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Expression of protamine-1 and -2 mRNA during human spermiogenesis

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During spermiogenesis, the histone-to-protamine replacement causes the compaction of the spermatid chromatin. The genes for protamines, *PRM-1* and *PRM-2*, are transcribed in round and elongating spermatids. The transcripts are stored in a translationally-repressed state by the binding of protein repressors before being translated in elongating and elongated spermatids. RNA extracts from homogenized whole testis samples supply only average data, and cell-specific and stage-specific expression cannot be addressed. Therefore, we used UV-laser-assisted cell-picking (UV-LACP) to select spermatids of defined differentiation steps. Subsequent reverse transcription–polymerase chain reaction (RT–PCR) with intron-spanning primer pairs allowed the detection of DNA-free and pseudogene-free *PRM-1* and *PRM-2* cDNA. Additional in-situ hybridization with digoxygenin-labelled cRNA probes exhibited *PRM-1* and *PRM-2* mRNA from step 1/2 spermatids to step 4 spermatids, but not in elongated spermatids. RT–PCR revealed amplicons for *PRM-1* and *PRM-2* in all spermatids except step 3 round spermatids. Applying proteinase K digestion, *PRM-1* and *PRM-2* transcripts were also detected in step 3 spermatids indicating that protein repressors may bind to both *PRM-1* and *PRM-2* mRNA in step 3 round spermatids. These data demonstrate that the combination of UV-LACP and non-radioactive in-situ hybridization appear to be a suitable approach for the study of cell-specific and stage-specific gene expression during spermiogenesis.

Key words: human testis/in-situ hybridization/laser-assisted cell-picking/protamines/spermiogenesis

Introduction

In round spermatids, both histones and non-histone proteins are replaced by transition proteins. In elongating spermatids, transition proteins are removed from the condensing chromatin and are replaced by protamines constituting the nuclear proteins of elongated spermatids and mature spermatozoa (Hecht, 1989a,b, 1990a,b; Oliva and Dixon, 1991; Dadoune, 1995; Siffroi *et al*., 1999; Steger, 1999). In man, the histone-to-protamine exchange is only ~85% complete (Tanphaichitr *et al*., 1978; Gatewood *et al*., 1987; Prigent *et al*., 1996).

Protamine-bound DNA is the most tightly compacted DNA (Pogany *et al*., 1981; Ward and Coffey, 1991) causing cessation of transcription in elongating spermatids. Thus major modifications in both nuclear and cytoplasmic structures continue throughout spermiogenesis; stringent temporal and stage-specific gene expression via transcriptional and translational control mechanisms is of fundamental importance to ensure complete differentiation of round spermatids into mature spermatozoa. In transgenic mice, premature expression of protamine-1 causes precocious nuclear condensation and arrests spermatid differentiation (Lee *et al*., 1995).

Specific sequences in the 3′-untranslated region (3′-UTR) of *PRM-1* mRNA cause delayed translation of both a human growth hormone reporter mRNA (Braun *et al*., 1989) and a human green fluorescent protein reporter mRNA (Schmidt *et al*., 1999). Since transgenes lacking these sequences showed no delayed translation (Braun, 1990; Fajardo *et al*., 1997), these sequences appear responsible for masking *PRM-1* mRNA. In round spermatids, most of the mRNA is stored as translationally inactive ribonucleoprotein (RNP) particles, involving protein repressors binding to specific sequences located in the 3′-UTR or to the 160–180 residues counting poly-A tail of the transcripts (Stern *et al*., 1983; Hecht, 1989a,b, 1990a,b; Morales *et al*., 1991; Dadoune, 1995; Kleene, 1996; Steger, 1999). Translation subsequently takes place in elongating and elongated spermatids after mRNA undergo a partial poly-A shortening by deadenylation. Before or during translation, mRNA for both protamines are subjected to a shortening process, namely from 0.62 to 0.45 kb for *PRM-1* mRNA and from 0.9 to 0.7 kb for *PRM-2* mRNA (Domenjoud *et al*., 1991). It has been shown that 85–95% of *PRM-1* and *PRM-2* mRNA with poly-A tails of 160–180 residues are translationally inactive, whereas 80–95% of *PRM-1* and *PRM-2* mRNA with poly-A tails of 30 residues are translationally active (Kleene *et al*., 1984, Heidaran and Kistler, 1987; Kleene, 1989, 1993).

