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Localization of the 16S mitochondrial rRNA in the nucleus of mammalian spermatogenic cells

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Amplification of RNA from human sperm heads yielded a fragment of 435 bp that shares 100% identity with a central region of the 16S mitochondrial rRNA. The nuclear localization in the sperm of the mitochondrial RNA was confirmed by in-situ hybridization. These results, together with the localization of the 16S mitochondrial rRNA in mouse sperm, are the first demonstration that the organelle transcript is a normal component of the mammalian gamete. The possibility that the nuclear mitochondrial RNA arises from nuclear transcription of a mitochondrial pseudogene was ruled out. To determine when during spermatogenesis the mitochondrial RNA is localized in the nucleus, in-situ hybridization of mouse and human testis was carried out. The nuclei of spermatogonia, spermatocytes and round and elongated spermatids were all positively stained. In human spermatocytes, the nuclear staining pattern was fibrillar, suggesting an association of the mitochondrial transcript with the meiotic chromosomes. These results indicate that early in spermatogenesis and before the onset of meiosis, the 16S mitochondrial rRNA is localized in the nucleus of spermatogenic cells, suggesting a process of translocation of the transcript from the mitochondria.

Key words: human/mitochondrial RNA/mouse/sperm/spermatogenesis

Introduction

The presence of RNA in the nucleus of mammalian gametes has been confirmed by different approaches. Using the immunogold procedure for electron microscopy, the nuclear localization of RNA in the mature sperm of rat and human has been demonstrated (Pessot *et al*., 1989). Later, we reported that U1 and U2 small nuclear RNA, transcripts involved in pre-mRNA splicing, were confined to the rat sperm nucleus (Concha *et al*., 1993). The first mRNA identified in the nucleus of human sperm corresponded to the proto-oncogene c-myc (Kumar *et al*., 1993). Since then, several mRNA have been identified in the nucleus of mature sperm of rodents and humans (Chiang *et al*., 1994; Miller *et al*., 1994; Rohwedder *et al*., 1996; Kramer and Krawetz, 1997; Miller, 1997). Moreover, the presence of RNA in the nucleus of the male gamete seems to be of universal occurrence. Consistent with this, the mature pollen grain contains a store of presynthesized mRNA (Mascarenhas, 1993), and we have reported the presence of U1 small nuclear RNP in the generative and vegetative nuclei of the pollen grains of *Pinus radiata* and *Viburnum tinus* (Concha *et al*., 1995).

To characterize other RNA enriched in sperm, we used a differential screening strategy of a mouse testis cDNA library. Thus, we identified a transcript, referred to as chimeric mitochondrial RNA, containing an inverted repeat of 121 nucleotides covalently bound to the 5′ end of the 16S mitochondrial rRNA (Villegas *et al*., 2000, 2002). Most interesting was the nuclear localization of this novel RNA in mouse sperm (Villegas *et al*., 2000). To determine whether the unusual nuclear localization of this mitochondrial transcript is a peculiarity of the mouse sperm or if it also occurs in the sperm of other species, we investigated the localization of the 16S mitochondrial rRNA in the human gamete. Amplification by RT–PCR of RNA from isolated human sperm heads and in-situ hybridization confirmed the nuclear localization of the mitochondrial transcript. The possibility that the 16S mitochondrial rRNA found in the nucleus is the result of nuclear transcription of a mitochondrial pseudogene (Hirano *et al*., 1997; Wallace *et al*., 1997; Kindmark *et al*., 2001) was ruled out. These results suggest that the mitochondrial RNA is translocated from the organelle to the sperm nucleus. Thus, we also investigated at which stage of spermatogenesis this transfer process takes place. In-situ hybridization of human and mouse testis revealed that the 16S mitochondrial rRNA is localized in the nucleus of several types of spermatogenic cells, including spermatogonia, spermatocytes and round and elongated spermatids. Altogether, these results strongly suggest that the 16S mitochondrial rRNA is transferred from the organelle to the nucleus of spermatogenic cells by an unknown mechanism of RNA translocation and that this event occurs early in spermatogenesis prior to the meiotic prophase.

Materials and methods

Human sperm samples

Permission to use human semen samples from healthy donors and adult human testis biopsies was granted by the board of The Ethical and Biosafety

Committee of Bios Chile I.G.S.A., and Fundación Ciencia para la Vida. The semen samples were allowed to liquefy for 1 h at room temperature, and then centrifuged at 600 *g* (Kubota 7930, rotor RS-200G) for 10 min at 4°C. The sediment was resuspended in phosphate-buffered saline (PBS) and centrifuged as described previously. The sperm were again resuspended in 0.5×PBS and centrifuged again at 600 *g* for 10 min and resuspended in PBS. The final suspension of human sperm was checked by phase contrast microscopy.

