The lack of protamine 2 (P2) in boar and bull spermatozoa is due to mutations within the P2 gene

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ABSTRACT

The nuclei of spermatozoa in all mammals examined so far contain P1 protamine. A second protamine variant, protamine P2, has to date been isolated only from human and murine spermatozoa where it represents the major fraction of basic nuclear protein. In order to elucidate the reason for this unusual distribution of the protamine variants among mammals we have investigated the expression of protamine P2 in boar and bull. It can be shown that also in these species protamine 2 is transcribed and translated on low levels. Various mutational events though have altered the primary structure of the protein: In boar, a deletion of 8 aminoacids has removed a sequence motif from the amino-terminus of the molecule, which highly probable is of functional relevance. The bovine sequence, as a consequence of numerous point mutations has accumulated neutral and hydrophobic aminoacids which reduce the affinity of the protamine 2 to DNA.

INTRODUCTION

In mammalian spermatogenesis chromatin structure of the germ cells is drastically changed during the elongation of the spermatids, when histones are replaced by transition proteins and protamine, the predominant nuclear proteins in mature spermatozoa. Protamine-DNA-interaction leads to a highly condensed parallel aggregation of smooth nucleoprotamine fibers, which substitutes for the beaded nucleosomal organisation of the chromatin found in somatic cells and round spermatids (1,2,3).

While in most mammals examined so far only one protamine variant is present in spermatozoa, two protamines were found in mouse and human (2,4,5). Both of the protamines are highly basic proteins of approximately 50 aminoacids and are very rich in arginine and cysteine, while histidine is only present in protamine 2. Although protamine function has not yet been completely elucidated and still little is known about the role of protamine 2, it has been learned that protamine binds to the DNA mainly by electrostatic interactions of its arginines. The neutralization of the DNA then allows the tight aggregation of the nucleoprotamine, which is further stabilized by inter- and intramolecular disulfide bonds (3, 6–8).

Transcription of the protamines is limited to round spermatids and the transcripts are stored as RNPs for several days until biosynthesis begins in elongated spermatids (9,10). In contrast to protamine 1, protamine 2 is synthesized as a precursor protein of roughly twice the length of its mature form and is bound to the nuclear DNA previous to its maturation by proteolytic cleavage. Possibly therefore a short time lag is observed between the occurence of the mature protamine 2 as compared to protamine 1 in spermatid nuclei (11–13).

Although protamine 2 to date has not been isolated from any species but mouse and human, where it even represents the major component of the basic protein in sperm nuclei, the gene is also transcribed in hamster, rat, monkey, ram and cat (14, unpublished results). Furthermore, at the genomic level cross hybridizing sequences have been detected in all 3 mammalian subclasses (15). Here we present the cDNA sequences for protamine 2 of boar and bull and a study of the expression of the gene in spermatids. It is shown that, although the gene product is not present in mature spermatozoa (16,17), protamine 2 is transcribed and translated on low levels. It is very likely that due to various mutational events the mature protein is not functional and therefore is superseded by protamine 1 in both species.

MATERIALS AND METHODS

cDNA library construction and screening

The library construction was performed as described earlier (18). In the case of bull, the DNA was copied from RNA of spermatids with oligo d(T) priming and was cloned into λgt 11. The boar cDNA library was constructed by the use of poly $(A)^+$ RNA from total testis, random hexanucleotide primer $(dN)_6$ and the vector λgt 10. Screening was carried out with a nick-translated cDNA probe of human protamine 2 (12) under conditions of moderate stringency. The clones isolated were subcloned into pUC9 (boar) and pBluescribe™ (Stratagene) (bull) and sequenced by the chain termination method with Sequenase™. Sequence data were analyzed by the help of the DNA*™ program.

