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Structure of the rRNA Genes in the Hamster Sperm Nucleus

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ABSTRACT: We have examined the structure of the major ribosomal RNA (rRNA) genes in the hamster sperm nucleus, using fluorescent *in situ* hybridization (FISH). The rRNA genes are present as tandemly repeated clusters located at the telomeric ends of the short arms of five pairs of acrocentric chromosomes in the Syrian golden hamster (as they are in humans). In somatic cells, these five chromosome pairs come together to form the nucleolus, the site of rRNA synthesis. The nucleolus remains intact through S phase of the cell cycle, breaking apart only during late G_2 and mitosis when the chromosomes condense. Mammalian sperm nuclei are the final products of meiotic division and morphological differentiation that includes a dramatic chromatin condensation. Consequently, it was not immediately obvious whether the rRNA genes would be condensed into a nucleolus-like structure in the mature spermatozoa, or separated, as they are in mitotic chromosomes. We found that of 117 sperm nuclei examined, 91.5% contained between two and five FISH signals for the rRNA gene clusters, and 64.0% contained four (29%) or five (35%) signals. In decondensed hamster sperm nuclei, the rRNA hybridized signals were separated into independent strands. These data collectively indicate that the chromosomes containing the rRNA genes are not bound together into a pre-nucleolar structure in fully condensed mammalian sperm nuclei.

Key words: Nuclear matrix, nucleolus, DNA structure, DNA loop domains.

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We have been studying the organization of DNA within the hamster sperm nucleus. Previously, we began to define the structural constraints and plasticity of this organization (Ward and Coffey, 1991; de Lara et al, 1993). In this work, we have focused on the three-dimensional organization of the five chromosome pairs that make up the nucleolus in somatic cells. Because the mammalian sperm nucleus is too highly condensed to visualize internal structures by conventional electron microscopy (Lalli and Clermont, 1981), it is not clear whether a nucleolus or a nucleolus-like structure exists in mature spermatozoa. Therefore, we examined this structure in spermatozoa using fluorescent *in situ* hybridization (FISH) to directly visualize the ribosomal RNA (rRNA) genes that are the chromatin components of this structure.

The nucleolus is the largest visible structure within somatic cell nuclei, and it is the site of rRNA synthesis (Bouteille and Hernandez-Verdun, 1979; Thiry and Goessens, 1992). Three of the four major rRNA types in mammals, the 28S, the 18S, and the 5.8S rRNAs, are transcribed from a single gene that is repeated 300–400 times in humans (Henderson et al, 1972). These repeated genes are located on the ends of five different pairs of acrocentric chromosomes in both humans (Tantravahi et al, 1976) and in the Syrian golden hamster (Wahl et al, 1983). During rRNA transcription, the ends of these chromosomes come together to form the nucleolus (Anastassova-Kristeva, 1974; Goessens and Lepoint, 1974). In mitotic chromatin preparations, the rRNA genes can be identified by silver staining, and they have been termed nucleolar organizing regions, or NORs (Fischer et al, 1991). The nucleolus of somatic cells changes structure during the cell cycle. It is a single, prominent sphere that contains all the rRNA genes from late G_1 through G_2, breaking apart into 10 separate NORs (as the chromosomes condense) only during mitosis (Anastassova-Kristeva, 1974; Goessens and Lepoint, 1974).

The structure and activity of the nucleolus have been examined in early spermatogenesis. Transcription of rRNA has been shown to increase markedly between leptotene and zygotene spermatocytes, decreasing again in late pachytene (Tres, 1975). Stahl et al (1991) examined the structures of the nucleoli that form during this time, demonstrating that the most prominent nucleoli form during leptotene. Spermatids contain a unique "padlock" nucleolus (Czaker, 1985; Sousa and Carvalheiro, 1994), and evidence for the post-meiotic reactivation of rRNA transcription has recently been demonstrated in these cells (Schmid et al, 1982; Haaf et al, 1988; Dadoune et al, 1994).

