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The Sperm Nuclear Matrix is Required for Paternal DNA Replication

Jeffrey A. Shaman, Yasuhiro Yamauchi, and W. Steven Ward*

From the Institute for Biogenesis Research, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii, 96822

Abstract The mammalian sperm nucleus provides an excellent model for studying the relationship between the formation of nuclear structure and the initiation of DNA replication. We previously demonstrated that mammalian sperm nuclei contain a nuclear matrix that organizes the DNA into loop domains in a manner similar to that of somatic cells. In this study, we tested the minimal components of the sperm nucleus that are necessary for the formation of the male pronucleus and for the initiation of DNA synthesis. We extracted mouse sperm nuclei with high salt and dithiothreitol to remove the protamines in order to form nuclear halos. These were then treated with either restriction endonucleases to release the DNA not directly associated with the nuclear matrix or with DNAse I to digest all the DNA. The treated sperm nuclei were injected into oocytes, and the paternal pronuclear formation and DNA synthesis was monitored. We found that restriction digested sperm nuclear halos were capable of forming paternal pronuclei and initiating DNA synthesis. However, when isolated mouse sperm DNA or sperm DNA reconstituted with the nuclear matrices were injected into oocytes, no paternal pronuclear formation or DNA synthesis was observed. These data suggest that the *in situ* nuclear matrix attachment organization of sperm DNA is required for mouse paternal pronuclear DNA synthesis. J. Cell. Biochem. 102: 680–688, 2007.

Key words: nuclear matrix; DNA replication; DNA loops; sperm chromatin

The mammalian sperm nucleus provides an excellent model for studying the relationship between the formation of nuclear structure and the initiation of DNA replication. Sperm chromatin is so highly condensed by protamines, which replace the histones during spermiogenesis, that it is essentially inert, with no DNA replication or transcription possible [Balhorn, 1982; Zirkin et al., 1982]. However, the evolutionary pressures that have condensed mammalian sperm DNA to this degree could not sacrifice the essential elements necessary for that DNA to remain functionally accessible to the oocyte after fertilization. In this respect, the mammalian sperm cell is also a good model for identifying these essential components of eukaryotic chromatin structure. After fertilization, the sperm chromatin decondenses, and histones replace the protamines to form nucleosome-based chromatin structures [Perreault and Zirkin, 1982]. A pronucleus forms around the paternal DNA, while a second pronucleus is formed around the oocyte DNA. Both then undergo one complete round of DNA replication before the first zygotic cell division [Adenot et al., 1997; Aoki and Schultz, 1999]. This functional separation of the paternal and maternal chromatin into two distinct pronuclei in the mammalian one cell embryo allows for the examination of the early events exclusive to the paternal chromatin.

This unique biology of fertilization has inspired several laboratories to use the oocyte and various combinations of extracted sperm or isolated DNA to study nuclear structure and DNA replication. The most extensively studied system is *Xenopus* oocytes and oocyte extracts.

Jeffrey A. Shaman and Yasuhiro Yamauchi contributed equally to this work.

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*Correspondence to: W. Steven Ward, Institute for Biogenesis Research, John A. Burns School of Medicine, University of Hawaii, 1960 East-West Road, Honolulu, HI 96822. E-mail: wward@hawaii.edu

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Isolated DNA injected into these oocytes or incubated with oocyte extracts initiated the formation of psuedonuclei and the DNA even began replication [Blow and Laskey, 1986; Newport, 1987; Laskey et al., 1991]. However, this DNA replication was very inefficient. Demembranated Xenopus sperm nuclei incubated with *Xenopus* oocyte extracts decondense and undergo one full round of DNA replication [Laskey et al., 1991] and it was eventually shown that in order for DNA replication to begin at the normal origins, DNA must be presented to the oocyte "in the form of an intact nucleus" [Gilbert et al., 1995]. These data suggest that the sperm cell provides some structural information to the oocyte that is required for the proper replication of the paternal DNA.

