Gene expression in mouse spermatogenesis during ontogenesis

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Abstract. In this study, we evaluated the expression of genes probably involved in spermatogenesis in the mouse. We examined cytosolic chaperonin theta subunit (CCTθ), Ngg1 interacting factor 3 like 1 binding protein 1 (NIF3L1 BP1) and apolipoprotein H (ApoH) expression during mouse ontogeny using RT-PCR. Testicular tissue was obtained from mice 3, 6, 8, 10, 12, 14, 18, 20 and 40 (adult) days after birth. For each mouse, one testis was used for histological examination, whereas RNA was extracted from the controlateral testis for expression analysis. RT-PCR analysis showed that CCTθ gene expression was low until day 10, but increased drastically afterwards. At this age, spermatocytes started to be present in the mouse testis. Therefore, CCT protein could be involved in chromatin packaging and remodeling during spermiogenesis, as also suggested by other studies. NIF3L1 BP1 expression increased steadily during ontogenesis reaching maximum levels in the adult mouse when all germ cell stages are present. This finding suggests that NIF3L1 BP1 is a gene not expressed by a specific germ cell type. ApoH expression was very low or absent during prepuberal stages, whereas it was detectable in the adult testis when spermatogenesis was completed. This suggests that ApoH may be involved in clearing apoptotic bodies during spermatogenesis since apoptotic events increase during spermatogenesis. This study contributes to understanding the role played by genes important for spermatogenesis.

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Introduction

Spermatogenesis is a complex biological process controlled by many genes which start acting in the prenatal life and regulate the production of mature spermatozoa in adults. Although an important part in spermatogenesis regulation is played by the so-called azoospermia factor (AZF) genes located on the Y chromosome long arm (Yq11) (1), other genes are involved in this process, but the role of some of them is not known yet (2,3). The aim of many studies has been to contribute to the understanding of which genes are important for spermatogenesis. We focused our attention on the expression of the following three genes: cytosolic chaperonin θ subunit (CCTθ), Ngg1 interacting factor 3 like 1 binding protein 1 (NIF3L1 BP1) and apolipoprotein H (ApoH).

CCTθ and NIF3L1 BP1 are two genes which we found, by differential display mRNA technique, to be differently expressed in the testicular biopsy of a patient with obstructive azoospermia and normal spermatogenesis, utilized as control, compared with the biopsy of an azoospermic patient with Sertoli cell-only syndrome (SCOS) and Y chromosome microdeletion (AZFb and AZFc regions). RT-PCR results showed that significant CCTθ and NIF3L1 BP1 expression occurred in the control testis, whereas a lower expression was present in the testis of the patient with SCOS.

The human CCTθ gene maps on chromosome 21q22.11. It codifies a subunit of the hetero-oligomeric molecular chaperone (CCT), a member of the chaperonin family, which plays an important role in the refolding of denaturated protein and in the folding of actively synthesized protein in the cytosol of mammalian cells (4-7). Soues and colleagues unveiled two main cytoplasmic localizations of CCT during spermatogenesis: the centrosome and the microtubules of the 'manchette', a structure unique to male germ cells, both of which are essential for spermatid differentiation (8).

The human NIF3L1 BP1 gene maps on chromosome 3p14.1 and encodes a recently characterized protein bearing a putative leucine zipper domain necessary for interaction with NIF3L1. This is a protein which prevents NIF3L1 BP1 from binding DNA (9).

The human ApoH gene maps on chromosome 17q23. The gene encodes a serum glycoprotein whose physiological role has not been clarified. *In vitro*, there is evidence that this protein binds anionic phospholipids, platelets, heparin, DNA and mitochondria (10,11). ApoH is involved in blood coagulation processes with an inhibitory effect on ADP-mediated platelet aggregation and on prothrombinase activity (12,13). In addition, it has a role in lipoprotein metabolism (14). Although the major site of ApoH synthesis is the liver, its expression seems to be ubiquitous (15). Our previous study (15) suggested that ApoH is implicated in membrane remodeling, clearing apoptotic bodies (16,17) and proliferation processes. RT-PCR showed that ApoH gene expression is up-regulated in human SCOS testis compared with the control.