Isolation of sperm heads

Rat and mouse sperm were isolated as described previously (Vera *et al*., 1984; Villegas *et al*., 2000, 2002). To isolate sperm heads, the sediment containing the sperm was resuspended in 500 µl of digestion buffer (10 mmol/l Tris–HCl, pH 8.0, 10 mmol/l EDTA, 50 mmol/l NaCl and 2% SDS) after which 6 µl of 20 µg/ml of proteinase K were added. The mixture was incubated for 10–15 min at 50°C (von Beroldingen *et al*., 1989), diluted with 10 volumes of PBS and centrifuged at 3000 *g* for 10 min (Kubota 7930, rotor RS-200G). The heads were washed twice with PBS and the final preparation was monitored under phase microscopy and used immediately for extraction of RNA.

RNA extraction

RNA extraction was performed as described (Chomczynszki and Sacchi, 1987; Villegas *et al*., 2000, 2002). Sperm or purified sperm heads were homogenized in 5 ml of a solution containing 4 mol/l guanidinium isothiocyanate, 1% Sarkosyl, 0.1 mol/l 2-mercaptoethanol and 25 mmol/l sodium citrate adjusted to pH 7.5. The mixture was incubated for 15 min at 37°C, after which one volume of water-saturated phenol (pH 5.2) and 0.2 volumes of chloroform were added, with vortexing after each addition. The mixture was centrifuged at 5000 *g* for 10 min at room temperature, and the aqueous upper phase was collected and precipitated with one volume of isopropyl alcohol. After 2 h at –20°C, the RNA was recovered by centrifugation at 10 000 *g* for 20 min at 4°C (Villegas *et al*., 2000). Finally, the RNA pellet was washed twice with 70% ethanol and dissolved in sterile water treated with 0.1% diethylpyrocarbonate (Sambrook *et al*., 1989). The RNA concentration was determined spectrophotometrically at 260 nm.

RT–PCR reaction

Synthesis of cDNA was carried out in a final volume of 20 µl containing 50–100 ng of total RNA from whole sperm, sperm heads or human testis (Clontech Labs, USA), 4 µl of 5×First Strand Buffer (250 mmol/l Tris–HCl pH 8.3, 375 mmol/l KCl, 15 mmol/l $MgCl_2$), 2 µl 0.1 mol/l dithiothreitol (DTT), 50 ng of random hexamers and 1 µl 10 mmol/l dNTP. The mixture was incubated for 10 min at 80°C and chilled on ice for 5 min. After a short spin, 40 IU of RNase OUT™ (Gibco, BRL) and 200 IU of M-MLV reverse transcriptase (Gibco, BRL) or 200 IU of SuperScript II (Gibco, BRL) was added to the mixture and incubated for 10 min at 25°C followed by 50 min at 37°C. For PCR, 2–5 µl of the cDNA reaction mixture was mixed with 5 µl of 10×PCR Buffer (200 mmol/l Tris HCl pH 8.4, 500 mmol/l KCl) 1.5 µl 50 mmol/l $MgCl_2$, 1 µl 10 mmol/l dNTP, and 50 pmol of antisense and sense primers. The mixture was denatured for 5 min at 95°C followed by 30 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C. After a final extension at 72°C for 10 min, 10 µl of the amplification reaction mixture was analysed by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized under UV light.

The primers used and their position in the corresponding human genes were as follows. 16S mitochondrial rRNA (Anderson *et al*., 1981): P1: 5′ GTAGGCCTAAAAGCAGCCACCAA (2170–2192); P2: 5′ TGATTATGCTACCTTTGCACGGT (2582–2604); P3: 5′ ACCGTGCAAAGGTAGCATAATCA (2582–2604); P4: 5′ GCTAAACCTAGCCCCAAACC (1671–1689); P5: 5′ AAACCCTGTTCTTG-GGTGGGTG (3208–3229). 12S mitochondrial rRNA (Anderson *et al*., 1981): P6: 5′ CCAAACTGGGATTAGATACCCCA (1064–1087); P7: 5′ TTGCTGAAGATGGCGGTATATA (1257–1276). Pro-opiomelanocortin exon 3: P8: 5′ TACTCCATGGAGCACTTCCGC (521–541); P9: 5′ TCTGGCTCTTCTCGGAGGTCA (828–848) (Takahashi *et al*., 1983). β-globin: P10: 5′ TGGCCAATCTACTCCCAGG (12–30); P11: 5′ GGAAAATAGACCAATAGGCAG (333–353) (Marotta *et al*., 1977).

