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Regulation of hsp expression during rodent spermatogenesis

K. D. Sarge* and K. E. Cullen

Department of Biochemistry, College of Medicine, University of Kentucky, 800 Rose Street, Lexington (Kentucky 40536-0084, USA), Fax + 1 606 323 1037, e-mail: kdsarge@pop.uky.edu

Abstract. Spermatogenesis is the process by which immature male germ cells, through a complex series of events involving mitosis, meiosis, and cellular differentiation, eventually become mature spermatozoa capable of fertilizing an ovum. This process involves the developmental progression of male germ cells through a number of spermatogenetic cell types, each of which is characterized by unique features of morphology, cellular associations, and specialized functions. The unique features of each germ cell type are dictated, to a large degree, by the patterns of protein expression characteristic of each cell type. This review will examine two different aspects of the regulated expression of heat shock proteins in spermatogenic cells. First, we will review studies showing that the expression of several different members of both the hsp70 as well as hsp90 families of heat shock proteins is regulated during the differentiation of these cells. Second, we will review studies which have examined the induction of hsp expression in spermatogenic cells following exposure to elevated temperatures. Next, we will review the role of the transcription factors, heat shock factor 1 (HSF1) and HSF2 in the regulation of expression of hsps in the testis. One interesting and unique function of the male reproductive system in many species is the maintenance of the testes at a temperature below that of the other tissues of the animal. The importance of precise thermoregulation of the testis is evidenced by the fact that even slight elevations of scrotal temperature are associated with infertility. The results of recent studies have suggested a potential involvement of the cellular stress response in the mechanism responsible for these inhibitory effects of elevated testis temperature on spermatogenesis. Possible mechanisms are discussed.

Key words. Heat shock protein (hsp); regulation; spermatogenesis; differentiation; development; heat.

Introduction

Spermatogenesis is the process by which immature male germ cells, through a complex series of events involving mitosis, meiosis, and cellular differentiation, become mature spermatozoa. This process begins when spermatogonial cells initiate several rounds of mitosis to exponentially increase their number. Following spermatogonial proliferation and differentiation, the mature spermatogonia divide, forming primary spermatocytes. The spermatocytes undergo meiotic division, during which the genetic material is recombined and segregated, resulting in haploid spermatids. The maturation of spermatids into spermatozoa, called spermiogenesis, involves major morphological transformations, including the development of a flagellum and an acrosome, as well as the condensation of the chromosomal material within the nucleus. At this point, the spermatozoa are equipped to reach and fertilize an ovum. The unique features of each of these germ cell types are dictated by the patterns of protein expression characteristic of each cell type.

The expression of heat shock proteins (hsps) is a highly regulated event throughout the process of spermatogenesis. As in most cell types, within the cells of the testes, hsps are rapidly synthesized in response to adverse environmental stimuli such as elevated temperature. The heat-induced hsps play a vital cytoprotective role in preventing irreversible damage to cellular proteins by binding to unfolded or partially malfolded peptides to retard thermal denaturation and aggregation of cellular proteins. In addition to their stress-related cytoprotective function, hsps are also involved in many normal cellular processes. These include folding and assembly of nascent polypeptides, oligomerization, and intracellular protein transport. Thus, some hsps are constitutively expressed in the testis while others are induced by stressful conditions such as exposure to elevated temperature as well as in response to normal cell functions, such as growth and development or progression through the cell cycle. It has been hypothesized that hsps are expressed in spermatogenic cell types in order to provide for their specialized needs [1, 2]. During the process of spermatogenesis germ cells undergo dramatic alterations in gene expression, which result in changes in both the types and amounts of proteins present in each germ cell type [3–5]. The testis-specific patterns of hsp expression have been suggested to serve a molecular chaperone function that allows spermatogenic cell types to accommodate the unique set of proteins synthesized during the development and differentiation of these cells.

* Corresponding author.