Altogether these findings suggested that CCTθ, NIF3L1 BP1 and ApoH genes may be involved in the regulation of spermatogenesis. We therefore evaluated their expression in the mouse testis explanted at different ages. This model was chosen because murine spermatogenesis is homologous to the human one and gene products are often similar. In addition, murine testes have, at different ages, a homogenous germ cell population (18) which may hint at the possible function of these gene products.

Materials and methods

Testicular samples. CD1 SPF/VAF mice were sacrificed 3, 6, 8, 10, 12, 14, 18, 20 and 40 (adult) days after birth. For each mouse, one testis was used for histological examination, whereas RNA was extracted from the controlateral one for mRNA expression analysis. Animals used in this study were maintained and sacrificed according to procedure described in the NIH guide for the care and use of laboratory animals.

Tissue preparation and histological examination. The testes were excised, decapsulated and immersed in a fixative containing 2.5% gluteraldehyde and 2% paraformaldehyde in phosphate buffer pH 7.4 for 2 h at 4˚C. After timing, specimens were post-fixed in 1% osmium tetroxide for 1 h at 4˚C. Finally, samples were embedded in Epon resin (TAAB, UK). Sections (0.7 μm) were stained with Richardson's stain (19) and examined by light microscopy.

RNA extraction and RT-PCR. Total RNA was extracted from decapsulated testis by the acid guanidinium thiocyanate method (20). The concentration of RNA was determined by spectrophotometry at 260 nm absorbance. The contaminated DNA was removed by DNAse I (Gibco BRL) digestion. One μg of total RNA was transcribed into cDNA by incubation at 37˚C for 60 min in 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 μg random esamers (Pharmacia, Uppsala, Sweden) and 200 U Moloney murine leucemia virus reverse transcriptase (RT) (Amersham, Cleveland, OH) in a total volume of 20 μl. The PCR reactions were performed in a 50 μl mix containing 1X PCR buffer (Invitrogen), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP (Invitrogen), 1 μM of each primer, 2.5 U of TaqDNA polymerase, recombinant (Invitrogen) and 5 μl of cDNA.

Thermocycling conditions consisted of an initial denaturation of 5 min at 94˚C, 35 cycles of 1 min at 94˚C, 1 min at specific primer annealing temperature (62˚C for CCTθ: forward 5'-gcctgtcagtatcagaggta-3', reverse 5'-taca gagtcgtctaggatcc-3'; 59˚C for NIF3L1 BP1: forward 5'-gctga agttcatggatgg-3', reverse 5'-gatgttgcttcctaccac-3'; 55˚C for ApoH: forward 5'-tattcctgcttgtgctcg-3', reverse 5'-ggcaagaagg accaagtt-3'). As an endogenous internal control, the phosphoglycerate kinase-1 gene (PGK-1) was co-amplified (PGK: forward 5'-aggtgctcaacaacatgg-3', reverse 5'-ccagtcttggcatt ctca-3'). RT-PCR products were separated by electrophoresis in a 1% agarose gel in TBE 1X buffer. The ethidium bromide-stained gels were visualized using an ultraviolet light source and photographed on a gel video imager. Amplified products for CCTθ, NIF3L1 BP1, ApoH and PGK-1 genes were respectively 611, 398, 374 and 162 bp. The same experiment was repeated in order to confirm the RT-PCR results.