Isolation of human sperm genomic DNA

To isolate the highly polymerized DNA from human sperm, the mitochondrial sheath of the sperm tail was released by using a treatment employed for the isolation of the fibrous sheath (Olson *et al*., 1976; Brito *et al*., 1989; Brito and Burzio, 1995). The sperm were isolated as described above and resuspended in 5 ml of a solution containing 50 mmol/l Tris–HCl pH 8.0, 1% Triton X-100 and 2 mmol/l dithiothreitol (DTT) and incubated for 12 min at room temperature. After centrifugation at 3000 *g* for 10 min, the sediment was resuspended in 30 ml of 2.2 mol/l sucrose in PBS and centrifuged at 9000 *g* for 30 min at 4°C (Sorvall, rotor HB-6). The sediment, containing the heads, the fibrous sheath and the axonemes (Brito *et al*., 1989; Brito and Burzio, 1995), was resuspended in PBS and centrifuged at 3000 *g* for 10 min. This washing step was repeated twice to eliminate the sucrose. The sediment was resuspended again in the same solution containing 1% Triton X-100 and 2 mmol/l DTT and incubated for an additional 10 min at room temperature. After a second centrifugation step through a 2.2 mol/l sucrose cushion as described above, the final pellet was washed three times with PBS and resuspended in 6 ml of the 4 mol/l guanidinium isothiocyanate solution described above (Chomczynszki and Sacchi, 1987; Villegas *et al*., 2000, 2002). The DNA was extracted using the standard phenol/chloroform procedure (Sambrook *et al*., 1989).

The DNA was dissolved in TE buffer (10 mmol/l Tris–HCl, pH 7.5 and 1 mmol/l EDTA), electrophoresed on a 0.5% agarose gel for 2 h at 60 V and stained with ethidium bromide. The major band of DNA migrating ~1 cm from the well was cut and the genomic DNA recovered using the Concert™ Rapid Gel Extraction System (Gibco, BRL) according to the manufacturer's instructions. The elution step was performed by incubating the spin cartridge for 3 min with 50 µl of TE buffer warmed to 80°C, followed by centrifugation. This step was repeated twice, and the final pool of 150 µl containing the genomic DNA was precipitated with 2.5 volumes of ethanol at –20°C (Sambrook *et al*., 1989). After 3 h at –20°C the DNA was recovered by centrifugation, washed twice with 70% ethanol and resuspended in 50 µl of nuclease-free water.

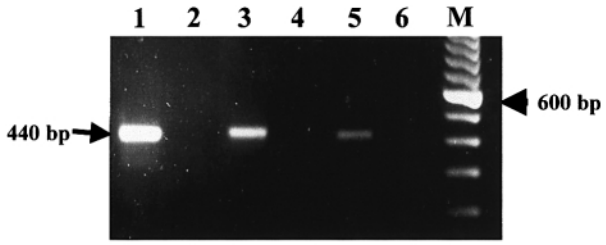

Figure 1. Presence of the 16S mitochondrial rRNA in human sperm. Total RNA isolated from heads of mouse (lane 1) or rat sperm (lane 3) or from whole human sperm (lane 5) was amplified by RT–PCR using the primers P1 and P2. The fragment of 440 bp was not amplified when the reaction was performed without reverse transcriptase (lanes 2, 3 and 6). The position of the 440 bp amplicon is indicated at the left. M: 100 bp ladder.

In-situ hybridization

In-situ hybridization was carried out as described previously (Concha *et al*., 1993; Villegas *et al*., 2000). Smears of human sperm on slides previously coated with a mussel adhesive protein (Burzio *et al*., 1997) were air-dried for 30 min at room temperature. The smears were treated with 15 mmol/l 2-mercaptoethanol in PBS, pH 7.4, for 10 min at room temperature and fixed in 4% paraformaldehyde for 10 min. The slides were washed three times with PBS for 10 min and incubated in 2×SSC (2×SSC: 0.3 mol/l NaCl, 30 mmol/l sodium citrate, pH 7.0) for 10 min at room temperature. Prehybridization was performed for 30 min at 37°C in a mixture containing 4×SSC, 10% dextran sulphate, 100 µg/ml yeast tRNA and herring sperm DNA, 50% formamide and 1×Denhardt's solution (0.2 mg/ml Ficoll type 400, 0.2 mg/ml polyvinylpirrolidone, 0.2 mg/ml BSA). Hybridization was performed for 12 h at 37°C with 100 µl/slide of the prehybridization mixture containing 3.5 pmol of antisense or sense probes previously labelled at the 3′ end with digoxigenin-11-dUTP (Boehringer Mannheim, Germany) and 17 IU of terminal transferase (Gibco, BRL) as described previously (Concha *et al*., 1993; Villegas *et al*., 2000). Then, the smears were washed first with 2×SSC and with 1×SSC for 10 min at room temperature followed by 0.5×SSC for 30 min at 45°C and finally, with 0.2×SSC for 10 min at room temperature. After the washing steps, the smears were incubated for 30 min in a blocking buffer (1% BSA, 0.3% Triton X-100 in PBS) followed by 2 h at room