This review will focus on the patterns and mechanisms of the regulated expression of hsps in spermatogenic cells. First, we will review studies showing that the expression of several members of both the hsp70 and hsp90 families of heat shock proteins is regulated during the differentiation of male germ cells. Second, we will review studies which have examined the induction of hsp expression in spermatogenic cells following exposure to elevated temperatures. Third, we will examine the roles of HSF1 and HSF2 as regulators of hsp expression in spermatogenic cell types. Finally, we will discuss interesting new results which have demonstrated a lowered temperature set-point for HSF1 activation in male germ cells. One unique feature of the male reproductive system in many species is the maintenance of the testes at a temperature below that of the other tissues of the animal. Even slight elevations in scrotal temperature are associated with male infertility. The results of recent studies have suggested a potential involvement of the cellular stress response in the mechanism responsible for these inhibitory effects of elevated testis temperature on spermatogenesis. Possible mechanisms are discussed.

Developmental regulation of hsp expression in spermatogenic cell types

Many hsps have been identified and characterized in cells of mouse testis (see table 1 and fig. 1). Some of these hsps are constitutively expressed, some are developmentally regulated, some are specific to testis, and some are only expressed in response to exposure to elevated temperatures. The most abundantly expressed hsps in the testis are those belonging to the hsp70 family. One of the most abundant is the developmentally regulated P70 protein, which first appears in mice at day 17. P70 from spermatogenic cells was originally recognized for its similarities to hsp70. However, peptide mapping proved it to be unique, and unlike hsp70, P70 is not heat-inducible [6]. The *hsp70.2* gene codes for the P70 protein, which is predominantly found in pachytene spermatocytes, but also in round spermatids, residual bodies and cytoplasmic fragments [1, 2, 7]. The expression of the P70 protein in rat, also referred to as hst70 [8] is first detected on postnatal day 22, with upregulation to adult levels after day 28. It has been identified in purified populations of adult rat pachytene spermatocytes, round spermatids, and elongating spermatids, but is absent in post-testicular sperm [9, 10].

Another widely recognized testis-specific hsp is the heat shock cognate 70 kDa protein. Messenger RNA from the hsc70t gene has been shown to accumulate mainly in early round spermatids, but, the protein does not accumulate until elongation of the spermatids [11, 12]. It was once thought that the mRNAs of the proteins necessary for spermiogenesis must be synthesized prior to meiosis, so this was an important finding because it showed that messenger RNA could be synthesized by round spermatids.

The expression of another member of the hsp70 gene family, 73T, is believed to be germ cell specific, as it was not detected in mutant mice devoid of germ cells [13]. It is produced by germ cells in the presence or absence of heat shock, and is synthesized in both meiotic and post-meiotic germ cells, but not spermatogonia. It is possible that the 73T protein, which is expressed at its highest levels in haploid germ cells, is the product of a 2.7 kb transcript described by Zakeri and Wolgemuth [14] which they refer to as hsp70.1 (see also ref. 13).

There are two main proteins belonging to the hsp90 family that are expressed in cells of the mouse testis, hsp84 and hsp86. Despite the fact that they share high sequence homology, studies have revealed that the hsp84 and hsp86 genes exhibit very distinct patterns of expression in developing spermatogenic cells. The mRNAs for both genes are expressed in mouse embryos before initiation of testicular development [15, 16]. During postnatal development, however, hsp86 expression increases during testicular development whereas hsp84 expression decreases. Gruppi et al. [15] observed hsp86 expression throughout the male germ cell lineage, with elevated levels during meiotic prophase. They also reported that hsp84 was expressed predominantly by somatic testicular cells. The distinct patterns of expression for these two hsps suggest they have distinct functional roles during spermatogenesis. Hsp90 has been shown to interact with steroid hormone receptors and to be important for the formation of the functional forms of these receptors. Therefore, it is likely that regulated expression of hsp90 proteins in cells of the testis may be required for the proper regulation of testis cell functions by steroid hormones such as testosterone. In rats, a testis-specific 105 kDa protein has been identified by its cross-reactivity to an anti-hsp90 antibody [17]. Though very similar to hsp90, it does not appear in testis until age 5 weeks, compared to 3 weeks for hsp90. In addition, it appears to be germ cell specific, though it has not yet been characterized in specific purified spermatogenic cell populations [18].

