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Stage and developmental specific gene expression during mammalian spermatogenesis

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ABSTRACT Spermatogenesis is a complex developmental process which involves amplification of germinal stem cells, their differentiation into spermatocytes, meiotic division and finally transformation into mature spermatozoa. Therefore, spermatogenesis provides an interesting system for examining the regulation of gene expression during development and differentiation. The genes expressed during spermatogenesis can be divided into two main groups: diploid and haploid expressed genes. In this review we report about the regulation of expression of a diploid expressed gene, namely the proacrosin gene, and that of a haploid expressed gene, the transition protein 2 gene.

KEY WORDS: *spermatogenesis, gene regulation, transgenic mice, DNA-protein interaction*

Introduction

The highly specialized spermatozoon is the result of the unique developmental process of spermatogenesis. Spermatogenesis in mammals takes place within the seminiferous epithelium lining the seminiferous tubule. The seminiferous epithelium is comprised solely of spermatogenic cells and Sertoli cells. The Sertoli cells envelop and support the germinal cells, providing the framework and environment for their development in the seminiferous tubule. Although spermatogenesis encompasses three phases of germ cell development, namely from spermatogonia to spermatocytes to spermatids, all of the different germ cell types are constitutively present in the testis of the adult male (McCarrey, 1993). Therefore spermatogenesis presents a unique opportunity to study cell commitment and differentiation.

From a genetic point of view, spermatogenesis can be divided into two parts, namely the diploid and the haploid phase. During the diploid phase two meiotic divisions occur resulting in round haploid spermatids. During the haploid phase, which is called spermiogenesis, the morphological and functional characteristics of the spermatozoon are determined (Clermont *et al.*, 1993). The basic features of spermiogenesis are common to all mammals. Acrosome development and flagellar formation begin in round spermatids (Escalier *et al.*, 1991), while in elongating spermatids the nucleus condenses and the cells become highly polarized (Hamilton and Waites, 1990). Spermiogenesis lasts about 2 weeks in mouse, 3 weeks in rat and 5 weeks in human.

Furthermore, as compared to other cell types in the body, the differentiation process occurring during spermatogenesis is unique because primary spermatocytes are genetically diploid but functionally tetraploid, round spermatids are genetically and functionally haploid while elongating spermatids become functionally anucleate due to ongoing condensation of the chromatin resulting in an extreme packaging of the DNA in the nucleus of the mature spermatozoon. Nevertheless the structures which are formed during spermiogenesis require the synthesis of new proteins. Because the progressive condensation of the chromatin shuts off all RNA synthesis it was assumed that protein synthesis during spermiogenesis and the differentiation of the haploid spermatids is mainly dependent on stored mRNAs derived from the diploid phase of spermatogenesis. Numerous genetic observations supported the hypothesis of diploid control of spermiogenesis and the results of autoradiographic and biochemical studies, especially in mouse spermatogenesis, have demonstrated that a considerable proportion of RNA produced in meiosis is preserved until late spermiogenesis (Hecht, 1986). However, during the last few years, the active participation of the haploid genome in sperm differentiation has been evaluated in mouse, rat, bull and human. In 1977 we presented the first evidence for gene transcription in haploid spermatids (Schmid *et al.*, 1977). We demonstrated the postmeiotic expression of ribosomal RNA genes during male gametogenesis. In the following years, using cDNA probes for germ cell specific genes, the haploid expression of protamines, transition proteins, specific isotypes of actin, α- and ß-tubulin and for protooncogenes has been demonstrated (Hecht, 1993).

Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; TNP, transition protein; UTR, untranslated region.

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We have isolated and characterized the cDNAs and the genes for several male germ cell specific genes including protamines (Klemm *et al.,* 1989, Domenjoud *et al.,* 1990; Kremling *et al.,* 1992), transition proteins (Luerssen *et al.,* 1988, 1989; Kremling *et al.,* 1989, Keime *et al.,* 1992), proacrosin (Adham *et al.,* 1989, 1990; Keime *et al.,* 1990; Klemm *et al.,* 1990, 1991a; Kremling *et al.,* 1991a,b) and mitochondrial selenoprotein in different mammals. Furthermore, transcriptional and translational regulation of proacrosin and transition protein 2 were studied.