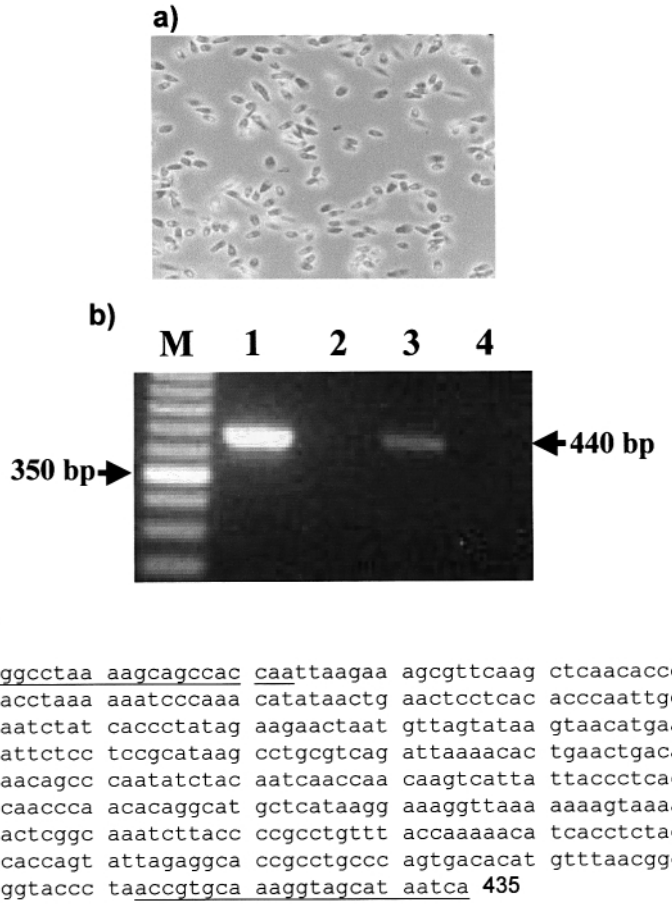

Figure 2. Presence of the 16S mitochondrial rRNA in human sperm heads. The sperm heads were obtained after treatment with proteinase K. (**a**) Phase contrast of the isolated sperm heads (×1000). (**b**) Amplification by RT–PCR using primers P1 and P2 of RNA from human testis (lanes 1 and 2) and from human sperm heads (lanes 3 and 4). M: 50 bp ladder. The amplification was negative when the reaction was carried out without reverse transcriptase (lanes 2 and 4). (**c**) Sequence of the 435 bp amplicon obtained after cloning of the amplification fragment of human sperm head RNA. The underlined sequences correspond to primers P1 and P2.

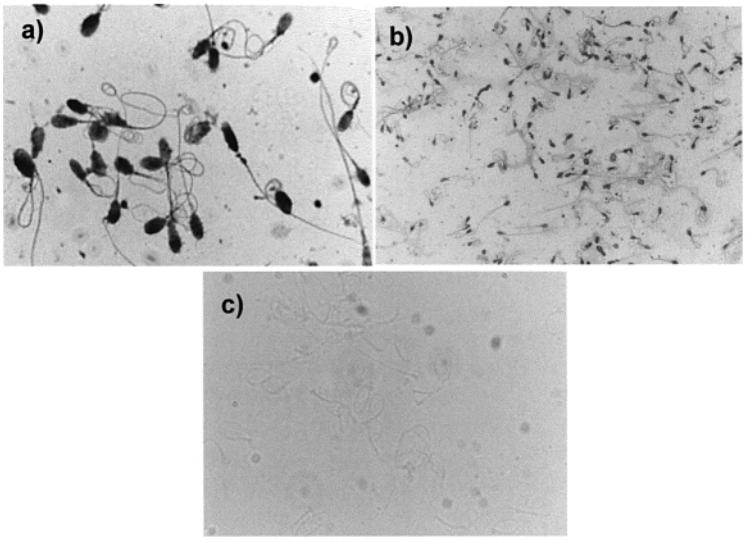

Figure 3. Localization of the 16S mitochondrial rRNA in human sperm. In-situ hybridization was carried out with whole sperm smears using an antisense (**a** and **b**, primer P2) or sense probe (**c**, primer P3), previously labelled with digoxigenin. Original magnifications: (**a**, **c**) ×1000; (**b**) ×200.

temperature with a monoclonal anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim, Germany) and diluted 1:500 in blocking buffer. Finally, the smears were washed twice with PBS and the colour reaction was carried out with 5-bromo-4-chloro-3-indolyl phosphate/Nitroblue Tetrazolium substrate mixture (DAKO, CA, USA) for 30 min at room temperature as previously described (Villegas *et al*., 2000).

