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Chromatin Structure of Telomere Domain in Human Sperm

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Telomeres in human sperm nucleus are clustered at the nuclear periphery. Chromosomes in the sperm are highly condensed with protamines, however, a small portion of DNA remains associated with histones; the role of the nucleohistone is unknown. To examine structure of the telomeric chromatin, the sperm nuclei were treated with micrococcal nuclease. Chromatin released by the digestion was free from protamines, but contained histones and revealed nucleosomal organization. It was enriched with telomeric DNA organized into closely spaced nucleosomes with a periodicity of 148 ± bp. Thus, while the most of the sperm genome is packed into extremely dense nucleoprotamine structure, at least a part of the telomeric DNA is arranged into nucleosomes and can be released by the nuclease. We suggest that telomeres might be among the first structures in the sperm nucleus that respond to oocyte signals for male pronucleus development at fertilization.

Key Words: **telomere; chromatin; histones; sperm.**

Telomeres are specialized nucleoprotein structures located at the ends of eukaryotic chromosomes. In most organisms they are composed of variable numbers of tandem G-rich repeats (TTAGGG in vertebrates) in the strand that points 5′ → 3′ towards the chromosomal end. They carry a number of diverse functions that are essential for eukaryotic cell maintenance. Telomeres preserve chromosome integrity (1, 2), they conduct chromosome arrangement and proper segregation in meiosis and mitosis (3, 4). In normal somatic cells, telomeres shorten with each cell division due to inability of conventional DNA polymerase machinery to replicate 3′ end of the template DNA. Telomere shortening is thought to serve as mitotic clock and tumour suppressor mechanism.

It has been shown that in mammalian somatic cells majority of the telomere DNA is organized into closely spaced nucleosomes (5, 6). Nothing is known about chromatin structure of sperm telomeres. At the same time, a number of features clearly distinguish sperm and somatic telomeres from each other. First, unlike somatic telomeres, telomeres in sperm do not shorten with age and are preserved for transmission of intact chromosomes over generations. The length of telomeric DNA in human sperm is 10–20 kb while in somatic cells, it is 5–10 kb (7–9). Second, proteins TRF1/2 and Ku, associated with telomeres in somatic cells are not present in human sperm. Instead, novel sperm-specific telomere binding proteins were found (10, 11). Third, telomeres during spermatogenesis migrate towards nuclear membrane and form telomere associations (10, 12–14). This feature of sperm nuclear architecture has been proposed to be important for fertilization and early development (12, 15–17).

In the process of spermatogenesis, dramatic chromatin reorganization takes place concomitant with spatial rearrangement of chromosomes. Testis-specific variants of histones, transitional proteins, and finally protamines replace somatic histones, which results in a highly condensed state of sperm chromatin (18). Importantly, in human sperm, unlike sperm of other mammals studied, a small portion of residual core histones (10–15%) remains associated with the chromatin (19–21). Linker histone H1 was not detected (21, 22) and nucleosomes were found to be closely spaced with a periodicity of about 150 kb (23). A suggestion has been made that the residual histones in human sperm may mark genes for early expression in the embryo (24, 25). However, no specified DNA sequence has been viewed as a nucleosomal ladder.

In this study, we have analyzed human sperm telomeric chromatin using micrococcal nuclease digestion and demonstrated that at least a part of telomeric DNA is organized in nucleosomes. Less compact structural

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state of the nucleohistone chromatin together with the close proximity of telomeres to the sperm nuclear surface can be related to specific functions of telomeres in spermatozoa. We suggest that telomeres can be the chromosomal domains that participate in initiation of sperm nucleus reorganization after fertilization.

MATERIALS AND METHODS

Preparation of sperm nuclei, nuclease digestion and chromatin fractionation. Human semen was obtained from healthy donors and kept frozen until used. To prepare crude nuclei, the semen was thawed, filtered through cheesecloth, centrifuged for 10 min at 3500 rpm; pelleted sperm cells were washed twice with PBS-1 mM PMSF. To lyse the cells, they were incubated on ice for 10 min in the above buffer supplemented with 0.5% Triton X-100. The nuclei were pelleted by centrifugation at 2000 rpm for 10 min, washed two times with PBS-1 mM PMSF, suspended at a concentration of 3–5 mg of DNA per ml in PBS-1 mM PMSF supplemented with 10 mM DTT, and incubated at 37°C for 30 min. Following incubation, $CaCl_2$ and micrococcal nuclease (Worthington) to a concentration of 0.6 mM and 30 U per 1 mg of DNA, correspondingly, were added, and digestion was performed at 37°C for time indicated. Digestion was stopped by adding EDTA to a concentration of 5 mM. The digested nuclei were centrifuged at 10,000 rpm for 3 min to separate released chromatin fragments (S fraction) from the pelleted fraction (P).