Recently, protamine-2 has been recommended as spermatogenic cell marker for molecular diagnosis of spermatogenesis in non-obstructive azoospermia providing valuable information about the existence, the stage of differentiation and the physiology of spermatids and spermatozoa (Lee *et al*., 1998). Although protamine-1 and -2 proteins are known to be present from step 4 elongating spermatids to step 8 elongated spermatids (Roux *et al*., 1987, 1988; LeLannic *et al*., 1993; Lescoat

et al., 1993; Prigent *et al*., 1996; Siffroi *et al*., 1999) and transcripts for *PRM-1* and *PRM-2* have been observed in human spermatids (Wykes *et al*., 1995, 1997; Saunders *et al*., 1996; Siffroi *et al*., 1999), mRNA expression has not been assigned to the various steps of spermatid differentiation.

In the present study, the cell-specific and stage-specific expression of both *PRM-1* and *PRM-2* mRNA were investigated to gain further insight into the regulation of gene expression in haploid spermatids during normal spermiogenesis. Non-radioactive in-situ hybridization as well as UV-laser-assisted cell-picking (UV-LACP) followed by reverse transcription–polymerase chain reaction (RT–PCR) analysis, recently demonstrated as appropriate methods for the study of testicular tissue (Steger *et al*., 1998, Pauls *et al*., 1999), were applied and compared regarding the sensitivity of both methods.

Materials and methods

Testicular tissue

Extraction of RNA was performed on four testes from two orchidectomized men with prostatic carcinoma, aged 52 and 72 years. The patients were not treated with any drugs prior to orchidectomy. For UV-LACP, two testes from a 63 year old man with epididymitis and two testes from a 63 year old man with prostatic carcinoma were snap-frozen and stored in liquid nitrogen. For in-situ hybridization, 20 testicular biopsy specimens from 10 men (aged 30–45 years; mean 37.2 years) with obstructive azoospermia were fixed by immersion in Bouin's fixative and embedded in paraffin using standard techniques. All patients revealed normal endocrine values and histologically normal spermatogenesis (score ⩾8, according to Holstein and Schirren, 1983).

UV-LACP, first strand cDNA synthesis, and RT–PCR

For UV-LACP, 5 µm cryostat sections were mounted onto glass slides, stained with haematoxylin for 45 s, and immersed in absolute ethanol. Spermatids from defined stages of the seminiferous epithelial cycle were collected using the UV-laser MicroBeam system (Palm GmbH, Wolfratshausen, Germany) and the inverted Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany). The isolated cell profiles were harvested by a sterile injection needle mounted on a digitally controlled micromanipulator (Palm GmbH), transferred into a reaction tube containing 10 µl of first strand buffer (52 mmol/l Tris–HCl, 78 mmol/l KCl, 3.1 mmol/l $MgCl_2$, pH 8.3), snap-frozen, and stored at –80°C until further processing.

Prior to cDNA synthesis, reaction tubes with picked cell profiles were incubated at 70°C for 10 min and then rapidly cooled on ice-water. For proteinase K (Sigma, Deisenhofen, Germany) digestion, the picked cell profiles were treated with either 100 µg/ml proteinase K or 400 µg/ml proteinase K to release RNA from RNA-binding proteins. After incubation at 53°C for 30 min, samples were incubated at 99°C for 10 min to destroy proteinase K.

First strand cDNA synthesis was performed using the GeneAmp RNA–PCR Kit, according to the manufacturer's instructions (Perkin Elmer, Foster City, CA, USA). 2 µl each of $MgCl_2$ (25 mmol/l) and 10× PCR buffer II (100 mmol/l Tris–HCl, 500 mmol/l KCl, pH 8.3), 1 µl each of dNTPs (5 mmol/l each), random hexamers (50 µmol/l), and MuLV reverse transcriptase (50 IU), and 0.5 µl of RNase inhibitor (10 IU) were added to 10 µl of first strand buffer containing the picked cell profiles, and incubated at 20°C for 10 min, at 43°C for 75 min, and at 99°C for 5 min.