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In this study, we examined the structure of the rRNA genes in mature sperm nuclei. We were particularly interested to determine the structural constraints of the five acrocentric chromosomes that contained the rRNA genes during the tremendous condensation of the haploid genome that accompanies the final stages of spermatogenesis (Lalli and Clermont, 1981). We tested whether these five chromosomes were bound together in a pre-nucleolar state or whether they were packaged separately within the sperm nucleus. Fully condensed sperm nuclei were examined by FISH using the hamster 28S rRNA gene as a probe. We found that the five major rRNA gene clusters in the sperm nucleus were packaged completely independently of each other, suggesting that the formation of a single nucleolus is not necessary for proper sperm DNA packaging.

Materials and Methods

Preparation of Fibroblast Cells and Mitotic Chromosomes

Primary hamster tailskin fibroblasts were obtained by cutting the tail and stripping the skin with dissecting scissors. Both the tail and the skin were incubated with 1 mg/ml of collagenase in α-minimum essential medium (α-MEM) without serum for 1 hour at 37°C, and then incubated in medium with 10% fetal bovine serum in a tissue culture flask until the cells were growing. The cells were then replated onto slide chambers. For examination of nuclei, the slides were fixed as described below. For chromosomes, the cells were treated with 0.0225 μg/ml colcemid for 4 hours when they reached approximately 50% confluence. The medium was then washed out 4 ml per slide of 75 mM KCl was added and incubated at 37°C for 35 minutes, 2 ml of 3:1 methanol:acetic acid was added to each slide chamber, and the slides were incubated for 2 minutes at room temperature. Slides were then fixed in four washes of 3:1 methanol:acetic acid, the first two at 1 hour and the last two for 45 minutes, all at room temperature. The chromosomes were then spread by gently forcing a stream of humidified air onto the slide.

Preparation of Sperm Nuclear Structures

All sperm nuclear structures were prepared and fixed as previously described (de Lara et al, 1993). Briefly, hamster spermatozoa were isolated from Syrian golden hamster caudae epididymides and washed in either 0.25% nonionic detergent NP-40 for decondensed nuclei or 0.5% sodium dodecyl sulfate (SDS) for condensed nuclei and nuclear matrices. These decondensed nuclei and nuclear matrices were prepared by extraction in 2 M NaCl, 25 mM Tris, pH 7.4, and 10 mM dithiothreitol, on ice. Condensed nuclei were extracted with 300 mM $CaCl_2$ and 10 mM dithiothreitol, on ice. All three types of nuclear preparations were then placed onto glass slides, dried overnight, and fixed in 3:1 methanol:acetic acid.

Fluorescent In Situ Hybridization (FISH)

FISH was performed as described previously (de Lara et al, 1993). The Syrian golden hamster 28S rRNA probe was provided by Dr. Geoffry Wahl. This probe was previously used for *in situ* hybridization of hamster chromosomes (Wahl et al, 1983).

Results

rRNA Genes in Fibroblast Nuclei, Mitotic Chromosomes, and Sperm Nuclei

The structure of the 28S rRNA genes in mitotic chromosomes can be seen in Figure 1A–C. The 28S rRNA gene clusters (green signal) can be seen positioned on 10 homologous pairs of chromosomes (Fig. 1C, arrows). Figure 1D demonstrates examples of more extended chromosomes, where the 28S rRNA hybridization signal is visible at the telomeric ends. A large fibroblast nucleus containing a single, large hybridization signal for the 28S rRNA probe, most likely representing a single nucleolus, is shown in Figure 1E and F. Fibroblast nuclei that have been treated with colcemid (see Materials and Methods section) are shown in Figure 1G. Because colcemid treatment blocks cells in mitosis, and because these nuclei did