Analysis of micrococcal nuclease digestion products. To prepare DNA for separation on agarose gel, SDS and proteinase K were added to each chromatin fraction and DNA was isolated by phenol-chloroform extraction followed by ethanol precipitation. Purified DNA was resolved in 2% agarose gel in TAE buffer. Alternatively, 6× DNA loading buffer containing 6% SDS was added to the chromatin which was loaded directly onto the gel; in this case, TAE buffer was made 0.1% in SDS. Our previous data showed that the DNA electrophoretic pattern did not change with the method of sample preparation. After electrophoresis, DNA was stained with ethidium bromide and gels were photographed using UV transilluminator. When SDS was present during electrophoresis, it was washed out with 50% ethanol before staining with ethidium bromide. The gels were incubated in 0.25 N HCl for 15 min, soaked in transfer buffer (0.4 N NaOH, 0.6 N NaCl) and DNA was blotted onto nylon membrane Hybond N+ (Amersham) by capillary transfer.

DNA probes and antibodies. Plasmids pTH12 and pTH2Δ with inserts corresponding to human telomere DNA and sequence of human subtelomere subset respectively, were kindly provided by Dr. T. de Lange (Rockefeller University). Microdissected DNA corresponding to the region q22B of chromosome 6 was kindly provided by Dr. M. Bittner (NIH). Rabbit polyclonal antibodies against purified calf thymus histone fractions were a gift from Dr. E. Bers (St. Petersburg University).

Southern hybridization, estimation of percentage of telomeric DNA released by micrococcal nuclease. DNA inserts were cut out, gel-purified and labeled with [α-^{32}P] as described in (9). Hybridization was performed according to Church and Gilbert (26) in 7% SDS, 0.5 M sodium phosphate buffer (pH 7.2), 1 mM EDTA at 65°C for 8 h. After hybridization, membranes were washed four times for 20 min at 65°C in 1× SSPE-0.5% SDS. Hybridization signals on Southern blots were quantified on phosphoimager (GS-525 Molecular Imager system, Bio-Rad). The integral intensities of the signals on electrophoretic lanes were determined using Molecular Analyst (Bio-Rad) software.

Determination of nucleosomal DNA repeat length. Values of nucleosomal DNA repeat lengths were obtained from the slope of the regression line constructed as a plot of fragment size versus band number. DNA fragments in multiples of 123 bp (Gibco BRL) were used as a standard.

Protein analysis. Two polyacrylamide gel electrophoretic systems were used. Histones were analyzed in the presence of SDS (27), protamines (insoluble in SDS) were separated in acetic acid-urea (AU) system (28) with a modified preparation of the samples. To minimize the loss of the material, proteins were not extracted from chromatin; instead, electrophoretic samples were prepared by adding an equal volume of the corresponding sample buffer to the whole nuclei or chromatin fractions. For SDS electrophoresis, the samples were boiled for 3 min in the standard Laemmli sample buffer to denature DNA. For AU gels, the sample buffer consisted of 0.9 N acetic acid, 8 M urea, supplemented with 2% CTAB (cetyl trimethyl ammonium bromide, a cationic detergent that precipitates DNA from the nucleoprotein complex) was added.

RESULTS AND DISCUSSION

Nucleosomal Organization of Human Sperm Telomeric Chromatin Released by Micrococcal Nuclease

Human sperm nuclei were treated with micrococcal nuclease (MNase) for various length of time. Chromatin fraction released by the digestion was separated from the rest of the nucleus by centrifugation. DNA from the soluble chromatin in supernatant (S fraction) and from the chromatin remaining in the pellet (P) fraction was fractionated on agarose gel, stained with ethidium bromide, followed by transfer to a nylon membrane. Micrococcal digest of HeLa nuclei was taken as a reference. Detection of telomeric DNA on the membrane was performed using labeled telomeric sequence as a probe. Figure 1 shows MNase products at four successive steps of the digestion. Telomeric DNA is present in both chromatin fractions, S and P (Fig. 1B). The major portion of the telomeric DNA remains in the pellet and appears on the membrane as a smear. As estimated by quantification of hybridization signals on the blot (see Materials and Methods), about 5–8% of total telomeric DNA is released by micrococcal nuclease. At the very early stage of the digestion released telomeric DNA appears on the blot as a smear that has the strongest intensity in the region of a mononucleosome and extends up to a fragment size corresponding to a 10-mer of HeLa nucleosomes. The bands are almost indiscernible due to the strong background. As the digestion proceeds, the background diminishes and electrophoretic pattern of the telomeric DNA acquires a character of nucleosomal ladder with 5-mer being the longest MNase product in this experiment. The amount of released chromatin did not increase with increase of digestion time, as judged by Southern hybridization. Instead, when digestion was prolonged beyond 30–40 min, only mononucleosomal DNA band, which was becoming weaker with digestion time, could be detected on a blot (data not shown). The general character of digestion kinetics was essentially the same in different experiments. However, the tim-