On the structure and function of proacrosin and germ cell specific basic nuclear proteins

Proacrosin

Acrosin (EC 3.4.21.10) is a serine protease that is localized in the sperm acrosome as an enzymatically inactive zymogen proacrosin, and is released as a consequence of the acrosome reaction. Acrosin is composed of two chains, a light chain and a heavy chain, which are connected via disulfide bridges. The light chain of approximately 23 amino acids contains a single carbohydrate attachment site and represents the activation peptide of the corresponding proenzyme. The heavy chain is of approximately 300-400 amino acid residues, and its N-terminal part contains the typical structural elements of a serine protease including the catalytic triad. A C-terminal extension of the heavy chain is rich in proline (18-34%), a feature that is unique to the acrosin-type of protease. The structure of the proline-rich domain is highly variable among the acrosin molecules from diverse species (Klemm *et al.,* 1991b).

Proacrosin is believed to play an important role in the initial stage of fertilization by providing both a trypsin-like serine protease activity and a lectin-like carbohydrate binding activity (Jones *et al.,* 1988). Because the proline-rich domain shows lower homology between the different species than the proteolytic domain, it has been suggested that the proline-rich domain could be implicated in species-specific recognition and binding of sperm to the zona pellucida of the ovum. Furthermore, this domain with its high proline content and its similarity to DNA-associated and/or DNA-binding proteins was suggested to be involved in gene regulation processes during early embryogenesis (Klemm *et al.,* 1991b).

The cDNA of proacrosin was isolated from different species (Adham *et al.,* 1989b, 1990; Klemm *et al.,* 1990, 1991a). Using the cDNAs we have isolated the genes for proacrosin of mouse, rat, boar, bull and human (Keime *et al.,* 1990; Kremling *et al.,* 1991a,b, 1994a). The exon-intron structure of the proacrosin genes is given in figure 1. The coding region of the proacrosin gene encompasses 5 exons. In mouse and rat an additional exon of 65 bp was identified in the 5'untranslated region. The exons of the proacrosin gene are arranged in two clusters, which are separated by a large intron. The proacrosin gene was assigned to human chromosome 22, region q13-qter, to mouse chromosome 15, band E/F and to rat chromosome 7 (Adham *et al.,* 1989a, 1991; Kremling *et al.,* 1991a,b).

Basic nuclear proteins (protamine 1 and 2, transition protein 1 and 2)

In mammals, early spermatids contain a mixture of somatic as well as germ cell-specific variants of histones. During the elongation and condensation of the spermatid nucleus, the histones are replaced by spermatid-specific transition proteins (TNPs) and finally by protamines, which are the principal basic nuclear proteins of mature sperm (Hecht, 1989; Balhorn, 1989). Protamines are small molecules, containing a large amount of arginine and cysteine, bind to DNA through hydrophobic and electrostatic interactions and package DNA into the form known as nucleoprotamine. As a consequence of the interaction occurring between DNA and protamine, the phosphodiester backbone of DNA is neutralized, the fiber nucleoprotamine condenses and the genome is rendered inactive (Balhorn, 1989).

