DNase I activity in pig MII oocytes: implications in transgenesis

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Abstract

Several reliable methods to produce transgenic animals utilize the male genome. After penetration into oocyte, sperm DNA undergoes dramatic conformational changes that could represent a great opportunity for exogenous DNA to be integrated in the zygote genome. Among the enzymes responsible for sperm remodeling, a nuclease could be involved. The presence of a DNase I in oocytes has not been much investigated. To date, an immunolocalization of DNase I has been reported only in rat immature oocytes and the presence of nuclease activities has been shown in avian oocytes.

The present study was conducted to verify whether a DNase-I like activity is present in MII mature pig oocytes. To do this, oocyte extracts were assessed for nuclease activity by a plasmid degradation assay and by zymography; these analyses evidenced a 33 kDa, Ca\(^{2+}\)/Mg\(^{2+}\) dependent DNase I-like activity that was inhibited by Zn\(^{2+}\). A further identification of DNase I was achieved by Western blot, immunofluorescence and RT-PCR experiments. Moreover, the presence of the enzyme activity was confirmed by the rapid degradation of exogenous DNA microinjected into the ooplasm. Finally, the exogenous DNA transferred to oocyte by spermatozoa during sperm mediated gene transfer in vitro fertilisation protocol seemed to be protected from DNase I degradation and to persist in the ooplasm till 6h.

These results, together with the high efficiency of sperm based transgenesis methods, suggest that the association with spermatozoa protects exogenous DNA from nuclease activities.


Introduction

Transgenesis represents an important tool in basic research as well as in livestock production. Several reliable methods to produce transgenic animals utilize the male genome for exogenous DNA integration. Male pronuclear injection is currently the most often utilized method to generate transgenic animals, but the rate of successful incorporation of the exogenous genes into the host genome is extremely low in farm animals (Pursel et al. 1989, Krimpenfort et al. 1991). Moreover, exogenous DNA injection into the ooplasm does not result in transgenic progeny (Page et al. 1995). Recently, a new possibility to produce transgenic animals is represented by somatic nuclear transfer, utilizing a selection of transgene-transfected donor cell (Lee et al. 2005). Other transgenesis methods utilizing the male gamete as a vector of exogenous DNA have been developed; their high efficiency could suggest a protection mechanism of exogenous DNA from degradation. Sperm mediated gene transfer (SMGT) is based on sperm ability to bind and internalize exogenous DNA (Lavitrano et al. 1989) and is extremely efficient for transgenic pig generation (Lavitrano et al. 2002, 2003). Metaphase II transgenesis is based on the co-injection of sperm head and exogenous DNA into MII mouse oocytes (Perry et al. 1999).

After penetration into the ooplasm, sperm DNA undergoes a deep remodeling starting with the sperm head decondensation, throughout a demethylation/methylation process, till the genomes fusion (McLay & Clarke 2003, Spinaci et al. 2004). In mouse zygotes, multiple DNA breaks have been shown in the decondensing sperm head, being interpreted as a transient process due to the substantial chromatin rearrangement (Kiessling & Markoulaki 2000). In the same year Seo and colleagues (2000) demonstrated that the co-injection of exogenous DNA with restriction endonucleases improves the frequency of DNA integration into the host genomes. Therefore the dramatic conformational changes of paternal DNA could be a great
opportunity for exogenous DNA, if located in proximity of sperm DNA, to be integrated in the zygote genome. Among other enzymatic activities, nucleases are also involved in DNA repair systems. A strong DNA repair function has been shown in Xenopus laevis oocyte (Ackerman & Lakoucheva 2000) and a rigorous checkpoint of DNA damage has been shown in MII mouse oocytes before syngamy (Perry 2000). To date, the presence of DNase I in the oocyte has been evidenced by immunolocalization in rat immature oocytes (Boone & Tsang 1997) while the nuclease activity referred to DNase I and II has been shown in avian oocytes (Stepińska & Olszańska 2001, 2003). The hypothesized function in avian oocytes is the control of polyspermic fertilization that is a physiological phenomenon in avian species, even if only one spermatozoon is selected for fertilization. Polyspermy is generally considered as a pathological phenomenon impairing mammalian embryo development; however, a high incidence of polyspermic fertilization has been reported in pig (for a review see Sun & Nagai 2003); an early block of polyspermy ensures monospermic fertilization in mammals (Galeati et al. 1991, Yanagimachi 1994).

