A fraction of mouse sperm chromatin is organized in nucleosomal hypersensitive domains enriched in retroposon DNA

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SUMMARY

We have characterized a nuclease hypersensitive chromatin fraction from murine spermatozoa. Endogenous nuclease activity can be induced in mouse epididymal spermatozoa by appropriate stimuli and cause the localized degradation of chromosomal DNA. Based on these observations, we have isolated nuclease hypersensitive chromatin regions released from spermatozoa in the supernatant of pelleted sperm cells, and have cloned and characterized the DNA. Gel electrophoresis of end-labelled released DNA fragments showed a typical nucleosomal distribution. Peripherally distributed nucleohistones were visualized by immunofluorescence in sperm nuclei, and histones were identified by western blot in sperm chromatin. Moreover, the released DNA is enriched in retroposon DNA from a variety of families. FISH and immunofluorescence analysis showed that retroposon DNA and nucleohistone chromatin co-localize and are both peripherally distributed in nuclei of spermatozoa. In contrast, a major satellite DNA probe, used for control, co-localizes with highly condensed chromatin in the central region of sperm nuclei. The nuclear Ran and RCC1 proteins were also visualized in the dorsal margin of sperm nuclei, and were abundantly released with the hypersensitive chromatin fraction. Together, these results indicate that nucleohistone chromatin fraction(s) with typical features of ‘active’ chromatin are present in murine spermatozoa, are hypersensitive to nuclease cleavage, enriched in retroposon DNA and organized in nucleosomal domains. These observations suggest that nucleohistone domains identify a fraction of the sperm genome which may be functional during early embryogenesis.

Key words: Hypersensitive, Sperm chromatin, Nucleosome, Retroposon

INTRODUCTION

Spermatozoa are traditionally regarded as metabolically inactive cells, whose exclusive function lies in the contribution of the paternal genome to the diploid zygote. This view rests upon the observation that these cells have essentially developed a large head, containing the male genome in a tightly packed structure, and a flagellum that acts as the motorial apparatus; the cytoplasmic portion of sperm cells is extremely reduced and lacks most, if not all, metabolic functions characteristic of somatic cells.

In the last few years, we have devoted much effort to clarify the molecular mechanism of the interaction of sperm cells with exogenous DNA molecules underlying sperm-mediated gene transfer (reviewed by Spadafora, 1998). While studying this process, we have observed unexpected features of spermatozoa that have deeply modified our view that these cells are metabolically inert. Briefly, we have found that the binding of exogenous DNA molecules to sperm cells is mediated by a specific class of DNA-binding proteins (Zani et al., 1995), and is modulated by a conserved glycoprotein abundant in the seminal fluid of mammals or associated to spermatozoa of lower organisms (Lavitrano et al., 1992; Zani et al., 1995). A portion of sperm-bound DNA is internalized within sperm nuclei (Francolini et al., 1993) in a process mediated by CD4 molecules (Lavitrano et al., 1997). The internalized foreign DNA reaches the nuclear scaffold, in close contact with the chromosomal DNA, becomes heavily rearranged and eventually undergoes recombination events that cause the integration of exogenous sequences in the sperm genome (Zoraqi and Spadafora, 1997). Consistent with those observations is the finding that the interaction of exogenous DNA with sperm cells activates endogenous nuclease in a DNA dose-dependent manner (Maione et al., 1997). Sperm nucleases heavily degrade the foreign DNA and can also locally degrade sperm chromosomal DNA, suggesting that discrete sites of hypersensitivity exist within the tightly packed chromatin of mature sperm cells. Thus, otherwise silent enzymatic activities
are triggered in spermatozoa upon interaction with foreign DNA molecules. With lower efficiency, the activation of sperm nuclease and the ensuing cleavage of hypersensitive sites can also be induced in the absence of added exogenous DNA, by prolonging the incubation of spermatozoa in medium for several hours (Maione et al., 1997).

Here we report the molecular characterization of nuclease-hypersensitive domains released from mouse sperm chromatin upon activation of endogenous nuclease. These chromatin domains contain histones that are assembled with the DNA in a typical nucleosomal organization, and are enriched in retroposon DNA sequences. Retroposon DNA shows a specific peripheral distribution in sperm nuclei. In addition, we have found that mature spermatozoa contain both the Ran GTPase and its guanine exchange factor, the regulator of chromosome condensation (RCC1) protein. Both components of the Ran signalling network were partly released together with the nuclease-hypersensitive fraction. Together, these results support the conclusion that the highly compact nuclei of mature spermatozoa contain nucleohistone domains preferentially associated with retroposon DNA and closely resembling the ’active’ chromatin conformation of somatic cells.

MATERIALS AND METHODS

Preparation of sperm cells
Mouse epididymal sperm cells were prepared from CD1 strain males and incubated in fertilization medium (FM) (Whittingham, 1971) as described elsewhere (Maione et al., 1997). FM contains 120 mM NaCl, 25 mM NaHCO3, 2.5 mM KCl, 2 mM CaCl2, 0.5 mM MgCl2 and is supplemented with 4 mg/ml BSA. The osmolarity ranges from 275 to 290. Particular care was taken to prepare pure sperm cell samples and avoid contamination by somatic cells. To that aim, we avoided squeezing of epididymis and instead collected spermatozoa that were spontaneously released through holes made by simply puncturing the epididymis with a needle. After a 30-60 minute ‘swim-up’ selection step of mobile cells, the purity of sperm cell preparations was checked by direct observation under a phase contrast microscope. Preparations that contained somatic cells were discarded. A more accurate determination was obtained by staining whole cell nuclei with DAPI and examination under a fluorescence microscope: under these conditions, hook-shaped spermatozoa were clearly distinguishable from contaminating somatic cells.