While several such experiments have been performed with lower vertebrates, very few studies on the requirements of pronuclear formation and of paternal DNA replication have been done in mammals. We have demonstrated that the only major somatic chromatin structure that is maintained in mammalian spermatozoa during spermiogenesis is the organization of DNA into loop domains attached to a proteinaceous nuclear matrix [Ward and Coffey, 1991; Klaus et al., 2001]. We suggested that sperm DNA loop domain organization provides necessary structural information for proper DNA replication and for embryonic development, and this has been supported by several recent studies. The mouse sperm nuclear matrix confers all the structural organization necessary for the oocyte to replicate the paternal DNA [Mohar et al., 2002]. Furthermore, the bases of the mouse sperm DNA loop domains have functional topoisomerase II [Shaman et al., 2006], as has been shown to be the case for somatic cells [Earnshaw et al., 1985; Cockerill and Garrard, 1986; Gromova et al., 1995]. Finally, it has recently been demonstrated in Xenopus that the sperm cell provides a unique DNA loop domain structure that is necessary for the proper development of the embryo, long after the first round of DNA replication is complete [Lemaitre et al., 2005]. This latter work suggests that sperm DNA loop domain organization may also play a role in transcriptional regulation.

These data suggest that the sperm nuclear matrix plays a role in both the proper formation of the paternal pronucleus and in the organization of DNA for replication. We tested this directly by injecting various forms of mouse sperm nuclear matrices into oocytes and following the formation of the paternal pronucleus and the paternal DNA replication.

MATERIALS AND METHODS

Animals

B6D2F1 (C57BL/6J $\times$ DBA/2) mice were obtained at 6 weeks of age from National Cancer Institute (Raleigh, NC). All mice used in this study were fed *ad libitum* with a standard diet and maintained in a temperature and light-controlled room (22°C, 14 h light /10 h dark), 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 in 1985). The protocol for animal handling and treatment procedures was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii.

Collection and Preparation of Spermatozoa for ICSI

Mature spermatozoa were extracted from the caudal epididymides and vas deferens of freshly sacrificed ~8 week old B6D2F1 mice and collected in a modified HZCB (mHCZB; [Yamauchi et al., 2007]. The solution was then supplemented with SDS to 0.5% and incubated for 5 min at room temperature. A half volume of mHCZB was added and then the complete solution was over-laid on a cushion of 550 mM sucrose and either 440 mM Tris or 10 mM Tris and 65 mM KCl. The sample was then centrifuged at 13,000 $\times$ g for 10 min to pellet the spermatozoa which were then resuspended in 2 M NaCl and 1.0 mM DTT and incubated at room temperature until halos were visible by ethidium bromide staining and viewing with an epifluorescent microscope; consistently between 15 and 25 min. The sperm cells were then centrifuged at 9,000 $\times$ g for 6 min and resuspended in TE buffer. This was supplemented with either 100 U of BamHI, 60 U each of EcoRI and HindIII, or 5 μg of DNaseI and incubated for 1.5–2 h at 37 °C before being used for ICSI. To visualize these resultant sperm nuclear halos, they were stained with ethidium bromide and viewed with a fluorescent microscope using the appropriate filter. To resolve

the DNA, sperm halos were incubated with digestion buffer and separated by Field Inversion Gel Electrophoresis (FIGE) as previously described [Shaman et al., 2006]. To quantitate the DNA released by restriction enzyme treatment, the same digestion buffer supplemented with Proteinase K was added to sperm halos and subsequently resolved on a 1% agarose gel. The relative percentages of nuclear matrix-bound and restriction enzyme-released DNA were quantified by densitometric analysis using the Kodak ID Image Analysis, Version 3.4 (Eastman Kodak, Rochester, NY).

Collection of Oocytes

Mature females, 8–12 weeks old, were induced to superovulate with i.p. injections of 5 IU eCG and 5 IU hCG given 48 h apart. Oviducts were removed 14–15 h after the injection of hCG and placed in HCZB [Kimura and Yanagimachi, 1995]. The cumulus-oocyte complexes were released from the oviducts into 0.1% of bovine testicular hyaluronidase (300 USP units/mg) in HCZB medium to disperse cumulus cells. The cumulus-free oocytes were washed with HCZB medium and used immediately for ICSI.