The first aim of this study was to verify whether swine MII oocytes contain DNase I, the second one was to check if exogenous DNA could be protected when introduced into the oocyte.

**Materials and Methods**

**MII oocytes in vitro production**

Swine ovaries were collected at a local abattoir and transported within 2 h to the lab in a thermostatic box at 30°C in physiological saline. Cumulus-oocyte complexes (COCs) from follicles 4–6 mm in diameter were aspirated using a 18-gauge needle attached to a 10 ml disposable syringe. The aspirates were collected in 20 ml plastic tubes (LP Italiana, MI, Italy) and were allowed to settle for 10 min after aspiration; the bottom volume was then distributed in petri dishes (90 mm, Nunclon, Denmark). Under a stereomicroscope, intact COCs were selected and transferred into another petri dish (35 mm, Nunclon) prefilled with 2 ml of PBS supplemented with 0.4% BSA (Sigma Chemical Company, St Louis, MO, USA). After four washes in NCSU 37 supplemented with 5.0 mg/ml insulin, 0.6 mM cysteine, 1.0 mM glutamine, 10 mg/ml epidermal growth factor, 50 μM mercaptoethanol and 10% pig follicular fluid (IVM—in vitro maturation—medium), groups of 50 COCs were transferred to a Nunc 4-well multidish and cultured at 39°C in a humidified atmosphere of 5% CO₂, 5% O₂. For the first 24 h of in vitro maturation the medium was supplemented with 1.0 mM db-cAMP, 101U/ml eCG (equine chorionic gonadotrophin, Folligon Intervet Int. Boxmeer, Holland) and 101U/ml hCG (human chorionic gonadotrophin, chorulon, Intervet). After 24 h, COCs were transferred to fresh IVM medium and cultured for further 24 h (Funahashi & Day 1997). At the end of the maturation period, only the oocytes presenting the first polar body (MII oocytes) under stereomicroscope visualization were utilized for the subsequent experiments. MII oocytes were recovered and transferred to PBS supplemented with hyaluronidase (0.1%) in order to remove cumulus cells by repeated pipetting through a small bore pipette. The oocytes were then transferred into 100 μl droplets of pre-warmed 0.5% pronase in modified PBS supplemented with 0.4% BSA in order to remove the zona pellucidae.

In order to evaluate the average proportion of oocytes that had reached nuclear maturation, part of the IVM oocytes (5 each well) were fixed at room temperature in 25% (v/v) acetic acid in ethanol, stained (Lacmoid, Sigma) and examined under a phase-contrast microscope at × 400 magnification.

