Characterization and Inducibility of hsp 70 Proteins in the Male Mouse Germ Line
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Abstract. The properties and inducibility of the heat shock protein 70 (hsp 70) gene products were examined during differentiation of mouse testicular cells by one and two-dimensional gel electrophoresis and immunoblotting. Low levels of the 72- and 73-kD heat shock proteins normally found in mouse cell lines were detected in the mouse testis. A novel isoform with a relative molecular mass of 73 kD (called 73T) was also observed, in the presence or absence of heat shock. 73T was shown to be produced by germ cells since it was not detected in testes from mutant mice devoid of germ cells. Furthermore, 73T was found only in adult mouse testicular cells, not in testes from animals that lack meiotic germ cells. 73T was synthesized in enriched cell populations of both meiotic prophase and postmeiotic cells, but was not inducible by in vitro heat shock. In the adult testis, low levels of the bona fide 72-kD heat-inducible (hsp72) were induced in response to elevated temperatures. In contrast, in testes from animals in which only somatic cells and premeiotic germ cells were present, there was a substantial induction of hsp 72. It is suggested that hsp 72 is inducible in the somatic compartment and possibly in the premeiotic germ cells, but not in germ cells which have entered meiosis and which are expressing members of the hsp 70 gene family in a developmentally regulated fashion.

Prokaryotic and eukaryotic organisms activate a set of highly conserved genes in response to external stress. These genes, called heat shock (hsp) genes, are also expressed during normal embryonic development in many organisms such as Drosophila (Dura, 1981), frogs (Graziosi et al., 1980; Bienz, 1984), sea urchins (Giudice et al., 1980), and mice (Bensaude et al., 1983; Bensaude and Morange, 1983). In particular, hsp have been shown to have unique patterns of expression in meiotic cells. Small hsps are expressed during sporulation in yeast (Kurtz et al., 1986) and in growing oocytes and spermatocytes of Drosophila (Zimmerman et al., 1983; Glaser et al., 1986). hsp 70 genes have been shown to be expressed in the germ line of Drosophila (Bonner et al., 1984; Kurtz et al., 1986), mice, rats, and humans (Zakeri and Wolgemuth, 1987; Zakeri et al., 1988b).

Our previous studies on the expression of hsp 70 genes have revealed that distinct members of this family are abundantly expressed in the mouse testes in the absence of stress and that their expression is under developmental regulation. We initially detected a testicular hsp 70, designated hsp 70.1, which was expressed at highest levels in populations of germ cells that have entered the haploid stage (Zakeri and Wolgemuth, 1987). This novel hsp 70 transcript likely gives rise to functional protein since it was associated with polyribosomes in testicular cells (Zakeri et al., 1988a).

We have also isolated, sequenced, and characterized the pattern of expression of a new member of the mouse hsp 70 gene family (Zakeri et al., 1988b). This distinct member of the hsp 70 gene family, designated hsp 70.2, was shown to not be activated in mouse cell lines in response to heat shock, but rather in response to developmental cues. This hsp 70 transcript was also regulated in a unique manner in the male germ line, being most abundant in meiotic prophase spermatocytes (Zakeri et al., 1988b).

In the present study, we have focused on characterizing the hsp 70 gene products synthesized in male mammalian germ cells at specific stages of spermatogenic development. In particular, we have examined whether hsp 70 gene products in germ cells were produced only in response to developmental cues and/or whether they could be induced by external stress as well.

Materials and Methods

Tissues and Cells
Swiss Webster male mice were used as the source of all normal mouse tis-
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Results

Detection of hsp 70-related Proteins in Adult Mouse Testes

To detect de novo synthesized hsp 70 proteins, mouse testes were incubated in media containing [35S]methionine. Labeled hsp 70 proteins were characterized by two-dimensional gel electrophoresis and compared with the patterns of hsp 70 proteins synthesized in mouse L cells. Both nonheat-shocked L cells and testicular cells synthesized proteins of 73 kD (hsp 73) in size (Fig. 1, A and C). Low levels of the 72-kD hsp (hsp 72) were detected in testicular cells (Fig. 1 A). An additional protein of ~73 kD (hsp 73T), which migrated differently than hsp 73, was observed only in the adult mouse testes (Fig. 1 A). The levels of hsp 73 and hsp 73T did not appear to increase when the testicular cells were subjected to heat shock (Fig. 1 B). Only a slight induction of hsp 72, which is induced at high levels in mouse L cells (Fig. 1 D), was observed in testicular cells (Fig. 1 B).