Mitochondria of spermatogenic cells must undergo rapid morphological changes during spermatogenesis. One group has looked at the distribution of mitochondrial hsp60 and its role during various developmental stages of spermatogenesis [19]. The results of their study demonstrated increased levels of hsp60 protein in cells at the mitotic stages of development. This makes biological sense because mitochondrial hsp60 functions as a molecular chaperone in the process of protein assembly and import into the mitochondria, and therefore these proliferating spermatogenic cells would require more hsp60 to accommodate the need to generate new mitochondria for daughter cells. Expression of hsp60

Table 1. Heat-inducible vs. developmentally controlled hsp expression.

Heat-inducible	Developmentally regulated
hsp70 [6, 7]	hsc70t [11, 12]
hsp72 [13]	rhst70 [9, 10, 38]
74 kDa [28]	hsp70.1 [2, 13, 14]
67 kDa [28]	hsp70.2 [1, 2, 6, 7, 10]
	hsc71 [6, 7, 26]
	73T [13]
	hsc74 [6, 7]
	hsp84 [15, 16]
	hsp86 [15, 16]
	*mt*hsp60 [19]

was limited to Leydig cells, Sertoli cells, and in early spermatogenic cells, but not in germ cells with condensed mitochondria.

Heat-responsive expression of hsps in spermatogenic cells

The heat-inducible expression patterns have been spermatogenic cells. As reviewed above, there are a number of hsps which are expressed in a developmentally-regulated manner, but whose levels are not increased by heat treatment. However, there is another group of hsps whose expression in spermatogenic cells does increase following exposure to elevated temperature. In many species, testis temperature must be precisely maintained at a temperature significantly below that of tissues in the main body cavity. In the mouse, testis temperature is approximately 30 °C, while tissues in the main body cavity are maintained at approximately 37 °C [20]. The importance of precise thermoregulation in testis is evidenced by the fact that even slight elevations in temperature can have a significant inhibitory effect on spermatogenic cell function, and are a major cause of male infertility [21–25]. Therefore, in light of the extreme sensitivity of testis cell types to elevated temperature, it is especially important to characterize the heat-induced expression of hsps in these cell types.

Several studies have been performed to address the question of the stress-responsiveness of hsp expression in spermatogenic cell types, but have reached different conclusions. It has been suggested by the results of some studies that the expression of the bona-fide heat-inducible hsp72 is increased in the somatic compartment of the testis and possibly premeiotic germ cells, but not in germ cells which have entered meiosis [13]. However, this conclusion differs from the findings of earlier studies, in which two-dimensional analysis of radiolabeled proteins of heat-treated, isolated meiotic and postmeiotic germ cells revealed an increase in levels of the heat-inducible hsp72 protein [6, 7]. There are several aspects of these studies which may have contributed to the different findings. First, the heat-treatment conditions employed differed between studies. Second, a lack of specific probes complicated the analyses by making it more difficult to be certain of induction of the heat-inducible hsp70 mRNA and protein because of recognition of closely related mRNAs and proteins encoded by other members of the mammalian hsp70 gene family.

Allen et al. [6] identified three HSP70-related proteins in unstressed mixed populations of germ cells; the spermatogenic cell specific P70 and heat shock cognates hsc71, further characterized by Maekawa et al. [26], and hsc74. However, when exposed to a temperature of 42.5 °C for 10 minutes, isolated populations of a number of germ cell types, including pachytene spermatocytes and round spermatids, also expressed an additional protein, the heat-inducible hsp70. This result is significant because pachytene spermatocytes and round spermatids have previously been shown to be especially sensitive to heat treatment [27]. Therefore, at