Intracytoplasmic Sperm Injection (ICSI)

ICSI was carried out as described recently by Szczygiel and Yanagimachi [Szczygiel and Yanagimachi, 2003]. Briefly, a small drop of treated sperm suspension was mixed thoroughly with an equal volume of HCZB containing 12% (w/v) polyvinyl pyrolidone (PVP, M_r 360 kDa) immediately before ICSI. ICSI was performed using Eppendorf Micromanipulators (Micromanipulator TransferMan, Eppendorf, Germany) with a Piezo-electric actuator (PMM Controller, model PMAS-CT150, Prime Tech, Tsukuba, Japan). A single sperm head or halo was drawn into the injection pipette and injected immediately into an oocyte. As a control, we injected the restriction enzyme reaction solution, without sperm or DNA, into oocytes.

Sperm DNA Isolation, Quantitation and Injection

2 M NaCl- and 1.0 mM DTT- extracted sperm halos were made and treated with restriction enzyme as described above. To isolate matrix-associated DNA, the samples were then centrifuged and the pellet was retained and washed with digestion buffer supplemented with Proteinase K. After incubating for 1–2 h at 55°C, the DNA was subjected to standard phenol:chloroform extraction and ethanol precipitation. The resultant DNA was quantitated using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the concentration adjusted with TKB and PVP in order to inject 3.3 pg of DNA (the amount in one spermatozoon) per 6 pl injection [Kimura and Yanagimachi, 1995] As a control, we injected 3.3 pg of Salmon Sperm DNA Solution (GibcoBRL).

Embryo Culture

After ICSI, the oocytes were cultured in 50 μl droplets of CZB overlaid with mineral oil for 5 h at 37°C, 5% CO_2 in air.

Assessment of DNA Replication

DNA replication analysis was done as described in [Ajduk et al., 2006; Yamauchi et al., 2007]. Briefly, oocytes which had been subjected to ICSI were incubated in CZB with 10 μM 5-bromo-2-deoxyuridine (BrdU) for 30 min at 7 h after ICSI, fixed with 2.5% paraformaldehyde in PBS (with 0.5 M NaOH, pH 7.3) for 15 min at room temperature, washed in PBS containing 10% fetal bovine serum (FBS) and 0.2% Triton X-100 (TX-100), and then blocked in the same solution for 30 min at 37°C. The oocytes were then washed in PBS containing 2% FBS and 0.1% TX-100 (PBS-2% FBS/0.1% TX-100) and then incubated in drops of anti-BrdU antibody conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, Oregon), diluted 1:19 in PBS-2% FBS/0.1% TX-100 for 1 h at 37°C. Oocytes were placed on poly-Lysine (1 mg/ml) coated microscope slides and covered with VectaShield mounting media containing propidium iodide (Vector Laboratories, Burlingame, CA) and examined using a fluorescence microscope fitted with the appropriate filters.

RESULTS

Sperm Halos Preparations

Mouse sperm nuclei that were extracted with 2 M NaCl and 2 mM DTT, as described in the Methods, appear as nuclear halos when stained with ethidium bromide (Fig. 1B). This is because the protamines have been extracted from the chromatin so that the DNA is devoid of all bound protein except for the attachment sites of the DNA to the nuclear matrix. The DNA

extrudes from the nucleus as loop domains. We then treated sperm nuclear halos with various nucleases to reduce the amount of DNA that remained associated with the nuclear matrix. Treatment with BamHI resulted in visibly smaller halos than untreated nuclear halos (Fig. 1C), and treatment with EcoRI and HindIII resulted in still smaller halos (Fig. 1D). Densitometric readings of ethidium bromide-stained 1% agarose gels, which was used to quantitate the amount of DNA released by the restriction enzyme digestion, show that approximately 20% and 50% of the sperm genome is removed by BamHI and EcoRI/HindIII treatment, respectively. When nuclear halos were treated with DNaseI no halo was visible (Fig. 1E). To verify the extent of DNA digestion, we subjected the spermatozoa to field inversion gel electrophoresis (FIGE) after incubation in a digestion buffer that released the DNA from the proteinaceous nuclear matrix. DNA in control and sperm nuclear halo preparations was of high molecular weight and remained in the wells of the gel (Fig. 2, lanes 2 and 3). DNA from spermatozoa treated with BamHI was broken into fragments ranging in size from 30 to 5 kb, which, while being much smaller than undigested chromatin, was larger than would be expected from purified mammalian DNA that was digested with BamHI (Fig. 2, lane 4). It has been reported that restriction endonuclease digestion of matrix associated DNA results in larger than expected fragments, and while the exact reason is still unknown it is most likely related to the complex structure of the nuclear matrix [Razin and Gromova, 1995; Razin et al., 1995]. For this study, the important point is that the DNA was only partially digested by BamHI. EcoRI and HindIII treated spermatozoa generated smaller fragments with an average size of about 7 kb (Fig. 2, lane 5). DNase I treated spermatozoa had no DNA that was detectible by FIGE (Fig. 2, lane 6).