The 73T Protein Is a Member of the hsp 70 Family

To determine if hsp 73T is antigenically related to the members of hsp 70 family, labeled proteins were separated by two-dimensional gel electrophoresis and analyzed by immunoblotting (Fig. 2). Monoclonal antibody N-27, which recognizes both the heat-inducible and the nonheat-inducible members of the hsp 70 family (Minota et al., 1988; Welch, W. J., unpublished observations), reacted with hsp 72 and hsp 73 as well as hsp 73T (Fig. 2, b and c). The same set of proteins was detected with other antibodies (data not shown), including antibody 7.10, which recognizes various members of the hsp 70 family in insect and mammalian cells (Velasquez et al., 1983).

An attempt was made to use these antibodies to localize hsp 70-related proteins by immunocytochemistry. The monoclonal antibody N27 detected proteins that were evenly distributed in all testicular cells (data not shown). This was not surprising given the fact that, as mentioned above, several hsp 70 proteins are recognized by this antibody, and in any given cell, one or all of the proteins may be present.

hsp 73T Is Synthesized by Germ Cells and Its Synthesis Is Developmentally Regulated

To determine if the hsp 73T protein was present in the somatic cells or in the germ cells of the testes, we examined the synthesis of the novel hsp 73T in testes from mutant strains of mice. Testes of homozygous W/Wmice are devoid of germ cells, but do contain a normal interstitium with well-formed seminiferous tubules containing Sertoli cells (Coulombre and Russell, 1954). Examination of proteins synthesized by testicular cells from homozygous W/Wmice versus normal littermates revealed that the germ cell deficient testes did not produce the hsp 73T protein, although both hsp 73 and hsp 72 proteins were synthesized (Fig. 3, a and b). This result indicated that hsp 73T is synthesized in the germinal compartment of the adult testes.

The adult mouse testis contains germ cells at different stages of development, from spermatogonial stem cells to spermatooza. We prepared enriched populations of germ cells at specific stages of spermatogenesis to determine...
which cell types were actively synthesizing the hsp 73T protein. Samples of meiotic prophase spermatocytes (predominantly in the pachytene stage of meiosis), postmeiotic early (round) spermatids, and residual bodies were incubated with [35S]methionine, and the synthesized proteins were analyzed by two-dimensional electrophoresis. All the meiotic and postmeiotic cell populations examined synthesized hsp 73T, in addition to hsp 73 and low amounts of hsp 72 (Fig. 4, a, c, and e). This indicated that meiotic and postmeiotic spermatogenic cells could synthesize 73T.

To determine if hsp 73T was also synthesized in premeiotic stages of spermatogenic differentiation, we took advantage of the biological features of testicular development in neonatal mice. Testes from day 7-8 neonates contain all the typical somatic cells including Leydig and Sertoli cells, as well as primitive type A spermatogonia and more advanced type A and B spermatogonia (Nebel et al., 1961). Proteins from the testes of day 7-8 animals were labeled with [35S]methionine and analyzed by two-dimensional gel electrophoresis as described above. The hsp 72 and hsp 73 seen in the mouse L cells and adult testes were also detected among the labeled proteins of day 7-8 neonatal testes (Fig. 5 a). However, even after long exposure of the autoradiograms, no hsp 73T was detected in the immature, day 7-8 testes. These results suggested that the hsp 73T is not synthesized by premeiotic germ cells.

**Induction of Specific hsp 70 Proteins in Testicular Cells by Heat Shock**

We then examined the heat inducibility of the hsp 70 proteins within various populations of testicular cells. Enriched populations of spermatogenic cells were heat shocked, labeled, and analyzed by two-dimensional gel electrophoresis (Fig. 4, b, d, and f). hsp 73 and hsp 73T, present in the non-stressed cells, were not induced after heat shock. However, in all the cell types examined there was a slight induction of hsp 72. The level of hsp 72 induction in the enriched germ cell populations was comparable to that observed for hsp 72 induction in the total testis samples. Since each of the enriched germ cell fractions had a low level of contamination with somatic cells, we suggest that the somatic, not germinal cells, were responsible for the appearance of the heat-inducible protein.