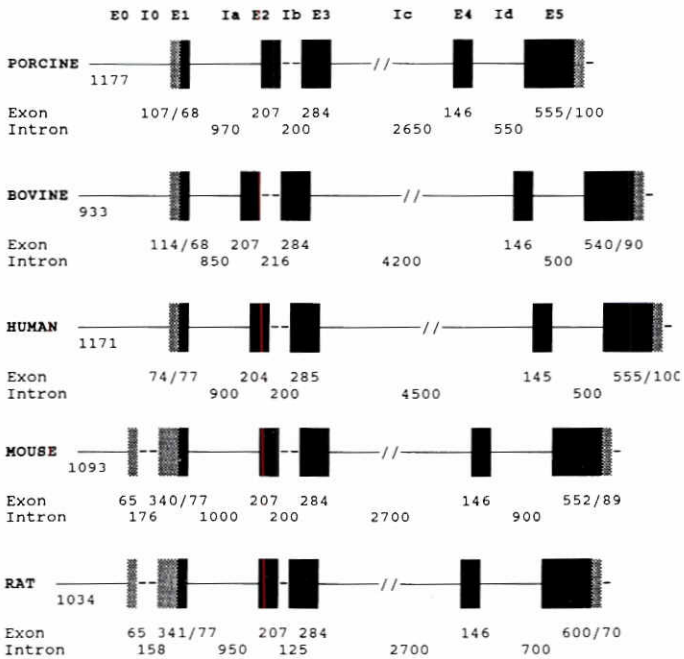

Fig. 1. Exon-intron structure of the proacrosin gene of different mammalian species. *The solid bars indicate the amino acid coding regions. The lines indicate the intron and untranscribed region and shaded boxes represent the untranslated region. The numbers indicate the length of the fragments in bp.*

The cDNAs and genes of protamine 1 and 2 have been isolated from human, bovine, rat and porcine (Lee et al., 1987a,b; Klemm *et al.,* 1989; Domenjoud *et al.,* 1990; Maier *et al.,* 1990; Reinhart *et al.,* 1991; Keime *et al.,* 1992b, Kremling *et al.,* 1992). The genes for protamine 1 and 2 both consist of two exons. Both genes are clustered in the genome of human, mouse, rat, bovine and porcine (Fig. 2). Sequence analysis of the protamine 2 cDNAs of bull and boar, which lack the gene product in the mature sperms showed that mutations and deletions are present in a region, which is probably of functional relevance (Maier *et al.,* 1990). Furthermore it was found that protamine 2 of the bull exists in two variants differing in length in the testis.

cDNA and genomic clones for transition protein 1 and 2 of several species have been isolated (Luerssen *et al.,* 1989, 1990; Kremling *et al.,* 1989; Kim *et al.,* 1989, 1992; Schlüter *et al.,* 1992). Transition protein 1 encodes a protein of 54 amino acids, which is highly conserved among mammals. From the cDNA of transition protein 2 a polypeptide of approximately 140 amino acids can be deduced. The genes for both transition proteins are

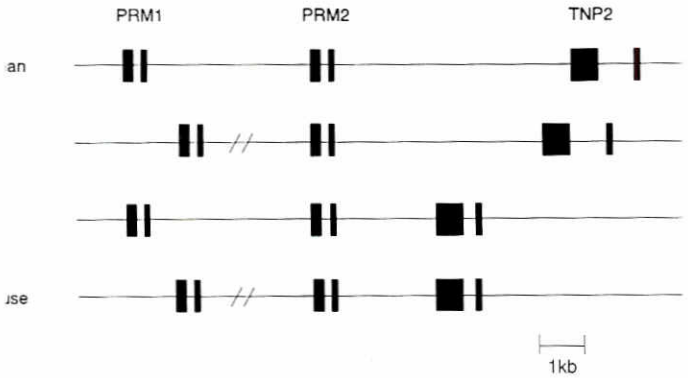

Fig. 2. Genomic organization of the genes for nuclear proteins protamine 1 and 2 (PRM1, PRM2) and transition protein 2 (TNP2) of different mammals. *The solid bars indicate the coding regions and the lines represent the untranslated and flanking regions.*

interrupted by one single intron. The gene for transition protein 2 was found to be closely linked to the protamine cluster in human, mouse, rat, bovine and porcine (Fig. 2), whereas the gene for transition protein 1 is located on another chromosome (Engel *et al.*, 1992). The chromosomal localization of the protamine/transition protein 2 cluster is in human and mouse on chromosome 16, in rat on chromosome 10 (Adham *et al.*, 1991). The gene for transition protein 1 is located on chromosome 2 in human (Luerssen *et al.*, 1990), on chromosome 1 in mouse (Lueressen *et al.*, 1990) and on chromosome 9 in rat (Adham *et al.*, 1991).