For in-situ hybridization of adult human or mouse testis, the tissue samples were fixed in Bouin's and paraffin-embedded. Sections of ~5 μm were fixed

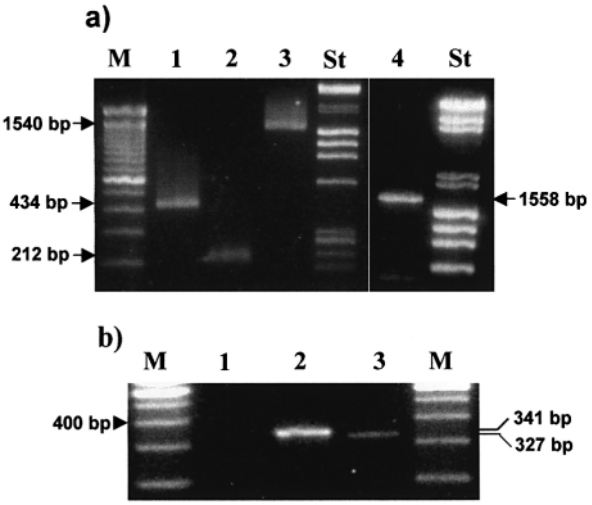

Figure 4. Absence of the sequences of the 16S mitochondrial rRNA in the nuclear genome of the human sperm. (**a**) DNA was prepared from the heads–fibrous sheath fraction and used as template for PCR to amplify the 435 bp fragment of the 16S rRNA (lane 1; primers P1 and P2), a 212 bp fragment corresponding to part of the 12S rRNA (lane 2; primers P6 and P7), an amplicon of 1540 bp corresponding to a segment of the 12S rRNA, the $tRNA^{Val}$ and part of the 16S rRNA genes (lane 3, primers P6 and P2), and an amplicon of 1558 bp corresponding to the entire 16S gene (lane 4, primers P4 and P5). M: 100 bp ladder. Lanes St: λ-DNA-HindIII/ϕX-174 DNA-HaeIII standards. (**b**) Amplification was carried out with the highly polymerized sperm DNA. Lane 1: attempted amplification of the 435 bp fragment of the 16S rRNA gene. Lanes 2 and 3: amplification of a 341 bp fragment of the β-globin gene (primers P10 and P11) and a 327 bp fragment of the pro-opiomelanocortin gene (primers P8 and P9) respectively. M: 100 bp ladder. The size of each fragment is shown at the sides of the gels.

on slides coated with polylysine, and after incubation for 1 h at 60°C the paraffin was removed by four washes of 15 min each with xylol. The sections were air-dried and washed four times with PBS, incubated in 0.2 mol/l HCl for 10 min at room temperature and then thoroughly washed with PBS. The sections were then subjected to in-situ hybridization as described above. Control sections were incubated overnight at room temperature in a humid chamber with a solution containing 1 mg/ml of RNase A (Sigma, MO, USA) in PBS, and then subjected to the same in-situ hybridization protocol.

Cloning of PCR fragments

Amplified DNA fragments were purified using the Wizard purification system (Promega, WI, USA) and cloned into the vector pGEM®-T Easy (Promega) as described (Villegas *et al*., 2000, 2002). The ligation product was used to transform 100 µl of *Escherichia coli* HB-101 competent cells according to standard protocols (Sambrook *et al*., 1989). A volume of 50–100 µl of the transformation mixture was plated onto LB agar plates containing 100 µg/ml of ampicillin, 160 µg/ml of X-Gal and 0.4 mmol/l IPTG and incubated overnight at 37°C. At least 20 white colonies were selected randomly and grown overnight in 5 ml of LB broth plus 100 µg/ml of ampicillin. Plasmids were purified using the Concert Rapid Miniprep System (Gibco, BRL), and the presence of the insert was confirmed by PCR, using primers P1 and P2. Sequencing of both strands of the insert was carried out using the forward and reverse M13 primers and the dideoxy-chain termination method (Sanger and Coulson, 1975) with a Perkin-Elmer ABI Prism 310 Genetic Analyzer (Villegas *et al*., 2000, 2002).

Results

Amplification from total human sperm RNA by RT–PCR using primers P1 and P2, which are homologous between human, rat and mouse, was expected to yield a fragment of ~440 bp corresponding to a central region of the 16S mitochondrial rRNA. As shown in Figure 1, RT–PCR on RNA from isolated mouse and rat sperm heads (lanes 1 and 3) as well as RNA from whole human sperm (lane 5) produced the expected fragment of 440 bp. No amplification was obtained when the reaction was carried out without reverse transcriptase (Figure 1, lanes 2, 4 and 6).

Since in mouse sperm the 16S mitochondrial rRNA was found in the nucleus (Villegas *et al*., 2000), the same amplification protocol was repeated but using RNA obtained from isolated human sperm heads as template. Treatment of human sperm with proteinase K and DTT (von Beroldingen *et al*., 1989) dissolved the sperm tails leaving the heads with normal morphology and without any apparent tail contamination (Figure 2a). After amplification of RNA obtained from sperm heads and from human testis, a single fragment of ~440 bp was obtained (Figure 2b, lanes 1 and 3). Once again, no amplification was obtained when the reaction was carried out without reverse transcriptase (Figure 2b, lanes 2 and 4), indicating that the amplification was not due to contamination with mtDNA. To confirm the identity of this amplicon, the fragment was cloned in pGEM-T and sequenced. As shown in Figure 2c, the sequence of a representative clone proved to be 100% identical to the sequence of the human 16S mitochondrial gene between positions 2170 and 2604 (Anderson *et al*., 1981).