The Requirement of Sperm Nuclear Matrix and DNA in Male Pronuclear Formation

We next investigated what is required of the sperm nucleus for male pronuclear formation and DNA replication by injecting the nuclear halo preparations described above into oocytes. Pronuclear formation was monitored by phase microscopy and DNA replication was detected by incubation with BrdU. Seven hrs after microinjection, when the male and female pronuclei were separate and had started DNA replication, embryos were fixed and examined for pronuclear formation, DNA replication, and DNA staining. Embryos lacking BrdU positive female pronuclei were discarded from the study. Normal ICSI controls formed male pronuclei that were of normal size, began DNA synthesis, and had homogenous DNA staining throughout (Fig. 3A–C). Embryos formed from the microinjection of undigested sperm halos, sper-

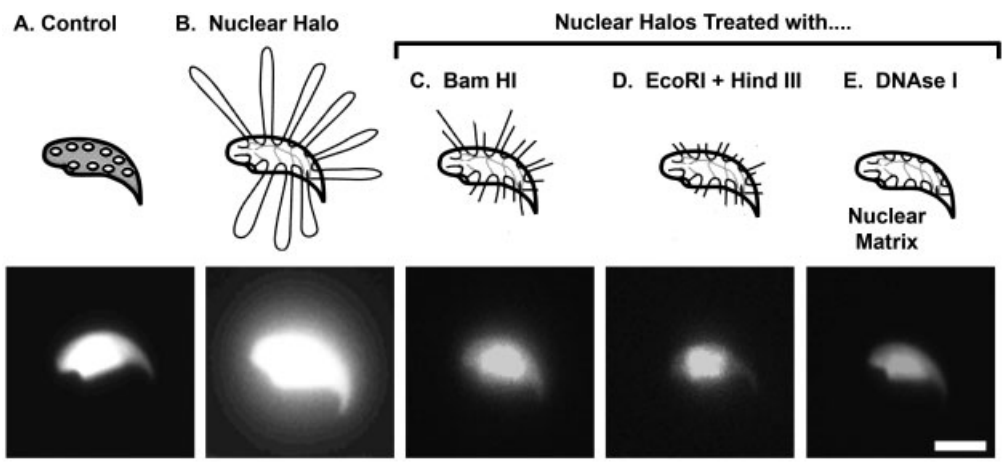

Fig. 1. Images and schematic representations of treated mouse spermatozoa that were injected into mouse oocytes. Spermatozoa were extracted from the caudal epididymus and vas deferens of B6D2F1 mice. Sperm nuclear halos were made by removing sperm membranes with SDS and extracting DNA-bound histones and protamines with salt and DTT. This allowed for the spreading of DNA radially from their attachments at the nuclear matrix. Subsequent incubation with DNase I digested all DNA, thus producing nuclear matrices. Incubating sperm nuclear halos with the restriction enzyme BamHI produced nuclear matrixes with some of the DNA removed; incubation with the restriction enzymes EcoRI and HindIII left nuclear matrixes with half as much DNA still attached. Bar = 5 μm.

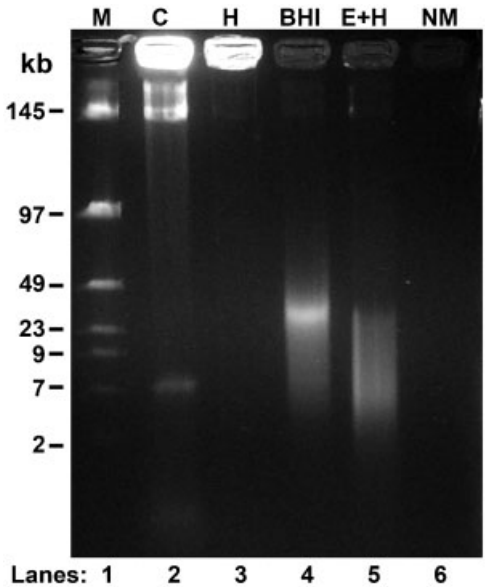

Fig. 2. Field Inversion Gel Electrophoresis separation of spermatozoa used in ICSI. Mouse nuclear halos were treated with various nucleases, then plugged in agarose, digested with SDS, and electrophoresed on FIGE. Lane 1, molecular weight markers; lane 2, control spermatozoa; lane 3, untreated nuclear halos; lane 4, halos treated with BamHI; lane 5, halos treated with Eco R1 and HindIII; lane 6, halos treated with DNAse I.