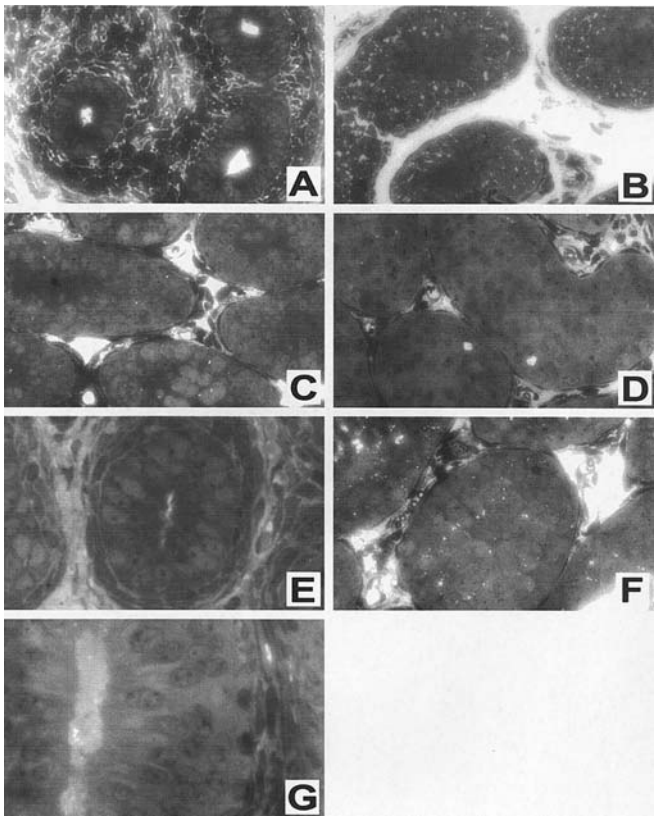

Figure 1. Microphotographs of mouse testicular tissue explanted at different ages showing a homogeneous cellular populations of Sertoli cells and primitive type A spermatogonia (6 days) (A), type A and B spermatogonia (8 days) (B), primary spermatocyte at preleptotene, zhygotene, pachytene stages (10, 12, 14 and 18 days) (C, D and E, respectively), secondary spermatocytes and haploid spermatids (18 and 20 days) (F and G, respectively).

Results

Murine testes at different ages had homogeneous cellular populations of Sertoli cells and primitive type A spermatogonia (3 and 6 days), type A and B spermatogonia (8 days), primary spermatocyte at preleptotene, zhygotene, pachytene stages (10, 12, 14 and 18 days), secondary spermatocytes and haploid spermatids (18 and 20 days) (Fig. 1).

During the development of the prepuberal mouse, CCTθ, NIF3L1 BP1 and ApoH genes were differently expressed. CCTθ gene was barely expressed in the mouse testis at 3, 6

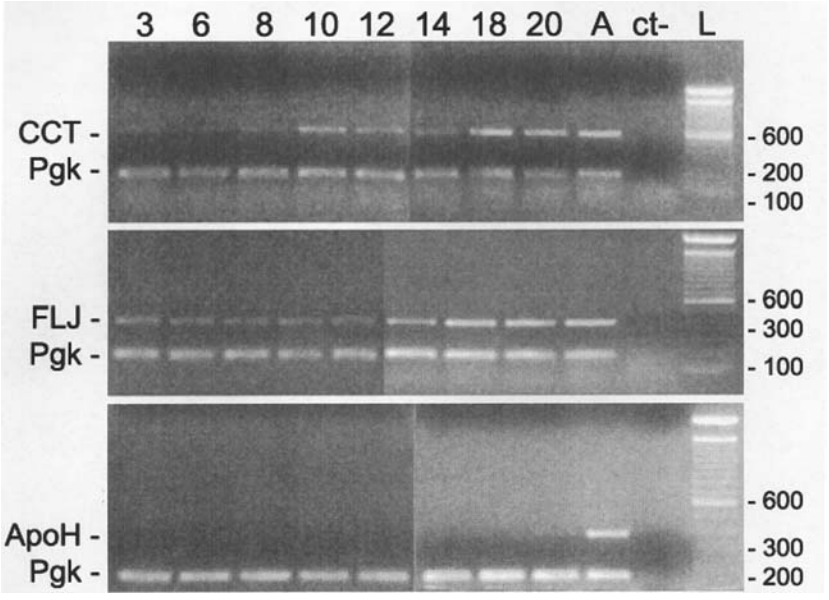

Figure 2. Expression by RT-PCR of cytosolic chaperonin θ subunit (CCT) (upper panel), Ngg1 interacting factor 3 like 1 binding protein 1 (NIF3L1) (FLJ) (middle panel) and apolipoprotein H (ApoH) (lower panel) genes in mouse testicular tissue explanted at different ages post-natally. A, adult testis; Pgk-1, internal control; ct-, no DNA.