Isolation of hypersensitive genomic DNA
Nuclease-sensitive DNA was isolated from the supernatant of samples containing at least 25×10^7 epididymal spermatozoa after incubation in FM for 3 to 5 hours, as specified in the figure legends. A sediment of non-mobile cells was discarded while mobile sperm cells were centrifuged for 4 minutes at 9000 rpm in a microfuge; the supernatant was collected and the DNA purified by repeated phenol/chloroform extraction. The amount of recovered DNA was generally too low for optical density measurements and its concentration was estimated by ethidium bromide incorporation in comparison to known standards under a UV transilluminator. DNA released in the supernatant was end-labelled using the T4 poly nucleotide kinase (Amersham) and 32P-labelled γATP, and electrophoresed through 1.2-1.7% agarose gels. Gels were fixed for 30 minutes in 10% acetic acid, 20% methanol, dried and exposed to a Kodak Biomax MS film.

Partial libraries construction and clone sequence analysis
Two partial genomic libraries were constructed using the released DNA from the sperm cells. The first library was constructed by digesting the released DNA with EcoRI/BamHI and ligating the restricted products to EcoRI/BamHI-digested pBluescriptII SK +/- vector. The second library was obtained from released DNA fragments which were blunt-ended by incubation with the Klenow polymerase (Amersham) and all four nucleotides (20 minutes at room temperature), followed by the addition of T4 polymerase (Amersham) and prolonging the incubation for 20 minutes at 12°C. The DNA was chloroform-extracted, ethanol precipitated, resuspended and ligated in EcoRV-restricted pBluescriptII SK +/- vector. Both ligations were used to transform competent DH5 Escherichia coli cells. Transformed colonies were randomly selected and plasmid preparations were sequenced. Sequences are available in the EMBL database under the accession numbers indicated in Table 1. Sequence homologies were searched in the GenBank and EMBL nucleotide databases using the BLAST and FASTA programs, as well as the RepeatMasker and CENSOR program (Jurka et al., 1996).

Isolation of nuclei from spermatozoa, DNA extraction and Southern blot analysis
Sperm nuclei were prepared essentially as described by Balhorn et al. (1977), with minor modifications (Maione et al., 1997) using cetlytrimethylammonium bromide (CTAB) detergent (Aldrich). DNA was extracted from sperm nuclei and from mouse tissues by conventional methods. DNA aliquots of 15 μg were restricted, fractionated through 1% agarose gels and blotted on Hybond nitrocellulose membranes (Amersham). Filters were hybridized with radiolabelled probes using the random priming kit (Amersham). A 60-oligonucleotide from the murine LINE/L1 ORF2 (positions 5641-5700) (Naas et al., 1998) was used as the probe to hybridize the filter in Fig. 6B and was end-labelled using the T4 polynucleotide kinase. Pre-hybridizations and hybridizations were routinely performed in 1 M NaCl, 20 mM NaP (pH 7), 0.1% NaPP and 1% NaodSO4 at 60°C. Hybridizations with the oligonucleotide were also performed at 42°C. Dot blot analysis were performed as described (Forlani et al., 1998) and filters were hybridized as above. Results were quantified by 32P-Phosphor-Imager counting of the filters.

Western blot analysis
Western blot analysis of histones was carried out using protein extract from both sperm nuclei and from the supernatant of incubated spermatozoa. To minimise contamination of samples by BSA, the concentration of BSA in FM was lowered to 0.2 mg/ml to collect spermatozoa. Nuclei were lyed at room temperature in 1 ml of HAP 2000 buffer containing 2 M NaCl, 20 mM NaP (pH 6.6), 1 mM DTT, 0.1 mM PMSF for 30 minutes with gentle agitation. In order to remove chromosomal DNA, 0.5 ml hydroxyapatite (Type II, Bio-Rad) pre-equilibrated with HAP 2000 were added to lysed nuclei and incubated overnight at 4°C under low speed magnetic stirring. Hydroxyapatite was removed through a short microfuge spin and the supernatant was extensively dialysed against sterile water at 4°C. Proteins were lyophilized and solubilized in SDS loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% BPB and 10% glycerol). Supernatant samples were similarly prepared, except that concentrated stock solutions were used to bring the concentration to HAP 2000. Samples were electrophoresed through 17% SDS-PAGE and electrobotted onto nitrocellulose membranes (Amersham). Western blot analysis of Ran and RCC1 were performed on lysed spermatozoa. Cells were suspended in 10% SDS, vortexed for 2 minutes, then made 1% SDS with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% Na deoxycholate), vortexed again for about 2 minutes and incubated on ice for 10 minutes. After lysis, the suspension was sonicated for 5-10 seconds prior to addition of standard loading buffer. The supernatant from at least 10^7 sperm cells was concentrated through 3-KDa cut-off Microcon filters (Amicon Inc., Beverly, MA) and directly mixed with loading buffer. Extracts from sperm pellets and supernatant samples were fractionated by 12% PAGE and transferred to PVDF (polyvinylidenefluoride) membranes (Amersham). Membranes were
blocked in 5% low-fat milk in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) at 4°C overnight, then incubated for 2 hours at room temperature with the following primary antibodies in 5% milk/TBST (w/v): anti-histone pan antibody (Boehringer Mannheim), 1 mg/ml; anti-Ran antibody, 0.2 mg/ml, and anti-RCC1 antibody, 0.5 mg/ml (both from SantaCruz Biotechnology). Anti-histone pan was detected using 0.2 mg/ml anti-mouse secondary antibody conjugated with horseradish peroxidase (Dako Corp., Carpinteria, CA), while anti-Ran and anti-RCC1 were detected using 0.1 mg/ml horseradish peroxidase-conjugated anti-goat secondary antibody (SantaCruz Biotechnology) and revealed with the enhanced chemiluminescence detection system (ECL reagents, Amersham)

**Immunofluorescence of mouse sperm cells and isolated nuclei**

Intact mouse sperm cells and isolated nuclei were prepared as described above. Cells or nuclei were collected by low-speed centrifugation and resuspended in Ca²⁺ and Mg²⁺-free phosphate buffered saline (PBS) and immediately smeared on precleaned coverslips. Approximately 10⁶ sperm cells were spread on each coverslip. Samples were let to dry for 1 hour at room temperature, then rehydrated in PBS for about 5 minutes. Cells were fixed in freshly prepared 4% (w/v) formaldehyde/PBS for 15 minutes, rinsed in PBS 3 times for 5 minutes each, and incubated in 0.2 M glycine in PBS for 30 minutes at 37°C. Coverslips were then blocked in 20% FBS (fetal bovine serum)/PBST (PBS containing 0.05% Tween-20) for 30 minutes, and incubated with the following primary antibodies in 5% FBS/PBST for 45 at 37°C minutes: anti-histone H1 antibody (Upstate Biotechnology), 1 mg/ml; anti-Ran and anti-RCC1 antibodies, 10 μg/ml each. Anti-mouse (10 μg/ml, Vector) or anti-goat (4 μg/ml, SantaCruz Biotechnology) FITC-conjugated secondary antibodies were used to detect, respectively, anti-histone H1 and both the anti-