matozoa treated with 2 M NaCl and 2 mM DTT, were indistinguishable from those of control microinjections with respect to pronuclear size, DNA replication, and DNA staining (Fig. 3D–F). Virtually all of the BamHI sperm halos that were injected into oocytes supported DNA replication (Table I). Interestingly, male pronuclei from microinjections with sperm halos treated with the restriction enzyme BamHI, i.e., sperm with an intact nuclear matrix but only ~80% of intact DNA, also resembled control embryos in both the size and shape of the pronucleus (Fig. 3G) and in DNA replication (Fig. 3H). Microinjections of EcoRI and HindIII treated sperm halos formed normal-sized male pronuclei as judged by phase microscopy (Fig. 3J), but the DNA and sites of replication were restricted to a small area within the pronucleus; an area larger than the injected spermatozoa but of similar shape (Fig. 3K and L). Although the BrdU staining was weaker in these oocytes, all EcoRI and HindIII treated sperm halos (i.e., those with only ~50% of intact sperm DNA) supported DNA synthesis after injection (Table I).

Male Pronuclei from EcoRI and *Hin*dIII Treated Sperm are of Normal Size, but DNA and Replication is Constrained to a Small Volume Within the Pronucleus

Further examination of male pronuclei formed from the microinjection of sperm halos treated with EcoRI and HindIII show that while the pronucleus resembles that of control embryos, the DNA and replication sites are restricted to a small volume within the pronucleus. Of note, the area of replication, while restricted to the pronucleus, appeared to occupy a volume slightly larger than the volume occupied by the detectable DNA (compare Fig. 4B with 4C, and 4E with 4F).

The Sperm Nuclear Matrix and Attached, Endogenous DNA is Required for Male Pronuclear Formation

These data above suggested that a sperm nuclear matrix associated with only a fraction of the total genomic DNA was enough for the mouse oocyte to form a male pronucleus and initiate DNA replication. However, previous work with Xenopus oocytes suggested that purified DNA, alone, could initiate the formation of pseudopronuclei in amphibians [Newport, 1987]. It was therefore possible that the pronuclei that formed when BamHI treated halos were

TABLE I. Results of Treated Mouse Spermatozoa Micro-Injected into Mouse Oocytes

		BrdU of male pronucleus			
Treated sperm	N	Normal (%)	Negative (%)	Weak (%)	Intact male PN
Halo	55	49 (89)	6 (11)	0 (0)	55 (100)
BamHI halo	47	37 (79)	8 (17)	2 (4)	45 (96)
EcoRI and HindIII halo	23	0 (0)	0 (0)	23 (100)	23 (100)
Nuclear matrix	23	0 (0)	23 (100)	0 (0)	0 (0)
Total BamHI DNA	19	0 (0)	19 (100)	0 (0)	0 (0)
Matrix BamHI DNA	11	0 (0)	11 (100)	0 (0)	0 (0)
Salmon sperm DNA	10	0 (0)	10 (100)	0 (0)	0 (0)
NM + M BamHI DNA	14	0 (0)	14 (100)	0 (0)	0 (0)

Only those oocytes in which the female pronucleus had normal BrdU staining were assayed for male pronuclear formation and staining.