Using the GeneAmp RNA–PCR Kit, according to the manufacturer's instructions (Perkin Elmer), RT–PCR conditions were 1× 2 min 45 s 94°C/60× 45 s 94°C, 45 s 58°C, 45 s 72°C/1× 7 min 72°C/4°C. 8 µl of cDNA were added to 4 µl 10× PCR buffer II, 3

Table I. Primer pairs (Biosource, Ratingen, Germany) used for reverse transcription–polymerase chain reaction (RT–PCR) amplification of *PRM-1* cDNA and *PRM-2* cDNA (Domenjoud *et al.*, 1990).

Protamine 1 (1 intron between bp 112 and 113)		
Forward primer	5′GCCAGGTACAGATGCTGTCGCAG3′	exon 1, bp 4–26
Reverse primer	5′TTAGTGTCTTCTACATCTCGGTCTG3′	exon 2, bp 132–156
		PRM-1 amplicon: 153 bp
Protamine 2 (1 intron between bp 271 and 272)		
Forward primer	5′GTGAGGAGCCTGAGCGAACGC3′	exon 1, bp 16–36
Reverse primer	5′TTAGTGCCTTCTGCATGTTCTCTTC3′	exon 2, bp 285–309
		PRM-2 amplicon: 294 bp

Table II. Detection of *PRM-1* mRNA and *PRM-2* mRNA after in-situ hybridization (ISH) and of *PRM-1* cDNA and *PRM-2* cDNA after RT–PCR from tissue samples collected by UV-laser-assisted cell-picking (UV-LACP). Percentages of positive spermatids (spd) (mean ± SD) and of positive RT–PCRs (total RT–PCR reactions in brackets) for various steps of spermatid differentiation are shown. Values in parentheses are percentages

	round spd step 1/2	round spd step 3	Elongating spd step 4	Elongated spd step 5/6	Elongated spd step 7/8
Protamine 1					
ISH (%)	61.9 ± 18.4	80.5 ± 9.2	61.9 ± 7.7	0	0
UV-LACP	13 (46.1)	12 (8.3)	12 (50.0)	14 (71.4)	13 (46.1)
Proteinase K 100 µg/ml	8 (25.0)				
400 µg/ml	7 (100)				
Protamine 2					
ISH	53.2 ± 14.3	76.4 ± 4.0	68.5 ± 8.9	0	0
UV-LACP	11 (72.7)	8 (25.0)	7 (100)	12 (75.0)	12 (75.0)
Proteinase K 100 µg/ml	8 (37.5)				
400 µg/ml	6 (50.0)				

µl $MgCl_2$ (25 mmol/l), 8 µl dNTPs (5 mmol/l each), 0.5 µl AmpliTaqGold^{TM}, 1.5 µl of each primer (10 µmol/l), and ultrapure H_2O to a final volume of 50 µl. The DNA sequences for human PRM-1 and PRM-2 (Domenjoud *et al*., 1990) were generated using the primer pairs listed in Table I.

The primer pairs employed in this study flanked the single intron of *PRM-1* DNA and *PRM-2* DNA to preclude genomic DNA amplification within the expected product length of 153 bp (*PRM-1* cDNA) and 294 bp (*PRM-2* cDNA). To exclude genomic DNA or pseudogene amplification, genomic DNA was isolated from similar testicular tissue with the QIAmp Blood Kit (Qiagen, Hilden, Germany). PCR analysis was carried out with the same primer pairs applied in RT–PCR. Amplification products were visualized by agarose gel electrophoresis and ethidium bromide staining. For each RT–PCR, controls were performed by omitting reverse transcriptase or by using primary spermatocytes instead of spermatids.

Cloning of human PRM-1 and PRM-2 cDNA, and digoxigenin-labelled cRNA probes for PRM-1 and PRM-2 mRNA

Total RNA was extracted from testicular tissue with Trizol Reagent, according to the manufacturer's protocol (Life Technologies, Eggenstein, Germany). First strand cDNA synthesis was performed using the Superscript II Kit, according to the manufacturer's instructions (Life Technologies).

The cDNA clones for human *PRM-1* and *PRM-2* (Domenjoud *et al*., 1990) were generated using RT–PCR (1× 3 min 95°C/35× 1 min 95°C, 1 min 69°C, 2 min 72°C/1× 10 min 72°C/4°C) with the primer pairs listed in Table I.