### Table 1. Characterization of clones derived from the nuclease hypersensitive DNA fraction

**A) Hypersensitive DNA sequences cloned after EcoRI+BamHI digestion**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Accession</th>
<th>Sequence length (bp)</th>
<th>Repeat family</th>
<th>Repeat size (bp)</th>
<th>%</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>MMU6827</td>
<td>265</td>
<td>LINE/L1</td>
<td>136</td>
<td>50.9</td>
<td>3’ end of L1 repeat</td>
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<tr>
<td>S6</td>
<td>MMU6828</td>
<td>452</td>
<td>LINE/L1</td>
<td>123</td>
<td>27.2</td>
<td>3’ end of L1 repeat</td>
</tr>
<tr>
<td>S7</td>
<td>MMU6723</td>
<td>1187</td>
<td>LINE/L1</td>
<td>251</td>
<td>21.15</td>
<td></td>
</tr>
<tr>
<td>S8</td>
<td>MMU6829</td>
<td>747</td>
<td>LINE/L1</td>
<td>485</td>
<td>40.85</td>
<td>3’ end of L1 repeat</td>
</tr>
<tr>
<td>S17</td>
<td>MMU6830</td>
<td>413</td>
<td>LINE/L1</td>
<td>485</td>
<td>40.85</td>
<td>3’ end of L1 repeat</td>
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<tr>
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<td>MMU6721</td>
<td>479</td>
<td>LINE/L1</td>
<td>119</td>
<td>15.9</td>
<td>3’ UTR</td>
</tr>
<tr>
<td>Total A</td>
<td></td>
<td></td>
<td></td>
<td>1660</td>
<td>53.04</td>
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</table>

**B) Hypersensitive sequences cloned after filling-in ends generated by endogenous nucleases**

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<tr>
<th>Sb9</th>
<th>MMU238777</th>
<th>1592</th>
<th>LTR</th>
<th>109</th>
<th>6.85</th>
<th>R-LTR 11A</th>
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<tbody>
<tr>
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<td>139</td>
<td>R-SINE1</td>
<td>109</td>
<td>78.4</td>
<td></td>
</tr>
<tr>
<td>Sb15</td>
<td>MMU238774</td>
<td>217</td>
<td>LINE/L1</td>
<td>215</td>
<td>99.1</td>
<td>ORF2</td>
</tr>
<tr>
<td>Sb24</td>
<td>MMU238775</td>
<td>208</td>
<td>LINE/L2</td>
<td>126</td>
<td>60.6</td>
<td>MIR2/LINE2, 3’ end</td>
</tr>
<tr>
<td>Sb28</td>
<td>MMU238776</td>
<td>343</td>
<td>LTR</td>
<td>295</td>
<td>86</td>
<td>LTR homology, RMR 17B</td>
</tr>
<tr>
<td>Sb31</td>
<td>MMU238777</td>
<td>453</td>
<td>SINE B1</td>
<td>36</td>
<td>7.95</td>
<td></td>
</tr>
<tr>
<td>Sb36</td>
<td>AJ1133875</td>
<td>690</td>
<td>LINE/L1</td>
<td>145</td>
<td>21</td>
<td>5’ UTE/ORF1</td>
</tr>
<tr>
<td>Sb38</td>
<td>AJ1133876</td>
<td>723</td>
<td>HERV</td>
<td>174</td>
<td>25.2</td>
<td>ORF 2</td>
</tr>
<tr>
<td>Sb45</td>
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<td>R-SINE1</td>
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<td></td>
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<td>AJ13883</td>
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<td>Sb51</td>
<td>AJ133878</td>
<td>135</td>
<td>LINE/L1</td>
<td>51</td>
<td>37.8</td>
<td>3’ end of L1 repeat</td>
</tr>
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<td>AJ133879</td>
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<td>SINE B2</td>
<td>124</td>
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<td>Sb54</td>
<td>AJ133879</td>
<td>924</td>
<td>SINE B3</td>
<td>66</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>Sb54</td>
<td>AJ133879</td>
<td>924</td>
<td>SINE B1</td>
<td>90</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>Total B</td>
<td></td>
<td></td>
<td></td>
<td>5794</td>
<td>36.6</td>
<td></td>
</tr>
<tr>
<td>Total (A+B)</td>
<td></td>
<td></td>
<td></td>
<td>8924</td>
<td>42.36</td>
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</table>

**C) Other clones**

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<thead>
<tr>
<th>Clone</th>
<th>Accession</th>
<th>Sequence length (bp)</th>
<th>Repeat</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sb2</td>
<td>AJ133881</td>
<td>360</td>
<td>0</td>
<td>1-170: identity with AF082077 (murine PAK gene)</td>
</tr>
<tr>
<td>Sb21</td>
<td>AJ133880</td>
<td>342</td>
<td>0</td>
<td>Similar to human AC002301 (pos. 63430-63680)</td>
</tr>
<tr>
<td>Sb25</td>
<td>AJ133884</td>
<td>105</td>
<td>0</td>
<td>Unidentified sequence, one ORF</td>
</tr>
<tr>
<td>Sb26</td>
<td>AJ133882</td>
<td>104</td>
<td>0</td>
<td>Unidentified sequence, one ORF</td>
</tr>
</tbody>
</table>

**Summary of sequenced clones (A+B+C)**

<table>
<thead>
<tr>
<th>No. clones</th>
<th>Sequenced bp</th>
<th>Repeats (bp)</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>21</td>
<td>9835</td>
<td>3780</td>
<td>38.43</td>
</tr>
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</table>