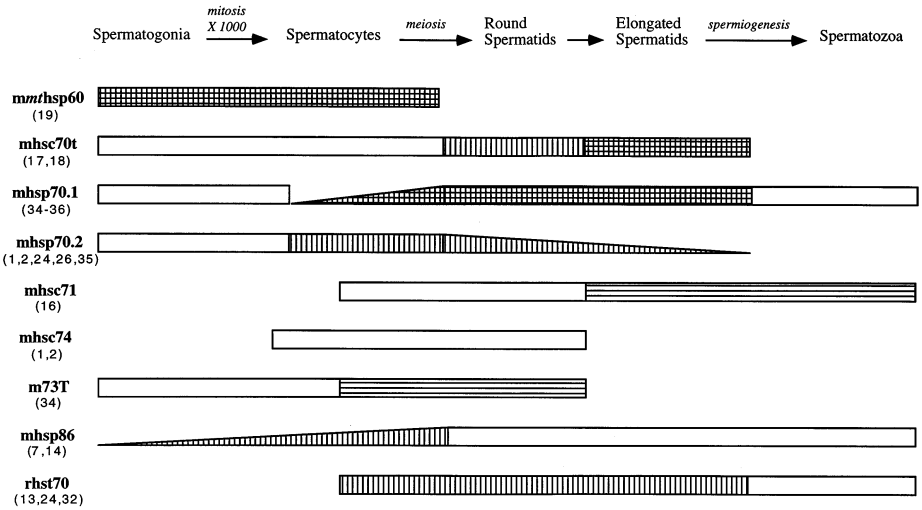

Figure 1. Summary of developmental regulation of Hsps in spermatogenic cell types. Absence of boxes indicates lack of analysis for that particular spermatogenic cell type. ▥ indicates the expression of RNA, ▤ the expression of protein, and □ indicates neither RNA nor protein was detected.

least under these treatment conditions, heat-induced expression of hsps does not appear to protect male germ cells from the harmful effects of elevated temperature on spermatogenic cells.

Zakeri et al. [13] observed the expression of a 72 kDa (heat inducible hsp70) and a 73 kDa (cognate hsp70) protein in non-heat-shocked and heat-shocked enriched testicular cell types. In addition, they observed a second 73 kDa (73T) protein only in adult mouse testis cells. The levels of the 73 kDa protein and the 73T protein did not change following heat treatment, whereas levels of the 72 kDa protein did increase slightly. They attributed the low levels of induction of hsp72 to low levels of somatic cells in their cell preparations, suggesting hsp72 was being induced in the somatic cells and/or in non-meiotic germ cells, in contrast to the conclusions drawn by Allen et al. [6, 7]. These differences were explained by overlapping electrophoretic patterns in the previous studies as well as non-specific antibodies.

Another group examined hsp expression in defined cell associations of dissected sections of seminiferous tubules. When protein expression patterns from tubules at different stages were compared following incubations at temperatures of 32 °C or 38 °C, a 74 kDa and a 67 kDa protein were only observed after the 38 °C treatment of testis from age 6 day mice [28]. In adult tissue, the mRNA encoding these proteins was expressed following heat treatment. However, they were not able to detect translation of these proteins in the 38 °C-treated cells. The reason for this result is not yet understood. A 36 kDa protein was observed to be strongly induced in a time and temperature dependent manner and hypothesized to be involved in intercellular signaling to report the cellular stress. The mRNA for a third protein, 28 kDa in size, was produced in all tubule sections at normal scrotal temperatures, with the highest levels produced in tubule sections which were enriched in elongating spermatids. Following heat treatment, this 28 kDa protein was not efficiently translated in the elongating stage sections.

The expression of hsps in germ cells is mediated by HSF1 and HSF2

Initiation of the stress response pathway occurs in response to specific signals, such as heat or other stressful environmental conditions. When cells are exposed to these stress signals, the inactive cytoplasmic form of the heat shock factor 1 (HSF1) protein is converted to the active DNA-binding form. Activation of HSF1 to the DNA-binding form is mediated by heat-induced conversion of the inactive HSF1 monomer to the DNA-binding trimer, and is also accompanied by increased phosphorylation and translocation to the nucleus. Following nuclear localization, activated HSF1 trimers bind to hsp gene promoters to stimulate transcription of these genes. However, hsp expression in mouse cells is also mediated through a second heat shock factor, HSF2, which is believed to function as a regulator of hsp expression during spermatogenesis and in other processes of cellular differentiation and development [29, 30].