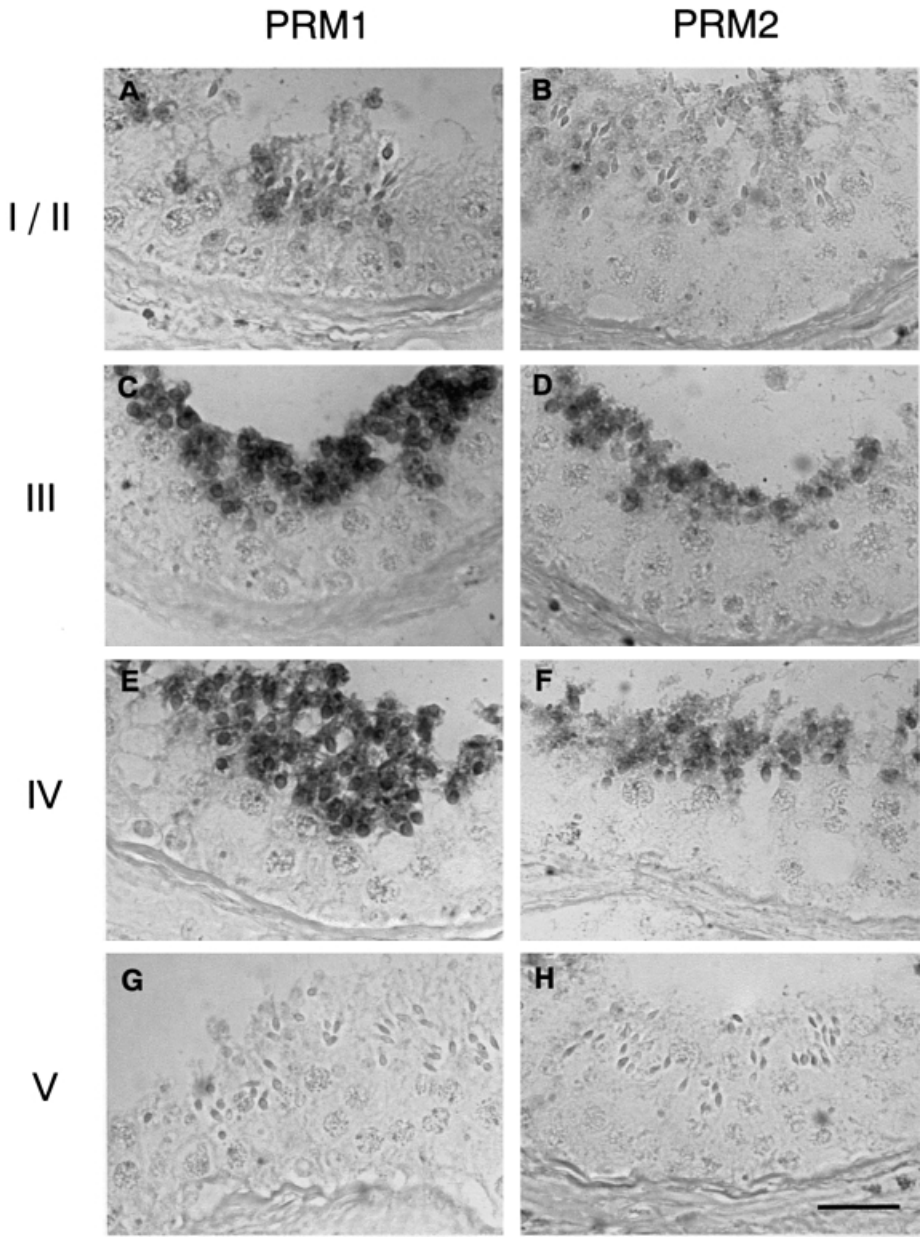

Figure 1. Non-radioactive in-situ hybridization using a digoxygenin (DIG)-labelled *PRM-1* cRNA probe (left column) and a DIG-labelled *PRM-2* cRNA probe (right column). Both protamines presented a similar staining pattern with protamine-2 exhibiting less intensive signals than protamine-1. (**A**, **B**) During stage I/II of the seminiferous epithelial cycle, step 1/2 round spermatids displayed only a weak signal, whereas step 7/8 elongated spermatids were completely negative. While (**C**, **D**) step 3 round spermatids (stage III) as well as step 4 (**E**, **F**) elongating spermatids (stage IV) revealed a strong signal for both protamine-1 and protamine-2, step 5 (**G**, **H**) elongated spermatids (stage V) were again completely negative. Bar = 10 µm.