...
that are induced in response to the interaction with foreign
Previous experiments identified sperm endogenous nucleases
nucleosomal pattern
Cleavage of sperm chromatin by endogenous
RESULTS
in PBST. DNA was counterstained with 0.5 µg/ml DAPI, and samples
were mounted in Vectashield antifade mounting medium (Vector Labs
Inc, Burlingame, CA). Images were taken using a Zeiss microscope
configured for epifluorescence with a x100 oil immersion objective
1.3 N.A. CCD camera (Photometrics, Tucson, AZ) and IP-Lab
software were used to digitize images. Images were pseudocolored
using the Adobe Photoshop program.
Fluorescent in situ hybridization (FISH) analysis
Sperm nuclei were isolated and washed twice in 1 mM Tris (pH 7.8)
by centrifuging for 4 minutes at 3000 rpm at room temperature. 30-
40 µl of suspension, containing about 2x10^7 nuclei, were smeared on
microscope slides and air-dried. FISH staining was performed as
previously described (Boyle et al., 1990; Cimini et al., 1996) with
minor modifications. Briefly, slides were dehydrated by sequential
immersions in 70%, 90% and 100% ethanol and air-dried. Samples
were denatured for 2 minutes at 76°C (NIH/3T3 cells), or for 4
minutes at 81°C (sperm nuclei), using 70% deionized formamide
(Kodak) in 2x SSC. After denaturation, samples were again
dehydrated in ethanol. A 69-bp oligonucleotide from the murine major
satellite DNA (5’-TATGGCCAGGAAAACTGAAAAAGGTGGG-
AAAAATTTAGAAATGTCCACTGTGGACGTGGAA TA TGGCAA
G-3’) was labelled using biotin-16-dUTP (Boehringer) and terminal
transferase. A 251-bp LINE/L1 ORF2 probe derived from the first
LINE/L1 element of clone S7 (see Fig. 7), was labelled using also
biotin-16-dUTP and a nick-translation kit (Boehringer). 300 ng of
each probe and 10 µg of sonicated salmon sperm DNA were ethanol-
precipitated and directly resuspended in the hybridization mixture.
Hybridization was carried out overnight at 37°C using 50 ng of
satellite, or 100 ng of LINE/L1. DNA probes in 12 µl of hybridization
mix (50% formamide, 2x SSC, 10% Dextran Sulphate) per slide. Probes
were denatured for 8 minutes at 80°C. After hybridization,
slides were washed three times in 50% formamide/2x SSC at 42°C
for 5 minutes, followed by three washes in 1x SSC at 42°C. Slides
were rinsed in 4x SSC/0.1% Tween-20 and subsequently blocked in
3% BSA (Sigma) at 37°C for 40 minutes. Probes were detected using
6.5 µg/ml FITC-avidin DCS (Vector Laboratories) in detection buffer
containing 1% BSA at 37°C for 40-60 minutes. Slides were then
rinsed three times in 4x SSC/0.1% Tween-20 at 42°C. Chromosomal
DNA was counterstained with 0.2 µg/ml DAPI (Sigma) for 10
minutes. Slides were rinsed in 4x SSC/0.1% Tween-20 and 2x
SSC/0.05x Tween-20, and finally mounted in Vectashield mounting
medium (Vector Laboratories). Cells were visualized using ultraviolet
excitation and hybridization signals were analysed under blue-violet
illumination under a Zeiss Axiohot microscope. Photographic
images were recorded with a cooled CCD camera (Photometrics) and
processed using the Adobe Photoshop software.

RESULTS
Cleavage of sperm chromatin by endogenous
nucleases releases DNA fragments with a
nucleosomal pattern
Previous experiments identified sperm endogenous nucleases
that are induced in response to the interaction with foreign
DNA molecules (Maione et al., 1997). These nucleases are
Ca^2+ dependent and are sensitive to compounds capable of
modulating apoptosis in somatic cells. Sperm nucleases cause
a partial degradation of chromosomal DNA, followed by
the release of cleaved DNA fragments from the cells to the
medium, suggesting that the interaction of mature sperm cells
with exogenous DNA triggers an active process resembling
apoptosis. Endogenous nucleases can be similarly activated
after prolonged incubation of sperm cells in fertilization
medium (FM) for several hours (Maione et al., 1997).

In order to characterize the released fragments from sperm
cells upon endogenous nuclease activation, the experiments
outlined in Fig. 1 were designed. Briefly, mouse epididymal
sperm cells were incubated in FM at 37°C for 3-5 hours,
centrifuged, and the supernatants were processed for DNA
extraction; the extracted DNA was characterized by parallel
Southern blot analysis and end-labelling experiments.
The DNA released from sperm cells upon nuclease
activation represents a small fraction of the sperm genome. Fig.
2 shows a dot blot quantitative analysis carried out by
hybridizing a total genomic DNA probe to the DNA released
from 16x10^6 spermatozoa (slot 1), standards corresponding to
increasing amounts of mouse sperm genomic DNA (slots 2-4)
and DNA extracted from sperm pellets (slots 5 and 6). The
proportion of hypersensitive DNA released in the supernatant
relative to that retained in the pellet was approximately

![Fig. 1](image1.png)

**Fig. 1.** Outline of the general procedure used for the preparation of the chromatin fraction released from spermatozoa in the medium
upon incubation. Both the DNA and the protein component were
extracted from the chromatin released in the supernatant. The DNA
was analyzed by gel electrophoresis, cloned and sequenced; proteins
were analyzed by western blot using anti-histones antibodies.