It is possible that the presence of the 16S mitochondrial rRNA in the human sperm heads might arise from contamination with mitochondrial RNA released from the organelle after treatment with proteinase K or from contaminating white blood cells. Therefore, whole human sperm smears were subjected to in-situ hybridization using primers P2 and P3 labelled with digoxigenin as probes (Concha *et al*., 1993; Villegas *et al*., 2000). An intense staining of the nucleus of the sperm was obtained after hybridization with the antisense probe (Figure 3a), and low magnification revealed that all cells were labelled (Figure 3b). In contrast, no staining was found when the hybridization was carried out with the sense probe (Figure 3c), indicating that the target of the antisense probe was RNA.

The unusual nuclear localization of the 16S mitochondrial rRNA might be explained by an unknown mechanism of translocation of the transcript from the organelle to the nucleus. Alternatively, the presence of this RNA in the nucleus could result from nuclear transcription of a mitochondrial pseudogene. To explore this possibility, isolation of human sperm nuclear DNA was carried out. Treatment of sperm with a solution containing 1% Triton X-100 and DTT releases the mitochondrial sheath and dissolves the outer dense fibres, leaving the sperm heads, the fibrous sheath and the axonemes as the only remaining structures (Olson *et al*., 1976; Brito *et al*., 1989; Brito and Burzio, 1995). When the DNA prepared from this fraction was amplified by PCR, the fragment of 440 bp (Figure 4a, lane 1) and an amplicon of 1558 bp corresponding to the full length of the 16S mitochondrial gene were obtained (Figure 4a, lane 4). Moreover, an amplicon of part of the 12S gene (Figure 4a, lane 2) and an amplicon of 1540 bp including part of the 12S, the $tRNA^{Val}$ and part of the 16S genes (Figure 4a, lane 3) (Anderson *et al*., 1981) were also obtained. These results indicate that the heads were contaminated with some mitochondria or mtDNA. To overcome this problem, nuclear DNA was purified by electrophoresis on agarose gels and elution of the highly polymerized DNA from the gel. Using this DNA as template for PCR, a fragment of 341 bp corresponding to the human β-globin (Figure 4b, lane 2) and an amplicon of 327 bp corresponding to part of exon 3 of the human pro-opiomelanocortin gene were amplified (Figure 4b, lane 3). In contrast, no fragment of 440 bp corresponding to the 16S mitochondrial rRNA was obtained (Figure 4b, lane 1). The same results were observed using sperm genomic DNA preparations obtained from three different donors (data not shown).