Further support for the contribution of the heat-inducible hsp 72 protein by the somatic and/or premeiotic stage germ cells was obtained by subjecting testes of neonatal day 7-8 mice to heat shock. Testes from animals of this age contain all somatic cell types and a large proportion of sper-
Figure 2. Immunoblot detection of hsp 70 products synthesized in total mouse testicular cells. Labeled proteins from total intact mouse testes were separated by two-dimensional gel electrophoresis and transferred to nitrocellulose filters for analysis by immunoblotting, using antibody N27 (Minota et al., 1988). (a) Autoradiograph of labeled proteins which are detected by immunoblotting in b. The proteins migrating at 72 and 73 kD and other 70-kD proteins in the cells are readily detected. (c) A second immunoblot analysis of proteins from mature testis is shown in which higher concentration of the antibody was used and which resulted in enhanced sensitivity of detection of the proteins of the hsp 70 family.

matogonial cells, but lack meiotic stage cells. The known heat shock-inducible family member, hsp 72, was highly induced in the immature day 7–8 testes (Fig. 5 b), whereas it was only slightly induced in the adult testes (Fig. 1 b) or in purified meiotic and postmeiotic cells (Fig. 4, b, d, and f). hsp 72 was also highly induced in germ cell deficient testes (data not shown). These results suggested that hsp 72 gene was being induced in the somatic cells and/or in nonmeiotic germ cells.

**Inducibility of hsp 70 Transcripts**

To examine which hsp 70 mRNAs were inducible upon heat shock, RNA was isolated from mouse L cells and testis in the presence or absence of heat shock. The RNAs were analyzed by Northern blot hybridization with two probes. (a) Probe pMHS 213, a cDNA for the heat-inducible mouse hsp 68 gene, which also recognizes a developmentally induced, haploid spermatid-abundant mRNA of 2.7 kb in size (Zakeri and Wolgemuth, 1987). We refer to the gene recognized by this probe at high hybridization stringency as HSP 70.1. (b) Probe pM1.8, a genomic mouse clone which encodes a gene expressed at high levels within meiotic prophase spermatocytes and to lesser extent in later meiotic cells. This gene, which has been shown by sequence analysis to be a bona fide hsp 70 gene family member, is designated HSP 70.2 (Zakeri et al., 1988b).

Both pMHS 213 and pM 1.8 recognized two induced transcripts of ~3.4 and ~2.4 kb in size in heat shocked L cells (Fig. 6a, lane 2), which were not detected in the absence of heat shock (Fig. 6, lane 1). In the testis they recognized an abundant transcript of ~2.7 kb in the RNA isolated from both heat-shocked and nonheat-shocked testis (Fig. 6, b and c, lanes 1 and 2). As a control, RNA was isolated from mouse testes which were not subjected to manipulation (Fig. 6, b and c, lanes 3). There was no difference in the level of the 2.7-kb testicular transcript detected by these probes in any of the testicular RNAs, nor did we detect any new transcripts after heat shock.

We then extended our investigation to the testis of neonatal, day 7–8, mice, in which hsp 72 protein had been shown to be highly inducible (Fig. 5, b and c). Our probes detected very low levels of two transcripts of 3.4 and 2.4 kb in size in nonheat-shocked immature testes, and as predicted, failed to detect any of the meiotic and postmeiotic specific transcripts of 2.7 kb in size (data not shown). Upon heat shock, the 3.4-kb transcript was readily detectable (Fig. 7, lane 2). Although not clearly resolved in the Northern blot shown in Fig. 7, the 2.4-kb heat-inducible hsp 70 transcript was apparently induced as well. In addition, we saw no induction of the germ cell-specific 2.7-kb transcripts. This suggested that the 2.7-kb transcript, which is not normally synthesized in this cell population, did not become activated as a result of heat shock. Our results further suggested that the 72-kD protein seen in this cell population was not synthesized from the testis-specific 2.7-kb transcripts.

**Discussion**

**Detection of a Novel Member of the hsp 70 Gene Family in Spermatogenic Cells**

The present study extends our previous observations on the developmental stage specific expression of distinct members of the hsp 70 gene family in the mouse testis (Zakeri and Wolgemuth, 1987; Zakeri et al., 1988a, b) to the protein level. One- and two-dimensional gel electrophoretic analysis of newly synthesized proteins combined with immunoblotting revealed the presence of a unique hsp 70 protein of ~73 kD, designated hsp 73T, in the adult testis. Three sets of observations support the contention that 73T was synthesized by meiotic and postmeiotic germ cells. First, the protein was not detected in immature testes, which lack meiotic and postmeiotic germ cells. Second, it was not detected in testes from...
mutant strains of mice which are deficient in germ cells. Third, enriched populations of meiotic prophase spermatocytes, round spermatids, and elongating spermatids and cytoplasmic fragments and residual bodies all synthesized 73T.