![Fig. 2](image2.png)

**Fig. 2.** Dot blot hybridization assay and quantitative estimate of the
DNA released from mouse epididymal spermatozoa after three hours
of incubation in FM at 37°C. Lane 1: released DNA from 16x10^6
spermatozoa; lanes 2-4: DNA standards corresponding to 10 ng
(lane 2), 1 ng (lane 3) and 0.1 ng (lane 4) of mouse sperm genomic DNA;
lanes 5-6: serial dilutions of the DNA extracted from the sperm
pellet: 1.5x10^-4 (lane 5) and 1.5x10^-3 dilution (lane 6). The filter
was hybridized overnight with uniformly labelled mouse sperm
DNA, washed and exposed to a Fuji RX film. After exposure, slots
were cut out with a razor blade and counted in a scintillation counter.
Retroposon DNA in sperm nucleosomal chromatin

quantified by counting the radioactivity associated with the hybridizing dots: as can be seen in the example shown in Fig. 2, the hybridization signal of the released DNA (slot 1) falls within the linear range of the DNA standards (slots 2-4); the released DNA after three hours of incubation ranged from 0.03% (as in the experiment shown in Fig. 2) to 0.1% of the DNA retained in the sperm pellet in independent experiments.

Fig. 3. Analysis of the DNA released from sperm cells upon endogenous nuclease cleavage. (A) Lane 1: Southern blot analysis of the DNA released from 1.6x10⁸ sperm cells incubated in FM for five hours and electrophoresed through a 1% agarose gel. The filter was hybridized with uniformly labelled sperm genomic DNA. (B) Lane 1: released DNA from spermatozoa incubated for five hours as in A was end-labelled and fractionated through a 1.7% agarose gel. (C) Lanes 1-2: the supernatant DNA from an independent experiment was end-labelled as for B, except that sperm cells were incubated for three (lane 1) and five (lane 2) hours and electrophoresed through a 1.2% agarose gel. Lanes M in A and C contain λ HindIII end-labelled restriction fragments as markers; numbers on the left indicate marker sizes in Kbp. Lane M in B contains a 123-bp multimerized repeat DNA ladder.

Fig. 4. Histone detection in sperm chromatin. Western blot analysis of protein extracts from the supernatant fraction from 5x10⁶ spermatozoa after 5 hours incubation in FM (Spn) and from pelleted sperm cells (P), after incubation with a monoclonal antibody against all histones.

Fig. 5. Immunofluorescence localization of H1 histone in intact sperm cells (upper row) and in isolated nuclei (lower row). (a,c,f,h) DAPI staining of the DNA (pseudo-colored in red); (b and g) negative control using the secondary antibody only; (d and i) anti-H1 antibody; (e) merging of pictures c and d; (j) merging of pictures h and i.
DNA is shown in Fig. 3A: the released DNA migrates in a continuous, smeary pattern ranging from over twenty to about two kb (lane 1). Unexpectedly, however, when the same DNA was end-labelled at the cleavage ends produced by endogenous nucleases, fractionated on an agarose gel and directly exposed to a film, radioactive DNA fragments appeared to be distributed in a typical nucleosomal ladder (B, lane 1). The molecular mass of end-labelled fragments corresponded to that expected of multiples of 175-190 bp, i.e. the typical length of mononucleosomal DNA; discrete fragments in B are clearly visible up to the heptamer. A full ladder of nucleosome multimers was only visible in certain DNA preparations, presumably resulting from limited nuclease digestion. In other experiments only monomers, dimers and trimers were detected (C, lanes 1 and 2). The extent of chromatin cleavage by endogenous nucleases appears to be a variable parameter among different sperm cell preparations.

**Mouse sperm chromatin contains nucleohistone domains**

The finding that a nuclease hypersensitive fraction of the sperm genome is organized in nucleosomes suggests that histones are components of mouse sperm chromatin. To address this question, we carried out western blot assays of protein extracts from both pelleted spermatozoa and from the supernatant containing the hypersensitive chromatin fraction. A monoclonal antibody against all five histones (anti-histone pan antibody) was used. As shown in Fig. 4, all histone species are distinguishable both in the sperm pellet and in the supernatant, indicating that histones were partly released with the nuclease-sensitive DNA.

Immunofluorescence experiments were then carried out using either whole spermatozoa (Fig. 5, upper panel) or purified sperm nuclei (Fig. 5, lower panel), to localize histones in sperm chromatin. Monoclonal antibodies, either against all five histones (anti-histone pan) or against histone H1, were initially tested. Since the monoclonal antibody against histone H1 gave a higher signal-to-noise ratio than the anti-histone pan antibody in immunofluorescence experiments, we elected to use anti-H1 thereafter. Fig. 5d shows that anti-H1 stains the sperm head, while DAPI staining (Fig. 5a and c) clearly defines the hooked shape of murine intact spermatozoa. As can be seen from the merged picture (Fig. 5e), histone H1 is concentrated in a narrow area of the sperm head, corresponding to the subacrosomal segment. No staining was detected in control experiments with the secondary antibody alone (Fig. 5b). The experiments were repeated with purified nuclei from spermatozoa. Reactivity to the anti-H1 antibody revealed that nucleohistone domains were peripherally distributed around the nucleus, while being less abundant or absent in the central region of the nucleus (Fig. 5i and j); that region was instead intensely fluorescent after DAPI staining (Fig. 5f and h), and therefore appears to contain highly condensed chromosomal DNA. Together, the results in Fig. 5 show that histones are indeed present in sperm chromatin: in intact cells they are accessible to antibody recognition in the subacrosomal segment of the head which is not protected by the acrosome, and are redistributed to a peripheral location in purified nuclei.

**Sperm chromatin hypersensitive sites fall within genomic sequences enriched in retroposon DNA**

In order to establish whether the DNA released in the supernatant had a heterogeneous composition or was enriched in particular sequences, Southern blot experiments were carried out using this DNA to probe total genomic DNA. Fig. 6A shows two discrete hybridization bands, sized as 4 and 0.5 kb (lane 1) over the background of mouse genomic DNA after BamHI digestion. That pattern was reminiscent of that characterizing the LINE/L1 retroposon family (Fanning, 1983; Voliva et al., 1983). To further verify that similarity, a filter containing genomic DNA restricted with BamHI was hybridized with a 60 nucleotide-long probe derived from ORF2 of the mouse LINE/L1 element (Naas et al., 1998). The hybridization pattern in B confirmed the similarity between the hybridizing targets of both the supernatant and the LINE/L1 probes: the 4 kb band is well evident, whereas the 0.5 kb fragment is not visualized, since the 60-bp probe does not encompass that region.