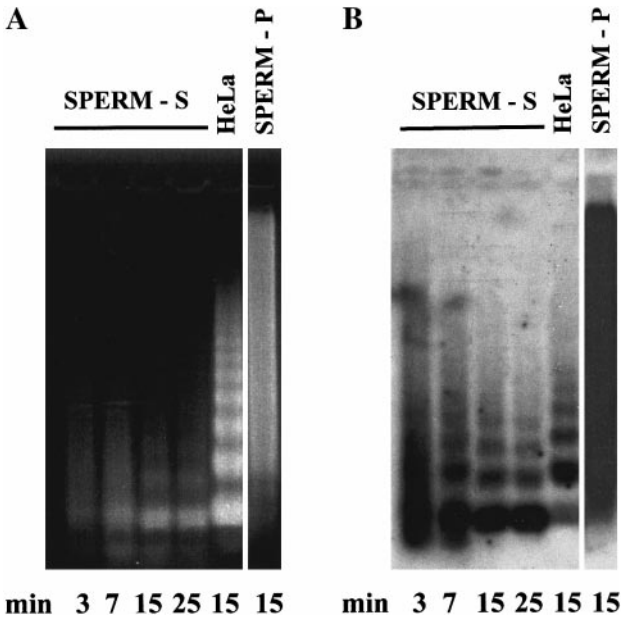

FIG. 1. Time-course of human sperm nuclei digestion with micrococcal nuclease. (A) Bulk DNA separated on 2% agarose gel and stained with ethidium bromide. (B) Southern hybridization. DNA from the gel shown in (A) was transferred onto a membrane and hybridized with ^{32}P-labeled telomeric DNA. S and P, DNA from the supernatant and pellet fractions, correspondingly. DNA pattern of P fraction did not noticeably change with time of digestion. HeLa DNA is taken as a reference.

ing and extent of cleavage varied, presumably due to variation in response of different sperm samples to the treatment with reducing agents. The latter was necessary to make sperm chromatin accessible to MNase. In different experiments, the size of the largest telomeric DNA nucleosomal fragments observed on a blot varied from a 3–4 mer to an octamer.

Nucleosomal repeat length of 148 ± 6 bp was estimated for sperm telomeric DNA using 123 bp repeat as a standard. In HeLa cells, nucleosomal repeat of telomeric DNA was estimated as 155 ± 5 bp (Fig. 1B, right lane), which is in a good correlation with data of other authors (5, 6). Nucleosomal repeat of telomeric DNA in most cells examined so far is significantly smaller than that of the bulk DNA (5, 6); compare also mobility of bulk and telomeric HeLa DNA in Figs. 1A and 1B. In contrast, in human sperm, bands of the bulk DNA nucleosomal ladder revealed by ethidium bromide coincide in their position with those detected by the telomeric probe on the Southern blot. At the same time, it can be seen, that on the blot the ladder is much more pronounced, indicating that the released nucleosomes contain significant amount of telomeric DNA. This finding was corroborated by comparing intensities of the electrophoretic bands using HeLa nucleosomal DNA as a reference. The amount of human sperm DNA from the chromatin released by micrococcal nuclease applied on the gel is much (more than an order) less than that from HeLa, as can be seen from ethidium bromide staining (Fig. 1A). At the same time, the hybridization signal is of the similar intensity suggesting that in human sperm, the soluble chromatin fraction is enriched with telomeric DNA.