and 8 days after birth, when Sertoli cells and type A and B spermatogonia were present. Its expression was increased at 10, 12 and 14 days after birth, when spermatocytes in several stages of meiosis were also present. CCTθ gene expression further increased in day 18 and 20 mouse testes when round spermatids appeared as well as in the adult mouse testis (40 days) when all spermatogenesis cellular populations were present (Fig. 2, upper panel).

NIF3L1 BP1 was steadily expressed at low levels in mouse testes at 3, 6, 8, 10, 12 and 14 days post-natally, while it increased in the testes of 18- and 20-day-old mice and reached maximum levels in the adult mouse, at which point all germ cell stages are present (Fig. 2, middle panel).

ApoH expression was absent or very low until 18 days after birth and was barely detectable at 20 days, whereas it was strongly expressed in the adult testis, at which point all stages of spermatogenesis are present (Fig. 2, lower panel).

Discussion

Spermatogenesis is a complex proliferative and differentiative process which involves the interplay of many gene products, most of which are still unknown. In this study, we evaluated the testicular expression of CCTθ and NIF3L1 BP1 genes because, by differential mRNA display technique, they had a lower expression in the testicular biopsy of a patient with SCOS compared with that of a patient with obstructive azoospermia and normal spermatogenesis (unpublished data). In addition, we studied ApoH as a possible 'candidate' spermatogenesis gene, since its product has been involved in membrane remodelling, lipid trafficking and clearing apoptotic bodies (16,17,21).

Cytosolic chaperonin θ subunit. We found that the CCTθ gene had a low expression pattern at the age of 3, 6 and 8 days post-natally, when only spermatogonia are present, whereas its expression increased drastically at day 10, when spermatocytes appeared in the testis, and remained steady afterwards, when spermatids and spermatozoa are present. The expression of the CCTθ gene in the mouse has recently been reported by Shima *et al* using an Affymetrix gene chip containing 36,000 transcripts (22). This study showed CCTθ expression increasing progressively during post-natal development until day 20 but had a lower expression in the adult mouse while, in our model, CCTθ expression remained elevated in the adult. This divergent result could depend on the different type of mouse used in the two studies. However, further studies will be necessary to verify the correctness of the data and, most importantly, to evaluate protein expression.

CCT is a member of the chaperonin protein family, which includes GroEL of bacteria, HSP60 of mitochondria, Rubisco subunit binding protein of plastids and archa group II chaperonins (4,6). These stress-inducible proteins act as molecular chaperones for the recovery of proteins denaturated by stress (7). In particular, CCT is a molecular chaperone that plays important roles in cell growth by assisting in the folding of actin, tubulin and other cytosolic proteins in the presence of ATP. All CCT subunits contain several highly conserved motifs for ATP binding, but the overall amino-acid sequence identity is only of 30%, suggesting that each subunit has a specific function (23). Although tubulin and actin chains are the main substrates, CCT has been shown to bind a large panel of unrelated unfolded polypeptides of cytosolic origin, such as α-transducin, cofilin and cyclin E (24).

Kubota and colleagues showed, by Western and Northern blot analysis, that all of the subunits are required for CCT function and that CCT expression levels vary greatly among different mouse tissues and cultured cells (23). Yokota and colleagues found that CCT subunits are highly expressed in growing mouse tissues, such as the testis (24).

Apart from cell growth, a tissue-specific requirement of CCT is also possible in particularly differentiated tissues, such

as the testis: CCT is probably necessary to produce tubulins of sperm tail microtubules in testicular germ cells (23).

Soues and colleagues studied CCT cellular distribution throughout rat spermatogenesis by immunofluorescence (IF) and immunoelectron microscopy (IEM) using an anti-CCTα antibody (8). The CCTα subunit was first identified in mouse testis (25), where its expression increases at the onset of meiosis and sperm differentiation (26,27). The results of Soues *et al* indicated that, at the beginning of spermiogenesis, CCT was mainly associated with centromeres of early round spermatids where nucleation of microtubules occurs primarily (8).