Asymmetric PCR and sequencing of the product

The PCR was performed in 40 cycles as described (19). We used a programmable robot arm (Rob 3, P+P Elektronik Nürnberg, FRG) and a cycle profile of 93°C for 30 sec, 55°C for 40 sec and 65°C for 90 sec. In a final volume of 50 μl the reaction mix

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contained 500 ng porcine liver DNA, 1.5 mM dNTP, 50 pM primer 1, 1 pM primer 2 and 2.5 units of Taq polymerase (BRL). 1×buffer was 16,6 mM ammonium sulfate, 67 mM Tris/HCl pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 6,7 μM EDTA, 170 μg/ml BSA and 10% DMSO. The reaction products were purified by ethanol precipitation and sequenced according to the method of Winship (20) with the primer used in limited concentration in the PCR. Four reactions were carried out with DNA from two different pigs and each of the primers used once in limited and once in excess concentration.

RNA preparation for Northern blot hybridization

RNA isolation, Northern analysis and reprobing of the blots were as described (21). For RNaseH digestion 20 μg of total RNA were annealed to 100 ng oligo d(T) (Pharmacia) at 65°C and digested with 20 units of RNase-H following the recommendations of the supplier (BRL). Polysomes were prepared according to the protocol of Clemens (22).

In situ hybridization

Boar testis was fixed in 4% paraformaldehyde and embedded in paraffin. For the labeling of the cDNA the random primer method was choosen (23). All other steps were carried out as described by Rentrop et al. (24).

In vitro transcription and translation

The protamine 2 cDNA of bull and human (12) was subcloned in the pBluescribe™ transcription vector (Stratagene). This construct was used for mRNA production using T7 RNA polymerase (Boehringer Mannheim) according to the manufacturer's procedures. mRNA at 1 μg was translated in a rabbit reticulocyte lysate (New England Nuclear) following the manufacturer's prescriptions.

In a second translation assay protamine 2 transcripts were used, which were purified from testicular RNA of bull and human by hybrid selection. Following the protocol of Parnes et al. (25) 20 μg of the protamine 2 cDNAs were dotted to Hybond N membranes (Amersham) and hybridized for 12 h at 42°C to 1 mg of the homologous total testicular RNA. The RNA fraction obtained by this method was entirely used in one translation assay.

Translation was stopped with 3 μg of RNase. Proteins were then extracted by TCA precipitation after a reducing treatment: The translation mix was incubated with 6 M urea/100 mM trisHCl pH 8.0/0.28M 2-mercaptoethanol. After 1 h at 56°C 1 μl ethylenimine was added and incubation was continued for 1 h at room temperature. Proteins were then precipitated with 20% TCA, washed with acetone and resuspended in 0.2 M HCl/0.28 M 2-mercaptoethanol/8 M urea.

Electrophoresis (26) was carried out in a 15% polyacrylamide gel containing 4 M urea. The gel was fixed, treated with En^{3}hance (NEN) and vacuum dried prior to autoradiography at −70°C (10 d).

RESULTS

Isolation and identification of cDNA clones coding for protamine 2 of boar and bull

Using a nick-translated probe of the human protamine 2 cDNA isolated earlier (12), cDNA libraries of bull and boar testes were screened for crosshybridizing clones. While from the bovine library one clone (600bp) of nearly full length was obtained, two non crosshybridizing cDNA clones (211bp and 218bp) were isolated from the randomly primed library of boar testis. Sequence analysis revealed that the clones were coding for the 3′- and 5′ regions of the protamine 2 transcript but did not show overlapping sequences (Fig. 1). No further cDNA clone could be isolated. In order to link the cDNA sequences found in the library, oligonucleotides (22mer) from either side of the gap were synthesized and 500 ng of porcine DNA were subjected to asymmetric PCR and sequencing. The nucleotide sequence of the porcine protamine 2 transcript was then deduced from the sequences of the cDNA clones isolated, connected by the PCR products obtained in four indepedant assays using DNA probes from two individuals and each of the primers once in excess and once in limited concentration.

The sequences are given in figure 1. On the cDNA level, homology with the protamine 2 clones already isolated from mouse and human is 72% and 71%, respectively, for the porcine protamine 2 and 67% with respect to the bovine cDNA. Between the bovine and porcine sequences homology is 80%.