**Zymography**

Zymography was conducted according to the method by Boone et al. (1995), after partial modification. MII pig oocytes were washed in 400 μl of ice-cold Ca²⁺/Mg²⁺-free DPBS (Dulbecco’s Phosphate Buffered Saline, Cambrex Bio Sciences, Verviers, Belgium) containing the protease inhibitor PMSF (Phenylmethanesulfonyl fluoride, Sigma) then they were lysed in 400 μl of ice-cold buffer (10 mM MgCl₂, 0.25% Nonidet P40, 2 μl PMSF). The suspension was incubated for 60 min at 4°C with gentle rotatory shaking and centrifuged 2 min at 10 000 g. The supernatant was collected and dried under vacuum speed centrifuge. The resulting pellet was re-suspended in Tris buffer (0.35 M NaCl, 1 mM EDTA, 200 mM TrisCl, pH 7.4) and the protein content was determined by Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules CA, USA). Seventy micrograms of protein extracts (n = 200 oocytes) were added with 2 volumes of Laemmli Buffer (Bio-Rad), incubated at 65°C for 10 min and loaded onto standard polyacrylamide gels containing calf thymus DNA (250 μg/ml) in both stacking (4%) and resolving gels (12%). Purified DNase type I (1 ng) from bovine pancreas (Sigma) was loaded on the same gel as control. Electrophoresis was carried out at 4°C at 150 V for 1.5 h in Tris—Glycine—SDS Buffer (Bio-Rad). The gels were soaked in several changes of TE buffer (50 mM Tris pH 7.5, 1 mM EDTA), stained with ethidium bromide (0.5 μg/ml TE buffer) and densitometrically measured by FluorMultimager (Bio-Rad) under u.v. light. The gels were then soaked in TE buffer added with Ca²⁺ (2 mM) and Mg²⁺ (5 mM) or Ca²⁺ (2 mM), Mg²⁺ (5 mM) and Zn²⁺ (2 mM) and incubated at 37°C with gentle shaking. The incubation media were changed and the nuclease activity was densitometrically monitored every 12 h until 120 h of incubation. The nuclease activity was detected, in zymographic assay, as a dark area on a fluorescent background following exposition to u.v. light. Three gel replicates were done with protein extracts derived from groups of 200 oocytes for each treatment in each replicates.
Plasmid degradation assay

The DNase activity of the oocytes was assayed by digestion of EGFP plasmid (Enhanced Green Fluorescent Protein-N1 vector, 4.7 kb, Clontech, Palo Alto, CA, USA). Three replicates were done with groups of MII oocytes (n = 40 or n = 100-total n = 280 per replicate). Oocytes were rinsed with 0.9% NaCl, placed in microtubes and lysed in nuclease/protease free H2O2 (Eppendorf, Hamburg, Germany) by multiple pipetting. Cellular lysates were then incubated (1 h at 37°C) with 250 ng of undigested plasmid in 50 mM Tris–HCl pH 7.4, with or without Ca2+/Mg2+ (2 mM CaCl2, 5 mM MgCl2). The reactions were stopped by the addition of EDTA. Pancreatic bovine DNase I (3530 U/mg, Worthington Biochemical Corporation Lakewood, NY, USA) was used as positive control. Samples were then subjected to TAE agarose gel (0.8%) electrophoresis.

Western blot analysis

MII oocytes (n = 40–50 per lane in triplicate) were lysed in SDS buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 20% glycerol). Proteins obtained from oocytes and 10 μg of bovine pancreatic DNase I (control) were separated on NuPage 10% Bis-Tris Gel (Invitrogen-Gibco, Paisley, UK) for 50 min at 200V and electrophoretically transferred onto a nitrocellulose membrane. Blots were washed in PBS and protein transfer was checked by staining the nitrocellulose membranes with 0.2% Ponceau Red. Non-specific protein binding on nitrocellulose membranes was blocked with 5% milk powder in PBS-T20 (Phosphate Buffer Saline-0.1% Tween-20) for 1 h at RT. The membranes were then incubated with a 1:500 dilution of anti-bovine DNase I antibody (Upstate Biotechnology Incorporated, NY, USA cat.#06-479) in Tris buffered saline-T20 (20 mM Tris–HCl, pH 7.4, 500 mM NaCl, 0.1% T-20) overnight at 4°C. After several washings with PBS-T20 membranes were incubated with the secondary biotin-conjugate antibody and then with a 1:1000 dilution of an anti-biotin horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology Inc, Beverly, MA, USA). The Western blots were developed using a chemiluminescent substrate (Pierce Biotechnology). The Western blots were developed using a chemiluminescent substrate (Pierce Biotechnology). The Western blots were developed using a chemiluminescent substrate (Pierce Biotechnology). The Western blots were developed using a chemiluminescent substrate (Pierce Biotechnology).