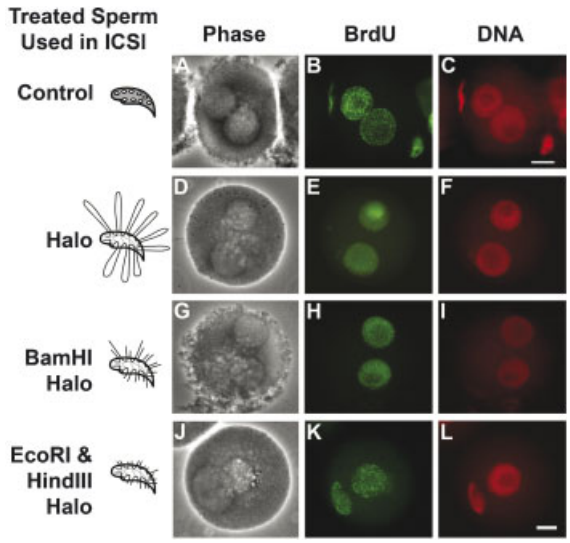

Fig. 3. Pronuclear Formation and DNA Synthesis in Oocytes Injected with Sperm Nuclear Halos. Mouse oocytes were microinjected with treated spermatozoa and activated with $SrCl_2$. 7 h after injection, the embryos were incubated with BrdU to identify areas of active DNA synthesis. The DNA was counterstained with PI. ICSI with normal spermatozoa (Control), sperm nuclear halos (Halo; prepared by removing membranes, histones, protamines, and non-nuclear matrix bound proteins), sperm nuclear halos treated with the restriction enzyme BamHI (BamHI Halo; sperm with less than the normal amounts of DNA), and sperm nuclear halos treated with EcoRI and HindIII (EcoRI&HindIII Halo). Bar = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

injected into oocytes (Fig. 2G–I) required only the DNA that was associated with the nuclear matrix, and not the nuclear matrix itself. To test this, we injected one sperm-equivalent (~3.3 pl) of either of two different fractions of sperm DNA. The first sample was prepared by digesting nuclear halos with BamHI and isolating the total DNA for injection. The second was prepared by isolating only the matrix associated fraction of this digestion. We chose these DNA preparations because their size made it much easier to manipulate than chromosomal DNA. Moreover, we had already demonstrated that BamHI halos could form pronuclei, so if DNA alone was required, the DNA from BamHI-digested sperm halos should be able to form the pronuclei.

We found that neither isolated BamHI-digested sperm DNA, isolated BamHI-digested matrix-associated DNA, or sheared salmon sperm DNA elicited the formation of pronuclei when injected into oocytes (Fig. 5D–F, Table I). In these experiments, the female pronuclei did form normally, serving as internal controls. As a control, we also injected nuclear matrices devoid of any DNA into oocytes and found that these could not initiate the formation of pronuclei, either (Fig. 5A–C, Table I). Finally, we tested whether sperm nuclear matrices that were reconstituted with matrix associated DNA could initiate the formation of pronuclei. Isolated BamHI-digested sperm DNA that was mixed with DNAse I treated nuclear matrices and injected into oocytes could not initiate pronuclear formation (Fig. 5G–I). Oocytes that were injected only with the supernatant of BamHI halos, as a control, survived demonstrating that there were no harmful effects of the buffers used to make the BamHI digested halos. These results suggested that in the mouse, male pronuclear formation required

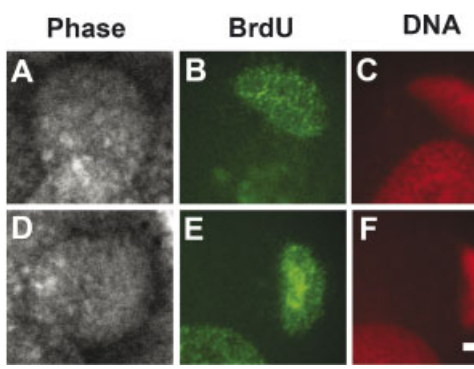

Fig. 4. Pronuclear Formation and DNA Synthesis in Oocytes Injected with DNA and Nuclear Matrices. Embryos from oocytes that were microinjected with DNase I treated sperm nuclear halos (Nuclear Matrices), BamHI halo DNA (BamHI DNA), or Nuclear Matrices and BamHI DNA together. Bar = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

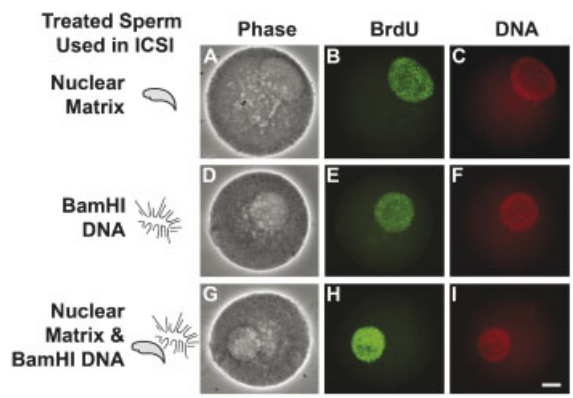

Fig. 5. Enlargement of male pronuclei in embryos fertilized with EcoRI & HindIII treated spermatozoa. Bar = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the in situ DNA loop attachment organization, and that this cannot be reconstituted properly in vitro by mixing the two components.