The protamine 2 cDNAs isolated from mouse and human show open reading frames of similar lengths, coding for proteins of 106 and 101 aminoacids, respectively. In contrast, from the porcine consensus sequence a peptide of only 91 aminoacids with 36 arginine and 5 cysteine residues is deducable while the bovine

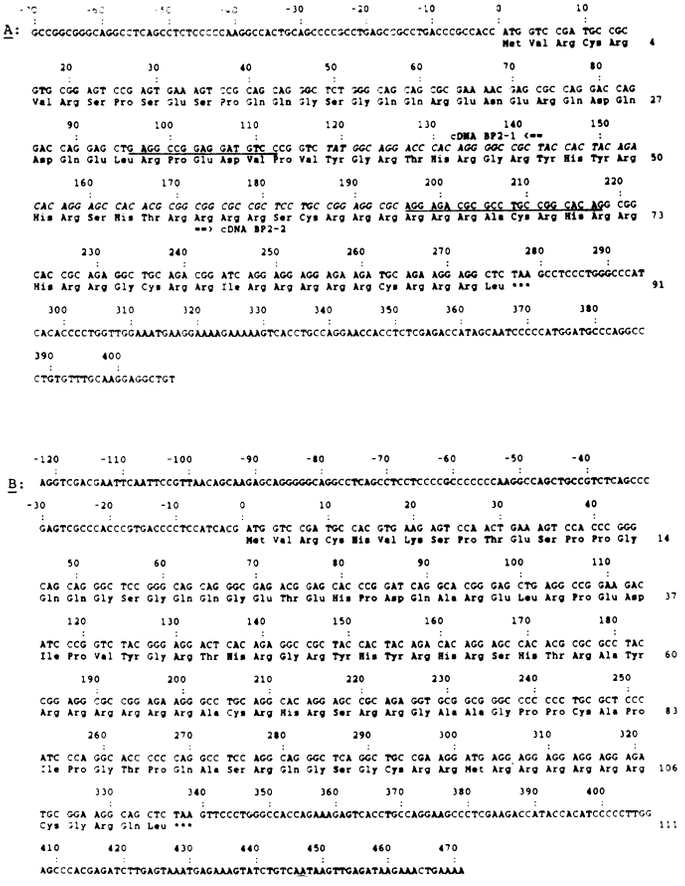

Figure 1: Nucleotide- and deduced aminoacid sequences of boar and bull protamine 2 cDNA.
A: Boar. The sequences of the cDNA clones BP2-1 and BP2-2 isolated from our randomly primed cDNA library are indicated by arrows. The sequence elements used as primers in the PCR are underlined. The fragment obtained from direct sequencing of PCR products is printed in italic letters.
B: Bull. The polyadenylation signal is underlined.

cDNA codes for a sequence of 111 aminoacids, 29 of which are arginines and 5 are cysteines. An alignment of the aminoacid sequences deduced from boar and bull cDNA to the primary structures of the protamine 2 precursors of mouse and human results in 65% and 49% homology with respect to the porcine sequence and 47% and 36%, respectively, for the bovine sequence (Fig. 2). The aminoacid sequences of boar and bull show 75% similarity.

The difference in length of the deduced primary structures as compared to the sequences of the protamine 2 precursors of mouse and human in the case of the boar cDNA is caused by a deletion of 27 bp in the central region of the transcript. In the bovine cDNA, due to numerous point mutations the reading frame of the transcript has been shifted to a posterior stop codon. Nevertheless, the similarity of the sequences indicates that the cDNAs isolated are indeed coding for the protamine 2 of boar and bull, although the protein is absent in the mature spermatozoa of both species.