RT-PCR

Total RNA isolation was performed from groups of MII oocytes (n = 100 in triplicate) using RNase Micro Kit and treated with RNase-free DNase set (Qiagen Sciences Inc, MD, USA) according to the manufacturer’s instructions. Purified RNA was reverse-transcribed to cDNA with iScript cDNA Synthesis Kit (Bio-Rad) for 1 h at 42°C. A reaction without reverse transcriptase was performed to determine DNA contamination. Oligonucleotide primers used for amplification of DNase I were designed using Primer Select software (DNASTAR Inc. WI, USA) based on porcine pancreatic DNase I sequence (AN AB048832). The upstream primer (5’-CTGGCCCTGTCCCTGAGAATAGCA-3’) is identical to nucleotides 164–187 and the downstream primer (5’-CTGGGTGGAGGGGGAGGAGAACT-3’) represents the reverse complement of nucleotides 547–527; the specific primers, located on exon II and V respectively, gave rise to a fragment of 384 bp. PCR conditions and specificity were checked on cDNAs derived from porcine pancreas and corpus luteum mRNAs. The PCR reaction mixture (25 μl) consisted of 2.5 μl cDNA, 0.3 μM each primer, 0.3 mM dNTPs, 1 mM MgSO4, 2.5 μl 10 × PCR Buffer, 0.25 μl Taq polymerase platinum Pfx (Invitrogen, Paisley, UK). PCR cycling conditions were: initial denaturation for 2 min at 94°C followed by 40 amplification cycles at 94°C for 15 s and at 68°C for 30 s. Aliquots (2.5 μl) of MII oocytes PCR products were submitted to a further 20 cycles of amplification. Amplicon specificity was confirmed by sequencing (MWG DNA Sequencing Service). RNA integrity was tested by RT-PCR of porcine β-actin. Amplicons were analyzed on a standard 2% agarose gel stained with ethidium bromide.

Immunofluorescence

All the procedures were carried out at room temperature unless otherwise specified. The oocytes were fixed leaving them free floating in cold methanol for 30 min and then blocked in PBS containing 10% FCS and 0.2% Triton X-100 for 30 min. Subsequent manipulations and antibody dilutions were performed in blocking solution. Specific anti-bovine DNase I antibody (cat.#06-479; Upstate Biotechnology Incorporated, NY, USA) was added at the appropriate dilution (1:150). Incubation was carried out overnight at 4°C. After extensive washing, oocytes were incubated with a goat-anti-rabbit FITC-conjugated secondary antibody (Sigma) for 1 h. Control cells were treated similarly with the omission of primary antiserum. Oocytes were then mounted on a drop of Vectashield with propidium iodide (Vector Laboratories, Burlingame, CA, USA). Images were obtained using a Nikon digital camera installed on a Nikon epifluorescence microscope (× 400).

Exogenous DNA microinjection

In order to evaluate the microinjected amount of exogenous DNA, EcoRI linearized EGFP plasmid was diluted (1:1; v:v) with radioactive nucleotide (Redive adenosine 5’-[32P]-triphosphate, Amersham Biosciences, Bucks, UK), at the final concentration of 2 mg/ml. Approximately 2 μl of DNA solution were microinjected into the cytoplasm of MII pig oocytes using a Leitz Axiowert microscope with Narishige micromanipulators. The entire procedure of microinjection was carried out at 25°C. During microinjection, MII oocytes were held under mineral oil in DPBS Ca2+/Mg2+-free microdrops. After injection MII oocytes were incubated for 5 or 15 min at 39°C.
or at room temperature (25°C). Groups of microinjected oocytes were immediately recovered as control. In some experiments pEGFP was added with 2 mM ZnCl₂ before microinjection. Five microinjection sections (replicates) were done with three groups of 10 undamaged oocytes for each treatment in each replicates. Oocytes were subjected to zona removal, washed and recovered in 15 µl of PBS and heated at 95°C for 10 min. Seven µl of each sample were added to 4 ml liquid scintillation counting cocktails Ultima Gold (Perkin Elmer, MA, USA) and counted for 10 min in a α-counter (Packard 1600 TR). Only the samples presenting a radioactivity ranging between 350 and 500 c.p.m. were utilized. The remaining amount of each sample was separated on agarose gel (0.8%) at 100 V for 2 h with TAE buffer (0.04 M Tris, 0.02 M acetic acid, 1 mM EDTA).