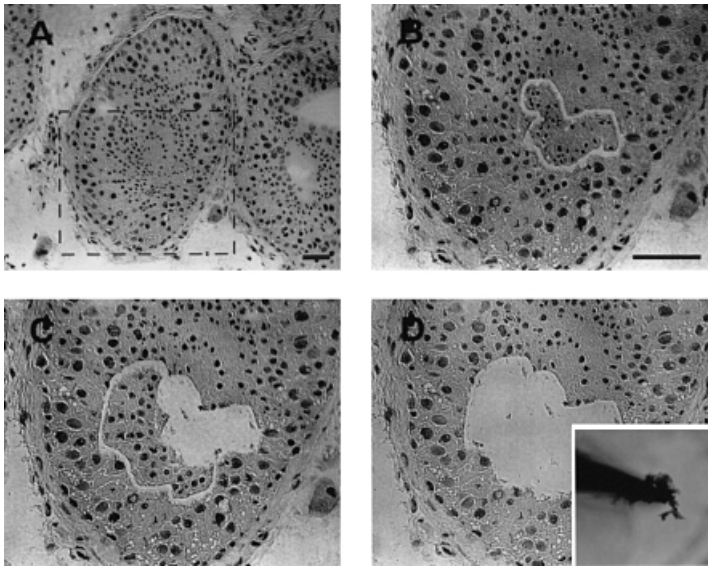

Figure 2. (**A**) UV-laser-assisted cell-picking (UV-LACP) in a seminiferous tubule with stage I/II of the seminiferous epithelial cycle exhibiting two generations of spermatids, step 1/2 round spermatids and step 7/8 elongated spermatids. Rectangle in (**A**) indicates section of **B–D**. First, step 7/8 elongated spermatids were cut out by UV-laser (**B, C**). Then, step 1/2 round spermatids were cut out by UV-laser (**C, D**). The isolated cell profiles were harvested by a sterile injection needle (Insert) mounted on a digitally controlled micromanipulator and directly transferred into a reaction tube. Bar = 30 μm.

A 156 bp RT–PCR product of the human *PRM-1* cDNA and a 294 bp RT–PCR product of the human *PRM-2* cDNA were subcloned in pGEM-T (Promega, Heidelberg, Germany). The plasmids were transformed in the XL1-Blue *Escerichia coli* strain (Stratagene, Heidelberg, Germany) and extracted by column purification, according to the manufacturer's instruction (Qiagen).

In-vitro transcription of digoxygenin (DIG)-labelled cRNA was performed using the RNA–DIG Labelling Mix (Boehringer Mannheim, Mannheim, Germany) and RNA-polymerases T7 and SP6. Prior to cRNA synthesis, the vectors containing the *PRM-1* and *PRM-2* inserts had been digested with *Nco*I or *Not*I (New England Biolabs, Schwalbach, Germany) for the production of sense cRNA (*Nco*I) or antisense cRNA (*Not*I). After phenol extraction, the dried pellet was reconstituted in 100 μl RNase-free water. The concentration of the DIG–RNAs was estimated by a semiquantitative dot blot test (Jackson, 1991).

In-situ hybridization

In-situ hybridization was performed as previously reported (Steger *et al*., 1998). Briefly, 7 μm sections from Bouin-fixed and paraffin-embedded tissue samples were mounted on slides coated with aminopropyltriethoxysilane (Sigma). Deparaffinized and rehydrated tissue sections were digested with proteinase K (20 μg/ml 1× PBS) for 30 min at 37°C, post-fixed in 4% paraformaldehyde for 10 min and prehybridized in 20% glycerol for 30 min. Sections were then incubated with the DIG-labelled sense and antisense cRNA probes. Both *PRM-1* cRNA and *PRM-2* cRNA were used at a dilution of 1:100 in hybridization-buffer containing 50% deionized formamide, 10% dextran sulphate, 2× sodium chloride/sodium citrate (SSC), 1× Denhardt's solution, 10 μg/ml salmon sperm DNA (Sigma) and 10 μg/ml yeast t-RNA (Sigma). Hybridization was performed overnight at 37°C in a humid chamber containing 50% formamide in 2× SSC.

Post-hybridization washes were performed, according to a previously described method (Lewis and Wells, 1992). Tissue samples were incubated overnight at 4°C with an anti-DIG Fab-antibody conjugated to alkaline phosphatase (Boehringer Mannheim). Staining was visualized with NitroBlue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (KPL, Gaithersburg, MD, USA) in a humid chamber and protected from light. Finally, sections were mounted in Glycergel (Dako, Hamburg, Germany). For each test, control incubations were performed using DIG-labelled cRNA sense probes.