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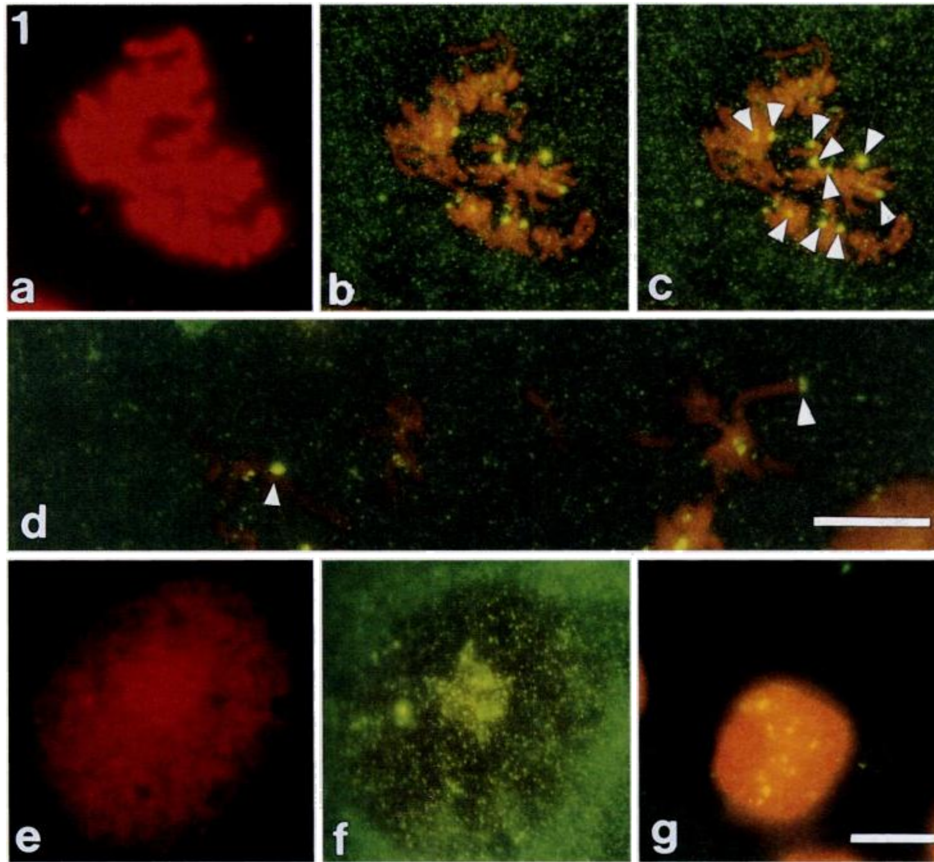
FIG. 1. Distribution of rRNA genes in mitotic chromosomes and fibroblast nuclei. (A–C) Hamster tail fibroblast mitotic chromosomes were hybridized to the 28S rRNA probe. Micrographs of the same preparation were taken with a red filter for total DNA, stained with propidium iodide (A), and with the green filter to visualize the hybridization signal (B and C). The 28S rRNA genes appear as yellow-green dots. The signals that were scored as positive in B are indicated with the arrow in C. (D) An enlarged micrograph of a mitotic preparation that contained extended chromosomes hybridized to the 28S rRNA probe. In two of the chromosomes, the 28S rRNA signal can be seen clearly localized at the telomeric ends of the chromosomes (arrows). (E, F) A fibroblast nucleus shown with propidium iodide staining in red (E), and with the 28S rRNA hybridization in green (F). The 28S rRNA signal is present as a single large nucleolus. (G) A fibroblast nucleus in which the 28S rRNA signals are separated into several dots, indicating the disruption of the nucleolus. Bar = 10 μm; all figures were at the smaller magnification shown in G except D, which is shown at a slightly larger magnification.

FIG. 2. Distribution of the 28S rRNA in hamster sperm nuclei. Fully condensed hamster nuclei were hybridized with the 28S rRNA probe by FISH. A and C show two different micrographs, and B and D show the same micrographs, with the signals that were scored as positive marked with arrows. Bar = 10 μm.

FIG. 4. rRNA genes in decondensed hamster sperm nuclei and in sperm nuclear matrices. (A, B) Micrographs of the same decondensed nucleus taken with the red (total DNA) and green (28S rRNA genes) filter. The rRNA genes are separate strands positioned at different points of the decondensed nuclei. The nuclear annulus is the bright red structure in B. (C) A second example of a decondensed sperm nucleus hybridized to the 28S rRNA gene. (D) Hamster sperm nuclear matrices hybridized to the 28S rRNA probe. Each green signal represents a single 28S rRNA loop domain emanating from the nuclear matrix. Note that the signals are bound to different areas of the sperm nuclear matrix, further supporting the conclusion that the five gene clusters are separate. Bar = 10 μm.



