pre-activating the nuclease, but rather that EGTA chelated to Ca^{2+}activated EAN. To test the lower activation threshold of EAN, Pel B (obtained from the vas deferens) was frozen at -80ºC, thawed, and centrifuged at 26,000 x g for 10 minutes at 4ºC; giving rise to the supernatant Pel B-S. Next, 3 µl aliquots were incubated simultaneously with 1.25 µg DNA, 10 mMCa^{2+} and increasing concentrations of EGTA (0.5, 1.0, 1.5, 2.0, and 2.5 mM) (Fig. 3.23a, lanes 3-7). The lowest concentration of EGTA needed, simultaneously with 10 mM Ca^{2+}, to activate EAN activity was between 1.0mM and 1.5mM (Fig. 3.23a, lanes 4 and 6). Since the concentration of Ca^{+2} was almost tenfold higher than that of EGTA, most of the EGTA was saturated with Ca^{+2}. However, as in all previous

Figure 3.23. Luminal Fluid Pellet Derived Supernatant: EAN Activation Thresholds



(A) Pel B-S was prepared as described in Figure 3.19 and aliquots were then incubated at RT for 10 minutes with increasing concentrations of EGTA: 2, 4, 6, 8, and 10 mM (lanes 3 – 7). Five µl of each solution, an un-incubated aliquot of Pel B-S (lane 2), and a solution containing only plasmid DNA (lane 1) were then assayed for nuclease activity in the presence of 10 mM Ca^{2+}. All samples were analyzed by conventional agarose electrophoresis.

(B) Using the the same prepared solution of Pel B-S from 3.23A; 5 µl aliquots were separately assayed in solutions of 2.5 mM EGTA with 1, 2, 4, 8, and 10 mM Ca^{2+} (lanes 2 – 6, respectively). A solution without Pel B-S (lane 1) was assayed without Ca^{2+} but with 2.5 mM EGTA as a control. All samples were analyzed by conventional agarose electrophoresis.

experiments, without any EGTA at all, there was no activity (Fig. 3.23a, lane 2) indicating that EGTA and Ca^{+2} were both still required. This experiment suggests that EAN is activated by EGTA chelated with Ca^{+2}.

Next we varied Ca^{+2} concentrations in the presence of a constant 2.5 mM EGTA (Fig. 3.23b, second gel, lanes 1-6). EAN from the same preparation of Pel B-Sup used above was not pre-activated with EGTA, but simply added to a buffer containing EGTA, Ca^{+2} and DNA. Under these conditions, we would expect a little less than half of the EGTA to be chelated by Ca^{+2}, but all of the Ca^{+2} to be chelated. We found that EAN could digest DNA in the presence of as low as 2 mM Ca^{+2} (Fig. 3.23b, lane 3). But, because the large majority of Ca^{+2} is chelated to EGTA, once again the most likely possibility is that EAN is activated by Ca^{+2}-chelated EGTA.

Together, these experiments suggested that the mechanism by which EGTA functions are that EGTA chelated to Ca^{+2} somehow activates the nuclease.

Chapter 4. Discussion

In this work, we have described a novel nuclease, EAN for EGTA Activated Nuclease, that digests DNA in the presence of EGTA and Ca^{2+} in concentrations at which EGTA is saturated by Ca^{2+}. This is a unique characteristic in nucleases, as no other enzyme that digests DNA requires the presence of both chelators and ion. This discovery has important implications for both reproductive biology and for nucleic acid biochemistry.

4.1. EAN's Requirement for Both EGTA and Ca^{2+} is Unique Among Nucleases.

EAN is activated by both EGTA and Ca^{2+}, together. There are several nucleases that require binding directly to Ca^{2+} to digest DNA [53, 83, 85]. However, these Ca-dependent nucleases are all inhibited by the presence of EGTA or EDTA, as are most other nucleases, because these chemicals chelate divalent cations that are required co-factors for the nuclease activity [85, 100-102]. This inhibition of nuclease activity is so universal that most buffers used to store DNA include at least EDTA [Maniatis]. There are some bacterial nucleases that can digest DNA in the presence of EDTA, but even these are more active in the presence of the divalent cation, in this case, Mg^{2+} [103-106]. EAN clearly requires the presence of both EGTA and Ca^{2+} (Fig. 3.18).