Quantification and statistical analysis

In sections subjected to in-situ hybridization, the ratio of labelled to unlabelled spermatids was determined in 10 seminiferous tubules from each biopsy. For stage-specific quantification (Clermont, 1963), this ratio was determined in 10 seminiferous tubules for each stage of the seminiferous epithelial cycle. Data were analysed using Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

In-situ hybridization visualized a stage-specific expression of *PRM-1* and *PRM-2* mRNA (Table II; Figure 1). In stage I/II of the seminiferous epithelial cycle containing two generations of spermatids, namely steps 1/2 round spermatids and step 7/8 elongated spermatids, 62 and 53% of step 1/2 round spermatids revealed a weak signal for *PRM-1* and *PRM-2* respectively, whereas step 7/8 elongated spermatids were completely negative for both *PRM-1* and *PRM-2* (Figures 1 A, B). 80.5% (*PRM-1*) and 76% (*PRM-2*) of step 3 round spermatids (stage III; Figures 1 C, D) and 62% (*PRM-1*) and 68.5% (*PRM-2*) of step 4 elongating spermatids (stage IV; Figures 1 E, F) exhibited a strong signal for both *PRM-1* and *PRM-2*. Step 5 elongated spermatids (stage V; Figures 1G,H) were again completely devoid of both *PRM-1* and *PRM-2* mRNA. Summarized, ~68% and ~66% of steps 1–4 spermatids revealed signals for *PRM-1* and *PRM-2* mRNA respectively. Differences between steps 1/2, step 3, and step 4 spermatids were not significant. Control incubations with DIG-labelled *PRM-1* and *PRM-2* cRNA sense probes were completely negative (data not shown).