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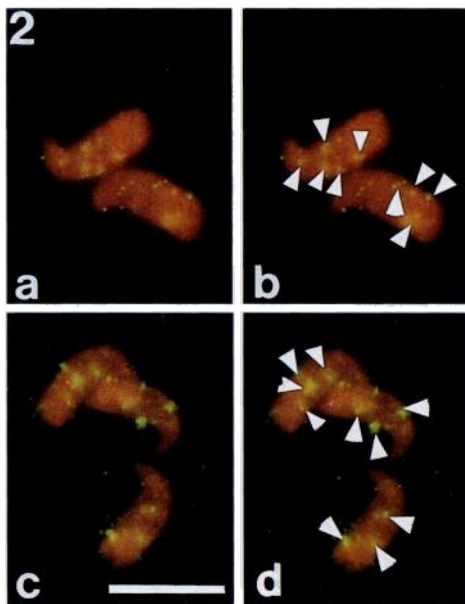

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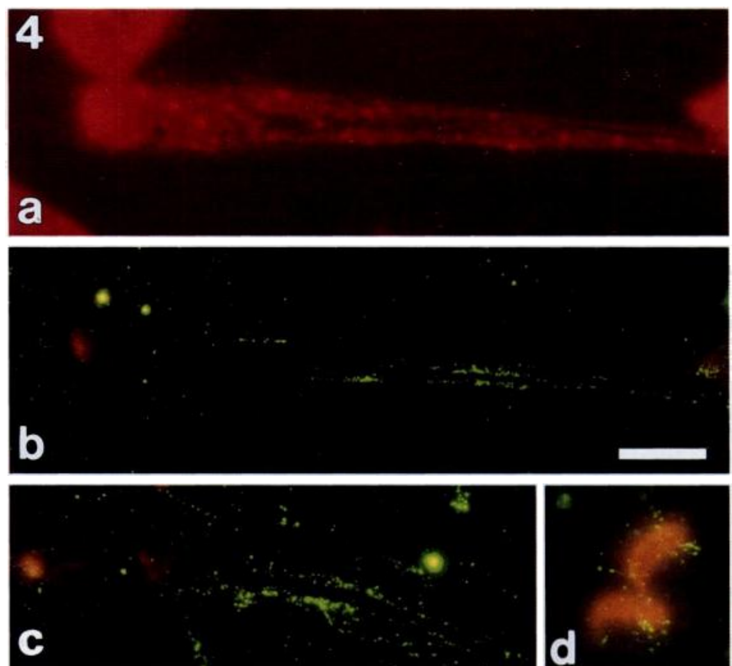

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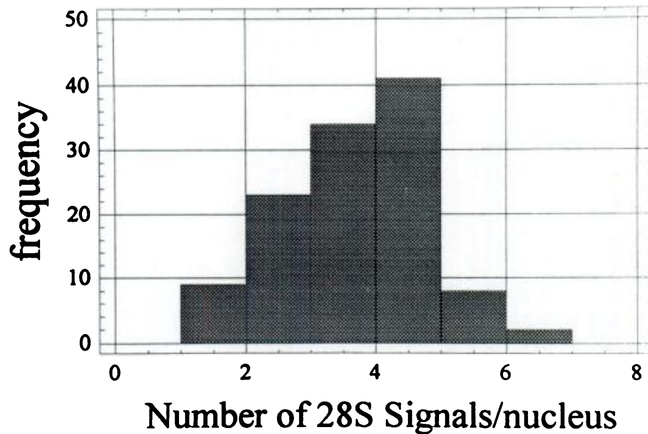

FIG. 3. Number of 28S rRNA FISH signals in hamster sperm nuclei. The number of 28S rRNA hybridization signals (such as those shown in Fig. 1F and G) were counted in 117 sperm nuclei and plotted as a histogram.

not progress to chromatin condensation, it is likely that they were in stage G_2 of the cell cycle. The 28S rRNA signals in these nuclei were separated into individual spots, similar to the structure expected for the nucleolus that occurs just before mitosis.