The divalent cation requirement of EAN is also unique. Many nucleases, the best known of which is DNase 1, require Mg^{2+} [107-109], but Mg^{2+} cannot

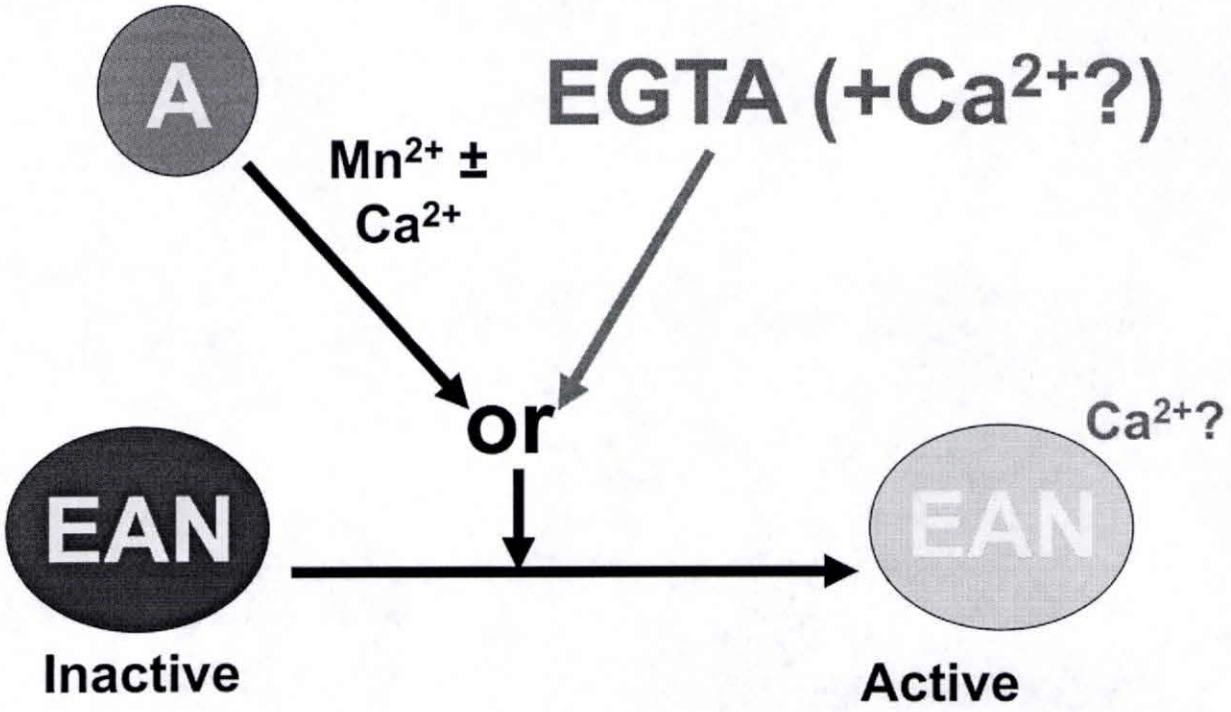
activate EAN under any conditions, with or without EGTA [89]. In addition to the Ca-dependent nucleases already mentioned, there are also nucleases that require both Ca^{2+} and Mg^{2+} [110], but these, too, are inhibited by EDTA.

Moreover, at least one of these, DNASIL3, is actually inhibited by 10 mM Ca^{2+} and 10 mM Mg^{2+}, the same concentrations of cation that we routinely used for EAN. EAN can digest DNA in the presence of between 2 and 30 mM Ca^{2+} or Mn^{2+}, but not with Mg^{2+} [89]. It is clear that EAN does not have a specific requirement for one particular divalent cation, other than that Mg^{2+} is not active. DNASIL3 has been isolated from bull semen, and we therefore thought it might be EAN, but EAN's requirement for EGTA and its ability to digest DNA in the presence of high concentrations of divalent cation, and the inability of Mg^{2+} to activate EAN all suggest EAN is a different enzyme. EAN's requirement for both a chelator (EGTA or EDTA) and a divalent cation (Ca^{2+} or Mn^{2+}) in the same buffer makes it a unique nuclease. The fact that it requires the same chemical that most researchers use to inhibit nucleases may have contributed to the fact that it was not yet described by any other laboratory.

4.2. EAN's Relationship to MCN.

In this study we have described two nucleases, EAN and MCN. Several lines of evidence support a model in which MCN is the normal pathway for activation of EAN, and EGTA short-circuits this endogenous pathway (Fig. 4.1). The first piece of evidence is that both MCN and EAN fractionate in the same way. In each

Figure 4.1. Model for EGTA Activation of EAN (EGTA Activated Nuclease)



fraction derived from luminal fluid (Pel A, Sup A, Sup B, Pel B) EAN activity was always present, however, MCN activity was found in Sup A (from which Sup B and Pel B are derived), in Sup B and Pel B, and conditionally in sperm, only when pre-incubated with Sup A and Mn^{2+} and Ca^{2+}.