The agarose gels were transferred to Hybond N⁺ membrane (Amersham Biosciences Freiburg, Germany) by capillary blotting in 10× SSC (Invitrogen). The filters were hybridized at 37°C for 2 h in DIG easy hybridization buffer (Roche Diagnostics GmbH, Germany) and hybridized at 37°C for 16 h with 25 ng/ml of EGFP DIG-labeled (DIG high prime labelling and detection starter kit, Roche). The blots were washed twice with SSC 2X, 0.1% SDS at room temperature and twice with 0.5× SSC, 0.1% SDS at 68°C. The chemiluminescent detection was performed using anti-DIG alkaline phosphatase conjugated antibody (1:10 000; Roche) and densitometrically quantified (Fluor5Multimag, BioRad).

### Sperm mediated gene transfer

Semen was collected from a boar of proven fertility and prepared according to the method by Lavitrano et al. (2003) after partial modification. Briefly, sperm cells were resuspended in swine fertilisation medium (SFM: 11.25 g/l glucose, 10 g/l Na citrate 2H₂O, 4.7 g/l EDTA 2H₂O, 3.25 g/l citric acid H₂O, 6.5 g/l Trizma, pH 6.8) supplemented with 6 mg/ml BSA (Fraction V. Miles-Kankakee, IL, USA). Sperm cells were coincubated for 2 h at 16°C with pEGFP (10⁹ sperm cells/5 µg DNA per ml).

To evaluate the uptake of exogenous DNA by sperm cells, aliquots (0.5 ml) of the suspension were removed after 0, 5, 15, 120 min and 24 h of coincubation. Semen aliquots were washed twice with PBS and resuspended in 0.7 ml lysis buffer (2 M NaCl, 25 mM TrisCl pH 7.4, 40 mM DTT), incubated at 4°C for 16 h and then submitted to a phenol/chloroform DNA extraction. EcoRI digested DNA (800 ng) was separated on agarose gel (0.8%) (100 V, 2 h) in TAE buffer.

SMGT treated and control sperm cells were extensively washed then resuspended with pre-equilibrated warm IVF medium. Four groups of fifty matured oocytes, freed from cumulus cells by repeated pipetting, were transferred into 500 µl IVF medium containing 2.5 × 10⁶ sperm/ml. After 1 h of co-culture, oocytes were transferred to fresh IVF medium. After 6 h oocytes were removed from fertilization medium, washed four times in DPBS Ca²⁺/Mg²⁺-free and subjected to zona removal. To check the fertilization efficiency, one group of oocytes was recovered 18–20 h after the beginning of fertilization, fixed, stained and observed as described above to evaluate pronuclei formation. Pools of 100 fertilized oocytes were subjected to DNA extraction using extraction DNA wizard (Promega Corporation, WI, USA). EcoRI digested DNA was separated on agarose gel, and submitted to hybridization analysis as above described. The experiment was done in triplicate.

### Statistical analysis

Data were analyzed by one-way ANOVA using the SPSS program Version 8.0 (SPSS Inc, Chicago, IL, USA). A probability of P < 0.05 was considered significant. Data represent mean± s.e.m. of 5 separate replicates.

### Results

The average proportion of oocytes that had reached MII stage after IVM was 97.2±0.6% (total number of evaluated oocyte = 496)

### DNase I activity in MII oocytes

Zymography of MII oocyte extracts identified a band of nuclease activity clearly detectable in all replicates when incubated in presence of Ca²⁺/Mg²⁺ at 37°C. The molecular weight of the observed bands was almost identical to that of the purified DNase I (33 kDa) (Fig. 1A). The activity of purified DNase I was detectable as a faint band already after 24 h of incubation while the nuclease activity of proteins extracted from oocytes needed a longer incubation period being clearly detectable after 120 h. The activation was impaired by the addition of ZnCl₂, a DNase I inhibitor (Fig. 1B).