As reviewed above, several members of the heat shock protein family display developmental or cell-type specific expression patterns during spermatogenesis. One possibility is that HSF2 may function to regulate the expression of hsp genes during germ cell differentiation. HSF2 mRNA expression in mouse testis was shown to be regulated in a developmental, cell-type, and stage dependent manner [29]. HSF2 mRNA first appears in testis between day 14 and 21 of post-natal development. In adult testis, HSF2 mRNA is found at highest levels in spermatocytes and round spermatids. In addition, this factor was localized to the nuclei of these cells and exhibited unusual constitutive DNA-binding activity. A recent development in the study of HSF2 regulation is the finding that mouse cells express two isoforms of the HSF2 protein, which are generated by alternative splicing of the HSF2 pre-mRNA [31]. Interestingly, this alternative splicing of HSF2 is regulated during testis postnatal development, with a switch in expression from predominantly HSF2-β in day 7 testis to primarily HSF2-α in adult testis. The biological significance of the regulated alternative splicing of HSF2 in spermatogenic cells is that these two proteins are functionally different. Transfection experiments revealed that the HSF2-α isoform has 2.6-fold transcriptional activity than the HSF2-β isoform [31]. Taken together, these results provide support for a role for HSF2 as an important regulator of hsp expression during mouse spermatogenesis.

Lowered temperature threshold for hsp expression in male germ cells

A unique feature of the male gonads in many species is their location outside the main body cavity [20]. In mice, testis are maintained at 30 °C, 7 °C lower than the normal core temperature within the body cavity, where tissues such as liver are stored. Though it has been known that HSF1 temperature set-point for activation varies between species, until recently it was not known whether the set-point was identical in all cells of a single organism. The unique feature of the lower normal growth temperature of testis cells provided a unique opportunity to investigate this question. Sarge et al. demonstrated that HSF1 was activated in testis cells at a significantly lower temperature than that observed for HSF1 activation in cells of mouse liver, demonstrating that the temperature set-point for HSF1 activation does not have a fixed value in a given species [32]. These studies were extended to show that the difference in

activation set-point was not due to a simple difference in normal temperature between the tissues. It was found that HSF1 from pachytene spermatocytes was activated at a threshold temperature of 35 °C, whereas HSF1 from the somatic testis cells was activated at 42 °C [33], demonstrating that the phenomenon of reduced HSF1 activation temperature is a unique feature of male germ cells. In addition, this study also showed that the HSF1 activated in the male germ cells at the lower temperature set-point of 38 °C was capable of mediating a productive cellular stress response in these cells.

The cellular stress response and the inhibition of spermatogenesis

The importance of precise thermoregulation of the testis is evidenced by the fact that even slight elevations of scrotal temperature, caused by conditions such as varicocele, cryptorchidism, sauna use, and even the wearing of tight, insulating clothing, are associated with male infertility [21–25]. Studies on the effects of temperature on the testis [34] have revealed that virtually all testis cell types are affected by temperature elevation. However, the underlying mechanism for the inhibitory effects of elevated temperature on the process of spermatogenesis has not been elucidated.

The fact that the stress response is mounted at temperatures which have been shown to inhibit spermatogenesis raises an interesting hypothesis concerning the mechanism by which heat inhibits spermatogenesis. It may be that it is the induction of the cellular stress response in male germ cells following exposure to elevated temperature which is in fact responsible for the heat-induced inhibition of spermatogenic cell functions, thereby causing male infertility. In most cell types, the heat-induced hsps play a vital cytoprotective role by binding to denatured proteins which result from adverse environmental conditions and aiding in their refolding back to the native functional state [35]. However, the induction of the stress response has also been shown to have other effects on cells, and some of these effects could be responsible for heat-induced loss of spermatogenic cell function. For example, induction of hsp expression has been shown to significantly inhibit the progression of cells through mitosis [36]. It is clear that any reduction in the rate of cell division of early spermatogenic cell types would have a significant inhibitory effect on the net production of spermatozoa. Therefore, we hypothesize that it may be the induction of hsp expression, via its inhibition of cell division, which is responsible for the inhibitory effects of elevated temperature on spermatogenic cells that culminate in male infertility.