Transcriptional and translational regulation of the genes for proacrosin and transition protein 2

It is now well established that the regulation of gene expression occurs at the level of gene transcription and mRNA translation. For the regulation of gene transcription in a specific temporal and spatial manner the formation of a transcription initiation complex between the DNA sequence elements, usually located in the 5'-flanking region of the gene, and specific transcription factors is necessary (Latchman, 1992). Different techniques such as transgenic mice, DNaseI-footprinting and *in vitro*-transcription, have already been used to identify the cis-acting DNA sequences, which are required for specific gene expression in the male germ cells (Stewart *et al.*, 1988; Robinson *et al.*, 1989; Gebara and McCarrey, 1992; Zambrowicz *et al.*, 1993). Another approach for identification of these DNA motifs is the comparison of the 5'flanking regions of different germ cell specific expressed genes. It can be suggested that genes expressed in male germ cells are activated by a common regulatory or signalling mechanism, possibly involving identical transcription factors. If this is the case, germ cell specific genes would be expected to share common DNA-binding sites for such factors. Till now, several DNA sequences which are suggested to be involved in the regulation of specific expression of genes in male germ cells, have been identified (Hecht, 1993).

In recent years we have used different techniques to identify the cis-acting DNA sequences and trans-acting nuclear factors which are involved in the regulation of the proacrosin gene. In different mammalian species, biosynthesis of the zymogen proacrosin was found to start in early round spermatids which are haploid spermatogenic cells (Flörke *et al.*, 1983). While during mouse and rat spermatogenesis proacrosin gene transcription is first observed in pachytene spermatocytes, which are diploid spermatogenic cells (Kremling *et al.*, 1991b; Nayernia *et al.*, 1993, 1994a,b), in bull, boar and human proacrosin gene transcription is reported to first occur in haploid round spermatids (Adham *et al.*, 1989b). Transgenic approaches have been used to demonstrate that 2.3 kb of proacrosin 5'flanking sequence is sufficient to confer germ cell specific expression on the CAT reporter gene (Nayernia *et al.*, 1992). The CAT gene is first transcribed in pachytene spermatocytes while enzyme activity is first detected in round spermatids. The mRNA for the proacrosin-CAT transgene and for the endogenous mouse proacrosin gene were found for the first time in the testis of 17-day-old mice but the CAT protein was first observed in testis of 21-day-old mice. This correlates with the appearance of pachytene spermatocytes and early spermatids during testicular differentiation, respectively. These studies showed that 2.3 kb of the 5'flanking region of the proacrosin gene directs not only the specific expression of this gene in male germ cells but is also able to regulate the transcription and translation during testicular development and germ cell differentiation. For detailed analysis additional transgenic lines have been generated which included deletions in the 5'flanking region (Nayernia, *et al.*, 1994a). The analysis of transgenic lines harboring 900 bp of 5'flanking region demonstrated that the spatial and temporal expression of this transgene mimics the expression of the transgene which contains 2.3 kb of the 5'flanking region. The shortening of the 5' flanking region from 900 bp to 400 bp upstream of ATG results in a decrease of CAT activity in the male germ cells of transgenic mice. Therefore, it can be concluded that the DNA sequences located between the 400 bp and 900 bp upstream of ATG can function as enhancer elements. For further characterization of DNA cis-acting elements we sequenced about 1 kb of the 5' flanking region of the proaccrosin gene of different mammals. It is possible that a common cis-acting DNA element in the 5' flanking region of the proacrosin gene could be recognized by specific transcription factors which are involved in the regulation of this gene. We compared the 5' flanking region of the proacrosin gene of different mammals, and we identified the conserved DNA motifs in this region (Table 1). Using this approach and DNase I-footprint experiments with different fragments from the 5'flanking region of the rat proacrosin gene and testicular nuclear proteins we were able to identify DNA sequences which could be involved in the regulation of the proacrosin gene (Kremling *et al.*, 1994b). These DNA sequences are shown in Table 1. The mutational analysis of these DNA sequences and generation of transgenic animals are necessary to demonstrate whether any of these sequences function as cis-acting regulatory elements.