Plasmid DNA after 1 h incubation with MII lysates, in conditions optimal for DNase I activity, showed a shift

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**Figure 1** Zymographic analysis of DNase I activity of oocyte protein extract compared with purified DNase I from bovine pancreas. (A) Gel incubated with Ca²⁺(2 mM) and Mg²⁺(5 mM) at 37°C. Bovine pancreatic DNase I (lane a; 1 ng), porcine MII protein extract (lane b; 70 µg). (B) Gel incubated with Ca²⁺(2 mM), Mg²⁺(5 mM) and Zn²⁺(2 mM) at 37°C. Bovine pancreatic DNase I (lane a; 1 ng), porcine MII protein extract (lane b; 70 µg). Images were acquired after 120 h of incubation.
from the supercoiled to the linear form. The degradation level was related to MII oocyte number (Fig. 2A). Figure 2B shows the time course of plasmid digestion by purified bovine DNase I.

**DNase I presence in MII oocytes**

Western blot analysis showed that anti-bovine DNase I antibody recognizes a band of approximately 33 kDa in protein extracts of both porcine corpus luteum and MII oocytes (Fig. 3A). The apparent molecular weight was the same as that of purified bovine pancreatic DNase I.

RT-PCR experiments showed a single band of expected length (384 bp) both on pancreas and corpus luteum, as well as on MII oocytes. To obtain an amplicon clearly visible on agarose gel from MII oocytes mRNA, it was necessary to submit the cDNA to a reamplification (Fig. 3B). The sequence of amplicon confirmed the specificity of PCR reaction with 100% homology with the porcine DNase I mRNA (GenBank Accession No. AB048832). The DNase I immunoreactivity was found to be evenly distributed in the whole oocyte cytoplasm without any preferential localization (Fig. 4). DNA staining with propidium iodide confirmed that oocytes have reached MII stage. No label was detectable when the second antibody alone was used.

**Degradation of exogenous microinjected DNA**

Radioactivity detected in undamaged MII oocyte groups utilized was 425 ± 70 c.p.m., showing that the amount of microinjected DNA was constant (approximately 4 pg DNA/2 pl per oocyte).

Southern blot hybridization analysis showed a degradation of microinjected exogenous DNA when oocytes were incubated 5 or 15 min at 39°C, with a residual percentage of exogenous DNA in relation to control of 13.51% ± 7.3 and 15.47% ± 9.2 respectively (Fig. 5A and B). On the contrary, microinjected oocytes incubated at a lower temperature (25°C) did not show any significant reduction in exogenous DNA content.

ZnCl$_2$ significantly protected microinjected linearized exogenous DNA ($P < 0.05$) (Fig. 5C–D).

**Persistence of exogenous DNA in MII oocytes fertilized with SMGT treated spermatozoa**

DNA extracted from sperm cells coincubated with exogenous DNA showed a clear band consistent with the linearized plasmid (4700 bp) (Fig. 6A). DNA uptake took place already after 5 min; no signs of plasmid degradation were detectable in samples up to 24 h of co-incubation at 16°C (Fig. 6A).

The fertilization rate was 62.3 ± 5.1% and 61.8 ± 4.2% in control and SMGT experiments respectively, while polyspermy rate was around 28% in both conditions.

Southern blot of DNA extract from oocytes 6 h after fertilization showed a band ascribable to plasmid DNA (Fig. 6B). Different groups of fertilized oocytes presented different amounts of plasmid DNA.

