Spatial organization of repetitive DNA sequences in the bovine sperm nucleus

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Summary

During spermatogenesis, DNA in the sperm head becomes more tightly condensed as histones are replaced by protamine-like molecules. In this article, the question is asked whether, during the production of this highly differentiated cell, controls are imposed on the spatial organization of DNA within the nucleus. Heads from bull spermatozoa were isolated by a technique that removed the plasma membrane and acrosomal contents, and the DNA was induced to decondense by addition of 2-mercaptoethanol and trypsin. Under these conditions, decondensation was induced in all regions of the head. To determine whether there was any spatial restraint on packaging of the genome, three DNA probes were used (pl.709–512, containing an interspersed repetitive sequence; pCSIH, containing a copy of the major bovine centromeric satellite sequence; p18s and p28s, containing the 18S and 28S ribosomal genes) that might be expected to hybridize to different regions. Results showed that the interspersed repetitive probe hybridized to all regions of the head, whereas the ribosomal and centromeric probes hybridized to sequences that were largely confined to the equatorial region of the sperm. We conclude that organization of the genome in the bovine sperm nucleus is not random.

Key words: sperm nucleus, genome organization, in situ hybridization.

Introduction

The genome of eukaryotic cells is complexed with a variety of soluble nuclear proteins, such as histones, polymerases and transcription factors. At certain points physical contact is also made with components of the nuclear scaffold and the nuclear envelope (Moroi et al. 1981; Jackson et al. 1984; Gasser and Laemmli, 1986). Several lines of evidence indicate that these structural attachment sites impose constraints on the organization of the genome. In studies of Drosophila salivary gland polytene nuclei, Sedat and colleagues found that centromeric DNA was attached to the nuclear membrane at one pole and telomeric DNA at the opposite pole, perhaps preserving the orientation generated during anaphase (Agard and Sedat, 1983; Mathog et al. 1984). By contrast, Manuelidis found cell type-specific organization of DNA in cells of the mouse nervous system; using in situ hybridization experiments, it was found that centromeric sequences capped the nucleoli in Purkinje cells, whereas the same sequences were dispersed around the nuclear membrane in granule neurons (Manuelidis, 1984). Furthermore, recently it has been shown that individual chromosomes occupy cohesive domains in interphase nuclei (Lichter et al. 1988). Clearly, an order is imposed on the genome organization in at least some somatic cells. We have investigated the organization of the genome in the sperm cell nucleus with a view to understanding the changes in chromatin organization during spermatogenesis and fertilization.

For at least three reasons, a requirement for defined sequence organization is likely to be intensified in sperm nuclei. First, the volume of the sperm nucleus is about 21 μm³ (a bovine sperm nucleus is approximately 7 μm x 3 μm x 1 μm), less than 5% of a 'typical' somatic nucleus (r=5 μm, volume=520 μm³), presumably requiring very tight and careful packing of chromatin into the sperm head. Second, during spermatogenesis, somatic histones are removed from the DNA and progressively replaced by protamine-like molecules (highly basic, arginine-rich proteins of about 5000 to 10,000 molecular weight) that bind tightly to the genome, inducing dramatic chromatin condensation (Marushige and Marushige, 1975; Loir et al. 1986; Poccia, 1986). This condensation is secured by the formation of disulphide cross-links between protamines lying in the minor groove of the DNA, with the result that the sperm nucleus has about the same volume as would be occupied by 3.3 pg of haploid DNA alone (see Balhorn, 1982). This progressive condensation is presumably carefully orchestrated during spermatogenesis. Third, at fertilization these constraints are relieved as the sperm head decondenses and the paternal genome is liberated in the egg cytoplasm. During this process, disulphide cross-links are reduced, protamines hydrolysed and rapidly replaced by somatic histones and within hours the male pronucleus has formed (Yanagimachi and Noya, 1970; Marushige and Marushige, 1978; Perrault et al. 1987; Schatten and Schatten, 1987).