During the second phase of spermiogenesis, in elongating spermatids, CCT was associated with the manchette, a structure present during the period of maximum morphogenetic change. This cone-shaped microtubular structure is held by a perinuclear ring of proteic material and it seems implicated both in sperm head nuclear shaping and in caudal redistribution of the cytoplasm.

Molecular chaperone involved in tubulin folding localizes near the manchette because this is a long-lived structure which lasts for almost a week in rodents and thus may need a safeguard system for stabilization and/or renewal. During spermatid maturation, the centrosomes and manchette disorganize themselves and the cytoplasmic pool of CCT shed residual bodies, such as unpolymerized tubulin and most cytoplasmic components, unessential to mature spermatozoa, although a subpopulation of them may still contain these proteins as demonstrated by Western blotting.

IEM allowed the detection of CCTα in the cell nucleus associated with heterochromatin. This localization was not restricted to germ cells but was also observed in Sertoli and Leydig cells. Soues *et al* hypothesized that CCT may be implicated in the maintenance and remodeling of heterochromatin. CCT probably assists the folding of substrates in the nucleus and possibly nuclear tubulin or actin, putative proteins of the nuclear matrix and functional or structural proteins involved in DNA remodeling (8).

Our data indicated that CCTθ subunit expression (and, therefore, presumably also CCT) increased from the moment in which spermatocytes appear. This observation is not in contrast with Soues' results since most of the cells studied by Soues *et al* (8) were of germinal origin: at least 80% of the cells isolated from testes were spermatids and 99-100% of the cells isolated from epididymes were spermatozoa. In addition, we studied CCTθ mRNA expression but not protein localization. CCTθ gene expression further increased when round spermatids appeared and during spermiogenesis, the third phase of spermatogenesis characterized by cytodifferentiation of spermatids. This is an elaborate process that includes nuclear shaping and DNA condensation, flagellum formation, redistribution of cytoplasmic organelles and shedding of the cytoplasm, ending with the release of viable spermatozoa into the lumen of the seminiferous tubules. All these morphological changes are linked to a complete reorganization of the microtubular cytoskeleton which demands the presence of CCT that may play an essential role in spermatogenesis.

Ngg1 interacting factor 3 like 1 binding protein 1. NIF3L1 BP1 expression increased progressively during germ cell maturation, reaching a maximal expression in the adult mouse, at which point all spermatogenetic cell types are present. This finding suggested that NIF3L1 BP1 is a gene which is not expressed by any specific germ cell type. The constant increment of expression observed in this study indicates that this protein could be important for maturation events. Shima *et al* (22), in their expression array study, reported the expression profile of the NIF3L1 gene that codifies for the NIF3L1 protein which interacts with the NIF3L1 BP1 protein in the cell cytoplasm. Interestingly, the expression profile of NIF3L1 shows an opposite pattern with respect to that of NIF3L1 BP1.

The human NIF3L1 BP1 was first described by Kleiderlein and colleagues studying cDNA libraries from human brain (28). Tascou *et al* (9) characterized human and mouse NIF3L1 BP1 proteins, which show 90% homology at the nucleotide level and 97% homology at the amino-acid level. Both represent novel proteins bearing in the carboxy-terminal region a putative leucine zipper-like domain essential for interaction with the NIF3L1 protein. This leucine zipper motif is adjacent to a putative DNA-binding domain consisting of two clusters of amino acids. Deletion analysis showed the relevance of the zipper domain for interaction with NIF3L1 which thereby prevents NIF3L1 BP1 binding to chromosomal DNA (9).