To unambiguously establish the sequence identity of the hypersensitive DNA, we constructed two partial genomic libraries from the supernatant released DNA: the first library was constructed by restricting released DNA fragments with EcoRI/BamHI and cloning into compatible sites in a plasmid vector (clones from this library are collectively indicated with S); the second one was constructed by filling-in the ends of released DNA fragments without previous restriction (see Materials and Methods for details), and ligating the blunt-ended fragments into the EcoRV site of the vector. This strategy was followed to eliminate any preferential sequence selection that might have been generated by a restriction enzyme bias, and resulting clones are indicated with Sb. 21

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**Fig. 6.** The DNA released in the supernatant contains LINE/L1 DNA sequences. Southern blot analysis of mouse sperm DNA restricted with BamHI and hybridized with supernatant DNA (A, lane 1), and with a 60-nt oligonucleotide derived from LINE/L1 ORF II (B, lane 1). Lanes M contain λ HindIII end-labelled restriction fragments; numbers on the left indicate marker sizes in Kbp.
clones were randomly selected (5 from the S, and 16 from the Sb libraries) and fully sequenced. Features depicted by sequence analysis identify three groups, two of which (A and B, representing 17 clones out of 21) contain retroposon DNA, as specified in Table 1.

Quantitative and qualitative differences characterize the clones from both libraries. All S clones (group A) contain LINE/L1 sequences; clone S8 also contain SINE elements. Retroposon sequences (i.e. pooled LINE/L1 and SINE elements) from clones of this group account for 53.04% of the total DNA sequences (3130 bp), with LINE/L1 in significant excess (41.3%) over SINE (11.69%). Truncated LINE/L1 elements show a variable degree of substitutions within the matching regions compared to the canonical LINE/L1 element, whereas a region from clone S7, indicated as ORF2 in Table 1, is 99% identical to the canonical ORF2 from the mouse LINE/L1 element. Indeed, virtual translation of this sequence indicate an uninterrupted open reading frame potentially coding for a functional portion of reverse transcriptase. Other LINE/L1 sequences share homologies with the non-coding 3' end of the element. Out of the sixteen selected blunt-ended clones (Sb series), twelve (listed in group B) contain retroposon sequences. These clones show an overall lower content (36.6%) of retroposon DNA compared to S series clones in group A. LINE/L1 sequences are less represented, accounting for 12.28% of total DNA in this group of clones, which is comparable to the level of representation of other retroposon families, i.e. SINE (8.4%), RSINE (6.5%), and LTR (7%). Two blunt-ended clones, i.e. Sb15 and Sb36, show again complete identity with ORF2. The 4 Sb clones classified as group C do not contain retroposon DNA; a common feature is the presence of hypothetical ORFs, with the exception of Sb46. Actually, the 5' end of Sb2 is identical to the murine gene encoding the Cdc42/Rac effector kinase PAK-A. In addition, clone Sb21 shows homologies with a human clone carrying SINE/Alu elements on either side of the region of similarity. Altogether, 9835 bp from nuclease hypersensitive DNA were sequenced, 3780 of which (corresponding to 38.43%) were found to be of retroposon origin. A schematic representation of clones containing retroposon sequences from both libraries is shown in Fig. 7. Taken together, the sequencing results show that hypersensitive sperm chromatin domains are significantly enriched in retroposon DNA.

**LINE/L1 DNA co-localizes with the nucleohistone chromatin fraction in sperm nuclei**

To localize retroposon DNA sequences in sperm nuclear chromatin, a 251-bp fragment, derived from the LINE/L1 element in clone S7, was used as the probe in FISH experiments. Fig. 8b show that the periphery of nuclei is specifically and intensely stained by the LINE/L1 probe. In contrast, the central area, which is intensely stained by DAPI (Fig. 8a), does not appear to contain a significant amount of LINE/L1-homologous sequences. The specificity of the LINE/L1 probe hybridization was controlled on mouse NIH/3T3 metaphase chromosomes (Fig. 8d): as expected (Boyle et al., 1990), hybridization signals were spread throughout all chromosome arms but not in centromeric heterochromatic regions. Parallel experiments using a major DNA satellite probe (Horz and Altenburger, 1981) gave clearly restricted hybridization signals in the central area of nuclei (Fig. 8f), indicating that satellite DNA, differently from

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**Fig. 7.** Schematic representation of seventeen randomly selected clones from two partial libraries of sperm-released DNA. S clones derive from a EcoRI/BamHI restriction fragments library (group A in Table 1), Sb clones from a blunt-ended fragments library (group B in Table 1). All clones contain retroposon DNA (solid boxes) flanked by unidentified mouse sequences (lines). Boxes identify retroposon families as indicated.
LINE/L1, localizes to the sperm nuclear region of highest condensation. To control the hybridization specificity, we verified that the satellite probe exclusively hybridized with centromeric regions on NIH/3T3 metaphase chromosomes (Fig. 8h), as expected (Joseph et al., 1989). Thus, mature mouse spermatozoa comprise a nucleohistone fraction, a significant component of which is represented by retroposon DNA sequences that specifically co-localize with histones in the sperm nuclear periphery.