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                                        leader sequence | mature protein

                   10v       20v       30v       40v    |50v       60v
HUMANP2   VRYRVRSLSERSHEVYRQ-QLHGQEQGHHGQEEQGLSRMHVEVYERTH|GQS-HYRRRH--CSRR
MOUSEP2   VRYRMRSPSEGPH-Q-GPGQDHEREEQG--QG-QGLSPERVEDYGRTH|RGHHHH--RHRRCSRK
BOARP2    VRCRVRSPSESP--QQGSGQQRENERQD--QD-QELRPEDVPVYGRTH|RGRYHY--RHR--SHT
BULLP2    VRCHVKSPTESPPGQQGSGQQGETEHPD--QA-RELRPEDIPVYGRTH|RGRYHY--RHR--SHT

                   70v       80v       90v       100v      110v
HUMANP2   RLHRIHRRQHRSCRRRKRRSCRHRRRHRTGCRT-RKR-TCRRH
MOUSEP2   RLHRIHKRR-RSCRRRRRHSCRHRRRHRRGCRRSRRRRRCRCRKCRRHHH
BOARP2    -------RRRRSCRRRRRRACRHRR-HRRGCRRIRRRRRCRRRL
BULLP2    RAYR--RRRRRACRHRSRRGAAGPPCAPIPGTPQASRQGSGCRRMRRRRRRCGRQL
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Figure 2: Alignment for maximal similarity of the primary structures of protamine 2 precursors deduced from cDNA sequences of human (12), mouse (11), boar and bull. The cleavage sites of the precursorprotein (putative in boar and bull) are indicated by a solid line. Note the motif R-L-H-R-I-H (underlined) conserved in mouse and human, which is deleted in the bovine and porcine sequence.

Gene expression of protamine 2 in boar and bull

As already known for rodents and human (14, 12), also in boar and bull protamine 2 is expressed exclusively in the testis as a transcript of roughly 900 bp in size. While in mouse and human abundance of both protamine transcripts is in the same order of magnitude (14, unpublished results), the level of transcription of the protamine 2 gene in boar and bull is 10 to 20 fold lower as compared to protamine 1.(Fig 3., note that an approximately 5 fold increase in autoradiograph exposure time was needed to obtain a reasonable hybridization signal with protamine 2).

This result is substantiated by in situ hybridizations of paraffin sections of boar testis, which were performed under identical conditions with homologous cDNA probes of equal lengths for both protamines (27). Although results are only semiquantitative the difference in abundance of both types of protamine in boar is significant. Furthermore it is demonstrated that transcription of both protamines is limited to spermatids (Fig.4).

It is interesting to note that the hybridization signals with both protamine probes do not form sharp bands, but are extended to a minor class of transcripts, which is slightly smaller in size (Fig.3). As alredy known in other species also in boar and bull this size variation of the protamine transcripts is caused by a partial deadenylation of the mRNA which occurs concomitant to the initiation of protamine biosynthesis (Fig.5). The association of the transcripts with polysomes shows that very likely protamine 2 in boar and bull is translated in vivo. Furthermore, the bovine cDNA proved to be functional with respect to its transcriptional and translational competence in vitro. Translation of protamine 2 mRNA isolated from total testicular RNA by hybrid selection only with the human probe resulted in reasonable protein synthesis, while the signal obtained with the bovine mRNA was hardly visible, which is most probable due to its low quantity (Fig. 6).