The second line of evidence that MCN and EAN are the same nuclease activated by different pathways is that they both digest sperm chromatin via a topoisomerase II intermediary. When nuclease activation is studied in a physiologically relevant setting, as in the sperm cell when digesting chromatin DNA, both MCN and EAN models have similar digestion patterns in that both initially execute reversible 50 kb cleavages prior to irreversible digestion [89]. In the somatic cell, topoisomerase II cleaves nuclear DNA at inter-nucleosomal regions, during apoptosis and mitosis, resulting in 50 kb fragments [91], as such, the reversible fragmentation most likely occurs through topoisomerase II [14] and always precedes irreversible digestion by the nuclease, which suggests that the two enzymes are somehow coordinated. A similar phenomenon has been described in the case of topoisomerase II working in conjunction with another nuclease; DNASIL3 [66]. In our work this is most clearly demonstrated by the fact that even the reversible 50 kb fragmentation requires EGTA for activity.

As our data show evidence that Mn^{2+} and Ca^{2+} pretreatment of sperm, when in the presence of Sup A, gives rise to MCN, yet without the pretreatment no MCN is detectable. However, since EAN activity is always present in sperm it is

suggestive that Mn^{2+} and Ca^{2+} pretreatment may simply move an activator into the sperm cell. The activator may not necessarily be a protein but another cofactor.

EAN activity rests on the use of EGTA along with other divalent cations, however EGTA is not a natural cofactor usually found in cells. So, even if MCN and EAN are different enzymes, it is likely that EAN is naturally activated by something other than EGTA. These data support, but not prove, that MCN and EAN are the same enzyme activated by either an endogenous (MCN) or artificial (EAN) mechanism.

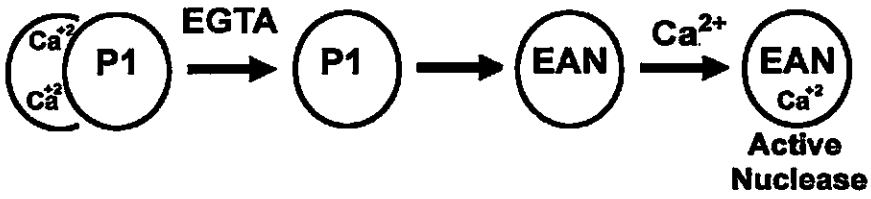
4.3. Model for EGTA Activation of EAN.

As discussed earlier, the use of a chelating chemical for the suppression of nuclease activity is normally used when handling DNA since most nucleases require a divalent metal cation for activity. In the few instances were nucleases have been described as being resistant to chelating agents, the activity exhibited in the presence of its required cation was higher than when compared to its activity with the chelating chemical [103-106]. Given that EGTA activation is so unique, the question arises as to how does EGTA activate EAN. We will discuss several models, all depicted in Fig. 4.2.

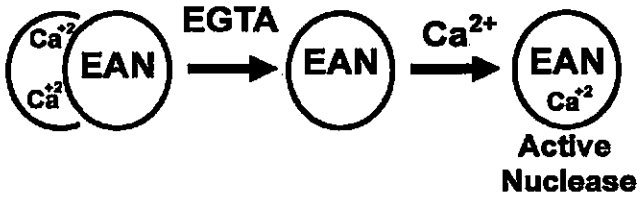
Model 1: We initially interpreted our data to suggest that EGTA activated a signal transduction mechanism, possibly through the release of a protein bound to a calcium binding protein, such as calmodulin [111, 112]. We tested our model by

Figure 4.2. Various Models of EAN Activation

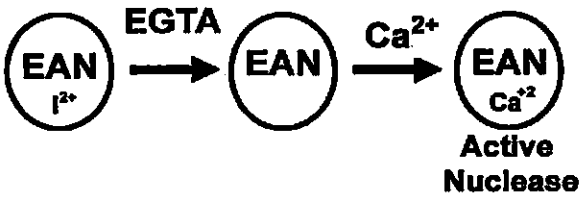
Model 1: EGTA Activates a Signal Transduction Pathway



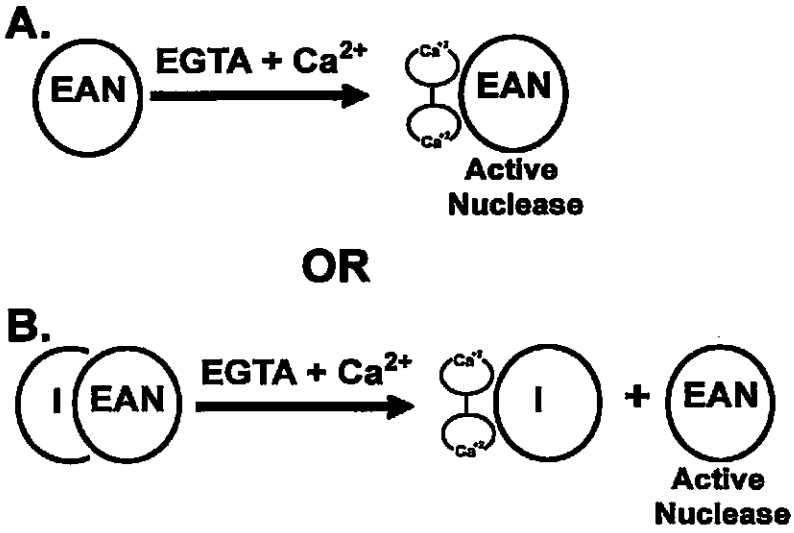
Model 2: EGTA Releases an Inhibitory Binding Protein from EAN