A major question then arises as to how these changes in density, organization and DNA:protein interactions are orchestrated. In particular, is the condensation reaction during spermatogenesis entirely a random process or is there a long-range order within the nucleus so that specific
DNA sequences are localized in particular regions of the head? During decondensation, are certain DNA sequences liberated first in a pre-determined fashion and are these sequences important for the establishment of a male pronucleus? To answer these questions, we have initiated investigations into the organization of DNA and chromatin in the sperm nucleus using in situ hybridization techniques. In these studies, we have used DNA probes for interspersed repetitive, centromeric and ribosomal DNAs to locate these sequences in the nucleus of bovine spermatozoa.

Materials and methods

DNA probes
Plasmid p1.709-512, an interspersed bovine repetitive sequence containing a bovine Alu-like sequence was a gift from Dr Andrej Plucienniczak (Skowronski et al. 1984). A cloned sequence derived from the major bovine 1.716 g cm⁻³ satellite DNA was kindly supplied by Dr Roizès (Pages and Roizès, 1984). This clone, termed here pCSH, contains a single copy of the 1.4 kb (1 kb = 10⁶ base-pairs) centromeric EcoRI satellite sequence. Plasmids p28 and pEC10 were obtained from Marianne Salditt-Georgieff (Rockefeller University) and were originally isolated by Dr Norman Sonnheim (SUNY, Stony Brook). p28 contains a SalI-EcoRI fragment of 4.8 kb containing all the sequence of 28 S rRNA; p10 contains a 1.8 kb SalI-EcoRI fragment that includes the 5'-most 1.1 kb of 18 S rRNA. Each insert was purified by agarose gel electrophoresis (Vogelstein and Gillespie, 1979).

Preparation of sperm heads
Frozen semen, collected from Friesian bulls of proven fertility, was used throughout these experiments. Semen was thawed at 20°C for 5 min and then washed three times in phosphate-buffered saline (PBS: 170 mM NaCl, 3.4 mM KCl, 1 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.2). Pelleted sperm were then resuspended in 1 ml TNE (50 mM Tris–HCl, pH 7.5, 10 mM NaCl, 10 mM Na₂EDTA) and sonicated for 15 s at full power using an MSE sonicator. 2-Mercaptoethanol was added to 10 mM and heating to 65°C for 10 min. Labelled DNA was purified by alkali digestion using a Sephadex G50 (Pharmacia) column.

Labeling of DNA and Southern hybridizations
DNA was prepared from mercaptoethanol-treated bovine, murine and ovine sperm using proteinase K digestion followed by phenol/chloroform extraction. Purified DNAs were digested with EcoRI, electrophoresed and blotted using standard protocols (Maniatis et al. 1982). Probes labelled with [³²P]dCTP using random primer labelling reactions, were hybridized at 65°C in 5XSSPE (see below), 1% SDS at 65°C and washed according to standard procedures (Maniatis et al. 1982).

Labelling of DNA and in situ hybridization
Labelling and hybridization was carried out essentially as described by Singer and Ward (1982). Between 100 ng and 500 ng of plasmid insert were labelled by nick translation (Bigby et al. 1977) in a reaction mixture using biotinylated dUTP (Sigma Chemical Co.) and dATP (BRL). After incubation at 14°C for 2 h, the reactions were terminated by the addition of Na₂EDTA to 10 mM and heating to 65°C for 10 min. Labelled DNA was purified using a Sephadex G50 (Pharmacia) column.

Endogenous peroxidases in dried sperm heads were inactivated by incubating the slides in 3% hydrogen peroxide, 97% methanol for 10 min at room temperature, followed by a rinse in 100% ethanol and air drying. DNA was denatured by incubating the slides in 70% NaOH for 5 min at room temperature, followed by a rinse in 2X SSC (1X SSC is 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), dehydrated through an ethanol series and air dried.