NIF3L1 is a protein strongly conserved during evolution from bacteria to mammals (29). The NIF3L1 gene is ubiquitously expressed with strong overexpression in the spermatogonia-derived cell line, GC-1 spg, and in the teratocarcinoma cell line, F9 (30). The cellular localization of NIF3L1 BP1 expression is comparable with that of the NIF3L1 protein. Both proteins are ubiquitously expressed and both proteins are present in the cytoplasm. NIF3L1 BP1 is also present in the nucleus of the cell, but its amino-acid sequence does not show any nuclear localization signal (NLS); therefore, the protein would be able to diffuse into the nucleus, for example, through the nuclear pore complex or by co-transport with a nuclear protein. However, the interaction of NIF3L1 and NIF3L1 BP1 takes place exclusively in the cytoplasm of the cell. Although, in some tissues, NIF3L1 BP1 works as a repressor (29), its specific cellular function remains to be determined (31,32). The reported higher expression in the spermatogonia (9) suggests a greater involvement of NIF3L1 BP1 in the initial fundamental steps of germinal cell maturation. However, we could not confirm this observation in the mouse testis.

Apolipoprotein H. ApoH expression was very low or absent during the early stages of spermatogenesis, whereas it was detectable when mature germ cells and, therefore, spermatozoa were present. A similar ApoH gene expression profile has been reported by Shima and co-workers (22); in their analysis, ApoH gene expression increased strongly when round spermatids appeared in the testis.

The interactions of ApoH with phospholipids have been considered as a basic mechanism related to its physiological and pathogenic functions and it is reported that ApoH is one of the major proteins appearing in the very rapidly cleared large liposomes that contain phosphatidylcholine, cholesterol and negatively charged phospholipids (phosphatidylserine, phosphatidic acid or CL) (16,17). The ability of ApoH to preferentially bind negatively charged phospholipids has been demonstrated by several laboratories.

Apoptotic bodies have been observed in germ cells of normal testes but not in Sertoli cells, and apoptosis is considered a regulatory mechanism of spermatogenesis in normal and pathological conditions (33). Using several *in vivo* model systems, Balasubramanian *et al* (34) have shown that endogenous ApoH binds apoptotic cells *in situ* irrespective of the triggering pathway; their data provide evidence for a physiological role of ApoH in the recognition and disposal of apoptotic cells *in vivo*. The increased apoptotic processes observed during spermatocyte and spermatid maturation (35-37) suggest that ApoH may perform its biological functions as a 'cleaner', purging the plasmic liposomes coming from senescent and apoptotic cells in mature adult testes, during the late phase of spermatogenesis when its expression increases.

In addition, it is possible to speculate that the expression of ApoH may also increase during inflammatory processes of the male genital tract, which are characterized by an increased apoptotic rate due to reactive oxygen species overproduction by leukocytes and spermatozoa (38). However, the up-regulated ApoH expression found in a patient with SCOS compared to a patient with obstructive azoospermia and normal spermatogenesis suggests that ApoH plays additional function(s) at the testicular level in humans, which deserves further investigation. In this regard, it is noteworthy that a glycoprotein highly homologous to ApoH, isolated from the human follicular fluid, was able to increase the straight line velocity (VSL) and the amplitude of lateral head displacement (ALH) of spermatozoa obtained from normozoospermic men (39).

Moreover, Aleporou-Marinou *et al* (39) proposed that the purified ApoH-like protein may have a local regulatory effect on the mitotic proliferation of granulosa cells. This hypothesis is in agreement with our previous study (40) performed on a rat liver regeneration model. In this work, it was clearly documented that ApoH expression is related to the proliferative status of hepatic cells, being the mRNA and protein expression higher in mitotic elements, and that the protein behaves as a survival factor for HepG2 cells in culture.

In conclusion, CCTθ, NIF3L1 BP1 and ApoH genes are differently expressed in the mouse testis during ontogeny and seem to regulate different aspects of spermatogenesis. In particular, it may be hypothesized that CCTθ participates in chromatin packaging and remodeling during spermiogenesis; NIF3L1 BP1 plays a role in cell differentiation but is not specific of any stage of spermatogenesis, and ApoH may be involved in clearing apoptotic bodies during the last phase of spermatogenesis. The murine model proved useful in studying the mechanisms which regulate spermatogenesis.

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