These results indicate that transcription and translation of the

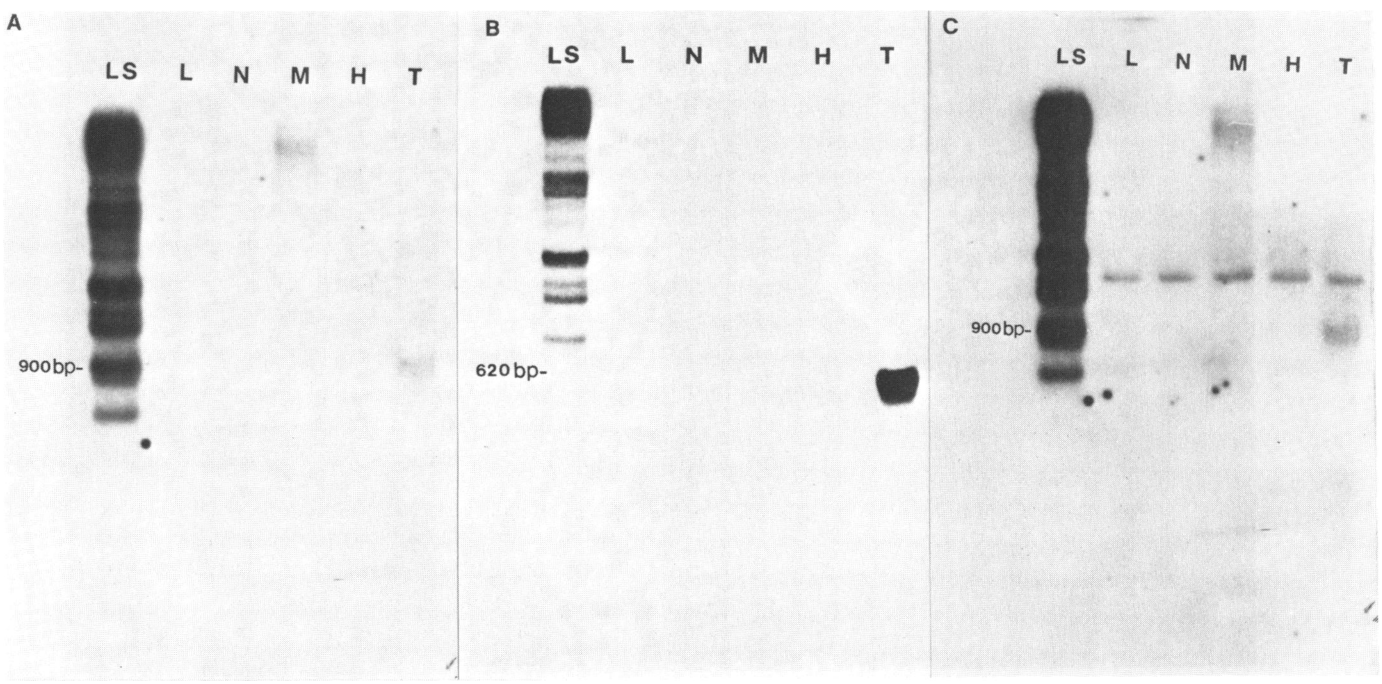

Figure 3: Northern blot hybridization of total RNA (20 μg) from boar liver (L), kidney (N), spleen (M), brain (H) and testis (T) to 100 ng of the porcine protamine cDNAs (10^7 cpm/μg) under conditions of high stringency. A: Protamine 2 cDNA (clone BP 2-1). Exposure time 36h. B: Protamine 1 cDNA (25). Exposure time 7h. C: Internal hybridization control with α-Actin cDNA.