**Correlation of hsp 70 Gene Products at the Level of RNA and Proteins**

We have previously shown that at least two distinct members of the hsp 70 gene family are expressed in a developmentally regulated pattern during mouse spermatogenesis (Zakeri and Wolgemuth, 1987; Zakeri et al., 1988b). The mRNA produced by the gene designated HSP 70.2 is most abundant in meiotic prophase spermatocytes and appears to decrease in abundance as the cells progress through spermiogenesis (Zakeri et al., 1988b). In contrast, the similarly sized transcripts corresponding to the gene designated HSP 70.1 begin to accumulate in round spermatid stages and are quite stable, being readily detected in RNA from elongating spermatids and residual bodies (Zakeri and Wolgemuth, 1987). We do not know if either, both, or neither of these members of the hsp 70 gene family give rise to the 73T protein. In vitro translation or translation of full-length transcripts of these genes in mammalian or baculovirus expression systems may resolve this question.

**Inducibility of the hsp 70 Family Gene Products in Testicular Cells**

In addition to 73T, the adult mouse testis synthesized low levels of the typical (somatic) hsp 70 proteins, hsp 73 and hsp 72. When adult testes were subjected to heat shock in the in vitro system, the levels of hsp 73 and 73T did not appear to change, whereas the level of hsp 72 was slightly increased. The response of the cells within the immature testes or testes from germ cell deficient mice was markedly different in that hsp 72 was strongly induced. Since hsp 72 induction occurred at high levels in testes lacking germ cells, we suggest that the somatic compartment, which includes Leydig cells, peritubular cells, Sertoli cells, etc., was responsible for this induction. The low level of induction of the hsp 72 protein detected in the total adult testis after heat shock would be consistent with the fact that the mature gonad is made up of a small proportion of somatic cells relative to germ cells (<10%; Meistrich, 1977). Similarly, the low level of hsp 72 induction observed in the enriched populations of spermatogenic cells could have been due to the small proportion of somatic cells relative to germ cells (5–8%) which routinely contaminate our enriched fractions of spermatogenic cells (Wolgemuth et al., 1985). In the immature testes, it is possible that the spermatogonial stem cells could also respond to heat shock by synthesizing hsp 72, although we have no direct evidence supporting or refuting this possibility. Hence we suggest that the slightly increased levels of hsp 72 protein seen in the adult testis and in the various enriched germ cell populations after heat shock were due to synthesis by the somatic cells, and not the germ cells.

At the level of RNA analysis, neither the germ cell–specific nor the somatic transcripts appeared to be induced in the heat-shocked adult testis. The slight induction of hsp 72 protein in adult testicular cells upon heat shock could thus be under translational control. Alternatively, since the somatic cells are such a small proportion of the adult testicular compartment and the levels of the 2.7-kb germ cell-specific transcript so abundant, detection of the somatic hsp 70 transcripts may be masked. In contrast, in the immature testis, heat shock induction of what appeared to be the typical heat-induced transcripts was evident. Although we can not as yet correlate directly specific transcripts with their corresponding proteins, our RNA analyses further support the observation that hsp genes are noninducible in meiotic germ cells.

Our interpretation that meiotic and postmeiotic germ cells do not respond to heat shock by synthesizing hsp 72 is in contrast to the interpretation reported by Allen et al. (1988a, b). It is possible that the exact conditions for inducing the heat

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**Figure 3.** Detection of hsp 70 proteins in germ cell deficient testes. Proteins from intact adult testes from a fertile offspring from a W/+ x W/+ mating (a) and the homozygous, infertile W/W~ littermate (b) were labeled as described in Materials and Methods, analyzed by two-dimensional gel electrophoresis, and labeled proteins were detected by autoradiography. No 73T protein is detected in the germ cell deficient testes sample.
Figure 4. Synthesis of hsp 70 gene products in enriched populations of germ cells of different stages of spermatogenic differentiation. Enriched spermatogenic cell populations were obtained as described by Wolgemuth et al. (1985). Cells were heat shocked, labeled and analyzed by two-dimensional gel electrophoresis as described in Materials and Methods and detected by autoradiography. a, c, and e represent proteins synthesized in the absence of heat shock; b, d, and f depict the proteins labeled when cells were subjected to heat shock. a and b contained populations of cell enriched for meiotic prophase spermatocytes, predominantly in the pachytene stage; c and d contained early, round spermatids; and e and f contained residual bodies. The position of actin is indicated by the open triangle and the letter a.