Biotinylated probes were denatured in the presence of salmon sperm DNA at 100°C for 10 min, quick-chilled and then mixed with SSPE–dextran sulphate to give final concentrations of 0.4–2 μg ml⁻¹ probe, 0.1 μg ml⁻¹ Salmon sperm DNA, 10% dextran sulphate, 4XSSPE (1XSSPE is 150 mM NaCl, 10 mM Na₂HPO₄, 1 mM EDTA). A 60 μl sample of this mixture was added to each slide, coverslips were applied, and the slides were then incubated for 16 h at 60°C in a moist chamber. After hybridization, the slides were washed twice in 2X SSC, 0.1% Triton X-100 and once in 0.1X SSC, 0.1% Triton X-100 each for 5 min at room temperature. After washing for 10 min at 50°C in 0.1X SSC, 0.1% Triton X-100, the slides were incubated for 5 min at room temperature in 0.1X SSC, 0.1% Triton X-100, 0.5% bovine serum albumin (BSA; Sigma, Fraction V).

Streptavidin conjugated to horseradish peroxidase (SA-HRP; Amersham) was diluted 1:200 in 0.1X SSC, 0.1% Triton X-100. A 100 μl sample of diluted SA-HRP was added to each slide, coverslips applied and the slides incubated for 60–90 min at 37°C in a moist chamber. After washing for 5 min each at room temperature, the slides were incubated in 0.1X SSC, 0.1% Triton X-100, 5% BSA, peroxidase activity was detected by staining in 0.5 μg ml⁻¹ diaminobenzidine (DAB), freshly prepared in 0.1X SSC, 0.1% Triton X-100, 0.009% hydrogen peroxide for 30–40 min in the dark. After rinsing in deionized water, the slides were stained in haematoxylin and eosin.

Results

Morphological examination of bovine sperm head decondensation
When washed bovine sperm heads are incubated in buffer containing reducing agents, an endogenous proteolytic activity hydrolyses the reduced protamine subunits and causes decondensation initially in the posterior region of the head (Fig.1A,B; Marushige and Marushige, 1978). However, for in situ hybridization it is important that DNA from all regions of the sperm nucleus is simultaneously available to the hybridization probes; the endogenous proteolytic decondensation clearly does not satisfy this requirement. We therefore investigated the effects of the addition of exogenous proteases on the decondensation reaction.

Preliminary experiments showed that decondensation of the sperm nucleus required the presence of a serine protease, as Steptococcus aureus V8 protease (0.2 mg ml⁻¹) was without detectable effect. However, the addition of pancreatic trypsin to a final concentration in the range 0.05 mg ml⁻¹ to 1 mg ml⁻¹ in the presence of 10 mM 2-mercaptoethanol induced decondensation uniformly and evenly around the entire head (Fig. 1C,D,E,F). Below 0.05 mg ml⁻¹ of added trypsin, the pattern of decondensation was intermediate between the full proteolysis found for high trypsin concentrations and the endogenous reaction. At higher concentrations, the first events detected at the light microscope level were lifting or peeling away of most or all of the membranes from the
Fig. 1. Phase-contrast microscopy of bovine sperm heads. Heads were prepared as described in Materials and methods and incubated at 23°C in TNEd containing 10 mM 2-mercaptoethanol. A,B. Endogenous reaction of heads incubated in the absence of added trypsin. After 50 min at room temperature, the postacrosomal region of the head starts to swell (A) and by 70 min most of the head has decondensed. Note that the baseplate (arrow) remains associated with the head (cf. Yanagimachi and Noda, 1970). C-F. Decondensation in the presence of added trypsin (0.2 μg/ml). At early times some or all membranes of the head lift away from the nuclear contents; the nucleus itself becomes fenestrated (C, 25 min). As the reaction proceeds, progressively more of the chromatin is decondensed, producing a loose aggregation of DNA surrounding a central core (D, 30 min; E, 35 min), which is also finally decondensed (F, 40 min). Bar, 10 μm.