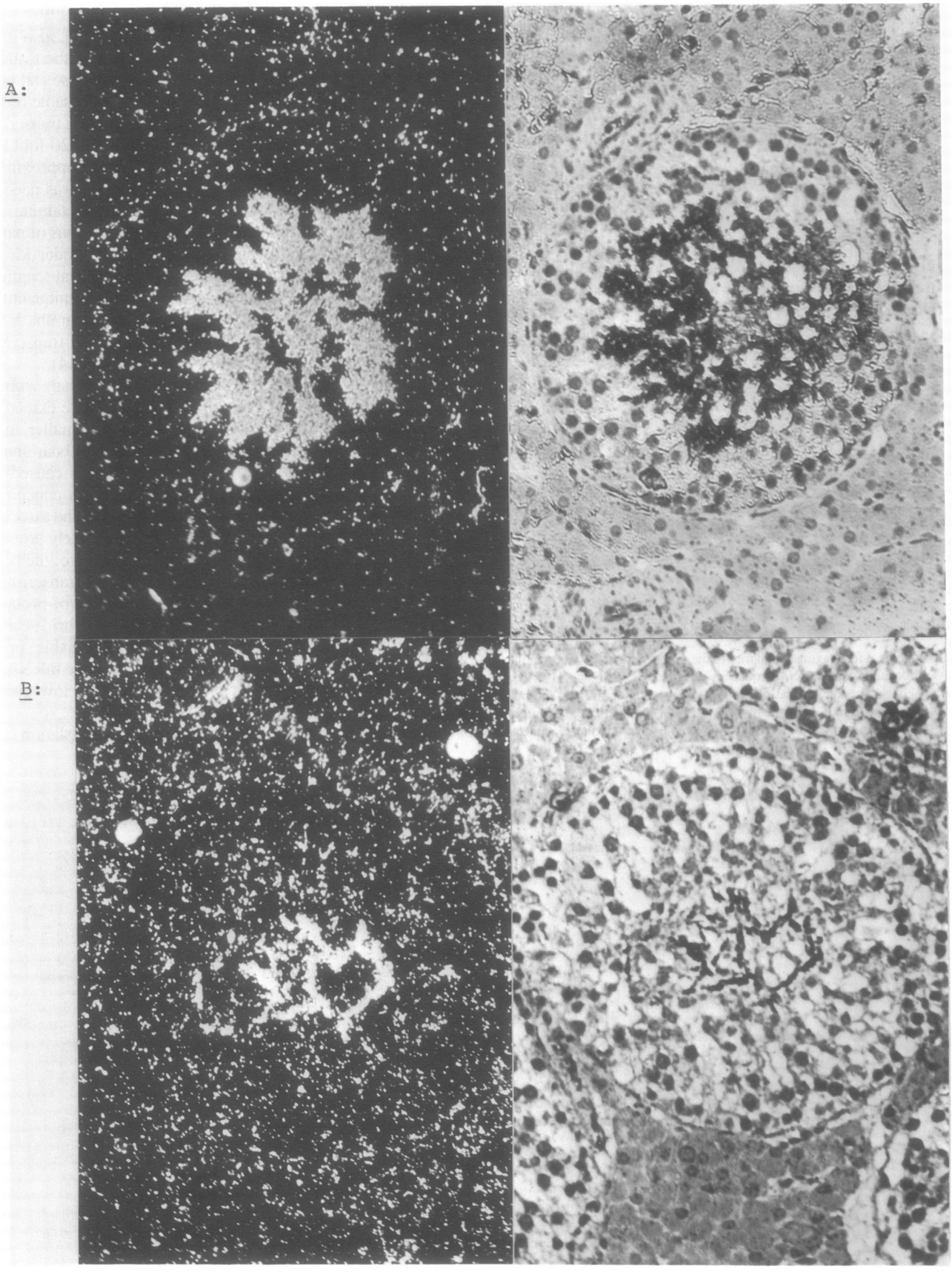

Figure 4: In situ hybridization of 5 ng of boar protamine P1 (25) and P2 cDNAs to paraffin sections of boar testis. Both cDNAs were labeled to similar specific activities (10^8 cpm/μg) and hybridization conditions were identical. Note that hybridization signals are limited to spermatids.
A: Dark field and bright field micrograph with protamine 1 cDNA, *B*: Dark field and bright field micrograph with protamine 2 cDNA.