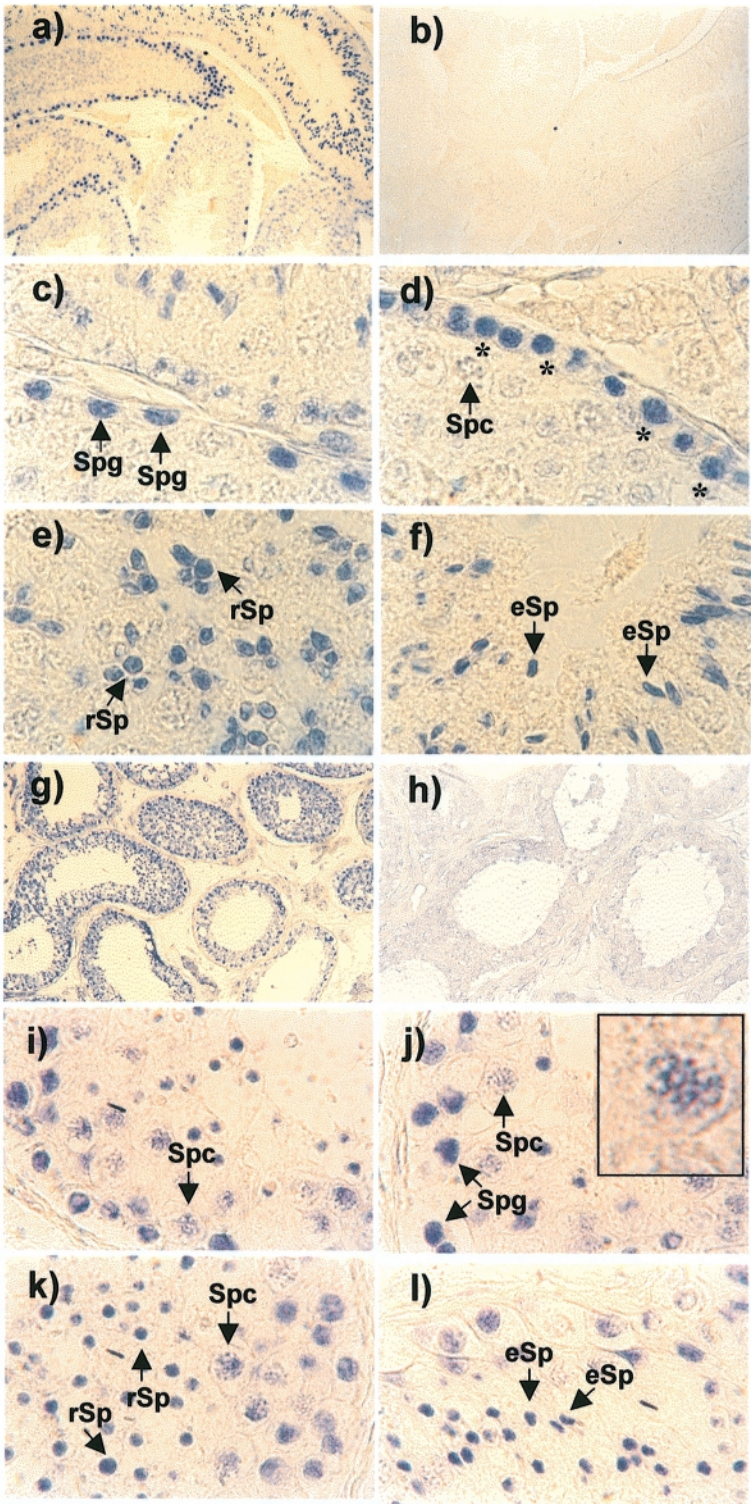

Figure 5. Nuclear localization of the 16S mitochondrial rRNA in mouse and human spermatogenic cells. Sections of ~5 µm of adult mouse testis (**a**–**f**) or human testis (**g**–**l**) were deparaffinated and subjected to in-situ hybridization using as a probe primer P2 labelled with digoxigenin. (**a**) Low magnification showing nuclear staining of several types of mouse spermatogenic cells. (**b**) A control section treated with RNase A prior to in-situ hybridization revealed total absence of hybridization. High magnification revealed the strong staining of mouse spermatogonia nuclei (Spg) (**c** and **d**), as well as of the nuclei of round (rSp) and elongated (eSp) spermatids (**e** and **f** respectively). Weak staining of the spermatocytes (Spc) was also evident. The asterisks in **d** indicate either spermatogonia or preleptotene spermatocytes. (**g**) Low magnification showing nuclear hybridization of several types of human spermatogenic cells. A control section treated with RNase A prior to in-situ hybridization revealed no staining (**h**). (**i**–**l**) High magnification showing the strong nuclear staining of human spermatogonia (Spg) and round (rSp) and elongated (eSp) spermatids. The insert in **j** shows the fibrillar staining pattern of the human spermatocyte (Spc) nuclei. Original magnifications: (**a**, **b**, **g** and **h**) ×200; others ×1000.

It was pertinent to ask if this unusual nuclear localization of the 16S mitochondrial rRNA also occurs in other spermatogenic cells. Accordingly, in-situ hybridization was carried out with adult mouse testis and normal human testis sections. As shown in Figure 5a, strong staining was observed in the nuclei of mouse spermatogenic cells at different stages of development. At high magnification, the

nuclear staining was clearly observed in spermatogonia (Figure 5c), and in round (Figure 5e) and elongated spermatids (Figure 5f). The nuclear staining of spermatocytes was positive but less strong (arrows in Figure 5d). The round stained nuclei shown in Figure 5d (see asterisks) might correspond to either spermatogonia or preleptotene spermatocytes. In contrast, sections treated with RNase prior to in-situ hybridization revealed total absence of staining (Figure 5b). The same results were obtained with human testis sections (Figure 5g), and again no hybridization was observed after treatment with RNase (Figure 5h). The nuclei of spermatogonia (Figure 5j), round and elongated spermatids (Figure 5k and l) as well as spermatocytes (Figure 5i–k) showed strong staining. Notice the fibrillar staining of the spermatocyte nuclei (insert, Figure 5j).

Discussion

The results presented here, together with those reported previously (Villegas *et al*., 2000, 2002), are the first demonstration that the 16S mitochondrial rRNA is a normal component of the nucleus of human and mouse spermatogenic cells. The localization in the nucleus of the human sperm was demonstrated by RT–PCR amplification of RNA extracted from purified sperm heads and confirmed by in-situ hybridization in samples from at least 25 different donors.

This novel localization could be explained as the result of transcription of a nuclear mitochondrial pseudogene. The presence of mitochondrial pseudogenes in the nuclear DNA has been reported in several organisms (Jacobs and Grimes, 1986; Smith *et al*., 1992; Arctander, 1995), including humans (Hirano *et al*., 1997; Wallace *et al*., 1997). But more pertinent is a report indicating that 14 722 bp of the mitochondrial genome is inserted in human chromosome 17 (Kindmark *et al*., 2001). In general, this macro mitochondrial pseudogene has an identity of ~83% with various mitochondrial genes, and 87% identity with the 16S mitochondrial gene (Kindmark *et al*., 2001). Since the amplicon of 440 bp obtained with RNA from isolated human sperm heads has a 100% identity with the sequence of the human 16S mitochondrial rRNA (Anderson *et al*., 1981), one could argue that the nuclear mitochondrial RNA reported here does not result from the transcription of a pseudogene. However, to formally rule out this possibility, a method was developed to isolate highly polymerized nuclear human sperm DNA, involving isolation of sperm heads and purification of the DNA by electrophoresis on agarose gels. Using this DNA purified from three human sperm donors as the template for PCR, no amplification product of the 16S gene or the 440 bp fragment corresponding to the same sequence were obtained. In contrast, regions of the β-globin and pro-opiomelanocortin genes were successfully amplified, indicating that the failure to obtain the corresponding amplicon of the putative 16S mitochondrial pseudogene was not due to problems of experimental design such as amount of template, magnesium concentration or annealing temperature. Therefore, the evidence strongly indicates that the presence of the 16S mitochondrial rRNA in the nucleus of human sperm is not the result of transcription of a mitochondrial pseudogene.