**In situ hybridization with a bovine interspersed repetitive sequence**

The bovine 1.709 satellite sequence is composed of about 70,000 copies of an imperfect 3808 bp repeat unit (Macaya et al. 1978). This unit contains several distinct repetitive elements, including bovine Alu-like sequences that are dispersed in the bovine genome (Fig. 3A). We used a cloned copy of the repetitive sequence, clone p.709-512 (Skowronski et al. 1984), for in situ hybridization experiments. Bovine sperm heads were treated with trypsin for varying lengths of time and then hybridized with biotinylated p.709-512. Hybridization of the probe was detected by reaction with SA-HRP and staining with DAB. We found that peroxidase activity was detected over all regions of the sperm head, with no apparent preferential localization after either limited or extensive digestion with trypsin (Fig. 3B,C). We measured the location of these deposits along the anteroposterior axis on one hundred randomly selected sperm heads and plotted these data as a histogram of percentage of stain deposits against distance from the baseplate (Fig. 6A, below). This demonstrated that staining was distributed apparently at random along the head and, furthermore, that sequences throughout the sperm nucleus were available for hybridization.

**In situ hybridization with a bovine centromeric repetitive sequence**

The bovine genome contains several hundred thousand copies of an imperfect satellite repeat sequence, which is defined by its buoyant density in cesium chloride; these satellite sequences are located mainly at or near the surface of the nucleus (Fig. 1C) and, shortly thereafter, the chromatin became patchy in appearance as peripheral regions of the nucleus decondensed (Fig. 1D). This reaction proceeded centripetally, so that a central core of condensed chromatin became progressively smaller (Fig. 1E) until eventually all internal structure was lost (Fig. 1F). At this stage, the reaction mixture became highly viscous with all the characteristics of partly solubilized, high molecular weight DNA.

At the ultrastructural level, it was clear that the majority of tails were detached by sonication and that the plasma membrane covering the head was lost, together with the outer acrosomal membrane and the contents of the acrosome (Fig. 2A). The inner acrosomal membrane appeared to remain reasonably intact and covered the nuclear envelope. However, in the posterior part of the cell the nuclear envelope was directly exposed.

The first observable change during trypsinization was a lifting of the nuclear membrane from the entire surface of the head. Shortly afterwards, a peripheral reticulum of DNA fibres formed with many fibres apparently remaining attached to a nuclear envelope component and being pulled out radially from the nucleus (Fig. 2B). Later, the network of fibres became more extensive and relaxed with the central core becoming markedly disperse in all but the most posterior region (Fig. 2C). This network was of apparently similar density along the whole length of the head, from which we conclude that trypsin digestion initially liberated DNA from all peripheral regions of the head to similar extents. However, DNA in the core and in the most posterior regions overlying the baseplate was liberated only after extensive digestion. We therefore used heads that had been trypsin-treated for varying lengths of time for the following in situ hybridization experiments.
Fig. 2. Electron microscopy of bovine sperm heads. Trypsin-treated heads were fixed at various times and prepared for electron microscopy as described in Materials and methods. A. Electron micrograph sections of sperm heads after sonication. The outer (small arrow) and inner (large arrow) acrosomal membranes are both preserved in the equatorial region. The inner acrosomal membrane and nuclear membrane (open arrow) appear to be intact. B. Initial stages of head decondensation (38 min in 0.05 mg ml\(^{-1}\) trypsin). The membranes (large arrowheads) have lifted away from the head, apparently pulling strands of DNA (small arrowheads) with them. Tangential sections indicate the network of DNA fibres in peripheral regions (open arrowhead). C. Later stages of head decondensation (42 min at 0.06 mg ml\(^{-1}\) trypsin). DNA from all regions of the head is decondensed, producing a coarse meshwork of fibres. The central core is reduced in all regions except the most posterior part near the baseplate (large arrowhead). Bar, 1 \(\mu m\).

Fig. 3. Hybridization of bovine 1.709 g cm\(^{-3}\) satellite DNA to a Southern blot of DNA and to trypsin-treated bovine sperm heads. Filter hybridizations were performed as described in Materials and methods. Sperm heads were treated with trypsin and smeared on poly-L-lysine-coated slides. All samples were hybridized with biotinylated, purified insert from p1.709-512 and, after washing and reaction with SA-HRP, stained for peroxidase activity with DAB (Materials and methods).