It has been shown earlier that in HeLa cells, especially in those with short telomeres, subtelomeric chromatin can contribute to a nucleosomal ladder pattern observed with telomeric probe (6). Although telomeres are elongated in human sperm (7, 9), subtelomeric contribution cannot be excluded. Therefore, subtelomeric chromatin was examined using pTH2Δ probe that corresponds to a subtelomeric repeat which is at least 4 kb long and located directly adjacent to the telomeric repeat region at several chromosome ends in human cells. The same membrane was successively probed with subtelomere-specific sequence and then with the telomeric probe (Figs. 2A and 2B). Subtelomeric probe revealed some periodicity in the DNA from the S fraction (Fig. 2A), however, somewhat less pronounced than when using telomeric probe (Fig. 2B). In the beginning, at the stage of digestion, when telomeric DNA reveals nucleosomal character, subtelomeric DNA appears as a smear. Therefore, it is likely that only nonperiodically cut (if any) sub-telomeric DNA could contribute to telomeric DNA electrophoretic pattern. Although this has not been quantified, rough estimation, made using HeLa DNA as a reference, indicates that telomeric DNA is more abundant in the released chromatin than adjacent to it subtelomeric sequences.

Earlier we have shown, that in human sperm, chromosomes are arranged in a very specific manner with their centromeres buried clustered inside the nucleus, and telomeres located close and possibly anchored to the nuclear membrane (10, 12). The proximity of the telomeres to the nuclear surface, presumably being related to their specialized function(s) in sperm (not yet fully determined), can be reflected in the specific structural features of the telomeric chromatin revealed by MNase. If the suggestion is true, response to MNase treatment in the chromatin located at a distance from the chromosomal ends would be different.

To examine this possibility, we used microdissected DNA probe q622B corresponding to the region of the chromosome 6 located closer to its centromeric part. Only very weak hybridization was detected in the S fraction released from sperm while HeLa digest demonstrated a strong signal with pronounced nucleosomal pattern (Fig. 2C).

Analysis of three chromosomal regions using MNase showed that telomeric DNA prevailed in the chromatin fraction released by the digestion and that telomeric chromatin in the released fraction had the most distinct (among three examined) periodic organization. The major portion of the telomeric chromatin, although accessible to MNase (as evidenced by the extended

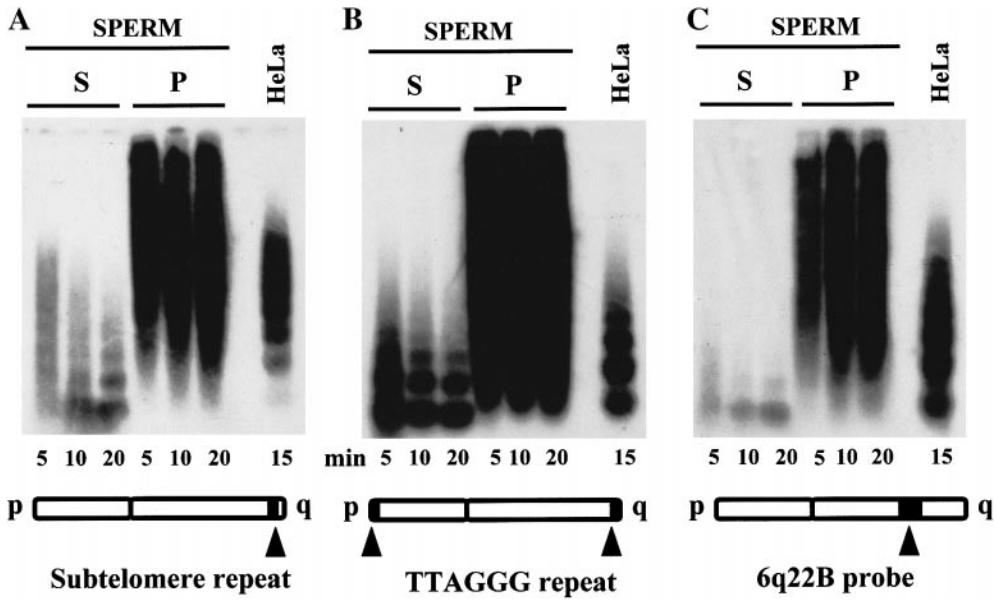

FIG. 2. Micrococcal nuclease digests of three chromosomal domains. Southern hybridization. (A) Subtelomeric probe; (B) The same membrane was stripped and hybridized with the telomeric probe; (C) Identical blot was hybridized with 6q22B microdissected probe. Schematic presentation of the chromosomal areas corresponding to the origin of the hybridization probes is shown below each panel. Lane designations are as in Fig. 1.

smear pattern of its DNA), remained unreleased. The diffuse electrophoretic pattern might suggest that this chromatin does not contain nucleosomes. However, analysis of the nuclear proteins distribution between the chromatin fractions presented below evidences that other possibilities may as well be considered.