If the 16S mitochondrial rRNA is present in the nucleus of human and mouse sperm, it is pertinent to ask at what time during spermatogenesis does this localization process take place. The results presented here indicate that the RNA is localized in the nuclei of round and elongated spermatids, the direct precursors of the gamete. It is important to note that in-situ hybridization with 20 mer antisense probes corresponding to positions 1685, 2170 and 2930 of the mtDNA (Anderson *et al*., 1981) yielded the same results (data not shown), suggesting that the complete mitochondrial 16S mitochondrial rRNA is present in the nuclei of spermatogenic cells. Although the hybridization signals obtained with mouse spermatocytes were weak, the nuclear staining of human spermatocytes was unequivocal. It is interesting to note that the staining pattern found in the nuclei of spermatocytes was not homogeneous, as was that observed in spermatogonia, spermatids and spermatozoa. The pattern revealed a fibrillar staining, suggesting a putative association of the mitochondrial transcript with the meiotic chromosomes. The function of this association during meiosis is not clear at the present time.

The strong staining of the nuclei of mouse and human spermatogonia indicates that the localization process occurs during spermatogonial proliferation and differentiation and before the onset of meiosis (Clermont, 1972). The same results of in-situ hybridization were obtained with rat testis (data not shown). It would be interesting to study the localization at earlier stages of spermatogenesis in mouse fetal testis or in testes obtained from 10–20 day old mice (Clermont, 1972).

The above discussion strongly suggests that the nuclear localization of the 16S mitochondrial rRNA in spermatogenic cells is the result of an intriguing process of translocation of the transcript from the organelle to the nucleus. This hypothesis leads to several important questions; for example, how the organelle regulates the exit of the 16S mitochondrial rRNA without affecting the number of copies of the RNA needed to assemble the mitoribosomes for normal mitochondrial translation (Wallace, 1992; Taanman, 1999; Suzuki *et al*., 2001). Another intriguing question is about the mechanism used by the RNA to exit the mitochondria. The extramitochondrial localization of the 16S mitochondrial rRNA described here is not unique, since the same transcript has been found consistently in the cytoplasm of *Drosophila* and *Xenopus* embryos (Kobayashi *et al*., 1993, 1998). In these cases, the 16S mitochondrial rRNA is tightly associated to the polar granules of the germ plasma (Kobayashi *et al*., 1993, 1998). In embryos of the ascidian *Halocynthia*, the mitochondrial 16S rRNA is localized in the myoplasm of precursor muscle cells (Oka *et al*., 1999). However, the mechanism involved in the export of the transcript from the organelle is not understood.

At present, the significance of the nuclear localization of the 16S mitochondrial rRNA in spermatogenic cells or in the sperm nucleus is not clear. As discussed previously, the cytoplasmic localization of the 16S mitochondrial rRNA in embryos of *Drosophila* and *Xenopus*, tightly bound to polar granules (Kobayashi *et al*., 1993, 1998) is an essential event for pole cell formation and development of the germ line and abdomen formation (Blackler, 1958; Okada *et al*., 1974; Lehmann and Nüsslein-Volhard, 1986; Kobayashi and Okada, 1989; Kobayashi *et al*., 1993, 1998). Injection of an anti-16S rRNA ribozyme into cleavage embryos of *Drosophila* demonstrated that the rRNA is actively involved in the generation of pole cells, the progenitors of the germ line (Iida and Kobayashi, 1998). Also, it was suggested that the localization of the 16S rRNA in the myoplasm of precursor cells of muscle in embryos of the ascidian *Halocynthia roretzi*, might play an important role in development and differentiation (Oka *et al*., 1999). Therefore, one is tempted to speculate that the function of the mitochondrial transcript in the human sperm nucleus might be related to the determination of the germ line after fertilization. Recently, we have reported that the mouse chimeric mitochondrial RNA is edited from U to C (Villegas *et al*., 2002). In this regard, and related to the above proposition, it is important to mention that a high proportion (75%) of the RNA in mouse sperm is edited compared with 55% in testis and ~10% in somatic tissues, suggesting that this modification of the transcript might be necessary for its function in the gamete (Villegas *et al*., 2002).

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