The 28S rRNA gene clusters in hamster sperm nuclei appeared to have distribution patterns similar to those of fibroblast nuclei in late G_2 (Fig. 2). In most of the sperm nuclei illustrated, five clear hybridization signals were evident (Fig. 2B and D, arrows). Five signals, rather than 10, were seen because sperm nuclei are haploid and contain only half the number of rRNA gene clusters. Because of the flat, asymmetric shape of hamster sperm nuclei, we were able to compare the patterns of the 28S rRNA signals from one nucleus to the next using the various aspects of the nuclear structure as independent reference points. We found that no clear pattern of the different signals could be elucidated (e.g., compare the 28S rRNA signals in two nuclei shown in Fig. 2A). We quantitated the number of 28S rRNA signals in 117 nuclei (Fig. 3). None of the sperm nuclei examined had only one 28S rRNA signal, and 83.8% had three to five signals. The number of nuclei containing more than five signals was low (8.5%).

rRNA Gene Structure in Decondensed Nuclei and Sperm Nuclear Matrices

To confirm that the five 28S rRNA gene clusters were separated in sperm nuclei we examined their distribution in two types of sperm nuclear preparations. First, we partially decondensed the sperm nuclei using NP-40, followed by a reducing reagent and a high salt concentration to remove the protamines (as described in the Methods). All of the structural components of the sperm nuclei dissipated during these extractions, except for the nuclear annulus (Ward and Coffey, 1989). In the two nuclei shown in Figure 4B and C, the 28S rRNA genes were visible as independent, linear signals within the decondensing nucleus. This suggests that they were not clustered together when the nuclei were condensed. Furthermore, the rRNA genes were located at positions far removed from the annulus, suggesting that the rRNA genes are not bound to the nuclear annulus.

We next examined the 28S rRNA genes in nuclear matrix–halo preparations. In these nuclear extractions, the protamines were also removed but the structural component of the sperm nucleus, the nuclear matrix, remained intact (Ward et al, 1989). The DNA remained associated with the nuclear matrix as loop domains, evidenced by a fluorescent halo surrounding the nuclear matrix (Ward et al, 1989; de Lara et al, 1993). Individual rRNA gene loop domains were visualized by hybridizing the 28S rRNA probe to such nuclear matrix preparations (Fig. 4D). The loop domains emanated from different sites along the sperm nuclear matrix, once again suggesting that the 28S rRNA genes were positioned at different points within the sperm nucleus.

Discussion

The nucleolus is the largest and most conspicuous structure in eukaryotic cell nuclei, and its function and structural components have been well characterized (Schwarzacher and Wachtler, 1991). The most interesting aspect of the nucleolus in this study is that it is formed by the coalescing of the telemeric ends of five pairs of chromosomes that contain the rRNA genes. Because the function of the nucleolus is the active transcription of rRNA, one might not expect any type of nucleolar structure to be present in sperm nuclei (Stewart et al, 1984). However, the lack of nucleoli in sperm cells has not been confirmed to our knowledge, and there were at least two possible explanations that might explain the existence of a nucleolar-like structure in spermatozoa. First, the association of the five chromosomal ends that contain the rRNA genes might have contributed to sperm DNA condensation. Second, the rRNA genes might have formed a pre-nucleolar complex to aid the formation of the nucleolus in the paternal nucleus (Chartrain et al, 1987).

The data presented in this work suggest that a nucleolus or pre-nucleolar structure that contains all five rRNA gene clusters does not exist in mature sperm nuclei. In most of the nuclei examined (64%), at least four and sometimes five distinct FISH signals could be seen that were separated from each other. This was further confirmed by examining the FISH signals for the 28S rRNA in fully decondensed nuclei and in nuclear matrix preparations. In the decondensed nuclei, the rRNA hybridization signals were clearly separated from each other, unfolding

into individual strands in different areas of the nucleus. In the nuclear matrix preparations, the hook-shaped structure of the sperm nucleus was preserved even though the protamines were extracted (Ward et al, 1989). Here, all of the sperm DNA was present as loop domains attached at their bases to the nuclear matrix. The 28S rRNA gene clusters were more fully exposed and, therefore, more fully hybridized, and they could be seen emanating from different areas of the sperm nuclear matrix. This means that the loop domain attachment sites were located in different areas of the nucleus, again indicating that the rRNA genes were not organized together in one area. Taken together, these data indicate that the five 28S containing rRNA gene clusters are separated in the hamster sperm nucleus, and they are therefore not organized into a nucleolus or nucleolus-like structure.