Employing UV-LACP, cell profiles of 10–20 spermatids of

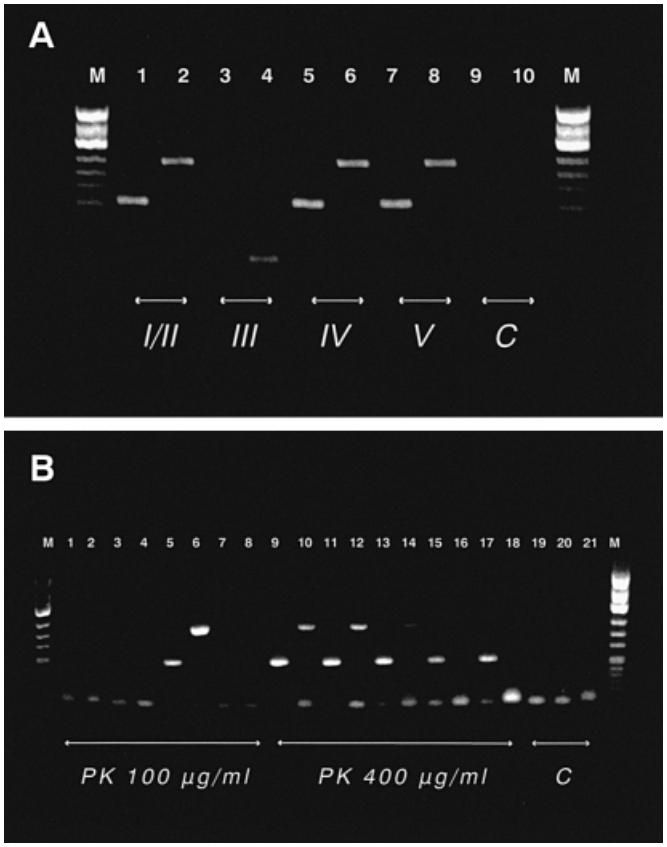

Figure 3. Agarose gel electrophoresis after reverse transcription–polymerase chain reaction (RT–PCR) from tissue samples collected by UV-laser-assisted cell-picking (UV-LACP). (**A**) Stage-specific detection of *PRM-1* cDNA (lanes 1, 3, 5, 7) and PRM-2 cDNA (lanes 2, 4, 6, 8). Probes layered on lanes 1/2, 3/4, 5/6, and 7/8 contained the same cell profile and the same master mix and differ only in the applied primer pairs. Notice the lack of amplicons for both *PRM-1* and *PRM-2* in stage III of the seminiferous epithelial cycle (lanes 3 and 4). I–V: stage of the seminiferous epithelial cycle; C = control (primary spermatocytes; lanes 9 and 10); M = 1 kb DNA-ladder. (**B**) Detection of *PRM-1* cDNA (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17) and *PRM-2* cDNA (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18) in step 3 round spermatids (stage III of the seminiferous epithelial cycle) after proteinase K (PK) digestion using a concentration of 100 µg/ml (30 min, 53°C; lanes 1–8) and 400 µg/ml (30 min, 53°C; lanes 9–18). C = control (primary spermatocytes; lanes 19–21); M = 1 kb DNA ladder.

a defined step of differentiation were cut out of the seminiferous epithelium (Figures 2A–D). Subsequent RT–PCR analysis of these cell profiles revealed transcripts of both *PRM-1* and *PRM-2* in all steps of spermatid differentiation. However, we noticed considerable variation among different UV-LACP processed cell profiles in the efficiency of RT–PCR amplification (Figure 3A). With the exception of step 3 round spermatids, the average success of RT–PCR amplification of *PRM-1* and *PRM-2* transcripts was 53 and 81% respectively (Table II). In steps 1/2 round spermatids, revealing a weak staining by in-situ hybridization, 46% of RT–PCR samples (*PRM-1*) and 73% of RT–PCR samples (*PRM-2*) displayed positive signals. In step 4 elongating spermatids, revealing a strong staining by in-situ hybridization, 50% of RT–PCR samples (*PRM-1*) and 100% of RT–PCR samples (*PRM-2*) exhibited positive signals. In elongated spermatids, which were completely negative by in-situ hybridization, 71% (step 5/6) and 46% (step 7/8) of RT–PCR samples were positive for *PRM-1*, while 75% (step 5/6) and 75% (step 7/8) of RT–PCR samples were positive for *PRM-2*. In step 3 round spermatids, which revealed a strong staining by in-situ hybridization, only 8% (*PRM-1*) and 25% (*PRM-2*) of RT–PCR samples exhibited positive signals. However, in step 3 round spermatids, positive signals for both *PRM-1* and *PRM-2* cDNA could be obtained and, therefore, the discrepancy between in-situ hybridization and RT–PCR analysis could be removed when cell profiles were digested with proteinase K prior to first strand cDNA synthesis and RT–PCR amplification (Table II; Figure 3B). Using 100 µg/ml proteinase K and 400 µg/ml proteinase K, 25 and 100% of RT–PCR showed positive signals for *PRM-1*, while 37.5% and 50% of RT–PCR showed positive signals for *PRM-2* respectively. Primary spermatocytes which are known to be devoid of protamines, revealed no positive signals and, therefore, served as negative control (Figures 3A,B).

Discussion

The human genes *PRM-1*, *PRM-2*, and *TNP-2* exist as a linear array on chromosome 16p13.3 (Domenjoud *et al*., 1990, Schlüter *et al*., 1992) being regulated as a single genetic unit (Choudhary *et al*., 1995). Applying radioactive in-situ hybridization, *PRM-2* mRNA was shown to be the most abundant transcript in human testis. *PRM-1* and *TNP-2* mRNA were present in approximately equal quantities (Wykes *et al*., 1995). These results are in contrast with those of others suggesting that *TNP-2* mRNA is expressed at a very low level in human testis (Schlueter *et al*., 1992, 1993). The human *TNP-2* gene differs from that of other mammalian species, that have been investigated so far, by the absence of the conserved 5′GCCATCAC3′ nucleotide sequence in the 3′-UTR (Schlueter *et al*., 1992, 1993). The difficulty in demonstrating *TNP-2* transcripts in human testis may be due to insufficient storage of *TNP-2* mRNA as RNP particles. Using Northern blot analysis and fluorescence in-situ hybridization, *PRM-2* mRNA was again the most abundant transcript in human testis. Comparatively, transcripts for *PRM-1* and *TNP-2* were present at a level of ~50% and ~3% of that of *PRM-2* mRNA (Choudhary *et al*., 1995). Applying non-radioactive in-situ hybridization, *TNP-2* mRNA cannot be demonstrated in human testis (Steger *et al*., 1998).