**Ran and RCC1 proteins are associated with sperm chromatin and are released after nuclease digestion**

Results thus far identify a small fraction of the sperm chromatin that has typical features of functionally active domains in somatic cells. We wished to establish whether nuclear components other than histones were also present in sperm nuclei, and, if so, whether they were released with the nuclease-sensitive fraction. We analysed the RCC1 regulator of chromosome condensation, one of the most abundant chromatin-bound protein in somatic cells (reviewed by Dasso, 1993), and its molecular partner, the small Ran GTPase, over which RCC1 acts as the guanine exchange factor. Ran and RCC1 are major components of a GTP-mediated signalling system that regulates many functions in somatic cells including DNA replication, nuclear structure, nuclear transport, chromatin condensation and cell cycle progression (reviewed by Rush et al., 1996). Specific antibodies against murine Ran and RCC1 proteins were used to analyse whole-mount sperm preparations by immunofluorescence. These experiments revealed that both RCC1 and Ran were present in spermatozoa. RCC1 showed a low level of abundance and was only detectable in a proportion of cells (Fig. 9d); Ran was
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comparatively more abundant and was easily visualized in most, if not all, sperm cells (Fig. 9f). Interestingly, in those sperm cells where both were visibly expressed, RCC1 and Ran proteins co-localized in a specific peripheral compartment of sperm heads (compare the signals in Fig. 9d and f, and DAPI-stained cells in Fig. 9c and e). When spermatozoa were incubated in FM for 5 hours, Ran molecules appeared to be displaced from sperm cells (Fig. 9h), despite the overall integrity retained by sperm cells under these conditions as revealed by DAPI staining (Fig. 9g). RCC1 displacement could not be similarly assessed by immunofluorescence due to its low overall abundance in spermatozoa. To further confirm the immunofluorescence results, sperm cells were exposed to prolonged incubations in buffer, centrifuged, and both the sperm pellet and supernatant fractions were processed for protein extraction to be analyzed in western blot assays. The results of those experiments, shown in Fig. 10, confirmed that both RCC1 and Ran are present in mature sperm cells; in addition, both molecules were found in high proportions in the supernatant together with the active chromatin fraction.

DISCUSSION

Mammalian sperm heads contain highly condensed chromatin, resulting from the tight interaction between DNA and protamines (Ward and Coffey, 1991). The nuclear matrix constitutes a third component and plays an essential role: according to a widely accepted model (Ward, 1993) the chromosomal DNA/protamine complex is organized in loop domains whose basis are attached to the nuclear matrix. These three components are assembled in an extremely compact structure which is thought to be incompatible with genomic activity. However, the finding that otherwise silent enzymatic functions are activated in the nuclei of sperm cells exposed to the interaction with foreign DNA molecules (Maione et al., 1997; Zoraqi and Spadafora, 1997) has lead us to reconsider our view of sperm chromatin. The finding that sperm endogenous nuclease can locally degrade chromosomal DNA (Maione et al., 1997), and the ensuing release of chromatin fragments from sperm cells, suggested that hypersensitive sites exist in sperm chromatin, where the DNA is accessible to nucleases. The present results contribute to define the molecular structure of hypersensitive sites. We report here that nuclease hypersensitive chromatin domain(s) (i) localize in the sperm nuclear periphery, (ii) are significantly enriched in retroposon sequences, (iii) interact with histones in a typical nucleosomal conformation, and (iv) associate with Ran and RCC1 proteins.

Released chromatin after endogenous nuclease cleavage represents a subfraction of the nucleohistone component of sperm nuclei, as indicated by the partial retention of histones in sperm pellets after solubilization of the hypersensitive chromatin. Quantitative estimates indicate that the solubilized DNA does not exceed 0.1% of total genomic DNA. This DNA is organized in a typical nucleosomal ladder, migrating as multiple units of 175-190 bp in length (Fig. 3B). In some experiments, the ladder extended up to heptamers, while in others digestion was more extensive. The different degrees of digestion likely reflect the differential extent to which nucleases are activated in sperm cells in response to prolonged incubation; though experimental conditions were identical in all experiments, sperm nucleases may be differently activated due to intrinsic differences between cell populations and/or among donor animals.

Fig. 9. Immunofluorescence localization of Ran and RCC1 proteins in sperm cells. (a,c,e,g) DAPI staining; (b) control experiment using the secondary antibody only; (d) anti-RCC1 antibody; (f and h) anti-Ran antibody on intact sperm cells (f) and on pelleted sperm cells after 3 hours of incubation in FM (h).

Fig. 10. Detection of RCC1 and Ran in sperm chromatin. Western blot analysis of protein extracts from: lane P, pelleted sperm cells; lane S, supernatant from 107 spermatozoa; the filter was simultaneously incubated with anti-Ran and anti-RCC1 antibodies. Numbers on the left show the migration of molecular mass markers, indicated in kDa.
Histones can be distinguished in western immunoblotting assays of both sperm nuclei and released chromatin upon nuclease digestion (Fig. 4), suggesting that they are components of the hypersensitive chromatin. By in situ immunofluorescence, histones detection was restricted to the subacrosomal segment of the head in intact spermatozoa (Fig. 5d and f) and showed a peripheral distribution within a narrow margin around nuclei (Fig. 5i and j). These different staining patterns may reflect the protective effect exerted by the acrosome over the accessibility of internal nuclear structures. It is also possible that the nucleohistone component undergoes a redistribution during the preparation of nuclei. In intact cells, the fluorescent pattern follows the profile of the portion of the nucleus which protrudes from underneath the acrosome (Eddy, 1988), while in nuclei the detergent removes the acrosome, as well as membranes, due to its swelling effect, and hence may cause a redistribution of the nucleohistone domains. We have observed that the extent of redistribution is correlated with the time of exposure of spermatozoa to DTT and CTAB detergent during the procedure of nuclei preparation (data not shown). The peripheral redistribution of histones in nuclei indicates that they are excluded from the area with the highest degree of DNA condensation, which is instead located around the central-apical portion of sperm heads (Fig. 5f and h). These different distributions indicate that the bulk of compact sperm chromatin does not comprise the histone component. Our microscopical localization of nucleohistones in murine sperm nuclei are consistent with previous results by Soon et al. (1997) in marsupial spermatozoa: the authors reported a peripheral localization of the five (including H1) histones; peripheral nucleohistones actually constituted a specific, low-density chromatin fraction in the species under examination.

Mammalian spermatozoa were previously reported to contain core histones: earlier reports showed that sperm nuclei of rams (Uscheva et al., 1982) and humans (Gatewood et al., 1987, 1990), maintained variable amounts of chromatin subfractions in which the original histone component had not been replaced by protamines during spermiogenesis. In human spermatozoa, a subset of nucleohistones was reported to package the DNA in a sequence-specific manner (Gatewood et al., 1987); histones and DNA were found to be organized in a typical nucleosomal conformation resembling that of somatic chromatin (Banerjee et al., 1995). Our present observations provide the first characterization of histones and DNA sequences organized in nucleosomes in the sperm chromatin of mouse, as depicted in a functional assay of hypersensitivity to nuclease cleavage. Previous reports (Gatewood et al., 1990) failed to detect H1 in human spermatozoa. Various possible explanations may account for this discrepancy: firstly, it is possible that H1 is indeed a chromatin component in murine and marsupial, but not in human, spermatozoa; secondly, the techniques employed to assess histone identity have a different sensitivity: we have presently carried out immunodetection assays using monoclonal antibodies, while in earlier reports histones were recognized by gel staining (Gatewood et al., 1990).