A. Hybridization of p1.709-512 to Southern blot of bovine, murine and ovine DNA digested with EcoRI. The probe hybridizes to a smear of genomic DNA fragments, indicating the presence of interspersed repetitive sequences in the 1.709 g cm\(^{-3}\) satellite sequence. The bands represent cleavage at internal EcoRI sites in the 3.8 kb repeat unit. B,C. Hybridization of p1.709-512 to trypsin-treated bovine sperm heads. Peroxidase activity is detected in all regions of the heads. Treatment with 0.1 mg ml\(^{-1}\) trypsin for 27 min (B) or 32 min (C). Bar, 10 \(\mu m\).

Fig. 8. Hybridization of bovine 1.709 g cm\(^{-3}\) satellite DNA to a Southern blot of DNA and to trypsin-treated bovine sperm heads. Filler hybridizations were performed as described in Materials and methods. Sperm heads were treated with trypsin and smeared on poly-L-lysine-coated slides. All samples were hybridized with biotinylated, purified insert from p1.709-512 and, after washing and reaction with SA-HRP, stained for peroxidase activity with DAB (Materials and methods).

A. Hybridization of p1.709-512 to Southern blot of bovine, murine and ovine DNA digested with EcoRI. The probe hybridizes to a smear of genomic DNA fragments, indicating the presence of interspersed repetitive sequences in the 1.709 g cm\(^{-3}\) satellite sequence. The bands represent cleavage at internal EcoRI sites in the 3.8 kb repeat unit. B,C. Hybridization of p1.709-512 to trypsin-treated bovine sperm heads. Peroxidase activity is detected in all regions of the heads. Treatment with 0.1 mg ml\(^{-1}\) trypsin for 27 min (B) or 32 min (C). Bar, 10 \(\mu m\).

centromeres (Fig. 4A; see Kurnit et al. 1973). We used a probe derived from pCSIH of Roizes et al. (Pages and Roizes, 1984), which represents one member of this satellite family, in hybridizations with trypsin-treated heads. When heads were lightly decondensed (after 25–30 min incubation with 0.1 mg ml\(^{-1}\) trypsin) we found that this probe hybridized principally about half-way along the length of the head and across the entire width of the head. In the majority of cases, and especially at early incubation times, there was little hybridization to other regions of the head (Fig. 4C,D). The location of peroxidase staining along
Fig. 4. Hybridization of bovine 1.715 g cm\(^{-3}\) satellite DNA to bovine metaphase chromosomes, to a Southern blot of DNA and to trypsin-treated bovine sperm heads.

A. Hybridization of pCSIH to bovine metaphase chromosomes. The peroxidase activity is localized over the centromeres of chromosomes, confirming previous observations (Kurnit et al. 1973). B. Hybridization of pCSIH to Southern blot of bovine, murine and ovine DNA digested with EcoRI. C–E. Hybridization of clone H to trypsin-treated sperm heads. Peroxidase activity is localized over the equatorial region of the sperm head, occupying most of the width of the head. In some cases, hybridization occurs to other sites; this is more common after extensive trypsin digestion (arrowheads in E). Treatment with 0.1 mg ml\(^{-1}\) trypsin for 26 min (C, D) or 35 min (E). Bar, 10 µm.

Discussion

The imposition of order on the genome in the nucleus might be required, among other roles, for efficient movement of chromosomes within the cell and to elicit appropriate levels of gene expression. Here we have investigated genome organization in the bovine sperm nucleus with the ultimate aim of defining the steps involved in condensation and decondensation of the paternal genome during gametogenesis and fertilization. The especially compact packaging of the mature sperm head might be expected to impose rigid constraints on genome organization. In addition, spermatozoa offer the advantages that they are readily prepared as a pure single cell suspension and are unusual among mammalian nuclei in having a polarity along the anteroposterior axis. Thus, there are several attributes of sperm nuclei that are suitable for the study of genome organization. To permit access of the DNA probes to the condensed genome, however, it was necessary to decondense the sperm heads partially.