In support of this hypothesis, preliminary studies have shown that the threshold temperature for induction of the cellular stress response in isolated pachytene spermatocytes is 35 °C [32, 33], a temperature within the range of temperatures known to cause male infertility. This lowered threshold of the cellular stress response in male germ cells is an important and unique finding, because all other mouse cell types studied thus far only induce the stress response at temperatures of 41 °C and above. In addition, previous results have suggested that pachytene spermatocytes and round spermatids, two spermatogenic cell types which are particularly heat-sensitive [27], express hsps in response to heat treatment. Thus, the heat-inducible hsps are not adequately performing their cytoprotective function in these cells.

A second possibility is that elevated temperatures cause inhibition of germ cell functions by inhibiting the expression or function of proteins essential for the viability or development of one or more spermatogenic cell types. One obvious possible mechanism is that elevated temperatures may simply cause partial denaturation of thermolabile essential spermatogenic cell proteins, thus leading to loss of cell viability or function. Another possibility is that heat exposure of male germ cells may inhibit the expression of genes encoding essential proteins. In support of this latter possibility, previous studies using cultured mouse and human cell lines have shown that exposure of these cells to elevated temperature causes a rapid and near-absolute inhibition of both mRNA splicing and protein synthesis. The only exception to these heat-induced blocks in gene expression is the induction of heat shock proteins, which are able to surmount the blocks at both of these steps (reviewed in ref. 27).

Summary and future directions

In order to fully understand the significance of both normal and heat-induced hsp expression in testis cells, future studies must determine the function of each hsp that is expressed in these cells. The fact that many of the hsps are only expressed in certain spermatogenic cell types suggests that they play specific roles in the development and function of these cell types. A common method for defining a protein's function is to inhibit the expression or function of that protein and look for abnormalities. Unfortunately, the most simple methodologies may not be used since male germ cells cannot be cultured in vitro. However, antisense technology may be used to develop transgenic mice lacking in testis-specific hsps. Monitoring the process and efficiency of spermatogenesis in these mice should yield important information concerning the function of the blocked protein's function. In addition, the expression patterns of hsps in the testis need to be further characterized, particularly the hsps for which different studies have revealed discrepancies. Testis-specificity, testis cell specificity, and developmental regulation all reveal information about the functions of these hsps.

The mechanisms which control the expression patterns of each of these hsps also need to be elucidated. The genes for mouse hsp70.2 [2] and hsc70t [11], rat hst70 [8] and other hsp70 family members [37] have already been cloned, and HSE-like sequences have been identified in their 5′ upstream regions. However, the single HSE elements in the mouse hsp70.2 and the rat hst70 gene are considered non-functional with respect to heat-inducibility as they do not exactly match the consensus sequence. Thus, they are probably not controlled by HSF1. HSF2 has been shown to bind to the promoter of the developmentally regulated hsp70.2 gene, and it is expression in the testis is controlled in a developmental, cell-type-dependent, and spermatogenesis stage-dependent manner [29]. These data suggest that the regulation of the transcription factor HSF2 itself may be responsible for the developmental control of hsp expression.

It is likely that there are testis and cell-type specific *trans*-acting factors which limit the expression of many of the hsps and the HSFs to the testis and to specific spermatogenic cell types. These putative factors may be identified by promoter deletion or mutation analysis. Again, transgenic mice with mutated promoter sequences may be used to identify important sequences. Wisniewski et al. [38] have established transgenic mice by injecting fertilized eggs with a construct containing 0.8 kb of the 5′ upstream region of the rat hst70 gene fused to a reporter gene and determined that the 0.8 kb upstream region is sufficient to direct spermatogenesis-specific transcription. Once essential promoter regions have been identified, in vitro transcription analyses and transfection studies will facilitate further characterization of the *cis*- and *trans*-acting factors which are responsible for the testis-specific and spermatogenic cell type specific patterns of hsp expression. These studies will almost certainly reveal unique features of transcriptional regulation in spermatogenic cells.

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