In this study, we investigated the expression of both *PRM-1* and *PRM-2* mRNA during normal spermatogenesis. In contrast to previous studies (Choudhary *et al*., 1995; Wykes *et al*., 1995, 1997; Saunders *et al*., 1996), we were able to demonstrate cell-specific and stage-specific expression applying non-radioactive in-situ hybridization and UV-LACP followed by RT–PCR analysis.

PRM-1 and *PRM-2* mRNA was demonstrated in the cytoplasm from steps 1/2 round spermatids to step 4 elongating spermatids. While both mRNA revealed only weak hybridization signals in step 1/2 spermatids, strong staining was observed in step 3 and step 4 spermatids. Therefore, translation of the corresponding proteins, P1 and P2, known to be present in the

nucleus from step 4 to step 8 spermatids (Roux *et al*., 1987, 1988; LeLannic *et al*., 1993; Lescoat *et al*., 1993; Prigent *et al*., 1996; Siffroi *et al*., 1999), appears to be delayed.

In haploid spermatids, translational arrest is due to the storage of mRNA as RNP particles (Stern *et al*., 1983; Penttilä *et al*., 1995; Kleene, 1996) in chromatoid bodies which can frequently be observed in round spermatids (Biggiogera *et al*., 1990; Moussa *et al*., 1994). The translational arrest is caused by the binding of protein repressors to the 3′-UTR or the poly-A tail of mRNA. Numerous mouse mRNA-associated proteins have already been identified (Grange *et al*., 1987; Kwon and Hecht, 1991, 1993; Berger *et al*., 1992; Murray *et al*., 1992; Kwon *et al*., 1993, Fajardo *et al*., 1994; Kleene *et al*., 1994; Gu *et al*., 1995; Schumacher *et al*., 1995a,b; Lee *et al*., 1996).

Step 3 round spermatids exhibited a strong signal using in-situ hybridization, but in contrast displayed nearly no signal applying the more sensitive RT–PCR technique. However, amplification of both *PRM-1* and *PRM-2* cDNA could be obtained when cell profiles were subjected to proteinase K treatment prior to first strand cDNA synthesis and RT–PCR amplification. This is in line with data obtained by in-situ hybridization where paraffin sections had also been subjected to proteinase K digestion prior to hybridization of the DIG-labelled cRNA probe. These data suggest a tight binding of protein repressors preventing the transcripts of *PRM-1* and *PRM-2* from being translated in step 3 round spermatids.

For step 1/2 and step 4 spermatids, data obtained by UV-LACP RT–PCR were in line with those obtained by in-situ hybridization. RT–PCR producing no amplification products may be explained by the selection of spermatids being devoid of transcripts for *PRM-1* and *PRM-2*. During normal spermiogenesis, only 68 and 66% of round and elongating spermatids revealed hybridization signals for *PRM-1* and *PRM-2* mRNA respectively.

RT–PCR positive signals in step 5/6 and step 7/8 elongated spermatids being completely negative using in-situ hybridization may be caused by remnants of untranslated mRNA, which can only be detected by the more sensitive RT–PCR technique. Both *PRM-1* and *PRM-2* mRNA have been demonstrated even in epididymal and ejaculated human spermatozoa (Pessot *et al*., 1989; Kumar *et al*., 1993; Miller *et al*., 1994; Miller, 1997; Wykes *et al*., 1997).

Applying non-radioactive in-situ hybridization, both protamines exhibited an identical localization with protamine-2 showing less intensive signals than protamine-1. However, quantitative evaluation revealed no significant differences in the distribution of *PRM-1* and *PRM-2* mRNA. These results are in contrast with previous studies (Choudhary *et al*., 1995; Wykes *et al*., 1995) which demonstrated that, in human testis, *PRM-1* mRNA is present at a level of ~50% of that of *PRM-2* mRNA. These data correspond with the results obtained by RT–PCR following UV-LACP. Here, the average success of RT–PCR amplification (except step 3 round spermatids) of *PRM-1* and *PRM-2* transcripts was 53 and 81% respectively.

In conclusion, our data demonstrate that the combination of non-radioactive in-situ hybridization and UV-LACP RT–PCR is a suitable approach for the study of cell and stage-specific gene expression during spermiogenesis. Furthermore, the inclusion of proteases more specific than proteinase K prior to first strand cDNA synthesis may contribute to our understanding of the timing and functional interaction of protein repressor molecules to mRNA transcripts.

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