**Discussion**

The present study demonstrates the presence in pig MII matured oocytes of an endonuclease activity that is bio-chemically, immunologically and functionally superimposable to DNase I. The specific mRNA as well as the protein has been detected by means of different techniques: RT-PCR showed a clear single band of expected length even if the DNase I mRNA seems to be present in a low copy number. To detect the band under u.v. light, a re-amplification of the product (total 60 cycles) was necessary. The utilization of a Platinum pfX high fidelity Taq polymerase only allowed the amplification of the
specific band, the nucleotide sequencing confirmed that it
corresponded to the porcine DNase I mRNA. The
immunological characterization showed a weak band
consistent with the molecular weight of pancreatic DNase
I (33 kDa) by Western blot and a diffuse localization in
the whole ooplasm by immunofluorescence. We set up
different kinds of experiments to identify DNase I activity
in MII oocytes. Zymography experiments again confirmed
the expected molecular weight. All results evidenced that
the enzymatic activity requires Ca\(^{2+}\)/Mg\(^{2+}\), incubation

![Figure 5](image)

**Figure 5** Persistence of exogenous DNA microinjected into porcine
MII oocyte. (A) Example of Southern blot analysis of pEGFP microinjected
into MII pig oocytes (n = 5). Microinjected MII oocytes without
incubation (lane a; control), microinjected MII oocytes incubated for
5 min at 39°C (lane b), microinjected MII oocytes incubated for 15 min
at 25°C (lane c), microinjected MII oocytes incubated for 15 min at
39°C (lane d), DNA molecular weight marker III, DIG-labeled (lane e).
(B) Densitometric evaluation of microinjected DNA (mean ± S.E.M
resulting from five different experiments). Columns with different
superscripts indicate significant difference (P < 0.05). (C) Example of
Southern blot analysis of microinjected pEGFP into MII pig oocytes
(n = 5). Microinjected MII oocytes without incubation (Lane a; control),
microinjected MII oocytes incubated for 5 min at 39°C (lane b),
microinjected MII oocytes incubated for 5 min at 39°C in presence of
2 mM Zn\(^{2+}\) (lane c), DNA molecular weight marker III, DIG-labeled
(lane d). (D) Densitometric evaluation of microinjected DNA (mean ±
S.E.M resulting from five different experiments). Columns with different
superscripts indicate significant difference (P < 0.05).

![Figure 6](image)

**Figure 6** Persistence of plasmid DNA on SMGT experiments.
(A) Example of Southern blot hybridization of DNA extracted from
8 x 10^5 spermatozoa for plasmid DNA detection. Purified pEGFP
(Lane a; 4 ng), DNA molecular weight marker III, DIG-labeled (lane b),
immediately after exogenous DNA addition (lane c; no uptake is
observed). Co-incubation at 5, 15, 60 min and 24 h (lanes d, e, f and
g, respectively). (B) Example of Southern blot hybridization of DNA
extracted from oocytes 6 h after fertilization. pEGFP (Lane a; 4 ng),
DNA molecular weight marker III, DIG-labeled (lane b), DNA
extracted from three different groups of oocytes (lanes c, d and e;
n = 100) fertilized with SMGT-treated spermatozoa, DNA extracted
from 10^5 and 10^2 SMGT-treated spermatozoa (lanes f and g,
respectively).
temperature of 37°C and neutral pH, moreover, this activity is inhibited by the addition of Zn\(^{2+}\).

These biochemical characteristics are very similar to those generally reported for the mammalian DNase I (Mori et al. 2001).

Our study documents, for the first time, on in vitro matured pig oocytes, the presence of an active DNase I. The Western blot results taken together with the fact that detection of the mRNA required reamplification, show that the protein is present in low quantities. Though functional experiments demonstrated that the enzyme could be biologically significant, even if we don’t know how much DNase I an oocyte needs, for it to be biologically significant. Moreover, experiments on in vivo matured oocytes are needed in order to verify eventual artefacts produced by the in vitro system. Previous findings in rat oocytes evidenced an immunolocalization of DNase I only in oocytes derived from immature preantral follicles, while oocytes from antral follicles did not stain for DNase I (Boone & Tsang 1997). A more recent study (Stępińska & Olszańska 2003) failed to detect nuclelease activities in ovulated mouse oocytes, whereas two endonucleases (DNase I and II) have been demonstrated in avian oocytes.