The shock response varied slightly and thus elicited different responses. There are other features of the two sets of studies to consider, however. The two-dimensional electrophoretic system used in the present study was suitable for clear separation of all the different hsp 70 family members, in contrast to the overlapping patterns of the previous studies (Alien et al., 1988a, b). Secondly, the observation that inducible hsp 72 was expressed in postmeiotic germ cells (Allen et al., 1988a, b) relied on antibodies which are now known to recognize multiple hsp 70 proteins in murine tissues, not only the heat-inducible protein hsp 72 (Zakeri, Z., F., W. J. Welch, and D. J. Wolgemuth, unpublished observations). Finally, since the procedures used for obtaining populations of spermatogenic cells (Allen et al., 1988b) were similar to those used in the present studies, and are known to yield low levels of contaminating somatic cells, the previous study may also have been complicated by the presence of hsp 72 ensuing from somatic cells.
The present observations also suggest that the spermatogenic cells which are producing certain hsp 70 family proteins in response to developmental cues are unable to respond or respond very poorly to heat shock. Hsp 70 proteins have been shown to be induced as a normal consequence of differentiation during sporulation and meiosis in yeast and to be noninducible by heat shock during this period (Kurtz et al., 1986). The inability to respond to heat shock by increased hsp 70 expressing in germ cells may be implicated in the susceptibility of mammalian germ cells to elevated temperatures. Spermatogonial stage germ cells have been shown to be more resistant to elevated temperatures than are pachytene spermatocytes and early spermatids (Young, 1927; Rock and Robinson, 1965), which our observations suggest are unable to synthesize bona fide hsp 72. Preovulatory mouse oocytes are also highly sensitive to elevated temperatures, and they synthesize low levels of members of the hsp 70 gene family (Curci et al., 1987).

An inability to respond to heat shock by cells which are concomitantly expressing developmentally regulated hsp 70 proteins has also been observed in early embryos of many species, including mice. Exposure to heat does not induce an enhanced synthesis of bona fide heat shock proteins in cleavage stage embryos of Drosophila (Dura, 1981; Graziosi et al., 1980), sea urchins (Roccheri et al., 1981, 1982), frogs (Bienz, 1984; Helkkila et al., 1985), and mice (Bensaude et al., 1983; Bensaude and Morange, 1983). Thus during normal development and differentiation, the expression and inducibility of members of hsp gene families change and are different from the patterns observed in cell lines.

Figure 6. Northern blot hybridization analysis of RNA from heat-shocked testes. Total RNA was isolated from normal adult mouse testes subjected to conditions as described in Materials and Methods and as noted below. The Northern blot hybridization analysis used 32P-labeled probes from pMHS213 (a 1.3-kb Hind III/Eco RI insert) or pM1.8 (a 1.8-kb Bam HI/Eco RI insert) as noted. Both probes detect multiple HSP 70 gene family members (Zakeri and Wolgemuth, 1987; Zakeri et al., 1988b). (a) Lane 1, 20 μg RNA from mouse L cells; lane 2, 20 μg RNA from heat-shocked mouse L cells. b and c contain 30 μg RNA per lane as follows: lane 1, testicular RNA at 33°C; lane 2, testicular RNA at 42°C; lane 3, RNA from testes which were frozen immediately and not subjected to manipulation before extraction of the RNA. b was hybridized with pMHS 213 and c with pM1.8. Exposure times: a, 1 d; b and c, 2 d.

Figure 7. Northern blot hybridization analysis of RNA from heat-shocked immature mouse testes. RNA was isolated and analyzed as described in the legend to Fig. 6. The probe used was pMHS 213. Lane 1 contains RNA isolated from adult mouse testis maintained at normal temperature. Lane 2 contains RNA isolated from day 7 neonatal mouse testes after heat shock at 42°C for 90 min followed by a 2-h recovery. Each lane contains 20 μg total RNA. Exposure time, 5 d.
In addition, thermosensitivity and the ability to become thermotolerant also change. For example, while very early embryos appear extremely sensitive to elevated temperatures, such sensitivity decreases sharply at the blastula stage. Moreover, it is at the blastula stage that the cells now acquire an ability to induce heat shock gene expression in response to classical stress exposures (e.g., Bienz, 1984; Graziosi et al., 1980; Heikkila et al., 1985; Rocchetti et al., 1981). Our data indicate that similar changes in the heat and developmental inducibility of heat shock gene expression occur during germ cell differentiation. An understanding of the relationship between thermosensitivity and the presence or absence of hsp family members will be aided by determination of their function in cells in which they are expressed in the absence of heat shock as well as in response to exogenous stress.

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