DISCUSSION

Our previous work demonstrated that sperm nuclear halos injected into mouse oocytes formed normal paternal pronuclei, and could complete one round of DNA synthesis and form normal mitotic chromosomes [Mohar et al., 2002]. This suggested that the sperm nuclear matrix and its associated DNA were sufficient for pronuclear formation, but did not reveal whether both components were required. The data presented here suggest that the formation of the male pronucleus in the mouse requires both the sperm nuclear matrix and the matrix associated DNA. Pronuclei still formed when ~20–50% of the sperm DNA was eliminated by restriction endonuclease digestion, but the DNA alone could not stimulate pronuclear formation. Thus, pronuclei can form and DNA synthesis can be initiated in the paternal pronucleus of the mouse oocyte even with severely degraded DNA. However, this is true only if the in situ DNA loop attachments to the nuclear matrix are preserved.

The fact that DNA alone could not stimulate pronuclear formation or DNA synthesis contrasts with a series of experiments performed in *Xenopus* that suggest that DNA, alone, can do both in amphibians [Newport, 1987; Laskey et al., 1991; Ullman and Forbes, 1995]. However, DNA replication in these experiments was not nearly as efficient as demembranated sperm cells in which the chromatin remained organized into higher domains by the nuclear matrix. Furthermore, recent experiments have provided further evidence that the structural organization that is naturally present in Xenopus sperm nuclei is very important for regulating DNA synthesis [Gilbert et al., 1995; Lemaitre et al., 2005]. Therefore, even in Xenopus, it is clear that the sperm cell provides important structural information. Our data suggest that the mammalian oocyte may simply be more sensitive to this requirement for the proper sperm chromatin structure and does not initiate DNA synthesis at all if the sperm DNA is not associated with its sperm nuclear matrix in the native form.

This latter conclusion is also supported by our attempt to reconstitute the sperm nuclear matrices with isolated BamHI-digested DNA, which also failed to induce formation of the paternal pronucleus. In the experiments depicted in Figure 3G–H and Figure 5A–C, the same components were injected into oocytes, but in the first one the DNA and the nuclear matrix were associated in their native form. The exact nature of the proteins that contribute to the formation of eukaryotic DNA loop domains remains a mystery. Our work suggests that, at least for the sperm cell, it is not possible to reconstitute a functioning DNA attachment site to the nuclear matrix by incubating isolated DNA and nuclear matrices in vitro. The requirement of the sperm nuclear matrix with its associated DNA to initiate paternal pronuclear DNA synthesis is consistent with a large body of literature that suggests that mammalian DNA replication occurs on the nuclear matrix [Vogelstein et al., 1980; Jackson and Cook, 1986; Gerdes et al., 1994; Dijkwel and Hamlin, 1995; Lemaitre et al., 2005]. We previously suggested that the sperm nuclear matrix provides the oocyte with a structural template for the replication of the paternal DNA after fertilization [Ward, 1994; Sotolongo and Ward, 2000]. Recent work in *Xenopus* supports this prediction by demonstrating that sperm DNA loop domains are already organized for proper DNA replication [Lemaitre et al., 2005]. The evidence presented in this work demonstrates that even when the sperm nuclear matrix retains only ~50–80% of the total DNA, replication can be initiated as long as the DNA is associated with the nuclear matrix in its native form. These data support the hypothesis that the sperm cell provides the developing embryo with not only the genetic material from the father, but also with the structural framework that is necessary for proper development.