We previously reported that foreign DNA molecules internalized in sperm nuclei integrate in one or few genomic sites (Zoraqi and Spadafora, 1997). The present finding that nucleosomal domains harbour hypersensitive sites, unlike the compact nucleoprotamine structure of sperm chromatin, offers an ideal substrate as the preferential target site of integration of foreign DNA sequences: the tightly protamine-packaged chromosomal DNA is virtually unaccessible to foreign molecules; however, restricted portions of the sperm genome organized in nucleosomal domains appear to be selectively exposed.

Sequence analysis of the nuclease-sensitive DNA released in the supernatant depicted retroposon sequences from several families among randomly selected clones (Table 1 and Fig. 7). The enrichment in retroposon DNA in the hypersensitive fraction poses the intriguing question as to whether sperm hypersensitive chromatin domain(s) should be regarded as the graveyard of retroposon fossils, or whether these sequences may instead have a functional role. LINE/L1 expression is an event directly correlated with transposition and actively takes place in germ cells (Branciforte and Martin, 1994), in teratocarcinoma cell lines (Dargon et al., 1990) and in mouse embryos (Packer et al., 1993). Taken together, these results suggest that the retroposon DNA in the hypersensitive chromatin fraction may be in a potentially ‘active’, transpositionally competent, state. LINE/L1 DNA co-localizes with the nucleohistone component of sperm chromatin in the nuclear periphery (Figs 5 and 8). In contrast, satellite DNA is located in the heavily condensed central region of the sperm nucleus.

In principle, transposition requires a full-length LINE/L1 element (6-7 kb) containing an intact open reading frame (Boeke, 1997). The supernatant DNA which we have cloned and sequenced was cleaved by endogenous nucleases into rather short fragments. Therefore, the experimental conditions of DNA isolation and library construction did not enable us to establish whether full-length LINE/L1 element(s) are released in the supernatant. This question will require screening of a mouse genomic library using non-retroposon DNA sequences identified in hypersensitive clones as probes. Thus far, it is of note that, out of eight LINE/L1-containing clones, three contain an uninterrupted open reading frame potentially coding for a functional reverse transcriptase domain (Table 1). Curiously, all cloned LINE/L1 sequences released from sperm cells exhibit homology with the ORF2 and/or with the 3’ end portion of the element, with the sole exception of a 145-bp sequence in clone Sb36 overlapping with the 5’UTR/ORF1. It is possible that the 5’ ends of LINE/L1 are hypersensitive to nuclease activity compared to the rest of the element, and are therefore extensively digested, while the central and 3’ portion would survive nuclease exposure. Alternatively, the sperm nucleohistone chromatin fraction may be selectively enriched in ORF2 sequences rather than containing the entire LINE/L1 unit.

In our characterization of potentially functional domain(s) in the sperm nucleus, we have also examined the distribution of two nuclear components of the Ran GTPase signalling network, i.e. the Ran GTPase itself and its guanine exchange factor, RCC1. Ran is a predominantly nuclear protein, yet does not bind the DNA, and shuttles between the nucleus and the cytoplasm in somatic cells (reviewed by Rush et al., 1996). RCC1 does instead directly interact with the DNA and is implicated in several aspects of nuclear organization (reviewed by Dasso, 1993; Huang et al., 1997). We have found that Ran and RCC1 are both present in sperm nuclei. Ran was found to be present in excess over RCC1 in our western immunoblotting...
experiments, consistent with quantitative estimates in somatic cells and oocytes (Bischoff and Ponstigl, 1991; Nicolas et al., 1997). In situ immunofluorescence assays showed that, in cells where both molecules could be detected, they were distributed peripherally to the sperm nucleus and specifically co-localized in the dorsal margin (Fig. 9). Both molecules were found to be associated with the hypersensitive chromatin released from sperm cells in western blot assays (Fig. 10). The finding that most of the Ran protein is released in the supernatant may not be surprising, given the intrinsic shuffling function of this protein; the finding that a fraction of the nuclear pool of RCC1 was also released with the hypersensitive chromatin suggests that RCC1 is associated, at least in part, with active chromatin domains.

Taken together, the present results show that potentially active chromatin domains are present within the almost crystalline structure of sperm nucleoproteamines. Interestingly, Ward and colleagues (1996) reported that three housekeeping genes (i.e. CAD, 55 rRNA and the class I L1.6 gene from the major histocompatibility complex) exhibit preferred areas of localization in hamster sperm nuclei: in all three cases, the depicted areas were described by the authors as very narrow margins at the periphery of the sperm nucleus. These findings, together with the results reported here, may suggest that the sperm nuclear periphery, which contains nucleohistones, may recruit coding genes (Ward et al., 1996) and retroposon sequences (this paper), whereas ‘non informative’ DNA sequences may be structured in a tightly compact organization in the central area of the sperm nucleus. The significance of these functional compartments in spermatozoa is a challenging question. A recent analysis of globin gene organization in sperm cells and early embryos (Gardiner-Garden et al., 1998) shows that both the ε- and γ-globin genes are actively expressed already in the yolk sac, and both are associated with histones in human spermatozoa, unlike β and δ-globin genes, which are silent in embryos. Genetic activities, i.e. DNA synthesis coupled to the activation of certain genes (Tesari and Kopecny, 1989; Matsumoto et al., 1994; Boulin et al., 1995; Aoki et al., 1997) take place in the male pronucleus of the zygote soon after sperm penetration in the oocyte and are instrumental for the acquisition of transcriptional competence in the embryo. It is tempting to suggest that hypersensitive chromatin domains characterized here, though representing a quantitatively minor fraction, identify functional compartments of sperm nuclei that are preset to support genetic activities during fertilization and/or in early steps of embryonic development.

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