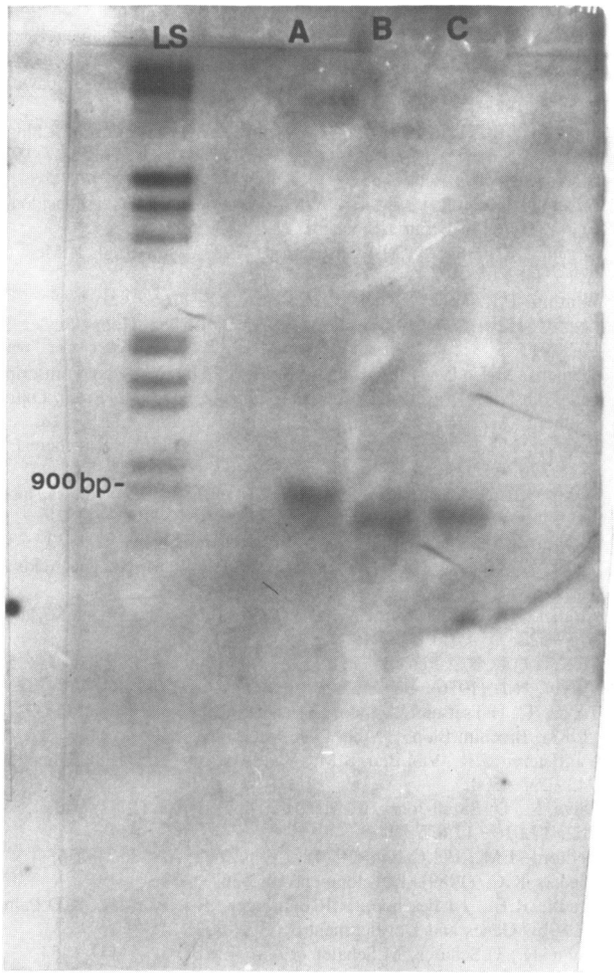

Figure 5: Northern blot hybridization of RNA-preparations (20 μg) from boar testis to 100 ng of the boar protamine 2 cDNA BP2-1 (10^7 cpm/μg). A: Total RNA, B: RNA deadenylated with RNase-H, C: RNA isolated from polysomes.

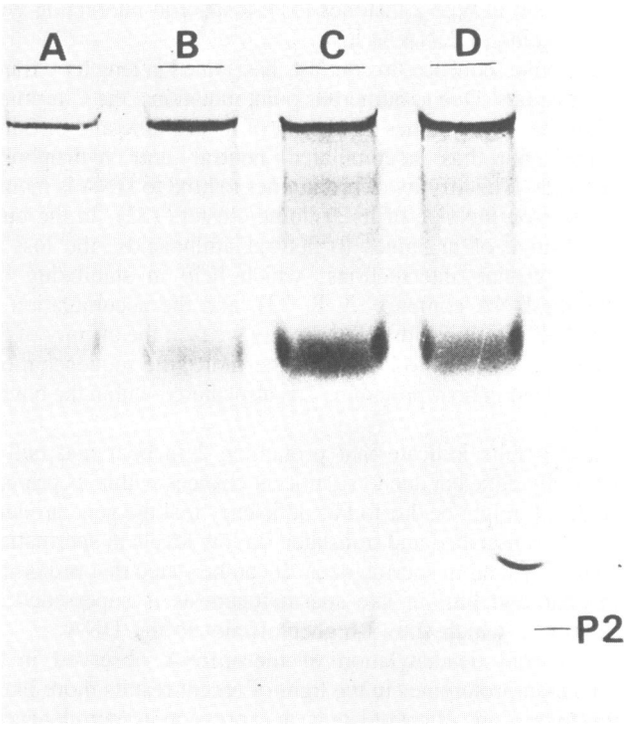

Figure 6: In vitro translation of mRNA probes for bovine (lanes A and C) and human (lanes B and D) protamine 2. For one assay mRNA was isolated from 1 mg of total testicular RNA by hybrid selection (lanes A and B). The second reaction was carried out with 1 μg of mRNA transcribed from cDNA (lanes C and D).

non-functional protamine 2 in boar and bull is regulated in a similar way as it is the case for the protamines when functional.

DISCUSSION

Protamine 2 has to date been isolated only from murine and human spermatozoa, where it represents the major fraction of nuclear protein (13, 28, 29). In the present study we have demonstrated that the protamine 2 gene is transcribed and translated as well in boar and bull, where, like in most other mammals, the gene product is not present in the sperm nuclei (16, 17, 30). This complete absence can hardly be explained by only quantitative differences in gene expression of the protamines. More likely, protamine 2 in boar and bull is superseded by protamine 1 and degraded during the late steps of spermiogenesis.