Our hypothesis that the sperm nuclear matrix contributes to the paternal pronucleus structure is supported by our experiments with HindIII and EcoRI digested halos. These halos represented the minimum amount of DNA associated with the sperm nuclear matrix that could still support pronuclear development. Nuclear halos treated with two restriction endonucleases, HindIII and EcoRI, retained only ~50% of the total sperm DNA, but still supported DNA replication. In these oocytes, the paternal pronuclei appeared normal by phase microscopy, but the DNA was sequestered in one oblong area of the pronucleus that

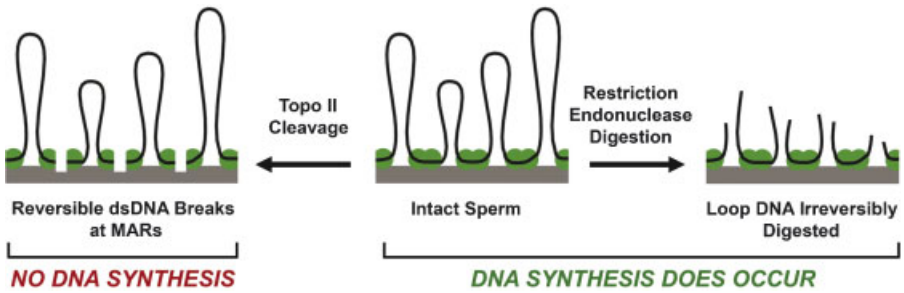

Fig. 6. Comparison of endogenous TOP2B mediated sperm DNA cleavage with exogenous restriction endonuclease treatment of sperm halos. The endogenous TOP2B of sperm nuclei can be induced to cleave all the sperm DNA at the bases of the loop domains [Shaman et al., 2006; Yamauchi et al., 2007]. This TOP2B-mediated cleavage does not eliminate any DNA sequence from the sperm chromatin and is reversible. When spermatozoa with TOP2B cleavage are injected into oocytes, paternal pronuclei do form, but there is no DNA replication [Yamauchi et al., 2007]. Here we have shown that in contrast, when much of the sperm DNA is removed by restriction endonuclease digestion, the paternal DNA is still replicated after ICSI. This suggests that the intact attachment sites of DNA to the nuclear matrix are more important for the initiation of DNA synthesis than the integrity of the DNA, itself. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

resembled a decondensed sperm head (Fig. 4). This suggests that the oocyte provides an important contribution to the structure of the paternal pronucleus that aids in defining its total size. The sperm nuclear matrix contributes something to the structure, as well, possibly the internal components of the nuclear matrix on which DNA replication actually occurs.

A recent study from our laboratory demonstrated that reversible cleavage by sperm topoisomerase IIβ (TOP2B) resulted in complete inhibition of DNA synthesis of the paternal pronuclear DNA after ICSI, while the maternal pronucleus replicated its DNA normally [Yamauchi et al., 2007]. This contrasts sharply with the data in this work that paternal pronuclear DNA synthesis could still be initiated even when most of the sperm DNA was removed by restriction endonuclease digestion. The difference between the two studies, however, was that the TOP2B mediated DNA cleavage occurred at the bases of the DNA loop domain attachment sites, just where DNA synthesis would be expected to initiate. In this study, the DNA that was removed by restriction endonuclease digestion was in the extended DNA loops while the matrix attachment regions were preserved (Fig. 3B and C). The difference in the results of these two experiments therefore supports the importance of the sperm nuclear matrix/DNA attachment regions for the initiation of DNA synthesis in the developing zygote (Fig. 6). This conclusion is supported by the recent demonstration that the efficiency of DNA replication of Xenopus erythrocyte nuclei injected into oocytes can be vastly increased when the loop domain organization of the erythrocytes is first augmented by mitotic egg extracts to resemble the smaller loops of Xenopus sperm chromatin [Lemaitre et al., 2005]. In this system the proper attachment of the DNA to the nuclear matrix was required for DNA replication to occur. They also demonstrated that sperm cells have smaller loop domains. Our data further suggest that is the in situ organization of the matrix attachment regions, or MARs, with the nuclear matrix, and not the loop size, per se, that is critical for the initiation of DNA synthesis.

The data presented in this work demonstrate that in the mouse, both the sperm nuclear matrix and its associated DNA are required for pronuclear formation and for the initiation of DNA replication. The injection of restriction digested halos into oocytes will be an important model for understanding the contribution of the sperm nuclear matrix to the developing oocyte.

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