The pattern of 28S rRNA FISH signals is compatible with the observed behavior of nucleoli in spermatids, which are the haploid products of the last meiotic division and precursor to the spermatozoon. The round spermatid contains a distinct nucleolus, indicating that some, if not all, of these chromosomal ends are clustered together in a structural unit (Czaker, 1985; Sousa and Carvalheiro, 1994). Later stages of the spermatid do not exhibit a predominant nucleolus, suggesting that the structure has begun to segregate into separate chromosomes (Mirre and Knibiehler, 1985).

These *in situ* hybridization data do not, however, address the question of the presence of NORs in mature spermatozoa. NORs are the regions in the mitotic chromosomes that contain the rRNA gene clusters that are distinguished by their ability to bind Ag^{2+}, in a manner similar to nucleoli in interphase nuclei (Olson, 1990). It is thought that NORs contain a specific protein composition that is involved in the formation of nucleoli. In support of this, proteins, termed Ag-NOR proteins, have recently been identified that are present in nucleoli thought to be responsible for this Ag^{2+} binding (Roussel and Hernandez-Verdun, 1994). In most somatic cells, small nucleoli form around these NORs after mitosis and eventually fuse into one or two large nucleoli (Anastassova-Kristeva, 1974). It is not possible to determine from our data whether Ag-NOR or NOR-like structures exist in the mature sperm nuclei. However, we can conclude that the individual gene clusters are separated and have not fused to form a single nucleolus.

These data have two implications for packaging chromatin within mammalian sperm nuclei. The first is that the areas of the five chromosomes that contain the 28S rRNA gene clusters are not restricted to be positioned in the same location in the fully condensed sperm nucleus as they are in the nucleoli of somatic cell nuclei. As discussed above, this is probably related to the absence of rRNA transcription in sperm nuclei. The second implication is deduced from the absence of any particular pattern for the five 28S rRNA signals in the sperm nuclei. The hamster sperm nucleus is flat and asymmetrically shaped, so the position of FISH signals can be easily compared in different nuclei. We found no obvious similarities in the patterns of 28S rRNA signals in these experiments. It is possible that such patterns were distorted by the extraction and fixation procedures used to expose the sperm DNA for *in situ* hybridization, but this is unlikely because the nuclei retain the characteristic shape of the nucleus. Thus, the packaging of these five chromosomes within the hamster sperm nucleus seems to be relatively free of three-dimensional constraints that force them into defined positions within the sperm nucleus.

This conclusion does not suggest, however, that some structural constraints do exist that have not yet been identified or that are involved with other parts of the genome (Zalensky et al, 1995). Nor do these results conflict with previous data that suggest that individual loop domains are attached by specific sequences to the sperm nuclear matrix (Kalandadze et al, 1990; Ward and Coffey, 1990). But the data do indicate that there is a degree of plasticity in the positioning of chromosomes within the sperm nucleus. Similar data have been accumulated for the position of genes within somatic cell nuclei (Lawrence et al, 1993). This plasticity may seem surprising given the specificity of the morphological changes of the nucleus that accompany sperm differentiation. However, data from our laboratory and from others suggest that sperm chromatin organization is complex, with structural constraints at the level of protamine binding (Gatewood et al, 1987) and the attachment of DNA to the nuclear matrix (Kalandadze et al, 1990; Ward and Coffey, 1990). But the work presented here and other data (work in progress) suggest that there is more freedom at the level of the positioning of chromosomes within the sperm nucleus.

It is not yet clear what effects these different aspects of mammalian sperm chromatin structure have on function. But a model for sperm DNA organization is slowly emerging that may eventually be able to connect the high degree of sperm DNA packaging with its varied roles in fertility and embryogenesis.

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