In this case, at the least trace amounts of protamine 2 should be detectable transiently in spermatids. Actually, in electrophoretic investigations of proteins extracted from spermatids and spermatozoa of the boar, in spermatids a protein was found, which migrated close to the protamine 1 signal and was no more present when mature spermatozoa were investigated (2, 16, 30). It can be suggested that this protein fraction, counting for about 5% of the quantity of protamine 1, might represent the mature form of the porcine protamine 2, which shows similar properties in charge and weight and therefore can be expected to be found near the protamine 1 fraction in electrophoresis.

Unlike protamine 1, protamine 2 is synthesized in a precursor form and is cleaved to the mature protein by cleavage of its amino-terminal leader sequence (11, 12). Figure 2 shows an alignment of the protamine 2 precursors from mouse and human and sequences deduced from boar and bull cDNAs. Regions of clear homology within all 4 sequences are only found in the aminoterminus, were 5 out of 7 residues are conserved or equivalent in charge, and around the cleavage site of the mature protamine 2. The conservation of this region suggests that the processing of the protamine 2 precursor should not be disturbed in boar and bull. Within the mature protamine 2 from mouse and human, clusters of arginine and histidine are spread over the whole length of the molecule, interrupted in regular intervals only by small intersections of neutral aminoacids and cysteine. This primary structure found in the functional protamines is altered significantly in the bovine and porcine sequences as deduced from the cDNAs isolated:

The porcine protamine 2 exibits a deletion of about 8 aminoacids, which has removed parts of the first basic cluster in the amino-terminus of the molecule, while the central and carboxyterminal regions of the sequence are well conserved as compared to the murine and human protamines. Within the region deleted in boar, the functional protamines both exibit the remarkable sequence motif R-L-H-R-I-H (figure 2, underlined) which contains the only hydrophobic aminoacids present in the mature protamine 2 of mouse and human. It is very likely that this conserved motif plays an important role in protamine 2 function. Maybe due to the loss of this sequence element, which

is also found in type 2 histones (31), a specific interaction with other proteins or DNA is lost.

The bovine sequence, in contrast, has gained in length by frame shift mutations. Due to numerous point mutations, the C-terminal third of the sequence has lost many of its arginine and histidine residues, but has accumulated neutral and hydrophobic aminoacids. The affinity of protamines to bind to DNA is mainly dependent on the size of its arginine clusters (32). In the bull, the exchange of arginines to neutral aminoacids, the loss of regular cysteine intersections, which help in stabilizing the protamine-DNA complex (3, 8, 33), and the accumulation of hydrophobic aminoacids will severely weaken the affinity of the translational product to DNA. Furthermore, the sequence motif found deleted in boar protamine 2 is also altered within the bovine sequence.

These results indicate that protamine 2 in boar and bull is functionally deficient due to significant changes within its primary structure. It might be due to this deficiency that the gene product, although transcribed and translated on low levels in spermatids, is no more found in spermatozoa. It can be stated that protamine 2 in boar and bull in late spermatogenesis is superseded by protamine 1 which shows higher affinity to the DNA.

The partial deadenylation of the mRNA observed in all mammalian protamines in the light of recent results more likely seems to be a side effect of late gene expression in spermiogenesis but a significant feature of gene control (35): Molecular cloning of 3'untranslated sequences of protamine 1 has led to delayed translation of transgenes in the murine system (36) as well as 5'untranslated sequence motifs did in Drosophila (37). It long has been known that the activity of RNA polymerase (also poly (A) polymerase?) is ceased in elongating cell stages of spermiogenesis (38) and more likely to its inhibition, poly (A) sequences do promote efficient translation of mRNAs in multiple systems (39–41). It could therefore be due to the lack of competing poly (A) mRNAs that the protamine transcripts are translated in late spermiogenesis *although* they are partially deadenylated.

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