Model 3: EGTA Removes an Inhibitory Cation



Model 4: EGTA Chelated to Ca^{2+} Activates EAN



activating the putative signal transduction mechanism with EGTA, then removing EGTA, and adding Ca^{2+}. If this model was correct, removal of EGTA before the addition of Ca^{2+} should not prevent EAN activation because we would not expect such transduction mechanisms to operate in the reverse direction. However, we found no EAN activity in this experiment. We then tested the reversibility of the EGTA activation by again adding EGTA followed by Ca^{2+}, which resulted in nuclease activity. This suggested that a signal transduction model is not a correct depiction of the pathway mechanism.

Model 2: The idea that a calcium binding inhibitory protein binds directly to the nuclease was next explored. In this situation the nuclease requires calcium for activity and so addition of EGTA initially removes calcium from solution and so detaches the inhibitory protein from the nuclease. However, calcium is again needed to activate the uninhibited nuclease. We tested this model by adding EGTA to remove calcium, which theoretically should have released the nuclease. After removal of buffer solution, which included all added variables except the nuclease, calcium and DNA was then added. If this model was correct, the uninhibited nuclease would have been activated by calcium and DNA degradation would have ensued. However, the nuclease was not activated, but was activated by re-addition of EGTA along with calcium. Once again, the data do not support the model.

Model 3: It has been suggested that the nuclease is activated due to EGTA chelating an inhibitory divalent cation that contaminates our TKB buffer. This

model for nuclease activation may be possible however we view it as unlikely for two reasons. Our buffers are prepared using de-ionized water and the concentration of a contaminating ion needed to inhibit EAN would be unlikely. Secondly, in our last set of experiments, Ca^{2+} was added in high enough concentrations to saturate EGTA prior to the addition of nuclease, yet EAN was still active. This suggests that very little EGTA was available to chelate any other ions, including a potential unknown cation contaminant, which inhibits EAN.

Model 4: In every experiment involving nuclease in the presence of both EGTA and Ca^{2+}, concurrently, EAN was activated. We are left with the possibility that EAN is not activated by the addition, or removal, of divalent cations but by the chelated form of EGTA itself. Two possibilities are depicted in Fig. 4.2. Either EGTA chelated to Ca^{2+} binds directly to EAN, activating it (A), or the chelated EGTA binds to an inhibitory protein releasing it from inhibiting EAN. A third possibility, not shown, is that chelated EGTA binds to EAN competing off the inhibitory protein. We are currently testing this model. It is interesting to note that post-ribosomal modification of glutamic acid in certain proteins produce a modified reside called gamma-carboxy glutamic acid [113-119]. This residue is involved in protein-protein interactions, setting a precedent for our Model 4.

Our data clearly show that EAN can be activated by the presence of EGTA and Ca^{2+}, together, in concentrations of the ion that would be expected to be completing chelated in solution. In the course of our work, we rejected several more conservative models for the activation of EAN by EGTA (Models 1 and 2, and

variations thereof) and are left with only two possibilities, depicted as Models 3 and 4. For the reasons we discussed, we feel that our data support most strongly Model 4. But, this is clearly testable, and current experiments in our laboratory are directly focused on testing both of these models.

4.4. Significance of EAN.

EGTA activated nuclease, though activated by exogenous EGTA, may have the physiological purpose of degrading damaged sperm in the vas. As discussed earlier, mature spermatozoa may contain double strand breaks and EAN may serve the purpose of genetic quality control prior to ejaculation and the journey to the oocyte.

For clinical fertility, EGTA has been suggested as a preservative for mouse sperm [100, 102, 120-124] and might be used at some point for human sperm. The existence of EAN would suggest that the use of EGTA may have potentially harmful effects on the genetic integrity of stored spermatozoa under specific, yet unknown, conditions. Thus, the biochemical significance is that the EAN may represent a novel form of nuclease regulation that has not been seen before.

4.5. Summary

In this work we have identified a novel nuclease activity activated by EGTA and Ca^{2+}. Additionally, it is probable that the chelated form of EGTA may play an important role in the activation of EAN. Lastly, we have identified a fraction with a high specific activity, from which we hope to purify the enzyme.

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