Both in vivo and in vitro, the endogenous sperm decondensation reaction proceeds from the posterior half of the head to the anterior, acrosomal structures giving a marked regionalization to the DNA availability. Further...
Fig. 5. Hybridization of mouse ribosomal DNA to a Southern blot of DNA and to trypsin-treated bovine sperm heads. DNA or cells were prepared and hybridized as described in Materials and methods. A. Hybridization of p18 s and p28 s inserts to Southern blots of bovine, murine and ovine DNA digested with EcoRI. B–D. Hybridization of p18 s and p28 s to trypsin-treated sperm heads. Peroxidase activity is most frequently localized over the equatorial regions of the head, and most frequently to one side of the head; 0.1 mg ml⁻¹ trypsin for 30 min. Bar, 10 μm.

more, there appears to be a structural relationship between the sperm nuclear annulus and the genome, which may play a role in spatial organization of DNA (Ward and Coffey, 1989). It was therefore necessary to ensure that DNA decondensation proceeded equally throughout the head to allow uniform access of probes and thus obviate artifacts. This was accomplished by incubating the heads in TNEd containing 10 mM 2-mercaptoethanol and a final trypsin concentration in the range 0.02 to 0.5 μg ml⁻¹. In these conditions, decondensation of the heads began after about 15–30 min and was complete (with complete decondensation of DNA) after a further 20 min. Both phase-contrast microscopy and electron microscopy indicated that exogenous trypsin induced decondensation of DNA from all regions of the head. Electron microscopy in particular showed that a network of DNA fibres was liberated around the periphery and that the core of undecondensed DNA was gradually reduced during the incubation. The only region in which decondensation was significantly delayed was in the most posterior area, adjacent to the base plate. Over the remainder of the head the liberated DNA was evenly dispersed, from which we conclude that such preparations would provide a suitable substrate for *in situ* hybridization.

This conclusion was reinforced by the hybridization results obtained with p1.709–512. We found that this probe hybridized to all regions of the head. This indicated that sperm DNA was available throughout the head and that there was no specific localization of 1.709 satellite repeat sequences in the head. This does not, however, exclude a role for such sequences in ordering sperm nucleus packing, like that found in attachment to the nuclear membrane. We are currently carrying out extraction experiments to define the interactions between the genome and components of the sperm nucleus.

We found that the other two probes used in these experiments hybridized to specific regions of the sperm
nucleus. The pattern of localization was most apparent after limited digestion; after longer times, with more-extensive decondensation of DNA, hybridization of pCSIH in particular (the centromere probe) extended over a greater region. Electron microscopy of heads revealed that after extensive digestion a highly tangled network of fibres extending in many directions was produced. We conclude that the dispersal of the hybridization signal over an extended area in heavily digested sperm heads was principally due to the extensive liberation and folding of decondensed DNA.

Our major conclusion with the satellite DNA of pCSIH is that centromeric sequences are localized in the equatorial region of the sperm nucleus. Over three-quarters of the deposits of stain were found in the equatorial one-third of the head. By contrast, only about 10% of the deposits lay in each of the other two-thirds of the head. A very similar pattern of hybridization was found with the ribosomal DNA probes with more than 75% of the deposits in the equatorial third of the sperm head. In many mammalian species the ribosomal genes are located near the centromeres (O'Brien, 1987), and this co-localization of centromeric and ribosomal DNA may reflect this close linkage. However, we cannot exclude the possibility that the localization of ribosomal sequences in this region reflects a more fundamental biological significance.

A major question from a functional standpoint concerns the significance of organization in which centromeric DNA is post-acrosomal. On the basis of the events that occur during the endogenous in vitro decondensation reaction or during fertilization (e.g. see Schatten and Schatten, 1987), it is possible that this organization participates in the ordered release of chromosomes into the egg cytoplasm. During fertilization, the postacrosomal region decondenses first, and we surmise that centromeric and, perhaps, ribosomal sequences are liberated early in this reaction. Centromeric DNA is involved in formation of the kinetochore, a specialized structure that interacts with microtubules of the spindle to move chromosomes within the cell (e.g. see Mitchison, 1988), and the early release of centromeric DNA from the sperm nucleus may permit the rapid attachment of the chromosomes to the microtubule array and assist the ordered withdrawal of chromosomes from the head. Experiments in progress are designed to provide further information on the structural organization of sperm DNA and in temporal patterns of condensation and decondensation.

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