The regulation of gene expression during mammalian spermatogenesis occurs not only at the level of gene transcription but also for many germ cell specific expressed genes at the post-transcriptional level. It was shown that an unusually high proportion of testicular Poly $(A)^+$-RNA was nonpolysomal, and this is evidence for post-transcriptional regulation of most testis expressed genes (Hecht, 1993). The 5' and 3'-untranslated regions of mRNA are known to play an important role in post-transcriptional regulation of gene expression. For the protamine gene it was shown that the 3'-untranslated sequence from the mouse protamine gene delayed the translation of a human

growth hormone reporter gene in transgenic mice from early round spermatids to elongating spermatids where the protamine 1 mRNA is normally translated (Braun *et al.*, 1989). We have also observed a delay between transcription and translation for the proacrosin gene (Nayernia *et al.*, 1992). While the proacrosin-CAT fusion gene as well as the endogenous mouse and rat proacrosin genes are transcribed in pachytene spermatocytes, translation of the mRNAs first occur in early round spermatids which is four days later in germ cell development. This is an indication of translational control of proacrosin gene expression. Although the proacrosin-CAT fusion gene lacks the 3'untranslated region of the endogenous proacrosin-gene, this transgene is translationally regulated in an identical manner to the endogenous mouse proacrosin gene. Thus, the 5'untranslated region of proacrosin mRNA should contain a sequence for binding germ cell specific cytoplasmic proteins which hinders translation in spermatocytes or activates translation in spermatids. This assumption is supported by the result of our experiments that *in vitro* transcribed RNA of the 5'untranslated region is able to bind cytoplasmic proteins of germ cells (Nayernia *et al.*, 1994b). In contrast to the proacrosin gene, the posttranscriptional regulation of transition protein 2 is controlled by the nucleotide sequences, which are located in the 3' untranslated region (Schlüter *et al.*, 1993). The genes for transition proteins (TNPs) are exclusively expressed in round spermatids and mRNA is stored in a translationally repressed state until translation starts 4-6 days later. The human TNP2 gene, in contrast to its counterparts in other mammalian species, is expressed at a very low level. It is suggested that an intriguing deletion of the conserved motif GCYATCAY in the 3'UTR of the human gene influences the TNP2 mRNA level. This conserved 8bp motif is present in the 3'UTR of the TNP2 gene of mouse, rat, bull and boar which all express the gene at a high level. Storage of TNP2 mRNA in human testis would then fail due to the absence of the appropriate protein binding site. This hypothesis is supported by the result of RNA-bandshift experiments. Wildtype rat TNP2 3'UTR is able to bind a cytoplasmic protein factor from rat testis while a mutated 3'UTR, lacking the 8bp motif, is not able to bind the cytoplasmic protein extract. Further experiment will be aimed at identifying the regulatory elements controlling translation of the proacrosin and transition protein genes.

TABLE 1

THE PUTATIVE TESTIS SPECIFIC CIS-ACTING DNA ELEMENTS IN THE 5'FLANKING REGION OF THE RAT PROACROSIN GENE

DNA sequence	position	(upstream of ATG)	
C1: AGCTTTGTGAGGTCACAGCTTGCAGGCC	-17	to	-43
C2: GGGTGGGGGTGGG	- 78	to	-90
F3a: ATAAAGTGAGACGTCAGAAGG	- 247	to	-267
F3b: GCCAAGGATGAATGAAGGTC	- 271	to	-291
TS3: GCAGAACCCGATTTCTT	- 380	to	-396
F1: AACTTCAAAATGGCTCC	-457	to	-473
F7b: TGATAATCAGATATGTATAAATAAAGACG	-632	to	-661
F7a: ATCATAGTACGTGGCACGCACCTGTCATACTG	-666	to	-699
C3: GAGAGGATACCT	-727	to	-738
C4: AAGTAAGACATTCAGTTA	-1014	to	-1032

C, conserved elements; TS and F, footprint elements

Concluding remarks

It is suggested that in about 20% of infertile men, infertility is due to mutations in germ cell specific genes. However, in no case of morphological and/or functional sperm defect has the molecular nature of the defect been demonstrated at the DNA level. Even in patients with reduced acrosin activity or disturbances of chromatin condensation no mutations in the proacrosin gene or in the genes for protamines and transition protein 1 were found. It can be suggested that the functions of these genes are others than binding and penetrating the zona pellucida and condensation of the sperm chromatin, respectively. We therefore started to produce knock out mice for these genes. The evaluation of the resulting sperm phenotypes could help to analyse the genetic causes in patients with male fertility. Furthermore, we are continuing our efforts to isolate other genes specifically expressed in male gametogenesis. These genes can be used as probes for DNA-studies in infertile men.

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