Since an early block of polyspermy ensures monospermic fertilization, Stępińska & Olszańska (2003) suggest that the absence of DNase activity in mammalian oocytes was expectable. At the same time they suggest that endonucleases are involved in the degradation of supernumerary spermatozoa during polyspermic fertilization that is a physiological phenomenon in the avian species. They also hypothesize that the low efficiency of transgenic chicken production by DNA microinjection might be related to the presence of DNase in oocytes.

Our findings led us to hypothesize that the role of this enzyme in pig oocytes might be similar to that suggested for avian. The incidence of polyspermy in porcine eggs fertilized in vivo ranges from 5 to 35% and can reach 65% in in vitro fertilization trials (Mattioli et al. 1988, Sun & Nagai 2003). In this species, polyspermy is, at least in part, related to the number of capacitated spermatozoa available at the site of fertilization (Hunter & Nichol 1988); nevertheless, poly-pronuclear pig eggs can correct abnormal ploidy and develop to term (Han et al. 1999). Moreover, hypersensitivity of male DNA to exogenous DNase I during sperm decondensation has been reported in mouse oocyte; however, once pronuclei are fully formed, this sensitivity is completely abolished (Bizzaro et al. 2000). Therefore DNase activity in pig MII oocytes could be useful in control of polyspermy in an early phase, before pronuclei formation.

Our microinjection experiments showed that exogenous DNA is degraded, up to 85%, after 5 min of incubation at 39°C while incubation at 25°C is not effective. DNase I is likely to be involved: in fact, the presence of Zn\(^{2+}\) significantly protects exogenous DNA. This is an important issue and should be carefully considered, in that the persistence of microinjected DNA might be hopefully improved by adjusting the procedures (i.e. working at low temperatures or using specific DNase I inhibitors).

No signs of plasmid DNA degradation are observed after co-incubation with spermatozoa in SMGT experiments. These results suggest that nucleases, eventually present in fully mature spermatozoa (Pittoggi et al. 1999, Sakkas et al. 2002), are not effective in degrading exogenous DNA in our incubation conditions.

Finally, our experiments indicate that exogenous DNA delivered into pig oocytes by SMGT-treated spermatozoa is protected from the nuclelease activity of the ooplasm. As it is impossible to know the amount of oocytes fertilized by positive spermatozoa and the amount of DNA carried by a single spermatozoon, we could not quantify the residual exogenous DNA present in the oocyte 6h after fertilization.

During fertilization, male genome becomes relatively exposed and accessible (Perry 2000) and decondensing mouse sperm heads show DNA breaks (Kiessling & Markoulaki 2000) and hypersensitivity to DNase I (Bizzaro et al. 2000) thus representing a huge and unique opportunity for DNA integration. In ICSI-mediated trangenesis, the addition of Ca\(^{2+}\)/Mg\(^{2+}\)-dependent DNase I-like activity to suspensions of sperm cell and exogenous DNA, inhibits both transgenesis and DNA breaks in chromosomes, showing a positive correlation between incorporation of exogenous DNA and paternal chromosome breaks (Szczygiel et al. 2003). These results, together with the high proportion of innovative gene transfer methods involving sperm as a transgene transport system (Wall 2002) and their high efficiency in transgenesis, agree well with our findings about the presence of a Ca\(^{2+}\)/Mg\(^{2+}\)-dependent DNase in pig MII oocytes.

In conclusion, our data demonstrate the presence of an endogenous Ca\(^{2+}\)/Mg\(^{2+}\)-dependent DNase I-like activity in pig MII ooplasm that could likely be responsible for microinjected DNA degradation; in addition, our findings show that the association of exogenous DNA to spermatozoa in SMGT, could protect the DNA from the nuclelease degradation.

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