Distribution of Protamines and Histones between Human Sperm Chromatin Fractions

The major protein component (85–90%) of the human sperm chromatin is protamine (18, 20). Figure 3 shows that protamines are present in the P fraction, but they are completely absent from the S fraction, which can account for differential solubility of the fractions. While analyzing histones from human sperm, we observed that major electrophoretic bands in the histone area on a gel do not always coincide with the bands detected by anti-histone antibodies. Presence of other major proteins in the histone electrophoretic area has been demonstrated before (29). Therefore, to ensure reliable histone detection in the human sperm chromatin, we used anti-histone antibodies. Figure 4 shows that histones H2A, H2B, H3, and H4 are associated with the both, released and retained chromatin fractions. Antibodies against H1 histones from a number of sources did not react with human sperm proteins (data not shown). Presence of the histones was expected in the MNase released fraction since it contained nucleosomal DNA. Occurrence of histones in the chromatin remained in the pellet after MNase digestion indicates that P chromatin may contain nucleosomes. However, the periodical structure can be concealed by the presence of protamines.

Telomeres in human sperm are arranged in a specific manner: they are clustered and reside at the nuclear periphery (10, 12, 14). Data presented here demonstrates another feature that distinguishes telomeres from the rest of the sperm chromatin. While the most of the sperm genome is packed into extremely dense

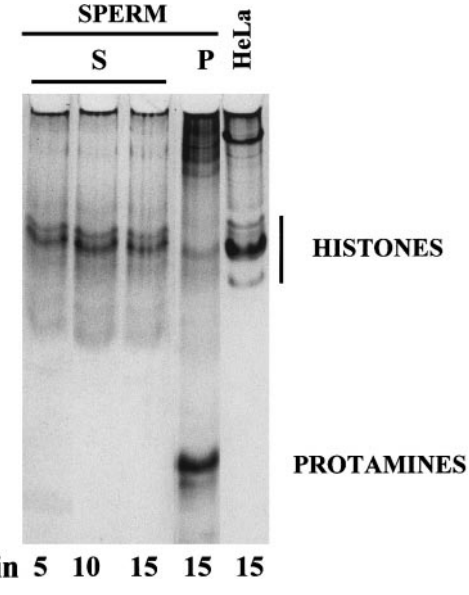

FIG. 3. Protamines are present in the pellet, but not in the supernatant fraction. Acetic acid-Urea-CTAB electrophoresis, Coomassie staining.

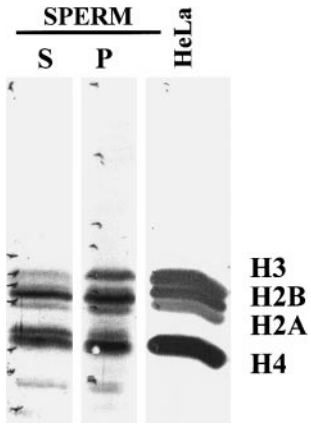

FIG. 4. Histones are present in both, supernatant and pellet fractions. Western blot of the proteins separated on 15% SDS–PAGE. Histones were detected using a mixture of antibodies against individual fractions (H3, H4, H2A, and H2B).

nucleoprotamine structure, at least a part of the telomeric DNA is organized into nucleosomes and can be released by MNase. The specific telomere organization is likely to be functional. What this specific organization could bring to a chromosomal domain? (i) Sperm nuclear periphery is the area that is most accessible to the egg's environment at fertilization. (ii) Nucleosomal organization may be advantageous for the chromosomal domain whose function is required at the initial stage of the male pronucleus development since the long and complex process of protamine removal is not needed here. We suggest that telomeres might be among the first structures in the sperm nucleus that respond to oocyte signals and start male pronucleus development at fertilization.

Although there is no data about telomere behaviour at fertilization, in our attempt to get insight into it, we find the following information could be taken into consideration: (i) Telomere–microtubule relationship (direct or mediated by nuclear envelope) has been shown or suggested in some cells (30–32). (ii) Telomere-led nuclear movement mediated by astral microtubules has been demonstrated in fission yeast (31–35). (iii) Male pronucleus development starts with sperm chromatin decondensation and microtubule-guided movement towards female pronucleus (36). We speculate that at fertilization, male pronucleus development and its microtubule-guided movement may be led by the telomeres.

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